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Osmotically Induced Response in Representatives of Halophilic Prokaryotes: the Bacterium *Halomonas elongata* and the Archaeon *Haloferax volcanii*

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***Haloferax volcanii* and *Halomonas elongata* have been selected as representatives of halophilic *Archaea* and *Bacteria*, respectively, to analyze the responses to various osmolarities at the protein synthesis level. We have identified a set of high-salt-related proteins (39, 24, 20, and 15.5 kDa in *H. elongata*; 70, 68, 48, and 16 kDa in *H. volcanii*) whose synthesis rates increased with increasing salinities. A different set of proteins (60, 42, 15, and 6 kDa for *H. elongata*; 63, 44, 34, 18, 17, and 6 kDa for *H. volcanii*), some unique for low salinities, was induced under low-salt conditions. For both organisms, and especially for the haloarchaeon, adaptation to low-salt conditions involved a stronger and more specific response than adaptation to high-salt conditions, indicating that unique mechanisms may have evolved for low-salinity adaptation. In the case of *H. volcanii*, proteins with a typical transient response to osmotic shock, induced by both hypo- and hyperosmotic conditions, probably corresponding to described heat shock proteins and showing the characteristics of general stress proteins, have also been identified. Cell recovery after a shift to low salinities was immediate in both organisms. In contrast, adaptation to higher salinities in both cases involved a lag period during which growth and general protein synthesis were halted, although the high-salt-related proteins were induced rapidly. In *H. volcanii*, this lag period corresponded exactly to the time needed for cells to accumulate adequate intracellular potassium concentrations, while extrusion of potassium after the down-shift was immediate. Thus, reaching osmotic balance must be the main limiting factor for recovery of cell functions after the variation in salinity.**

Osmoregulation in prokaryotes has captured major interest, not only to understand cell response and adaptation to varying environmental conditions but also because of the applied aspects of this field (such as interaction between microbe and plant or pathogenicity) (7). Osmoregulatory mechanisms have been studied in different groups of organisms, particularly *Escherichia coli* (6, 7). However, in the case of halophilic organisms, specially adapted to relatively high or even extreme osmotic conditions, osmoregulatory mechanisms are poorly understood. Most of the studies on halophilic prokaryotes have been centered on physiological aspects of their adaptation to salt (15, 19, 20, 24, 41), but aspects such as gene and protein expression, or global response, under different osmotic conditions are practically unknown.

Within the two prokaryotic domains *Bacteria* and *Archaea* (45), the species of the genera *Halomonas* and *Haloferax*, respectively, are the organisms with the widest growth spectra in relation to salt (16, 39, 40). The extremely halophilic archaeon *Haloferax volcanii* is able to grow over a range of NaCl concentrations from about 8% to saturation (29). The range supported by the bacterium *Halomonas elongata* is even wider, from 0.1% to saturation (40, 44). Considering the wide growth range of these organisms, their osmoregulatory mechanisms should be quite efficiently evolved. *H. elongata* and *H. volcanii* can thus be considered models for the study of adaptation to different osmolarities in halophilic prokaryotes.

Adaptation to such different osmolarities must imply rather complex physiological and molecular mechanisms. Accumulation of compatible solutes, different lipid compositions, and synthesis of new proteins are some examples. Accumulation of compatible solutes under high osmotic pressure conditions is a universal mechanism of adaptation (2, 7, 15). Most halophilic prokaryotes accumulate organic compatible solutes to a large extent (2, 15, 23). In *Halomonas*, these are mainly glycine betaine, ectoine, and hydroxyectoine (38, 46), in various concentrations depending on the osmolarity of the medium (46). The case of the haloarchaea (formerly halobacteria) is completely different. To compensate for the typically extreme saline conditions of their habitats, they accumulate not organic compatible solutes but high intracellular concentrations of potassium (up to 5 M) (1, 4). Modifications in lipid composition depending on salinity are also well known in halophilic bacteria (18, 36, 37), including *Halomonas* (42), and haloarchaea (21).

However, little information exists on proteins and genes involved in osmoregulation in these organisms. In the case of haloarchaea, Doolittle's group detected in *H. volcanii* a set of proteins induced upon salt dilution shock (10). Our own group described some genes with salt-dependent expression in *Haloferax mediterranei* (28) and global response at the transcriptional level in the *H. volcanii* genome at different salinities (11). Differential expression as a response to salinity in *Halomonas* is even less well understood. Vreeland and coworkers reported modifications of cell surface hydrophobicity (14, 43), antigenic character, and total protein pattern, depending on the NaCl concentration of the medium, in *H. elongata* (43).

We have examined the global response under osmotic stress conditions in both *H. volcanii* and *H. elongata*. Cells were grown at different salinities approaching the optimum, minimum, and maximum for each organism, and the protein synthesis pattern was analyzed. Aspects of the general response, such as protein synthesis, or cell recovery after either a down-

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shift or an up-shift of salinity conditions, have also been studied.

MATERIALS AND METHODS

Strains and culture conditions. *H. elongata* (ATCC 33173) was grown in CAS medium (40), containing (weight/volume) 0.1% yeast extract (Difco), 0.75% Casamino Acids (Difco), 0.5% Proteose Peptone (Difco), 0.3% sodium citrate, 2% $MgSO_4 \cdot 7H_2O$, 0.0005% $Fe(NH_4)_2(SO_4)_2$, and 0.75% K_2HPO_4, supplemented with different NaCl concentrations (0, 0.3, 6, 15, and 23%, corresponding roughly to 0, 0.05, 1, 2.5, and 4 M, respectively). Cultures were incubated at 30°C with vigorous shaking.

H. volcanii (strain DS2) cells were grown in complex medium containing 0.5% yeast extract (Difco) and different concentrations of a mixture of salts (SW) in the proportions found in seawater, as described previously (35) (30% SW containing [weight/volume] 23.4% NaCl, 1.95% $MgCl_2$, 2.9% $MgSO_4$, 0.12% $CaCl_2$, 0.6% KCl, 0.03% $NaHCO_3$, and 0.075% NaBr, corresponding to a total salt concentration of 4.5 M). Salt concentrations (10, 20, and 30% SW) approaching the minimum, optimum, and maximum for growth, but where growth is not significantly affected, were used. For studying the effect of organic osmolytes, a medium containing 10% SW was supplemented with 50% sucrose. The incubation temperature was 37°C. All cultures were incubated under similar conditions (100-ml cultures in 500-ml flasks, with orbital shaking at 200 rpm).

For the shift experiments, cells growing in the first medium were collected by centrifugation at 4,000 × *g* for 10 min at the incubation temperature and suspended in the second growth medium, reaching a similar culture volume. Media were always prewarmed and maintained at the growth temperature.

Growth measurements and total protein determinations. Growth was recorded by measuring both optical density at 600 nm (OD_{600}) and concentration of total proteins. Total protein determinations were performed with a Bio-Rad assay reagent (Bio-Rad Laboratories GmbH) as recommended by the manufacturer.

Labeling of cellular proteins. Proteins were labeled essentially as described by Daniels et al. (10). Cells from 1 ml of culture (OD_{600}, 0.4 to 0.8) were collected by centrifugation. Cell pellets were suspended in 500 μl of 1% (wt/vol) methionine assay medium (MAM; Difco), containing the corresponding salt concentration, after a wash in the same medium. Labeling of total proteins was initiated by adding 25 μCi of [^{35}S]methionine (>1,000 Ci/mmol; ICN Biomedicals, Inc.), and cells were incubated for 30 min (pulse-labeled for 15 min in shift experiments and labeled for 1 h for the two-dimensional gel electrophoresis). Cells were then collected by centrifugation, washed with a similar salt concentration solution, and lysed by suspending the pellet in 100 μl of 20 mM Tris-HCl (pH 7.5) containing RNase (20 μl/ml) and DNase (1.0 μg/ml). Lysates were incubated at 10°C for 30 min and then centrifuged to remove any remaining cells. Salts and nonincorporated radiolabeled methionine were removed by centrifugation through an Ultrafree-MC ultrafiltration system (Millipore Corporation) as recommended by the manufacturer.

Radioactivity incorporated into proteins was determined with a liquid scintillation counter (Beckman) by measuring the emission corresponding to a 5-μl sample diluted in 4 ml of scintillation cocktail (Beckman).

Isolation of cytoplasm and total membrane fractions. *H. elongata* cells were grown under different osmotic conditions, and cytoplasm and total membrane fractions were separated by a modification of the procedure described by Miller and Mekalanos (27), in which adequate NaCl concentrations were added to the cell suspension. Cells from the different cultures were pelleted by centrifugation (at 10,000 × *g* for 20 min at 4°C), washed, and suspended in cold HMNS buffer (50 mM HEPES [pH 7.4], 10 mM $MgCl_2$, 5% sucrose, plus NaCl in a concentration similar to that in the corresponding growth medium). After sonication and removal of unbroken cells (at 10,000 × *g* for 15 min), the lysate was centrifuged in a Kontron TFT-70 rotor at 12,000 rpm for 90 min at 5°C. The pellet (envelope fraction) was suspended in HMNS buffer. The supernatant (cytoplasm fraction) was further purified, removing contaminating membranes by incubation at 30°C for 2 h followed by centrifugation in the TFT-70 rotor at 15,000 rpm for 90 min at 5°C.

Protein gel electrophoresis. Samples for one-dimensional gel electrophoresis were prepared by adding to the protein extract an equal volume of Laemmli sample buffer (22) and boiling for 5 min just prior to loading. Samples loaded into a gel contained either equal amounts of total proteins or equals amounts of labeled extracts (labeled precursor incorporated), as measured previously.

Proteins were electrophoresed on sodium dodecyl sulfate (SDS)–10 (or 12) to 18% (or 20%) polyacrylamide linear gradient slab gels (0.75-mm thickness), overlaid with a 4% polyacrylamide stacking gel. The gradient gel was prepared by mixing 12 ml each of freshly prepared solutions A (10 or 12% acrylamide) and B (18 or 20% acrylamide) through a gradient mixer. Solutions A and B consisted of 0.375 M Tris-HCl (pH 8.8) and, on a weight-to-volume basis, 9.5% glycerol, 0.1% SDS, and 0.017% ammonium persulfate, plus the corresponding acrylamide-bisacrylamide concentration. TEMED (*N*,*N*,*N*′,*N*′-tetramethylethylenediamine) was added to both solutions A and B at 0.25 μl/ml just prior to gradient formation. The stacking gel contained 0.125 M Tris-HCl (pH 6.8) and (weight/volume) 4% acrylamide, 0.138% bisacrylamide, 0.1% SDS, 0.1% ammonium persulfate, and 5 μl of TEMED per ml. Electrophoresis buffer contained 20 mM Tris-glycine (pH 8.3) and 0.1% SDS. Polyacrylamide gel electrophoresis (PAGE) was performed at 40 V for 16 h followed by 300 V for 1 h.

Two-dimensional gel electrophoresis was performed essentially by O'Farrell's procedure (30). Samples were prepared by adding urea to saturation, 1% (vol/vol) Nonidet P-40 (Sigma), 100 mM dithiothreitol, and 2% (wt/vol) ampholines (Bio-Lyte 3/5 ampholyte; Bio-Rad). Samples were clarified by centrifugation just prior to loading. An SDS–10% polyacrylamide gel with a 4% polyacrylamide stacking gel was used for the second dimension. For isoelectric focusing of proteins, a mixture of Bio-Lyte 3/5 and Bio-Lyte 5/7 (Bio-Rad) was used.

Low-range SDS-PAGE molecular weight standards (Bio-Rad) were coelectrophoresed for calculation of molecular weights. Gels were stained with Coomassie brilliant blue R250. After destaining, gels were dried under vacuum at 80°C for 2 h with a gel drier from Bio-Rad, cooled, and exposed to Hyperfilm-βmax films (Amersham) at −70°C.

Densitometric analysis. For quantification of intensity of labeled proteins, a Bio Image-TDI (TDI, Madrid, Spain) image analyzer was used. Data were analyzed with a two-dimensional analyzer and whole-band analyzer software (provided by the manufacturer) for two-dimensional and one-dimensional gel electrophoresis, respectively.

Estimation of rates of total protein synthesis after the shift experiments. To determine the rates of total protein synthesis, samples extracted at different times after the shift were purified and the radioactivity was measured by liquid scintillation counting as described above.

Intracellular potassium determinations. Intracellular potassium determinations were performed essentially as described previously (31). Cells were harvested by centrifugation and washed with an NaCl solution of concentration similar to that in the culture medium. Lysis of cells was carried out by suspending them in distilled water. The K^+ concentration was measured by flame emission spectrophotometry in a Perkin-Elmer atomic absorption spectrophotometer. Protein determinations were performed as described above.

RESULTS AND DISCUSSION

Protein synthesis profiles in *H. elongata* under various salinity conditions. The only report on salt-dependent differentially expressed proteins in *Halomonas* refers to 60- and 45-kDa species detected from a total protein pattern as being highly abundant at low salinity (43). To identify osmoregulated proteins, we have studied here the protein synthesis profiles in *H. elongata* cells grown at different salinities (long-term response) and after a hypoosmotic and a hyperosmotic shock (short-term or transient responses). Since modifications in the cell surface (hydrophobicity and antigenic character) of the organism were previously described (14, 43), we also determined the cellular location (cytoplasm or envelope fraction) of these proteins. A general overview of the long-term response to different salinities is given in Fig. 1, and induction of protein synthesis after a shift to low- or high-salt conditions is shown in Fig. 2.

Haloadaptation (salt adaptation) could be indicated by a gradually higher accumulation of certain proteins in cells growing under gradually increasing salinities. We considered as high-salt condition related a set of proteins whose synthesis rates increased notably in high-salt media. Among such proteins were 39-, 24- and 15.5-kDa species. These proteins were not synthesized in the lowest salinities (0% NaCl [Fig. 1A]), were not detected or were poorly synthesized at low (0.3%) NaCl concentrations, but were synthesized at considerably increased rates at high salinities (Fig. 1A; see also Fig. 3A). All of these proteins related to the long-term response to high salt were also immediately induced upon hyperosmotic shock (Fig. 2B). When cells were transferred from low to high salinities, these high-salt proteins began to be synthesized within the first 15 min, and within 0.5 to 1 h after the up-shift, they reached synthesis rates close or equal to those of the high-salinity conditions (Fig. 2 and 3). In general, all hyperosmotic shock responses and long-term responses to high-salt conditions corresponded to the same proteins (the high-salt-related 39-, 24-, and 15.5-kDa proteins mentioned above and other 14- and 20-kDa species more gradually and slightly induced under increasing salinities, as shown in Fig. 1 and 2). It appears then that the putative role of all high-salt-related proteins might be

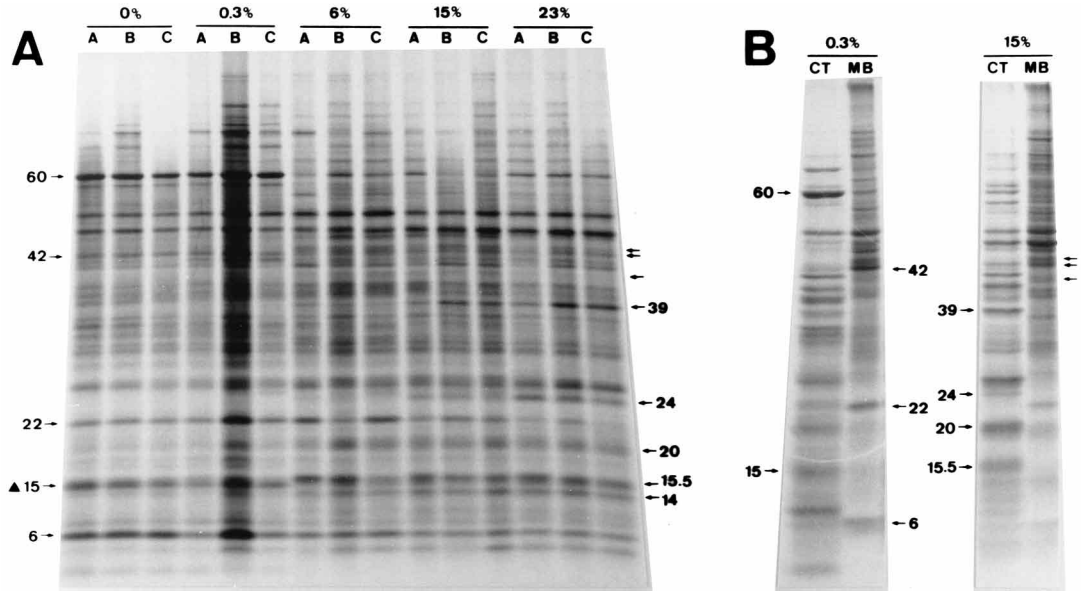

FIG. 1. Protein synthesis patterns at different salinities in *H. elongata*. (A) Cells grown at different NaCl concentrations (0, 0.3, 6, 15, and 23%) in CAS medium and in different growth stages (samples A, B, and C correspond to early, middle, and late logarithmic phases of growth, respectively) were transferred to 1% MAM containing the corresponding concentration of salts for labeling with [^{35}S]methionine for 30 min. The protein synthesis pattern was analyzed after PAGE on SDS–12 to 18% polyacrylamide linear gradient gels. Molecular masses (in kilodaltons) of low- and high-salt-related proteins are indicated. Proteins specific for low (0 and 0.3%) NaCl concentrations in the growth medium are marked with a triangle. A 22-kDa species responding transiently (Fig. 2) to low salt is also indicated for comparison with Fig. 2. (B) Labeled proteins from the cytoplasm (CT) and total membrane (MB) fractions from cells grown at different salinities are shown. Low- and high-salt-related proteins, as in panel A, are indicated in their corresponding cellular fractions. Electrophoresis was performed on SDS–12 to 18% polyacrylamide linear gradient gels.

important for both transient and long-term responses to salt. Apart from high-salt-related proteins, any other protein was transiently induced by hyperosmotic shock.

Low-salinity conditions had a dramatic effect on *Halomonas* protein synthesis, despite the fact that the growth rate for the lowest salinity (0% NaCl added to CAS medium) was similar to that of the high (15%) NaCl concentration and even higher than that of the highest (23%) NaCl concentration in the media and conditions used here. These low-salt (0 and 0.3% NaCl) conditions elicited sharp and even specific responses (Fig. 1 and 3B). Low salt specific was a 15-kDa protein, not detected at medium (6%) and high salinities but showing high synthesis rates at low salinity (0 and 0.3% NaCl) (Fig. 1A and 3). A sharp increase in protein synthesis under low-salt conditions was also shown by 60- and 42-kDa proteins. A more gradual and modest response was observed for a 6-kDa species. Synthesis of these proteins started rapidly after a hypoosmotic shock (Fig. 2B), but these low-salt-related proteins generally responded by gradually and slowly increasing synthesis rates after the down-shift (Fig. 3B). A particularly transient response to low salt was that of a 22-kDa protein (Fig. 2), a species whose synthesis rates in the long-term response stage were higher at low and higher still at medium (6%) salinities (Fig. 1A and 3C). The expression of the other low-salt-related proteins appeared to be more important in the long term than in the immediate response to low salt.

The more apparent responses in the adaptation to either low- or high-salinity conditions appear to occur in the cytoplasm. Most of the high-salt-related proteins (39-, 24-, 20-, and 15.5-kDa species) were isolated within the cytoplasm fraction (Fig. 1B). The most relevant, specific (15-kDa), and sharply induced (60-kDa) proteins responding to low-salt conditions were also cytoplasmic (Fig. 1B). However, changes also occurred in the membrane fraction, where we detected species of 42 and 6 kDa (induced by low salt) and 22 kDa (with a transient response to hypoosmotic stress and long-term response to medium and low salinities). The decrease of these membrane proteins at high osmolarities might be in accordance with the suggestion of Vreeland et al. (43), considering that the increasing hydrophilicity of the *H. elongata* cell surface at high salinity would be in accordance with a deficit in several proteins (as seen in other bacteria).

Proteins homologous to those previously described as having a role in osmoregulation in bacteria such as *E. coli* (7) could be expected to be found in this study. That would be the case, for instance, for the ProP protein and ProU system components (ProV, ProW, and ProX) induced at high osmolarities. These proteins could be identified as membrane associated. It is known, for example, that migration of the ProV protein corresponds to a molecular mass of 42 kDa (33) and that the ProP protein (54.8 kDa) shows a greater mobility than would be predicted by its molecular mass (8) (below the 42-kDa band corresponding to ProV) (26), as typically occurs with hydrophobic membrane proteins. It would not be easy to identify in this study those membrane-associated proteins by their mobilities. We were able to detect membrane-associated proteins migrating within a range of about 40 to 44 kDa (indicated by arrows in Fig. 1), which could include proteins related to salt adaptation such as ProP or ProV homologs. OsmY would migrate as a 22-kDa membrane protein (47). OsmC is a 14-kDa protein (13) and could perhaps correspond to that of a similar molecular mass detected here. Most of these proteins would have to be found within the membrane fraction. However, the most relevant responses to high-salt conditions corresponded to cytoplasmic proteins, many of which do not appear to correspond to proteins described for *E. coli* and related bacteria. This could be an indication that in *Halomonas*, other osmoregulatory mechanisms have evolved for salt adaptation.

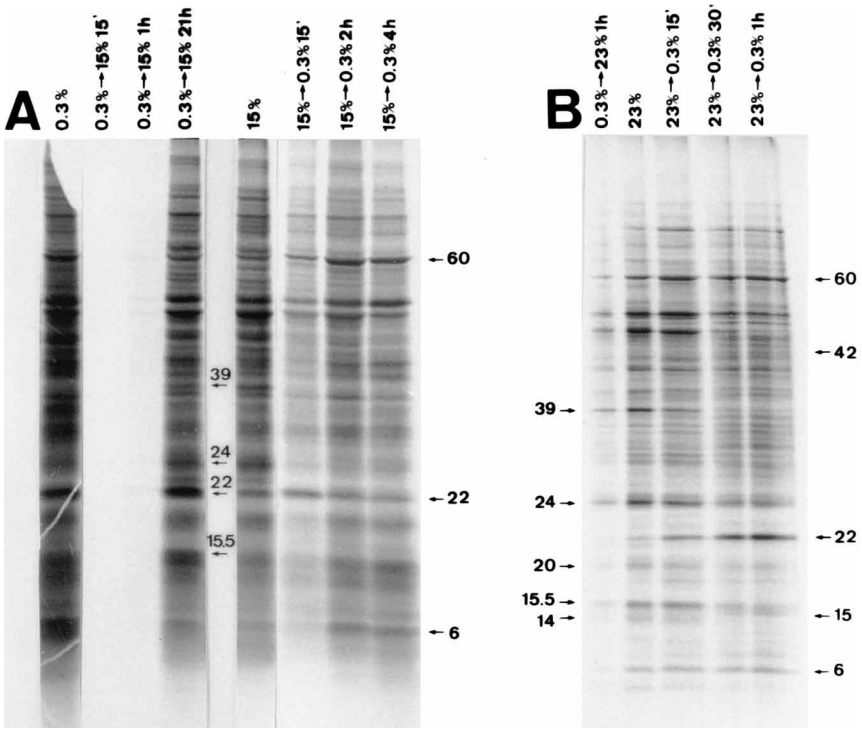

FIG. 2. Effects of variations of salinity on protein synthesis in *H. elongata*. Cells growing in log phase in normal *H. elongata* growth medium (CAS), at either low or high salinity, were shifted to the new salinity conditions (as indicated) in the same CAS medium. Cellular proteins were pulse-labeled for 15 min with [^{35}S]methionine in 1% MAM containing the corresponding concentration of salts. Times indicated are the end of the various labeling periods. (A) Protein synthesis pattern of cells after a shift from 0.3 to 15% NaCl medium and vice versa. Similar amounts of total proteins were loaded on the gel for each sample. Electrophoresis was performed on SDS–12 to 18% polyacrylamide linear gradient gels. (B) Protein synthesis pattern of cells after a shift from 0.3 to 23% NaCl medium and from 23 to 0.3% NaCl medium. Samples containing similar amounts of labeled proteins were loaded. Electrophoresis was performed on SDS–12 to 20% polyacrylamide linear gradient gels. Proteins responding to low- and high-salt conditions are indicated. Sizes are indicated in kilodaltons.

General response of *H. elongata* cells after variations in the salinity conditions. As we have already mentioned, after a variation in the salinity conditions, there was a rapid response of the long-term-related proteins. Synthesis rates of both sets of low-salt- and high-salt-related proteins started to increase rapidly after the shift. After analyzing the response of particular proteins, we describe below how cells recover after the salinity variation, whether they are able to start growing immediately after the shift, and how the protein synthesis machinery (total protein synthesis) could be affected by the stressing conditions due to the shift (transient osmotic imbalance, etc.).

When cells were subjected to a down-shift (from 15 to 0.3% NaCl), both growth (Fig. 4) and general protein synthesis (Fig. 2A) were maintained at high rates. As shown in Fig. 2A (shift from 15 to 0.3%), when similar amounts of total proteins were loaded in the gel, the total protein synthesis levels (observed as total amounts of radiolabeling) after the down-shift immediately reached the synthesis (radiolabeling) levels shown before the shift (15% sample). Thus, cells recovered quickly after the down-shift, and there was no apparent lag period.

On the contrary, a long period of time was needed for cells to recover after a shift to high-salt conditions. There was a long lag period during which general protein synthesis and growth were halted. There was a lag of about 20 h or more before cells were able to start growth after a shift from 0.3 to 15% NaCl (Fig. 4). Protein synthesis in general was low or almost undetectable during this lag period (Fig. 2A, where similar amounts of total proteins were loaded onto the gel). However, as mentioned above, an immediate synthesis of high-salt-related proteins was observed after the up-shift, even for the highly stressing shift from 0.3 to 23% NaCl (Fig. 2B, where samples with similar amounts of labeled precursor incorporated were loaded). Therefore, the long lag period of adaptation to the new salinity conditions cannot be explained by a delay in the induction of synthesis of the corresponding high-salt proteins.

To compensate for the extracellular salt concentrations, *Halomonas* accumulates high intracellular concentrations of compatible solutes, mainly glycine betaine, ectoine, and hydroxyectoine, in complex medium (38, 46). *Halomonas* would have to transport and probably synthesize glycine betaine and synthesize ectoine and hydroxyectoine (32, 38). In a complex medium, the main compatible solute at low-medium salinity (5% NaCl) is glycine betaine, while at higher salinities the increase in intracellular solute concentration is mainly related to ectoine and hydroxyectoine accumulation by synthesis (46), probably because of exhaustion of glycine betaine reserves in the medium. Accumulation of the appropriate compatible solutes by synthesis would thus play an important role in the adaptation to high salt concentrations. Potassium has also been shown to be necessary after hyperosmotic shock (9). Osmoregulatory mechanisms in *Halomonas* could be similar to those described for *E. coli*, in which the initial intracellular potassium acts as a signal to induce the osmoregulatory systems, finally being substituted by organic compatible solutes (7). This would imply a rather complex response, initially accumulating potassium and then transporting and particularly synthesizing the organic compatible solutes to a large extent, as needed in the high-osmolarity medium. Such a complex and slow process to

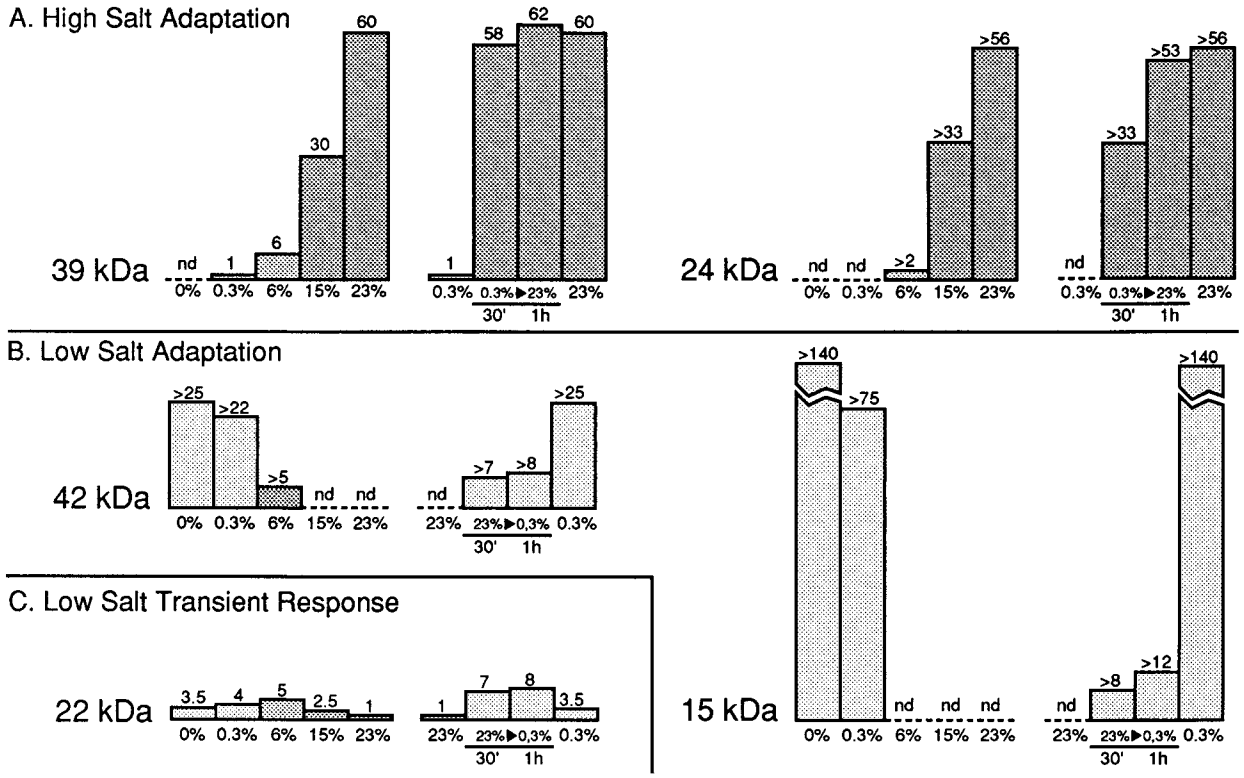

FIG. 3. Densitometric analysis of representative responses of proteins for low- and high-salt adaptation in *H. elongata*. A few proteins showing typical patterns of response to low or high salinities have been selected. Molecular masses of proteins are indicated. Data are shown as percentages of integrated intensity compared with the minimal values detected for that protein (minimal values always considered 1). When a protein was not detected (nd) at a certain salinity, the remaining values for that protein at the other salinities are considered as $>x$-fold with respect to the minimal value detected by the densitometer. (A) Typical responses for high-salt adaptation; (B) typical responses for low-salt adaptation; (C) transient strong response to low salinities by a 22-kDa protein (see text).

reach osmotic balance could explain the long lag period shown by *Halomonas* after a shift to high salinities.

Protein synthesis profiles in *H. volcanii* under various osmotic conditions. In the case of halophilic archaea, no previous work dealing with responses at the protein synthesis level to different osmotic conditions exists. The only report on proteins related to osmotic shock is that of Daniels et al. (10), in which the proteins induced under a heat shock and a hypoosmotic shock in *H. volcanii* were compared. A 91-kDa and a 79-kDa species induced by both heat shock and salt dilution shock and a 17-kDa protein induced by salt dilution but not by heat shock were detected (10). To more appropriately compare our results with those previously described (10), labeling of cellular proteins, either at constant salinities or after a salinity shift, was performed basically as described previously (10).

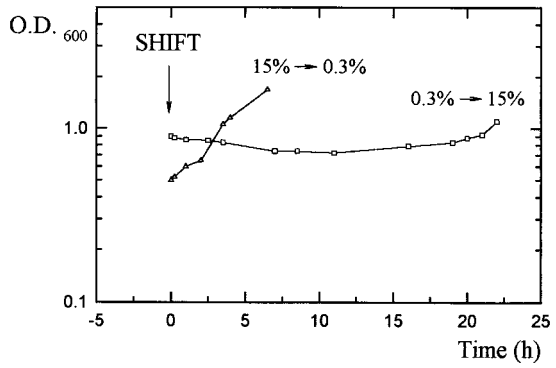

FIG. 4. Growth of *H. elongata* cells after a shift from low-salt to high-salt (0.3 to 15% NaCl) medium and from high-salt to low-salt (15 to 0.3% NaCl) medium. Cells growing in CAS medium in middle to late log phase were shifted to CAS medium with the new salt concentration.

We investigated the short-term (or transient) and long-term responses to different osmotic conditions in *H. volcanii*. Protein synthesis patterns in cells growing at low (10% SW) and high (30% SW) salinities (see Materials and Methods), approaching the minimum and maximum for growth but at which good growth still occurs, as well as the response after a sudden variation in the osmotic conditions of the medium were analyzed. A general overview of the response of *H. volcanii* to different osmotic conditions is presented in Fig. 5. Osmotic stress proteins (OSPs) with a transient response after a shift to low or high salinities, and other proteins showing a long-term response in the adaptation to certain salinity conditions, have been identified. Most of the OSPs described here may correspond to previously described heat shock proteins or in fact be general stress proteins (GSPs), as will be discussed below.

We also considered it worthwhile to analyze whether, or in what proportion, the response to high osmolarity in a halophilic archaeon, highly specialized to live in extreme environments where salts are usually in concentrations near saturation, was dependent on the nature of the solute. As shown in Fig. 6, the patterns of response to a hyperosmotic shock were quite similar for organic (sucrose) and inorganic solutes. After

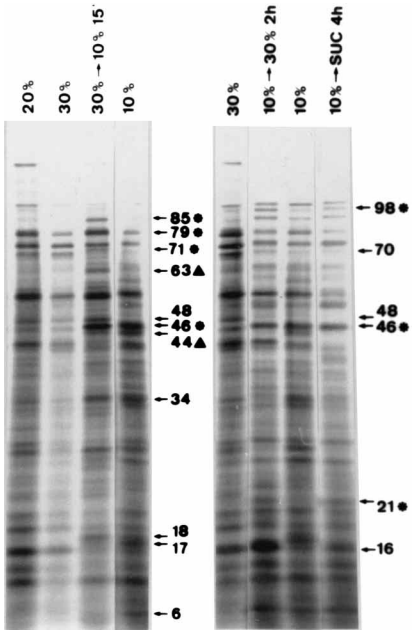

FIG. 5. Protein synthesis pattern of *H. volcanii* cells grown at different salinities (10, 20, and 30% SW) and after the shifts from high (30%)- to low (10%)-salt media and vice versa. A sample showing the effect of an increase in osmolarity by adding an organic compound (from 10% SW medium to 10% SW plus 50% sucrose medium) is also included. For detection of proteins synthesized in cells under either constant-salinity conditions or after a salinity shift, cells growing in complex medium were transferred to 1% MAM containing either the same or a different salt concentration, respectively, and pulse-labeled (15 min) with [^{35}S]methionine. Times indicated represent the ends of the various labeling periods. Samples with similar amounts of labeled proteins were electrophoresed on SDS–10 to 18% polyacrylamide linear gradient gels. Molecular masses (in kilodaltons) of proteins are indicated. Proteins specific for the low-salt response are marked with triangles among other low-salt-related proteins (indicated in the 10% SW medium lane). Proteins related to high salt, or high osmotic conditions in general, are indicated on the right side. Proteins showing transient responses induced by different stressing conditions and considered as corresponding to GSPs (see text) are marked with asterisks.

a shift from 10% salts to 30% salts or to 10% salts plus sucrose to saturation, the synthesis of typical low-salt proteins decreased simultaneously in both cases, while high-osmolarity proteins started to be synthesized. The only apparent differences correspond to a 42-kDa and a 55-kDa protein, whose synthesis rates were notably depressed under the high sucrose concentrations.

Hyperosmotic shock triggered a rather general stress response. Notable transient responses to hyperosmotic shock were those of 98-, 46-, and 21-kDa OSPs (or rather GSPs) (Fig. 5; see also Fig. 8C). All of them showed a transient synthesis rate enhancement (5- to 50-fold or more), dropping in the long-term response stage. The 98- and 21-kDa proteins could correspond to those of similar-molecular-weight heat shock proteins found previously in *H. volcanii* (10). Heat shock proteins of 98 and 18 to 22 kDa have also been found in other archaeal groups (methanogens and extreme thermophiles) (5), indicating their universality at least among archaea. Two-dimensional gel electrophoresis studies (data not shown) revealed that the 46-kDa OSP, induced under hyperosmotic shock and specially by high sucrose conditions, is in fact also induced after a hypoosmotic shock (Fig. 5 and 7B) and could correspond to DnaJ-like chaperones, as discussed below. Therefore, all of them can be considered to be GSPs.

Adaptation to grow at high (30% SW) salinities did not involve unique long-term responses. The 20 and 30% salt patterns were in fact rather similar (Fig. 5). We have, indeed, identified a set of proteins which might be related to the high-salt response (Fig. 5). The synthesis levels of these proteins increased with increasing salt concentrations, although the 20% salt levels were always near the 30% salt levels (frequently 10-fold or more with respect to 10% salt [Fig. 8A]). Proteins of relevance in the long-term response to medium-high salinities were a 16-kDa, a 48-kDa, and a set of around 70-kDa (72-, 70-, and 68-kDa) species (Fig. 5 and 7A). Most of them have been identified by two-dimensional gel electrophoresis (Fig. 7A), where we have distinguished two components of about 72 kDa and two proteins of about 48 kDa, with enhanced synthesis at medium-high salinities (48b and 48c in Fig. 7A, although 48c also showed a transient response to low salt in Fig. 7B [30 to 10% shift]).

The most noteworthy behavior corresponded to the low-salt adaptation process. Low-salinity (10% SW) conditions triggered certain unique responses as well as strong general stress responses. One-dimensional gel electrophoresis analysis (Fig. 5) had already pointed to this peculiar pattern of response. The more important long-term responses (10- to 100-fold or greater increase in synthesis rates) were shown by the 63-, 44-, 34-, 18-, 17-, and 6-kDa proteins (Fig. 5 and 7C, 10%). Among them, the 63- and 44-kDa species appeared to be low salt specific (Fig. 5 and 7C). Other proteins are also indicated in Fig. 7C. Most of these proteins overexpressed in the long-term

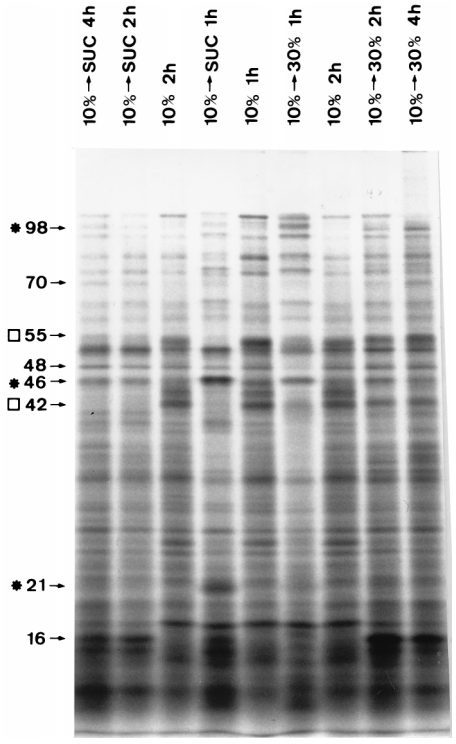

FIG. 6. Effect of an increase in osmolarity on the *H. volcanii* protein synthesis profile, depending on the nature of the solute. Cells were shifted from 10% SW complex medium to 1% MAM containing either 30% SW or 10% SW plus 50% sucrose (SUC samples). Control samples in which cells were transferred from 10% SW complex medium to 1% MAM containing a similar 10% SW salinity are also included. Cellular proteins were pulse-labeled for 15 min. Times indicated represent the ends of the various labeling periods. Samples of proteins with similar amounts of [^{35}S]methionine incorporated were electrophoresed on SDS–10 to 18% polyacrylamide linear gradient gels. Proteins which appeared to be depressed by addition of sucrose to the medium are marked with squares. Proteins related to high osmotic conditions are indicated as in Fig. 5.

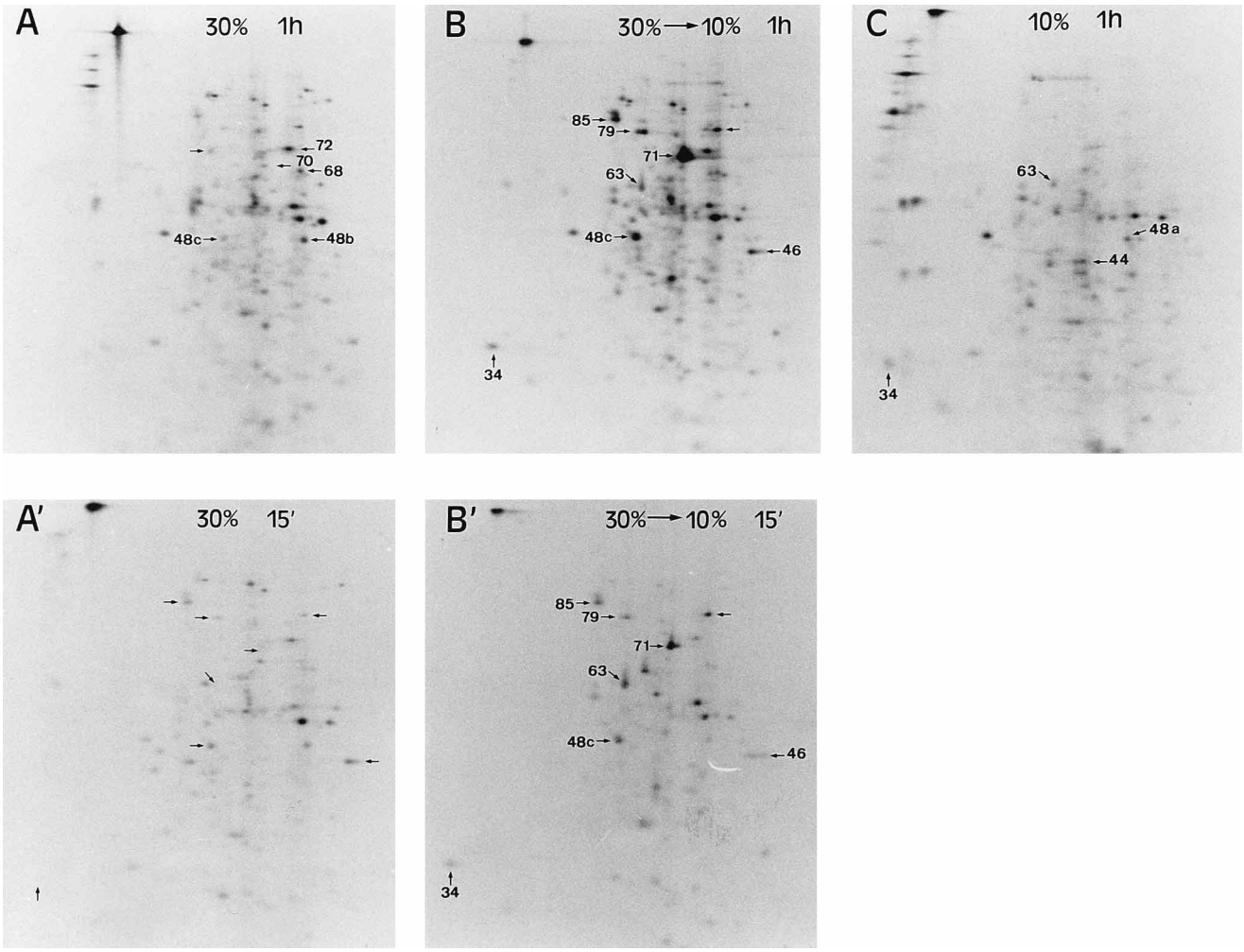

FIG. 7. Two-dimensional gel electrophoresis of *H. volcanii* proteins synthesized in cells growing at different salinities (30 and 10%) and after a hypoosmotic shock (shift from 30 to 10% salts medium). Cells growing in normal *H. volcanii* complex medium were transferred to 1% MAM containing the same or a different salt concentration (for constant salinity or salinity shift experiments, respectively) and then labeled with [^{35}S]methionine for the times indicated. (A) Cells growing in 30% SW medium were labeled for 1 h. Molecular masses (in kilodaltons) of some proteins involved in the long-term response to high-salt conditions are indicated. (B) Cells were labeled for 1 h after a shift from 30 to 10% SW medium. Molecular masses of proteins induced after the down-shift are indicated. (C) Cells growing in 10% SW medium were labeled for 1 h. Some proteins related to low-salt adaptation (long-term response) are indicated. (A′) Cells were labeled for 15 min in 30% SW medium. Proteins induced after a shift from 30 to 10% SW medium are indicated for comparison with panel B′. (B′) Cells were labeled for 15 min after a shift from 30 to 10% SW medium. Proteins induced after the down-shift are indicated as in panel B.

response to low salt increased their synthesis levels gradually after a down-shift (Fig. 8B), but particularly strong transient responses were shown by the 63-, 34-, and 18-kDa low-salt proteins (Fig. 8B).

Hypoosmotic shock also induced typical transient general stress responses, such as those of the 85-, 79-, 71-, and 46-kDa species (Fig. 5; Fig. 7B and B′). The 46-kDa protein, as mentioned above, was induced upon both hyper- and hypoosmotic shock. In general, the hypoosmotic shock transient response would reflect a general stress response. Actually a hypoosmotic shock might mimic a heat shock, particularly for a haloarchaeon, in the sense that most of the halophilic proteins could be partially denatured under a dilution or defect of the appropriate ion concentration (by water incoming after the down-shift). The 85- and 79-kDa species could correspond to the similar-molecular-weight heat shock proteins described for *H. volcanii* (10). Heat shock proteins with molecular masses of about 70 and about 45 kDa have been described (10, 12) for haloarchaeal species of the genera *Haloarcula* (*H. marismortui*) and *Halobacterium* (*H. trapanicum*) but not for *Haloferax* (*H. volcanii* and *H. mediterranei* R-4) (34) or for other *Haloarcula* (*H. hispanica* Y-27) (17) or *Halobacterium* (*H. salinarium*) species (10). However, our data suggest the presence of GSPs or chaperones of about 70 and 46 kDa in *H. volcanii*. A gene encoding a protein of about 70 kDa, homologous to the universally present HSP70 protein, has been found in the haloarchaeon *Haloarcula* (previously *Halobacterium*) *marismortui* (12). Besides, a DnaJ homolog with an approximate molecular mass of 43 kDa has been found in a methanogenic archaeon (25), and chaperones of about 46 kDa, also homologous to the *E. coli* DnaJ protein, are present in eukaryotes (3, 48), showing a transient accumulation in response to NaCl stress (48). The universal presence of such HSP70-like and DnaJ-like chaperones among haloarchaea, with a possible role under osmotic stress, is pointed out here.

All proteins with typical transient responses after a down- or up-shift must be considered GSPs of 98, 85, 79, 71, 46, and 21 kDa. Determination of the general stress character of these

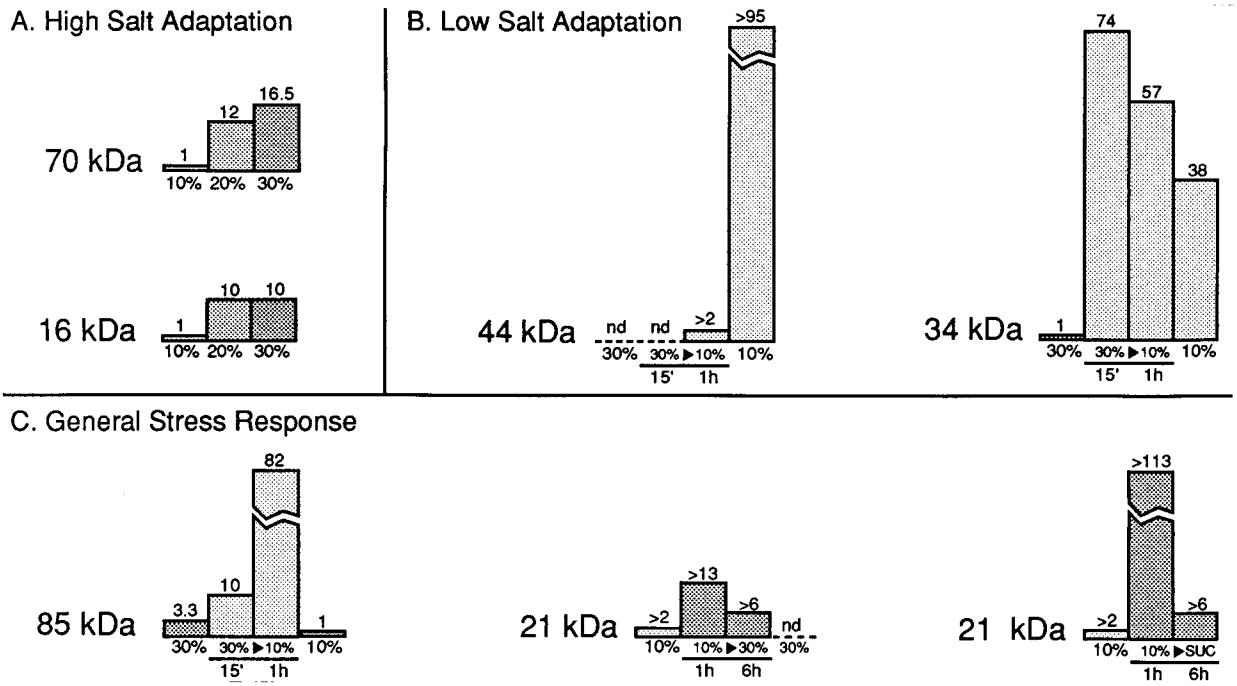

FIG. 8. Densitometric analysis of representative responses of proteins for low- and high-salt adaptation in *H. volcanii*. A few proteins showing typical patterns of response to low or high salinities have been selected. Molecular masses of proteins are indicated. Data are shown as in Fig. 3. (A) Typical responses for high-salt adaptation (long-term responses at different salinities); (B) typical responses for low-salt adaptation; (C) typical transient responses performed by GSPs upon hypo- or hyperosmotic shock.

proteins can be based not just on the coincidence of molecular mass ranges with described haloarchaeal heat shock proteins (98, 85, 79, 71, and 21 kDa) or universal chaperones such as the HSP70 homologs and 46-kDa DnaJ homologs (71 and 46 kDa) but particularly on the pattern of response observed here: (i) a typical transient response to stress and (ii) a response to different stressing conditions (see Fig. 8C for some examples). (i) All of them presented an immediate and strong transient response, with a time course pattern similar to that described for haloarchaeal heat shock proteins (reaching the maximum synthesis levels at about 1 h after the shift) (10). (ii) All of them showed to a higher or lower degree a response to both hypo- and hyperosmotic conditions in either the short-term or the long-term response stage. For example, the 85- and 79-kDa GSPs responded transiently to low-salt conditions while being accumulated at higher levels in the 20 to 30% salt medium than in the 10% salt medium as a long-term response (Fig. 8C). Therefore, all of these GSPs would respond to different stressing conditions.

In summary, we have distinguished different sets of proteins related to different osmotic conditions and showing typical patterns of response. First, the salt adaptation process was found to involve certain proteins with gradually higher synthesis levels for growth under increasing salinities (Fig. 8A). Second, low-salt adaptation involved strong responses and certain specific proteins (Fig. 8B). Finally, proteins with typical transient responses, elicited by either hypo- or hyperosmotic shock, in most if not all cases might correspond to GSPs (Fig. 8C).

From a general viewpoint, in *H. volcanii*, adaptation to low-salinity conditions triggered a wider, stronger, and more specific response in protein synthesis than adaptation to high-salt conditions. This is in agreement with the data obtained in previous work, in which we also detected a wider response and a much higher differential expression, at the transcription level, to low salinities in the *H. volcanii* genome (11).

General response of *H. volcanii* cells after variations in the salinity conditions. Despite the particular and strong response elicited by low salinity, which may perhaps reflect a difficult process of adaptation, *H. volcanii* cells were capable of an immediate recovery after a shift to low salinities. After a down-shift (from 30 to 10% salts), cells started growing rapidly (as seen by OD_{600} and total protein measurements [Fig. 9A]). General protein synthesis rates were also maintained after the down-shift, as seen by detection of labeling of proteins upon gel electrophoresis performed with similar quantities of total proteins (Fig. 10) and by direct measurement of incorporated labeled precursor (data not shown). Induction of particular low-salt-related proteins also occurred rapidly after the down-shift. Adaptation to low-salinity conditions thus appeared to be a fast process.

On the contrary, and as also happened with *Halomonas*, adaptation of *H. volcanii* cells to high salinities involved a much slower process. Growth was halted for a period of about 6 to 8 h after the up-shift (Fig. 9B). During this period, the general protein synthesis rates were very low (as shown in Fig. 10 for similar amounts of total proteins loaded). However, *H. volcanii* cells started synthesizing the high-salt proteins soon after the up-shift (as seen in gels with similar amounts of radiolabeled proteins [Fig. 5 and 6]). Therefore, as in *Halomonas*, the lag period in the adaptation to hyperosmotic conditions cannot be explained by a delay in the induction of the corresponding high-salt proteins.

In haloarchaea, the main compatible solute is potassium, the intracellular concentrations of which vary depending on the salinity of the medium (31). We have recorded intracellular potassium accumulation and observed that the lag period after the up-shift actually corresponds to the time needed for cells to accumulate the high intracellular potassium concentrations reached at the high-salt conditions (Fig. 9C). In the down-shift,

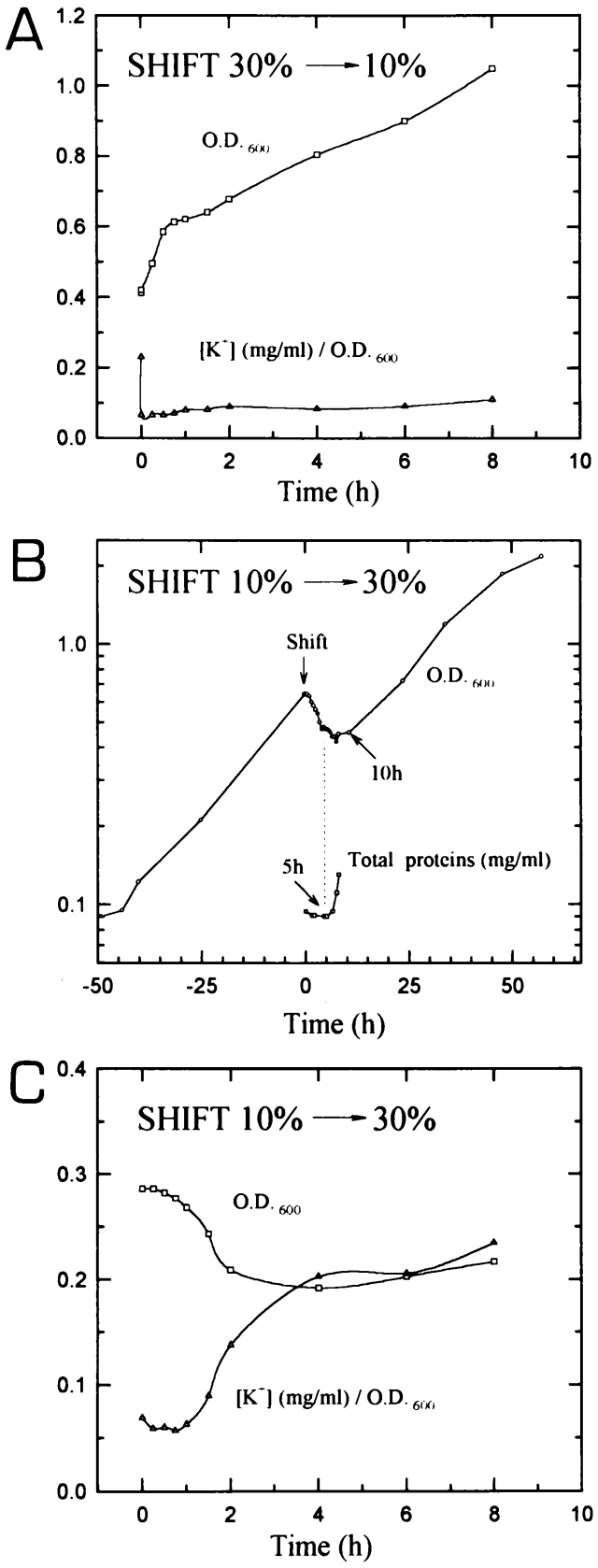

FIG. 9. Effects of variations of salinity on growth and intracellular potassium concentrations in *H. volcanii*. (A) Growth measurement and intracellular potassium concentrations of *H. volcanii* cells after a shift from 30 to 10% SW medium. Results for protein determinations (data not shown) were similar to those shown for OD_{600} measurements. (B) Effect of an increase of salinity (10 to 30% SW) on the growth of *H. volcanii* cells. (C) Growth and intracellular potassium concentrations of *H. volcanii* cells after a shift from 10 to 30% SW medium. Results for protein determinations (data not shown) were similar to those shown for OD_{600} measurements.

on the other hand, extrusion of potassium was immediate (Fig. 9A). We conclude, then, that reaching adequate compatible solute concentrations must be the main limiting factor for growth after the salinity variation.

Concluding remarks. The data obtained here may contribute to understanding the global response to different osmotic stress conditions in these representatives of halophilic prokaryotes. Further characterization of the proteins described could help to identify particular mechanisms of adaptation to hypo- versus hyperosmotic conditions. Particular sets of proteins possibly related to either high- or low-salt adaptation have been distinguished in *Halomonas* and *Haloferax* cells. Proteins whose synthesis rates were poor at low (near minimum) salinities while increasing notably at medium (near optimum) and high (near maximum) salinities can be considered possible candidates for studying mechanisms related to salt (or high-salt) adaptation. The fact that low-salt conditions had a dramatic effect in both organisms, with a sharp increase in synthesis of the low-salt-related proteins (some of them specific for low-salt adaptation), is remarkable. This sharp response to low salinities must be representative, given that the organisms are able to grow well at those low salt concentrations (growth rates in the low-salt conditions were similar to or even higher than those in the high-salt conditions assayed). Strong responses and also unique mechanisms may have evolved for low-salt adaptation, specially for haloarchaea, which are unable to grow below 8% salts and are specifically adapted for life in extreme hypersaline environments.

Upon osmotic stress, certain GSPs may play an important role. The general stress nature of responses to hypo- or hyperosmotic shock is particularly clear in the case of the GSPs described for *H. volcanii*. These GSPs show an immediate strong and transient response, are induced under both hypo- and hyperosmotic conditions (a fact which indicates a more general role of these proteins under stressing conditions), and could be related to haloarchaeal heat shock proteins or universal chaperones. Further studies on these general stress proteins would also be of interest to clarify the role of proteins conferring protection under different stress conditions or to investigate regulatory mechanisms in which different environmental factors may act as stimuli for expression of the same proteins. In this sense, our work reports a first indication of the possible haloarchaeal homolog (the 46-kDa GSP of *H. volcanii*) to the DnaJ-like chaperones. The description here of the 71- and 46-kDa GSPs from *H. volcanii* points not only to the universal presence of GSPs such as HSP70 and DnaJ homologs but also to a universal nature of their role in different organisms, as chaperones which might have an important function for proper protein folding under different (hypo- and hyperosmotic, heat) stressing conditions.

For both types of organisms, the recovery of cells after a hypoosmotic shock (a frequent natural event) was a fast process, while the reverse, adaptation to a salinity increase (a rather gradual event in nature), was a much slower process for the cells. This can be explained in terms of reaching the adequate concentration of compatible solutes. When cells are shifted from high to low salinity, the critical point would be that the cell envelope withstands the sudden increase in turgor

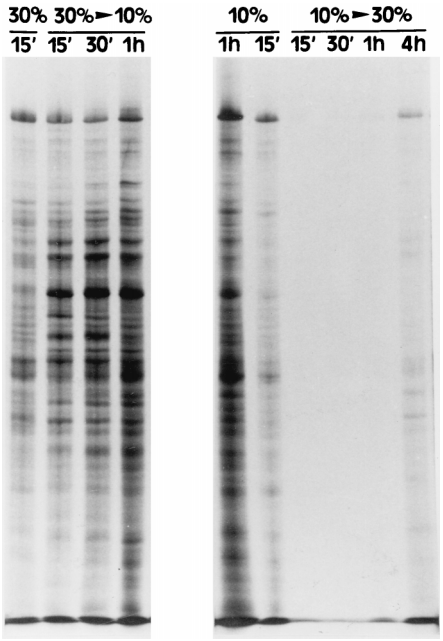

FIG. 10. Protein synthesis after a down-shift (30 to 10% SW medium) and an up-shift (10 to 30% SW medium) in *H. volcanii*. Similar quantities of total proteins were loaded in the gel for each sample, and electrophoresis was performed in SDS–10% polyacrylamide gels. For labeling of cellular proteins, cells grown in complex medium were suspended in 1% MAM containing the corresponding salt concentration and labeled with [^{35}S]methionine by incubation for the times indicated.

pressure. Once this point is overcome, reaching osmotic equilibrium would be an easy process. The release of compatible solute from cells, at least as shown in the case of *Haloferax*, is almost immediate, and in any case it is a thermodynamically favorable process. However, in the up-shift, due to the osmotic imbalance, the cell is probably dehydrated. The cell then must accumulate compatible solutes against a gradient (an energy-demanding process) until osmotic balance is reached. It appears reasonable that, as shown here, the efficiency of general protein synthesis would be affected during this lag period, although the particular high-salt-related proteins are induced rapidly. Reaching an adequate compatible solute concentration must be the main limiting factor for recovery of normal cell functions after the variation in salinity.

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