

Pressure-Induced Alterations in the Protein Pattern of the Thermophilic Archaeobacterium *Methanococcus thermolithotrophicus*

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Elevated hydrostatic pressure has been shown to affect the growth rate of the thermophilic methanobacterium *Methanococcus thermolithotrophicus* without extending its temperature range of viability. Analysis of the cell inventory after ≈ 10 h of incubation at 65°C and 50 MPa (applying high-pressure liquid chromatography and two-dimensional gel electrophoresis) proved that pressure induces alterations in the protein pattern and the amino acid composition of the total cell hydrolysate. Gels showed that after pressurization a series of (basic) proteins with a molecular mass in the range of 38 and 70 kilodaltons occurs which is not detectable in cells grown at normal atmospheric pressure. The question of whether the observed alterations are caused by the perturbation of the balance of protein synthesis and turnover or by the pressure-induced synthesis of compounds analogous to heat shock proteins remains unanswered.

Life on Earth is sensitive to environmental parameters, in that living systems are only able to maintain metabolic activity and viability within certain limits of temperature, pressure, water activity, pH, nutrient concentration, etc. The physiological state of organisms in their specific environment is tuned to a characteristic set of these parameters. Altering the conditions may cause physiological stress (10). This can be countered either by avoidance or by tolerance (5, 9).

Tolerance may be accomplished by mutative or regulatory adaptation, leading to altered protein structures or cellular components, such as antifreeze peptides or heat shock proteins. As indicated by temperature-sensitive mutants of microorganisms, single amino acid replacements in a single protein may be lethal. Thermotolerance requires the whole inventory of the cell to be adapted.

As suggested by the inhibitory effects of high pressure on the growth of nonadapted organisms, high hydrostatic pressure is expected to require similar adaptive efforts. Model studies on proteins from nonbarophilic organisms have shown that pressure is able to shift thermodynamic equilibria and to alter the rate of chemical reactions. Targets in this context are (i) protonation-deprotonation reactions, (ii) hydrophobic solvation, and (iii) dissociation-association (deactivation-reactivation) of oligomeric proteins (11, 12).

The key reaction of barosensitivity seems to be the pressure-induced disassembly of multimeric protein complexes, especially those involved in replication, transcription, and translation (22). From this, one can conclude that the stabilization of assembly structures of proteins is the basic mechanism of pressure adaptation.

The effect of pressure on the cell inventory of microorganisms has not been investigated so far. If the assembly structures involved in cell proliferation and protein translation were the only targets of high pressure, quantitative shifts in the protein pattern (proportional to the inhibitory effect on cell growth) should occur. Qualitative alterations in the ratio of proteins would indicate alterations in the balance of protein synthesis and turnover or induction of compounds analogous to heat shock proteins.

In the present study we investigated pressure-induced alterations of the protein pattern of the thermophilic archaeobacterium *Methanococcus thermolithotrophicus* (6). Previous experiments with this organism were designed to answer the question of whether high hydrostatic pressure has an effect on the range of viability of a thermophilic bacterium. As was found, pressures up to 50 MPa enhance the growth rate without shifting the optimum temperature of growth to higher values. Beyond 50 MPa, cell lysis was found to predominate (4). From results of these experiments, it has become clear that pressure affects cell growth in a complex way. Whether it does so at the DNA or protein level or whether membrane components are the key target has not been clarified. The results of this study indicate that high pressure causes qualitative alterations in the protein pattern analogous to the occurrence of heat shock proteins at elevated temperatures.

MATERIALS AND METHODS

M. thermolithotrophicus, a thermophilic lithotrophic methanogen, does not grow above 72°C. It grows with a doubling time of 60 min in shakers at 65°C, which is the optimal temperature (6). Inoculation of bacterial suspensions into 20 ml of medium was performed at 65°C in serum flasks containing a gas mixture of 80% H₂ and 20% CO₂ at 0.2 MPa (6). In order to accomplish constant pH at various temperatures and pressures (2), the standard medium (MG⁻) was supplemented with 0.12 M HEPES (*N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid).

MG⁻ medium consisted of 0.34 g of KCl, 4.30 g of MgCl₂ · 6H₂O, 3.46 g of MgSO₄ · 7H₂O, 0.26 g of NH₄Cl, 0.14 g of CaCl₂ · 2H₂O, 0.14 g of K₂HPO₄, 18.0 g of NaCl, 5.0 g of NaHCO₃, 10 ml of mineral salts plus 10 ml of vitamins as described by Balch et al. (1), 1.0 ml of Fe(NH₄)₂(SO₄)₂ (0.2%), 1.0 ml of resazurin (0.1%), 0.50 g of Na₂S · 9H₂O, 0.5 g of cysteine hydrochloride, 1.0 g of sodium acetate, and water to 1,000 ml.

The pH of the medium was adjusted to pH 6.9 before autoclaving and gas exchange at room temperature (cf. reference 3).

Substances (A-grade purity) were purchased from Sigma (Heidelberg, Federal Republic of Germany), Fluka (Basel,

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Switzerland), E. Merck AG (Darmstadt, Federal Republic of Germany), Roth (Karlsruhe, Federal Republic of Germany), and Zinsser Analytic (Frankfurt am Main, Federal Republic of Germany). [^{14}C]NaHCO₃ was obtained from Amersham (Braunschweig, Federal Republic of Germany), Ampholine (pH 3.5 to 10 and pH 5 to 7) from LKB (Munich, Federal Republic of Germany), DNase I (bovine pancreas) from Boehringer GmbH (Mannheim, Federal Republic of Germany), and molecular weight marker proteins (type high and low) and RNase A (bovine pancreas) from Sigma.

High-pressure autoclaves, as well as equipment for pressurization and pressure-temperature-pH control, were used as described previously (2, 3, 18, 21). In order to quantify bacterial growth at high temperature and high pressure, cell counting was performed in a Neubauer chamber with a 0.0025-mm² area and a 0.02-mm depth (phase-contrast microscope [Zeiss]).

Amino acid analyses were performed after derivatization with *o*-phthalaldehyde (OPA) by high-pressure liquid chromatography (HPLC) (Spectra Physics SP 8700 solvent delivery system with He degassing device and Rheodyne injection valve); separation on a Polyol-Si-100-RP-18.5- μm column (4.6 by 250 mm; flow rate, 2 ml/min; Serva, Heidelberg, Federal Republic of Germany). The detector was a filter fluorometer (Spectra Glow; Gilson) ($\lambda_{\text{exc}} = 360 \text{ nm}$, $\lambda_{\text{em}} = 455 \text{ nm}$); a Spectra Physics SP 4270 integrator was used.

Cell suspensions (3 ml) were centrifuged at $11,200 \times g$ for 15 min. The pellet was hydrolyzed with 50 μl of 5 N HCl at $\approx 110^\circ\text{C}$ for 24 h under reduced pressure. After evaporation of HCl, the hydrolysate was redissolved in 200 μl of H₂O and filtered (Millipore Corp., Bedford, Mass.).

In order to provide base-line separation of the peaks, the procedure described by Larsen and West (14) was modified as follows. Mercaptoethanol was substituted by ethanethiol. Samples of 20 μl were mixed with 40 μl of OPA reagent for 20 s; after 1 min, 20 μl of the OPA derivatives was injected. Solvents were as follows: A, 0.01875 M triethylamine acetic acid (pH 7.5); B, acetonitrile. Solvents A and B were present in the following ratios in the gradient: 0 min, 100% A; 38 min, 62% A and 38% B; 48 min, 30% A and 70% B; 48.1 min, 100% B. Sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis was performed as described by Laemmli (13).

Scanning of the gels was done with a laser densitometer (Ultrascan; LKB, Uppsala, Sweden). Two-dimensional gel electrophoresis was performed as described by O'Farrell and co-workers (19, 20). Since *M. thermolithotrophicus* does not grow on acetate or cysteine, radioactive labeling was done with NaH¹⁴CO₃. NaH¹⁴CO₃ (3.7 to 5.7 MBq) was added to 20 to 40 ml of MG⁻ medium containing 0.12 M HEPES plus 2 g of NaH¹²CO₃. Cells were grown in nickel tubes at 65°C without additional CO₂; the gas atmosphere contained only 0.2 to 0.4 MPa of H₂. The incubation time was 10 to 14 h.

Depending on the cell density, 10 to 40 ml of suspension (0.2 to 0.8 optical density units) was centrifuged (20 min, $12,000 \times g$) and suspended in 200 μl of sonication buffer (Tris hydrochloride plus MgCl₂ [pH 7.4]). After sonication (step 3) (type G 15 sonifier; Branson Sonic Power Co., Danbury, Conn.), the solution was incubated with 10 μl of RNase A-DNase I (1 mg/ml; 1:1) at room temperature for 20 min. After the addition of 190 mg of recrystallized urea, lysis buffer (5.4 g of urea, 2 ml of Nonidet P-40 [10%], 0.5 ml of β -mercaptoethanol, 0.4 ml of Ampholine [pH 5 to 7], 0.1 ml of Ampholine [pH 3.5 to 10], 2.4 ml of H₂O) was added (1:1). Isoelectric focusing of samples of 10 to 15 μl in tubes (inner

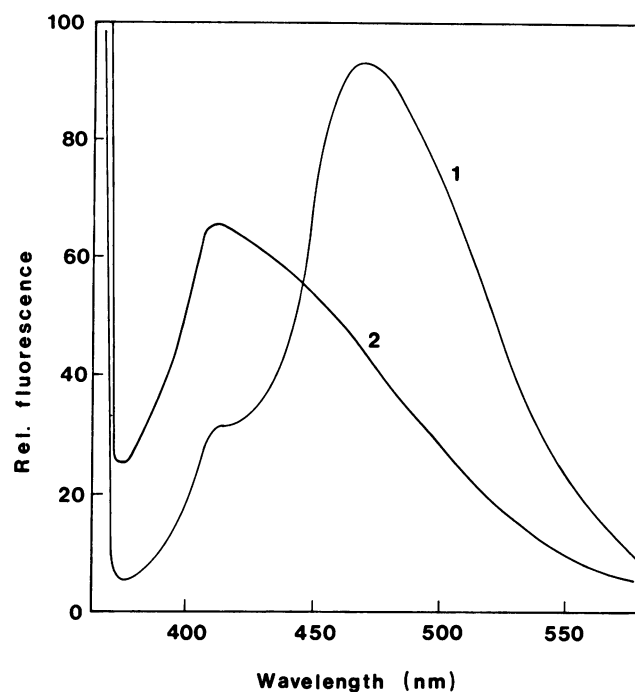


FIG. 1. Fluorescence emission spectra of the products of acid hydrolysis of nonpressurized (curve 1) and pressurized (curve 2) *M. thermolithotrophicus* cells. Cells were harvested after 24 h of incubation at 65°C, hydrolyzed in 6 N HCl, and subsequently dissolved in H₂O. Excitation wavelength (λ_{exc}) = 360 nm.

diameter, 1.5 mm; length, 150 mm) was performed in two subsequent steps: (i) 15 h at 400 V and (ii) 1 h at 800 V.

After the gels were transferred into test tubes and dialyzed for 30 min against SDS sample buffer (20 g of glycerol, 4.6 g of SDS, 1.51 g of Tris [titrated with HCl to a final pH of 6.8], 10 ml of β -mercaptoethanol, 200 μl of bromophenol blue [0.5%] in 200 ml of H₂O), the gels were placed on SDS-polyacrylamide gels and subjected to a second electrophoresis (7 W per gel). In order to allow the patterns obtained at various pressures to be compared, two gels were applied to each SDS-polyacrylamide gel electrophoresis in a tandem array, i.e., under identical conditions.

After silver staining, gels were treated with dimethyl sulfoxide containing 20% 1,4-di-[2-(5-phenyloxazolyl)]-benzene (POPOP) in order to reduce the time required for autoradiography. Autoradiography at -84°C over a period of 2 to 30 days was done with X-ray film (13 by 18 cm; Blue Base; Eastman Kodak Co., Rochester, N.Y.).

RESULTS AND DISCUSSION

Fluorescence emission spectroscopy was applied to monitor alterations in the cell inventory of *M. thermolithotrophicus* (Fig. 1). The emission spectra were compared after acid hydrolysis of cells that were incubated at normal atmospheric pressure and 50 MPa (24 h, 65°C), and a significant blue shift was observed. As determined by K. O. Stetter (unpublished data), the broad band at 380 to 440 nm cannot be attributed to factor F_{420} .

Drastic changes in the amino acid composition were indicated by HPLC analysis before and after pressurization (Fig. 2). Shifts refer mainly to those of Glu, Ser, and Arg. An extra peak was observed close to the retention volume of the acidic amino acids. Because of the short half-lives of the

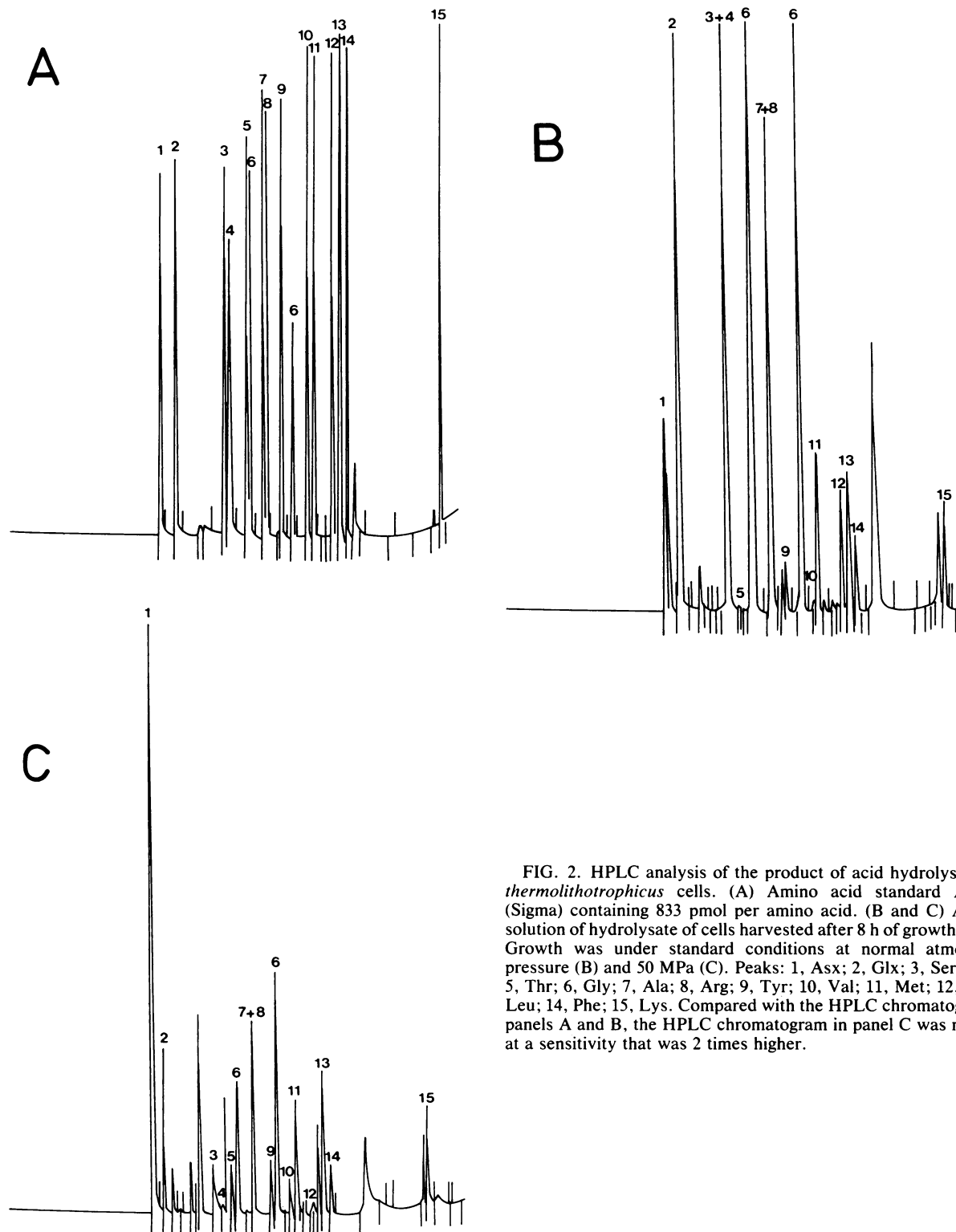


FIG. 2. HPLC analysis of the product of acid hydrolysis of *M. thermolithotropicus* cells. (A) Amino acid standard AA-S-18 (Sigma) containing 833 pmol per amino acid. (B and C) Aqueous solution of hydrolysate of cells harvested after 8 h of growth at 65°C. Growth was under standard conditions at normal atmospheric pressure (B) and 50 MPa (C). Peaks: 1, Asx; 2, Glx; 3, Ser; 4, His; 5, Thr; 6, Gly; 7, Ala; 8, Arg; 9, Tyr; 10, Val; 11, Met; 12, Ile; 13, Leu; 14, Phe; 15, Lys. Compared with the HPLC chromatograms in panels A and B, the HPLC chromatogram in panel C was recorded at a sensitivity that was 2 times higher.

decay products of phthalaldehyde derivatives of amines and amino acids, the functional group of the isoindole moiety was inaccessible to gas chromatography-mass spectrometry analysis (8).

In order to monitor the protein pattern of *M. thermolithotropicus* at various pressures, two-dimensional gel electro-

phoresis was applied. Since radioactive labeling could not be accomplished by growing the bacteria on [¹⁴C]acetate, [¹⁴C]formate, [³⁵S]sulfate, or [³⁵S]cysteine, [¹⁴C]sodium bicarbonate was used as the carbon source. Under optimum growth conditions, the medium contained ~150 mM CO₂ (~60 mM HCO₃⁻ plus ~90 mM CO₂); in order to provide

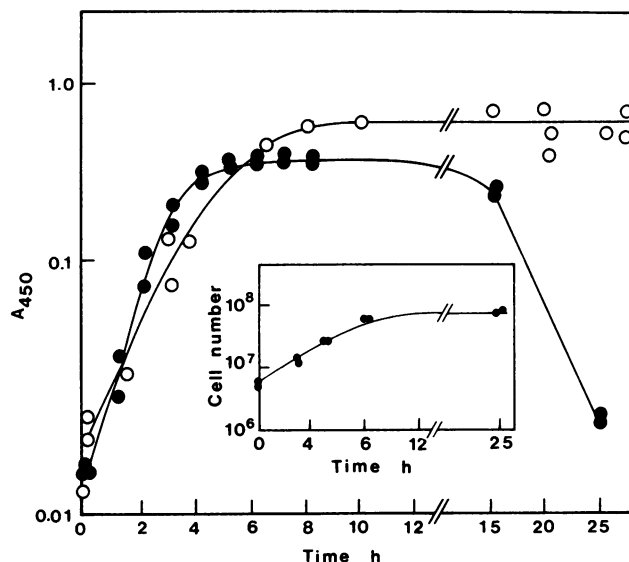


FIG. 3. Dependence of the growth of *M. thermolithotrophicus* on the concentration of NaHCO_3 at 0.2 MPa of H_2 and 65°C . NaHCO_3 was included at concentrations of 2 (●) and 5 (○) g/liter and at atmospheric pressure. (Inset) Growth curve of *M. thermolithotrophicus* in NaHCO_3 -poor medium (2 g/liter) at 50 MPa.

optimum labeling, the carbon source was restricted such that the cell density was just sufficient for determining the protein pattern after 3 to 30 days of exposure. *M. thermolithotrophicus* was grown under an H_2 atmosphere in the presence of HCO_3^- as the only carbon source (Fig. 3). Cell growth and HCO_3^- consumption were found to parallel each other. Optimum labeling (providing sufficient contrast and high resolution on gels described by O'Farrell and co-workers [19, 20]) was accomplished with 24 mM NaHCO_3 by applying a specific radioactivity of ~ 250 kBq/ml. In these experiments, proper osmotic conditions were maintained by adding 120 mM NaCl.

Comparison of extracts from cells harvested at various times during the logarithmic and stationary phases of growth

indicated that the protein patterns obtained after SDS-polyacrylamide gel electrophoresis and silver staining did not exhibit significant changes (densitograms not documented).

Under the conditions mentioned above (280 to 379 kBq/ml), 15 to 20% of the radioactive carbon was incorporated into the cells, independent of the pressure applied to the system.

Autoradiographic evaluation of one-dimensional SDS-polyacrylamide gels shows that the gel patterns were significantly changed on pressurization (Fig. 4). Alterations were mainly to proteins in the 40-kilodalton range. Since cells were harvested under optimum growth conditions (65°C , 50 MPa), artifacts caused by proteolytic or hydrothermal degradation could be excluded. Quantitative differences in the total protein concentration and the distribution of the various peaks also indicate that there were real pressure effects on protein expression or protein turnover.

This conclusion was confirmed on two-dimensional gels, as described by O'Farrell and co-workers (19, 20) (Fig. 5). From a comparison of the protein patterns obtained at 65°C in the stationary phase, high pressure was found to cause dramatic changes, preferentially in the range of basic proteins with molecular masses of 38 and 70 kilodaltons. This result corroborates the shift in the amino acid composition toward higher basicity mentioned above.

The overall characteristics of the two-dimensional patterns at normal atmospheric pressure and 50 MPa were closely related. Similarly, no differences in the morphological or metabolic properties of the bacterium under the two sets of conditions were detected. Therefore, the observed differences in the protein patterns cannot be attributed to different organisms. Rather, it must be concluded that elevated pressure induces alterations in the normal cell inventory, similar to the occurrence of heat shock proteins that have been observed at elevated temperatures (7, 15–17, 23, 24).

Conclusions. It is well established that elevated pressure affects the growth of microorganisms that are not adapted to high pressure (23). So far it has not been possible to attribute this overall effect to altered levels of specific cellular components. The reason is that high hydrostatic pressure may

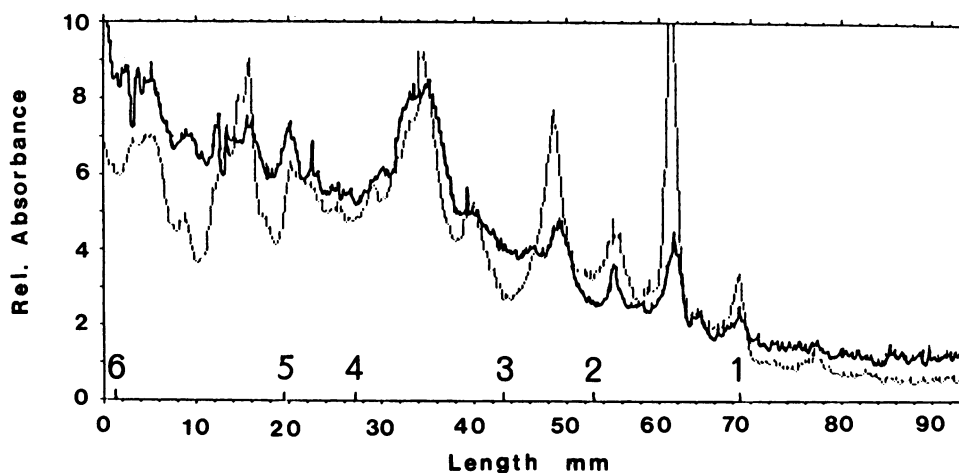


FIG. 4. Densitogram of ^{14}C -labeled extracts of *M. thermolithotrophicus*. Cell extracts were dialyzed for 15 h against sample buffer prior to SDS-polyacrylamide gel electrophoresis. Results for nonpressurized (thin lines) and pressurized (50 MPa) (heavy lines) cells are shown at the stationary state after 10 h at 65°C . Marker proteins (molecular weights) were as follows: 1, phosphorylase *b* (92,000); 2, bovine serum albumin (66,200); 3, ovalbumin (45,000); 4, carboanhydrase (31,000); 5, soybean trypsin inhibitor (21,500); 6, lysozyme (14,500).

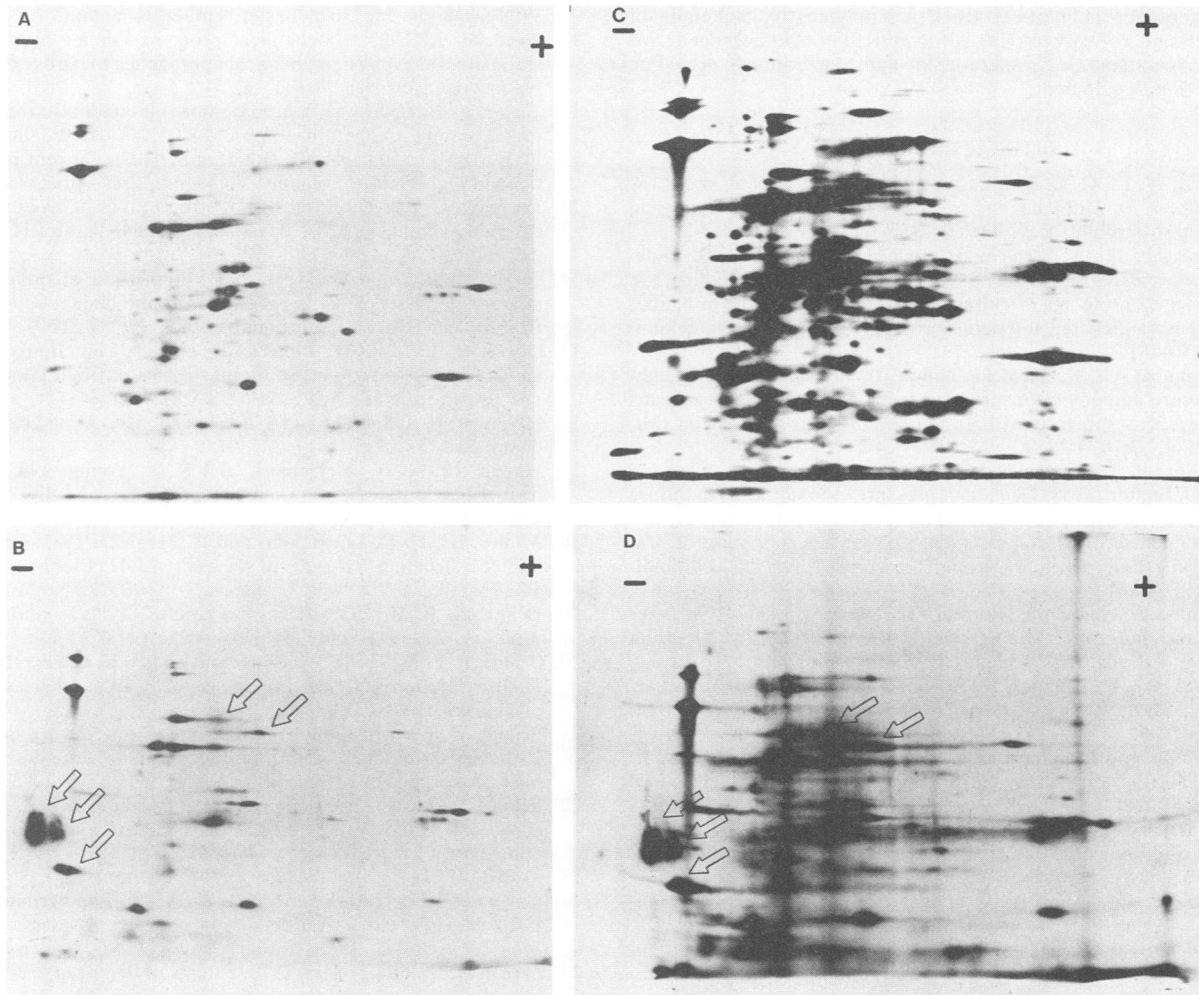


FIG. 5. Autoradiographic analysis of the two-dimensional gels described by O'Farrell and co-workers (19, 20) of nonpressurized (A and C) and pressurized (B and D) cultures of *M. thermolithotrophicus* after 10 h of incubation at 65°C. Growth conditions were as follows: NaHO₃-poor medium (2 g/liter) with 0.4 MPa of H₂ as the gas phase. (A and C) Normal atmospheric pressure, 1.86-kBq extract, after 4 days (A) and 14 days (C) of exposure; (B and D) 50 MPa, 0.68-kBq extract, after 14 days (B) and 30 days (D) of exposure.

affect the equilibrium and kinetic properties of a wide variety of cellular processes because of its effect on intermolecular interactions involving macromolecules and their relevant ligands (11, 12). Known reaction volumes (ΔV) and activation volumes (ΔV^\ddagger) suggest that the fundamental reactions connected with DNA replication and protein biosynthesis are the most sensitive targets for the action of pressure (9). Evidence gained from the experiments described here indicates that the inhibitory effect of high hydrostatic pressure on the growth of bacteria is reflected by shifts in the protein pattern of pressurized cells compared with that of the cell inventory at normal atmospheric pressure. There was not only a quantitative shift, in the sense that decreased cell proliferation was directly related to a decrease in overall protein translation. In addition, there were qualitative alterations in the relative amounts of proteins that included the expression of additional amounts of proteins that were not detectable in nonpressurized cells. Their chemical nature was reflected by a shift in the amino acid composition, as determined by comparing the total hydrolysate of pressurized cells with that of nonpressurized cells. Amines with an unknown chemical nature were observed apart from a significant increase in basic amino acids (Fig. 2). Since the protein

pattern remained unchanged over the whole period of bacterial proliferation, changes in the growth rate could not have been responsible for the observed effects. Indirect pressure effects, for example, increased substrate concentration or shifts of pH, were equally negligible because of the high buffer capacity of the growth medium and the high solubility of CO₂ and H₂ under the given experimental conditions (2). These conditions are crucial as far as temperature is concerned. At 65°C (50 MPa), enhanced bacterial growth was accompanied by changes in the protein pattern; on the other hand, lower and higher temperatures (56 and 75°C, 50 MPa) led to growth inhibition and alterations in the stability and morphology of the cells. These observations illustrate the complex mechanisms underlying the pressure-induced metabolic dislocation. Whether the *de novo* synthesis of specific pressure-induced shock proteins shows any analogy to the ubiquitous expression of heat shock proteins and how these proteins may affect the network of metabolic reactions require further investigation.

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