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Induction of Heat Shock Proteins DnaK, GroEL, and GroES by Salt Stress in *Lactococcus lactis*

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The bacterium *Lactococcus lactis* has become a model organism in studies of growth physiology and membrane transport, as a result of its simple fermentative metabolism. It is also used as a model for studying the importance of specific genes and functions during life in excess nutrients, by comparison of prototrophic wild-type strains and auxotrophic domesticated (dairy) strains. In a study of the capacity of domesticated strains to perform directed responses toward various stress conditions, we have analyzed the heat and salt stress response in the established *L. lactis* subsp. *cremoris* laboratory strain MG1363, which was originally derived from a dairy strain. After two-dimensional separation of proteins, the DnaK, GroEL, and GroES heat shock proteins, the HrcA (Orf1) heat shock repressor, and the glycolytic enzymes pyruvate kinase, glyceraldehyde-3-phosphate dehydrogenase, and phosphoglycerate kinase were identified by a combination of Western blotting and direct N-terminal amino acid sequencing of proteins from the gels. Of 400 to 500 visible proteins, 17 were induced more than twofold during heat stress. Two classes of heat stress proteins were identified from their temporal induction pattern. The fast-induced proteins (including DnaK) showed an abruptly increased rate of synthesis during the first 10 min, declining to intermediate levels after 15 min. GroEL and GroES, which also belong to this group, maintained a high rate of synthesis after 15 min. The class of slowly induced proteins exhibited a gradual increase in the rate of synthesis after the onset of stress. Unlike other organisms, all salt stress-induced proteins in *L. lactis* were also subjected to heat stress induction. DnaK, GroEL, and GroES showed similar temporal patterns of induction during salt stress, resembling the timing during heat stress although at a lower induction level. These data indicate an overlap between the heat shock and salt stress responses in *L. lactis*.

The rapidly increasing volume of data concerning the physiology and genetics of the gram-positive bacterium *Lactococcus lactis* is partly due to the importance of this organism in dairy fermentations. Aside from this, the bacterium is beginning to gain an autonomous status as a model organism in its own right, for several reasons. Primarily, it has a very simple type of metabolism and obtains most of its energy from lactic acid fermentation, which makes energy calculations fairly uncomplicated (3, 22). *L. lactis* is also very well characterized with respect to transport proteins and the energetics of translocation of the various sugars and amino acids (28). Of no less importance is that we know of both free-living prototrophic strains (15) and dairy strains which have been living in milk in an excess of sugar, vitamins, and amino acids for hundreds, even thousands of years. As a consequence of their plentiful life, the latter strains have aquired multiple auxotrophic phenotypes by the accumulation of a large number of point mutations in the genes for biosynthetic operons, such as the operons for the branched-chained amino acids and the amino acid histidine (10, 15). The comparison of wild-type and dairy strains of *L. lactis* can thus function as a model for studying the importance of specific genes and functions during life in an excess of nutrients.

We have initiated a study of the capacity of the *L. lactis* MG1363 to carry out directed responses to various stress conditions. MG1363 is a plasmid- and prophage-cured derivative of a dairy strain. We wanted to know whether the different global regulatory circuits, which are believed to be important during life in changing environments (26), are also important for growth in excess of nutrients and have been conserved in dairy strains. Initially, we have focused upon the responses to heat stress and salt stress, which are often applied to lactic acid bacteria during cheese manufacture. Elevated temperatures are applied to accelerate whey expulsion, and elevated salt concentrations are used for hardening of the cheese surface. The bacteria do not, however, encounter these stress conditions at a regular basis, since life in the cheese ecosystem is a dead end, with most or all bacteria being killed by autolysis after a few days. Lactic acid bacteria used for starter cultures are cultivated by repeated dilutions in complex media under more favorable conditions. The starter bacteria in dairy productions are likely to have been subjected to periods of acid stress and mild sugar starvation in their history but not to heat and salt stress.

Most organisms respond to heat shock by synthesizing a conserved set of proteins, including the DnaK-DnaJ-GrpE and GroEL-GroES chaperone complexes (38). Heat shock proteins are generally involved in the maturation of newly synthesized proteins, and they assist in the refolding or degradation of denatured proteins (14, 16). Accordingly, it is believed that their importance during heat stress is in rescuing or scavenging heat-denatured proteins. The heat shock response has been studied in most detail in *Escherichia coli* and *Bacillus subtilis*. In *L. lactis*, heat shock appears to elicit a similar response to that in other bacteria. It has been shown that proteins which cross-

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react with antibodies against DnaK, DnaJ, GrpE, and GroEL accumulate upon heat shock (2, 11, 35), and experiments with radioactive labelling of proteins during growth in enriched broth (M17) tentatively showed that the synthesis of at least 13 proteins is induced under heat shock conditions (35).

Salt stress induces a different set of proteins, which are mostly involved in the accumulation of osmoprotective substances (8, 9). Studies mostly with gram-negative bacteria have shown that the events following a hyperosmotic shock involve shrinkage of the cell (plasmolysis) followed by rapid influx of K^+ to balance the change in osmotic pressure (8, 9). This reversal of the normal size of the cell is termed deplasmolysis. The accumulated potassium is then exchanged by osmoprotectants such as proline or glycine betaine. Both the K^+ transporter and the proteins involved in the accumulation of osmoprotectants are among the salt-induced proteins (9).

In older reports on the salt shock proteins from *E. coli* and *B. subtilis*, it was claimed that neither of the heat shock proteins, DnaK or GroEL, was induced by the addition of salt to the growth medium of the bacteria (7, 17, 18, 33). Recently, however, it was found that DnaK is crucial for deplasmolysis in *E. coli* and that the protein is induced during the first phase of the salt shock response (24). The salt shock response of *L. lactis* has never been investigated in detail, but it has been found that proline and betaine transport is elevated during salt stress (25).

To analyze the induction of individual proteins in *L. lactis*, we have developed experimental conditions for [^{35}S]methionine labelling of polypeptides during growth in a chemically defined (SA) medium under standard and stress conditions. We have also developed a new procedure for the extraction of proteins and have used high-resolution separation of proteins by two-dimensional polyacrylamide gel electrophoresis (2-D PAGE). These conditions enabled us to obtain individual synthesis rates for a large number of separated proteins under both standard and stressed conditions.

We have analyzed the temporal changes in the synthesis rates for various proteins during heat and salt stress. Seventeen proteins showed a marked induction during heat stress. Eight proteins showed a rapid increase in the synthesis rate within the first 10 min after heat stress, to more than 10-fold-elevated levels, followed by a decrease to intermediate levels after 15 min. Interestingly, five of these proteins showed the same pattern of induction after entering salt shock, although at a lower level of induction. To identify the position of the DnaK, GroEL, and GroES chaperone proteins on the 2-D gels, we used Western blotting and direct N-terminal amino acid sequencing of separated proteins from the gels. The combined results revealed that DnaK, GroEL, and GroES are among the proteins which are induced under both heat and salt stress.

MATERIALS AND METHODS

Bacterial strains and growth conditions. *L. lactis* subsp. *cremoris* MG1363 (13) was used in the present study. In all experiments, MG1363 was grown in the morpholinepropanesulfonic acid (MOPS)-based chemically defined SA medium, which contains all necessary vitamins and amino acids (21), supplemented with glucose at 1% (GSA medium). GSA medium with a low methionine concentration (5 μg/ml; LM-GSA medium) was used for [^{35}S]methionine labelling of polypeptides. Cultures were grown in filled 50-ml flasks with slow magnetic stirring to prevent precipitation of the cells without extensive aeration of the culture.

[^{35}S]methionine labelling of polypeptides. The cells were grown exponentially at 30°C in LM-GSA medium to an optical density at 450 nm (OD_{450}) of 0.4 (about 4×10^8 cells per ml). Heat stress was applied by transferring the flask to 43°C, and salt stress was applied by the addition of prewarmed medium, containing 20% NaCl, to a final concentration of 2.5% NaCl. At various time points, 150 μl of culture was transferred to an Eppendorf tube containing 15 μCi (3 μl) of [^{35}S]methionine. After 10 min, unlabelled methionine was added at a final concentration of 0.8 mg/ml to complete the synthesis of labelled proteins. After 2 min of incubation, 10 μl of chloramphenicol (20 mg/ml) was added, and the cells were pelleted by centrifugation at 20,000 × *g* for 5 min. The cells were resuspended in 200 μl of an ice-cold solution of 0.9% NaCl containing 30% ethanol and repelleted. The added ethanol resulted in better adherence of cells to the centrifuge tubes. The pellet was stored at −80°C.

When the 150-μl sample at 0 min was transferred to 43°C in the presence of [^{35}S]methionine, the temperature shift occurred instantaneously, whereas the 50-ml culture required about 5 min for full temperature equilibration. Shortly after the shift to 43°C, before full temperature equilibration of the culture, the strain grew at a higher growth rate than at 30°C (see Fig. 1A), reflecting that the optimal growth temperature is above 30°C (unpublished data). After equilibration of the culture at 43°C, the growth slowed considerably, with a doubling time of 190 min.

2-D PAGE of labelled proteins. Frozen pellets were freeze-dried in a vacuum centrifuge, and cells were broken by grinding with a small number of acid-washed glass beads (150 μm) on the tip of a melted Pasteur pipette. A 20-μl volume of sample buffer (50 mM Tris/HCl, 0.3% sodium dodecyl sulfate [SDS], 0.6 M β-mercaptoethanol) was added, and the proteins were solubilized by boiling for 2 min followed by standing for 5 min at room temperature. The sample was chilled on ice, 30 U of nuclease (Benzonase; a gift from the Biotechnology Institute of Denmark) plus 1 μl of 300 mM $MgCl_2$ was added, and the sample was incubated on ice for 15 min. An 80-μl volume of ampholyte solution (54% urea, 240 mM β-mercaptoethanol, 2% Pharmalyte pH 3-10 [Pharmacia], 0.5% Triton X-100) was added. A 25-μl volume of this solution (one-quarter of the total extract) was analyzed by the Pharmacia 2-D electrophoresis system, as recommended by the supplier, with 11-cm Immobiline dry strips (pH 4 to 7) and ExelGel XL SDS 12 to 14% precast gels. The protein sample was applied to the alkaline end of the Immobiline strip. After SDS-PAGE (the second dimension), the plastic-supported gels were fixed in 10% acetic acid–40% ethanol for 10 min and then washed in 10% glycerol–40% ethanol for 10 min. The gels were placed on a glass plate and coated with a sheet of household cellophane, presoaked in glycerol-ethanol solution. The gels were dried at room temperature overnight, and the radioactivity distribution was monitored with a Packard Instant Imager or by autoradiography with X-ray films (Kodak X-Omat AR or AGFA Curix).

Western blot of 2-D gels. After SDS-gel electrophoresis, the gel was cut from its plastic support and the proteins were transferred to polyvinylidene difluoride (PVDF) membrane (Hybond PVDF; Amersham) by semidry electroblotting by the method of Ploug et al. (27), with 10 mM 3-(cyclohexylamino)propanesulfonic acid (CAPS; pH 11)–10% methanol as the transfer buffer. Chemiluminescence detection of heat shock proteins, with rabbit antibodies raised against the homologous proteins from *E. coli*, *B. subtilis*, or *B. stearothermophilus* and horseradish peroxidase-conjugated secondary antibodies, was performed with the Amersham enhanced chemiluminescence Western blotting analysis system. By stripping and reprobing, as recommended by the manufacturer, multiple rounds of detection were performed on the same membrane. Exposure times were usually around 15 s on AGFA Curix X-ray film.

N-terminal amino acid sequence determination. Cells from a 250-ml culture of MG1363 at an OD_{450} of 1, grown under the desired conditions, were concentrated by centrifugation and resuspended in 1 ml of Tris/HCl (pH 8.0). The cell paste was passed through a French pressure cell at 28,000 lb/in^2 and centrifuged at 20,000 × *g* for 10 min. A 340-μl volume of supernatant was added to 40 μl of a solution containing 0.5 M Tris/HCl (pH 8.0) and 3% SDS. Then 20 μl of β-mercaptoethanol was added, and the sample was boiled for 2 min. A 10-μl volume of a 300 mM $MgCl_2$ solution and 300 U of Benzonase were added, and the sample was digested for 15 min on ice. After the addition of 540 mg of ultrapure urea (Millipore), 20 μl of β-mercaptoethanol, 20 μl of Pharmalyte ampholyte pH 3 to 10 solution (Pharmacia), and 21 μl of 25% Triton X-100, the solution was brought up to 1 ml with water. Up to 25 μl of this sample was used in 2-D PAGE separation and was transferred to a PVDF membrane as described above. The PVDF membrane containing separated proteins was stained by the method of to Ploug et al. (27) for 1 min with Coomassie brilliant blue R-250 (0.1% in water containing 50% methanol [high-pressure liquid chromatography grade]), destained for 5 to 10 min in a solution of 40% methanol (high-pressure liquid chromatography grade) plus 10% acidic acid in water, and washed in ultrapure water. Stained protein spots were cut from the membrane and applied directly to a Procise-494 Sequencer (Applied Biosystems), as described by the manufacturer.

RESULTS

Conditions for growth, stress, and [^{35}S]methionine labelling of MG1363 in GSA medium. Since the multiple auxotrophic *L. lactis* subsp. *cremoris* MG1363 also has a requirement for methionine, it was important for the [^{35}S]methionine labelling to determine the lowest concentration of methionine which could be used without inducing starvation. The normal growth of MG1363 in GSA medium shows an exponential phase with a doubling time of approximately 55 min. When the cell density reaches an OD_{450} of 1.8, growth then enters sta-

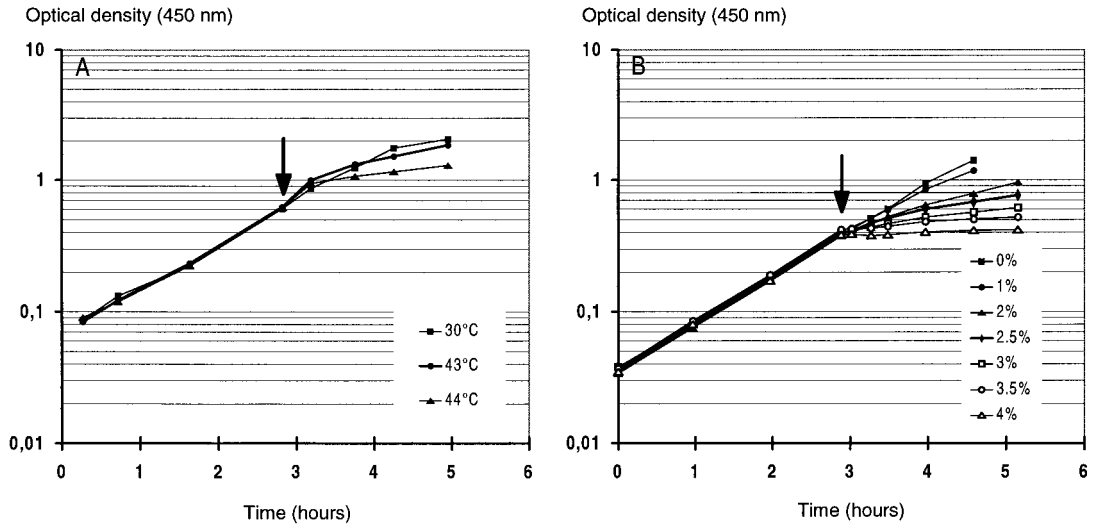

FIG. 1. Growth conditions. *L. lactis* MG1363 was grown in GSA medium at 30°C, in filled 50-ml flasks with slow magnetic stirring. Growth was monitored by measuring the OD_{450} of the culture. (A) Effect on growth of shifts to high temperatures. Cells were heat stressed at an OD_{450} of 0.6 by transferring the flask to a 42 or 43°C water bath. (B) Effect on growth of shifts to higher salt concentrations. Cells were salt stressed at an OD_{450} of 0.4 by slowly adding preheated medium containing 25% NaCl to the indicated final concentrations.

tionary phase (see Fig. 1A, 30°C). The stationary phase is presumably a result of the lowered pH of the medium, since the exponential phase can be prolonged by increasing the buffer capacity of the medium (21). To avoid oxygen toxicity (11), we routinely cultured the bacteria in filled 50-ml Erlenmeyer flasks, with slow magnetic stirring to ensure homogeneity of the culture. The growth rate of the cultures, with starting concentrations of methionine below 5 μg/ml, was constant with a doubling time of about 55 min, until the cells suddenly stopped growing, due to methionine depletion (data not shown). Above 5 μg of methionine per ml, no shortening of the exponential phase was seen, but the final yield was dependent on the methionine concentration at concentrations up to at least 7.5 μg/ml (data not shown). To avoid entering methionine starvation, all labelling experiments were performed at a starting concentration of 5 μg/ml, and labelling was performed at cell densities below $OD_{450} = 0.8$, well below the end of the exponential growth phase in this particular medium ($OD_{450} = 1.8$).

When cells are subjected to a severe stress, they usually shut off most metabolic activity and commit themselves to adaptive strategies. Consequently, they enter a physiological state with very little protein synthesis, very different from the normal growth physiology. To obtain a less severe depression of the physiology, we wanted to apply a stress level which would result in growth rates corresponding to 25 to 50% of the unstressed rate. In a series of growth experiments with strain MG1363, we determined that a reduction in the growth rate of this magnitude required a temperature shift from 30 to 43°C for temperature stress (Fig. 1A) and the addition of NaCl to 2.5% for osmotic stress (Fig. 1B). These conditions were chosen as standard stress conditions. When the stressed cultures were diluted and grown under the same conditions, growth stopped 3 to 4 h after the onset of stress (data not shown); therefore, neither of the stress conditions are mild enough to allow for continued exponential growth.

Reference map for lactococcal proteins. The standard procedure for the extraction of lactococcal proteins involves prolonged lysozyme treatment, beating with glass beads for 15 min, and then boiling for an additional 10 min, due to the extreme lysozyme resistance of the cells (35). In this study, we used a simple extraction procedure by freezing the cell pellets at −80°C after a wash in cold salt water. The frozen cell pellet was then freeze-dried in a vacuum centrifuge, where the evaporation kept the pellet frozen until it was thoroughly dried. The cell envelopes were broken by grinding the dried pellet with glass beads, and the proteins were solubilized by boiling with SDS buffer. This approach gives a more efficient breakage of the cells and results in cell extracts that have been exposed to subzero temperatures only during preparation, which may be important to avoid protein degradation.

Figure 2 shows an autoradiogram of a 2-D gel, with [^{35}S]methionine-labelled proteins from MG1363, grown in LM-GSA medium at 30°C (standard conditions). The intensities of the spots represent the synthesis rates of the particular proteins. In the present pH range from 4 to 7, more than 400 different spots appear on highly exposed autoradiograms. The extended range from pH 3 to 10 did not result in more than 5% additional spots (data not shown), and so the better separation in the pH 4 to 7 range was preferred to the full protein complement. The autoradiogram in Fig. 2 is positioned with the pH gradient rising from left to right and with increasing molecular weights from bottom to top. Overlaying the autoradiogram is a coordinate system, which is placed so that vertical lines runs through polypeptides with similar isoelectric points and horizontal lines run through polypeptides with same apparent molecular weight. The angle of the grid relative to the gel was determined from the comparative analysis of many autoradiograms, especially the streaks resulting from slightly overloaded gels. The direction of the streaking gives the paths of migration and thus the vectorial direction of the two axes. The migration pattern in the gel shown in Fig. 2 was found to be almost undistorted, and the gel was chosen as our reference gel. Molecular mass markers ran at the following *y*-axis values: 6.5 kDa, −10.0; 14.2 kDa, 7.5; 20 kDa, 28.0; 24 kDa, 46; 29 kDa, 53.5; 36 kDa, 66.0; 45 kDa, 77.0; 55 kDa, 85.5; 66 kDa, 94.0; 84 kDa, 100.5; 97 kDa, 105.0; 116 kDa, 108.0; 205 kDa, ca. 123; therefore, by using this dependence, the apparent molecular mass of any spot can be determined from its *y* coordinate (original data not shown, but the relative positions of the

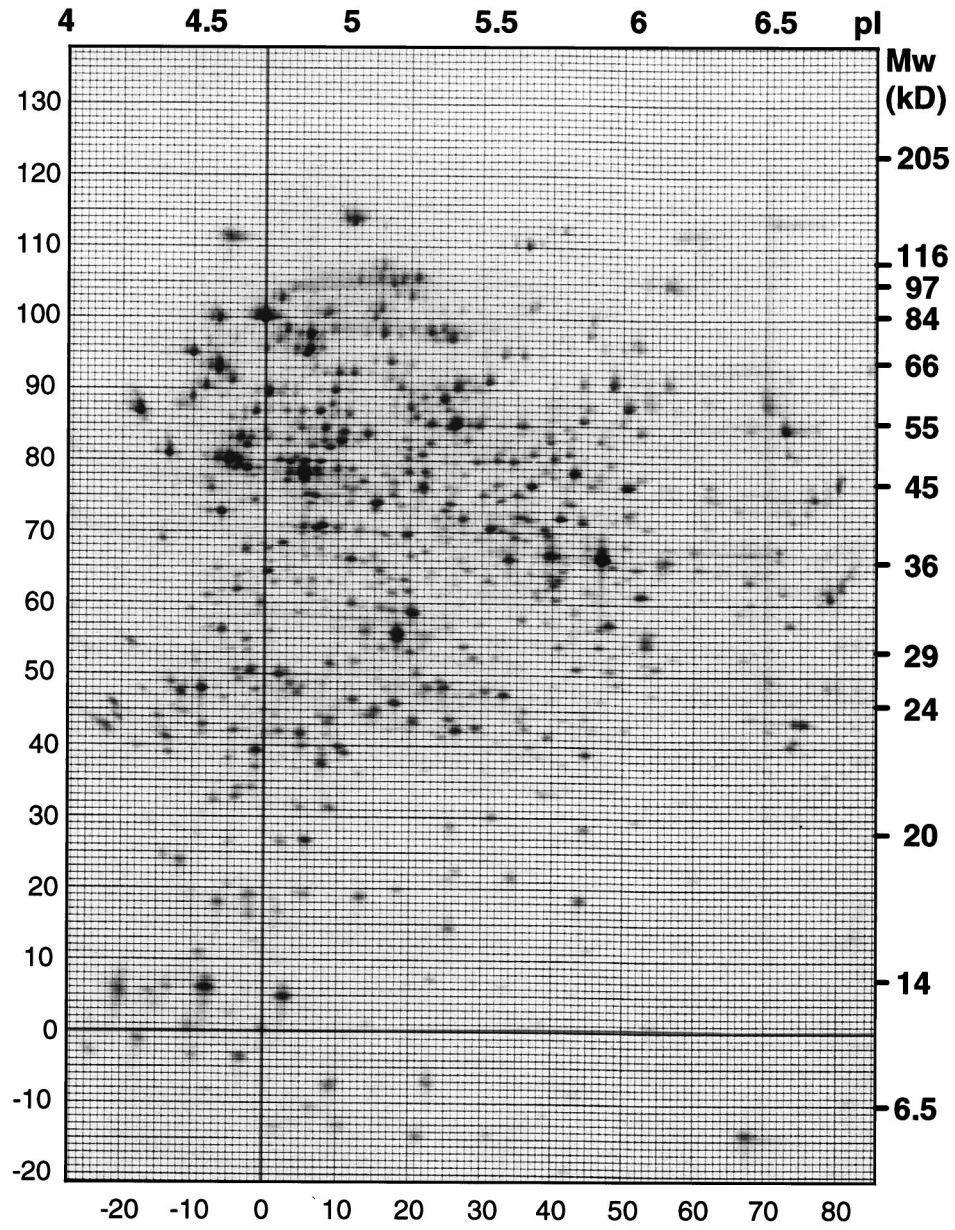

FIG. 2. Reference map of proteins from MG13632, separated by 2-D PAGE. The strain was grown exponentially at 30°C in LM-GSA medium to an OD_{450} of 0.4, and proteins were labelled for 10 min with [^{35}S]methionine followed by a 2-min chase with nonradioactive methionine. After 2-D PAGE separation by isoelectric point (pH 4 to 7) and molecular weight, an autoradiogram was produced from the gel. A coordinate system is superpositioned over the autoradiogram, so that vertical lines run through polypeptides with similar isoelectric points and horizontal lines run through polypeptides with similar molecular weights. Protein spots in the present study are assigned a coordinate, referring to the position in this map. The picture was scanned at 400 dpi with a Scan Jet 4c/T (Hewlett-Packard) scanner and the DeskScan II version 2.3 software. The TIF file was imported into Top Draw version 3.1 for addition of text. Mw, molecular mass (in kilodaltons).

marker proteins are marked in Fig. 2). If it is assumed that a linear pH gradient from pH 4 to 7 is formed over the entire range of the gel (as claimed by the manufacturer), a crude estimate of the pI value for a polypeptide can be obtained from its x coordinate by using the simple formula $pI \approx 4.7 + 0.025x$.

Identification of heat shock proteins by Western blotting. To identify known heat shock proteins among the spots on the reference gel, we used ECL Western blotting on the 2-D PAGE-separated proteins from a [^{35}S]methionine-labelled culture (labelled 0 to 10 min after transfer to 43°C) of MG1363, after transfer of the proteins from the gel to a PVDF membrane. Antisera against *B. subtilis* DnaK, DnaJ, GrpE, and HrcA (Orf39), *B. stearothermophilus* GroEL, and *E. coli* GrpE proteins were used.

Antibodies against the DnaK protein detected a single protein (Fig. 3B). By positioning the Western blot over an autoradiogram of the radioactive proteins on the membrane (Fig. 3A), the *L. lactis* DnaK protein was found to correspond to the protein at coordinate $\{-10.0, 95.0\}$ on the reference gel in Fig. 2. The position corresponds to a size of 70 kDa, in agreement with the calculated molecular mass of the *L. lactis* DnaK protein (12). Direct reprobing of the membrane with antibodies against GroEL, without stripping the membrane for antibodies against DnaK, gave the expected signal from DnaK and a new

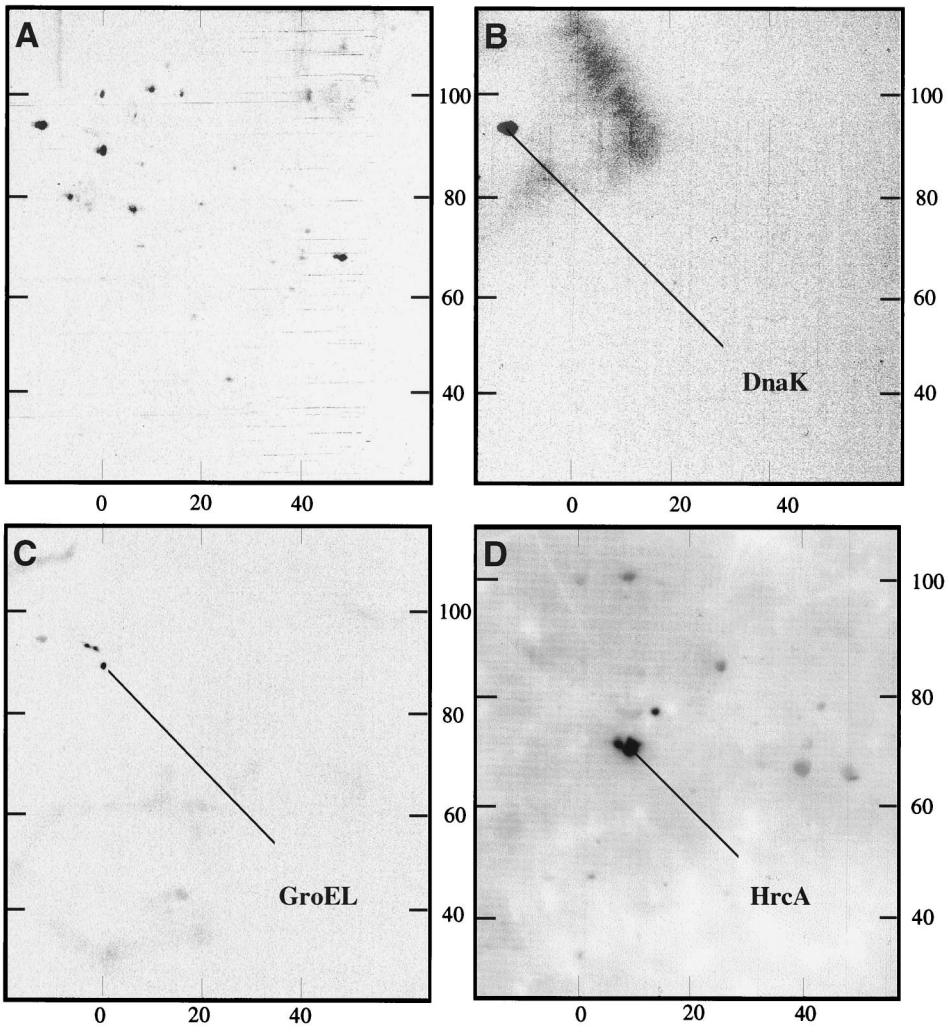

FIG. 3. Detection of DnaK and GroEL by Western blotting. Proteins from a heat-stressed [^{35}S]methionine-labelled (0- to 10-min) culture of MG1363 were separated by 2-D PAGE and transferred to a PVDF membrane. (A) Autoradiography of [^{35}S]methionine-labelled proteins from the membrane. (B to D) DnaK and GroEL homologs were visualized by the ECL Western blot detection system after cross-reaction with antibodies raised against purified *B. subtilis* DnaK (B), *B. stearothermophilus* GroEL (C), and *B. subtilis* HrcA (Orf39) (D) proteins. The membrane used in panel D contains a much higher protein concentration than the membrane used in panels A to C, resulting in a higher background of nonspecific binding to abundant proteins. The picture was scanned at 200 dpi with a Scan Jet 4c/T (Hewlett-Packard) scanner and the DeskScan II version 2.3 software. The TIF file was imported into Top Draw version 3.1 for addition of text.

signal corresponding to the coordinate {0.5, 89.5}. This position corresponds to a 60-kDa protein, in agreement with the size of the GroEL protein (2). Two slower-migrating protein spots can be seen both on the Western blot and on the autoradiogram of the [^{35}S]methionine-labelled proteins. These proteins cannot be found in any other gels, and so we expect them to be artifacts. No proteins on the gel shown in Fig. 3 cross-reacted with antibodies raised against the DnaJ protein. This could be expected, since the calculated pI for the *L. lactis* DnaJ protein (31) is slightly above pH 7 (pI = 7.1). However, by using 2-D gels with the extended pH range from 3 to 10 in Western blots, a smear of proteins, with an apparent molecular mass around 40 kDa and a pI above pH 7, cross-reacted with antibodies raised against DnaJ (data not shown).

For unknown reasons, antibodies raised against the GrpE protein from both *E. coli* and *B. subtilis* cross-reacted with a variety of proteins on gels with both narrow and wide pH ranges (data not shown). Antibodies raised against *E. coli* GrpE cross-reacted with proteins around coordinates {47.0, 66.0} and {40.0, 66.5} and a third protein positioned around coordinates {12.0, 66.0} The estimated molecular mass of all three proteins is around 38 kDa, and no proteins with an expected molecular mass of around 20.6 kDa (12) were observed. Western blots with antibodies raised against the *B. subtilis* GrpE protein also gave multiple signals (different from those obtained with *E. coli* CrpE), with a 34-kDa protein around coordinates {20, 59} as one of the major cross-reacting proteins on the gel (data not shown). It was, however, impossible to obtain a positive identification of GrpE from the blots. Identification of the HrcA heat shock regulatory protein with antibodies against the *B. subtilis* protein (encoded by *orf39* in the *dnaK* operon [34]), resulted in a signal from a 40-kDa protein (Fig. 4D) at a position around coordinates {8, 71}. The blot was performed with a 2-D gel, containing higher concentrations of unlabelled proteins, so that the identification of the protein spot had to rely upon Coomassie blue-stained proteins on the membrane, which is much less sensitive than the detection of radioactively labelled proteins. The size of the protein

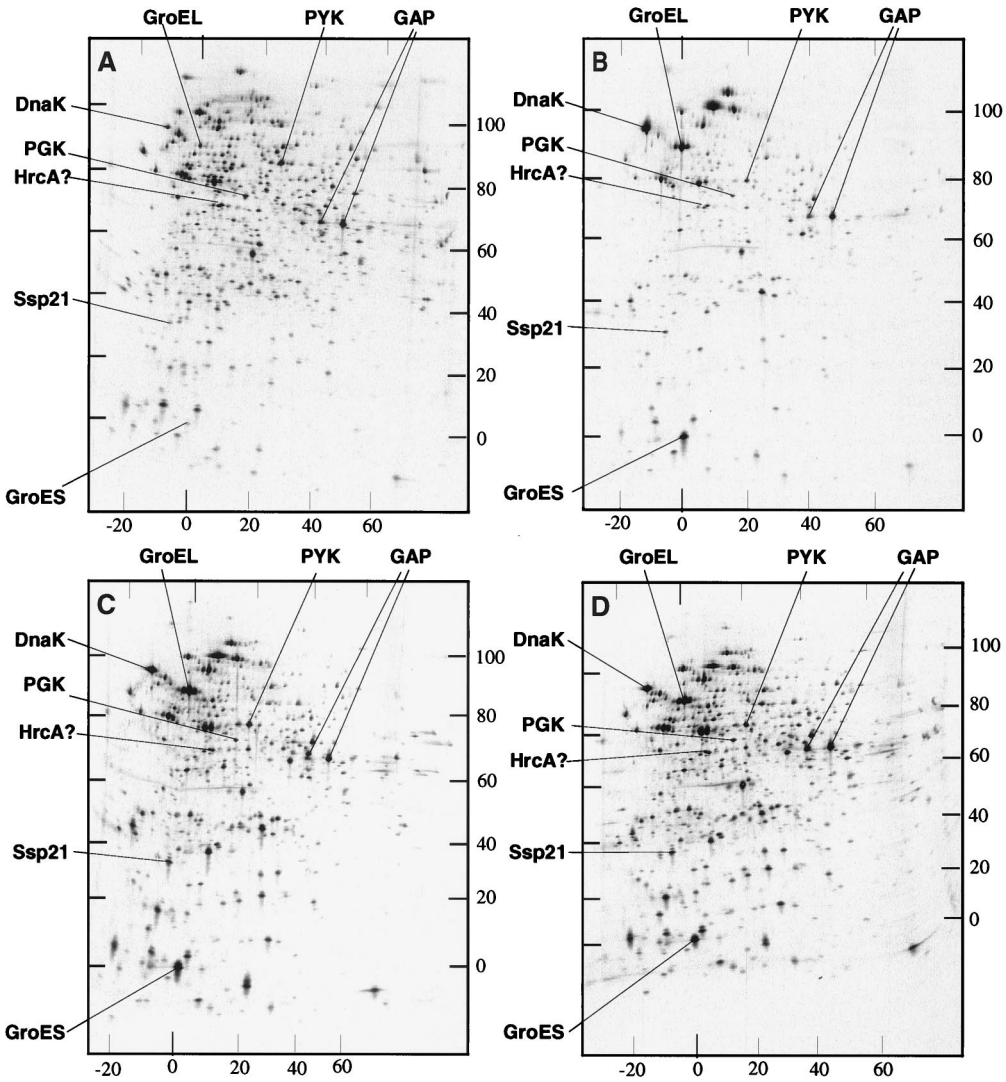

FIG. 4. 2-D PAGE analysis of [^{35}S]methionine pulse-labelled proteins of strain MG1363 subjected to heat stress. Bacteria were grown in LM-GSA medium at 30°C to an OD_{450} of 0.4 and were pulsed-labelled for 10 min before (A) and at 0 to 10 min (B), 15 to 25 min (C), and 30 to 40 min (D) after transfer of the culture flask to 43°C. The positions of DnaK, GroEL, GroES, heat shock repressor HrcA, salt stress protein Ssp21, glyceraldehyde-3-phosphate dehydrogenase (GAP), pyruvate kinase (PYK), and phosphoglycerate kinase (PGK) are indicated on the gels. The picture was scanned at 200 dpi with a Scan Jet 4c/T (Hewlett-Packard) scanner and the DeskScan II version 2.3 software. The TIF file was imported into Top Draw version 3.1 for addition of text.

is very close to the calculated 39 kDa for the *hrcA* gene product from *B. subtilis*, and so we believe that *L. lactis* harbors an HrcA-like repressor, which migrates around coordinates {8, 71}.

Identification of 2-D PAGE-separated proteins by N-terminal amino acid sequence determination. To identify a number of reference spots on the gel, we performed direct N-terminal sequencing of the separated proteins. To get enough material for the sequence determinations, we ran gels with higher protein loads. The protein gels described above each contained protein from approximately 2×10^7 cells. For the extraction of separated proteins, we used protein from about 10^9 cells per gel. After transfer of the proteins to a PVDF membrane and Coomassie blue staining, the regions of the membrane containing the desired protein spots were excised and used directly for automated Edman degradation. Highly expressed proteins were extracted from gels by using cells that were harvested 1 h after transfer to 43°C or after addition of 2.5% NaCl. Table 1 shows the N-terminal amino acid sequence for a number of extracted proteins together with their coordinates obtained from Fig. 2.

The protein running at coordinates {0, 0} was extracted from heat-stressed cells. From the size of the protein and its higher expression during heat stress (shown below), we expected it to be the GroES protein. Accordingly, the first five N-terminal amino acids are in complete agreement with the translated sequence of the *groES* reading frame from *L. lactis* (21a), except that the N-terminal formylmethionine residue must have been removed by posttranslational modification. To verify the positions of the DnaK and GroEL proteins on the 2-D gels, found by Western blotting, we extracted the proteins from gels by using heat-stressed cells and determined the three N-terminal amino acids. The identity of the sequences (Table 1) with the translation products from the *groEL* and *dnaK* (12) reading frames established the identity of the spots.

The two 37- to 38-kDa highly expressed proteins at coordinates {40.0, 66.5} and {47.0, 66.0} in Fig. 2, which cross-react-

TABLE 1. N-terminal amino acid sequences of proteins extracted from 2-D gels

Spot designation	Coordinate[a]		N-terminal amino acid sequence	Homologous protein[b]
	x axis	*y* axis		
GroES (Hsp11)	0.0	0.0	MLKPL	GroES (id)
GroEL (Hsp60)	0.5	89.5	SKE	GroEL (id)
DnaK (Hsp70)	−10.0	95.0	SKI	DnaK (id)
GAP (Pro38)	40.0	66.5	VVKVGINGFGRIGRL	Glyceraldehyde-3-phosphate dehydrogenase (id)
GAP (Pro37)	47.0	66.0	VVKVGINGFG	Glyceraldehyde-3-phosphate dehydrogenase (id)
PYK (Pro55)	26.5	85.0	MNKRVKIVST	Pyruvate kinase (id)
PGK (Pro44)	15.5	74	AKLTVKDVELKG	3-Phosphoglycerate kinase (high similarity)
Pro32	18.5	55.5	AIVSAEKFVQAA	None found
Pro48	5.5	78.5	TENEFE	None found
Pro49	−5.0	80.0	SIIDT	None found

[a] Coordinates refer to the positions of the protein spots on the map shown in Fig. 2.
[b] Identity (id) or similarity of the amino acid sequence to sequences in GenBank.

ed with antibodies raised against GrpE from *E. coli*, turned out to have identical N-terminal amino acid sequences (Table 1). The sequence was identical to the N-terminal part of glyceraldehyde-3-phosphate dehydrogenase, encoded by the *gap* gene from *L. lactis* LM0230 (5). The molecular mass of the enzyme was predicted to be 36.0 kDa (5), in good agreement with the molecular masses of the two proteins. We therefore conclude that the two proteins are different forms of glyceraldehyde-3-phosphate dehydrogenase, where the leftmost isoform could be deaminated, phosphorylated, or modified by chemical groups which shift the isoelectric point of the enzyme toward acidic pH.

When the amino acid sequence of the 55-kDa protein, at coordinates {26.5, 85.0} in Fig. 2, was used in a search for homologous proteins, the N-terminal sequence of pyruvate kinase, encoded by the *pyk* gene from *L. lactis*, showed up with 100% identity (23). The protein is abundant in cells grown under all conditions tested. In agreement with the predicted size of the protein from the 2-D gel, the calculated molecular mass of pyruvate kinase was reported to be 54.3 kDa (23).

Another protein, which was also found in high concentrations on all gels, runs as a 44-kDa protein around coordinates {15.5, 74}. When the N-terminal amino acid sequence, shown in Table 2, was analyzed for similarity to other proteins, triosephosphate isomerase (Tpi) from *Thermotoga maritima* (3-KMTIRDVDLKG-13) and phosphoglycerate kinase (Pgk) from *Penicillium chrysogenum* (6-KLPVTDVDLKG-16) showed up with high similarity, as possible homologs. Considering that the predicted N-terminal amino acid sequence of the *tpi* gene product (26.8 kDa) of *L. lactis* is MSRKPIIAGNW (6), this rules out the possibility that the 44-kDa protein is Tpi. However, the N-terminal amino acid sequence, MNKKTLKDID VKG, of the 42.5-kDa *Bacillus megaterium pgk* gene product (29) has a high degree of similarity with the 44-kDa protein. We therefore believe that the N-terminal sequence of Pro44 (A**K**L**T**V**KD**VEL**KG,** where underlined and boldface amino acids show identical and semiconservative substitutions, respectively, compared to the *B. megatarium* Pgk sequence) suggests that the protein is the phosphoglycerate kinase from *L. lactis*. Unfortunately, none of the remaining N-terminal sequences shown in Table 2 exhibited a high degree of similarity to proteins in the databases.

Temporal induction of proteins during heat stress. It is known from studies of a number of bacteria that heat shock proteins such as DnaK and GroEL accumulate most rapidly during the early phases of the heat shock response. After a rapid rise in the rate of synthesis of the proteins, which follows a shift to lethal high temperatures, the synthesis rate decreased after 10 to 15 min to intermediate levels (36, 37). To investigate whether this behavior is conserved in *L. lactis*, we conducted a series of pulse-labelling experiments of proteins from strain MG1363 during the first 40 min after a shift from 30 to 43°C.

By visual inspection of the autoradiograms shown in Fig. 4, representing the four labelling periods before (Fig. 4A), and from 0 to 10 min (Fig. 4B), 15 to 25 min (Fig. 4C), and 30 to 40 min (Fig. 4D) after transfer to 43°C, it is clear that heat stress results in a pronounced change in the protein synthesis pattern in the cells. A small subset of proteins are synthesized at a higher rate, while the rest are synthesized at a lower rate. At least 17 of the spots in Fig. 4A were enhanced under heat stress conditions. To quantify the net synthesis rates in the chosen 10-min time intervals, we determined the radioactivity distribution over the surface of the dried gels by using a Packard instant imager. Since smaller corrections had to be made for the efficiency of cell lysis and protein extraction, the radioactivity in each spot was normalized to the total radioactivity on the gel, i.e., the total protein synthesis rate in the cell.

The results are summarized in Table 2. The unknown protein spots are designated by Hsp (heat stress protein), Ssp (salt stress protein), or Pro (protein), followed by a number indicating the apparent molecular weight. The single Ssp is also a heat stress protein, but the suffix was used to indicate that the induction of the protein is more pronounced during salt stress (shown below). In Table 2, the positions of each of the proteins, identified by their coordinates on the reference map in Fig. 2, as well as the apparent molecular weight and a rough estimate of the isoelectric point, are also indicated. The estimated pIs of the known proteins are in very good accord with the pIs calculated from the amino acid sequence (Table 2).

When the time courses of the synthesis rates were compared for the 17 heat stress-induced proteins and a selection of other proteins, three different patterns of induction emerged. One group of proteins showed a high (more than 10-fold) immediate induction in synthesis rate during the first 10 min, followed by a decline to intermediate levels in the next labelling periods (Fig. 5A). DnaK, which shows a maximum 35-fold induction in its synthesis rate, is a typical member of this group. GroEL and GroES, with 45- and 35-fold maximal induction, respectively, also belong to the group of highly induced proteins, but their expression does not decrease with time to the same extent as that of the other proteins in Fig. 5A.

The second group of heat stress proteins, whose patterns of induction are shown in Fig. 5C, are induced only between two- and eightfold during heat stress at 43°C. These proteins show

TABLE 2. Properties of proteins separated by 2-D PAGE

Spot designation	Identified protein[a]	Coordinate[b]		Estimated value[c]		Induction[d]	
		x axis	*y* axis	pI	Mol mass (kDa)	Heat	Salt
Hsp9		23.0	−7.5	5.3	~8.5	+	−
Hsp11	GroES	0.0	0.0	4.7	~11	+++++	+
Hsp14		−8.0	6.0	4.5	14	+	+
Hsp15		29.0	7.0	5.5	15	++	−
Hsp17		−6.0	18	4.5	17	+	+
Hsp18		26.5	22.5	5.3	18	+	−
Hsp23		8.0	37.5	4.9	23	+	+
Hsp25		25.5	44.0	5.4	25	++	+
Hsp26		−15.0	44.0	4.3	26	++	+
Hsp39		40.0	68.0	5.7	39	+	−
Hsp42		41.0	72	5.7	42	+	−
Hsp48		20.0	78.5		48	+	+
Hsp60	GroEL	0.5	89.5	4.7	60	++++	+
Hsp70	DnaK	−10.0	95.0	4.4	70	++++	+
Hsp84		15.0	100.0	5.1	84	++	+
Hsp85		8.5	100.5	4.9	85	++++	+
Hsp100		13.0	105.0	5.0	100	+++	−
Ssp21		−7.0	32.5	4.5	21	+	++++
Pro5		67.5	−14.5	6.4	~5	−	−
Pro30		18.5	55.5	5.2	30	−	−
Pro32		21.5	58.5	5.2	32	−	−
Pro37	GAP	47.0	66.0	5.9	37	−	−
Pro38	GAP	40.0	66.5	5.7	38	−	−
Pro44	(PGK)	15.5	74.0	5.1	44	−	−
Pro48		5.5	78.5	4.8	48	−	−
Pro49		−5.0	80.0	4.6	49	−	−
Pro55	PYK	26.5	85.0	5.4	55	−	−

[a] Proteins were identified by Western blotting or direct N-terminal amino acid sequence determination. GAP, PYK, and PGK, glyceraldehyde-3-phosphate dehydrogenase, pyruvate kinase, and phosphoglycerate dehydrogenase, respectively.

[b] Coordinates refer to the positions of the protein spots on the map shown in Fig. 2

[c] pI ≈ 4.7 + 0.025*x*. The calculated pI and molecular mass for proteins with known amino acid sequences are as follows (from *L. lactis*, unless otherwise indicated): GroES (pI 4.8; 10.2 kDa), GroEL (pI 4.7; 57.2 kDa), DnaK (pI 4.5; 64.9 kDa), HrcA (Orf1) (pI 5.8; 39.9 kDa), GAP (pI 5.3; 36.1 kDa), PYK (pI 5.3; 54.0 kDa), PGK (*B. magatarium*) (pI 4.7; 42.5 kDa).

[d] Induction: −, below 2-fold; +, between 2- and 10-fold; ++, between 10- and 20-fold; +++, between 20- and 30-fold; ++++, between 30- and 40-fold; +++++, between 40- and 50-fold. Induction was determined from the relative synthesis rates (see the legend to Fig. 5), measured in duplicate experiments.

a gradual increase in synthesis rate during the first two labelling periods. None of these proteins have been identified.

The last group of proteins are not induced by heat stress (Fig. 5E). Either they are unaffected, as exemplified by the alkaline isoform of glyceraldehyde-3-phosphate dehydrogenase (GAP, Pro37), or their synthesis rate is reduced, exemplified by pyruvate kinase (PYK, Pro55) and Pro32.

Temporal induction of proteins during salt stress. The presence of 2.5% NaCl in GSA medium increased the doubling time of MG1363 to approximately 200 min. The shift was performed by carefully adding a prewarmed 25% NaCl solution in GSA medium to the growing cells, without aerating the culture. Figure 6 shows the 2-D PAGE-separated proteins from MG1363, labelled for 10 min with [^{35}S]methionine before (Fig. 6A) and from 0 to 10 min (Fig. 6B), 15 to 25 min (Fig. 6C), and 30 to 40 min (Fig. 6D) after the addition of NaCl. As found for the total protein synthesis rate during heat stress, the total incorporated [^{35}S]methionine into acid-precipitable material under salt stress dropped to about 50% of the preshift level during the 40 min after the addition of NaCl (data not shown).

From studies in *E. coli* and *B. subtilis*, it was expected that salt stress would induce a set of proteins unrelated to the heat stress proteins. Interestingly, however, this was not the case. Most of the proteins induced by salt stress were identical to the proteins induced by heat stress (Fig. 4A and C). DnaK, GroEL, GroES, and Hsp26, which were among the proteins that were highly induced under heat stress (Fig. 5A), showed the same pattern of induction during salt stress, although at a lower maximal level of induction (Fig. 5B). DnaK was synthesized at an eightfold-elevated level during the first 10 min after salt addition, while GroEL and GroES reached five- and sevenfold-elevated levels, respectively (Fig. 4B). The synthesis of Hsp26 was ninefold elevated.

Hsp84, Hsp85, and Hsp100, belonging to the same heat stress induction group as DnaK, GroEL, and GroES, showed a different pattern of salt stress induction. Their synthesis rates were elevated threefold during the first 10 min of salt stress. This level was maintained for the rest of the period (Fig. 5B).

The second heat stress induction group (Fig. 5C) was characterized by a gradual rise in synthesis rate to intermediate induction levels during the heat stress period. Members of this group showed a variety of induction patterns during salt stress (Fig. 5D). The level of the sole protein with a high induction level during salt stress, denoted Ssp21 for 21-kDa salt stress protein, gradually increased 35-fold during the 40-min period. Hsp17, which was fivefold induced during heat stress, showed a gradual rise to an eightfold-elevated level during salt stress. The rest of the proteins in the group showed either low induction (Hsp23, fourfold; Hsp48, threefold), no induction (Hsp42, Hsp18, and Hsp39), or depression (Hsp15 and Hsp9).

The selection of proteins in Fig. 5E, which showed no heat stress induction, also showed no salt stress induction (Fig. 5F).

DISCUSSION

In the present study, we have analyzed the temporal induction patterns of individual proteins in *L. lactis* during heat and salt stress. To obtain reproducible results, we have optimized growth conditions and procedures for the labelling, extraction, and 2-D separation of proteins. These procedures result in 2-D gels with sufficiently high resolution.

In previous reports on the heat shock response in derivatives of NCDO712 (i.e., MG1363, LM0230, and ML3), it was shown that the strains induce the synthesis of a number of proteins during heat shock in rich M17 broth. One report described the induction of at least 13 proteins after transfer from 30 to 42°C (35), while another report described the induction of 16 proteins after transfer from 30 to 40°C (2). By comparing the size of the heat-induced proteins with the size of proteins which gave positive signals in Western blots, DnaK, DnaJ, GroEL, and GrpE were found to be among these heat shock proteins (2, 35). It was also found that exposing cells to 42°C enhanced their thermotolerance to incubation at 50°C (35), which was ascribed to the accumulation of chaperones during the preconditioning step (35).

In the present report, we positively identified the positions of DnaK, GroEL, and GroES on 2-D gels in the pI interval between pH 4 and 7. We also identified the position of DnaJ at pH 8 to 10, in gels with a wider pH range. Unfortunately, GrpE could not be identified with certainty, due to the emergence of multiple signals in the Western blots. Because of the high synthesis rates of the glycolytic enzymes pyruvate kinase, glyceraldehyde-3-phosphate dehydrogenase, and phosphoglycerate dehydrogenase (Fig. 4 and 6), they could be extracted from

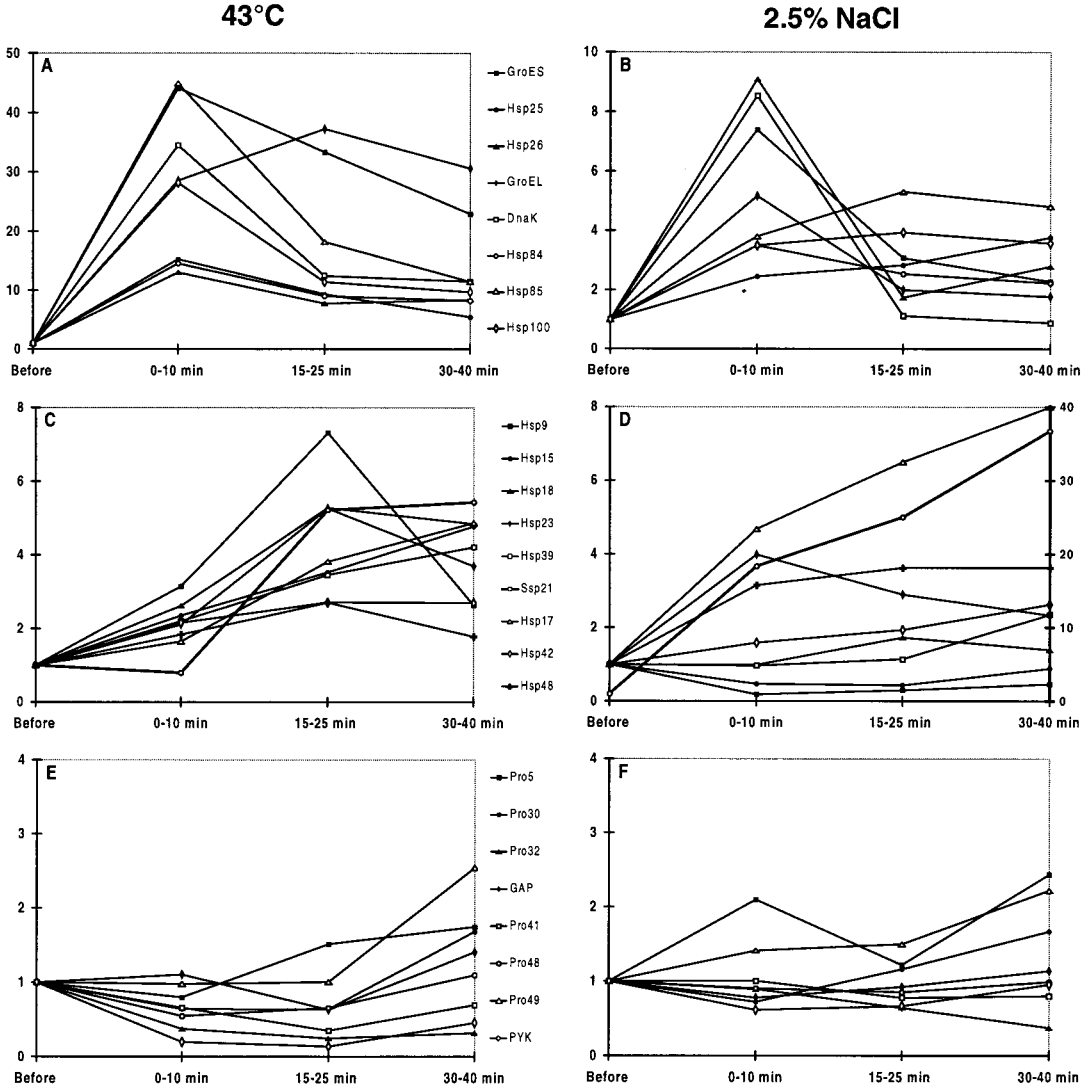

FIG. 5. Temporal induction of individual proteins during heat and salt stress. The radioactivity incorporated into individual 2-D PAGE-separated proteins was determined with a Packard Instant Imager. Values represent the mean values for duplicate experiments. All values were normalized to the total incorporated radioactivity (internal control) and are shown relative to unstressed values. (A, C, and E) Relative synthesis rate during heat stress at 43°C. (B, D, and F) Relative synthesis rate during salt stress at 2.5% NaCl. (A and B) Quickly induced heat stress proteins. (C and D) Slowly induced heat stress proteins. (E and F) Proteins which are not induced by heat stress. In panel D, the extended axis on the right side applies to Ssp21 only.

2-D gels and identified by their N-terminal amino acid sequence. Glyceraldehyde-3-phosphate dehydrogenase appeared to exist in two forms with equal molecular weights but different isoelectric points.

When the temporal induction of 24 individual proteins were analyzed during heat stress, three classes emerged: (i) proteins which are quickly induced during heat stress, (ii) proteins which are slowly induced during heat stress, and (iii) proteins which are not induced during heat stress. In assigning the proteins to the three classes, a twofold induction was chosen as the lower level of induction, based on the fact that the total protein synthesis rate dropped to 50% during both heat and salt stress (data not shown). A twofold induction in relative synthesis rates thus corresponds to an unaltered absolute synthesis rate. The proteins can be divided into three additional classes, based on their temporal induction during salt stress: (i) proteins which are quickly induced during salt stress, (ii) proteins which are slowly induced during salt stress, and (iii) proteins which are not induced during salt stress.

Table 3 shows the distribution of the proteins corresponding to these criteria. We have not been able to find any proteins which are induced solely by salt stress (induction classes IIIA and IIIB in Table 3 [also compare Fig. 4 and 6]). Whether such proteins do exist in *L. lactis*, as found in *B. subtilis* (17), and are not found on the gels because of the chosen experimental conditions is presently being investigated. All fast-induced heat stress proteins showed induction by salt stress as well (absence of proteins of class IC in Table 3). These results indicate an overlap between heat and salt stress induction in *L. lactis.*

Genetic basis for heat shock regulation. In *L. lactis*, the structural gene encoding DnaK is situated in the *orf1-grpE-dnaK* operon (12). Two controlling inverted repeat for chaperone expression (CIRCE)-like regulatory elements are present in the promoter region of the *dnaK* operon, and one CIRCE

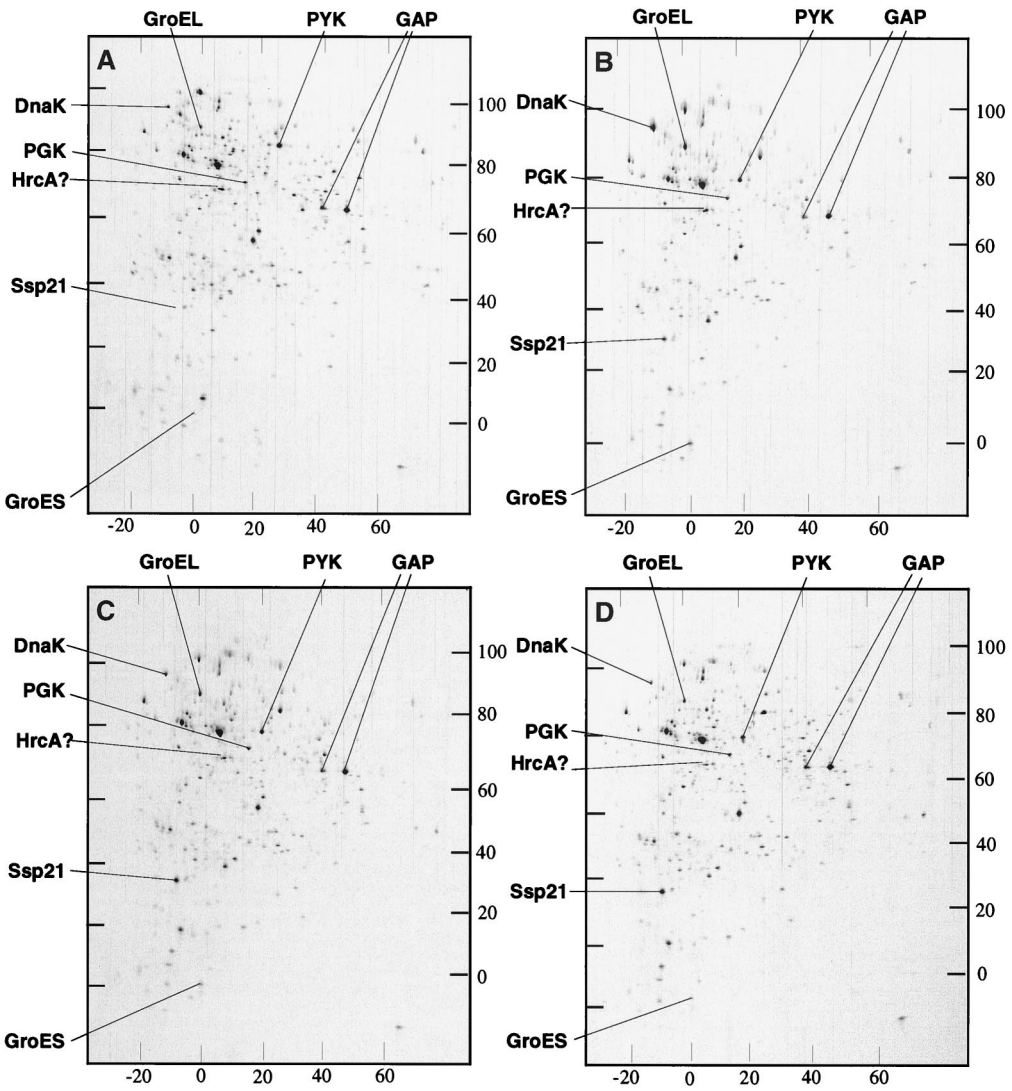

FIG. 6. 2-D PAGE analysis of [^{35}S]methionine pulse-labelled proteins of strain MG1363 subjected to salt stress. Bacteria were grown in LM-GSA at 30°C to an OD_{450} of 0.4 and were pulse-labelled for 10 min before (A) and at 0 to 10 min (B), 15 to 25 min (C), and 30 to 40 min (D) after the addition of NaCl to 2.5%. The positions of DnaK, GroEL, GroES, the heat shock repressor HrcA, salt stress protein Ssp21, glyceraldehyde-3-phosphate dehydrogenase (GAP), pyruvate kinase (PYK), and phosphoglycerate kinase (PGK) are indicated on the gels. The picture was scanned at 200 dpi with a Scan Jet 4c/T (Hewlett-Packard) scanner and the DeskScan II version 2.3 software. The TIF file was imported into Top Draw version 3.1 for addition of text.

element is present in the promoter region of the *groESL* operon (12, 21a). CIRCE elements have been found in the promoter region of heat shock genes, such as *dnaK*, *groEL*, and *groES*, from a variety of gram-positive bacteria and cyanobacteria (34). We are currently investigating whether all fast heat stress proteins (class I in Table 3) are regulated by CIRCE elements. Recently, it has been found that *orf39* in the *orf39-grpE-dnaK* operon of *B. subtilis* encodes a repressor protein, which binds to CIRCE elements (30, 39) and is responsible for the heat shock control of the genes. It is at present unknown how the DNA binding activity of the repressor is changed in response to elevated temperatures. The regulatory gene, which

TABLE 3. Distribution of proteins in regulatory classes[a]

Induction during salt stress	Induction during heat stress		
	Fast (I)	Slow (II)	None (III)
Fast (A)	DnaK, GroEL, GroES, Hsp26	Hsp23	
Slow (B)	Hsp84, Hsp85, Hsp100	Ssp21, Hsp17, Hsp48	
No (C)		Hsp9, Hsp15, Hsp18, Hsp39, Hsp42	GAP, PYK, Pro5, Pro30, Pro32, Pro41, Pro48, Pro49

[a] Data from Fig. 5.

was named *HrcA*, for heat regulation at CIRCE (30), is homologous to the *orf1* gene in the *L. lactis dnaK* operon (12). It is therefore expected that the expression of DnaK, GroEL, and GroES in *L. lactis* would be under the control of a repressor, encoded by the *orf1* gene.

A homolog of the HrcA regulatory protein was found in the present study with antibodies raised against the protein from *B. subtilis*. The protein had the expected molecular mass of about 40 kDa and was situated in the vincinity of coordinates {8, 71}. By comparing the area in Fig. 4C with that in Fig. 4A, it can be seen that a new protein appears on top of three closely migrating proteins. This protein is not expressed in Fig. 4A or B but is highly expressed in Fig. 4C and D. The resolution did not allow for the quantification of the radioactivity in the protein without contamination from the comigrating proteins, but it is clear that the protein at coordinates {71.5, 7.0} is a heat stress protein which is highly induced after 15 min. The combined results indicate that the protein could be the HrcA repressor, although it is peculiar in that its synthesis kinetics does not follow the DnaK pattern, since *hrcA* and *dnaK* are transcribed from the same promoter.

In a recent study, Arnau et al. (1) analyzed the transcription of the *dnaK* and *groESL* operons in strain MG1363 during heat stress. All conditions and techniques used in their study, relating to the growth and stress of the strain, were adopted from the present study. The kinetics of the appearance of mRNA for the major heat shock proteins are in agreement with the present protein data, except that a sharp drop in *groEL* mRNA was observed after 20 min. At present, we have no explanation for this difference in response.

Induction of heat shock proteins during salt stress. A significant finding in this study is that the classic heat shock proteins DnaK, GroEL, and GroES are induced during salt stress in *L. lactis*. The contrary has been shown for both *B. subtilis* and *E. coli* in older reports on the 2-D PAGE analysis of labelled proteins (7, 17–19). In *B. subtilis*, the labelling was performed from 10 to 20 min after the addition of salt. By using this labelling period, the authors might have missed the elevation in synthesis rate of DnaK and GroEL if the kinetics were similar to those in *L. lactis*, as the synthesis rate had resumed the preshift level between 10 and 15 min after the onset of stress (Fig. 5B). In another study, however, the negative results were confirmed, since no rise in the level of *dnaK* and *groEL* mRNA was detected from 3 to 20 min after the addition of salt. On the other hand, it has been shown that other heat shock proteins in *B. subtilis* are induced during salt shock (33) and that preconditioning of *B. subtilis* cells with 4% NaCl resulted in increased thermotolerance (32). It is interesting that this acquired thermotolerance is independent of the levels of DnaK and GroEL. In *E. coli*, the labelling was performed from 1 to 15 min after the addition of salt, so that any response towards the high osmolarity should have been reflected in the incorporated radioactivity in the spots.

The heat shock response in gram-negative bacteria such as *E. coli* is very different from the CIRCE-based heat shock regulation. In *E. coli*, the heat shock response is initiated by the sequestering (titration) of free DnaK-DnaJ-GrpE chaperone complex with denatured protein from the cytoplasm. Under unstressed conditions, the complex binds the heat shock-specific RNA polymerase subunit, sigma-32, and promotes its degradation (4, 20). The unavailability of the chaperone complex results in the accumulation of sigma-32 and increased transcription from heat shock promoters.

In a recent study, it was reported that a *dnaK*(Ts) mutant was impaired in K^+ uptake and deplasmolysis following NaCl addition at 30°C (24). By immunoblotting of the separated proteins from a wild-type strain, the authors found a two- to threefold increase in the level of DnaK between 1 and 5 min after the addition of salt. This moderate increase in the level of accumulated protein reflects a large increase in synthesis rate, since the bacterium has synthesized twice the amount of DnaK in 4 min that it normally synthesizes in one full generation time. The authors suggested an explanation for the requirement of the wild-type DnaK protein in deplasmolysis. They speculated that the defect could be due to the lack of the DnaK chaperone function in translocating protein components of the uptake system into the periplasmic space or in renaturation of membrane components after subjection to denaturing salt concentrations during plasmolysis. If the water efflux during plasmolysis lowers the water activity inside the cells of *E. coli* to a degree where both cytoplasmic and membrane proteins are denatured, this could promote the heat shock response.

One of the major differences between gram-negative bacteria like *E. coli* and gram-positive bacteria like *B. subtilis* and *L. lactis* is the more rigid cell wall and higher internal turgor pressure of the latter. As a consequence, water efflux is more difficult to analyze in gram-positive bacteria, where the cell shrinkage is less pronounced. In *E. coli*, the optical density of the bacterial culture increases considerably as a consequence of the cell shrinkage after the addition of NaCl (24). In the present study, we found that DnaK, together with GroEL, GroES, and a number of other heat stress proteins, is induced during salt stress in *L. lactis*. No elevation in OD was observed after the addition of 1 to 4% NaCl (Fig. 1A), indicating a major degree of cell shrinkage, and no difference was observed in the microscopic appearance of the cells (data not shown). Nevertheless, our working hypothesis is that the heat shock proteins are induced as a consequence of the accumulation of denatured proteins, resulting from a lowered water activity during plasmolysis of osmotically shocked cells.

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