# Stress proteins and cross-protection by heat shock and salt stress in *Bacillus subtilis*

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*Bacillus subtilis* induced a set of general stress proteins in response to a salt or heat stress. Cells subjected to a mild heat stress showed a protective response which enabled them to survive otherwise lethal temperatures (e.g. 52 °C). In a similar way bacteria were enabled to survive toxic concentrations of NaCl by pretreatment with lower salt concentrations. A mild heat shock induced a cross-protection against lethal salt stress. The pretreatment of cells with low salt, however, was less effective in the induction of thermotolerance than a preceding mild heat stress. Three stress proteins were identified on the basis of their N-terminal amino acid sequences as homologues of GroEL, DnaK and ClpP of *Escherichia coli*. The role of general and specific stress proteins in the induction of thermotolerance/salt tolerance and cross-protection is discussed.

## Introduction

Stress proteins are involved in the adaptation of bacteria to growth-limiting conditions which are quite common in nature (Hecker & Babel, 1988). This protective function is well established for heat shock proteins of *Escherichia coli*, a special group of stress proteins. These proteins are essential elements in the induction of thermotolerance, a protective response which is induced by a mild heat stress and which enables the bacteria to survive otherwise lethal temperatures (Neidhardt & VanBogelen, 1987). In *rpoH* mutants of *E. coli* neither induction of heat shock proteins nor of thermotolerance occurs (Yamamori & Yura, 1982).

Heat shock proteins are encoded by genes which need a specific sigma factor, sigma 32, for transcription. A subset of heat shock proteins, the chaperonines like GroEL, GroES or DnaK, DnaJ and GrpE, have fundamental functions for survival at very high temperatures (Kusukawa *et al.*, 1987; Bukau *et al.*, 1989; Ang & Georgopoulos, 1989). The heat shock proteins belonging to this sigma 32-dependent high-temperature regulon are, however, necessary but not sufficient for thermotolerance (VanBogelen *et al.*, 1987). Furthermore, the protection of *E. coli* against heat stress requires KatF, a

newly described sigma factor necessary for the expression of special genes during starvation (Lange & Hengge-Aronis, 1991*a*, *b*; McCann *et al.*, 1991).

The high-temperature regulon of *E. coli* is one of the best-known model systems for analysing the global control of gene expression (Neidhardt & VanBogelen, 1987). Surprisingly there is only limited information available on the structure and function of heat shock genes of *Bacillus subtilis* (Imanaka & Takagaki, 1988; Hearne & Ellar, 1989; Wetzstein & Schumann, 1990; Wetzstein *et al.*, 1990; Hecker & Völker, 1990; Miller *et al.*, 1991). However, there have been detailed studies on the physiology of stress protein synthesis triggered by environmental inducers (Richter & Hecker, 1986; Hecker & Richter, 1987; Hecker *et al.*, 1987, 1988; Dowds *et al.*, 1987; Hecker & Völker, 1990). On the basis of these studies we proposed a distinction between general stress proteins which are induced by general growth-limiting environmental signals like salt stress, oxygen limitation, nutrient starvation or heat stress, and specific heat shock proteins whose synthesis is stimulated exclusively by heat shock (Richter & Hecker, 1986; Hecker & Völker, 1990).

Among the environmental inducers, salt stress may be very common, especially for soil-living bacteria like *B. subtilis* (Hecker *et al.*, 1988). In agreement with this assumption, salt stress is a very good inducer of general stress proteins in *B. subtilis* (Hecker *et al.*, 1988), but not
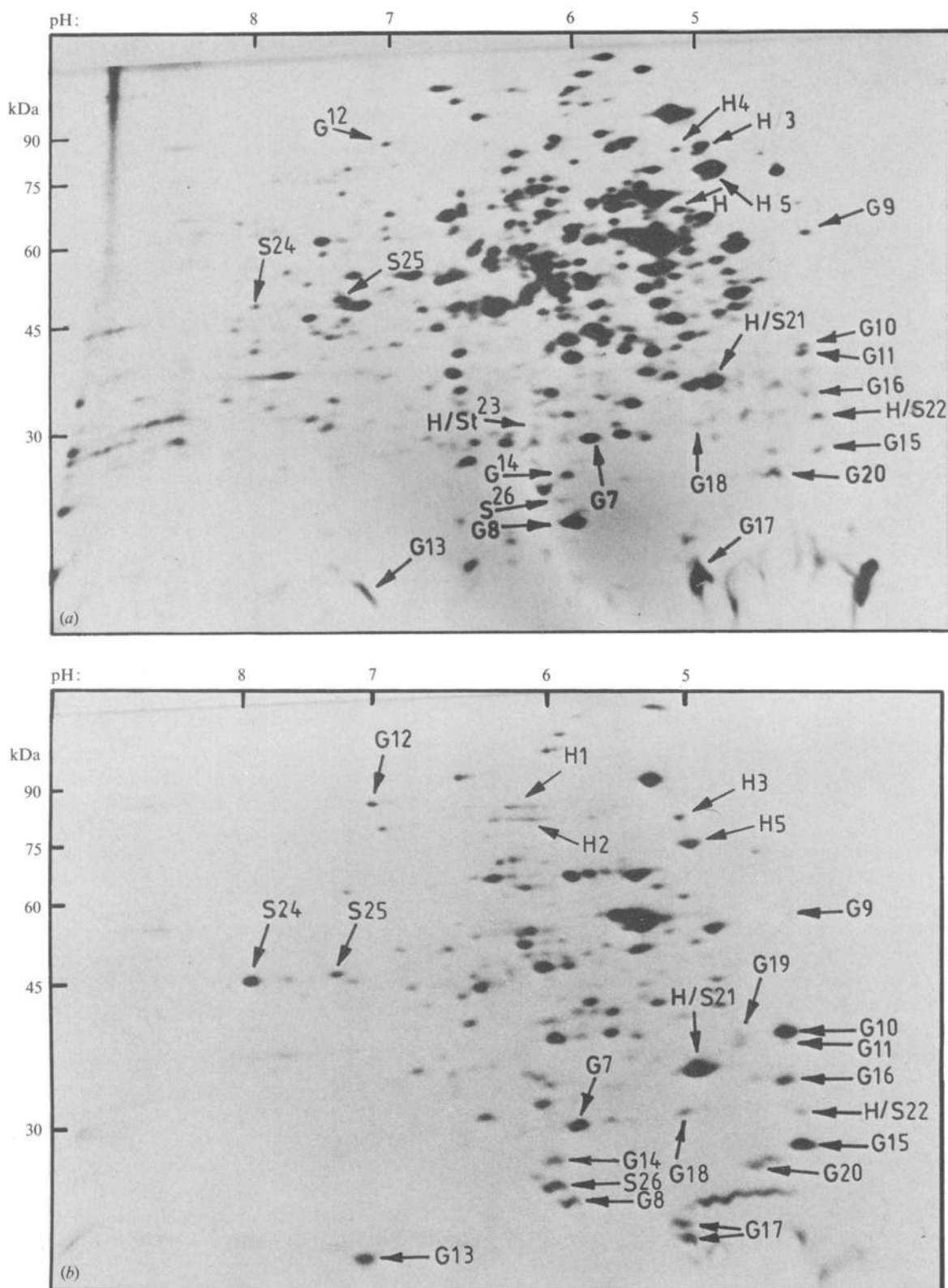
Fig. 1. Kinetics of synthesis of stress proteins in *B. subtilis* IS58 after salt stress. Bacteria were pulse-labelled with L-[$^{35}$S]methionine, (*a*) before and (*b–d*) at different times after imposition of salt stress for 10 min (*b*, 10–20 min; *c*, 30–40 min; *d*, 60–70 min). Cells were challenged by addition of solid NaCl to a final concentration of 6% (w/v). Proteins were separated and detected as described in Methods.
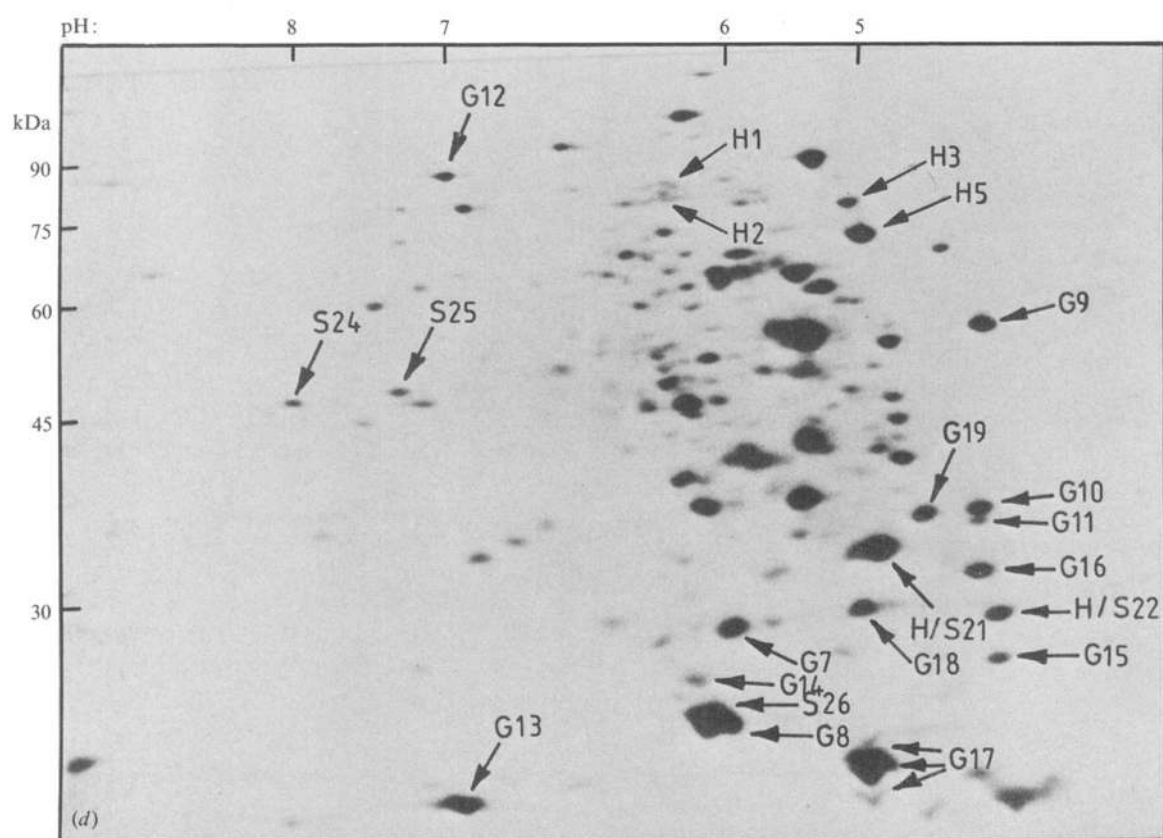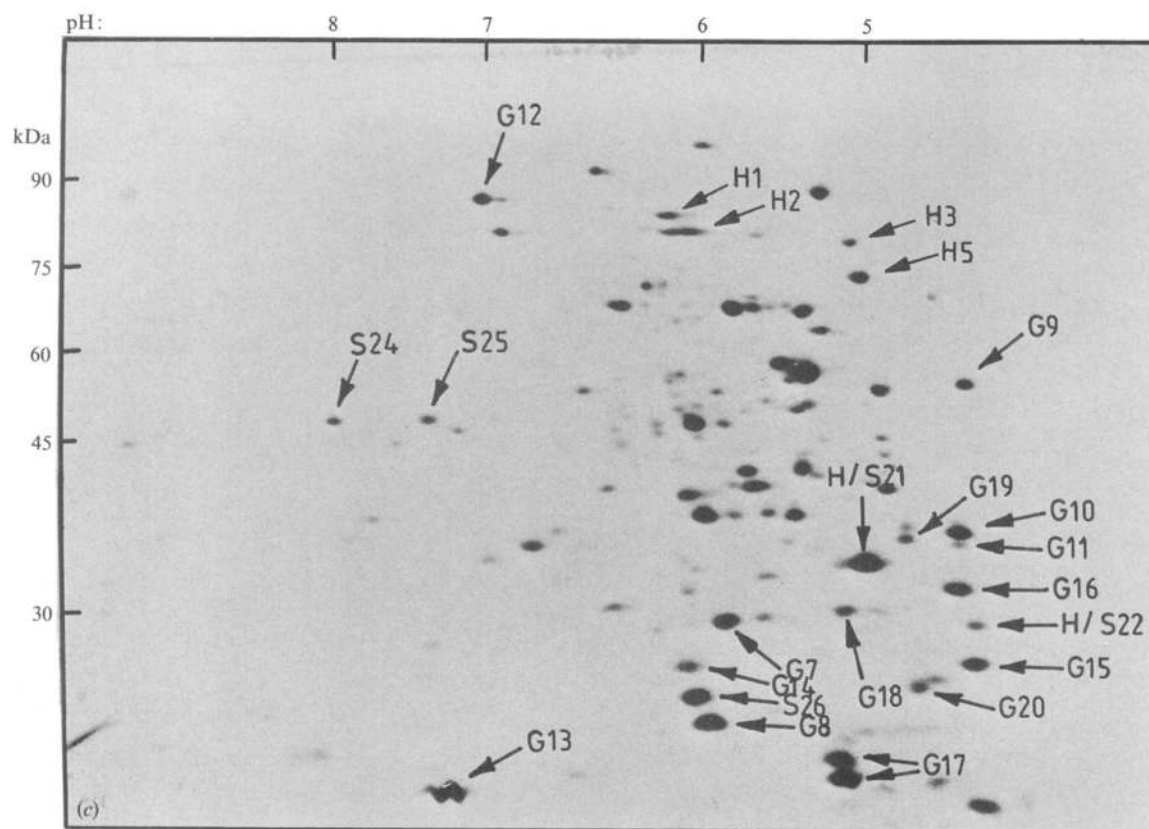
in *E. coli* (Clark & Parker, 1984). Specific heat shock proteins, however, are not induced by salt stress. In this paper we describe the interaction of heat and salt stress in the induction of stress tolerance in *B. subtilis*.

## Methods

*Bacterial strain.* The *Bacillus subtilis* strain IS58 (*trpC2 lys*; see Smith *et al.*, 1980) was grown in a minimal medium (Belitsky & Shakulov, 1980) as described earlier (Hecker *et al.*, 1988).

*Culture conditions/viability assay.* For salt stress or heat challenge, cultures were grown at 37 °C to a density of about $5 \times 10^7$ cells ml$^{-1}$. At this point the cells were either treated with solid NaCl to a final concentration of 2, 4 and 6% (w/v), or transferred to high temperature (48 °C, 52 °C) for at least 4 h. Growth was monitored by determination of $OD_{500}$. Viable counts were determined by plating appropriate dilutions on agar at 37 °C (2·4%, w/v, agar in minimal medium).

To study heat and salt resistance with and without preadaptation, exponentially growing cells ($5 \times 10^7$ cells ml$^{-1}$) were shifted from 37 °C to 48 °C for 30 min, before being challenged either at 52 °C or with 6% (w/v) NaCl followed by testing for cell viability. In other experiments, exponentially growing cells were treated with 2% (w/v) NaCl before being challenged at 52 °C or with 6% (w/v) NaCl (final concentration). Viability of preadapted and non-adapted cells was determined by plating dilutions on agar at 37 °C.

*Pulse-labelling and electrophoresis.* Labelling of cellular proteins with L-[$^{35}$S]methionine and separation of labelled proteins according to O'Farrell (1975) were carried out as described earlier (Hecker *et al.*, 1988).

*Microsequencing.* For the determination of the N-terminal amino acid sequences, appropriate proteins were cut from 25 two-dimensional gels, electroeluted and centrifuged onto an Immobilone-membrane (Millipore). Proteins were sequenced on an Applied Biosystems A473 protein sequencer.

## Results

*Induction of salt tolerance by preadaptation to 'low' NaCl concentrations*

The addition of NaCl to exponentially growing cells of *B. subtilis* induced general stress proteins, whereas specific heat shock proteins (e.g. H3, H5) were not induced (Hecker *et al.*, 1988; Fig. 1). General stress proteins were synthesized with very high intensity immediately after the addition of NaCl (6%, w/v). Most of the general stress proteins have a low molecular mass. Time-course studies revealed that this induction of stress proteins was maintained for at least 60 min. The rate of synthesis of some of them (e.g. proteins G9, G17, G8; Fig. 1) increased after the initial induction phase. Besides general stress proteins, salt-specific proteins were also induced (e.g. proteins S24, S25 in Fig. 1). Interestingly, after a strong salt stress (6%, w/v, NaCl final concentration) two proteins (H1, H2), first grouped as specific heat shock proteins (Hecker *et al.*, 1988), were transiently
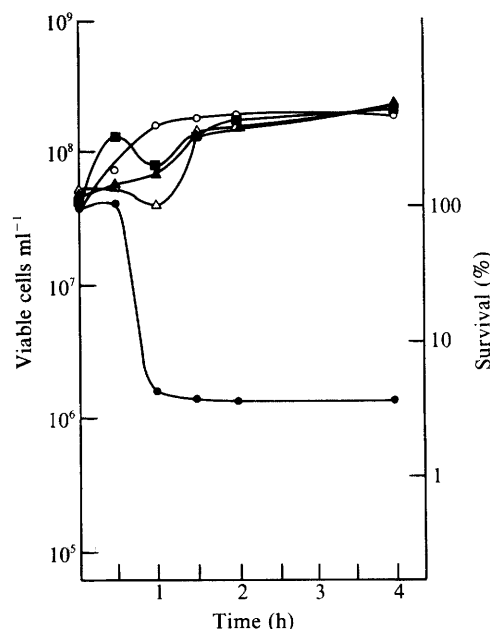


Fig. 2. Growth and viability of *B. subtilis* cells before and after salt stress. Cultures were grown to an $OD_{500}$ of 0·5 ($5 \times 10^7$ cells ml$^{-1}$) and challenged with NaCl (▲, 2%, w/v; △, 4%, w/v; ●, 6%, w/v). In the preadaptation experiment (■) solid NaCl was added (final concentration 6%, w/v) after 30 min treatment with low salt (2 %, w/v). Control cells were not stressed (○). Viability was determined by plating dilutions on agar. Survival of 100 % is equivalent to $5 \times 10^7$ cells ml$^{-1}$ in this and subsequent figures. For details see Methods.

induced. However, in these earlier experiments cells were treated with 4% (w/v) NaCl only.
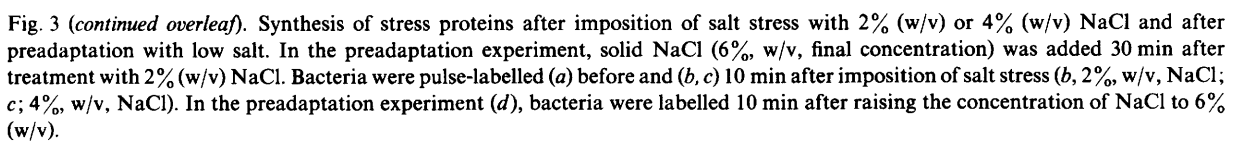
The viability of cells drastically decreased after addition of 6% (w/v) NaCl. However, after pretreatment with a low salt concentration for 30 min and addition of the remaining 4% (w/v) NaCl after this preadaptation period to reach the final concentration of 6% (w/v), *B. subtilis* cells survived the otherwise lethal treatment (Fig. 2). The pretreatment with low salt also induced stress proteins (Fig. 3).
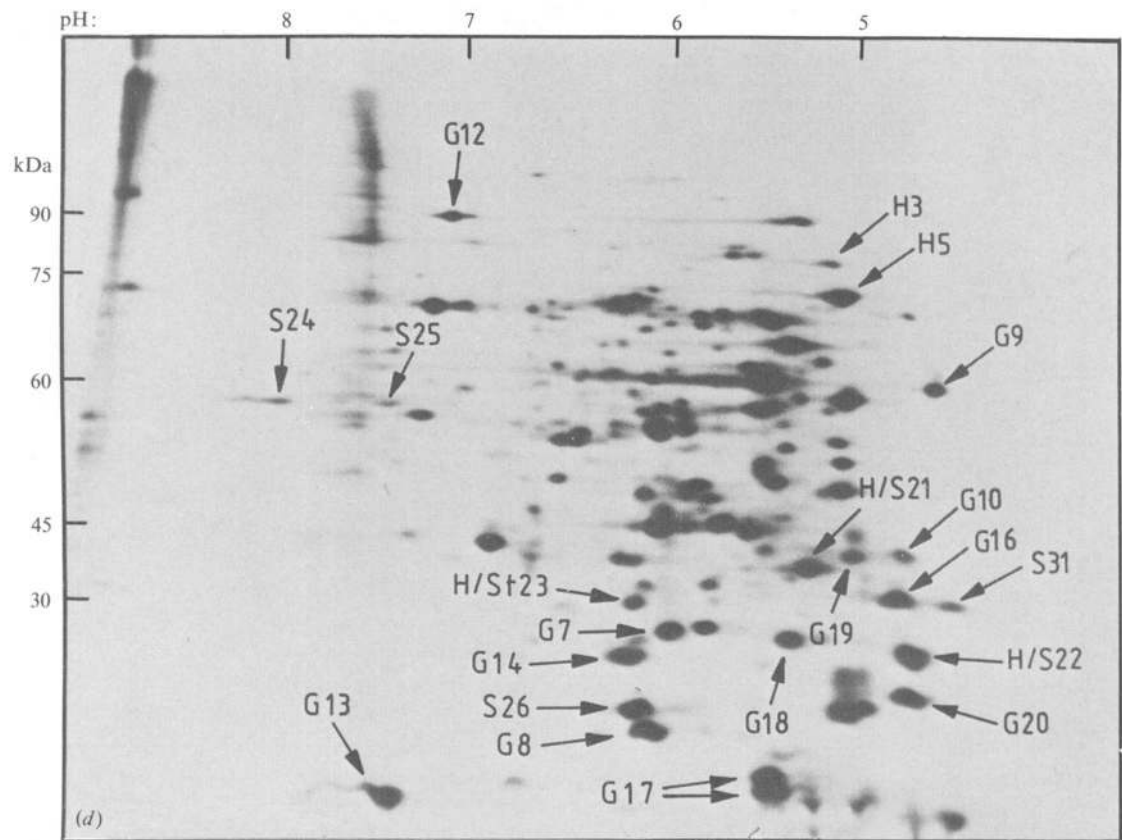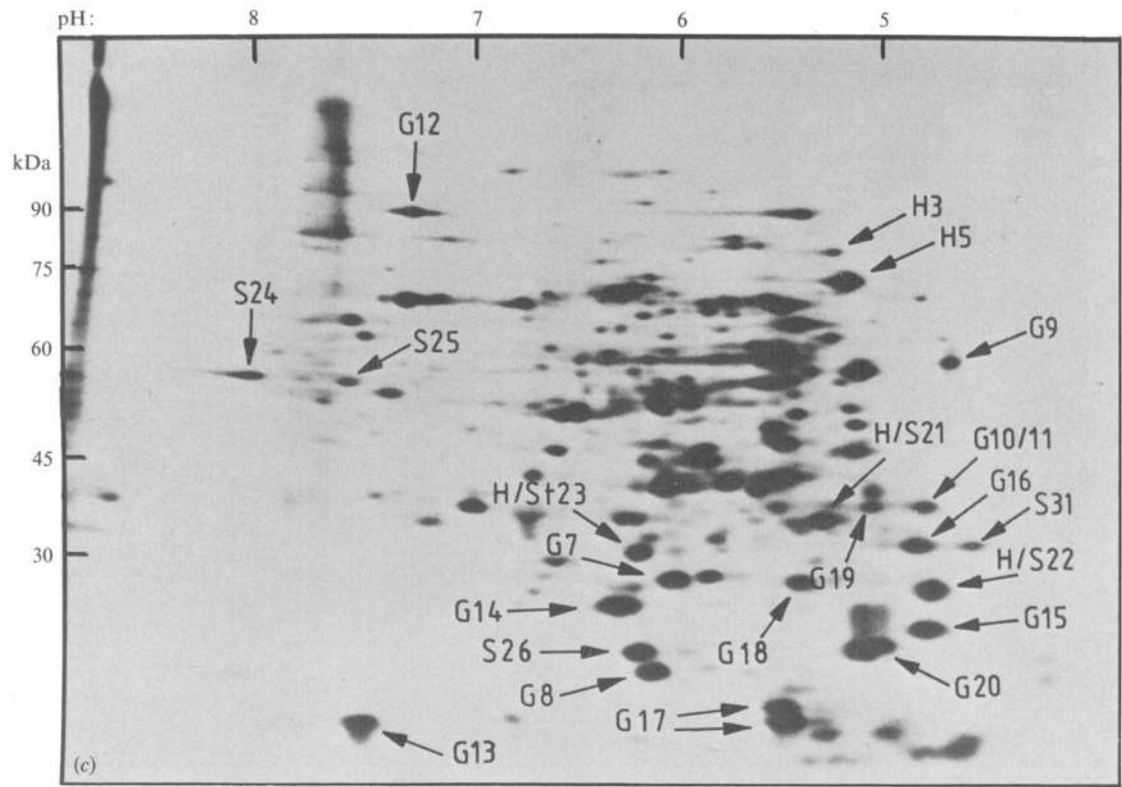
### Thermotolerance and preadaptation to a lethal temperature

Cells of *B. subtilis* IS58 cannot survive the temperature of 52 °C. However, cells exhibited a markedly enhanced survival at 52 °C after a 30 min preadaptation period at 48 °C (Fig. 4). During this short preadaptation period heat shock proteins were produced (Fig. 5; see Richter & Hecker, 1986).

### Induction of salt tolerance or thermotolerance by cross-protection experiments

A mild heat stress (30 min at 48 °C) was very effective in the induction of tolerance against otherwise lethal salt

Fig. 3 (*continued overleaf*). Synthesis of stress proteins after imposition of salt stress with 2% (w/v) or 4% (w/v) NaCl and after preadaptation with low salt. In the preadaptation experiment, solid NaCl (6%, w/v, final concentration) was added 30 min after treatment with 2% (w/v) NaCl. Bacteria were pulse-labelled (*a*) before and (*b, c*) 10 min after imposition of salt stress (*b*, 2%, w/v, NaCl; *c*; 4%, w/v, NaCl). In the preadaptation experiment (*d*), bacteria were labelled 10 min after raising the concentration of NaCl to 6% (w/v).
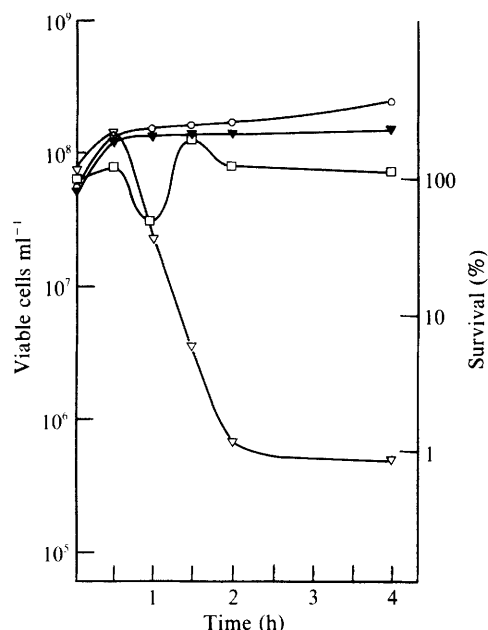
(c)



(d)

Fig. 4. Growth and viability of *B. subtilis* during heat stress. Exponentially growing bacteria were cultivated at 37 °C (○) or shifted to 48 °C (▼) and 52 °C (▽) or preadapted for 30 min at 48 °C and then transferred to 52 °C (■). For details see Methods and the legend of Fig. 2.

concentrations (Fig. 6a). A preadaptation to the lower salt concentration (4%, w/v) conferred only a partial protection against the lethal temperature of 52 °C (Fig. 6b).

*Identification of two heat-specific stress proteins and one general stress protein*

Since specific and general stress proteins seemed to be involved in the induction of stress tolerance, the N-terminal amino acid sequences of several stress proteins were determined as described in Methods. For three of the partially sequenced proteins, the heat-specific stress proteins H5 and H3 and the general stress protein G7, a homology search revealed strong homology with GroEL, DnaK and ClpP of *E. coli*, respectively (Fig. 7). For H3 and H5 the N-terminal amino acid sequences are in agreement with the data deduced from the DNA sequences of the *dnaK* locus and the *groESL* operon of *B. subtilis* (Wetzstein *et al.*, 1992; Schmidt *et al.*, 1992). G7, the ClpP homologue of *B. subtilis*, is missing the first 13 amino acids of the ClpP protein of *E. coli*.

## Discussion

Natural growth-limiting conditions, very common in nature, induce general stress proteins. In *B. subtilis* the same stress proteins are induced by a diverse range of

stresses such as heat, ethanol, hydrogen peroxide, high salt concentration, starvation for carbon or nitrogen sources, and oxygen limitation. It is tempting to speculate that these stress proteins may provide a general protection of the cell under adverse environmental conditions, but definitive evidence is still lacking. Besides these general stress proteins, all extracellular signals analysed in our studies induced a set of stress-specific proteins that may exert a specific protection against this signal and not against the others (e.g. osmotic stress – accumulation of osmoprotective substances; nutrient starvation – induction of proteins which can utilize alternative nutrient sources, etc.; see Hecker & Völker, 1990).

According to this general scheme, heat stress induced general stress proteins as well as specific heat shock proteins. The proteins H3 and H5, identified as DnaK and GroEL, belong to this group of specific heat shock proteins (Hecker & Völker, 1990). Salt stress induced not only some salt-specific proteins but also general stress proteins, although GroEL and DnaK were not induced. There is some evidence for a role of GroEL and DnaK in the growth and survival of *E. coli* at high temperatures (Kusukawa & Yura, 1988). However, definitive data on the function of stress proteins in the protection against 'physiological' stress are rare (Jenkins *et al.*, 1990, 1991; Meury & Kohiyama, 1991).

Preadaptation to a lethal thermal shock can be achieved by a pretreatment with a mild heat stress. The same is true for salt stress. Cells were enabled to survive toxic concentrations of NaCl by pretreatment with lower salt concentrations which induced general stress proteins. This finding might be taken as preliminary evidence that the function of at least some stress proteins is to protect the cells from damage by either lethal heat shock or lethal salt stress. However, the role of general stress proteins and of stress-specific proteins in the induction of thermotolerance or salt tolerance needs further investigation. As a first attempt to analyse this problem we studied the induction of stress tolerance by cross-protection experiments to determine whether a mild heat stress which did not induce salt-specific stress proteins provided a tolerance against lethal salt concentrations and vice versa. Cross-protection experiments with starving *E. coli* cells have been carried out by Matin and co-workers (Jenkins *et al.*, 1988, 1990, 1991; Matin, 1991), who found a starvation-induced cross-protection against other stresses.

*B. subtilis* cells incubated under toxic salt stress were killed more slowly if they had previously been subjected to either a mild salt stress or to a mild heat shock. Both pretreatments were similarly effective. These results may indicate that salt-specific stress proteins play an inessential role at least in this initial phase of salt tolerance.
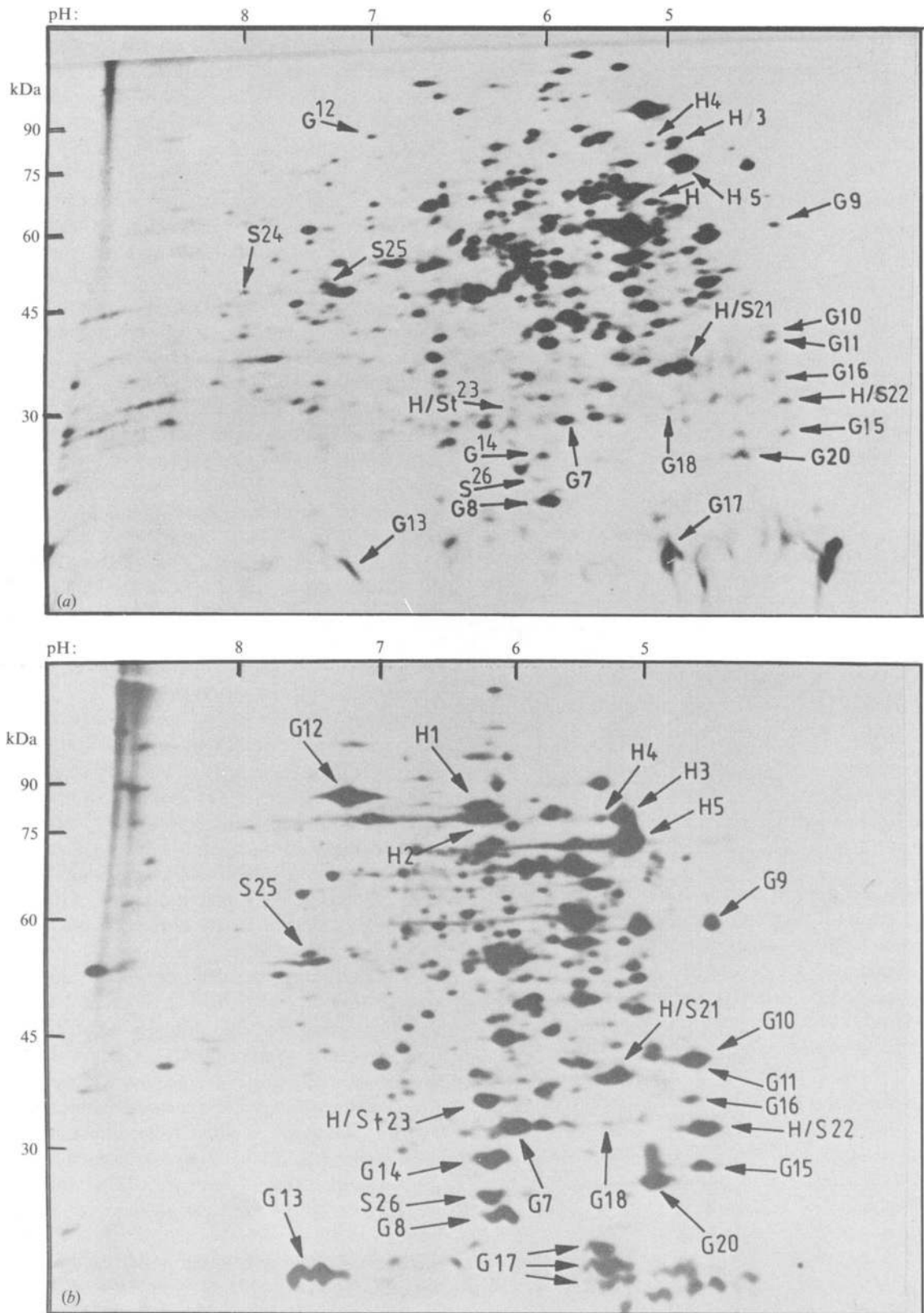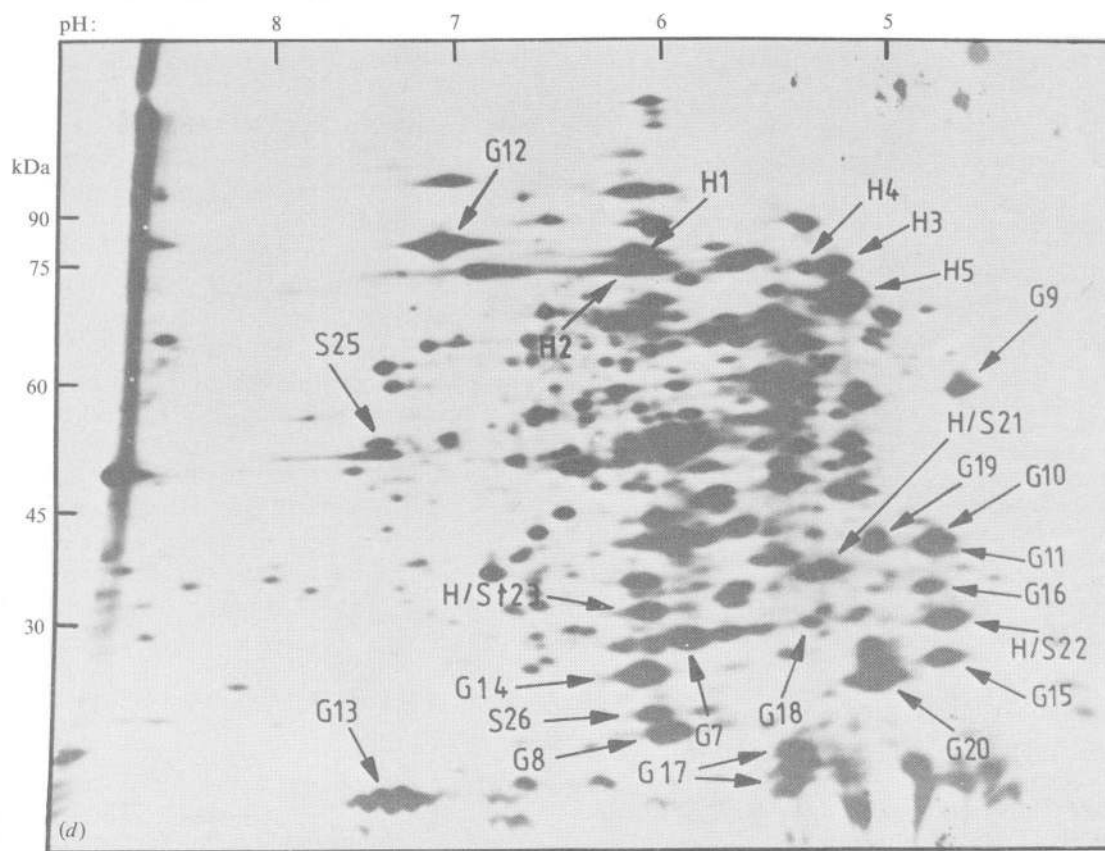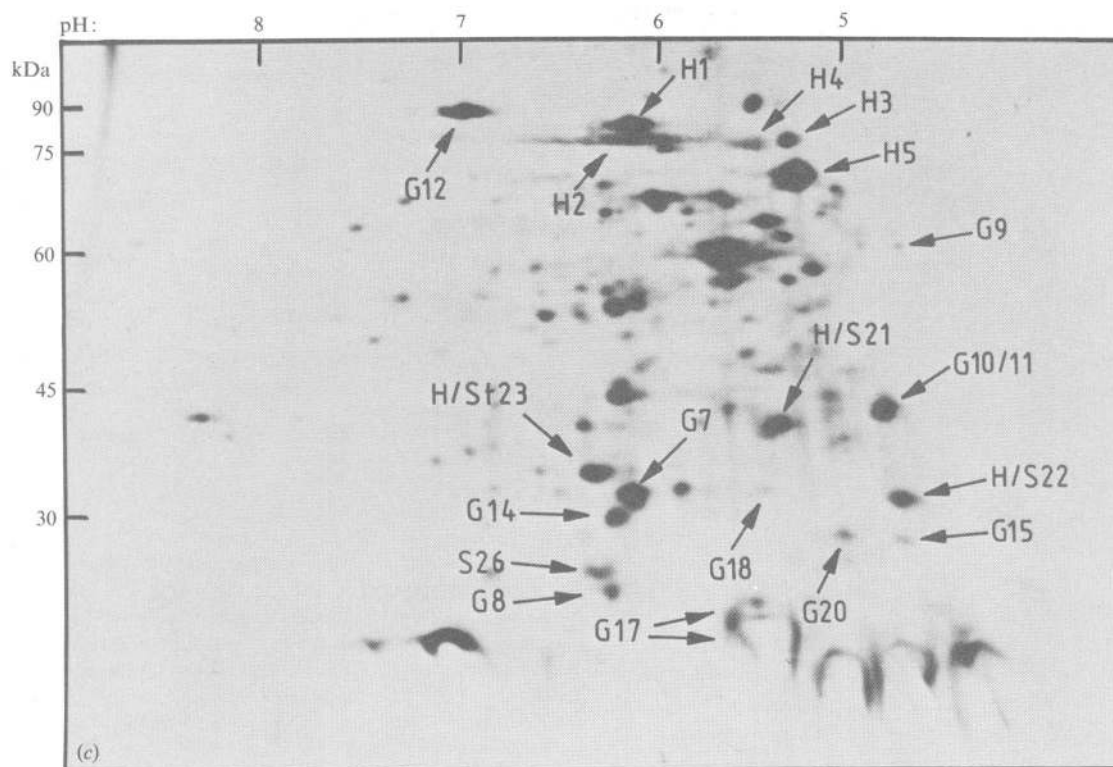
Fig. 5. Synthesis of stress proteins after heat stress and after the heat preadaptation. In this experiment the cells were preadapted for 30 min at 48 °C and then transferred to 52 °C. Pulse-labelling was carried out (*a*) before and (*b–d*) 10 min after the imposition of the final heat stress (*b*, 48 °C; *c*; 52 °C; *d*, 48–52 °C).
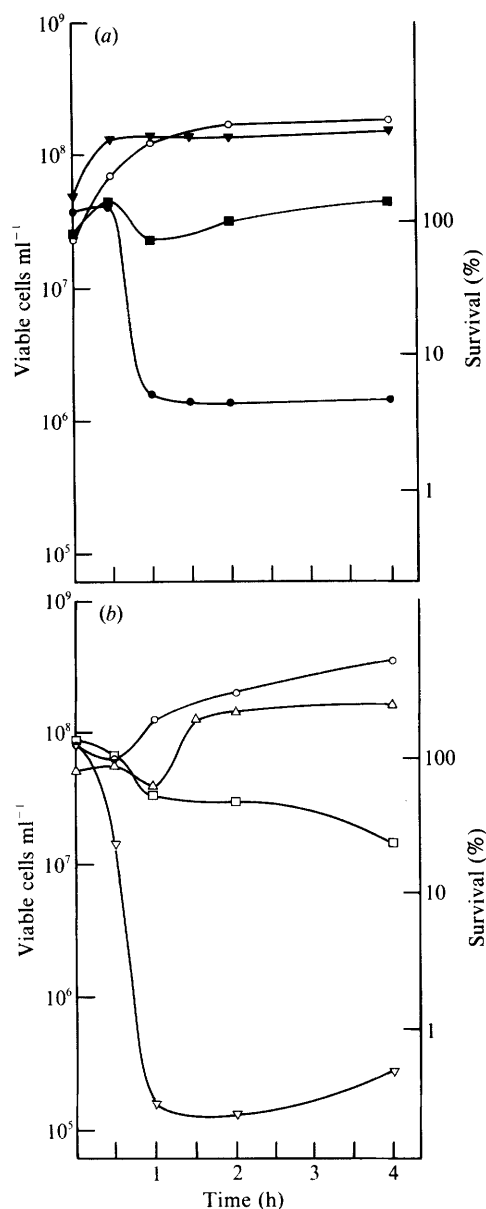
(c)



(d)

Fig. 6. Growth and viability of *B. subtilis* cells after (*a*) temperature-mediated salt tolerance and (*b*) salt-mediated thermotolerance. Cultures were grown at 37 °C (O) to an OD₅₀₀ of 0·5. (*a*) Viability was determined either after addition of solid NaCl to a final concentration of 6 % (w/v) (●) or after temperature shift up to 48 °C (▼). For the determination of cross-protection, exponentially growing cells were shifted for 30 min to 48 °C and solid NaCl (final concentration 6%, w/v) was added after this preadaptation period (■). (*b*) Viability was determined either after addition of solid NaCl (4 %, w/v) (△) or after a temperature shift up to 52 °C (▽). For cross-protection, cells treated with NaCl (4%, w/v, final concentration) for 30 min at 37 °C were shifted to 52 °C (□).



Fig. 7. Sequence alignment of the N-terminal sequences of the H5, H3 and G7 proteins of *B. subtilis* IS58 with their protein homologues. Amino acids are given in the standard one-letter code. Identical amino acids and conservative amino acid replacements are indicated by stars and colons, respectively. Numbers define the position of the residues in the complete protein. Sequence alignments: H5 aligned with *E. coli* GroEL (Hemmingsen *et al.*, 1988) and *B. subtilis* YB886 Hsp9 (Miller *et al.*, 1991); H3 aligned with *E. coli* DnaK (Bardwell & Craig, 1984); G7 aligned with *E. coli* ClpP (Maurizi *et al.*, 1990).

of specific heat shock proteins like GroEL or DnaK which may be essential for thermotolerance. Nevertheless, the different survival behaviour in pretreated and non-treated cells suggests an involvement of general stress proteins in the induction of thermotolerance and cross-protection.

The investigation of defined mutants in genes encoding general and specific stress proteins will provide exact information on the role of single stress proteins in stress tolerance. The genes coding for GroEL and DnaK have been cloned by Schumann and co-workers (Schmidt *et al.*, 1992; Wetzstein *et al.*, 1992). In the work described here these proteins have been localized on two-dimensional protein gels; mutants defective in these proteins will be studied in further experiments. Furthermore, we are currently cloning the gene encoding ClpP of *B. subtilis* using oligonucleotides deduced from the N-terminal amino acid sequence of the protein.

By contrast, the pretreatment of cells with non-toxic salt concentrations was less effective in the induction of thermotolerance than a preceding mild heat shock. This result might be explained by the failure of full induction

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