ACTA BIOCHIMICA POLONICA

Vol. 35 1988 No. 4

ANNA FABISIEWICZ and MIROSŁAWA PIECHOWSKA

HEAT-SHOCK PROTEINS IN MEMBRANE VESICLES OF *BACILLUS SUBTILIS*

Department of Molecular Biology, Institute of Biochemistry and Biophysics, Polish Academy of Sciences, Rakowiecka 36; 02-532 Warszawa, Poland

Received 19 May, 1988

Fractionation of *B. subtilis* cells after heat shock, from 37°C to 54°C, shows an increase in synthesis of proteins localized in cell membranes and a decrease in synthesis of proteins localized in cytosol. There is no such effect of heat shock at temperature of 45°C.

Autoradiograms of electrophoretically separated proteins, labelled during heat shock at 54°C, reveal 26 heat-shock proteins (hsps) in membrane vesicles and 11 hsps in cytosol, five of which are common to both fractions. Heat shock at 45°C induces 18 hsps localized in membrane vesicles and 13 hsps localized in cytosol, six of which are common to both fractions.

Results are interpreted as showing a relevant role of membrane proteins in cell response to shock at high temperature, pointing to two steps of defense against heat stress.

Heat-shock proteins are known to appear in response to various types of stress, but there is little knowledge of their localization within the cell [1 - 6]. This knowledge might lead to a better understanding of the role of particular proteins in survival of various organisms under stress conditons. Studies on cells of higher plants and animals have demonstrated the presence of stress proteins in different cellular compartments such as nuclei, chloroplasts, mitochondria, and ribosomes, whereas in cytosol only a few shock proteins have been found. Moreover, it seems of interest that proteins of the same type induced by various shock factors differ in localization; e.g. heat-shock proteins are known to occupy a particular site within the cell (for review see [2 - 6]).

Preliminary data on localization of heat-shock proteins in bacteria, namely membranes of *B. subtilis*, were presented by us in 1987 [7] and reported in the same year by Qoronfleh & Streips [8]. However, the

results reported by those authors differ rather widely from our own, probably due to differences in heat shock conditions. A more detailed description of our results is presented here.

MATERIALS AND METHODS

Bacterial strains and growth. Bacillus subtilis 168[+] (wild type) was grown in supplemented MOPS medium as described previously [9]. Methionine was omitted from the medium in the case of protein labelling with [^{35}S]methionine. Growth was followed by A_{420} measurements and viability was estimated by plating on Nutrient Broth Agar (Difco).

Heat treatment of cells and labelling of proteins. The temperature and duration of heat shock were chosen on the basis of results previously described [9]. The conditions for labelling of proteins were based on the kinetic studies of Todd *et al.* [10] who showed that maximum synthesis of hsps occurred within 5 - 15 min after shift of *B. subtilis* cells from 30°C to 43°C. In typical experiments 0.5 ml aliquots of a bacterial culture, grown exponentially at 37°C to a density of about 2×10^8 cells per ml, were transferred to Corex 15 ml-centrifuge tubes and then placed at 54°C or 37°C (control). After 1 min incubation [^{35}S]methionine was added to a final activity of 40 to 60 μCi per ml (see Results), and incubation continued for 4 min. Labelling was terminated by addition of 2.5 mg cold methionine, and incubation continued for 1 min, followed by rapid chilling in ice. In some experiments heat shock was performed at 45°C (that is within the range of growth temperature) and labelling of proteins started after 5 min incubation and continued for 10 min.

Isolation of cell membranes. Cell membranes were isolated in the form of membrane vesicles, by the method of Konings *et al.* [11], including digestion of cells with lysozyme (Sigma, Grade I), ribonuclease (bovine pancreas, Worthington), and deoxyribonuclease I (bovine pancreas, Sigma). Centrifugation and washing of cells and membranes was performed at 4°C, instead of at room temperature; hence sodium phosphate buffer was used in place of potassium buffer, to avoid precipitation of salts. Plating, after digestion with lysozyme, did not show any viable cells in the suspension of 4 mg wet weight of cells per ml buffer. Membrane vesicles were collected by centrifugation at $48\,200 \times g$, washed twice and suspended in 0.1 M sodium phosphate buffer, pH 6.6, containing 10 mM 2-mercaptoethanol and 0.5 mM phenylmethylsulfonyl fluoride (PMSF), in a volume 50 times smaller than that mentioned above.

The supernatant collected after the first centrifugation of membrane vesicles was called cytosol and analysed parallel to cell membranes.

Fractionation of cells was evaluated by estimation of NADH oxidase

activity, an enzyme marker of membranes [12]. Its presence was demonstrated in the sediment of membrane vesicles, but not in the supernatant, when assayed in a control culture growing continuously in the presence of [^{35}S]methionine. The enzymatic activity was measured by following the decrease in absorbance at 340 nm of a solution containing 150 μmol of sodium phosphate, pH 7.0; 0.2 μmol NADH, and the enzyme in the form of a suspension of membrane vesicles, or of cytosol. The samples tested contained the same amount of proteins estimated as trichloroacetic acid--precipitable radioactivity.

SDS-PAGE of proteins and their detection. Suspensions of membrane vesicles, or cytosol, were diluted twice with twofold concentrated SDS-sample buffer [14], and proteins denatured by heating in a boiling water-bath for 5 min. This procedure solubilizes membrane vesicles completely. Soluble proteins were resolved according to Laemmli [13] on SDS-polyacrylamide 12.5% slab gels (15 cm long, 0.95 mm thick). Equal amounts of radioactivity, usually 3×10^5 cpm (in trichloroacetic acid-precipitable form) were loaded on each well. Molecular mass markers were ^{14}C-methylated proteins (Amersham): myosin (200 kDa), phosphorylase *b* (92.5 kDa), bovine serum albumin (69 kDa), ovalbumin (46 kDa), carbonic anhydrase (30 kDa), and lysozyme (14.3 kDa). After electrophoresis, labelled proteins were detected by autoradiography on β-max films (Amersham).

Determination of radioactivity. The radioactivity of [^{35}S]methionine labelled proteins was determined in solutions. Three to five parallel samples were transferred onto Whatman 3MM filter paper discs followed by protein precipitation with trichloroacetic acid as described previously [14]. Radioactivity was counted with a type 1211 Rackbeta LKB Wallac scintillation counter.

RESULTS

Protein synthesis during heat shock. Samples withdrawn from a *B. subtilis* 168 culture growing exponentially at 37°C were subjected to heat shock at 54°C for 6 min. From the 2nd to the 5th minute of the shock (4 min) the cell proteins were labelled with [^{35}S]methionine, simultaneously with a control sample incubated at 37°C. After heat shock, the bacteria were collected and fractionated into membrane vesicles and cytosol. In both fractions the trichloroacetic acid precipitable radioactivity was determined after solubilization of proteins by boiling in Laemmli sample buffer [13]. The results presented in Table 1 were obtained in separate experiments on cultures differing in sensitivity to elevated temperature. These differences were manifested in variation of survival from 63% to less than 1% of the population, of initial densities from 3×10^7 to 2.5×10^8 per 0.5 ml, i.e. the volume of tested samples. In all populations studied, the amounts

Table 1

Synthesis of proteins by B. subtilis during heat shock

The number of cells in the Table is referred to the 0.5 ml sample subjected to heat shock. Conditions of shock, labelling of proteins, cell fractionation and radioactivity measurements were as described in Materials and Methods. Abbreviations used: m.v., membrane vesicles; sup., supernatant (cytosol); m.v.HS and sup.HS, the respective subcellular fractions obtained from cells subjected to heat shock; m.v.37 and sup.37, the respective subcellular fractions from the control cells incubated at 37°C.

No	Labelling (μCi per no. of cells)	Temp. (°C)	Survival (%)	Total radioactivity of cell fractions (cpm × 10^{-7})				
				m.v.	sup.	m.v. × 100 / m.v. + sup.	m.v. HS / m.v. 37	sup. HS / sup. 37
1	70 (3 × 10^7)	37	100	5.4	19	22	2.3	0.3
		54	63	12.5	5.5	70		
2	20 (7 × 10^7)	37	100	1.6	2.2	42	2.1	0.4
		54	11	3.4	0.8	81		
3	20 (25 × 10^7)	37	100	0.9	2.3	28	2.4	0.2
		54	5	2.1	0.5	82		
4	30 (15 × 10^7)	37	100	0.6	1.3	31	1.2	0.2
		54	>0.1	0.7	0.3	68		
5	45 (17 × 10^7)	37	100	1.7	5.7	23	0.7	0.9
		45	146	1.2	5.3	18		

of proteins synthesized at 54°C, and localized in membranes, was larger than that of controls labelled at 37°C (Table 1, nos. 1 - 4). Membrane vesicles from the populations in which survival ranged from 5% to 63% contained 2.1 to 2.4 times more newly synthesized proteins than control membranes. By contrast, membrane vesicles from the population in which less than 1% of cells had survived, contained only 20% more of these proteins as compared to controls. Expressing these differences in another way, we may say that membrane vesicles from cells subjected to shock at 54°C contained 68 to 82% of newly synthesized cellular proteins, whereas membrane vesicles from control cells contained only 22 to 42% of such proteins.

An opposite effect was observed in the cytosol fraction, *viz.* shock at 54°C caused a decrease in the amount of newly synthesized proteins by a factor of 2.5 to 5, as compared to the cytosol of control cells.

Heat shock under mild conditions, i.e. at 45°C, did not significantly affect the distribution of newly synthesized proteins between membrane vesicles and cytosol. In the vesicles isolated from shocked, as well as from control, cells, the amount of proteins synthesized during 10 min labelling corresponded to 20% of total labelled proteins (Table 1, no. 5). In the cytosol from cells shocked at 45°C the amount of newly synthesized proteins was also very similar to that found in cytosol of control cells (Table 1, no. 5).

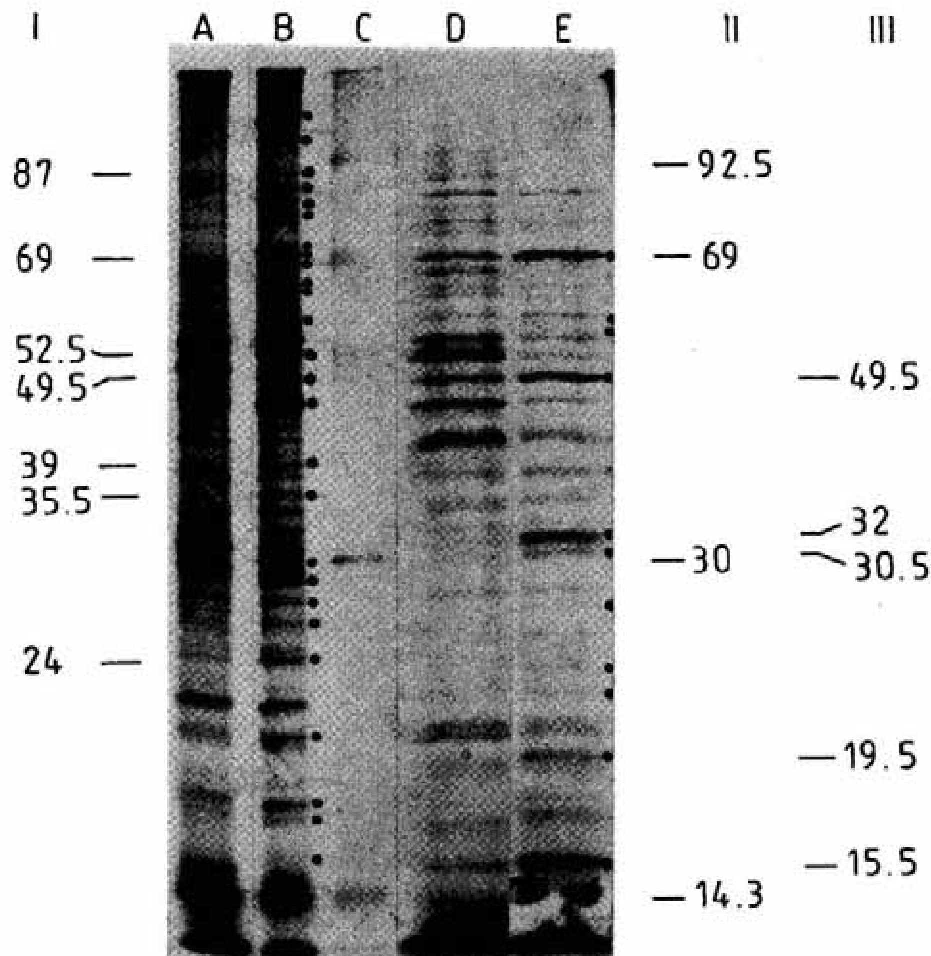

Plate 1. Autoradiogram of the SDS-polyacrylamide gel after electrophoretic resolution of ^{35}S-labelled proteins of the subcellular fractions of *B. subtilis* 168$^+$, synthesized during heat shock at 54°C. A, Proteins of membrane vesicles from the cells incubated at 37°C (control); B, proteins of membrane vesicles from the cells exposed to 54°C; C, standard proteins; D, cytosol proteins from the cells incubated at 37°C; E, cytosol proteins from the cells exposed to 54°C. All detected hsps are marked by dots. I and III, M_r values ($\times 10^{-3}$) of the main shock proteins in the membranes and cytosol, respectively; II, M_r values ($\times 10^{-3}$) of standard proteins. For details see Materials and Methods.

Electrophoretic pattern of membrane and cytosol proteins after heat shock at 54°C. From a *B. subtilis* culture growing at 37°C to a density of 14×10^7 cells per ml, samples were withdrawn and transferred to 54°C. During heat shock the proteins were labelled with [^{35}S]methionine. The cells were then collected and fractionated into membrane vesicles and cytosol (Table 1, exp. no. 2). The proteins of the two fractions were subjected to polyacrylamide gel electrophoresis. The results presented on Plate 1 show that heat shock

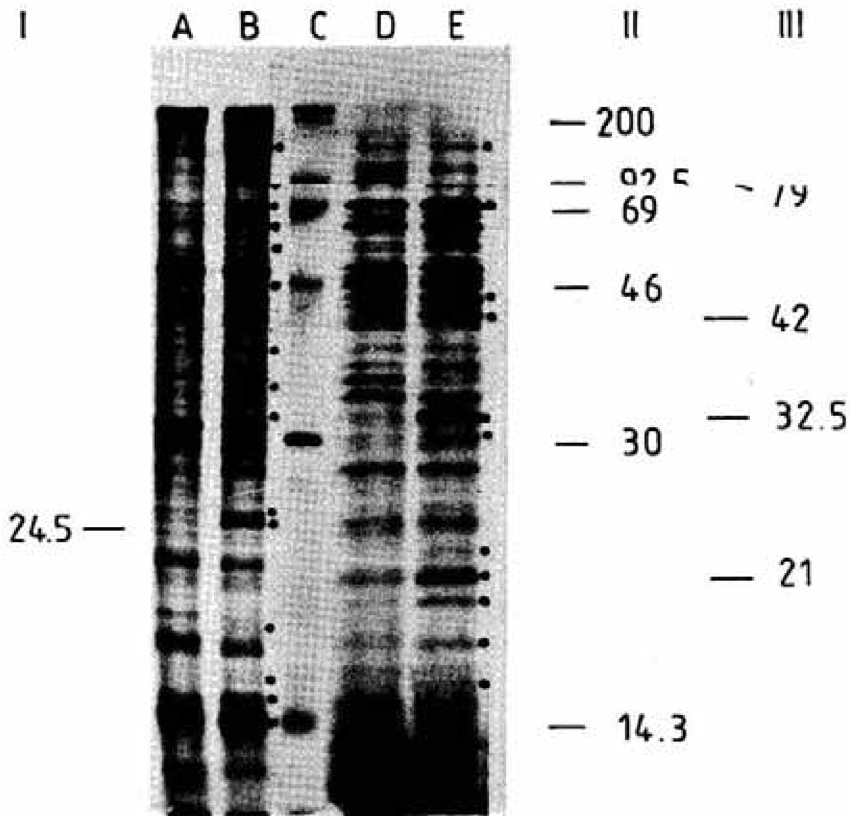

Plate 2. Autoradiogram of the SDS-polyacrylamide gel after electrophoretic resolution of ^{35}S-labelled proteins of the subcellular fractions of *B. subtilis* 168^{+}, synthesized during heat shock at 45°C. A, Proteins of membrane vesicles from the cells incubated at 37°C (control); B, proteins of membrane vesicles from the cells exposed to 45°C; C, standard proteins; D, cytosol proteins from the cells incubated at 37°C (control); E, cytosol proteins of the cells exposed to 45°C. I and III, M_r values ($\times 10^{-3}$) of the main shock proteins in the membranes and cytosol, respectively; II, M_r values ($\times 10^{-3}$) of standard proteins. For details see Materials and Methods.

evoked the appearance, or increases in intensity, of 26 protein bands in membrane vesicles, and 11 bands in cytosol. Five of the hsps are common to both, thus the total number of hsps was 32. Furthermore, as a result of heat shock, some of the protein bands become stronger in the membranes and weaker in the cytosol, e.g. those of 87, 83.5 and 52.5 kDa; by contrast, the band of 32 kDa became weaker in the membrane and stronger in the cytosol.

Hsps which, on the basis of their increased intensity or synthesis *de novo*, have been considered to be the major ones, either in the membranes or cytosol, are noted on the margins of the plate. These are the bands

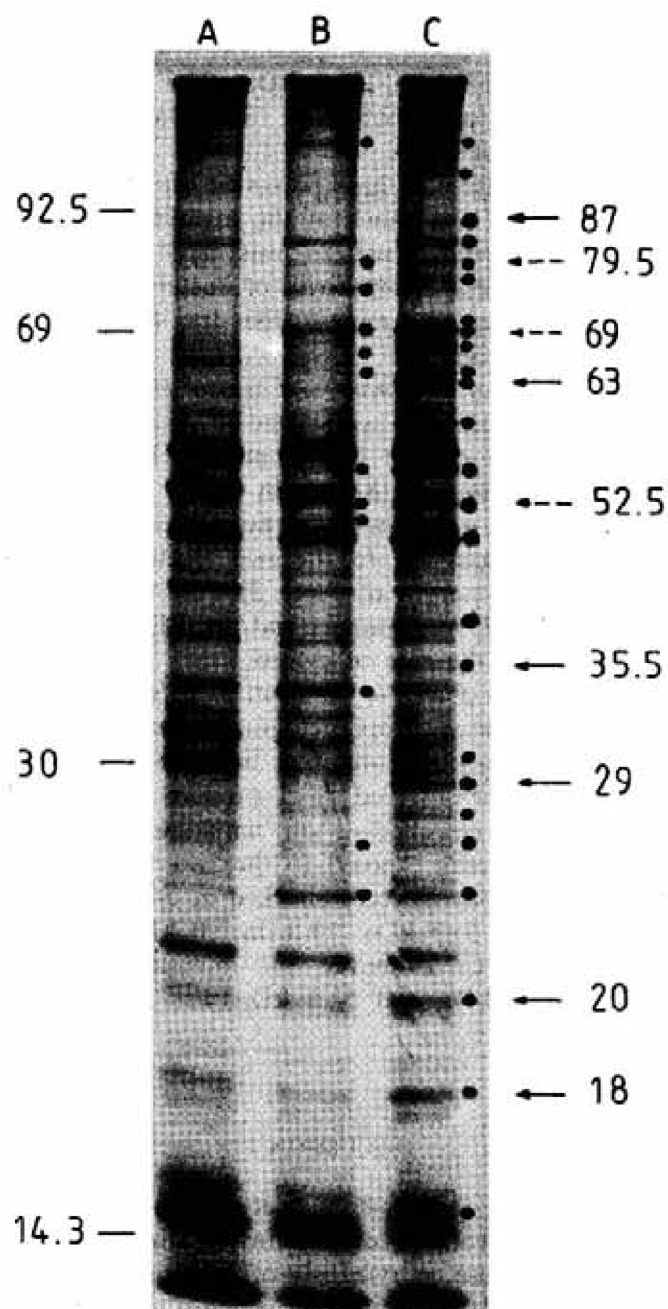

Plate 3. Autoradiogram of ^{35}S-labelled proteins of *B. subtilis* membranes, synthesized during heat shock at 45°C and 54°C, and subjected parallelly to electrophoretic separation on SDS-containing 12.5% polyacrylamide gel. For experimental details see Materials and Methods. Molecular weight values ($\times 10^{-3}$) of standard proteins are given on the left margin. Continuous arrows point to proteins induced at 54°C but not at 45°C; broken arrows point to those protein bands which are more intense after heat shock at 54°C than at 45°C.

of 87 kDa, 69 kDa, 52.5 kDa, 49.5 kDa, 35.5 kDa and 24 kDa in the case of membranes; and 49.5 kDa, 32 kDa, 30.5 kDa and 15.5 kDa in the case of cytosol.

The electrophoretic pattern of hsps presented on Plate 1 is characteristic of *B. subtilis* subjected to heat shock at the logarithmic phase of growth. Rather unexpectedly, the same pattern was most consistently observed irrespective of the differences in sensitivity of bacterial populations to heat shock at 54°C. These differences ranged from less than 1% to more than 50% survival after heat shock [9].

Electrophoretic pattern of membrane and cytosol proteins after heat shock at 45°C. The pattern of proteins synthesized at 45°C (cf. Table 1, no. 5) in membrane vesicles and cytosol is presented in Plate 2. It shows that the shock caused induction of 18 protein bands in membrane vesicles and 13 bands in the cytosol. The total number of shock proteins was 25 since 6 bands were common to the two fractions. The main shock proteins (marked on the margin of Plate 2) were those of 83 kDa and 24.5 kDa in the case of membranes, and 84 kDa, 79 kDa, 42 kDa, 32.5 kDa and 21 kDa in the case of cytosol.

Comparison of proteins found in B. subtilis cells subjected to 45° and 54°C. A comparison of the electrophoretograms presented in Plates 1 and 2 shows that, in the fractions obtained from cells exposed to higher temperature, the number of observed shock proteins was also higher. These differences become more conspicuous on simultaneous electrophoresis of membrane proteins from cells exposed to the two temperatures (Plate 3). The membrane vesicles from the cells shocked at 54°C contained six hsps, namely 87 kDa, 63 kDa, 35.5 kDa, 29 kDa, 20 kDa and 18 kDa which were not induced at 45°C, moreover, the intensity of the other three bands of 79.5 kDa, 69 kDa and 52.5 kDa was greater after exposure to 54°C than to 45°C.

DISCUSSION

The results presented above show that heat shock at 54°C causes a substantial increase in synthesis of proteins localized in cell membranes, and a simultaneous decrease in synthesis of proteins localized in cytosol. This was observed in *B. subtilis* populations at the stage of growth permitting 5 to 63% survival under shock conditions. With very low survival (less than 1% of cells) the increase in newly synthesized proteins was insignificant in cell membranes, and still distinctly lower in cytosol. By contrast, heat shock at a temperature in the range of growth (45°C) did not cause any distinct change in the distribution of the amount of newly synthesized proteins between cell membranes and cytosol.

Taken together with the known protective role of hsps [1 - 6], this indicates a relevant role for cell membrane proteins in protection of bacterial cells against the harmful effects of stress — a role visible under moderate, but not excessive, lethal conditions. This interpretation is supported by results of more detailed analysis involving electrophoretic resolution of hsps in cell fractions. More hsps were detectable in cell membranes than in the cytosol after heat shock at 54°C or 45°C; and the total number of hsps was higher at 54°C. The last observation is interpreted as revealing two stages of reaction to stress, each stage activated depending on the intensity of the stimulus acting on the cell.

Major hsps induced at 54°C are: 87 kDa, 69 kDa, 52.5 kDa, 49.5 kDa, 39 kDa, 35.5 kDa and 24 kDa proteins in cell membranes, and 49.5 kDa, 32 kDa, 30.5 kDa, 19.5 kDa, and 15.5 in cytosol; major hsps induced at 45°C are: 84 kDa, and 24.5 kDa in cell membranes, and 84 kDa, 79 kDa, 42 kDa, 32.5 kDa, and 21 kDa in cytosol. "Major" here means quantitatively dominant bands of radioactive proteins visible on electrophoretograms, but not necessarily those most important for the cell. In relation to this, it should be recalled that the very significant hsp factor δ^{32} — directly responsible for regulation of the heat shock response in *E. coli* [15], does not dominate quantitatively among other hsps.

Comparing our results with those obtained for *B. subtilis* by Qoronfleh & Streips [8] we limit ourselves to heat shock at 45°C as the closest to 48°C used by those authors. Such a comparison reveals some differences and some similarities as described below. We detected 25 hsps, 8 of them considered as prominent, of which two were localized in membranes, but 6 in the cytosol fraction. Qoronfleh & Streips detected 16 hsps, 4 of which were considered as prominent, and two of them in cell membranes and two in the cytosol fraction. Similar hsps were detected in the two laboratories, *viz.* hsp 65 kDa — localized in membranes, as well as hsps 42 kDa and 23 kDa — localized in cytosol, and detected in this work, were analogous to hsp 66 kDa — localized in membranes, and to hsps 40 kDa, and 23 kDa — localized in cytosol by Qoronfleh & Streips [8]. However, the relative proportions of hsps detected in the two laboratories were different so that, of the three hsps enumerated above, only one (42 kDa) was found as a prominent shock protein in our work, whereas all of them, plus hsp 97 kDa, were prominent in the results of Qoronfleh & Streips. These differences may be interpreted in the light of our results as showing that experimental conditions affect significantly induction of hsps and also their distribution among cell fractions.

Finally, it should be mentioned that fractionation of cells increases detectability of hsps as compared to analysis of whole lysates of cells performed in experiments preliminary to this work (not shown). Most probably this is due to a lower background in the case of proteins whose

synthesis is increased by heat shock in membranes and unchanged or decreased in cytosol or *vice versa*. Thus, fractionation of cells, as well as extreme conditions of heat shock, permitted us to detect 32 hsps in *B. subtilis* compared to 17 hsps found in *E. coli* — the highest number of hsps hitherto reported for bacterial cells [16].

REFERENCES

1. Neidhardt, F. C., VanBogelen, R. A. & Vaughn, V. (1984) The genetics and regulation of heat shock proteins. *Ann. Rev. Genet.*, **18**, 295 - 329.
2. Lindquist, S. (1986) The heat-shock response. *Ann. Rev. Biochem.*, **55**, 1151 - 1191.
3. Nagao, R. T., Kimpel, J. A., Vierling, E. & Key, J. L. (1986) The heat shock response: a comparative analysis. *Oxford Surveys of Plant molecular & Cell Biology* (Miflin, B. T., ed.) vol. 3, pp. 384 - 437. University Oxford Press.
4. Burdon, R. H. (1986) Heat shock and the heat shock proteins. *Biochem. J.*, **240**, 313 - 324.
5. Schlesinger, M. J. (1986) Heat shock proteins: the search for functions. *J. Cell Biol.*, **103**, 321 - 325.
6. Tanguay, R. M. (1985) Intracellular localization and possible functions of heat shock proteins; in *Changes in Eukaryotic Gene Expression in Response to Environmental Stress* (Atkinson, B. G. & Walden, D. B., eds.) pp. 91 - 113. Academic Press.
7. Fabisiewicz, A. & Piechowska, M. (1987) Heat shock proteins in *Bacillus subtilis* cell membranes. *18th FEBS Meeting, Ljubliana, Yugoslavia, June 28 - July 3*, Abstracts, WE 8.22 p. 164.
8. Qoronfleh, M. W. & Streips, U. N. (1987) Initial subcellular localization of heat-shock proteins in *Bacillus subtilis*. *FEMS Microbiol. Lett.*, **43**, 373 - 377.
9. Fabisiewicz, A. & Piechowska, A. (1988) Changes of sensitivity to heat shock during logarithmic growth of *Bacillus subtilis*. *Acta Biochim. Polon.*, **35**, 207 - 217.
10. Todd, J. A., Hubbard, T. J. P., Travers, A. A. & Ellar, D. J. (1985) Heat-shock proteins during growth and sporulation of *Bacillus subtilis*. *FEBS Lett.*, **188**, 209 - 214.
11. Konings, W. N., Bischop, A., Veenhuis, M. & Vermeulen, C. A. (1973) New procedure for the isolation of membrane vesicles of *Bacillus subtilis* and an electron microscopic study of their ultrastructure. *J. Bacteriol.*, **116**, 1456 - 1465.
12. Bishop, D. G., Rutberg, L. & Samuelson, B. (1967) The solubilization of the cytoplasmic membrane of *Bacillus subtilis* by sodium dodecyl sulphate. *Eur. J. Biochem.*, **2**, 454 - 459.
13. Laemmli, V. K. (1970) Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature (London)*, **227**, 680 - 685.
14. Piechowska, M., Sołtyk, A. & Shugar, D. (1975) Fate of heterologous deoxyribonucleic acid in *Bacillus subtilis*. *J. Bacteriol.* **122**, 610 - 622.
15. Grossman, A. D., Straus, D. B., Walter, W. A. & Gross, C. A. (1987) δ^{32} Synthesis can regulate the synthesis of heat-shock proteins in *Escherichia coli*. *Genes & Development*, **1**, 179 - 184.
16. VanBogelen, R. A., Kelley, P. M. & Neidhardt, F. C. (1987) Differential induction of heat shock, SOS, and oxidation stress regulons and accumulation of nucleotides in *Escherichia coli*. *J. Bacteriol.*, **169**, 26 - 32.