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ACQUISITION OF THERMOTOLERANCE DURING DEVELOPMENT OF *Blastocladiella emersonii*

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In the fungus *Blastocladiella emersonii* the synthesis of heat-shock proteins is developmentally regulated; particular subsets of heat-shock proteins are induced by heat shock during sporulation, germination and growth and some heat shock-related proteins are spontaneously expressed during sporulation (Bonato et al., 1987, Eur. J. Biochem., in press). Nevertheless, acquisition of thermotolerance can be induced at any stage of the life cycle. The development of thermotolerance is correlated with the enhanced synthesis of some heat-shock proteins: hsp 82a, hsp 82b, hsp 76, hsp 70, hsp 60, hsp 25, hsp 17b. Other hsps are not specifically involved in thermotolerance.

Elevated synthesis of heat-shock proteins (hsps) in response to a sudden increase in temperature or other environmental stresses appears to be a universal property of eucaryotic and procaryotic cells (1,2). The induction of hsps has been correlated with an increase in the capacity for thermotolerance. This phenomenon is defined as the induced capacity of cells to survive an otherwise lethal temperature after having been exposed to a heat shock or other stresses. A prior heat-shock treatment or treatment at intermediate temperatures appears to protect cells from death that might be caused by a subsequent, more severe heat challenge (3-8).

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A positive correlation between the ability of agents to induce hsps and to induce thermotolerance has been found in several organisms (5, 9-11). However, reports indicate that not all of the hsps need to be involved in this protection (7, 12). Evidence has also been presented indicating that no hsp synthesis is necessary for the induction of this acquired thermotolerance (13, 14).

In the aquatic fungus Blastocladiella emersonii the synthesis of hsps is developmentally regulated (15). Particular subsets of hsps are induced in each stage of the life cycle (sporulation, germination or growth), demonstrating a non-coordinate heat-shock gene expression. Further, heat shock-related proteins are spontaneously expressed at a high level during sporulation. Thus, we investigated the acquisition of thermotolerance and the synthesis of hsps in different stages of development to determine which heat-shock proteins play a role in the protecting effect.

MATERIALS AND METHODS

Culture conditions - Zoospores ($3x10^5$/ml) were inoculated into DM3 medium (16) followed by growth for 13 h at 19°C. Then, sporulation was induced by filtering vegetative cells (which are exponentially growing as a multinucleate cell) through a Nytex cloth (30µm), rinsing and resuspending in sporulation solution (1 mM Tris-maleate, pH 6.8, 1 mM $CaCl_2$) at the density of $5x10^5$ cells/ml. The progress and synchrony of sporulation was monitored by the formation of discharge papillae at the apical surface of sporulation cells and by the release of progeny zoospores. After 4h of starvation-induced sporulation, the released zoospores were filtered through Nytex cloth to remove sporangia ghosts. Zoospore germination was induced by inoculating zoospores at a density of $1x10^6$/ml in germination solution (1 mM Tris-maleate, pH 6.8, 1 mM $CaCl_2$, 10 mM $MgCl_2$, 50 mM KCl) or AL medium (15) which allows growth of germling cells. After 45 min of zoospore inoculation in either germination solution or AL medium virtually 100% of the cells are germlings.

The effect of heat shock on the viability of cells was determined as follows. Cell cultures at 27°C were transferred to either 38°C or 42°C or sequentially to 34, 38 and 42°C. At various intervals after the temperature shift, cell samples were removed and transferred to normal temperature (27°C). Cells were allowed to recover for 4 hours in which time starvation was induced. After 4 hours of starvation the number of zoospores released was determined. When the treatment was during sporulation, the number of zoospores was determined after 6 hours of recovery at 27°C.

One-and two-dimensional gel electrophoresis - Cells were labeled with 1-2 μCi/ml of [^{35}S]-methionine in sporulation solution or during germination in germination solution or low-methionine AL medium (5μM). Labeling was during the last 20 min (10 min in germination) of incubation at the indicated temperatures or during the entire period of thermotolerance. At the end of labeling period, cells were collected by centrifugation and protein extracts were prepared as described previously (17). The amount of radioactive methionine incorporated onto protein was determined by hot trichloroacetic acid precipitation on Whatman 3MM filters and scintillation counting immediately after lysis of the cells by sonication. Labeled extracts were analyzed by one-dimensional polyacrylamide gel electrophoresis (10%) or two-dimensional isoelectrical focusing (pH range of 5-8) as described (17).

RESULTS AND DISCUSSION

Blastocladiella cells at different stages of the life cycle (early sporulation, germination or early growth) were submitted to different conditions of hyperthermia. Cells were either exposed directly to 42°C or submitted to a pretreatment at 34°C, then to 38°C, and finally exposed to 42°C. The effect of these treatments on survival of the cells is shown in Fig. 1. Survival was determined by the ability to produce zoospores after transferring the heat-shocked cells to 27°C in order to continue the life cycle. During germination a preincubation at mild temperature (34°C for 15 min and subsequently 38°C for another 15 min) protects against the lethal effects of hyperthermia at 42°C. During growth and sporulation protection also occurs, although not so dramatic. When cells are heat-shocked at 38°C the viability is not affected (not shown).

To determine whether the development of thermotolerance is necessarily related to the synthesis of a set of specific hsps, we analyzed the pattern of protein synthesis during thermotolerance. Fig.2 shows the pattern of protein synthesis of growing cells exposed to different temperatures. The incorporation of [^{35}S] methionine into protein occurs at a high level when the incubation temperature is increased up to about 38°C; above 40°C there is a precipitous drop in incorporation. At 42°C cell protein synthesis

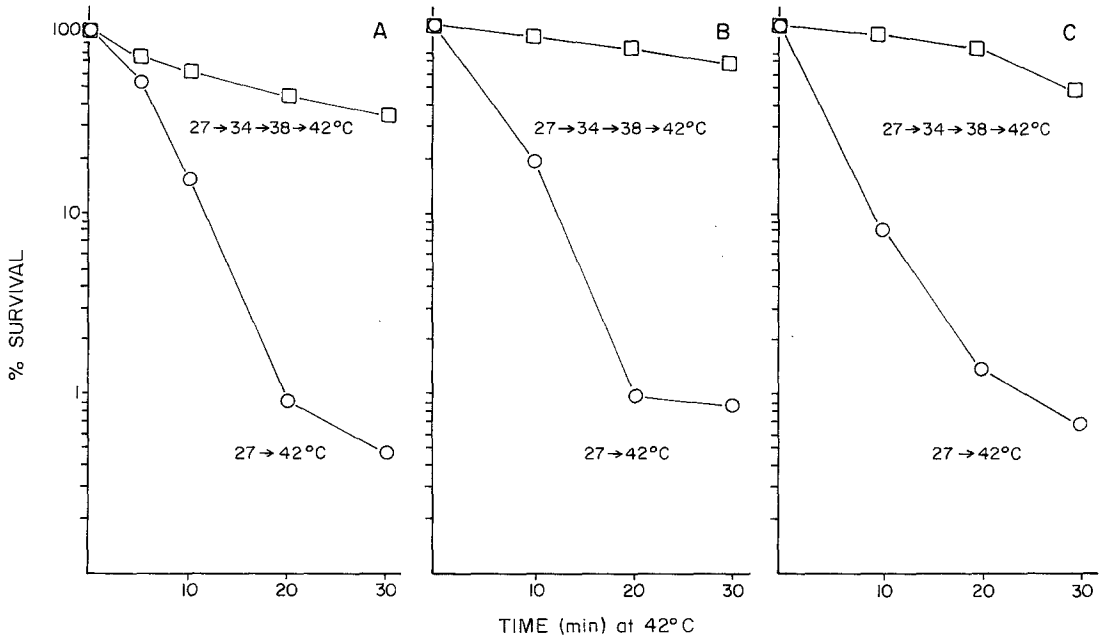

Fig.1. Heat-shock protection. (A) Sporulating cells were successively shifted to 34°C for 30 min, to 38°C for 30 min and finally to 42°C (□—□) or shifted directly to 42°C (o—o). (B) Zoospores induced to germinate in AL medium at 27°C during 15 min were shifted directly to 42°C (o—o) or successively to 34°C for 15 min, to 38°C for 15 min and then to 42°C (□-□). (C) Cells growing at 27°C in AL medium were shifted directly to 42°C (o—o) or successively to 34°C for 30 min, to 38°C for 30 min and finally to 42°C (□- -□). After the indicated times at 42°C, cells were returned to 27°C as described in Materials and Methods. 100% corresponds to control cells incubated at 27°C.

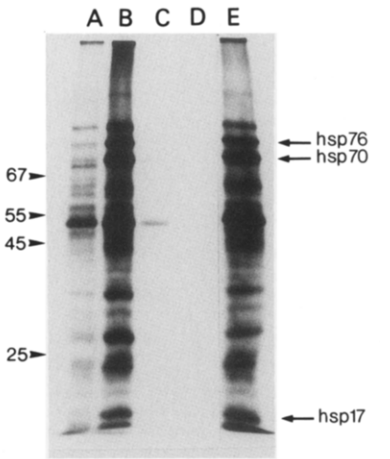

Fig. 2. One-dimensional gel pattern of proteins synthesized at different temperatures and during thermotolerance. Early growing cells were treated for 30 min at 27°C (A), 38°C (B), 40°C (C), 42°C (D) or treated for 30 min at 34°C, transferred to 37°C for 30 min and then treated for 30 min at 42°C (E). Equal amounts of proteins were applied in each lane.

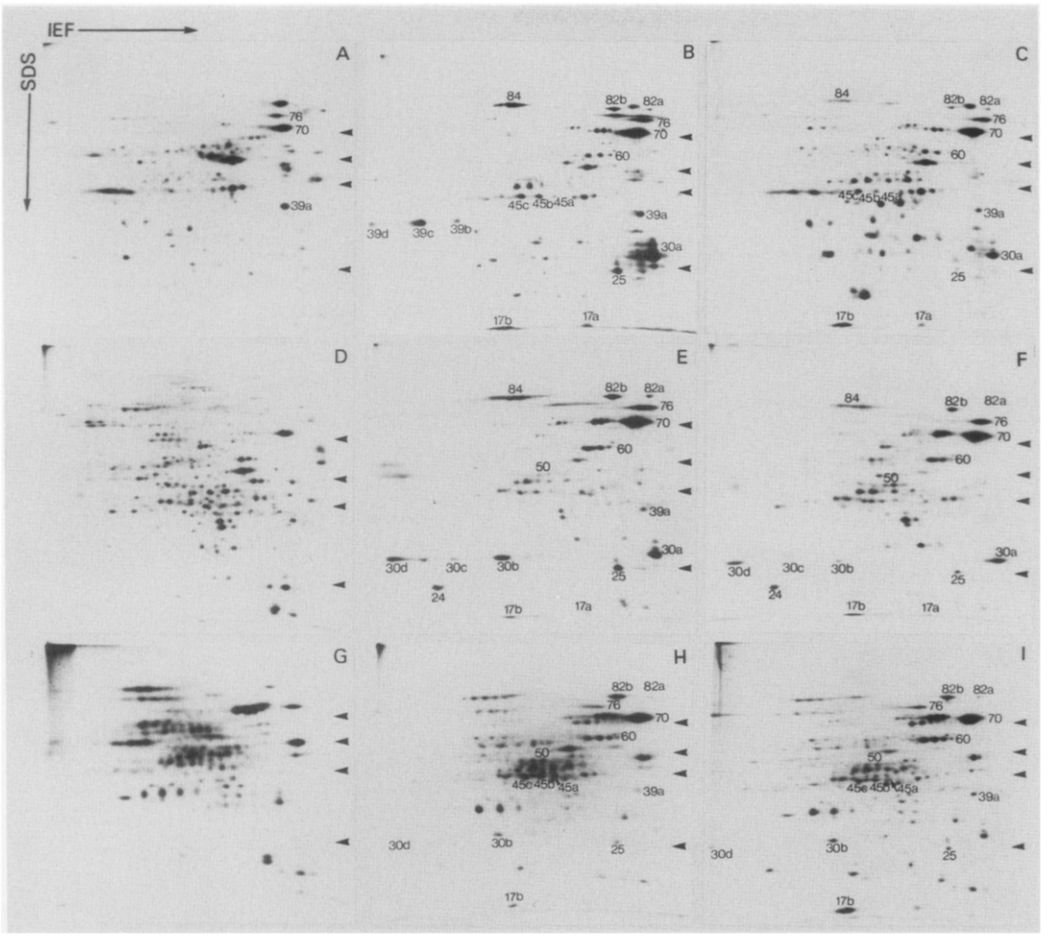

Fig. 3. Two-dimensional patterns of protein synthesis during thermotolerance induced at sporulation, germination or growth. Cells were induced to sporulate by starvation in sporulation solution; aliquots were kept at 27°C for 90 min (A) or heat-shocked at 38°C in the interval 60-90 min (B) or shifted successively to 34°C, 38°C and 42°C during the intervals 0-30 min, 30-60 min and 60-90 min, respectively, after the onset of sporulation[c] Zoospores were induced to germinate in inorganic solution and aliquots were kept at 27°C (D) or heat-shocked at 38°C during the interval 45-60 min (E) or transferred successively to 34°C, 38°C and 42°C during the intervals 15-30 min, 30-45 min, 45-60 min, respectively after the onset of germination (F). Early growing cells (120 min after inoculation of zoospores in AL medium) were kept at 27°C for 30 min (G), or heat-shocked for 30 min at 38°C (H), or transferred successively to 34°C, 38°C and 42°C for 30 min in each temperature (I). Arrowheads indicate molecular mass markers in descending order (67, 55, 45 and 25 kDa).

is decreased by >95%. Synthesis of specific hsps is seen when growing cells are exposed to 38°C for 30 min. Similar hsps are also seen when cells are initially exposed to 34°C, transferred to 38°C, and finally to 42°C. In Fig. 3, two-dimensional fluorograms of pro-

TABLE 1. HEAT-SHOCK PROTEINS INDUCED DURING THERMOTOLERANCE (TT) AT DIFFERENT STAGES OF THE LIFE CYCLE

hsps (kDa)	sporulation		late germination		early growth	
	38°C	TT	38°C	TT	38°C	TT
84	+	+	+	+	-	-
82a	+	+	+	+	+	+
82b	+	+	+	+	+	+
76*	+	+	+	+	+	+
70*	+	+	+	+	+	+
60	+	+	+	+	+	+
50	-	-	+	+	+	+
45a	+	+	-	-	+	+
45b	+	+	-	-	+	+
45c	+	+	-	-	+	+
39a*	+	+	+	-	+	+
39b	+	-	-	-	-	-
39c	+	-	-	-	-	-
39d	+	-	-	-	-	-
30a	+	+	+	+	-	-
30b	-	-	+	+	+	+
30c	-	-	+	+	-	-
30d	-	-	+	+	+	+
25	+	+	+	+	+	+
24	-	-	+	+	-	-
17a	+	+	+	+	-	-
17b	+	+	+	+	+	+

*these proteins are spontaneously synthesized during sporulation

teins synthesized during thermotolerance at different stages of the life cycle are compared to the patterns obtained at normal temperature and at 38°C. Table 1 summarizes these results. As shown previously, exposure of cells at 38°C induces the synthesis of specific sets of hsps depending on the developmental stage of cells. During thermotolerance the majority, if not all, of the hsps typical of either sporulation, or germination, or growth is also induced. The enhanced synthesis of several hsps: 82a, 82b, 76, 70, 60, 25, 17b correlates well with the development of thermotolerance. It is possible that the other hsps (84, 50, 45a, 45b, 45c, 39a, 39b, 39c, 39d, 30a, 30b, 30c, 30d, 24 and 17a) are not specifically involved in thermotolerance.

Since during sporulation heat shock-related proteins (hsp 76, hsp 70 and hsp 39a) are constitutively expressed (15), we

expected an increased thermoresistance of cells during sporulation. However, these cells are apparently as thermosensitive as growing or germinating cells. Thus, if these hsps play some role in thermoprotection they do that in concert with other hsps.

If synthesis of hsps is blocked with cycloheximide during the period at 34 and 38°C, then the cells are not protected from rapid killing at 42°C (not shown).

A strong correlation between the expression of the hsps and the development of thermotolerance has been found in several organisms (4, 7, 8, 10). The strongest evidence for a link between these two phenomena is found is Escherichia coli, where a strain with a mutation of the heat shock induction (hin) regulatory gene fails to synthesize hsps and is unable to acquire thermotolerance (11). In eucaryotes the evidence is less clear cut. Evidences have been presented indicating that no hsps synthesis is necessary for the induction of the acquired thermotolerance (13, 14). In contrast, some reports have specifically implicated a class of hsps (the small hsps) in thermoprotection (7, 12, 18), although recently it has been shown that the small hsp 26 is not required for thermotolerance in yeast (19). In Chinese hamster fibroblasts increased levels of the hsp 70 correlated well with the increased heat survival (20). In S. cerevisiae, the 48 kDa heat-shock protein may be responsible for thermotolerance (21, 22).

Thus, it is possible that most of the hsps are not specifically involved in thermotolerance. B. emersonii is a suitable organism to study the role of hsps on thermotolerance since differential expression of hsps occurs during heat shock at different stages of development and a spontaneous synthesis of some hsps is observed during sporulation (15). Our results demonstrate that only some of a group of 22 hsps are specifically involved in the phenomenon of acquisition of thermotolerance.

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