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Heat Shock Proteins of Vegetative and Fruiting *Myxococcus xanthus* Cells

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The heat shock response of *Myxococcus xanthus* was investigated and characterized. When shifted from 28 to 40°C, log-phase cells rapidly ceased growth, exhibited a 50% reduction in CFU, and initiated the synthesis of heat shock proteins (HTPs). Heat-shocked log-phase *M. xanthus* cells labeled with [^{35}S]methionine were found to produce 18 major HTPs. The HTPs, analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis and fluorography, were characterized with regard to molecular mass, subcellular location (periplasm, membrane, or cytoplasm), and temperature required for expression. Most HTPs were expressed at 36°C, the optimum growth temperature of *M. xanthus*. Cells preincubated at 36°C for 1 h before being shifted to 40°C demonstrated increased thermotolerance compared with cells shifted directly from 28 to 40°C. The HTPs produced by heat-shocked starvation-induced fruiting cells and glycerol-induced sporulating cells were also analyzed and characterized. Thirteen HTPs were detected in fruiting cells shifted from 28 to 40°C. Six of these HTPs were not seen in vegetative *M. xanthus* cells. Log-phase cells induced to sporulate by the addition of glycerol produced 17 HTPs after being shifted to 40°C. These HTPs were found to be a mixture of HTPs detected in heat-shocked log-phase cells and heat-shocked fruiting cells.

Myxococcus xanthus is a gram-negative, gliding, rod-shaped bacterium that is commonly found in soil where it grows by preying upon other microorganisms and by degrading complex organic matter (14, 29). When subjected to starvation on a solid surface, vegetative swarms of *M. xanthus* reverse their outward growth and migrate inwards to localized centers of aggregation, forming raised mounds of cells. Within these mounds, individual cells convert to ovoid or round, environmentally resistant myxospores. These mounds of myxospores are termed fruiting bodies (19). Rapid, synchronous myxospore formation may also be induced in vegetative *M. xanthus* cells by the addition of glycerol (to 0.5 M), dimethyl sulfoxide, or a variety of alcohols (6, 15, 30). In contrast to fruiting body formation which occurs over 48 to 72 h and requires a solid surface, glycerol-induced myxospores form within 3 h, do not aggregate, and do not require a solid surface (6). Glycerol-induced myxospores also lack some of the proteins characteristic of starvation-induced fruiting cells (15).

The intent of this investigation was to examine the response of *M. xanthus* to heat shock. It has been shown that organisms from all three urkingdoms respond to elevated temperatures by altering their patterns of growth and protein synthesis. Upon experiencing elevated temperatures, cells rapidly cease growth, repress the synthesis of most proteins, and begin to preferentially synthesize a small number of new proteins (21). This has been termed the heat shock response. Studies have shown that some of the genes coding for heat shock proteins (HTPs) are highly conserved across all three urkingdoms (1, 21). In this paper, we demonstrate that *M. xanthus* exhibits the heat shock response. HTPs produced by vegetative cells, starvation-induced fruiting cells, and glycerol-induced sporulating cells were analyzed and characterized with regard to molecular weight and localization in the periplasmic, membrane, or cytoplasmic fractions. We show that each cell type produces a similar but slightly altered set of HTPs. Additionally, we demonstrate that the expression of HTPs is temperature dependent. Further, we present data that suggest that preinduction of HTP synthesis protects *M. xanthus* cells from the lethal effects of incubation at elevated temperatures.

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MATERIALS AND METHODS

Cells and growth conditions. *M. xanthus* FB (DZF1), derived from DK101 (8), was used in all experiments. Vegetative cultures were grown in Casitone-yeast extract (CYE; Difco Laboratories, Detroit, Mich.) broth and maintained on CYE agar plates (4). Growth of vegetative cultures was monitored spectrophotometrically at 600 nm or by determination of the number of CFU per milliliter. To determine the number of CFU per milliliter, samples (0.1 ml) were withdrawn from cultures and diluted aseptically into sterile CYE broth, plated onto CYE agar plates, and incubated at 28°C for 5 days. Development of fruiting bodies was induced on clone fruiting (CF) agar (10). CF agar plates were routinely dried at 35°C for 3 to 4 days before use.

Heat shock conditions. Logarithmically growing *M. xanthus* cells (0.055 to 0.080 optical density [600 nm] units) were heat shocked by transfer to an incubator set to the appropriate elevated temperature (36, 40, or 42°C). For recovery studies, the cells were returned to 28°C immediately following the period at high temperature.

Isotope labeling conditions. Vegetative *M. xanthus* cells were prepared for labeling with [^{35}S]methionine by growing the cells at 28°C in A-1 medium (3) to a density of about 1.25 $\times 10^8$ cells per ml. The cells were centrifuged (10,000 $\times g$ for 5 min at 4°C), washed once in 10 mM Tris hydrochloride (pH 7.6)–8 mM $MgSO_4$ (TM buffer), and suspended to the same density in A-1 medium without methionine (A-1-met). Cells were then either kept at 28°C for 5 min or shifted immediately to an elevated temperature before being pulsed with 10 μCi of [^{35}S]methionine (specific activity, 1,123 Ci/mmol; New England Nuclear Corp., Boston, Mass.) per ml as indicated in the figure legends for each experiment. Developmental *M. xanthus* cells were prepared as described previously (23). After 20 h of development at 28°C on CF

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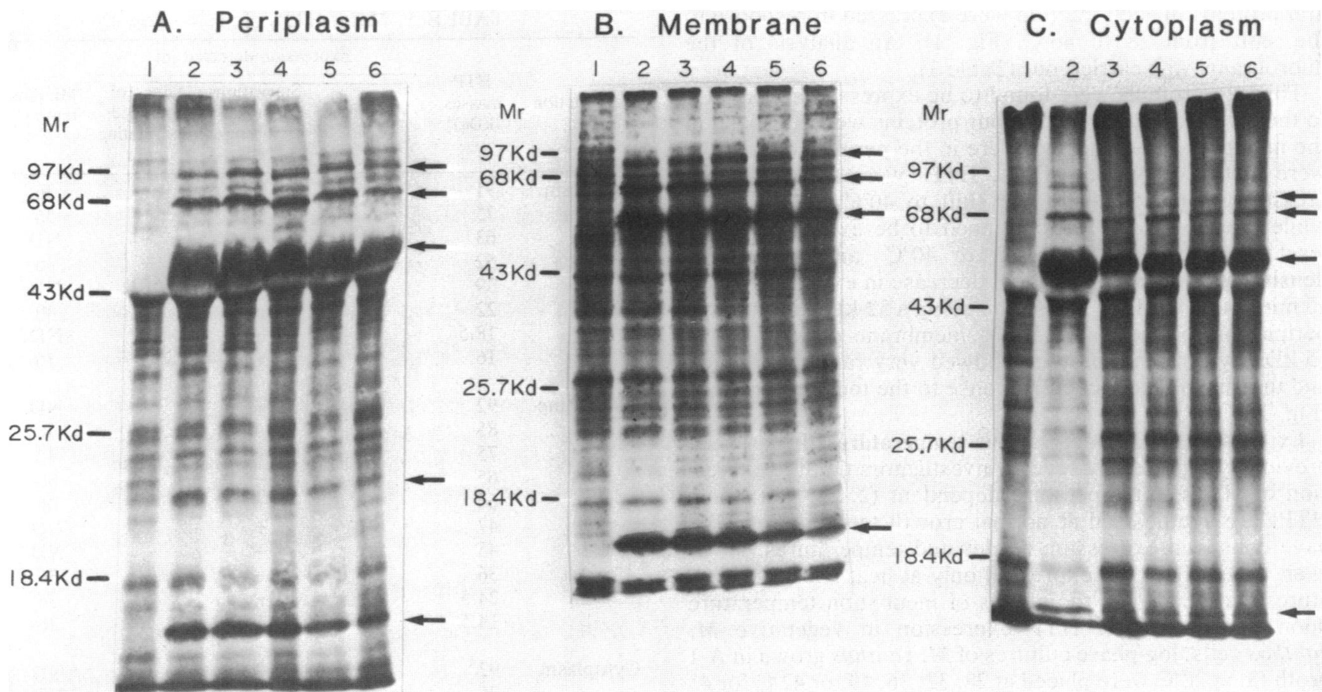

FIG. 1. The production of HTPs by vegetative *M. xanthus* cells at 40°C. A culture grown at 28°C in A-1 medium was suspended in A-1-met as described in the text. Half of the cells were placed at 28°C, and after 5 min a 1-ml sample was withdrawn and labeled with 10 μCi of [^{35}S]methionine for 10 min (lane 1). The other half of the cells were shifted to 40°C. Samples (1 ml) were withdrawn after 15 min (lane 2), 30 min (lane 3), 45 min (lane 4), 60 min (lane 5), and 90 min (lane 6) and labeled with 10 μCi of [^{35}S]methionine for 5 min. Labeled cells were harvested by centrifugation (8,000 × *g*, 5 min at 4°C) and fractionated to yield periplasmic (A), membrane (B), and cytoplasmic (C) fractions. Molecular masses are indicated in kilodaltons. HTPs are indicated by the arrows.

agar plates, the cells were harvested and resuspended in CF broth as described previously (24) and then either kept at 28°C for 5 min or placed at 40°C before being pulsed with 10 μCi of [^{35}S]methionine per ml as indicated in the figure legends. Vegetative *M. xanthus* cells growing in A-1 medium were induced to form myxospores by the addition of glycerol to 0.5 M (6). Such cells were prepared for labeling exactly as described above for vegetative cells, except that after suspension in A-1-met, glycerol was added to 0.5 M. Specific labeling conditions are given below (see legend to Fig. 5).

Cell fractionation procedures. The procedures described by Nelson et al. (22) were used to obtain the periplasmic, cytoplasmic, and membrane fractions of *M. xanthus* cells. The fractions were checked for cross contamination by assaying each for specific enzyme markers: alkaline phosphatase (periplasmic [5]), succinic dehydrogenase (membrane [17]), and glutamic dehydrogenase (cytoplasmic [7]). All fractions showed less than 10% cross contamination.

Electrophoretic methods. Polyacrylamide gel electrophoresis was carried out on 0.8-mm-thick 11% polyacrylamide slab gels, using the buffer system of Laemmli (18). Protein samples for polyacrylamide gel electrophoresis were concentrated by precipitation in 10% trichloroacetic acid. Equal amounts of radioactive material were loaded into each sample well. Polyacrylamide gels were usually fixed in a solution of 25% isopropanol and 10% acetic acid for 1 h before preparation for fluorography. Gels were prepared for fluorography by soaking in Fluoro-Hance (Research Products International Corp., Mount Prospect, Ill.) according to the instructions of the manufacturer.

RESULTS

Effect of elevated temperature on cell growth. The growth of *M. xanthus* cultures was followed at three temperatures: 28, 36, and 40°C. Cells grown at 28°C doubled every 6 h. When cultures were shifted from 28, to 36°C, the generation time decreased to 3.3 h. However, cells grown at 28°C and shifted to 40°C rapidly ceased growth and experienced a 50% decline in CFU within 60 min of the shift. The number of viable cells did not decrease further over the next 60 min at 40°C, and when the culture was shifted back down to 28°C, the cells immediately resumed growth. These data are consistent with those previously reported for *M. xanthus* by Janssen et al. (13), who reported that the optimal growth temperature was between 34 and 36°C. Since 40°C was only 4°C higher than the optimal growth temperature, it was chosen as the heat shock temperature for subsequent experiments.

Production of HTPs by vegetative *M. xanthus* cells. In *Escherichia coli*, the HTP regulon consists of genes coding for 17 proteins that are expressed in response to stress (e.g., elevated temperatures and ethanol [21]). To characterize the proteins expressed during heat shock in *M. xanthus*, aliquots of exponentially growing cells at 28°C were pulse-labeled with [^{35}S]methionine before and at various times after being shifted to 40°C. Subsequently, the labeled cells were fractionated to yield periplasmic, cytoplasmic, and membrane fractions. The proteins in each fraction were then analyzed by sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis and fluorography. The fluorograms show that sev-

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eral proteins in each fraction were expressed in response to the shift from 28 to 40°C (Fig. 1). An analysis of the fluorograms was carried out (Table 1).

Thirteen proteins were found to be expressed in response to the shift from 28 to 40°C. Four proteins were localized in the membrane fraction, five were in the periplasm, and five were in the cytoplasm. These HTPs were maximally expressed within 15 min of the shift to 40°C. Additionally, while most of the HTPs continued to be expressed for at least 90 min after the shift to 40°C, an analysis by densitometry showed a gradual decrease in expression after 15 min. Several of the HTPs, including a 52-kilodalton (kDa) periplasmic protein, a 14.5-kDa membrane protein, and a 55-kDa cytoplasmic protein, showed very rapid expression and unusual abundance in response to the temperature shift (Fig. 1).

Expression of HTPs at various temperatures. It has been previously reported by several investigators that the expression of HTPs is temperature dependent (2, 21, 25). Some HTPs are synthesized at normal growth temperatures, but have enhanced expression at elevated temperatures. However, other HTPs are expressed only at heat shock temperatures. To determine the effects of incubation temperature upon the pattern of HTP expression in vegetative *M. xanthus* cells, log-phase cultures of *M. xanthus* grown in A-1 broth (3) at 28°C were placed at 28, 32, 36, 40, or 42°C for 45 min and then pulse-labeled with [^{35}S]methionine. The labeled cells were fractionated to yield periplasmic, cytoplasmic, and membrane fractions. The proteins in each fraction were analyzed by SDS-polyacrylamide gel electrophoresis and fluorography. The fluorograms shown below (see Fig. 4) are the results of one such experiment and are summarized in Table 1. In general, HTPs produced by vegetative *M. xanthus* cells showed temperature-dependent expression (Fig. 2). HTPs were difficult to detect at temperatures below 36°C, and HTPs first detected at 36 or 40°C showed greatly increased expression at higher temperatures. Further, some HTPs were only detected at 42°C, the maximum temperature used in this study (Table 1).

Induction of thermotolerance by heat shock. To determine whether the synthesis of HTPs at 36°C could induce thermotolerance and protect *M. xanthus* cells from the lethal effects of heat shock at 40°C, the following experiment was carried out. An *M. xanthus* culture was grown at 28°C and divided in half. One half of the culture was kept at 28°C, while the other half was shifted to 36°C. After 1 h, both cultures were shifted to 40°C. Throughout the experiment the cultures were monitored for changes in CFU. *M. xanthus* cells incubated at 36°C for 1 h before being shifted to 40°C are more thermotolerant than cells shifted directly from 28 to 40°C (Fig. 3). These data suggest that the synthesis of HTPs by cells incubated at 36°C increases thermotolerance.

Heat shock response of *M. xanthus* cells during fruiting body formation. To determine whether developmental *M. xanthus* cells exhibited the same response to heat shock as vegetative cells, *M. xanthus* cells were allowed to fruit on CF agar at 28°C for 20 h. At this time the cells had begun to aggregate into mounds, but no spores were detected. Half the cells were then shifted to 40°C and after 30 min pulse-labeled with [^{35}S]methionine. The remaining cells were kept at 28°C and labeled with [^{35}S]methionine. The labeled cells were fractionated to yield periplasmic, cytoplasmic, and membrane fractions. The proteins in each fraction were analyzed by SDS-polyacrylamide gel electrophoresis and fluorography. In some experiments, protein extracts from both vegetative and fruiting cells were analyzed on the same gels to directly compare the HTPs (data not shown). Thirteen HTPs were detected in the heat-shocked, starvation-induced (20 h) fruiting *M. xanthus* cells (Fig. 4). Six of these proteins are common to both vegetative and fruiting cells, while the other seven are found only in developing cells (Table 1).

TABLE 1. Major HTPs of *M. xanthus*

Cell fraction	HTP masses (kDa)[a]	Expression detected in: Vegetative cells	Starvation-induced fruiting cells	Glycerol-induced sporulating cells	Minimum temp (°C)
Periplasm	91	+	+	+	36
	75	+	+	+	36
	63	−	+	+	ND[b]
	52	+	+	−	36
	39	−	+	−	ND
	22	+	−	+	40
	18.5	−	−	+	ND
	16	+	+	+	36
Membrane	92	−	−	+	ND
	85	+	−	+	36
	75	+	−	+	42
	65	+	−	−	36
	60	+	−	+	36
	47	−	+	−	ND
	45	−	+	−	ND
	36	+	−	−	36
	24.5	+	+	−	36
	14.5	+	−	+	36
Cytoplasm	92	−	+	+	ND
	75	+	+	+	ND
	69	+	−	+	40
	55	+	+	−	36
	50	+	−	−	42
	42	+	−	−	42
	25	−	−	+	ND
	23	−	+	+	ND
	20	−	−	+	ND
	16	+	−	−	36

[a] Molecular masses of proteins estimated by comparison to molecular standards separated on the same SDS-polyacrylamide gel.
[b] ND, Not determined.

Heat shock response of *M. xanthus* cells during glycerol-induced myxospore formation. *M. xanthus* cells can be induced to form myxospores while growing vegetatively by the addition of 0.5 M glycerol, dimethyl sulfoxide, or a variety of other alcohols (6, 16, 32). We therefore determined whether glycerol-induced cells exhibited a heat shock response. Exponentially growing cells in A-1 medium were incubated at 28°C. Glycerol was added to a final concentration of 0.5 M. Half of the *M. xanthus* cells were allowed to develop at 28°C for 60 min and then labeled for 30 min at 28°C with [^{35}S]methionine. These cells had begun to shorten, but no spores had formed. The remainder of the cells were incubated at 28°C for 30 min, shifted to 40°C for 30 min to induce the heat shock response, and then pulse-labeled with [^{35}S]methionine at 40°C for 30 min. The cells were harvested and fractionated to yield periplasmic, membrane, and cytoplasmic fractions. The proteins in each fraction were analyzed by SDS-polyacrylamide gel electrophoresis and fluorography. In some experiments, protein extracts from heat-shocked glycerol-induced cells were analyzed on gels containing protein extracts from either heat-shocked vegetative or fruiting cells to directly compare the HTPs (data not

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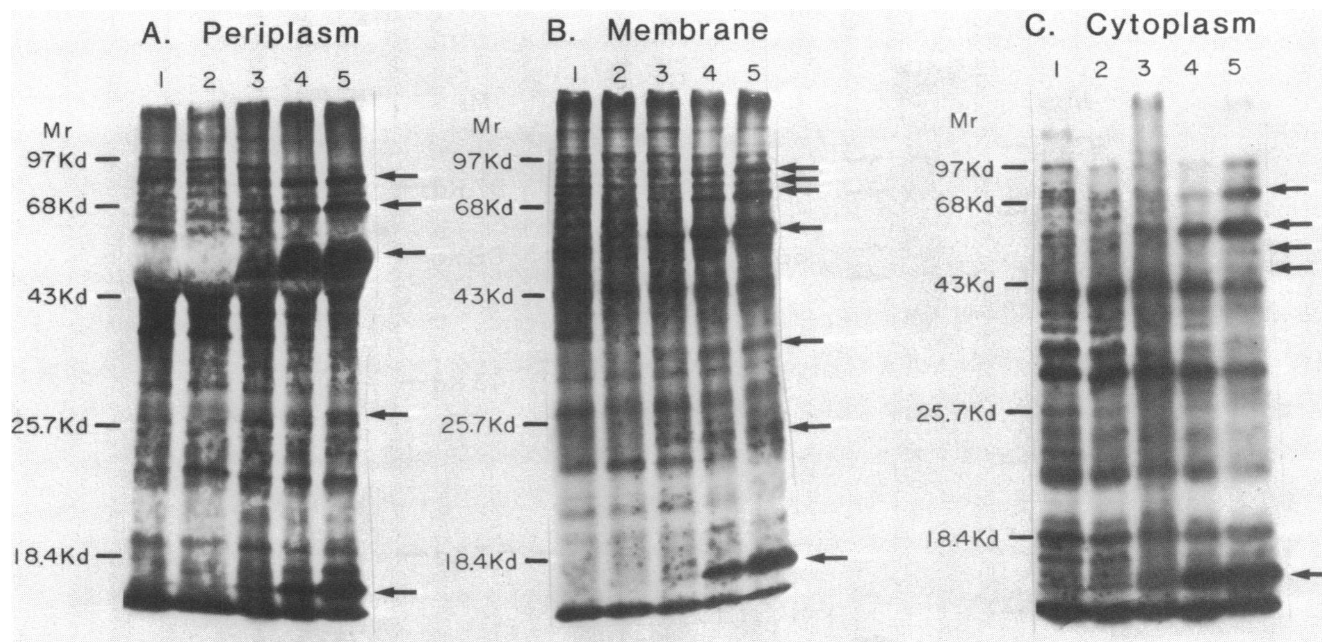

FIG. 2. Temperature-dependent production of HTPs by vegetative *M. xanthus* cells. Samples (1 ml) of a vegetative, log-phase culture were incubated at 28°C (lane 1), 32°C (lane 2), 36°C (lane 3), 40°C (lane 4), and 42°C (lane 5) for 45 min and then labeled with 10 μCi of [^{35}S]methionine for 10 min. Cells were harvested by centrifugation (8,000 × *g*, 5 min at 4°C) and fractionated to yield the periplasmic (A), membrane (B), and cytoplasmic (C) fractions. Molecular masses are shown in kilodaltons. HTPs are indicated by the arrows.

shown). Analysis of the fluorograms (Fig. 5) indicated that the HTPs detected in heat-shocked, glycerol-induced myxobacteria were similar to those detected in both vegetative cells and starvation-induced fruiting cells that had been heat shocked (Table 1). The heat shock response of glycerol-induced cells was a mosaic of the responses exhibited by vegetative and fruiting cells. Not all the vegetative specific HTPs and not all the fruiting specific HTPs were expressed in the glycerol-induced cells. Selected HTPs, some specific to vegetative *M. xanthus* cells, some specific to fruiting cells, and some common to both, were detected in the heat-shocked glycerol-induced cells.

DISCUSSION

Procaryotic and eucaryotic cells respond to incubation temperatures above their normal growth temperatures by altering their pattern of protein synthesis and growth (21). This response has been termed the heat shock response. The data presented here demonstrate that *M. xanthus* exhibits a heat shock response both during vegetative growth and during development. When the incubation temperature of *M. xanthus* cells was raised from 28 to 40°C, the cells rapidly cease growing and initiate the synthesis of HTPs (Fig. 1), while repressing the synthesis of many other proteins. The expression of some proteins appears unaffected. Typically, about 50% of the cells are killed during the first 60 min after the shift from 28 to 40°C; however, preincubation of the cells at 36°C before the shift to 40°C resulted in increased rates of survival (Fig. 3). This induction of thermotolerance has been observed in other organisms and has been attributed to protection afforded by the HTPs (8, 9, 11, 21, 25, 27, 28). Neidhardt et al. (20) have shown that *htpR* strains are unable to synthesize HTPs and are temperature sensitive. That *M. xanthus* cells express HTPs at 36°C (Fig. 2) is consistent with the idea that HTP expression induces thermotolerance.

The heat shock response of vegetative *M. xanthus* cells involves the induction of at least 18 HTPs. These proteins are cataloged with respect to their distribution in the periplasm, membranes, and cytoplasm (Table 1). In contrast, of the 17 HTPs detected in *E. coli* (21), only two, DnaJ and DnaK, have been localized to a subcellular fraction. Both are membrane associated (34, 35). We suggest that knowledge of the localization of HTPs will be useful in understanding the functions of the various HTPs, both in *M. xanthus* and in *E. coli*.

Perhaps the most interesting observations about the heat shock response of *M. xanthus* concern the HTPs detected in both types of developing cells and in vegetative cells. That

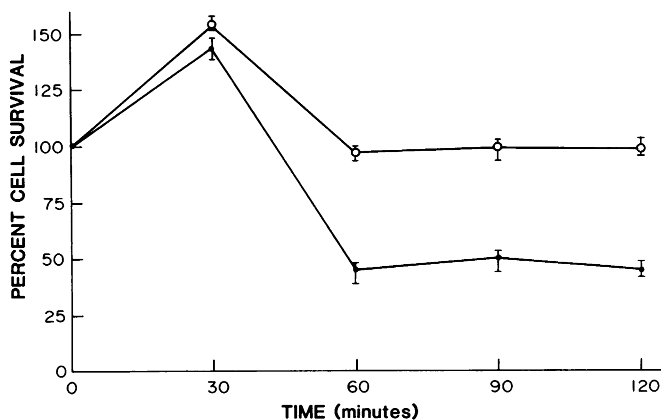

FIG. 3. Induction of thermotolerance in vegetative *M. xanthus* cells. A log-phase culture grown at 28°C in CYE medium was divided in half. The cultures were incubated at 28°C (●) or 36°C (○) for 60 min and then shifted to 40°C (0 min). Samples were withdrawn every 30 min to determine CFU. Each point represents the average from three separate experiments.

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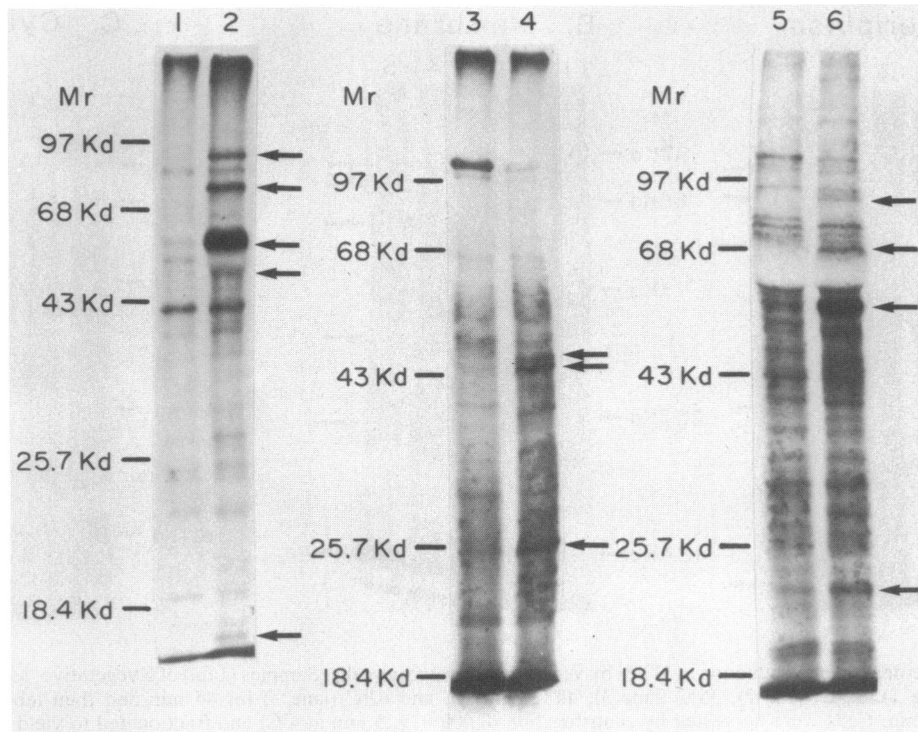

FIG. 4. The production of HTPs by starvation-induced fruiting *M. xanthus* cells. Cells allowed to develop on CF agar plates (1) at 28°C for 20 h were harvested and suspended in CF broth as described by Nelson and Zusman (23). Half of the cells were placed at 28°C and labeled with [^{35}S]methionine (10 μCi/ml) for 20 min (lanes 1, 3, and 5). The other half of the cells were shifted to 40°C. After 45 min at 40°C, the cells were labeled with [^{35}S]methionine (10 μCi/ml) for 15 min (lanes 2, 4, and 6). The labeled cells were then fractionated to yield periplasmic (lanes 1 and 2), membrane (lanes 3 and 4), and cytoplasmic (lanes 5 and 6) fractions as previously described (5). HTPs are indicated by arrows. Molecular masses are shown in kilodaltons.

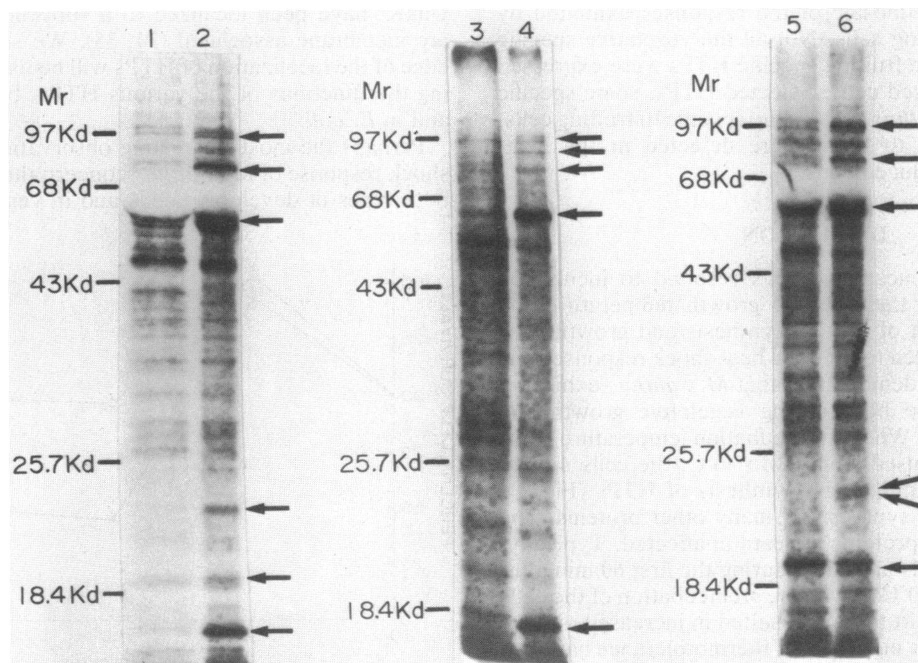

FIG. 5. The production of HTPs by glycerol-induced sporulating *M. xanthus* cells. Vegetative cells growing in A-1 broth at 28°C were induced to sporulate by the addition of glycerol to 0.5 M. After 30 min in 0.5 M glycerol, half the cells were shifted to 40°C for 30 min and then labeled with [^{35}S]methionine (10 μCi/ml) for 30 min (lanes 2, 4, and 6). The remaining cells were kept at 28°C. After 60 min they were labeled with [^{35}S]methionine (10 μCi/ml) for 30 min (lanes 1, 3, and 5). Cells were harvested and fractionated to give periplasmic (lanes 1 and 2), membrane (lanes 3 and 4), and cytoplasmic (lanes 5 and 6) fractions (5). HTPs are indicated by arrows. Molecular masses are shown in kilodaltons.

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the heat shock responses of the two developmental cell types are somewhat different from the response seen in vegetative cells is not altogether surprising. Vegetative and fruiting cells are different biochemically and physiologically (10, 25, 33); for example, vegetative cells are actively growing and carry out DNA replication (26, 29, 31). Fruiting cells are not growing and have completed DNA replication (26, 32, 33). Further, fruiting body formation bears a resemblance to the heat shock response. A new set of fruiting-specific proteins are rapidly induced by starvation, while many vegetative proteins are repressed, and others are unaffected (12). Cells that complete fruiting to become myxospores gain resistance to starvation and mild heat treatment (to about 60°C [30]). When the nutritional situation improves, the spores germinate and begin vegetative growth (30). Because glycerol-induced sporulation involves the use of logarithmically growing cells induced to form myxospores by the addition of glycerol, one might predict that analysis of HTPs produced in these cells would include HTPs common to both the vegetative and fruiting heat shock responses. This is, in fact, what is seen (Table 1).

Recently, it has been reported that sporulation in yeast and embryogenesis in *Drosophila melanogaster* involve the expression of some HTPs (16). These reports suggest that perhaps the heat shock response is an ancient, highly conserved pathway which has been exploited by many different organisms as a pathway for cellular development and differentiation. If this is the case in *M. xanthus*, it could be predicted that some HTPs should be expressed during the normal course of fruiting. That both starvation or exposure of moderate to high concentrations of alcohols or dimethyl sulfoxide result in the expression of HTPs in *E. coli* (21) supports this possibility. We are currently investing possible links between fruiting body formation and the heat shock response in *M. xanthus*.

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