

## NOTES

### Heat Shock Response of the Archaeobacterium *Methanococcus voltae*

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The general properties of the heat shock response of the archaeobacterium *Methanococcus voltae* were characterized. The induction of 11 heat shock proteins, with apparent molecular weights ranging from 18,000 to 90,000, occurred optimally at 40 to 50°C. Some of the heat shock proteins were preferentially enriched in either the soluble (cytoplasm) or particulate (membrane) fraction. Alternative stresses (ethanol, hydrogen peroxide, NaCl) stimulated the synthesis of subsets of the heat shock proteins as well as unique proteins. Western blot (immunoblot) analysis, in which antisera to *Escherichia coli* heat shock proteins (DnaK and GroEL) were used, did not detect any immunologically cross-reactive proteins. In addition, Southern blot analysis did not reveal any homology between *M. voltae* and four highly conserved heat shock genes, *mopB* and *dnaK* from *E. coli* and *hsp70* genes from *Drosophila* species and *Saccharomyces cerevisiae*.

Most of the studies of archaeobacteria have been concerned with their cytochemistry and physiology (8, 10, 25). Relatively little is known about the genomic organization, transcription, and regulation of gene expression in these microorganisms (5). Expression of genes easily modulated by environmental manipulations, such as the exposure of cells to rapid shifts in temperature (heat shock), would make ideal systems for studying transcriptional regulation. The heat shock response has been studied in numerous eukaryotes and eubacteria (4, 16-18, 20), as well as in three archaeobacteria (an extreme halophile, i.e., *Halobacterium halobium* [6], and the sulfur-dependent thermophiles *Sulfolobus acidocaldarius* [9] and *Sulfolobus* sp. strain B12 [23]). To date, this response has not been examined in any members of the methanogens. A general characteristic of the heat shock response is the rapid and usually transient induction of a limited number of proteins (heat shock proteins [HSPs]). In addition to the universal nature of this response, some of the HSPs exhibit high degrees of conservation across the eubacterial and eukaryotic kingdoms at both the amino acid and nucleotide levels (4). The heat shock phenomenon is very well studied (15, 16, 18, 20) and provides an established framework to study gene expression in *Methanococcus voltae*.

(Portions of this work were presented previously [7a, 7b].)

*M. voltae* cells were grown at 30°C as previously described (11) except that Balch medium III (3) was modified to decrease the content of yeast extract and Trypticase 10-fold (to 0.2 g/liter each) and supplemented with the essential amino acids leucine and isoleucine (26) at 0.5 and 1.0 g/liter, respectively. To study heat shock in *M. voltae*, cell samples (1.0 ml) were dispensed and temperature equilibrated at 30°C before shifting to 45°C. The cells were radiolabeled for 4 min with 25 µl of a 2×-concentrated, uniformly labeled <sup>14</sup>C-amino acid mixture (50 µCi/ml; Amersham Canada Ltd., Oakville, Ontario; catalog no. CFB.104) at intervals over an

incubation period of 1 h. The samples were then chased for 2 min by the addition of 0.5 ml of complete Balch medium III (i.e., containing 2 g [each] of yeast extract and Trypticase per liter). Protein synthesis was stopped by exposing the cells to air and chilling the samples on ice. Total cell protein was examined by electrophoresis through denaturing 12.5% polyacrylamide gels (12) and autoradiography. Since the upper temperature limit for growth in complex medium (Balch medium III [3]) is 49°C, with optimum growth occurring at 38°C (26), cell survival and growth at 45°C would be expected. The results (Fig. 1; Table 1) indicated that the induction of HSPs occurred between 3 and 6 min after shifting to 45°C, with the optimum induction of most HSPs occurring between 9 and 12 min. Eleven HSPs were induced in *M. voltae* (Table 1), as determined by one-dimensional sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis, with apparent molecular weights ranging from 18,000 to 90,000. Because of the presence of the regularly structured surface array protein (RS protein; *M<sub>r</sub>*, 76,000 [11]; indicated with an arrowhead in Fig. 1), which is the major cellular protein, it could not be determined if *M. voltae* possessed an HSP with an *M<sub>r</sub>* of 70,000, which in eubacteria (DnaK) and eukaryotes (HSP70) is one of the most highly conserved HSPs. The optimum temperature of HSP induction was found to be 40 to 50°C, with a protein induction pattern similar to that in Fig. 1 (data not shown). At 55°C, no incorporation of the radiolabel occurred. It is likely that cell death occurred at this elevated temperature.

Unlike the heat shock response of *Escherichia coli* (18), *M. voltae* did not exhibit an overall decrease in protein synthesis, with the exception of an apparent decrease in the synthesis of the RS protein later in the time course and at higher temperatures. In addition, the heat shock response of *M. voltae* was not rapidly transient as it is in *E. coli* (15, 18), since no appreciable decrease in HSP synthesis occurred over a 1-h period at 45°C (Fig. 1). This may be attributed to the actual lack of transiency or the physiological differences between these two organisms. It seems likely that, at some point after 60 min, the rate of HSP synthesis will decrease.

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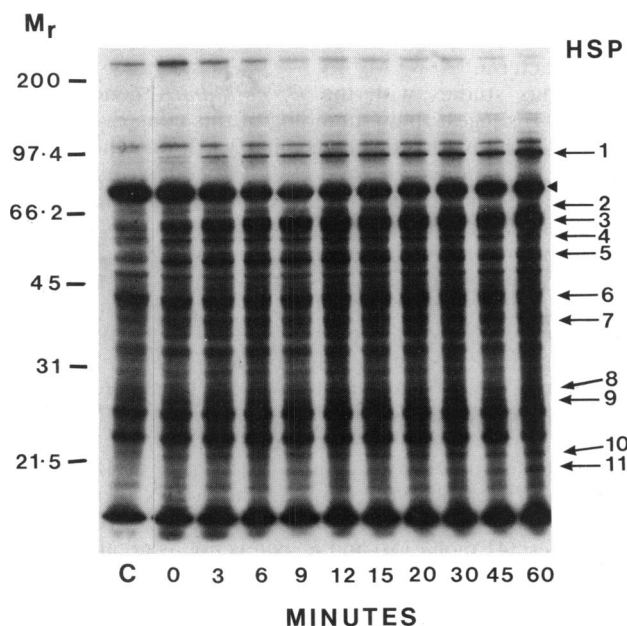


FIG. 1. Time course of the induction of the HSPs of *M. voltae*. *M. voltae* cells were shifted from 30 to 45°C and radiolabeled at the times indicated for 4 min with <sup>14</sup>C-amino acids. Total cellular proteins were analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (12.5% gel) and autoradiography (equal numbers of counts per minute were loaded per well). Lane C contains the control sample labeled at 30°C. Arrows indicate the readily discernible HSPs. Unnumbered arrowhead indicates the RS protein.

In the case of *H. halobium*, maximal expression of the heat shock response, at 60°C, occurred for 75 min before diminishing (6).

In an attempt to localize the HSPs, cells were preincubated for 10 min at 45°C and then radiolabeled as described above. The cells were lysed and centrifuged at 167,000 × *g* in a Beckman Airfuge centrifuge [Beckman Instruments (Canada) Inc., Mississauga, Ontario] for 30 to 40 min at 4°C. Particulate (e.g., membrane) and soluble (e.g., cytoplasm) fractions were obtained. Electrophoretic analysis of these samples (Fig. 2) revealed that certain HSPs were preferentially enriched in one of the fractions. When the RS protein was used as an indicator of the efficiency of cell fractionation, it was apparent that there was little contamination of the soluble fractions by the 76,000-molecular-weight RS

TABLE 1. Apparent molecular weights of the HSPs of *M. voltae*

Protein no.	Apparent mol wt (no. of determinations)
HSP-1.....	89,000 (22)
HSP-2.....	62,700 (10)
HSP-3.....	60,000 (15)
HSP-4.....	55,000 (7)
HSP-5.....	50,900 (19)
HSP-6.....	41,000 (21)
HSP-7.....	36,200 (5)
HSP-8.....	30,500 (12)
HSP-9.....	26,700 (8)
HSP-10.....	21,800 (8)
HSP-11.....	18,100 (7)

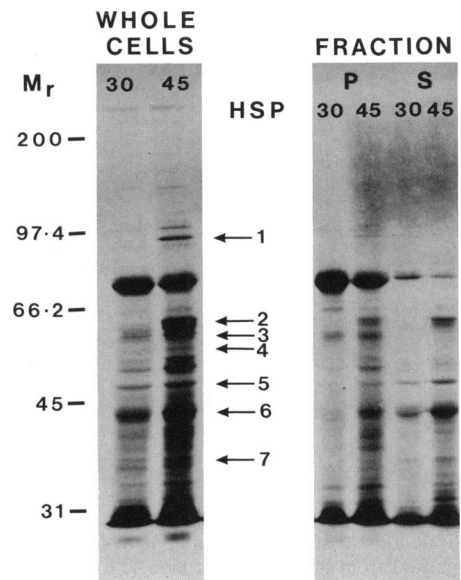


FIG. 2. Subcellular localization of *M. voltae* HSPs. *M. voltae* cells were shifted from 30 to 45°C and incubated for 10 min. The cells were radiolabeled and fractionated as described in the text. Samples were analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis and autoradiography. Arrows indicate the major HSPs. The arrowhead indicates a putative HSP masked by RS protein. P, particulate fraction; S, soluble fraction.

protein. The soluble fraction was enriched in HSP-5, -6, and -7. The particulate fraction was enriched in HSP-3 and -9. The tentative assignment of these HSPs to either the particulate or the cellular fraction may eventually suggest possible functions for these proteins.

In comparing *M. voltae* HSPs with major and highly conserved *E. coli* HSPs, proteins of comparable molecular weights can be found (Table 1), namely, HSP-1 (89,000) and Lon (*E. coli*) (18), and HSP-2 (62,700) and GroEL (18). HSP-2 was found in both fractions (Fig. 2), suggesting that this protein may be loosely membrane associated or of a high molecular weight such that it would partially cosediment with the membranes.

Since it is known (2, 19, 24) that HSPs are usually a subset of a general stress response, *M. voltae* was subjected to three additional stresses during two incubation periods, with 10 or 30 min of preincubation with each stress. Cells were exposed to ethanol (5 and 10% [vol/vol]), H<sub>2</sub>O<sub>2</sub> (50 and 100 μM), or NaCl (from an initial concentration of 0.4 M to a total of 0.6 and 0.8 M) and radiolabeled as stated. It can be noted in Fig. 3 that many proteins with *M<sub>r</sub>*s identical to those of the HSPs were induced by these stresses, with HSP-6 (*M<sub>r</sub>*, 41,000) appearing prominently in all cases. Exposure of cells to ethanol (Fig. 3, lanes 3 and 4) appeared to affect the cells in a manner comparable to heat (Fig. 3, lane 2). The protein profiles for both are quite similar, and ethanol apparently induced all of the HSPs. In addition, a protein of *M<sub>r</sub>* 58,000, specific to the ethanol stress response, was also induced. Oxidative stress induced by the addition of hydrogen peroxide (Fig. 3, lanes 5 and 6) resulted in protein profiles similar to the heat- and ethanol-induced stress response, although HSP-1 (*M<sub>r</sub>*, 90,000) was absent. The addition of higher concentrations of NaCl (from an initial concentration of 0.4 M to final concentrations of 0.6 and 0.8 M) greatly reduced overall protein synthesis in *M. voltae* (Fig. 3, lanes 7 and 8).

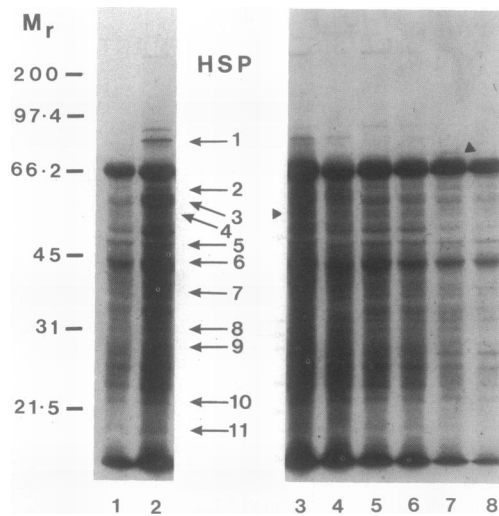


FIG. 3. Induction of stress proteins in *M. voltae* cells. *M. voltae* cells were shifted from 30°C (control, lane 1) to 45°C (heat shock, lane 2), incubated for 10 min, and radiolabeled. *M. voltae* cells (lanes 3 to 8) were stressed, incubated for 30 min, and radiolabeled. The stresses used were 5% ethanol (lane 3), 10% ethanol (lane 4), 50  $\mu$ M H<sub>2</sub>O<sub>2</sub> (lane 5), 100  $\mu$ M H<sub>2</sub>O<sub>2</sub> (lane 6), 0.6 M NaCl (lane 7), and 0.8 M NaCl (lane 8). Arrows indicate the major HSPs, and arrowheads indicate unique proteins.

The majority of the induced proteins migrated with apparent molecular weights identical to those of the HSPs. A protein of  $M_r$  80,000 which appeared to be specifically induced by salt stress can be noted just above the RS protein (Fig. 3, lane 7). This response is comparable to that of *E. coli* with respect to alternative stresses (24), in that a common subset of proteins is induced by various stresses. This may imply that these proteins have a fundamental role in stress response management. The induction of proteins unique to a particular stress can be attributed to the fact that each stress affects different cellular functions in addition to common functions. The unique proteins may be a specific defense mechanism to protect a susceptible cellular component and/or function.

Since some studies (7, 14, 19) have demonstrated serological relatedness between certain HSPs and in an attempt to further compare the heat shock response of *M. voltae* with that of *E. coli*, heat-shocked *M. voltae* cells, both whole and fractionated, were probed by using polyclonal antisera to two of the major *E. coli* HSPs, DnaK ( $M_r$ , 70,000) and GroEL ( $M_r$ , 65,000). However, no proteins of *M. voltae* reacted with the antisera under conditions in which the antibodies reacted specifically with the appropriate-molecular-weight proteins from *E. coli* (data not shown) and *Pseudomonas aeruginosa* (1) whole-cell preparations. This lack of immunological reactivity may not be unexpected considering the phylogenetic distance and the physiological differences between *M. voltae* and *E. coli*. Trent et al. (23) were also unable to demonstrate a cross-reaction between antibodies to DnaK and GroEL and proteins of a sulfur-dependent thermophilic archaeobacterium, *Sulfolobus* sp. strain B12. However, by using polyclonal antibodies against a 65-kDa mycobacterial protein antigen, Thole et al. (22) have shown cross-reactive proteins in a wide variety of prokaryotes, including two species of methanogens, *Methanobacterium thermoautotrophicum* and *Methanosarcina barkeri*.

Since this mycobacterial 65-kDa antigen is a GroEL homolog (21), it is possible that this protein also exists in the archaeobacteria.

Previous studies with the *E. coli* *dnaK* gene and *M. barkeri* (4) suggest that a homolog of this conserved HSP is present in the methanogens. Similar work on other archaeobacteria, *H. halobium* (6) and *Desulfurococcus mobilis* (13), also suggests the presence of these conserved genes in these divisions of archaeobacteria. By using a nonstringent DNA hybridization technique, genomic digests of *M. voltae* were probed with conserved heat shock genes *dnaK* and *mopB* from *E. coli* and *hsp70* from *Drosophila* species and *Saccharomyces cerevisiae*. No apparent homologs to any of these conserved HSPs could be detected in *M. voltae*. However, considering the difference in the moles percent G+C content between the DNAs of *M. voltae* (30 mol%) and *E. coli* (52 mol%), it might not be unexpected that we were unable to demonstrate homologous genes. In the case of *M. barkeri*, which has been shown to possess homologs to *dnaK* and *hsp70* (4), the difference in the moles percent G+C between *M. barkeri* (41 mol%) [5] and *E. coli* is not as great. It seems likely that *M. voltae* does possess homologous and conserved heat shock genes. However, simple probing might be insufficient to detect these genes, and more direct methods, such as biased probes to the mostly highly conserved regions of these genes with the subsequent isolation and cloning of the genes, might be required. These studies are currently under way.

The concept of the universality of the heat shock response has been supported by studies of a wide array of eukaryotes and eubacteria. The present study on *M. voltae* supports this concept and provides additional evidence of the presence of the heat shock response in the archaeobacteria. The response of *M. voltae* is similar, in some respects, to that of *E. coli*. The comparison of homologous proteins and genes provides a method of studying evolutionary relationships.

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