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The stress-shock response of the bacterium *Methylophilus methylotrophus*

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A sudden increase in the growth temperature of *Methylophilus methylotrophus* results in the synthesis of a number of unique proteins. The major heat-shock proteins have apparent molecular masses of 83, 78, 63, 60, 16 and 14 kDa. Other stress conditions elicit a similar response, although there are significant differences in the sets of proteins produced under the various conditions. Addition of methanol induces proteins identical in size to the heat-shock 83, 78, 63 and 14 kDa proteins and also induces unique 94, 36 and 29 kDa species. Addition of ethanol induces proteins identical in size to the 78 and 20 kDa heat-shock proteins and the 94 and 36 kDa methanol-induced proteins and an apparently unique 13 kDa species. Simultaneous exposure to elevated temperature and either methanol or ethanol resulted in the synthesis of all of the proteins induced by the separate treatments. The stress-shock proteins are differentially located in cytoplasmic, periplasmic and membrane fractions.

Heat shock; Methanol; Ethanol; Stress; *(Methylophilus methylotrophus)*

1. INTRODUCTION

The induction of a family of proteins in response to exposure to rapid temperature elevation has been observed in a wide range of organisms [1–3]. These so-called heat-shock proteins (hsps) are also induced by other changes in the environmental conditions [3–6] suggesting that activation of the hsp genes is part of a more general stress response phenomenon. (Note: in this paper the term hsp refers specifically to proteins induced by temperature shift.) There is now a good deal of evidence which implicates denatured or abnormal proteins as signals in the response [6–8], and one of the consequences of the heat-shock (stress-shock) response is an increased capacity to degrade abnormal proteins [6,7].

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The prokaryotic heat-shock phenomenon has been studied in greatest detail in *Escherichia coli* in which at least seventeen proteins have been shown to be induced [1,3,5]. A number of studies have now demonstrated a relationship between heat shock and proteolysis in *E. coli* [7,8] as well as in other organisms [9]. In *E. coli* the rapid turnover of some abnormal proteins can be reduced in host mutants which have an altered heat-shock response [10]. One of the *E. coli* heat-shock proteins is the *lon* gene product, Protease La, an ATP-dependent proteinase which is involved in the degradation of abnormal proteins [11]. Additionally, several other *E. coli* hsps may be proteinases or affect the activity of proteinases [3].

We are currently undertaking an investigation of the proteinases of another gram-negative bacterium, *Methylophilus methylotrophus*, an organism with a complex proteolytic system that has some similarities to that of *E. coli* [12,13]. As part of a study of the control of proteolysis and, in particular, the factors which might affect the

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stability of foreign gene products synthesised in *M. methylotrophus*, we wished to determine whether this organism responds to heat shock and other stress conditions in a manner similar to *E. coli*. Here we demonstrate that in common with other organisms, *M. methylotrophus* synthesizes a characteristic set of hsps. Two particularly noteworthy features are described. Firstly, the sets of proteins induced by different stresses were not identical. Secondly, stress-shock proteins were located in different subcellular fractions, with some of the proteins being found in more than one subcellular fraction.

2. EXPERIMENTAL

[^{35}S]Methionine was purchased from Amersham International (Amersham, England). All other reagents were of the highest purity obtainable.

Methylophilus methylotrophus AS1 was provided by Dr M. Worsey, ICI Corporate Bioscience Laboratory, Runcorn, England. The cells were grown in medium containing (per litre) 1.8 g $(NH_4)_2SO_4$, 0.2 g $MgSO_4 \cdot 7H_2O$, 1.4 g NaH_2PO_4, 1.9 g K_2PO_4, 0.98 mg $FeCl_2$, 0.02 mg $CuSO_4 \cdot 5H_2O$, 0.1 mg $MnSO_4$, 0.1 mg $ZnSO_4 \cdot 7H_2O$ and 1.8 mg $CaCO_3$. The pH was adjusted to 6.85 and methanol added to give a final concentration of 0.5% (v/v). The cultures were shaken in an orbital incubator at 150 rpm except during a temperature shift when a water bath was used. For routine maintenance the cells were grown at 37°C, but prior to stress treatment cultures were incubated at 30°C and incubation continued at this temperature unless otherwise indicated.

For labelling with [^{35}S]methionine, the cells were grown in 10 ml batches of medium in 20 ml fluted Erlenmeyer flasks. The following conditions were used to stress the cells: (i) temperature shift, 30–40°C (incubation in water bath); (ii) addition of 5% (v/v) methanol; (iii) addition of 5% (v/v) ethanol. [^{35}S]Methionine (55.5 kBq·ml^{-1}; 29.6 GBq·mol^{-1}) was immediately added and the cultures incubated for 30 min and then 0.5 ml aliquots were harvested by centrifugation (5000 × *g*, 5 min). The cell pellets were washed with successive treatments of ice-cold 10% (w/v) trichloroacetic acid, 5% (w/v) trichloroacetic acid and acetone. Samples were prepared for electrophoresis by resuspending the washed pellet in 25 μl of 10 mM Tris-HCl, pH 7.8, and 25 μl of a mixture containing 0.1 M Tris, 0.05 M H_2SO_4, 0.4% (w/v) SDS and 20% (v/v) glycerol (pH 6.1).

Cytoplasmic, periplasmic and membrane fractions were prepared from labelled cells by the method of Jones et al. [14]. The degree of purity was estimated from the distribution in fractions prepared from unlabelled cells of marker enzyme activities, namely glucose 6-phosphate dehydrogenase (cytoplasm), malate dehydrogenase (periplasm) and ascorbate-TMPD (*N,N,N',N'*-tetramethyl-*p*-phenylenediamine) (cytochrome *c*) oxidase (membrane). The periplasmic and membrane fractions contained 16 and 3%, respectively, of the total cytoplasm marker activity; 8 and 2%, respectively, of the total periplasm marker activity was present in the cytoplasmic and membrane fractions. No membrane marker activity was detectable in either the cytoplasmic or the periplasmic fractions.

Labelled samples were analysed by electrophoresis on 14% (w/v) polyacrylamide gels [15]. Molecular mass markers used were bovine albumin (66 kDa), egg albumin (45 kDa), pepsin (34.7 kDa), trypsinogen (24 kDa), β-lactoglobulin (18.4 kDa) and lysozyme (14.3 kDa). The gels were run at 6 mA for 12 h and then stained with 0.03% (w/v) Page blue 83 for 3 h, destained and treated with En^{3}Hance (NEN). Fluorograms were prepared by exposing Kodak X-ray film (X-Omat AR) to the gels for 3–4 days at −70°C.

3. RESULTS AND DISCUSSION

The stress-shock response of *Methylophilus methylotrophus* was determined by the standard procedure of examining fluorograms of proteins labelled with [^{35}S]methionine for a brief period after stress. Routinely, a labelling period of 30 min was used as the bacterium does not take up methionine rapidly. Fig.1 (lanes 1–4) shows the effect of three different stress treatments on the pattern of proteins synthesised. Heat shock (temperature shift from 30°C to 40°C) resulted in the induction of at least eight proteins (summarised in table 1). The molecular mass range for the *M. methylotrophus* hsps was similar, although not identical, to that of *Escherichia coli*, major hsps which have apparent molecular masses of 94.0

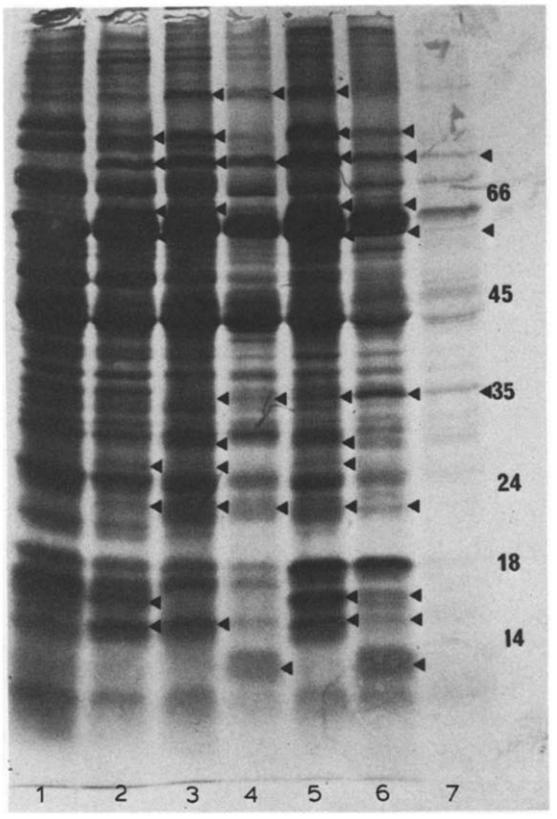

Fig.1. [^{35}S]Methionine incorporation into proteins in whole extracts of stress-shocked cells. Samples were prepared after 30 min treatment as described in section 2. The cells were kept untreated at 30°C (lane 1), shifted to 40°C (2), treated with methanol (3), treated with ethanol (4), shifted to 40°C and treated with methanol (5), shifted to 40°C and treated with ethanol (6) or treated with methanol and ethanol (both 5% v/v) (7). The position of protein standards is indicated (in kDa) on the right. The stress-shock proteins are arrowed.

(protease La), 70.3, 69.1, 62.9, 62.5, 48.5, 33.4, 25.3, 14.7 and 10.7 kDa [5]. In addition to the hsps some proteins which were synthesised during normal incubation at 30°C were also labelled in heat-shocked cells. The possibility that these were made during the period when the temperature was increasing was unlikely since the elevated temperature was attained within 1–2 min. Furthermore if samples were taken after 5 min, little labelling of protein had occurred and those proteins that were labelled resembled those seen in 30 min samples. It would appear that in this

Table 1

Stress-shock proteins of *Methylophilus methylotrophus*

Protein (kDa)	Stressing agent[a]	Subcellular location[b]
Proteins detected in whole cell extracts		
94	MeOH, EtOH	M
83	heat, MeOH	C, M
78	heat, MeOH, EtOH	C, P, M
63	heat, MeOH	C, P, M
60	heat	C, P
36	MeOH, EtOH	n.d.
29	MeOH	n.d.
27	heat, MeOH	n.d.
20	heat, MeOH, EtOH	M
16	heat	C, P
14	heat, MeOH	C, P
13	EtOH	n.d.
Proteins detected only in subcellular fractions		
>100 (X)	heat	M
55	heat	C
50	heat	C
45	heat	C

[a] Protein labelled after temperature increase (heat), or addition of methanol (MeOH) or ethanol (EtOH)

[b] Labelled protein detected in cytoplasmic (C), periplasmic (P) or membrane (M) fraction. n.d., not detectable in any subcellular fraction

organism heat shock does not result in the complete shut down of normal protein synthesis when hsps are made. The view that stress does not completely inhibit normal protein synthesis is supported by the results obtained following addition of methanol or ethanol, stressing agents which would have been immediately effective. During treatment with methanol and ethanol some, although not all of the *M. methylotrophus* hsps were synthesised. Other unique proteins were also produced (see table 1).

By using shorter labelling periods it was found that the stress-shock proteins were synthesised coordinately and that radioactivity could be first detected in the major proteins after 5 to 15 min of stress treatment (not shown). None of the proteins appeared to be precursors of other stress-shock proteins. The shorter labelling period revealed only one band not present after 30 min. This corresponded to a 77 kDa protein, which was detected

in methanol- or ethanol-treated cells after 5 min but had subsequently disappeared after 15 min.

The variation in the response of *M. methylotrophus* with the particular stress conditions is in contrast to the situation usually reported in other systems in which different treatments induce similar sets of proteins [3,5,8]. It is known that a number of agents can act as modulators in the heat-shock response [4]. To investigate this further, cultures were challenged with two stress conditions simultaneously. Fig.1 (lanes 5–7) shows that the combination of elevated temperature and either methanol- or ethanol-treatment resulted in the synthesis of all of the proteins induced by the individual treatments alone. Thus heat did not repress methanol- and ethanol-induced proteins, while the presence of the alcohols did not prevent the synthesis of heat-shock-specific proteins. This is consistent with the organism having more than one stress regulon with different signals controlling the expression of each one. This situation is closer to that now reported for other organisms including *E. coli* and *Salmonella typhimurium* in which variations in responses to different stress stimuli are apparent [16–18]. Functions for the stress-shock proteins are still being actively sought [9], and the diversity in the responses to the treatments raises questions about the role of any protein which is induced by one type of stress condition only, for example the *M. methylophilus* 60 and 16 kDa hsps and the 94 and 36 kDa proteins induced by methanol and ethanol.

To analyse the stress-shock response further, the pattern of induced proteins in three subcellular fractions was determined (fig.2). The location of the proteins differed significantly (summarised in table 1), although the nature of the stressing agent had no effect on the distribution of individual proteins. The 94 kDa protein induced by methanol and the 20 kDa hsp were detected only in the membrane fraction. Some of the 83 kDa hsp was associated with the cytoplasmic fraction, but it too was a major protein in the membrane fraction. The 78 kDa hsp was detected in all three fractions, but there was significantly less in the cytoplasmic than in the other two fractions. Because of the presence of a major constitutive protein of similar molecular mass it was difficult to assess precisely the distribution of the 63 and 60 kDa hsps, although the latter was apparently absent from the

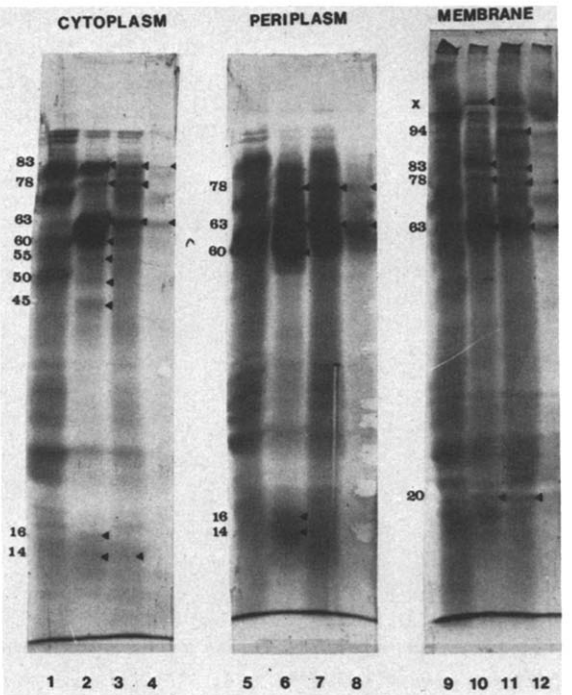

Fig.2. [^{35}S]Methionine incorporation into proteins in subcellular fractions prepared from stress-shocked cells. Samples were taken after 30 min treatment and cytoplasmic (lanes 1–4), periplasmic (5–8) and membrane (9–12) fractions prepared. The cells were kept untreated at 30°C (lanes 1,5,9), shifted to 40°C (2,6,10), treated with methanol (3,7,11) or treated with ethanol (4,8,12). The position and size (in kDa) of the stress-shock proteins are indicated: the molecular mass of one protein (X in lane 10) was too high to be estimated accurately.

membranes. The 16 and 14 kDa proteins were predominantly in the periplasmic fraction. The fractionation revealed additional hsps which were not detected in whole cell extracts, namely a high molecular mass protein (X) exclusive to membrane preparations and three others (45, 50 and 55 kDa) which were present only in the cytoplasmic fraction. In contrast, some of the minor stress-shock proteins detected in whole cell extracts were not apparent in any of the fractions.

In summary, the analysis of the stress-shock response of *M. methylotrophus* has highlighted two aspects which have until recently received little attention in other systems, namely the variation in

stress response with stressing agent and the cellular location of the stress-shock proteins. While the complexity of the stress-shock phenomenon revealed may make the determination of the molecular mechanisms involved more difficult, the knowledge that at least some proteins are specific to one stress condition and one subcellular fraction may prove invaluable in establishing their role in the response to and survival of stress.

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