# Heat-shock proteins during growth and sporulation of *Bacillus subtilis*

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Four major heat-shock proteins (hsps) with apparent molecular masses of 84, 69, 32 and 22 kDa were detected in exponentially growing stationary phase and sporulating cells of *Bacillus subtilis* heat-shocked from 30 to 43°C. The most abundant, hsp69, is probably analogous to the *E. coli groEL* protein. These proteins were transiently inducible by heat-shock. Partial purification of RNA polymerase revealed several other minor hsps. One of these, a 48 kDa polypeptide probably corresponds to $\sigma^{43}$. The synthesis of this polypeptide and at least two other proteins appeared to be under sporulation and heat-shock regulation and was affected by the *SpoOA* mutation.

Bacillus subtilis *Heat-shock protein* *Sporulation* *RNA polymerase* *Sigma factor* SpoOA

## 1. INTRODUCTION

The heat-shock response is a universal, cellular phenomenon that is characterised by the increased synthesis of a number of proteins upon a shift up in temperature and other stress stimuli (e.g. amino acid starvation, ethanol, ultraviolet light) [1,2]. It has provided an ideal experimental system to study eukaryotic gene expression [3] and recently, gene regulation in *Escherichia coli* [2].

The synthesis of the 17 heat-shock proteins (hsps) so far identified in *E. coli* is controlled by the heat-shock regulatory gene, *htpR* [4]. Recent results have implicated RNA polymerase as a major target for the regulatory mechanisms of the heat-shock response: the *htpR* gene product is a sigma factor ($\sigma^{32}$) [5] and in addition the major sigma subunit, $\sigma^{70}$, is a hsp [6]. Furthermore, the remarkably conserved heat-shock *dnaK* gene [7] encodes a negative modulator of the response, possibly affecting the activity of $\sigma^{32}$ [5,8].

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At least 5 sigma factors have been identified in *Bacillus subtilis* [9,10]. Their association with the core RNA polymerase enables the holoenzyme to recognise different promoters with characteristic consensus sequences. Hence changes in RNA polymerase specificity can at least partly account for the temporal gene regulation observed during the response of Bacilli to the environmental stress of nutrient deprivation, manifested by the cessation of growth and cell division and the initiation of sporulation.

Recently Briat et al., [11] showed that one of the minor sigma factors from *B. subtilis*, $\sigma^{28}$, has the same promoter specificity as the heat-shock regulator $\sigma^{32}$ of *E. coli*. They found however that 2 *B. subtilis* promoters [11] recognised by the $\sigma^{28}$-RNA polymerase were not under heat-shock control in *E. coli* and consequently suggested that additional factors are required for $\sigma^{32}$-RNA polymerase transcription in *E. coli*. In *B. subtilis*, transcription at the 2 characterised $\sigma^{28}$-specific promoters [11,12] requires the presence of several regulatory proteins encoded by the *spoOA* genes whose func-

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tion is essential for sporulation [13]. The aims of this study were to (a) characterise the hsps of *B. subtilis* during exponential growth, stationary phase and sporulation, (b) examine the hsps in a *spoOA* mutant and (c) to establish possible identities between hsps and sigma factors.

## 2. MATERIALS AND METHODS

### 2.1. *Bacterial strains and growth*

*E. coli* TG1 and *Streptomyces lividans* were gifts from Dr T. Gibson and Dr A. Roberts (MRC Laboratory of Molecular Biology, Hills Road, Cambridge, CB2 2QH). *B. subtilis* 168 Sueoka *(trp C2)* [14] and *B. subtilis* SL566 *(spoOA 34 phe-12 rif-2 tal-1)* [13] were gifts from Dr P. Piggot (National Institute for Medical Research, The Ridgeway, Mill Hill, London, NW7 1AA). *E. coli* was grown in M9 complete minimal medium [15], *B. subtilis* in modified Schaeffer's medium [14] supplemented with the appropriate amino acids at 50 $\mu$g/ml and *S. lividans* in YEME medium [16]. All strains were grown at 30°C prior to heat shock.

### 2.2. *Labelling*

Bacteria were grown exponentially in the appropriate medium to an absorbance of 0.5–0.6 at 600 nm. Aliquots (0.5 ml) were transferred to glass vials at 30 and 43°C and incubated for 5 min before the addition of [$^{35}$S]methionine (Amersham, final concentration 30 $\mu$Ci/ml). The incubation was continued for 10 min followed by the addition of unlabelled methionine (12.5 mg/ml) and trichloroacetic acid (12.5% final concentration). The cells were kept on ice for 30 min, the precipitate recovered by centrifugation (10 000 × *g* for 10 min at 4°C), washed with 1 ml of acetone and dried.

For partial purification studies (section 2.3) 40 ml of cell culture was pulse-labelled as described above, except that 7.5 $\mu$Ci/ml [S$^{35}$]methionine was used. Labelled cells were harvested and the pellet resuspended in 0.7 ml of 10 mM Tris-HCl buffer, pH 7.4, containing 10% glycerol, 0.5 mM dithiothreitol, 1 mM EDTA and 0.5 mM phenylmethylsulfonyl fluoride (PMSF), referred to as PMSF buffer. Cells were disrupted by vortex-mixing with glass beads [17,18]. Cell debris and membranes were removed by centrifugation (100 000 × *g* for 60 min at 4°C) and the supernatants subjected to heparin-agarose chromatography (section 2.3).

For the analysis of hsps during sporulation (fig.2) 0.5 ml cell culture were disrupted using glass beads as described [17,18] except that the cells, suspended in 130 $\mu$l PMSF buffer were vortex-mixed with 0.5 g glass beads in a 1.5 ml Minifuge tube. The disrupted cell suspension was treated with trichloroacetic acid as above.

### 2.3. *Heparin-agarose affinity chromatography*

DNA-binding proteins and RNA polymerase were partially purified by applying the supernatant recovered from 40 ml of disrupted cells (section 2.2) to a 0.8 ml heparin-agarose column (packed volume), as described in [19]. The column was then washed with 4 ml PMSF buffer containing 0.1 M KCl and eluted with 4 ml PMSF buffer containing 0.5 M KCl. The 0.5 M KCl elution fraction was treated with trichloroacetic acid as above.

Figure 1

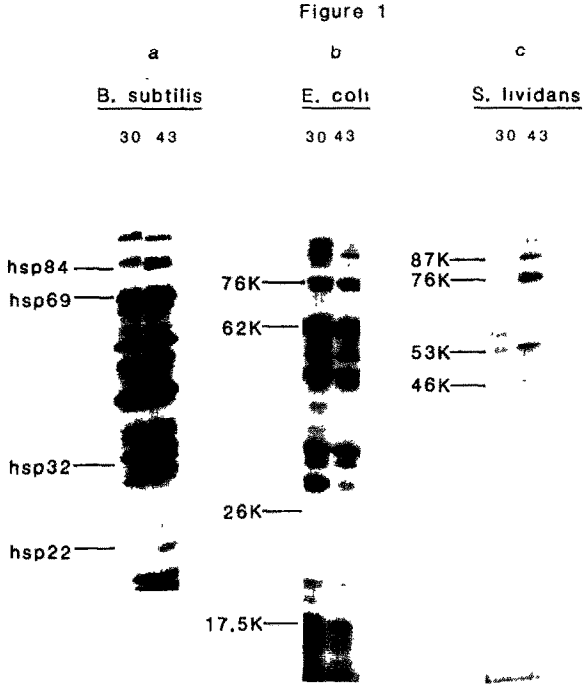


Fig.1. Fluorograms of SDS 13% polyacrylamide gels of $^{35}$S-labelled polypeptide synthesised during heat-shock in exponentially growing *B. subtilis* 168 Sueoka (track a), *E. coli* TG1 (track b) and *S. lividans* (track c). The lanes are marked 30 and 43 to indicate the normal growth temperature (30°C) and the temperature of induction (43°C). For experimental details, see section 2.

### 2.4. *Electrophoresis*

Polyacrylamide gel electrophoresis in the presence of SDS was conducted as described in [20], an acrylamide/*N,N'*-methylenebisacrylamide ratio of 100:1 being used. Gels were 13% unless otherwise stated. Trichloroacetic acid pellets were dissolved in SDS-sample buffer [21] except that the SDS concentration was 3%. Equal amounts of radioactivity ($10^4$–$10^5$ cpm) were loaded on each '30°C' and '43°C' track. After electrophoresis, labelled proteins were detected by fluorography [22]. Molecular mass markers were: $\beta$-galactosidase (116 kDa), phosphorylase (94 kDa), bovine serum albumin (68 kDa), glutamate dehydrogenase (56 kDa), creatine kinase (40 kDa), carbonic anhydrase (31 kDa), trypsin inhibitor (21 kDa) and ribonuclease (17 kDa).

## 3. RESULTS AND DISCUSSION

### 3.1. *Major hsps*

Fig.1 (track a) shows the 4 major hsps with apparent molecular masses of 84, 69, 32 and 22 kDa detected by one-dimensional electrophoresis in whole cells of *B. subtilis* growing exponentially and designated hsp84, hsp69, hsp32 and hsp22, respectively. A comparison of the relative amounts and apparent molecular masses of the hsps from *B.*

Figure 2

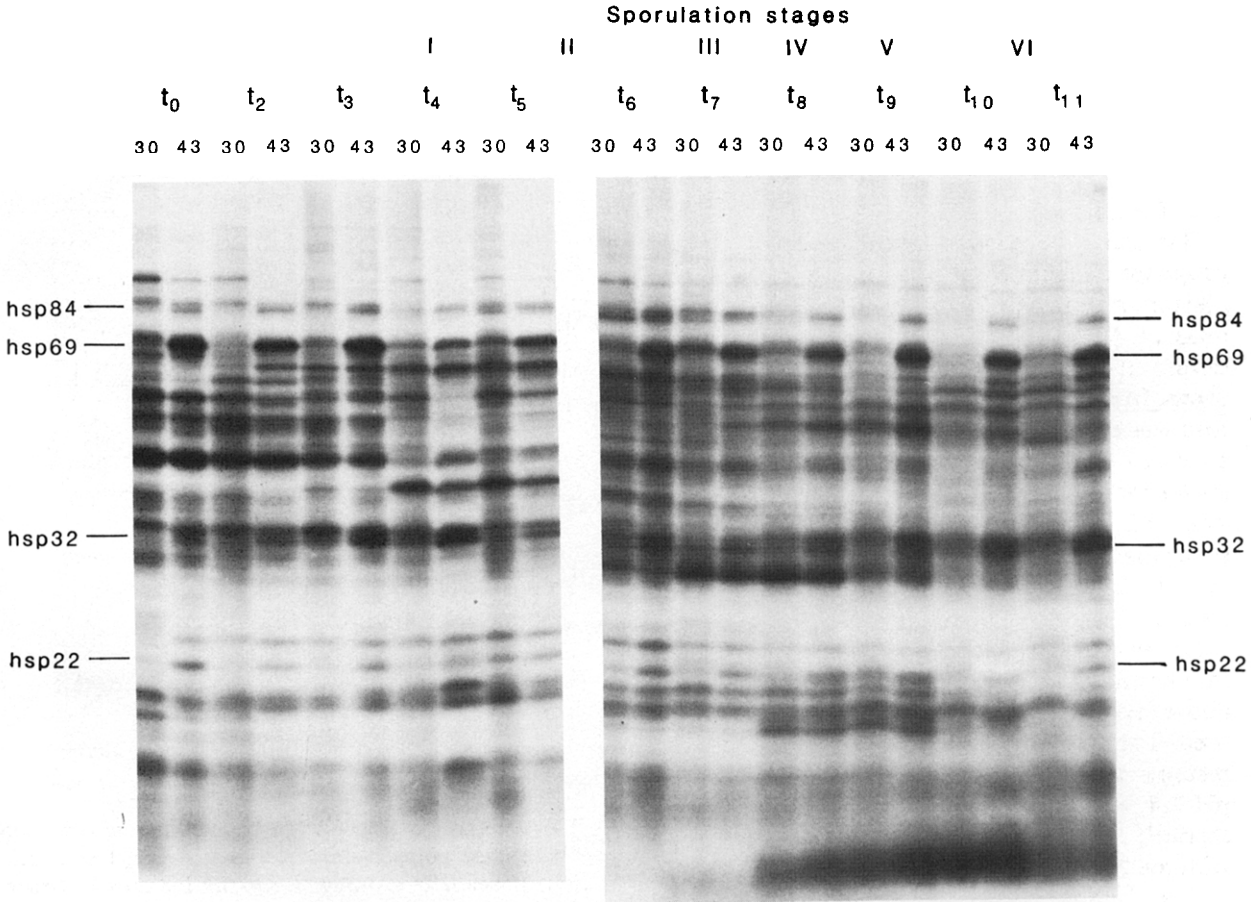


Fig.2. Fluorograms of SDS 15% polyacrylamide gels of $^{35}$S-labelled polypeptide synthesised during heat-shock in *B. subtilis* at the end of exponential growth ($t_o$), during stationary phase ($t_2$ and $t_3$) and during sporulation ($t_4$–$t_{11}$) as defined in section 3. Experimental details are given in section 2.

*subtilis* and *E. coli* (cf. fig.1 tracks a and b) suggests that the hsp69 and hsp84 of *B. subtilis* are analogous to the *groEL* (62 kDa polypeptide) and *dnaK* (76 kDa polypeptide) proteins of *E. coli*, respectively. A recent study of hsps in *Bacilli* [23] revealed that the most abundant hsp had an apparent molecular mass of 66 kDa which probably corresponds to a 65 kDa protein that cross-reacts with antibody against *E. coli groEL* protein [24]. We propose here that this polypeptide corresponds to the hsp69 shown in fig.1 (track a) which under our experimental conditions is the most abundant *B. subtilis* hsp and therefore may be equivalent to the *groEL* protein of *E. coli* and not the *dnaK* protein, the second most abundant hsp in *E. coli* [2] as suggested previously [23]. For comparison, fig.1 (track c) also shows the hsp profile of the mycelial Gram-positive bacterium *S. lividans*. Four proteins with apparent $M_r$ 87 000, 76 000, 53 000 and 46 000 were induced upon temperature shift up.

As found for *E. coli* [25] the heat-shock response in *B. subtilis* is transient. The 4 identified hsps are induced extremely rapidly as substantial synthesis was apparent after 0.5 min heat-shock. The response for these hsps is coordinated and reaches a maximum between 5 and 10 min followed by a rapid decline between 15 and 20 min (not shown).

The heat-shock response was examined during stationary phase and sporulation of *B. subtilis*

Figure 3

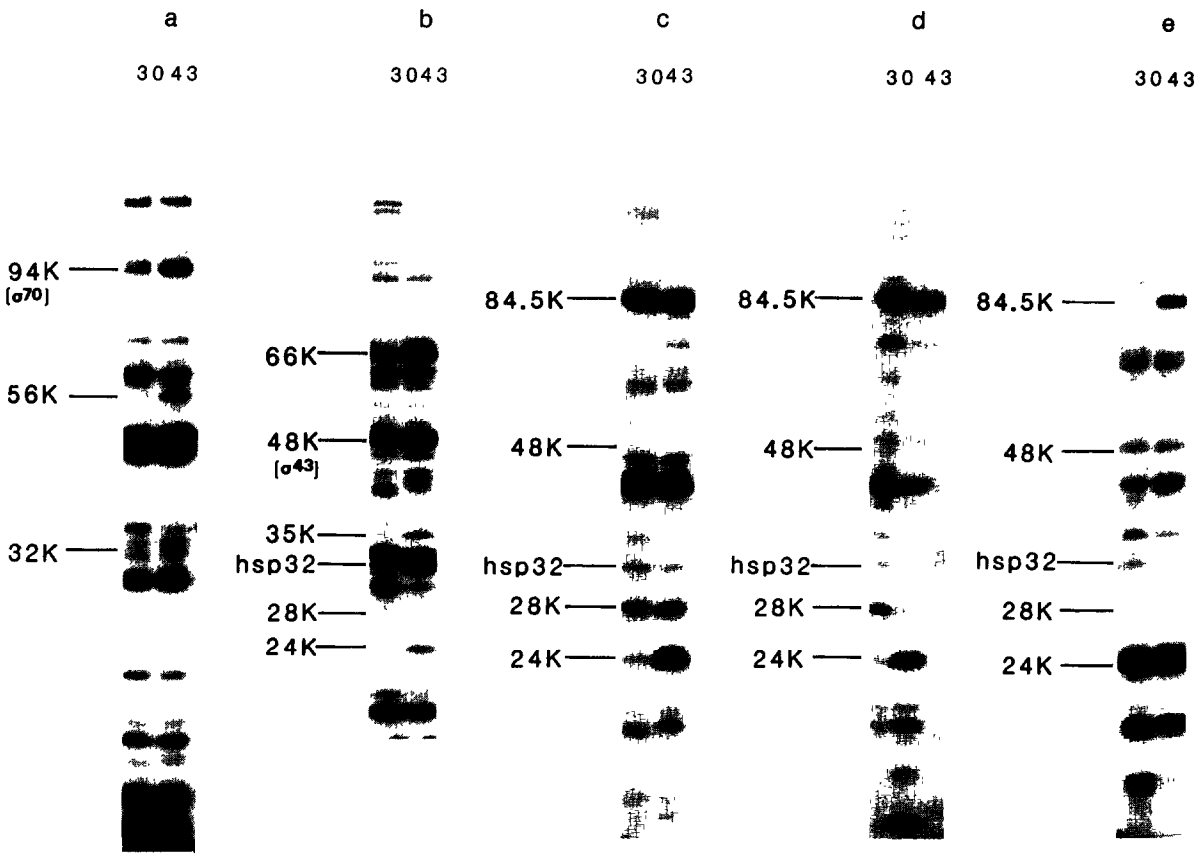


Fig.3. Fluorograms of SDS 13% polyacrylamide gels of $^{35}$S-labelled polypeptide synthesised during heat-shock and partially purified by heparin-agarose affinity chromatography from exponentially growing cells of *E. coli* (track a), *B. subtilis* (track b), and *B. subtilis* stage II sporulating cells (track c), stage V cells (track d) and *spoOA* mutant cells cultured for the same time as the stage II cells (track e). Experimental details are given in section 2.

(fig.2). By monitoring culture absorbance and the sporulation morphological stages [26] the hsps of cells were identified in cells at the end of exponential growth (defined as $t_0$), cells in stationary phase ($t_2$ and $t_3$) and in sporulation stages I–VI ($t_4$, $t_{5-6}$, $t_7$, $t_8$, $t_9$, $t_{10-11}$, respectively) where these stages are observed 4 h, 5 h, etc. after $t_0$. The major conclusion is that the synthesis of the 4 major hsps is heat-inducible throughout growth and sporulation. However, during early sporulation it appears that the constitutive levels of the major hsps especially hsp84 (fig.2, $t_5-t_7$) and hsp32 (fig.2, $t_6$–$t_8$) at 30°C increase. De-repression of heat-shock proteins during yeast sporulation has recently been reported [27]. Exponentially growing cells of the sporulation mutant *B. subtilis* SL566, which contains the most pleiotropic of the stage 0 mutations, *spoOA* possessed a heat-shock response similar to that of the sporulating strain 168 shown in fig.1a. Interestingly, the major hsp profiles of the *spoOA* mutant heat-shocked at the equivalent time to stage II were also similar to strain 168 at stage II (fig.2) except that the synthesis of hsp32 was fully constitutive and not heat-inducible (not shown).

### 3.2. *Heparin-binding minor hsps*

The effect of heat-shock on the synthesis of *B. subtilis* and *E. coli* heparin-binding proteins, among which are the subunits of RNA polymerase and other DNA binding proteins [19,28], was investigated. The major hsps, except *B. subtilis* hsp 32 did not bind to heparin-agarose. Fig.3 shows the 0.5 M KCl column fractions from exponentially growing *E. coli* and *B. subtilis* (tracks a and b, respectively). In *E. coli* 2 additional hsps, not visible in whole cells (fig.1, track b) and with apparent $M_r$ of 94 000 and 56 000, were detected (fig.3, track a). These probably correspond to $\sigma^{70}$ [6] and $\sigma$-prime [29], respectively. A third minor hsp (32 kDa) was also apparent.

In addition to hsp32, which bound weakly to heparin-agarose, *B. subtilis* (fig.3, track b) possessed 4 heparin-binding hsps previously undetected in whole exponentially growing cells (cf. fig.1 track a). Their apparent molecular masses were 66, 48 , 35 and 24 kDa. The 48 kDa polypeptide cosedimented with RNA polymerase on a 10–30% glycerol gradient strongly suggesting that this protein is $\sigma^{43}$, the major vegetative sigma factor (not shown). This is consistent with the recent report that the $\sigma^{43}$ gene is preceded by a sequence that is homologous to the $\sigma^{28}$-specific promoters [30].

The differences between the heparin-binding hsp profiles of *B. subtilis* exponentially growing cells (fig.3, track b), stage II sporulating cells (fig.3, track c), stage V sporulating cells (fig.3, track d) and the *spoOA* mutant cultured for the same time as the stage II cells (fig.3, track e) are pronounced. The synthesis of the 66, 35 and 48 ($\sigma^{43}$) kDa minor hsps is apparently repressed during sporulation. This supports the proposal that the $\sigma^{43}$ form of RNA polymerase is specifically reduced during sporulation [31]. As would be expected, $\sigma^{43}$ is detectable in the mutant *spoOA*, blocked in all sporulation-specific events (fig.3, track e). In whole sporulating cells, hsp32 is heat-inducible although its constitutive levels do increase (fig.2). It is noted that the increased synthesis of hsp32 at 43°C is only reflected in the heparin-agarose eluate from exponentially growing cells and not sporulating cells.

Fig.3 (tracks b–d) also shows that there is a sporulation-specific induction of an 84.5 kDa polypeptide that is not dependent on heat shock. At the normal growth temperature this polypeptide is not detectable in the asporogenic *spoOA* cells (fig.3, track e, 30°C lane) but is induced on temperature shift up (fig. 3, track e, 43°C lane). Since the *spoOA* allele is probably a missense mutation (Dr P. Piggot, personal communication) this result may reflect reactivation of the mutant *spoOA* protein at 43°C by direct temperature-dependent stabilisation or by association with one of the major hsps. The mutant strain SL566 is not, however, temperature-sensitive for sporulation [13]. The opposite holds for a 24 kDa heparin-binding hsp: it is heat-inducible during exponential growth and sporulation (fig.3, tracks b–d) but its synthesis in the *spoOA* mutant is constitutive (fig.3, track e). The synthesis of 2 other heparin-binding hsps, with apparent molecular masses of 40 and 19.5 kDa (not marked in fig.3) followed the same pattern as that of the 84.5 kDa and 24 kDa polypeptides, respectively. We also note in fig.3 a 28 kDa heparin-binding protein that is not a hsp and is only present in sporulating cells and not in exponentially growing cells or the *spoOA* mutant.

We conclude that the heat-inducible synthesis of

the 4 major hsps detected in *B. subtilis* 168 under these experimental conditions is not significantly affected by nutrient deprivation or sporulation. The constitutive levels of the major hsps did, however, increase during early sporulation. With the exception of hsp32, the partial de-repression of the hsps was not dependent on the function of the *spoOA* protein. Hsp32 and 2 other heparin-binding hsps (24 kDa and 19.5 kDa) were fully constitutive in the *spoOA* mutant cultured for the same time as a stage II culture. These results suggest that the synthesis of these hsps and the hsp identified as $\sigma^{43}$ are affected by the regulatory mechanisms operating during stationary phase and sporulation, possibly involving the *spoOA* gene product. Since the *spoOA* protein may interact with RNA polymerase in vivo [32] and is required for the transcription of 2 $\sigma^{28}$-dependent promoters [12] it seems likely that there is an interaction between heat-shock and stationary phase/sporulation regulation at the level of RNA polymerase. One possibility is that the *spoOA* gene product may modulate directly or indirectly the interaction of the different $\sigma$ factors with RNA polymerase.

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