

REVIEW ARTICLE

The heat shock stimulon of *Bacillus subtilis*

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ABSTRACT

The heat shock response is characterized by the transiently increased expression of a set of genes called heat shock genes. In *Escherichia coli*, the best studied eubacterial species, one major heat shock regulon with some 31 genes and two minor regulons are induced after heat stress; all three regulons are under the control of alternate sigma factors. In *Bacillus subtilis*, too, three classes of heat shock genes have been discovered, but two classes are most probably under negative control by two different repressors. Class I genes which form the CIRCE regulon are transcribed from σ^A -dependent promoters and are characterized by a 9-bp inverted repeat called CIRCE element which is located between their transcriptional and translational start points and is recognized by the repressor protein HrcA. The CIRCE element is highly conserved among eubacteria. The majority of the heat-inducible genes belongs to class II, and they are under the control of the alternative factor σ^B , whose activity is controlled by an anti-sigma factor. Class III genes encode predominantly ATP-dependent proteases or their regulatory ATPase subunits. They are transcribed from either one or two σ^A -like promoters or from one σ^A - and one σ^B -dependent promoter. For two members of class III, it could be shown that they are under negative control of a repressor. Whereas class I heat shock genes are induced only by heat or puromycin, those of class II and class III recognize additional stress factors such as ethanol, salt, or oxygen limitation for induction. All heat shock genes of *B. subtilis* form one large heat shock stimulon. It remains to be clarified whether the three classes are interconnected.

INTRODUCTION

Stress response involves the induction of a set of genes which help in the protection and adaptation of the organism in the new environmental conditions (Matin *et al.*, 1989). While part of the response appears to be specialized for a particular stress, there are also genes that respond to multiple stresses. Therefore, stress proteins fall into two groups: general stress proteins (GSPs) and specific stress proteins (SSPs). GSPs are characterized by their enhanced rate of synthesis after cells have been exposed to different environmental stimuli such as heat or salt stress, acid shock, treatment with ethanol, or starvation for glucose, oxygen, or

phosphate. On the contrary, SSPs exhibit an increased rate of synthesis after challenge by only one single stress factor, e.g., by heat. The protective function of stress proteins is well established for heat shock proteins, and the regulation of the heat shock response in eubacteria and mainly in *Bacillus subtilis* will be the subject of this review.

When cells of any organism are exposed to high temperature, the synthesis of a set of so-called heat shock proteins (HSPs) is rapidly induced. The primary structure of most HSPs appears to be highly conserved during evolution, suggesting that they serve similar functions in all organisms. The transient induction of HSPs represents an important protective and homeostatic mechanism to cope with the physiological and environmental stress at the cellular level. Most

HSPs are also synthesized under non-stress conditions, albeit at reduced rates, and research indicated that HSPs play fundamental roles in cell physiology in addition to their activity under stress conditions (for recent reviews on the different aspects of regulation and function of heat shock genes in pro- and eukaryotes see Morimoto *et al.*, 1990).

HSPs constitute a cellular system for folding, repair and degradation of proteins (Georgopoulos and Welch, 1993; Hendrick and Hartl, 1995). Their levels are tightly adjusted to the metabolic and environmental status of the cell by regulation at the transcriptional level. Most HSPs belong to one of two classes: molecular chaperones or proteases. Molecular chaperones are currently defined as a family of cellular proteins that mediate the correct folding of other proteins, and in some cases their oligomerization into polymeric structures, but are not themselves components of the final structures (Gatenby and Ellis, 1990). It is proposed that the essential function of molecular chaperones is to prevent the formation of incorrect structures, and proteases are involved in the degradation of misfolded (non-native) proteins. The general concept is that non-native proteins first interact with molecular chaperones which either mediate their refolding into the correct native state or pass them on to proteases for degradation. It is completely unknown as to which factors influence this decision and whether it involves additional factors yet to be determined.

The regulation of the heat shock response has been studied in detail in *Escherichia coli* (for recent reviews, see Bukau, 1993; Yura *et al.*, 1993). Most genes encoding cytosolic HSPs form a major regulon consisting of some 31 heat shock genes which are positively controlled at the transcriptional level by the alternate sigma factor σ^{32} , encoded by the *rpoH* gene, and therefore called the sigma-32 regulon. This sigma factor is required for the recognition of specific heat shock promoters by RNA polymerase, and the level and activity of σ^{32} is the limiting factor for the transcription of heat shock genes. At low temperatures ($\leq 30^\circ\text{C}$), the concentration of σ^{32} protein in *E. coli* is very low because of the weak translation of *rpoH* mRNA and the extreme instability of σ^{32} (approximately 1 min half-life). The low stability of σ^{32} is a consequence of its interaction with the DnaK chaperone machinery formed by the HSPs DnaK, DnaJ, and GrpE (Liberek and Georgopoulos, 1993) which seems to increase the accessibility of σ^{32} to proteases (Herman *et al.* 1995; Tomoyasu *et al.* 1995). Induction of the transcription of heat shock genes by stress is achieved through (i) stabilization of σ^{32} possibly by breakage of the DnaK/DnaJ- σ^{32} interaction, and (ii) increased σ^{32} synthesis

caused by derepression of *rpoH* mRNA translation (Nagai *et al.*, 1991; Yuzawa *et al.*, 1993). Down-regulation of transcription during the shut-off phase of the stress response is achieved through a decrease in the level and inactivation of σ^{32} (Straus *et al.* 1989). Components of the DnaK system have been shown to interact physically with σ^{32} in crude cell extracts and in a purified system (Gamer *et al.*, 1992; Liberek and Georgopoulos, 1993). Similarly, shutting off of the heat shock response followed by a rapid return to a new steady state also involves the DnaK/DnaJ/GrpE chaperones. Hence, the regulation of the DnaK chaperone machinery via σ^{32} is subjected to a negative feedback control mechanism. The signal for the sigma-32 regulon is probably an increased amount of non-native proteins that interact with the DnaK chaperone machinery and, as a consequence, sequester the chaperones. This heat shock regulatory system is known as the 'DnaK/DnaJ-titration model' (see below). Recently, it could be shown that the ATP- and Zn^{2+} -dependent FtsH protease anchored into the cytoplasmic membrane via two N-terminal transmembrane domains is involved in the degradation of σ^{32} (Tomoyasu *et al.* 1995). Notably, the *ftsH* gene is also part of the sigma-32 regulon.

Apart from the set of genes regulated by σ^{32} , there is an additional heat-shock regulon under the control of σ^E (σ^{24}) transcription factor. σ^E holoenzyme transcribes one of the promoters of the *rpoH* gene (Erickson and Gross, 1989) and the promoter of the *htrA* (*degP*) gene, coding for an ATP-independent endopeptidase localized in the cell envelope (Lipinska *et al.*, 1988; Strauch and Beckwith, 1988). Meccas *et al.* (1993) suggested that the σ^E regulon constitutes a complementary stress system that corresponds to changes in periplasm and outer membrane. σ^{54} is the third minor *E. coli* sigma factor which controls expression of genes involved in N-metabolism, but which also participates in the heat shock response. So far, only one operon (the *psp* operon) has been identified which is induced by temperature upshift and other diverse stress factors. This operon is under complex regulation by both positive and negative factors (Jovanovic *et al.*, 1996).

In summary, three distinct regulatory pathways are now known to control the transcription of the heat shock genes in *E. coli*. These three pathways are interconnected, as the gene for σ^{32} is transcribed by σ^E and the σ^{32} -controlled system down-regulates *psp* expression.

In 1989, we started to analyze the heat shock response in *B. subtilis*, the genetic model of the gram-positive bacteria. One of our motivations was to

answer the question whether in this microorganism the heat shock genes will also be regulated by alternate sigma-factors as it has been described for *E. coli*. Preliminary data argued against the existence of a σ^{32} -like factor in *B. subtilis* since promoter regions of the *E. coli* heat shock genes *dnaK*, *groE*, *lon*, and *htpG* fused to a reporter gene failed to exhibit an increased enzymatic activity after a thermal shock in *B. subtilis* (Wetzstein and Schumann, 1990). On the other hand, polyclonal antibodies raised against *E. coli* σ^{32} cross-reacted with a protein of about 50 kDa present in partially purified *B. subtilis* cell lysates (Schumann, W., unpublished results). During the following years, it turned out that there are three classes of heat shock genes regulated by different mechanisms at the level of transcription. While class I genes are negatively regulated by a repressor, class II positively by the alternate sigma factor σ^B , those of class III seem also to be controlled by a different repressor. At the moment, we cannot exclude that class III heat shock genes are regulated by more than one mechanism (see below). Whether there is a σ^{32} -like factor in *B. subtilis* will be decided by the end of 1996 when sequencing of the *B. subtilis* chromosome will be completed. In the following parts our current knowledge on the regulation of the three classes of heat shock genes will be reviewed. Chapters on the cellular thermometer and an outlook will complete this review. By comparison with an earlier review on the same subject published four years ago, the reader will be able to experience the progress achieved in this field of research (Schumann and Zuber, 1992).

The CIRCE regulon

Class I heat shock genes are composed of nine genes identified so far and are organized in two operons, the *dnaK* and the *groE* operon. Some years ago, we cloned and sequenced both operons and published that the *dnaK* operon consisted of the four genes *orf39*-*grpE*-*dnaK*-*dnaJ* (Wetzstein *et al.*, 1992). Since a function could be assigned to *orf39* meanwhile, this gene has been re-named as *hrcA* (see below). An extensive transcriptional

analysis of the *dnaK* operon using a *dnaJ* riboprobe revealed novel transcripts and suggested the existence of additional genes downstream of *dnaJ*. This hypothesis has been confirmed by sequencing of this region as part of the *Bacillus* genome sequencing project. The *dnaJ* gene is followed by three open reading frames denoted *orf35*, *orf28*, and *orf50* since they could encode proteins of potential molecular weights of 35, 28, and 50 kDa (Kobayashi, Y., personal communication).

While the proteins ORF28 and ORF50 do not reveal significant similarities to proteins in the data bases, ORF35 exhibits similarity to the PrmA protein of *E. coli*, a protein methyltransferase responsible for methylation of ribosomal protein L11 (Vanet *et al.*, 1994). Transcription of the *dnaK* operon turned out to be rather complex. The heptacistronic operon is preceded by a promoter denoted P_{A1} and recognized by the σ^A factor (Figure 1). This sigma factor represents the vegetative (housekeeping) sigma factor responsible for the expression of the majority of the *B. subtilis* genes (Haldenwang, 1995). In addition, there is an internal constitutive σ^A -dependent promoter (P_{A2}) located between *dnaK* and *dnaJ* (Figure 1).

In unstressed cells, several mRNA species can be seen in Northern blots: two large transcripts of 8.0 kb (encompassing the whole operon) and 7.9 kb (processing product of the 8.0-kb transcript lacking the coding region for *hrcA*) and three smaller transcripts of 3.9 (*hrcA* through *dnaK*; there is a potential

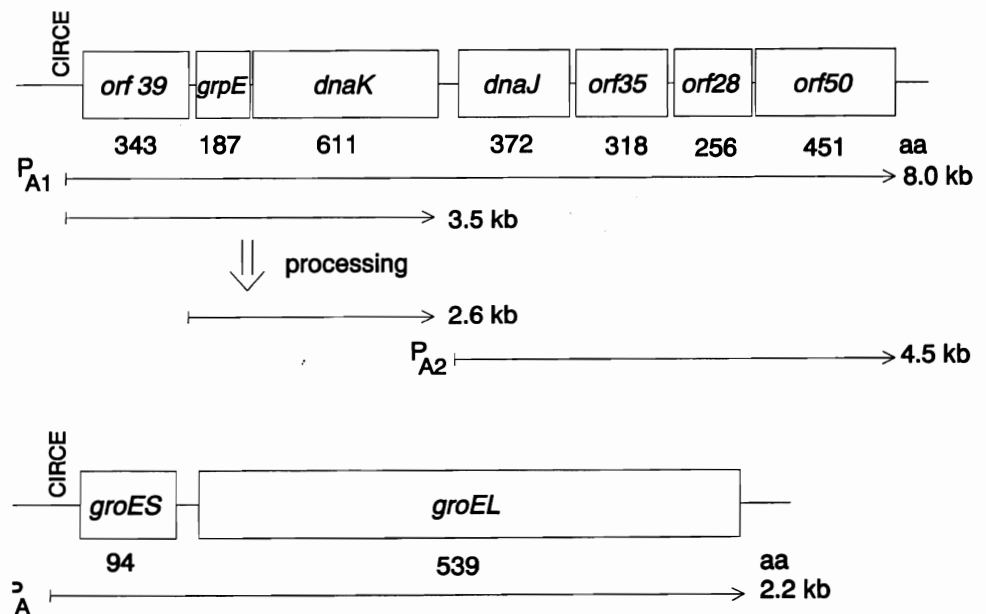


Figure 1 - Genetic organization of the class I heat shock operons *dnaK* and *groE*. The size of the different proteins is given in amino acids (aa), and the length of the mRNA species originating from the vegetative promoters P_A is indicated. The 2.6-kb transcript most probably results from processing of the 3.5-kb species. CIRCE stands for controlling inverted repeat of chaperone expression and acts as a binding site for the HrcA repressor.

rho-independent terminator between *dnaK* and *dnaJ*), of 4.0 kb (representing the four distal genes *dnaJ* through *orf50* expressed from the internal promoter P_{A2}) and of 2.6 kb (processing product of the 3.9-kb transcript). Upon temperature upshift, P_{A1} preceding the whole operon becomes activated resulting in a transient increase in the amount of transcripts originating at this promoter already at low temperatures. The *groE* operon is bicistronic and consists of the two heat shock genes *groES* and *groEL* (Schmidt *et al.*, 1992; Figure 1). There is only one transcript of 2.2 kb whose synthesis is transiently enhanced after heat shock at a σ^B -dependent promoter. The *groE* operon has independently been cloned and sequenced by Li and Wong (1992) and by Tozawa *et al.* (1992).

Expression of both the operons starts at a vegetative promoter recognized by the σ^A . These results were supported by the finding that the expression of the *dnaK* and *groE* operons was markedly reduced in a *sigA* mutant (encoding σ^A) after temperature upshift (Chang *et al.*, 1994). Furthermore, the *groE* promoter was shown to be transcribed by $E\sigma^A$ under both heat shock and non-heat shock conditions (Yuan and Wong, 1995b).

Downstream of these two promoters there is a perfect inverted repeat of 9 bp separated by a 9-bp spacer. In both operons, the sequence of the inverted repeat is identical while that within the spacer region is different. This inverted repeat is not specific for *B. subtilis* as it has been identified in more than 60 cases and in more than 30 different bacterial species (Hecker *et al.*, 1996). In all these cases, the inverted repeat is in front of either the *dnaK* or the *groE* operon. Since both operons encode molecular chaperones, we coined the expression CIRCE which stands for "controlling inverted repeat of chaperone expression" (Zuber and Schumann, 1994). The consensus sequence of CIRCE is TTAGCACTC-N₉-GAGTGCTAA, which is not always perfect and may contain up to four deviations. The spacer region always consists of 9 bp although the repeat might extend into both directions by up to 3 bp on each side. Some bacterial species contain two tandem copies of the element (e.g. *Streptomyces* and *Mycobacterium*). In most cases, where the transcription start site is known, the CIRCE element is located between the transcriptional and the translational start sites. The exceptions to this rule are the *dnaJ* gene of *Lactococcus lactis* and the *dnaK-dnaJ* operon of *Chlamydia trachomatis* where CIRCE is located upstream of the promoter (van Asseldonk *et al.*, 1993; Schmiel and Wyrick, 1994) whereas in *Mycobacterium leprae*, it overlaps with the potential promoter (Rinke de Wit *et al.*, 1992). The widespread occurrence of CIRCE

suggested to us that this DNA sequence could play an important role in the regulation of class I heat shock genes. To prove this assumption, 3 bp each within the left, the right or both arms of the CIRCE element preceding the *dnaK* operon were mutated by sequence-specific mutagenesis. Analyses of these three mutants revealed that the genes of the downstream operon were constitutively expressed at a significantly higher rate as compared to the wild-type strain, while expression of the *groE* operon remained unchanged (Zuber and Schumann, 1994). These results suggested that CIRCE acts as a negative *cis*-element, most probably as a binding-site for a repressor.

The first gene of the *dnaK* operon is *hrcA*, and recent experiments with *hrcA* mutants and complementation studies suggest that *hrcA* encodes a negative regulator of the CIRCE regulon. In a *hrcA::cat* insertion mutant, the unlinked *groE* operon is constitutively expressed at a high level at low temperatures while the genes *grpE* and *dnaK* were not expressed due to a polar effect of the *cat* insertion (Schulz *et al.*, 1995). Deletion of *hrcA* from the chromosome resulted in constitutive high expression of both the *dnaK* and *groE* operon at low temperatures. No further increase in the amount of *dnaK* operon-specific transcript could be measured in the deletion mutant after heat shock. In contrast, there was still an about two-fold increase in the amount of *groE*-specific mRNA suggesting an additional induction mechanism for this operon (Schulz and Schumann, 1996). Yuan and Wong (1995a) arrived at a similar conclusion. Addition of a wild-type plasmid-based copy of *hrcA*, fused to an inducible promoter, to wild-type cells largely prevented induction of class I heat shock genes if the heat shock was preceded by induction of *hrcA* (Schulz and Schumann, 1996).

Very recently, Yuan and Wong (1995a) published data strongly suggesting that *hrcA* codes for the repressor interacting with CIRCE. They could show that (i) seven point mutations resulting in an increased expression of the *groE* and *dnaK* operon at low temperatures mapped within *hrcA*; (ii) expression of a transcriptional fusion in *E. coli* where the controlling region of the *groE* operon was fused to a reporter gene was reduced upon adding *hrcA* to these cells, and (iii) crude extracts prepared from *E. coli* cells containing HrcA protein specifically retarded a DNA fragment with the CIRCE element. Experiments to purify HrcA in its native form failed so far. Overexpression is possible in *E. coli*, but the HrcA protein forms inclusion bodies, and we were not able to find conditions to renature them (Rudolph, M., unpublished results). In contrast, overexpression in *B. subtilis* was not observed.

Whereas the amount of *hrcA*-specific transcript increased significantly after induction, this increase was not accompanied by a comparable increase in the amount of HrcA protein suggesting either posttranscriptional regulation of *hrcA* or degradation of the protein (Schulz, A., unpublished observation).

An *hrcA*-like gene has been described in eight eubacterial species so far, namely *Clostridium acetobutylicum* (Narberhaus *et al.*, 1992), *Chlamydia trachomatis* (Schmiel and Wyrick, 1994), *Lactococcus lactis* (Eaton *et al.*, 1993), *Leptospira interrogans* (Adler, B., personal communication), *Mycoplasma genitalium* (Fraser *et al.*, 1995), *Staphylococcus aureus* (Ohta *et al.*, 1994), and *Caulobacter crescentus* (Roberts *et al.*, 1996). Alignment of the amino acid sequences of the HrcA proteins from these different bacterial species exhibits a low overall similarity. However, upon closer inspection of the amino acid sequences, three regions of increased similarity, tentatively designated as boxes A, B, and C can be detected. It is tempting to speculate that these boxes might be crucial to the function of these proteins. This hypothesis is strengthened by the finding that four out of the seven point mutations in *hrcA* exhibit relief of repression map either within box B or box C (Yuan and Wong, 1995a).

After a sudden rise in temperature, the amount of *dnaK*- and *groE*-specific transcripts increases transiently. This increase can be prevented if cells are treated with rifampicin prior to the heat shock (Schulz *et al.*, 1995), but it cannot be abolished if the cells are challenged with chloramphenicol before temperature upshift. We conclude from these results that the activity of the repressor is modulated in a way so as to allow a transient high level of transcription of the downstream operons and that this modification is independent of *de novo* protein synthesis. This means that the activity of the repressor is either regulated by a protein already present within the cell before heat shock or by an effector molecule which arises under the influence of thermal stress. Our current working model is illustrated in Figure 2.

The repressor protein HrcA will interact with a hypothetical protein HrcB which will modify the activity of HrcA in such a way to allow a transient high-level of transcription of the *hrcA*-controlled operons. Since addition of chloramphenicol cannot prevent this step, HrcB must already be present before temperature upshift. This in turn implies that HrcB is present in an inactive form, and we envisage three possibilities: (i) HrcB is held in the non-functional conformation by a third protein called tentatively HrcC; (ii) it exhibits an intrinsic inactivity under physiological conditions; (iii) it interacts with an effector molecule.

Upon heat shock, HrcB acquires its active form being either released from HrcC or it folds into its active conformation either by release or by interaction with an effector molecule. Active HrcB will then interact with HrcA mediating one of two alternative reactions: it could either inactivate HrcA thereby promoting its dissociation from CIRCE or, alternatively, turn it into an activator of transcription. In both cases, the result will be a burst of class I specific transcripts. But the latter possibility can be excluded since deletion of *hrcA* resulted in a high constitutive expression of the *dnaK* and the *groE* operons indicating that HrcA is not needed as an activator. Experiments are in progress to identify HrcB either at the genetic (by mutation) or the

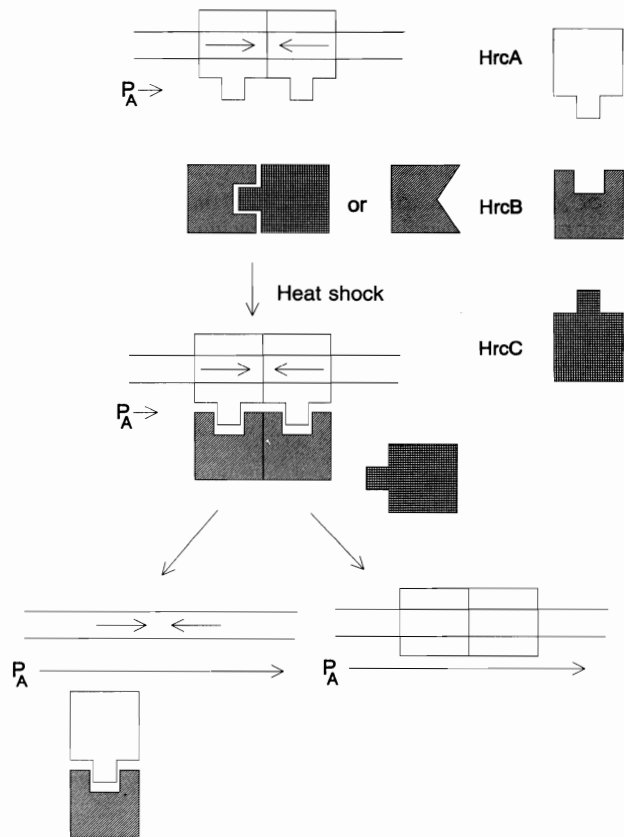


Figure 2 - Regulation of class I heat shock genes: a working hypothesis. At low temperatures, HrcA will interact with the CIRCE element symbolized by opposing arrows; whether it will bind as a dimer as shown here, is not known. Upon thermal stress, a hypothetical protein HrcB will become activated, interact with HrcA and thereby dissociate the repressor from its operator. This might be accomplished either by proteolysis or modification or protein-protein interaction. Since treatment of the cells with chloramphenicol prior to heat will not prevent induction of class I heat shock genes, HrcB must already be present within the cell albeit in an inactive form. Either HrcB is held in its inactive form by a third protein, HrcC, or it is intrinsically inactive but is activated by some effector molecule. Theoretically, HrcA can also be converted into a transcriptional activator, but that possibility can be ruled out by the fact that in the absence of HrcA class I heat shock genes are constitutively expressed at a high rate.

biochemical level (by interaction with HrcA).

Do class I genes belong to the group of GSPs or SSPs? Work in the group of M. Hecker at the University of Greifswald (Germany) has shown that they belong to the group of SSPs. When *B. subtilis* cells were challenged by different stress factors, it turned out that genes of the CIRCE regulon are induced by puromycin, but not by ethanol, glucose starvation, salt stress or H₂O₂ (Völker *et al.*, 1994; Maul *et al.*, 1995). This is in contrast to results obtained with *E. coli* where both operons are induced in addition by ethanol, oxidative stress and starvation.

The sigma-B regulon

The majority of the HSPs of *B. subtilis* are induced not only by heat but, in addition, by any of several environmental stimuli, e.g., ethanol, osmotic upshock or starvation for phosphate, glucose or oxygen (Hecker and Völker, 1990). These stress proteins are therefore classified as GSPs. By which mechanism are these more than 40 GSPs induced? N-terminal sequencing of some GSPs identified CtC and RsbW both of which have been described as σ^B -dependent (Völker *et al.*, 1994). σ^B had been discovered as a new sigma factor in 1979 by Haldenwang and Losick and was the first alternate sigma factor to be found in bacteria encoded by the gene *sigB*. Sequence analysis of the region surrounding *sigB* revealed the presence of seven additional open reading frames with the gene order *rsbR-rsbS-rsbT-rsbU-rsbV-rsbW-sigB-rsbX* (Figure 3). This large operon is transcribed from two different promoters: a σ^A -dependent promoter precedes the whole octacistronic operon (Wise and Price, 1995) while an internal σ^B -dependent promoter is located between *rsbU* and *rsbV* (see Figure 3). From this arrangement of promoters it can be deduced that a σ^B -dependent operon is embedded into a larger σ^A -dependent sub-operon. The activity of the σ^B -dependent promoter is completely eliminated in *B.*

subtilis strains with null mutations in the σ^B structural gene. Under normal growth conditions, transcription starts at the σ^A -dependent promoter ensuring low level of expression of the whole operon. If the cells are challenged with heat or other stress factors the σ^B promoter becomes activated by E σ^B .

The activity of σ^B is regulated in a complex way by the products of at least four genes of the large operon: *rsbU*, *rsbV*, *rsbW*, and *rsbX*. The data suggest that σ^B can be activated by different stimuli via two partially overlapping pathways, one dependent and the other independent of RsbU. Entry into stationary phase in rich medium, exhaustion of glucose, phosphate or oxygen, treatment of bacteria with an uncoupler of oxidative phosphorylation (CCCP) trigger activation of σ^B by the RsbU-independent pathway (Voelker *et al.*, 1995b). Environmental stresses such as heat shock, salt stress or ethanol stress constitute the second group of stimuli and require RsbU for the activation of σ^B .

Genes *rsbW* and *rsbV* control the activity of σ^B . The RsbW protein is an anti- σ factor, binds specifically to σ^B and, thereby prevents σ^B from interacting with RNA polymerase core enzyme (Benson and Haldenwang, 1993). RsbV is a positive regulatory

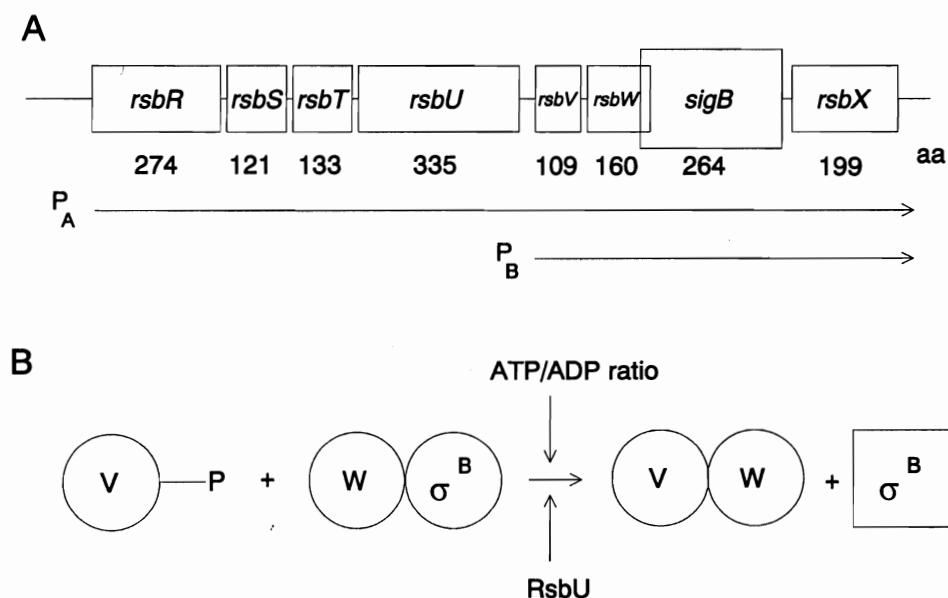


Figure 3 - Genetic organization of the *sigB* operon and a simplified regulation model to account for activation of σ^B . (A) The size of the different proteins is given in amino acids (aa), and the direction of transcription starting from the two promoters P_A and P_B is indicated. (B) In the presence of a high ATP/ADP ratio the anti-anti-sigma factor RsbV is present in its phosphorylated form (V-P), and the anti-sigma factor RsbW will sequester σ^B . Two different circumstances will lead to a dephosphorylation of RsbV which will then interact with RsbW, one independent and the other dependent of RsbU. If cells are treated with the uncoupler of oxidative phosphorylation CCCP, starved for glucose or phosphate or grown into stationary phase, the ATP level will drop thereby activating σ^B . Activation can also occur by heat, ethanol, or salt stress and involves RsbU which promotes RsbV-RsbW complex formation. The RsbU-dependent pathway is negatively regulated by RsbX, and it is unknown whether stress induction involves a release of RsbU from the negative regulation of RsbX or there occurs an independent activation of RsbU.

protein that antagonizes the RsbW block on σ^B (Benson and Haldenwang, 1992; Boylan *et al.*, 1992). Available evidence suggests a model whereby σ^B activity is controlled by the differential association of RsbW with either σ^B or RsbV (Benson and Haldenwang, 1993; Dufour and Haldenwang, 1994). The factors which determine whether RsbW will bind to either RsbV or σ^B are largely unknown. However, two conditions have been proposed to influence the decision about RsbW association: the ATP/ADP ratio and the phosphorylation state of RsbV. A high ATP/ADP ratio seems to favor RsbW binding to σ^B , while a low ratio results in preferential association of RsbW with RsbV. Stressors triggering a drop in ATP result in a shift of RsbW to RsbV, and the release of σ^B . In addition, RsbV can be phosphorylated by RsbW and the phosphorylated form of RsbV does not appear to bind to RsbW (Dufour and Haldenwang, 1994). The remaining two σ^B regulators, RsbU and RsbX, appear to influence the binding preference of RsbW in response to a subset of activation signals. RsbU, or a process dependent on RsbU, can promote RsbW binding to RsbV, thereby allowing $E\sigma^B$ formation. This binding is independent of the intracellular levels of ATP (Voelker *et al.*, 1995a), and RsbX behaves as a negative regulator of RsbU-dependent σ^B activation.

Class III heat shock genes

So far, four heat shock genes have been identified which neither belong to the CIRCE nor the sigma-B regulon. Therefore, these genes have been tentatively grouped into class III and it is not known whether they are all regulated by the same mechanism. These genes code for ATP-dependent proteases namely ClpP (Völker *et al.*, 1992), ClpC (Krüger *et al.*, 1994), Lon (Riethdorf *et al.*, 1994), and FtsH (Deuerling *et al.*, 1995), all of which belong in *E. coli* to the σ^{32} -regulon. These genes are induced by different stress factors and have, therefore, been regarded as GSPs.

The *clpC* gene is part of an operon containing six genes. Two promoters were mapped upstream of the first gene, recognized by σ^A and σ^B . Strong induction by heat, ethanol, and salt stress occurred at the σ^B -dependent promoter. However, in a *sigB* mutant, the σ^A -like promoter became inducible by heat and ethanol stress, completely compensating for *sigB* deficiency (Krüger *et al.*, 1996). A *clpC* mutant of *B. subtilis* is characterized by a drastically reduced tolerance to heat or osmotic stress (Krüger *et al.*, 1994; Msadek *et al.*, 1994). In addition, it has been reported that *clpC* is involved in the regulation of competence (Msadek *et al.*, 1994). The *lon* gene, most probably part of a bicistronic operon, is

induced by different stress factors (Riethdorf *et al.*, 1994). Furthermore, the prespore-specific sigma factor, σ^G , is a natural substrate of the Lon protease when it is prematurely produced (Schmidt *et al.*, 1994). The *ftsH* gene encodes an ATP- and Zn^{2+} -dependent protease which is anchored into the cytoplasmic membrane by two transmembrane domains near its N-terminus (Tomoyasu *et al.*, 1993). Two substrates have been identified being degraded by this protease in *E. coli*: the heat shock σ^{32} factor as already mentioned above (Herman *et al.*, 1995; Tomoyasu *et al.*, 1995) and λ protein cII (Herman *et al.*, 1993). Both these FtsH-substrate proteins do not occur in *B. subtilis*. We succeeded in isolating an *ftsH* knockout mutant. This mutant exhibits a pleiotropic phenotype: (i) it grows largely as filaments even under physiological conditions; (ii) it is extremely sensitive to heat and osmotic stress; (iii) it is not a major regulator of the heat shock response (see below); (iv) secretion of bulk exoproteins is severely impaired; (v) while secretion of α -amylase turned out to be normal that of subtilisin was prevented at the level of transcription, and (vi) the mutant failed to enter the sporulation program (Deuerling, E., Mogk, A., Purucker, M. and Schumann, W., unpublished results). These data point to a more general regulatory role exerted by the FtsH protein within the cell. The FtsH protein is regarded as a quality control protein for those proteins destined for insertion into or secretion through the cytoplasmic membrane either by folding of precursors into their native conformation by the chaperone activity of FtsH or by degrading them by the protease activity. Experiments are in progress to identify substrates of FtsH and to separate chaperone and protease activity by mutations.

Quite recently, we started analysis of another heat shock gene termed *htpG* which is neither preceded by a CIRCE element nor by a σ^B -dependent promoter as deduced from visual inspection of the DNA sequence upstream of the coding region of the gene. This assumption has been confirmed by showing that induction of *htpG* is neither impaired by mutations in *hrcA* nor in *sigB* (Schulz, A., unpublished results). Transcriptional fusions of different *htpG* promoter regions to a reporter gene revealed that this heat shock gene is under negative control and is only induced by heat and not by ethanol, salt, oxygen limitation or H_2O_2 (Schwab, S., unpublished results). These data suggest that *htpG* might be a member of a subclass of class III heat shock genes.

All these genes are preceded by a putative σ^A -dependent promoter, but whether the heat induction at these promoters acts via the same mechanism is unknown. In the case of *ftsH*, we could

map the same 5'-end of the potential *ftsH* transcript suggesting that identical transcriptional start sites are used before and after temperature upshock. Experiments are in progress to identify the *cis*-acting DNA sequences and the proteins involved in recognizing these regulatory sequences.

The cellular thermometer of the heat shock

How does the bacterial cell sense an increase in temperature? Which molecule serves as the sensor of heat? How does the sensor transmit the signal to the regulator of the heat shock response either an alternate sigma-factor or the repressor of the CIRCE regulon? It is generally accepted that the signal is a sudden increase in the amount of misfolded or/and partially denatured proteins collectively called non-native proteins. The formation of non-native proteins may result from one of several processes in *E. coli*: (i) heat shock; (ii) production of a nonsense fragment of β -galactosidase synthesis; (iii) accumulation of overproduced proteins as inclusion bodies; (iv) addition of ethanol; (v) production of puromycin fragments (Kanemori *et al.*, 1994); (vi) addition of certain antibiotics such as chloramphenicol or kanamycin (VanBogelen and Neidhardt, 1990); (vii) incorporation of amino acid analogues such as para-fluoro-phenylalanine into proteins (Babst *et al.*, 1996); (viii) production of heterologous proteins (Kanemori *et al.*, 1994); (ix) production of abnormal proteins (Parsell and Sauer, 1989). In addition, the loss of the 4.5S RNA results in the production of heat shock proteins (Bourgaize *et al.*, 1990), and some uncouplers of oxidative phosphorylation (e.g. CCCP) are known to induce HSP synthesis (Gage and Neidhardt, 1993). It is generally believed that these non-native cytoplasmic proteins will interact in *E. coli* with the DnaK chaperone machinery which will either promote their folding into the native state or deliver them to a proteolytic system. Several regulation models propose that the induction of the σ^{32} regulon relies on sequestering of the DnaK system away from σ^{32} through binding to non-native proteins accumulating during stress (Craig and Gross, 1991; Bukau, 1993; Gamer *et al.*, 1996).

At present, two different model systems are discussed to account for the sensor of heat: the DnaK chaperone machine or the ribosomes. In the first case, the free pool of DnaK serves as a cellular thermometer that monitors changes in cellular concentration of non-native proteins and thereby regulates the expression of Hsps (Craig and Gross, 1991). Alternatively, DnaJ rather than DnaK was postulated to play a key regulatory function in the heat shock

response (Gamer *et al.*, 1992). In the second case, ribosomes are assumed to serve as the sensor for heat shock (VanBogelen and Neidhardt, 1990). Here, details of the sensing and signaling mechanism are unknown. The authors suggested three possibilities for the biochemical nature of the ribosome-generated signal: (i) the ribosomes produce a small signal molecule; (ii) the state of the ribosome itself might be the signal; (iii) heat shock might result in a decrease in accuracy (misreading of the mRNA) thereby producing non-native proteins. A third sensor could be the cytoplasmic membrane which will certainly undergo changes after a heat shock. A possible involvement of the inner membrane in sensing heat stress has been discussed in the context of induction of the *psp* operon (Jovanovic *et al.*, 1996).

In the case of the CIRCE regulon, the DnaK chaperone machinery is not involved in sensing the heat shock, since mutations in any of the three genes do not alter the heat shock response (Mogk, A., unpublished observations). We favor the ribosome model and would like to suggest a fourth possibility for the biochemical nature of the ribosome-generated signal: upon a temperature upshift, a substantial amount of nascent polypeptide chains are released from the ribosomes which cannot fold into their native state and thereby attract the DnaK chaperone machinery. In the case of *B. subtilis*, there must be an alternative to the DnaK chaperone machinery recognizing these non-native polypeptides. The nature of this system is completely unknown at the moment.

Outlook

In *E. coli* and in *B. subtilis*, a heat shock is followed by an immediate and transiently enhanced expression of quite a number of heat shock genes which are regulated at the level of transcription. In *E. coli*, all heat shock genes are transcribed by RNA polymerase containing one of the three minor σ factors: σ^{32} , σ^E , or σ^{54} . Most of the heat shock genes are controlled by σ^{32} and constitute the sigma-32 regulon (Bukau, 1993; Yura *et al.*, 1993). A second class of heat shock genes is transcribed by RNA polymerase containing σ^E (Erickson and Gross, 1989). σ^{54} is responsible for transcription of the phage shock protein (*psp*) operon. This operon is induced by heat, ethanol, osmotic shock, and infection by the filamentous phage f1 (Brissette *et al.*, 1991). Therefore, in *E. coli* at least three distinct regulatory pathways are shown to control transcription of the heat shock genes.

In *B. subtilis*, heat shock genes belong to three classes, too, and are regulated by different mechanisms.

Class I heat shock genes constitute the CIRCE regulon. The *dnaK* and *groE* operons are preceded by typical σ^A -dependent promoters and regulated by a repressor. Further work will concentrate to identify (i) the protein(s) modulating the activity of the repressor HrcA, and (ii) the sensor which leads to heat induction of the CIRCE regulon. Most interestingly, the CIRCE regulon is induced only by heat and by addition of puromycin, but not by ethanol or other stress factors. In contrast, the genes of class II and III are also induced by other stressors such as ethanol or salt stress. Class II heat shock genes are positively controlled by the alternative sigma factor, σ^B . The σ^B regulon is by far the largest of the three classes and encompasses at least 40 members. This number is derived from the analyses of a *sigB* knockout mutant which failed to induce about 40 different proteins after heat or ethanol shock as revealed by 2D-protein analysis (Hecker, M., personal communication). σ^B controls a complicated network of genes, which fall into three groups: (i) genes which only depend on σ^B ; (ii) genes which are preceded in addition by a vegetative promoter P_A that contributes to the basal expression level; (iii) genes containing one (or more) additional promoter(s) which may also be stress-inducible. Here, further work is expected to elucidate the function of the proteins of the *sigB* operon which regulate the expression and activity of σ^B and also the expression of individual genes of the σ^B regulon in detail.

Regulation of the class III heat shock genes is relatively less studied. All genes which are not preceded by a CIRCE element or by a σ^B -dependent promoter are classified into this subgroup. So far, these genes encode either ATP-dependent proteases (*clpC*, *clpP*, *ftsH*, *lon*) or a molecular chaperone (*hspG*). If these genes are regulated by the same mechanism, they should contain a comparable regulator sequence up- or downstream of their P_A promoter. Though we carried out an intensive computer search, we failed to detect such a sequence. This could mean that either these genes are regulated by different mechanisms (which seems rather unlikely) or, alternatively, regulation is not exerted at the level of the primary DNA sequence. Here, the regulation mechanism of individual genes has to be studied to identify regulatory sequences. As already mentioned above, preliminary results indicate that *hspG* might be negatively controlled at the level of transcription, and that the regulatory sequence might be located upstream of the promoter (Schwab, S., unpublished results). Recently, the group of M. Hecker (Greifswald) succeeded in identifying a gene which most probably encodes a repressor responsible for controlling expression of *clpC* and some other genes of class III (Hecker, M., unpublished results).

What molecules sense heat? Is there only one sensor for all three classes of heat shock genes? What is the chemical nature of the signal generated by the sensor? Is there a crosstalk between the three classes? All these questions cannot be answered at the moment and await further intensive experimentation.

Circumstantial evidence suggests that the situation in other bacterial species might be more complicated. There are strong indications that some eubacterial species regulate some of their heat shock genes positively by σ^{32} and the others negatively by the HrcA repressor. In Table I, those bacterial species are listed where *rpoH* and *hrcA* or/and the CIRCE element have been described. This list also contains some bacterial species which have only one of the two mechanisms though a final conclusion can only be made when the complete chromosomal sequence will be available. For two bacterial species there are hints that their *groE* operon might be regulated by both mechanisms namely σ^{32} and HrcA. These are the *groE* operons of *Caulobacter crescentus* and *Bordetella pertussis* which are preceded by a σ^{32} -like promoter and by a CIRCE element (Roberts *et al.*, 1996; Fernandez and Weiss, 1995).

Table I - Occurrence of the two major heat shock regulation mechanisms among eubacteria.

Species	Regulator	
	Sigma-32	HrcA
<i>Escherichia coli</i>	+	-
<i>Citrobacter freundii</i>	+	-
<i>Enterobacter cloacae</i>	+	?
<i>Serratia marcescens</i>	+	?
<i>Proteus mirabilis</i>	+	?
<i>Zymomonas mobilis</i>	+	CIRCE
<i>Pseudomonas aeruginosa</i>	+	-
<i>Agrobacterium tumefaciens</i>	+	CIRCE
<i>Bradyrhizobium japonicum</i>	+	CIRCE
<i>Bacillus subtilis</i>	-	+
<i>Mycobacterium tuberculosis</i>	-	+
<i>Caulobacter crescentus</i>	+	+

+, Sigma-32 or HrcA is present; -, sigma-32 or HrcA is not present; ?, HrcA has not been demonstrated so far; CIRCE, this inverted repeat has been described and HrcA should also be present.

ACKNOWLEDGMENTS

I would like to thank Dr. A. Tripathi for helpful comments on the manuscript. The work carried out in my lab was supported by the Deutsche Forschungsgemeinschaft, by the CEC Biotech Grant BIO2 CT 920254, and by the Fonds der Chemischen Industrie.

RESUMO

A resposta ao choque de calor é caracterizada pela expressão temporariamente aumentada de um conjunto de genes chamados genes do choque de calor. Em *Escherichia coli*, a espécie eubacteriana mais bem estudada, um regulon do choque de calor importante com cerca de 31 genes e dois regulons menores são induzidos depois do estresse térmico; todos os três regulons estão sob controle de fatores sigma alternados. No *Bacillus subtilis*, também, três classes de genes de choque de calor foram descobertas, mas duas classes provavelmente estão sob controle negativo e dois repressores diferentes. Genes classe I que formam o regulon CIRCE são transcritos de promotores dependentes de σ^A e são caracterizados por um segmento de repetição invertido de 9 bp chamado elemento CIRCE, que se localiza entre seus pontos de início transcricional e translacional e é reconhecido pela proteína repressora HrcA. O elemento CIRCE está bem conservado entre as eubactérias. A maioria dos genes induzíveis pelo calor pertencem à classe II e estão sob o controle do fator alternativo σ^B , cuja atividade é controlada por um fator anti-sigma. Os genes classe III codificam predominantemente proteases dependentes de ATP ou suas subunidades de ATPase reguladoras. Eles são transcritos a partir de um ou dois promotores semelhantes a σ^A ou a partir de um promotor dependente de σ^A e de um dependente de σ^B . Para dois membros da classe III, poderia ser demonstrado que eles estão sob controle negativo de um repressor. Enquanto que os genes de choque de calor da classe I são induzidos apenas por calor ou por puromicina, aqueles das classes II e III reconhecem para a indução fatores de stress adicionais como etanol, sal ou limitação de oxigênio. Todos os genes do choque de calor do *B. subtilis* formam um grande "estimulon" do choque de calor. Resta ser esclarecido se as três classes são interconectadas.

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