
The GroE chaperonin machine is the major modulator of the CIRCE heat shock regulon of *Bacillus subtilis*

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Regulation of the heat shock response in bacteria has been studied extensively in *Escherichia coli* where heat shock genes are classified into three classes and where each class is regulated by a different alternate sigma factor. *Bacillus subtilis* serves as a second model bacterium to study regulation of the heat shock response in detail. Here, four classes of heat shock genes have been described so far where two are controlled by two different repressor proteins and the third by the alternate sigma factor σ^B . Class I heat shock genes consists of two operons, the heptacistronic *dnaK* and the bicistronic *groE* operon. Transcription of the *dnaK* operon is complex involving two promoters, premature termination of transcription, mRNA processing and different stabilities of the processed transcripts to ensure the appropriate amounts of heat shock proteins under different growth conditions. The translation product of the *hrcA* gene, the first gene of the *dnaK* operon, binds to an operator designated CIRCE element, and its activity is modulated by the GroE chaperonin system. We assume that the HrcA protein, upon *de novo* synthesis and upon dissociation from its operator, is present in an inactive form and has to be activated by the GroE chaperonin system resulting in an HrcA-GroE reaction cycle. Induction of class I heat shock genes occurs by the appearance of denatured proteins within the cytoplasm which titrate the GroE system. This results in accumulation of inactive HrcA repressor and thereby in induction of class I heat shock genes. Upon removal of the non-native proteins from the cytoplasm, the GroE chaperonin will interact with HrcA and promote folding into its active conformation resulting in turning off of class I heat shock genes. This mechanism ensures adequate adjustment of class I heat shock proteins depending on their actual need.

1. Introduction

The heat shock response involves the transient increased production of a set of proteins designated heat shock proteins (Hsps). It occurs in all organisms studied so far upon thermal upshock or exposure to other stress factors which will lead to the accumulation of denatured proteins. Most Hsps act either as molecular chaperones or as proteases which protect the cells from stress-induced damage by preventing denaturation of cellular proteins, reactivating once-inactivated proteins, and regulating the degradation of irreversibly denatured proteins (Parsell and Lindquist 1993). Expression of all heat shock genes is controlled at the level of transcription by an heat shock factor which is either an alternate sigma factor

or a repressor in prokaryotes or a transcriptional activator in eukaryotes. The activity of the heat shock factor is modulated by one or more Hsps through direct protein-protein interaction.

The best-studied prokaryotic system is the Gram-negative bacterium *Escherichia coli* where three classes of heat shock genes are regulated by three different alternate sigma factors (for a recent review, see Missiakas *et al* 1996). Besides alternate sigma factors, the HrcA repressor is another important heat shock factor which has been discovered in the Gram-positive bacterium *Bacillus subtilis*. In this article, we will shortly review regulation of the three classes of heat shock genes in *E. coli* followed by a detailed description of the *dnaK* operon transcription and of the modulation of the HrcA activity.

Keywords. Heat shock response; CIRCE; HrcA; GroEL; DnaK; repressor

2. The *E. coli* heat shock stimulon

In *E. coli* three classes of heat shock genes, two major and one minor class, have been described which are all positively controlled at the transcriptional level by three different alternative sigma factors thereby constituting two regulons and one operon designated the heat shock stimulon (table 1; Missiakas *et al* 1996). The two major classes are induced upon accumulation of denatured proteins in either the cytoplasm or in the periplasm. Class I heat shock genes are under the control of σ^{32} and form the largest heat shock regulon of *E. coli* with some 35 members also called the sigma-32 regulon. The activity of σ^{32} is modulated by the DnaK chaperone machine (Gamer *et al* 1992; Liberek *et al* 1992; Liberek and Georgopoulos 1993). A detailed description of the regulation of the genes of this regulon is presented by Nakahigashi *et al* 1998.

Class II heat shock genes form the sigma-E regulon, and this regulon is induced when non-native proteins accumulate within the periplasm. There are several possibilities how denatured proteins might be formed: (i) severe heat stress (>45°C); (ii) overproduction of outer membrane proteins; (iii) mutations in genes whose products act as protein folding catalysts, e.g., *surA* encoding a PPIase (Rouvière and Gross 1996). Under physiological conditions, σ^E is sequestered by an anti-sigma factor which is an integral inner membrane protein. Upon accumulation of denatured proteins within the periplasm, σ^E is released from its anti-sigma factor, interacts with the RNA polymerase core enzyme and directs expression of about ten genes of the sigma-E regulon. Shut-off

occurs upon removal of the non-native proteins from the periplasm and rebinding of σ^E to the anti-sigma factor. Two additional proteins are involved in these reactions, but their roles during the modulation of the activities of σ^E and its anti-sigma factor are not well understood (Missiakas *et al* 1997; De Las Peñas *et al* 1997).

As already mentioned, class III heat shock genes consist of only one single operon, the *psp* operon (for a recent review, see Model *et al* 1997). This operon has been identified as being induced after infection with phages containing single-stranded DNA such as M13 or fd. Later, it could be shown that production of gene IV protein encoded by these phages is sufficient to induce this operon (*psp* stands for *phage shock protein*), which is under the positive control by σ^{54} . Under physiological conditions, expression of this operon is prevented at the level of transcription by PspA acting as a repressor.

3. The *B. subtilis* heat shock stimulon

In *B. subtilis*, four different classes of heat shock genes have been described so far (table 2). While those of class I and III are under negative control by two different repressors, class II heat shock genes are positively controlled by the alternate sigma-factor σ^B . All those heat shock genes which neither belong to class I, nor to class II or III have been classified into class IV. Class IV genes are expressed from the vegetative promoter P_A , and *htpG* might be under negative control by a third repressor which is still unknown (Schulz *et al* 1997). This review will report in detail on the regulation of class I heat shock genes.

Table 1. The *E. coli* heat shock stimulon consists of three classes of heat shock genes which are regulated by three different alternate sigma factors.

Class	Sigma factor	Modulator	Number of heat shock genes
I	σ^{32}	DnaK chaperone machine	Some 35
II	σ^E (σ^{24})	Anti-sigma factor	About 10
III	σ^{54}	?	<i>pspABCDE</i>

4. Transcriptional organization of the *dnaK* and *groE* operons

Class I heat shock genes are composed of nine genes organized in two operons, the *dnaK* and the *groE* operon, forming the CIRCE regulon. Both operons are preceded by vegetative promoters P_A followed by a perfect 9 bp inverted repeat which has been designated CIRCE element and functions as an operator (Zuber and Schumann 1994). Under physiological conditions, expression of both operons is partially repressed due to the product of the

Table 2. The *B. subtilis* heat shock stimulon consists of at least four different classes of heat shock genes which are regulated by different mechanisms.

Class	Promoter(s)	Regulation type	Regulatory sequence	Modulator	Heat shock genes
I	P_A	Repressor: HrcA	CIRCE	GroE chaperonin	<i>dnaK</i> , <i>groE</i> operons
II	P_B	Sigma-B	Promoter	Anti-sigma factor	50–100 genes
III	P_A or/and P_B	Repressor: CtsR	CtsR box	?	<i>clpP</i>
IV	P_A	Repressor?	?	?	<i>clpX</i> , <i>lonA</i> , <i>lonB</i> , <i>htpG</i> , <i>ftsH</i> , <i>sigW</i> , <i>htrA</i> , <i>ahpC</i>

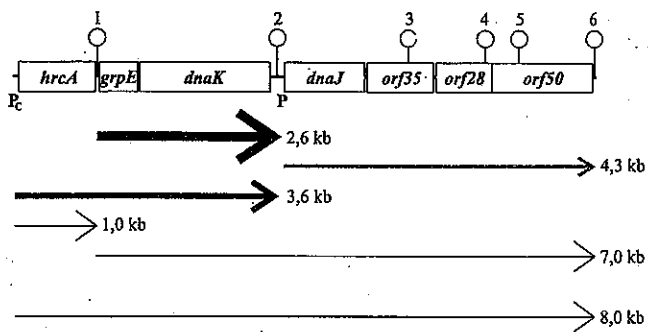


Figure 1. Transcriptional organization of the *dnaK* operon of *B. subtilis*. Transcription of the heptacistronic *dnaK* operon starts at two different promoters, designated P_C and P , as indicated by the arrows; the thickness of the arrows is indicative of the relative amount of the different transcripts. Most of the transcripts initiated at P_C will be terminated at a potential ρ -independent terminator located between *dnaK* and *dnaJ* (stem-loop structure 2); these transcripts will be processed at a site located between *hrcA* and *grpE* (stem-loop structure 1). The stem-loop structures 3 through 5 indicate potential degradation sites, 2 a processing site and 6 another putative ρ -independent terminator.

first gene of the *dnaK* operon, the HrcA protein, which binds to the CIRCE element. Consequently, initiation of transcription at the promoters upstream of the *dnaK* and *groE* operons is partially prevented (Yuan and Wong 1995a,b; Schulz and Schumann 1996; Mogk *et al* 1997).

The *groE* operon consists of the two genes *groES* and *groEL*, encoding the Hsp60 chaperonin and its Hsp10 co-chaperonin, respectively; both genes are transcribed as a bicistronic mRNA (Schmidt *et al* 1992). The *dnaK* operon is heptacistronic and contains the genes *hrcA*, *grpE*, *dnaK*, *dnaJ*, *orf35*, *orf28*, and *orf50* (figure 1). While genes *grpE*, *dnaK* and *dnaJ* code for the DnaK chaperone machine, the functions of the three distal genes are unknown. Knock-outs in these three latter genes neither evoke a temperature-sensitive phenotype nor do they influence the regulation of the heat shock response of the CIRCE regulon (Homuth *et al* 1997).

5. Transcription of the *dnaK* operon

Transcription of the *dnaK* operon is rather complex. The operon specifies three primary transcripts of 8.0, 3.6 and 4.3 kb. Two of these mRNAs (8.0 and 3.6 kb) are processed leading to processing products of 7.0, 1.0 and 2.6 kb. The amount of primary transcripts and processing products within the cells differs before and after thermal upshock. In addition, the stability of the different mRNA species varies considerably (figure 1; Homuth *et al* 1997; our unpublished results). The strongly heat-inducible 3.6 kb primary transcript initiated at the CIRCE-controlled promoter in front of *hrcA* is terminated at a potential

ρ -independent terminator structure located between *dnaK* and *dnaJ*. This transcript is highly unstable, and its processing leads to the formation of two products of 1.0 and 2.6 kb. Furthermore, the CIRCE element located near the 5' end of this mRNA decreases the stability of the transcript as published first for the *groE* mRNA (Yuan and Wong 1995a,b). Our analyses of the *dnaK* transcripts confirmed their results. The bicistronic 2.6 kb *dnaK-grpE* processing product exists within the cells in large amounts already at physiological temperatures and is only weakly induced after a heat shock. This observation can be explained by the fact that the processing event taking place between *hrcA* and *grpE* occurs with a constant, but low rate. The bicistronic 2.6 kb *dnaK-grpE* processing product represents the most stable mRNA species of the whole *dnaK* operon (our unpublished results). The monocistronic 1.0 kb *hrcA* processing product is present within the cell in only small amounts before heat shock and is strongly induced afterwards. This induction can be explained by enhanced novel mRNA synthesis and, in addition, by a transient stabilization of this highly unstable processing product (unpublished results).

Partial readthrough at the terminator structure between *dnaK* and *dnaJ* leads to the appearance of two large mRNA species where the 7.0 kb mRNA molecule represents the processed form of the 8.0 kb primary transcript (figure 1). Both mRNAs are highly unstable, independent of the CIRCE element and most probably due to endoribonuclease-recognition sites near three potential secondary structures as shown in figure 1 (stem-loop structures designated 3, 4 and 5). While the transcripts initiated upstream of the CIRCE-controlled σ^A promoter are heat-inducible, the second σ^A -dependent promoter upstream of the four distal genes is constitutive (Homuth *et al* 1997).

The functional consequences of this transcriptional organization for expression of the different genes of the *dnaK* operon can be summarized as follows: Under physiological conditions, the *hrcA* gene is expressed at a low level being consistent with its function as a repressor. After thermal upshock, this gene is strongly induced; quantitative slot-blot analyses revealed an induction factor of 10–11 for the *hrcA* mRNA (figure 2) which is accompanied by a concomitant increase in the amount of HrcA protein by a factor of about ten (Mogk *et al* 1997). Enhanced amounts of HrcA are needed for switch-off of the heat shock response. The DnaK and GrpE proteins being components of the DnaK chaperone machine are present in large amounts already under physiological conditions; this is ensured by the appearance of the stable 2.6 kb processing product. After heat shock, the total amount of *dnaK*- and *grpE*-specific mRNA increases by a factor of six. The DnaJ protein represents the third component of the DnaK chaperone system. But

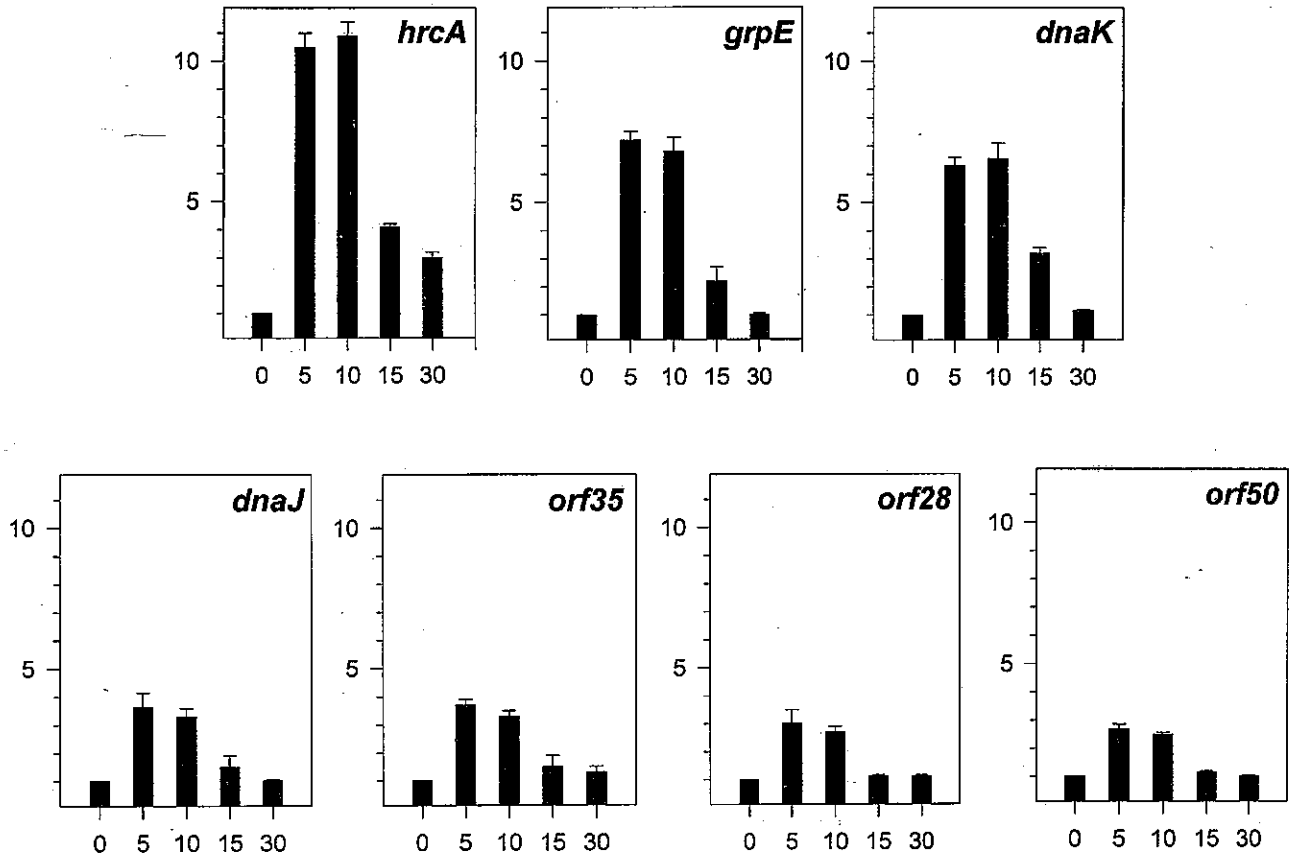


Figure 2. Schematic representation of the changes in mRNA level of the 7 genes of the *dnaK* operon before (0 min) and at different times after (5, 10, 15, and 30 min) heat shock from 37 to 48°C. Luminographs of slot-blot experiments were quantified with the WinCam software version 2.1 of Cybertech, Berlin, Germany.

in comparison to DnaK, it is needed in 3-fold lower amounts. This molar ratio is fine-tuned by partial termination between *dnaK* and *dnaJ* and by low stability of the *dnaJ*-encoding mRNA species; the *dnaJ* mRNA is 3-fold heat-induced. The three genes downstream of *dnaJ* are induced in a way similar to *dnaJ* (figure 2).

6. The GroE chaperonin machine modulates the activity of the HrcA repressor

Class I heat shock genes are under the negative control by a transcriptional repressor encoded by the *hrcA* gene, the first gene of the *dnaK* operon (figure 1). That *hrcA* codes for the repressor has been first shown by Yuan and Wong (1995). They screened for mutations affecting regulation of the *groE* operon and mapped them in *hrcA*. Furthermore, they could demonstrate that crude extracts prepared from an *E. coli* strain overproducing HrcA were able to specifically retard the mobility of a DNA fragment containing the operator, the CIRCE element. At about the same time, Schulz and Schumann (1996) could show that *hrcA* encodes a negative regulator of class I heat shock genes.

Transcriptional analysis of the class I operons indicated that their basal level is increased immediately after thermal shock. To allow a transient high level of expression of the two operons, the repressor must dissociate from its operator and be prevented from re-binding for at least 5 min. How is HrcA transiently kept in an inactive form? In *E. coli* and also in lower and higher eukaryotes it has been shown that non-native proteins formed after thermal upshock constitute the inducing signal. Therefore, we assume that in *B. subtilis*, too, preventing the repressor from binding to its operator is the consequence of accumulation of denatured proteins within the cytoplasm. Will these non-native proteins directly interact with HrcA thereby promoting its dissociation and keeping it in an inactive form or will they rather act in an indirect way? As mentioned above, in *E. coli* non-native proteins within the cytoplasm are recognized by the DnaK chaperone system. Therefore, we first asked whether in *B. subtilis* this system is also involved. We isolated knock-outs in *grpE*, *dnaK*, and *dnaJ* and tested them for their influence on the regulation of class I heat shock genes. It turned out that neither the basal level nor the induction behaviour in these three

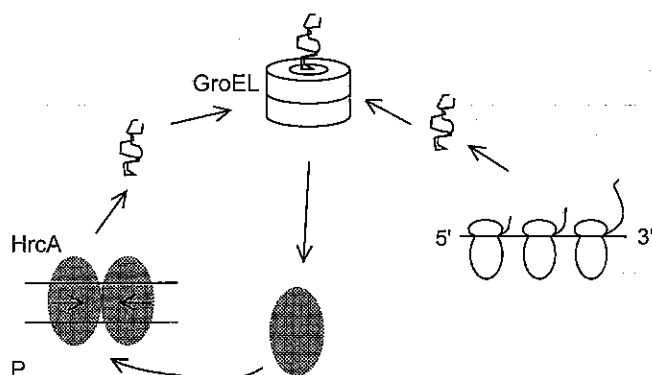


Figure 3. The HrcA-GroEL reaction cycle. We assume that newly synthesized HrcA polypeptide chains have to interact with the GroE system to gain their active conformation allowing them to bind to their operator. Upon dissociation from its operator, HrcA is present in an inactive form and has to bind to GroEL first to become reactivated.

mutant strains was altered. This clearly ruled out any influence of the *B. subtilis* DnaK chaperone system on the regulation of the *dnaK* and *groE* operons (Mogk *et al* 1997).

Since the *groE* operon is also part of the CIRCE regulon, we next asked whether the GroE chaperonin system might be involved in modulating the activity of HrcA. Since neither *groES* nor *groEL* can be inactivated (both genes are essential; Li and Wong 1992), we replaced its own promoter by a controllable promoter thereby allowing depletion of both proteins upon removal of the inducer (Mogk *et al* 1997). To easily monitor expression of the *dnaK* and *groE* operons, transcriptional fusions were constructed between either the promoter region of *dnaK* or *groE* and the reporter gene *bgab* which codes for a heat-stable β -galactosidase. These operon fusions were crossed separately into the *B. subtilis* strain carrying the *groE* operon under the controllable promoter. It turned out that upon depletion for the GroE proteins, both the *dnaK* and the *groE* operons were already expressed at physiological temperatures at a level exceeding that in the wild-type strain after heat induction (Mogk *et al* 1997). These data suggested to us that GroE acts as a modulator of the CIRCE regulon. To confirm these results we reasoned that overproduction of GroE proteins should affect expression of the class I operons adversely. The *groEL* gene was cloned in a high-copy-number plasmid under the control of an IPTG-inducible promoter, and expression of the *dnaK* operon was followed in the absence and in the presence of IPTG using the two transcriptional fusions. While a normal heat shock response was seen in the absence of IPTG, both the basal and the heat-induced level were severely reduced in the presence of IPTG (Mogk *et al* 1997).

To prove that HrcA interacts with its operator, we overproduced the protein in *E. coli* where it formed inclusion bodies. These inclusion bodies could only be dissolved in the presence of a chaotropic agent such as guanidinium hydrochloride or urea. Upon removal of the chaotropic agent either by dilution or by dialysis, the HrcA protein aggregated spontaneously. Therefore, it was almost impossible to obtain a bandshift between HrcA and a DNA fragment carrying the CIRCE element. Since GroEL seems to modulate the activity of HrcA (the role of GroES is not clear at the moment), we assumed a physical interaction between GroEL and HrcA. Therefore, we asked whether purified GroEL could prevent aggregation of HrcA. Indeed, upon dilution of HrcA from a high concentration of guanidinium hydrochloride into a chaotropic agent-free buffer, addition of GroEL in stoichiometric amounts largely prevented the formation of aggregates. This finding prompted us to test whether GroEL would also help to increase the amount of retarded DNA in a gel mobility shift assay. Indeed, the amount of retarded DNA was enhanced from 1 to 12% upon addition of GroEL (Mogk *et al* 1997). We concluded from these *in vitro* experiments that there is a physical interaction between HrcA and GroEL.

7. The HrcA-GroEL reaction cycle

The *in vivo* experiments revealed that after depletion of GroE proteins, high amounts of HrcA repressor accumulated within the cells, but the repressor was unable to interact with its operator to achieve repression. The *in vitro* experiments have demonstrated that GroEL prevents HrcA from forming aggregates and might in addition be involved in correct folding of the repressor. In the light of these results, the following scenario can be proposed for the molecular mechanism underlying the modulation of the HrcA activity. Whether the repressor binds as a dimer as shown in figure 3 is not yet known, but the structure of the operator consisting of two inverted repeats separated by a 9 bp spacer suggests binding as a dimer (or higher oligomer) where both monomers are on the same phase of the helix. Upon *de novo* synthesis, HrcA has to interact with GroEL to adopt its active conformation enabling it to dimerize and to bind to its operator. Upon dissociation, HrcA is unable to rebind to the CIRCE element, it needs activation through GroEL first. This model easily explains how the heat shock response is regulated at the molecular level. After thermal upshock, the amount of non-native proteins suddenly increases, thereby titrating GroEL which is, therefore, unable to reactivate HrcA. This model also explains why in the absence of GroEL, large amounts of inactive HrcA protein accumulate within the cells. Furthermore, it is postulated that the relative activity of HrcA is modulated by the GroE system in response to the amount of

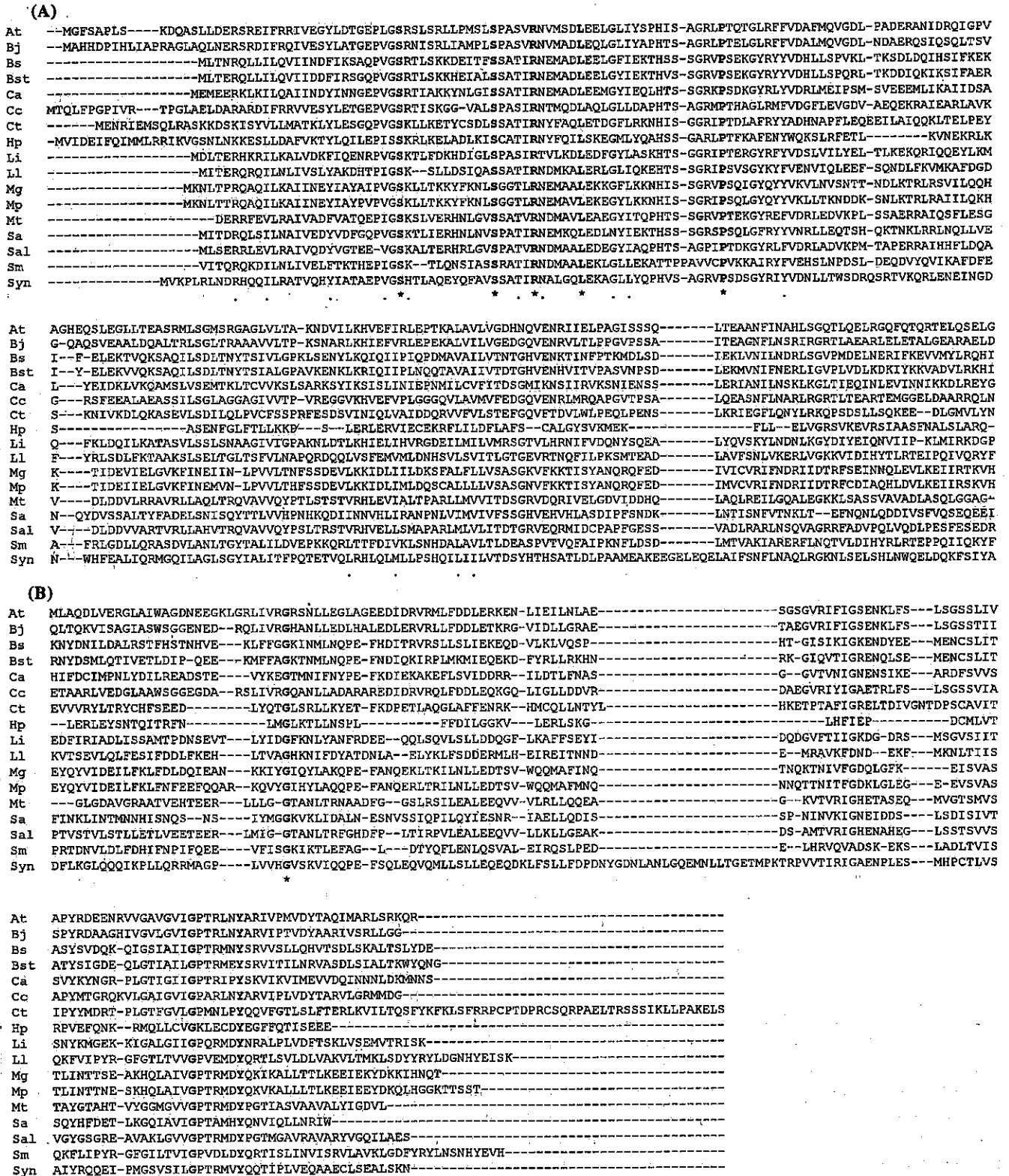


Figure 4. Alignment of HrcA amino acid sequences from 17 different bacterial species. Invariant amino acid residues are given in bold letters and marked by a star underneath the alignment whereas conserved residues are marked by a dot. At, *Agrobacterium tumefaciens*; Bj, *Bradyrhizobium japonicum*; Bs, *Bacillus subtilis*; Bst, *Bacillus stearothermophilus*; Ca, *Clostridium acetobutylicum*; Cc, *Caulobacter crescentus*; Ct, *Chlamydia trachomatis*; Hp, *Helicobacter pylori*; Li, *Leptospira interrogans*; Ll, *Lactococcus lactus*; Mg, *Mycoplasma genitalium*; Mp, *Mycoplasma pneumoniae*; Mt, *Mycobacterium tuberculosis*; Sa, *Staphylococcus aureus*; Sal, *Streptomyces albus*; Sm, *Streptococcus mutans*; Syn, *Synechocystis* PCC6803.

denatured proteins accumulating within the intracellular compartment.

8. The HrcA-CIRCE mechanism is not specific for *B. subtilis*

As already outlined above, the CIRCE element has been described in more than 30 different bacterial species so far implying that all these species also inherit the *hrcA* gene (Hecker *et al* 1996; Schumann 1996). Therefore, the HrcA-CIRCE mechanism seems to be more widespread than the sigma-32 mechanism. Most interestingly, there seem to be bacterial species which encode both mechanisms, and where that occurs, the *dnaK* operon is under σ^{32} control, while the *groE* operon is either only controlled by HrcA-CIRCE or in addition preceded by a σ^{32} -type promoter, thereby ensuring negative auto-regulation in both cases.

This regulation mechanism also allows specific induction of the *groE* operon when enhanced amounts of GroES and GroEL are needed. One example is *Rhodobacter sphaeroides* which contains two *groE* operons where *groESL*₁ is preceded by a CIRCE element (Lee *et al* 1997). GroES and GroEL synthesis parallels the regulated production of ribulose-1,5-bisphosphate carboxylase (RubisCO) under different growth conditions, suggesting important links among chaperonin synthesis, RubisCO control, and RubisCO function (Terlesky and Tabita 1991). We would like to suggest that in all those bacterial species where the *groE* operon is controlled by CIRCE and HrcA, it is needed for the assembly of some protein complex such as RubisCO in photosynthetic bacteria.

9. Conclusions and future work

In *B. subtilis* four classes of heat shock genes have been described to be regulated by different mechanisms. Class I genes consist of the *dnaK* and *groE* operons which constitute the CIRCE regulon. Both operons are regulated at the transcriptional level by the HrcA repressor which binds to the CIRCE element located in both operons between the transcriptional and translational start sites. The activity of the HrcA protein is modulated in a still unknown way by the GroE chaperonin system, and induction of the CIRCE regulon occurs by titration of the GroE system interacting with non-native proteins. Future work will concentrate on two different aspects: to elucidate (i) the domain structure of HrcA and (ii) the molecular mechanism of interaction between the GroEL and HrcA. To date, the primary sequence of HrcA from 17 different bacterial species is known. Alignment of the amino acid sequence of these proteins revealed about 20% identical and a further 10% similar amino acid residues bringing the overall similarity to

about 30% which is rather low for proteins of identical function. But all proteins contain two regions of increased similarity, one near the N-, the other near the C-terminus, designated box A and box B, respectively (Schulz *et al* 1995; see figure 4). Whereas box A exhibits similarity with a region within the DeoR family of DNA-binding proteins and might contain the DNA-binding motif (M Rudolph, unpublished results), box B might represent the multimerization domain. Experiments are in progress to introduce point mutations in both boxes by sequence-specific mutagenesis and to analyse the phenotype of these mutants.

Acknowledgments

We are indebted to K Nakahigashi and T Yura, F Narberhaus and P Mazodier for communicating their unpublished amino acid sequences of the HrcA proteins encoded by *Agrobacterium tumefaciens*, *Bradyrhizobium japonicum* and *Streptomyces albus*, respectively. The financial travel assistance of the German Ministry of Research to WS to attend the Workshop in Varanasi on "Molecular Biology of Stress responses" is gratefully acknowledged. Financial support for the work carried out in the laboratory is provided by the Deutsche Forschungsgemeinschaft and the Fonds der Chemischen Industrie.

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