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

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Class I heat-inducible genes in *Bacillus subtilis* consist of the heptacistronic *dnaK* and the bicistronic *groE* operon and form the CIRCE regulon. Both operons are negatively regulated at the level of transcription by the HrcA repressor interacting with its operator, the CIRCE element. Here, we demonstrate that the DnaK chaperone machine is not involved in the regulation of HrcA and that the GroE chaperonin exerts a negative effect in the post-transcriptional control of HrcA. When expression of the *groE* operon was turned off, the *dnaK* operon was significantly activated and large amounts of apparently inactive HrcA repressor were produced. Overproduction of GroEL, on the other hand, resulted in decreased expression of the *dnaK* operon. Introduction of the *hrcA* gene and its operator into *Escherichia coli* was sufficient to elicit a transient heat shock response, indicating that no additional *Bacillus*-specific gene(s) was needed. As in *B.subtilis*, the *groEL* gene of *E.coli* negatively influenced the activity of HrcA. HrcA could be overproduced in *E.coli*, but formed inclusion bodies which could be dissolved in 8 M urea. Upon removal of urea, HrcA had a strong tendency to aggregate, but aggregation could be suppressed significantly by the addition of GroEL. Purified HrcA repressor was able specifically to retard a DNA fragment containing the CIRCE element, and the amount of retarded DNA was increased significantly in the presence of GroEL. These results suggest that the GroE chaperonin machine modulates the activity of the HrcA repressor and therefore point to a novel function of GroE as a modulator of the heat shock response.

Keywords: CIRCE element/DnaK chaperone/GroE chaperonin/HrcA repressor

Introduction

Regulation of the heat shock response in bacteria has been studied most extensively in *Escherichia coli* (for recent reviews, see Bukau, 1993; Yura *et al.*, 1993; Georgopoulos *et al.*, 1994). The positive regulator of the major heat shock regulon, the sigma-32 regulon, is the σ^{32} subunit of RNA polymerase (RNAP), which confers to core RNAP the specificity to transcribe heat shock genes (Grossman *et al.*, 1984; Landick *et al.*, 1984; Cowing *et al.*, 1985). Induction of the heat shock response is achieved through a rapid increase in the levels of active σ^{32} because of enhanced synthesis and stabilization (Grossman *et al.*, 1987; Straus *et al.*, 1987; Tilly *et al.*, 1989). Genetic data show that at least three heat shock proteins, DnaK, DnaJ and GrpE, are involved in negative regulation of the heat shock response (Tilly *et al.*, 1983, 1989; Sell *et al.*, 1990; Straus *et al.*, 1990). Since these genes act synergistically in other reactions (reviewed by Georgopoulos and Welch, 1993), they are often referred to as the DnaK chaperone machine, where the DnaK protein is the major chaperone protein and GrpE and DnaJ act as co-chaperones (Georgopoulos, 1992). Mutants in any of the three genes overproduce heat shock proteins (HSPs) and are deficient in shut off of the heat shock response. At physiological temperatures, the DnaK chaperone system physically interacts with σ^{32} (Gamer *et al.*, 1992; Liberek *et al.*, 1992; Liberek and Georgopoulos, 1993) and this inhibits the heat shock response (Liberek and Georgopoulos, 1993; Gamer *et al.*, 1996).

In *Bacillus subtilis*, the heat shock response seems to be regulated differently. Three classes of heat shock genes have been described which are all regulated at the level of transcription (for recent reviews, see Hecker *et al.*, 1996; Schumann, 1996). While class I heat shock genes are under negative control by a repressor protein encoded by the *hrcA* gene (Yuan and Wong, 1995a; Schulz and Schumann, 1996), those of class II are positively regulated by the alternate sigma factor σ^{B} (Haldenwang, 1995). Heat shock genes belonging neither to class I or class II have been grouped into class III, including *clpP* (Völker *et al.*, 1994), *clpC* (Krüger *et al.*, 1994), *lon* (Riethdorf *et al.*, 1994), *ftsH* (Deuerling *et al.*, 1995) and *htpG* (Schulz *et al.*, 1997). Since the mechanism of regulation of these genes is largely unknown, class III might be heterogeneous. All heat shock genes together constitute the heat shock stimulon, and it is an open question whether there is cross-talk among the three classes (Schumann, 1996).

Nine class I heat shock genes have been identified so far and are organized in two operons, the heptacistronic *dnaK* and the bicistronic *groE* operon (Schmidt *et al.*, 1992; Homuth *et al.*, 1997). The *dnaK* operon consists of the genes *hrcA–grpE–dnaK–dnaJ–orf*35*–orf28–orf50* (Homuth *et al.*, 1997), where *hrcA* encodes a negative regulator of class I heat shock genes (Schulz and Schumann, 1996) which interacts with an operator sequence (Yuan and Wong, 1995a) composed of a perfect inverted repeat of 9 bp separated by a 9 bp spacer designated the CIRCE element (controlling inverted repeat of chaperone expression) by us (Zuber and Schumann, 1994). Transcriptional analysis of the *dnaK* operon revealed two σ^{A}-dependent promoters, one preceding the whole operon and being heat-inducible (Wetzstein *et al.*,

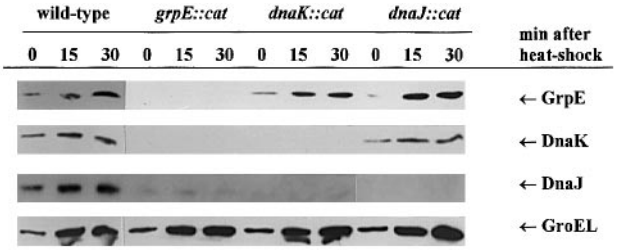

Fig. 1. Synthesis of class I heat shock proteins in wild-type and mutant strains. Total protein cell extracts were prepared from wild-type and three mutant strains LK06 (*grpE*::*cat*), BT02 (*dnaK*::*cat*) and GH05 (*dnaJ*::*cat*) grown at 37°C or subjected to a 15 or 30 min thermal upshift at 50°C. Cell concentrations were equivalent prior to extract preparations. Each set of samples was first loaded on an SDS–PAGE gel and stained with Coomassie brilliant blue to ensure that sample concentrations were equivalent. Samples run on an SDS–polyacrylamide gel were identified by immunoblot analysis. Western blots of the same membrane were performed sequentially using GrpE-, DnaK-, DnaJ- or GroEL-specific antisera.

1992), the other not being heat-inducible and located in front of *dnaJ* (Homuth *et al.*, 1997). The *groE* operon is bicistronic, as has been described for most bacterial species, and consists of the two genes *groES* and *groEL* whose products form the chaperonin machine. There is only one transcript whose synthesis is enhanced transiently after heat shock (Schmidt *et al.*, 1992) at a σ^A-dependent promoter (Yuan and Wong, 1995b).

The repressor protein HrcA and its operator CIRCE are the crucial elements in the regulation of class I heat shock genes. Whereas the *hrcA* gene has been described in 10 different species so far, the CIRCE element has been found >60 times in >30 bacterial species, suggesting that this regulation mechanism is widespread among eubacteria (Hecker *et al.*, 1996). Here, we address the question of how the HrcA repressor is transiently inactivated to allow enhanced expression of the genes of the CIRCE regulon. Our results unambigously show that the GroE chaperonin machinery is the major modulator of the CIRCE regulon.

Results

Genes grpE, dnaK and dnaJ are essential for growth and survival only at high temperatures

Recently, we reported on the isolation of a deletion/insertion mutation within the chromosomal *dnaK* gene (Schulz *et al.*, 1995). This mutant turned out to be viable and to form colonies in the temperature range 16–51°C, but was unable to grow at temperatures >51°C and exhibited a filamentous phenotype. Knockouts in genes *grpE* and *dnaJ* obtained by insertion of a *cat* cassette near their 5′ ends exhibited a comparable phenotype. Both genes could be inactivated, demonstrating that they are non-essential. To prove that insertion of the *cat* cassette led to inactivation of the three genes, the presence of their proteins was analysed by immunoblotting before and after exposure to heat. Low amounts of DnaK, DnaJ and GrpE were present in the wild-type strain 1012, which increased after thermal upshift (Figure 1). In the three knockouts, not only were the proteins encoded by the inactivated genes absent, but also those of the downstream genes, due to a polar effect exerted by the *cat* cassette (Figure 1). These results indicate that the genes encoding the DnaK chaperone machine are dispensable at temperatures below 51°C, which is in contrast to data published for *E.coli*, where *dnaK* and *dnaJ* null mutants grow extremely poorly at all temperatures (Bukau *et al.*, 1988) and where the *grpE* gene could not be deleted in a wild-type background (Ang and Georgopoulos, 1989).

Table I. The *grpE*, *dnaK* and *dnaJ* genes do not influence induction of the heat shock response of class I genes

Strain	Transcriptional fusion	U/mg of protein		
		0 min	15 min	30 min
		after heat induction		
1012	P_{hrcA}–*bgaB*	24	210	220
Δ*hrcA*	P_{hrcA}–*bgaB*	170	260	300
hrcA::*cat*	P_{hrcA}–*bgaB*	155	330	360
grpE::*cat*	P_{hrcA}–*bgaB*	27	224	184
dnaK::*cat*	P_{hrcA}–*bgaB*	22	180	190
dnaJ::*cat*	P_{hrcA}–*bgaB*	24	194	196
1012	P_{groE}–*bgaB*	80	280	300
Δ*hrcA*	P_{groE}–*bgaB*	280	580	550
hrcA::*cat*	P_{groE}–*bgaB*	200	440	520
grpE::*cat*	P_{groE}–*bgaB*	70	250	280
dnaK::*cat*	P_{groE}–*bgaB*	75	240	250
dnaJ::*cat*	P_{groE}–*bgaB*	85	240	260

Wild-type strain 1012 and several isogenic mutant strains carrying transcriptional fusions to the two class I operons were grown in LB medium to mid-exponential phase at 37°C and then heat-induced at 50°C. Samples were withdrawn immediately before (0 min) and at 15 and 30 min after temperature upshift, and β-galactosidase activities were determined as described (Mogk *et al.*, 1996).

The DnaK chaperone machine does not modulate regulation of class I heat shock genes

In *E.coli*, the DnaK chaperone system formed by the HSPs DnaK, DnaJ and GrpE negatively modulates transcription of the heat shock response by affecting the synthesis, activity and stability of σ^{32} (reviewed by Bukau, 1993; Yura *et al.*, 1993; Missiakas *et al.*, 1996). Here, we wished to determine whether these genes also regulate expression of the CIRCE regulon.

To assess expression of the *dnaK* and *groE* operons quantitatively *in vivo*, we measured the β-galactosidase activity of *hrcA–bgaB* and *groE–bgaB* operon fusions in the three knockout strains described above. These two transcriptional fusions have been integrated at the *amyE* locus of the *B.subtilis* chromosome to ensure unimpaired expression from the heat shock operons. As controls, the β-galactosidase activity was also determined in the wild-type strain 1012 and in two different isogenic *hrcA* null mutants. While the activity of the reporter enzyme increased ~10-fold in the wild-type strain after temperature upshift, high constitutive levels were observed in the *hrcA* deletion and in the *hrcA*::*cat* strains, which were comparable with the value observed 15 min after heat induction in the wild-type strain, thereby confirming the role of HrcA as a repressor of both operons (Table I). In the three mutant strains involving *grpE*, *dnaK* or *dnaJ*, both the basal and the induction levels were comparable with those measured in the wild-type strain (Table I). We conclude from these experiments that the DnaK chaperone machine in *B.subtilis* influences neither the basal nor the heat-induced level for the two operons of the CIRCE

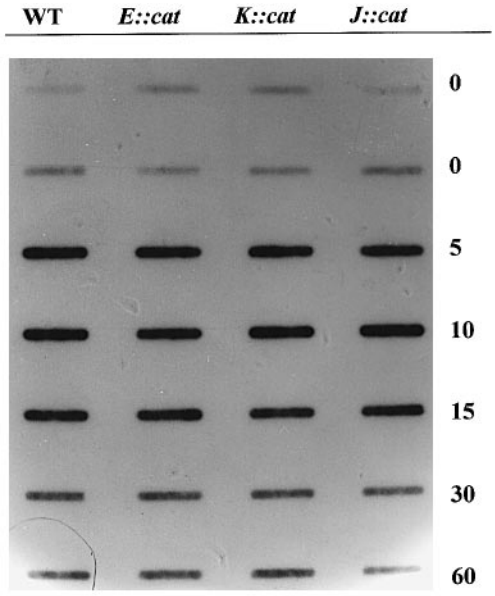

Fig. 2. Concentration of *groEL* mRNA in different *B.subtilis* strains. Slot-blot analysis of total RNA isolated before (0 min) and after a heat shock from 37 to 50°C (5, 10, 15, 30 and 60 min). *Bacillus subtilis* strains 1012 (WT), *grpE*::*cat* (*E*::*cat*), *dnaK*::*cat* (*K*::*cat*) and *dnaJ*::*cat* (*J*::*cat*) were analysed. Digoxigenin-labelled riboprobe RNA complementary to *groEL* mRNA was used as probe. Aliquots of 1 μg of total RNA were applied per slot.

regulon. We also tested for a putative influence of the DnaK chaperone machinery on expression of class II (*ctc–bgaB*) and III (*clpC–bgaB*) heat shock genes, and could not find any (data not shown).

To verify these results by an independent experiment, an immunoblot analysis was performed to determine the relative level of GroEL protein. Heat induction resulted in comparable increases in the amount of GroEL protein in the wild-type strain and in the three mutants (Figure 1). These results unequivocally demonstrate that induction of *groEL* occurred unimpaired in all three mutants, and confirm our previous conclusion that the DnaK chaperone machine influences neither the basal level nor the heat induction of class I genes.

Next, we tested whether the DnaK system is involved in turning off the CIRCE regulon by quantifying the amount of *groEL* mRNA by slot-blotting. In the wild-type strain, the level of *groEL*-specific transcript dramatically increased within the first 5 min after heat induction, peaked at ~10 min and then declined (Figure 2). The same transcription profiles were observed in *grpE*, *dnaK* and *dnaJ* null mutants. In summary, all these results clearly demonstrate that the DnaK chaperone system does not modulate expression of class I shock genes.

The amount of GroES/GroEL influences expression of the dnaK and groE operons

Because the DnaK chaperone system is not involved in regulating expression of class I heat shock genes, we searched for a possible role for the *groE* operon, the second operon of the CIRCE regulon. Since isolation of null mutations in either *groES* or *groEL* was not possible (Li and Wong, 1992), we decided to fuse the chromosomal copy of the *groE* operon to a controllable promoter,

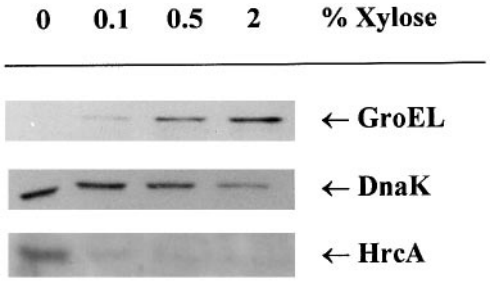

Fig. 3. Cellular levels of class I heat shock proteins depend on the amount of GroEL present in the cells. The amount of three different heat shock proteins was measured in *B.subtilis* strain AMX1 grown in the presence of different xylose concentrations. Other details are as for Figure 1.

thereby allowing depletion of the two proteins (strain AMX1). We have chosen a xylose-inducible promoter system where expression of the desired operon is negatively controlled at the level of transcription by the xylose repressor protein (Rygus *et al.*, 1991). The advantage of this promoter system is that it is tightly controlled in the absence of inducer and that its expression can be modulated by different xylose concentrations added to the medium (Kim *et al.*, 1996). When plated on LB agar in the absence of xylose, strain AMX1 failed to form colonies, confirming that expression of the *groE* operon is absolutely required for growth. When growth of AMX1 was followed in liquid LB medium, the generation time was strictly dependent on the concentration of added inducer at between 0.1 and 2.0% of xylose (data not shown).

Does the amount of GroEL protein within the cells correlate with the xylose concentration added to the medium? GroEL was not detectable in cells incubated for ~4 h in the absence of xylose, whereas addition of increasing amounts of inducer up to 2% resulted in a linear increase in the amount of GroEL (Figure 3), and the 2% value was slightly above the basal level found in wild-type cells and significantly less than the amount detected in heat-shocked cells (data not shown). These data clearly reveal that a certain amount of GroEL and, most probably, GroES protein is absolutely required for growth, thereby confirming the data published by Li and Wong (1992), and further demonstrate that the amount of GroEL in strain AMX1 can be manipulated by the xylose concentration in the medium.

The next step was to assess the basal level of expression of the *dnaK* and the *groE* operons in the presence of varying amounts of GroE proteins. Expression of the *lepA* gene (*lep* stands for leader peptidase) was included as a control since the *lepA* gene does not respond to a heat shock (Homuth *et al.*, 1996). This was achieved by transforming the *hrcA–*, *groE–* and *lepA–bgaB* fusions (all inserted at the *amyE* locus) separately into AMX1, resulting in strains AMX2, AMX3 and AMX4, respectively, and measuring the BgaB activity. In the absence of inducer, the β-galactosidase activity of *hrcA–bgaB* was 10.7-fold higher at 37°C as compared with its level in the uninduced wild-type strain, and reached a level comparable with that found either in the absence of repressor or 30 min after thermal upshift in the wild-type strain (Figure 4A). Growth in the presence of 0.1% xylose still produced a 3.8-fold increase in the basal level, and growth in the

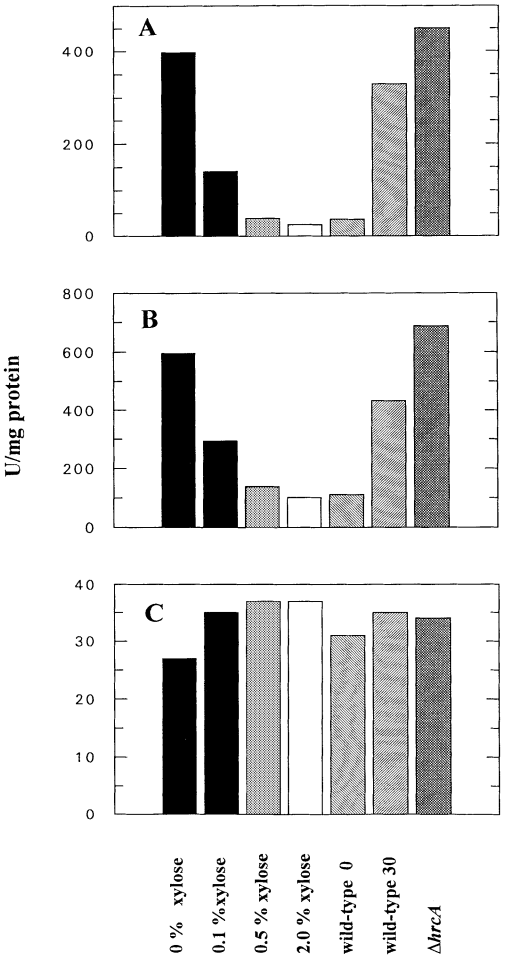

Fig. 4. The *groE* operon modulates expression of the *dnaK* operon. Expression of the three transcriptional fusions *hrcA–bgaB* (**A**), *groE–bgaB* (**B**) and *lepA–bgaB* (**C**) was measured in strain AMX1 in the absence (first column) or in the presence of increasing amounts of xylose (next three columns). Wild-type 0 and 30 mean strain 1012 before and 30 min after thermal upshift to 50°C. The data in the last column refer to 1012 carrying a deletion of *hrcA*.

presence of 0.5 or 2.0% xylose resulted in β-galactosidase activities comparable with those found in the uninduced wild-type strain. A thermal upshift in the absence of inducer produced only a slight induction of the operon fusion, most probably due to its already high expression, whereas heat induction in the presence of 0.5 or 2% xylose resulted in a normal heat shock response (data not shown). For *groE–bgaB*, a comparable expression pattern was obtained, resulting in an ~6-fold higher expression in the complete absence of xylose (Figure 4B). In contrast, the β-galactosidase activity in cells carrying the *lepA–bgaB* fusion remained about constant, independently of the xylose concentrations within the medium (Figure 4C).

In parallel, we measured the amount of DnaK and HrcA by immunoblotting. Whereas the amount of DnaK was high in the absence of GroEL (Figure 3), it decreased when the xylose concentration, and thereby that of GroEL, was enhanced. In accordance with these findings, the amount of HrcA repressor was also high when cells were grown in the absence of xylose (Figure 3). This result was astonishing since high amounts of repressor should result in strong repression of the *dnaK* operon. Since this was not the case, the repressor protein seems to be unable to interact with its operator. We conclude from these experiments that the *groE* operon modulates expression of the *dnaK* operon and of itself by acting as a negative regulator.

If reduced levels of GroE proteins enhance the basal level of expression of class I genes, overexpression of GroEL on the other hand should result in reduced expression of these genes. To test this assumption, *groEL* was fused to an isopropyl-β-D-thiogalactopyranoside (IPTG)-inducible promoter on the high copy number plasmid pREP9, and the new plasmid pREP9-*groEL* was transformed into three different reporter strains. Growth in the presence of 2 mM IPTG resulted in an ~25-fold overexpression of GroEL as compared with the amount present in wild-type cells before heat induction (data not shown). This high amount of GroEL reduced the basal level of *hrcA–bgaB* expression by a factor of ~4 and, in addition, greatly diminished the heat shock response by a factor of 4–5, but the induction factor remained the same in both strains (7- to 8-fold; Figure 5A). A comparable result was found for the *groE–bgaB* fusion, where the basal level of expression was also reduced ~4-fold, and the induction factor remained the same under both conditions (Figure 5B). When the *lepA–bgaB* fusion was analysed under these conditions, overproduction of GroEL did not influence its expression either before or after a heat shock (Figure 5B). These results are consistent with the conclusion that *groEL* acts as a negative modulator of class I heat shock genes. Taking all these results together, the GroE chaperonin rather than the DnaK chaperone machine modulates expression of the CIRCE regulon in *B.subtilis.*

A complete heat shock response can be obtained in E.coli in the presence of the HrcA repressor and its operator, the CIRCE element

After having identified the *groE* operon as a negative modulator of the CIRCE regulon, we asked whether additional gene(s) will influence expression of this regulon. To identify this gene(s), we decided to transfer the known components into *E.coli*, thereby creating a reporter strain and allowing screening for additional functions by transforming this strain with *B.subtilis* gene banks. Yuan and Wong (1995a) have already shown that a transcriptional fusion between the *groE* promoter and the *bgaB* reporter gene was expressed at a high level in *E.coli*; addition of *hrcA* on a second plasmid resulted in a drastic decrease in the β-galactosidase activity. They further reported that treatment of these cells with heat resulted in an increase in enzymatic activity, and speculated that the repressor protein itself might be temperature sensitive (Yuan and Wong, 1995a).

First, we performed similar experiments by constructing two different plasmids. One carried a transcriptional fusion between the *hrcA* promoter including the CIRCE regulatory element and *bgaB* (pAM100), the other contained, in addition, the *hrcA* gene expressed from its own promoter but devoid of the CIRCE element, resulting in constitutive expression of *hrcA* (pAM101). Both plasmids were transformed separately into *E.coli* strain Ω394, and β-galactosidase activities were measured in both strains before and

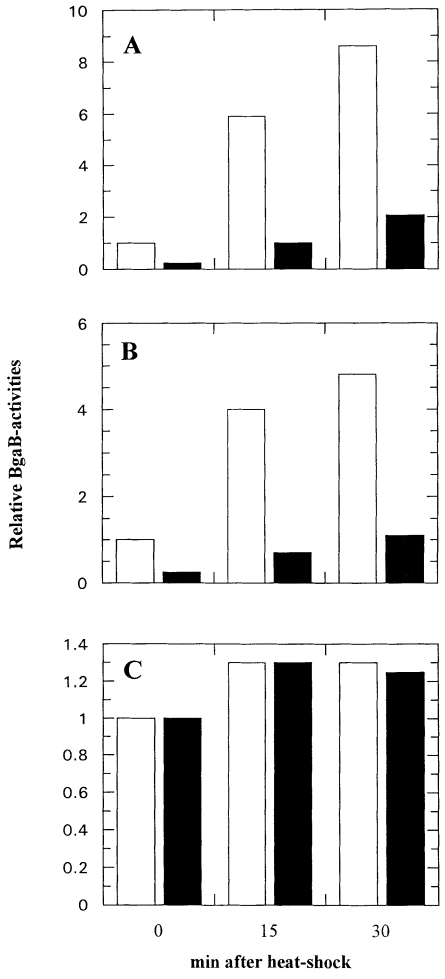

Fig. 5. Overproduction of GroEL leads to a reduced class I heat shock response in *B.subtilis*. Strain 1012 carrying transcriptional fusions between two different promoter regions and *bgaB* and pREP9-*groEL* were grown in the absence or presence of 2 mM IPTG to induce synthesis of GroEL. Strains were grown to mid-logarithmic phase, the BgaB activities were measured and the amount found in the absence of IPTG prior to heat shock was set as one. (**A**) Strain AM03 (*hrcA–bgaB*), (**B**) strain AM02 (*groE–bgaB*) and (**C**) strain AM04 (*lepA–bgaB*) carrying pREP9-*groEL*, respectively. Empty bars, no IPTG; closed bars, 2 mM IPTG added.

Table II. Heat regulation of class I genes occurs in *E.coli*

Strain	Plasmid	*hrcA* present	Relative β-galactosidase activities at		
			0	15	30
			min after heat shock		
Wild-type	pAM100	−	30	32	32
Wild-type	pAM101	+	1.0	4.3	5.1
dnaK756	pAM101	+	0.5	1.7	2.9
groEL140	pAM101	+	8.7	11.4	21.3
groES30	pAM101	+	19.2	27.9	32.4

Wild-type strain Ω394 and its isogenic derivatives were transformed with either pAM100 carrying only the *hrcA–bgaB* transcriptional fusion (no *hrcA* gene present) or pAM101 containing the fusion and the *hrcA* gene. The amount of β-galactosidase obtained in the wild-type strain in the presence of *hrcA* was set as one relative unit.

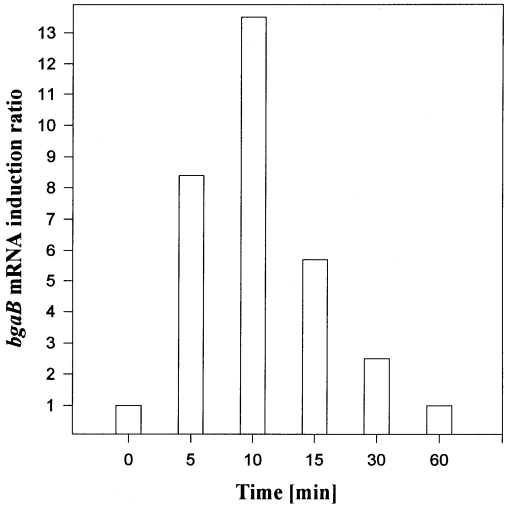

Fig. 6. The *dnaK* operon of *B.subtilis* is transiently heat induced in *E.coli*. Slot-blot analysis with RNA isolated from *E.coli* strain Ω394 carrying pAM101 grown in LB to mid-logarithmic phase at 30°C and at different times after a shift to 42°C. The *bgaB*-specific mRNA was detected using digoxigenin-labelled complementary riboprobe RNA, and the amount present before heat induction was set as one.

after temperature upshift. The enzymatic activity found with pAM101 prior to heat induction was set at one relative unit. In the absence of *hrcA*, ~30 relative units of β-galactosidase were measured, which did not increase after thermal upshift, as expected (Table II). Upon addition of *hrcA*, the enzymatic activity dropped to one relative unit in *E.coli* cells grown at 30°C and increased ~5-fold after a heat shock to 42°C, thereby confirming the results of Yuan and Wong (1995a). The failure to obtain full induction of the operon fusion after a heat shock can be explained by the fact that the level of HrcA protein is strongly enhanced due to the absence of its operator on pAM101, as visualized by immunoblotting (data not shown).

What happens to the repressor protein after a heat shock in *E.coli*? Will it be inactivated irreversibly due to an intrinsic temperature sensitivity, as suggested by Yuan and Wong (1995a)? If this should turn out to be the case, a prolonged incubation of *E.coli* cells carrying pAM101 at 42°C should not result in a shut off of the *bgaB* reporter gene. To answer this question, we measured the amount of *bgaB* mRNA after a heat shock from 30 to 42°C. A rapid increase after thermal upshift was followed by a decline (Figure 6), as has already been reported for the *dnaK* operon in *B.subtilis* (Wetzstein *et al.*, 1992). Since *hrcA* on pAM101 is not subject to down-regulation (its operator is not present), we additionally infer from this result that turning off of the heat shock response is independent of regulated synthesis of HrcA. Measuring the amount of HrcA protein present before and after temperature upshift by immunoblotting in *E.coli* wild-type cells revealed that it remained constant (Figure 7). Most importantly, these data clearly argue against an intrinsic temperature-sensitivity of HrcA. They further show that a heat shock response comparable with that found in *B.subtilis* can be obtained in *E.coli* in the presence of just the HrcA protein and its operator. We conclude that additional *B.subtilis*-specific factors are not involved in regulation of the HrcA activity.

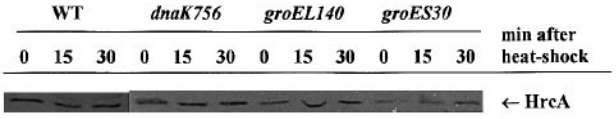

Fig. 7. The amount of HrcA protein remains constant in *E.coli* wild-type and in different isogenic mutant strains. Other details are as for Figure 1.

Will the amount of β-galactosidase be influenced by the *dnaK* or *groE* allele in *E.coli* cells? To answer this question, the plasmid pAM101 carrying *hrcA* and the transcriptional *hrcA–bgaB* fusion was transformed into different mutant strains. The *dnaK756* allele reduced expression of the *bgaB* gene 2-fold before and after heat shock, but the induction factor remained identical. In contrast, two different *groE* alleles dramatically increased expression of *bgaB* 10- to 20-fold (Table II). In all three mutants, the amount of HrcA protein remained constant (Figure 7). We conclude that, though the repressor is present, it is inactive. These data are in agreement with those found with *B.subtilis* and further underline the role of the GroE chaperonin machine in modulating expression of class I heat shock genes of *B.subtilis*.

Addition of GroEL partially suppressed aggregation of HrcA

All these *in vivo* data point to a physical interaction between the HrcA repressor and GroEL. To examine this assumption, the influence of GroEL on HrcA protein was studied *in vitro* using purified components. To facilitate purification of HrcA, the *B.subtilis hrcA* gene was cloned into an expression vector allowing its overexpression in *E.coli*. Upon addition of the inducer IPTG, large amounts of HrcA protein were synthesized and formed aggregates. We tried unsuccessfully to prevent formation of inclusion bodies by varying the temperature and IPTG concentration or by fusing HrcA to thioredoxin (R.Emmerich, unpublished results). The inclusion bodies could be dissolved in denaturing solvents containing 6 M guanidinium hydrochloride or 8 M urea. Upon removal of the chaotropic agent either by dialysis or by dilution into non-denaturing conditions (several buffers were tried), the HrcA protein aggregated spontaneously. Similar observations have been made by S.-L.Wong also for HrcA of *B.subtilis*, by T.Ohta for HrcA of *Staphylococcus aureus*, and by H.Bahl for HrcA of *Clostridium acetobutylicum* (personal communications). As an alternative, we cloned the *hrcA* homologue from *Bacillus stearothermophilus* because this protein from a thermophilic organism should be more stable. When this gene was introduced into an *hrcA* deletion strain of *B.subtilis*, it conferred a normal heat shock response, indicating that both genes are interchangeable (Mogk and Schumann, 1997). Upon overproduction in *E.coli*, the HrcA protein of *B.stearothermophilus* formed inclusion bodies as well, but some protein remained soluble (data not shown). Therefore, all the following experiments were performed with HrcA of *B.stearothermophilus* carrying an N-terminal His_6 tag and purified under denaturing conditions. The *hrcA* gene of *B.stearothermophilus* encoding the His-tagged repressor turned out to fully complement a *B.subtilis hrcA* deletion (data not shown).

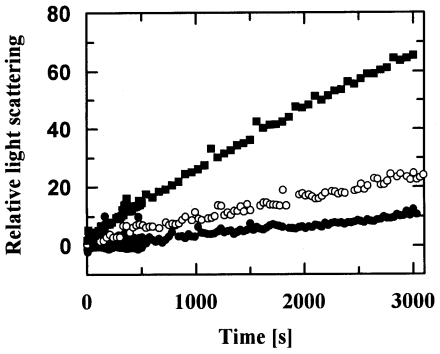

Fig. 8. GroEL retards aggregation of HrcA. Unfolded HrcA (solubilized in 8 M urea) was diluted to a final concentration of 0.4 μM in 10 mM Tris–HCl, pH 8.0, 100 mM Na phosphate buffer, 0.5 M urea, equilibrated at 15°C in the absence (■) or presence of 0.15 (○) or 0.75 μM (●) of GroEL. The increase in light scattering at 500 nm is shown as a function of the incubation time. The light scattering signal remained unchanged when only GroEL was present.

To study whether GroEL binds to HrcA and thus suppresses aggregation, we followed the time course of aggregation in the absence or presence of GroEL by the increase in light scattering (Buchner *et al.*, 1991). Denatured HrcA, dissolved in 8 M urea, was diluted to 0.4 M urea and a final HrcA concentration of 0.4 μM. In the absence of GroEL, HrcA aggregated and the light scattering increased strongly with time (Figure 8). In the presence of 0.15 μM GroEL (the molar ratio GroEL to HrcA was 1:3), aggregation and thus light scattering was reduced 3-fold. When GroEL was present in a super-stoichiometric concentration (0.75 μM), aggregation was decreased further (~6-fold), but not completely suppressed (Figure 8). Addition of 1 mM ATP had no influence on this behaviour (data not shown). We conclude from these data that GroEL interacts with HrcA and thus prevents aggregation of HrcA *in vitro* in a concentration-dependent manner.

Purified HrcA repressor retards the CIRCE element and retardation is significantly increased in the presence of GroEL

Since we have shown that GroEL could largely prevent the aggregation of HrcA, we asked whether it would also be able to promote its renaturation to a functional state. Active HrcA should be able to interact with its operator. Therefore, HrcA protein was incubated with a 120 bp DNA fragment with the operator sequence in the centre and end-labelled with ^{32}P. The reaction mixture was then loaded on a native polyacrylamide gel, and retardation of the DNA fragment was analysed. Addition of HrcA solubilized in 8 M urea resulted only in a very faint bandshift (Figure 9, lane 2; ~1% of the DNA retarded), while GroEL alone did not retard any fragment (lane 3). Incubation of the labelled DNA fragments with both HrcA and GroEL (molar ratio 1:2) resulted in a low, but significant retardation of ~12% of the fragments (lane 4). This retardation could be prevented by adding a 10-fold excess of unlabelled fragment (lane 5), but not by unspecific DNA (lane 6). Addition of ATP to the reaction mixture further increased the amount of retarded fragment to ~27% (lane 7). We conclude from these results that the HrcA preparation contained only very low amounts of

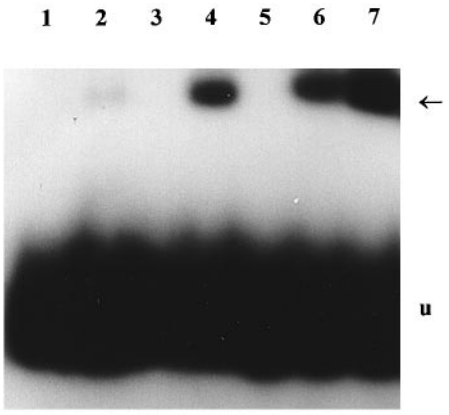

Fig. 9. Gel mobility shift analysis of the CIRCE element. DNA-binding reactions were performed with a 120 bp fragment recovered from pAM22 and labelled with [α-^{32}P]dATP. The position of unbound fragments is indicated by 'u', and the gel retarded bands are marked by an arrow. All lanes contain 0.2 pmol of end-labelled fragment and unspecific DNA (1 μg of sonicated herring sperm DNA) in gel shift buffer; lanes 2–7 contained in addition 0.5 μM of HrcA diluted from a stock solution in 8 M urea to a final concentration of 0.5 M urea (molar ratio of DNA to HrcA was 1:35). Lane 1, no protein; lane 2, HrcA; lane 3, 1 μM of GroEL; lane 4, GroEL and HrcA (molar ratio of GroEL to HrcA was 2:1); lane 5, HrcA, GroEL and a 10-fold excess of cold 120 bp fragment; lane 6, GroEL, HrcA and 4 μg of unspecific DNA; lane 7, HrcA, GroEL and 1 mM ATP.

active repressor able to bind specifically to its operator. The fraction of active HrcA protein could be increased 10-fold in the presence of GroEL which, besides preventing aggregation, also promotes folding. This interpretation is sustained by the finding that addition of ATP increased the amount of retarded fragment and thereby the amount of renatured protein.

Discussion

In *E.coli*, the regulation of the major σ^{32} heat shock regulon is thought to be well understood: σ^{32} has been identified as a positive regulator and the DnaK chaperone system as a negative regulator, and denatured protein acts as a signal to induce the heat shock response. In *B.subtilis*, the situation is more complex, and three classes of heat shock genes encoding cytoplasmic proteins have been identified (Hecker *et al.*, 1996; Schumann, 1996). Class I heat shock genes are under the negative control of the HrcA repressor interacting with the CIRCE element. These heat shock genes could not only be induced by a thermal upshock, but also by ethanol, inclusion bodies (data not shown) or puromycin (Hecker *et al.*, 1996), strongly suggesting that, as reported for *E.coli*, denatured proteins form the inducing signal. Here, we addressed the question of how the activity of this repressor protein is regulated in response to a heat shock. It has to be modulated in such a way as to allow a transient induction of class I heat shock genes.

We first tested whether the DnaK chaperone machine might be involved. In strains where all three genes coding for the DnaK system have been inactivated separately, we found no influence on the regulation of the CIRCE regulon. Instead, when expression of the *groE* operon was shut off, the *dnaK* operon was expressed at a high level at all temperatures, suggesting that *groE* acts as a negative modulator of the *dnaK* operon. In the complete absence of GroE proteins, the BgaB activity of transcriptional *dnaK–bgaB* and *groE–bgaB* fusions was comparable with that found in an *hrcA* deletion strain and in the wild-type strain 30 min after heat induction, though the amount of HrcA was higher than in the wild-type strain. On the other hand, when GroEL was overproduced from a high copy number plasmid, both the basal and the heat-induced levels were significantly reduced, indicating that increased amounts of GroEL within the cells will lead to a pre-adaptation of the system. This effect was specific for heat shock genes since the *lepA* gene, not subject to heat regulation, did not respond to either depletion or over-production of GroEL. We also analysed expression of class II and III operon fusions under conditions of GroESL depletion and found that the basal level was increased 2- to 3-fold, but the heat induction factor was not altered (data not shown). Therefore, titration of GroES/EL is specific for class I heat shock genes.

We established a genetic system in *E.coli* to screen for additional factor(s) which might be involved in the regulation of class I heat shock genes in *B.subtilis*. This system consisted of the two known *B.subtilis*-specific components, namely the *hrcA* gene and its operator fused to the reporter *bgaB* gene. Both components turned out to be sufficient to elicit the complete heat shock response. This remained unchanged when functional DnaK was absent in *E.coli* (data not shown). On the contrary, mutant alleles of either *groES* or *groEL* resulted in a dramatic increase in the basal level of reporter gene expression, suggesting that the HrcA protein is inactive. In *E.coli*, σ^{32} did not respond to the same alleles of *groE* (Strauss *et al.*, 1990). High constitutive expression of the *hrcA–bgaB* fusion in the absence of GroE proteins cannot be based on enhanced amounts of aggregated cytoplasmic proteins within *groES* or *groEL* mutants (Gragerov *et al.*, 1992), but should be the consequence of a direct interaction between GroE and HrcA. Furthermore, these data strongly suggest that no additional *B.subtilis*-specific genes are involved in the modulation of the heat shock response, although the participation of additional component(s) cannot rigorously be excluded. Such components must, however, be present and functional in both *B.subtilis* and *E.coli*.

To demonstrate a specific interaction between purified repressor protein and its operator, the HrcA protein was first overproduced in *E.coli* where it formed inclusion bodies. These could be dissolved by a chaotropic agent but, upon removal of this agent, HrcA aggregated spontaneously. This aggregation could be largely prevented by addition of purified GroEL, indicating that HrcA is a substrate for GroEL. A comparable observation was made when HrcA and GroES/GroEL were co-overproduced in *E.coli*; the amount of HrcA remaining in the soluble fraction was significantly enhanced (data not shown). Purified denatured HrcA was able to refold and specifically to retard a DNA fragment containing its operator, and the amount of DNA retarded was significantly increased in the presence of GroEL and enhanced a further 2-fold after addition of ATP. GroEL alone did not retard the DNA fragment nor did it act as a co-repressor, since the retarded fragment migrated to the same position in the absence or

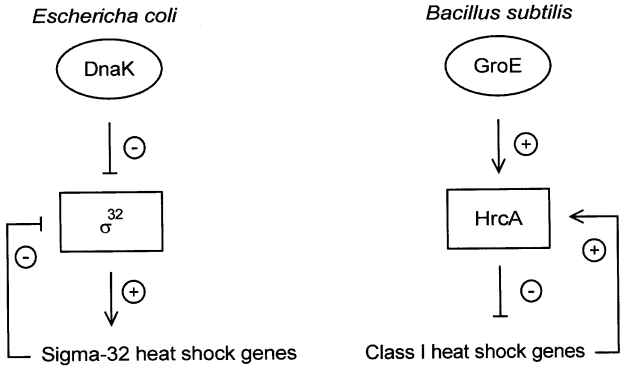

Fig. 10. Model comparing the main features of the two major regulation mechanisms for major heat shock genes in eubacteria. An arrow symbolizes positive, a bar negative regulation. For details, see Discussion.

Table III. Regulation of the *dnaK* and *groE* operons by the two major heat shock regulation mechanisms in different bacterial species

Species	*dnaK* operon	*groESL* operon
Escherichia coli	σ^{32}	σ^{32}
Bacillus subtilis	CIRCE	CIRCE
Agrobacterium tumefaciens	σ^{32}	σ^{32}/CIRCE
Bradyrhizobium japonicum	σ^{32}	σ^{32}/CIRCE
Caulobacter crescentus	σ^{32}	σ^{32}/CIRCE
Streptomyces coelicolor	IR[a]	CIRCE
Synechococcus sp. PCC7942	σ^{32}	CIRCE
Zymomonas mobilis	σ^{32}	CIRCE

[a]Inverted repeat.
References for *E.coli*: Bardwell and Craig, 1984; Hemmingsen *et al.*, 1988; for *B.subtilis*: Schmidt *et al.*, 1992; Wetzstein *et al.*, 1992; for *A.tumefaciens*: Segal and Ron, 1993, 1996; for *B.japonicum*: F.Narberhaus, personal communication; Fischer *et al.*, 1993; for *C.crescentus*: Avedissian *et al.*, 1995; Avedissian and Gomes, 1996; for *S.coelicolor*: Duchêne *et al.*, 1994; Bucca *et al.*, 1995; for *Synechoccocus*: Webb *et al.*, 1990; Nimura *et al.*, 1994; and for *Z.mobilis*: Michel, 1993; Barbosa *et al.*, 1994.

presence of GroEL. We conclude from these results that GroEL is able not only to prevent aggregation but also to promote renaturation of the HrcA protein.

How can all these observations fit into a working model for the regulation of the heat shock response? Such a model should account for (i) a direct interaction between HrcA and the GroE proteins leading to activation of the former, and (ii) the presence of non-native proteins interacting with GroE. A thermal upshift generates an increase in the concentration of non-native proteins, which temporarily deplete the free pool of GroE proteins. Since GroE proteins are necessary for activating HrcA, depletion of cells for GroE will lead to an increase in inactive HrcA and thereby result in induction of class I heat shock genes. If cells are artificially depleted for GroE, the amount of inactive HrcA will increase and lead to an induction of class I genes. Therefore, we consider GroE as the cellular thermometer in *B.subtilis*.

How might GroE influence the activity of HrcA? Theoretically, three possibilities can be envisaged: (i) HrcA could be a substrate for GroE and need the chaperonin for correct folding or/and multimerization; (ii) GroE could influence the activity of an as yet unknown protein which in turn will activate HrcA; (iii) upon dissociation from its operator, HrcA might form aggregates which need GroE for resolution. The experimental finding that GroEL prevented aggregation of HrcA *in vitro* indicates that GroEL can interact directly with the repressor without the interference of any other protein. This will not exclude the possibility that, *in vivo*, additional protein(s) might modulate the HrcA–GroEL interaction. However, we favour a direct interaction between HrcA and GroE, where the interactions between HrcA and the CIRCE element, on one hand, and that of HrcA and GroE, on the other hand, reflect dynamic equilibria. Thus, HrcA should not bind very tightly to its operator, which is confirmed by the finding that the promoter of the *groE* operon is strong under non-inducing conditions, reflecting weak repression. Our central assumption is that HrcA is inactive after dissociation from its operator and needs GroE for activation. Recently, a similar observation has been published by Babst *et al.* (1996) for *Bradyrhizobium japonicum*. In this bacterial species, five copies of the *groESL* operon have been detected (Fischer *et al.*, 1993). In a $groESL_4$ knockout mutant, expression of this operon, as measured by a transcriptional fusion, was significantly increased (Babst *et al.*, 1996). The authors postulated that the stability and/or activity of the repressor protein (its gene has not yet been identified) was enhanced by the chaperonin, thereby constituting a feedback mechanism.

The main features of the interactions between HrcA and GroE are summarized diagrammatically in a working model and compared with the situation in *E.coli* (Figure 10). It should be emphasized that additional heat shock genes in *E.coli* and in *B.subtilis* are regulated by different mechanisms (Missiakas *et al.*, 1996; Schumann, 1996). Though the regulation in both bacterial species is different, involving σ^{32} and the DnaK chaperone machine in *E.coli* and HrcA and the GroE chaperonin system in *B.subtilis*, they share two similarities: (i) in both species, a chaperone (chaperonin) system acts as the main modulator and thereby as the cellular thermometer by influencing the activity of a DNA-binding protein, and (ii) both mechanisms ensure autoregulation of the system where the molecular chaperone will be titrated by non-native proteins.

The *hrcA* gene and the CIRCE element are not specific for *B.subtilis*. While the CIRCE element has been reported to be present in >30 different bacterial species (Hecker *et al.*, 1996), the *hrcA* gene has been isolated and sequenced from 10 species so far, and it can be assumed that it will be found in all those species where a CIRCE element has been described. While *E.coli* is the prototype organism where both the *dnaK* and the *groE* operons are positively controlled by σ^{32}, *B.subtilis* is the prototype organism where both operons are negatively regulated by HrcA (Table III). There are bacterial species which inherit both mechanisms. While in these species the *dnaK* operon is always under σ^{32} control, the *groE* operon is controlled by HrcA (Table III), ensuring autoregulation in both cases. Exceptions to this rule are *Streptomyces albus* and *S.coelicolor* (and presumably other *Streptomyces* species), where the *dnaK* operon is also under negative control of a repressor which is different from HrcA (Bucca *et al.*, 1995; Grandvalet *et al.*, 1997). We suggest that the activity

Table IV. Bacterial strains

Strain	Relevant genotype	Reference
E.coli		
Ω394	F$^-$ *sup°* *galKTE* P_R-λ*cI857* *indl*-P_L-*Nam7,53* *thr::*Tn*10*	Gragerov *et al*. (1992)
Ω419	Ω394 *dnaK756*	Gragerov *et al*. (1992)
Ω425	Ω394 *groEL100*	Gragerov *et al*. (1992)
Ω427	Ω394 *groES30*	Gragerov *et al*. (1992)
M15(pDMI.1)	lacI^q Km^R	LeGrice (1990)
B.subtilis		
1012	*leuA8* *metB5* *trpC2* *hsrM1*	Saito *et al.* (1979)
AM02	*groE–bgaB* at *amyE*; derivative of 1012	Mogk *et al.* (1996)
AM03	*hrcA–bgaB* at *amyE*; derivative of 1012	Mogk *et al.* (1996)
AM04	*lepA–bgaB* at *amyE*; derivative of 1012	Mogk *et al.* (1996)
AMX1	*groE* operon fused to xylose-inducible promoter; derivative if 1012	this study
AMX2	*hrcA–bgaB* at *amyE*; derivative of AMX1	this study
AMX3	*groE–bgaB* at *amyE*; derivative of AMX1	this study
AMX4	*lepA-bgaB* at *amyE*; derivative of AMX1	this study
AS01	*hrcA* deletion derivative of 1012	Schulz and Schumann (1996)
BT02	*dnaK*::*cat*; derivative of 1012	Schulz *et al.* (1995)
LK06	*grpE*::*cat*; derivative of 1012	this study
GH05	*dnaJ*::*cat*; derivative of 1012	this study

of this repressor, HspR, is modulated by the DnaK chaperone system. In the cases of *Caulobacter crescentus* and *Agrobacterium tumefaciens*, the *groE* operons seem to be under dual control by σ^{32} and by HrcA (Segal and Ron, 1993; 1996; Reisenauer *et al.*, 1996; Roberts *et al.*, 1996; Wu and Newton, 1996). As already mentioned, an even higher level of complexity occurs in *B.japonicum*, where five homologues of the *groE* operon and three *rpoH* genes have been reported (Fischer *et al.*, 1993; Babst *et al.*, 1996; Narberhaus *et al.*, 1997). While a CIRCE element precedes three of these operons, a σ^{32}-like promoter is in front of the fourth copy.

This report is the first to identify GroE as a regulator of the heat shock response. Further work will concentrate on the characterization of the native state of the active repressor (monomer, dimer or higher oligomer) and its domain structure. It should contain at least a DNA-binding domain and an additional domain responsible for interaction with GroEL; in addition, it might contain a dimerization or multimerization domain. The alignment of the amino acid sequences of the different known HrcA proteins revealed three regions of increased homology (Schulz and Schumann, 1996). It is tempting to speculate that these regions are part of the two or three domains.

Materials and methods

Bacterial strains and growth conditions

The bacterial strains used in this study are listed in Table IV. Cultures were grown at ambient temperatures under aeration in Luria-Bertani (LB) medium. When necessary, LB was supplemented with ampicillin (200 μg/ml), neomyin (20 μg/ml) or chloramphenicol (Cm, 5 μg/ml). Growth was monitored by measuring the optical density at 578 nm (OD_{578}).

Construction of chromosomal insertion mutations

To obtain *grpE*::*cat* and *dnaJ*::*cat* insertions, both genes and their flanking regions have been generated by PCR using chromosomal DNA as template. Both amplicons were ligated separately into pBluescript KSII$^+$ (Statagene), resulting in p*grpE* and p*dnaJ*, followed by insertion of a *cat* cassette recovered as a *Bam*HI–*Hin*dIII fragment from pUC18Cm (Beall and Lutkenhaus, 1989) and blunt-ended into the unique *Nae*I or *Eco*47III site of *grpE* and *dnaJ*, respectively (p*grpE::cat* and p*dnaJ::cat*). In both plasmids, transcription of *grpE* or *dnaJ* and *cat* occurs in the same direction. To recombine the *cat* cassette into the chromosomal *grpE* and *dnaJ* genes, *B.subtilis* 1012 was transformed separately with p*grpE::cat* and p*dnaJ::cat*. Transformants were selected on plates containing Cm, and chromosomal DNA was screened by PCR for replacement recombination at the *grpE* and *dnaJ* locus, respectively (data not shown). One of each of these recombinants, LK06 (*grpE::cat*) and GH05 (*dnaJ::cat*), was kept for further studies. To bring the *groE* operon under a controllable promoter, a 180 bp fragment (corresponding to nucleotides 168–352 in Schmidt *et al.*, 1992) was generated by PCR using chromosomal DNA as template, flanked by *Bam*HI sites and ligated into the *Bam*HI site of pX2 (pX2-ES). Plasmid pX2 resulted from ligation of the *Bam*HI–*Bgl*II fragment carrying the *xylR*-P_{xylA} region recovered from pLK04 (Kim *et al.*, 1996) into the *Bam*HI site of pJH101 (Ferrari *et al.*, 1983). Next, pX2-ES was transformed into *B.subtilis* 1012, and transformants were selected in the presence of 5 μg of Cm/ml and 0.5% xylose to ensure expression of the *groE* operon. Integration of pX-ES at the *groE* locus was verified by PCR. To measure expression of different heat shock genes in the presence of varying amounts of GroE proteins, the transcriptional fusions *hrcA–bgaB*, *groE–bgaB* and *lepA–bgaB* were transformed into AMX1, resulting in strains AMX2, AMX3 and AMX4, respectively.

Construction of plasmids

To obtain increased amounts of GroEL protein within *B.subtilis* cells, the *groEL* gene was generated by PCR and inserted into the expression vector pREP9 (LeGrice, 1990), thereby fusing the structural genes to an IPTG-controllable promoter (pREP9-*groEL*). Plasmid pAM100 carries the *hrcA–bgaB* transcriptional fusion (Deuerling *et al.*, 1997). In another step, *hrcA* was added as a *Sma*I–*Bam*HI PCR fragment to this operon fusion in such a way that it was expressed from its own promoter, but devoid of the CIRCE element (pAM101). Here, chromosomal DNA of strain SW01 was used. In this strain, the CIRCE element preceding the *dnaK* operon had been deleted (S.Wallinger, unpublished). To generate a *bgaB* riboprobe, this gene was generated by PCR flanked by *Bam*HI sites and inserted into pBluescript SKII$^+$. To obtain controllable overexpression of HrcA in *E.coli*, *hrcA* of *B.stearothermophilus* (Mogk and Schumann, 1997) was generated by PCR, flanked by *Bam*HI sites and inserted into pDS56 (Hochuli *et al.*, 1988), resulting in pAM20. In parallel, the *hrcA* gene was cloned into pUSH1 (Schön and Schumann, 1994), allowing IPTG-dependent expression of *hrcA* with an N-terminal His_6 tag (pAM21). The His_6-tagged HrcA is fully active *in vivo*. This has been shown by expressing this gene from pAM21 in a *B.subtilis* AS01 deleted for its own *hrcA* gene and carrying the P_{hrcA}–*bgaB* transcriptional fusion. This strain exhibited a normal heat shock response (data not shown). pAM22 contains a 120 bp *Bam*HI–*Kpn*I fragment, with the CIRCE element preceding the *groE* operon located in the centre

(nucleotides 61–180 from the published DNA sequence; Schmidt *et al.*, 1992) cloned into pBluescript SKII$^+$.

DNA manipulations

For restriction digests, ligation, transformation, agarose gel electrophoresis and Southern transfer, standard procedures were followed (Sambrook *et al.*, 1989). For hybridization, the non-radioactive DNA labelling, hybridization and detection kit (Boehringer Mannheim) was used according to the directions given by the manufacturer.

Analysis of transcription

Preparation of total RNA, hybridization and slot-blot analysis were performed as described (Homuth *et al*., 1997). In all slot-blot experiments, 150 ng of total RNA per slot was used. As hybridization probes, digoxigenin-labelled RNAs complementary to the transcripts were used. These had been synthesized *in vitro* with T7 RNA polymerase (Boehringer Mannheim, DIG-RNA Labeling Kit) from linearized plasmids.

Overexpression and purification of GrpE and DnaJ and antibody production

To facilitate the overproduction and purification of GrpE and DnaJ, both genes were first amplified by PCR using chromosomal *B.subtilis* DNA and primers with *Bam*HI sites at their termini. The two amplicons were cleaved with *Bam*HI and cloned into *Bam*HI-linearized pDS56 (Hochuli *et al.*, 1988), resulting in plasmids pDS56-*grpE* and pDS56-*dnaJ*. These plasmids were then introduced separately into *E.coli* M15(pDMI.1) for overproduction of His_6-GrpE and His_6-DnaJ. Both His-tagged proteins were purified from IPTG-induced cells (1 mM IPTG for 3 h) as described (Hochuli *et al.*, 1988) and were used to raise polyclonal antibodies in rabbits. These primary antibodies were used at the following dilutions: anti-GrpE at 1:250 and anti-DnaJ at 1:2500. Antibodies raised against the *B.subtilis* HrcA, DnaK and GroEL proteins have already been described (Schulz *et al.*, 1995) and were used at dilutions of 1:500, 1:10 000 and 1:20 000, respectively.

SDS–PAGE and immunoblot analysis

Sample preparation for SDS–PAGE and immunoblot analysis were performed as described previously (Homuth *et al.*, 1996). Five μg of total cellular protein were applied per lane. Polyclonal sera against HrcA, GrpE, DnaK, DnaJ and GroEL, a donkey anti-rabbit IgG horseradish peroxidase conjugate (Amersham) and a chemiluminescence reaction (Amersham, ECL system) were used for visualization of cross-reacting material.

β-Galactosidase assay

β-Galactosidase activities were assayed at 55°C as described (Mogk *et al.*, 1996), using *o*-nitrophenyl-β-D-galactopyranoside (ONPG) as substrate. All assays were repeated at least three times and yielded comparable results. The data from one experiment are given.

Purification of HrcA

His_6-tagged HrcA of *B.stearothermophilus* was overproduced in *E.coli* containing pAM20 and purified under denaturing conditions essentially as described for GroES (Schön and Schumann, 1994). The purified protein was dissolved in 10 mM Tris–HCl, 100 mM Na phosphate buffer, pH 8.0 and 8 M urea. Purification of GroEL has already been described (Walter, 1996).

Light scattering

Light scattering during the aggregation of HrcA was followed essentially as described (Höll-Neugebauer *et al.*, 1991) using a Hitachi F-4010 fluorescence spectrometer equipped with a temperature-controlled cell holder and a magnetic stirrer. The excitation and emission wavelengths were set at 500 nm, with bandwidths of 5 nm in both cases. The solutions were stirred with a small magnetic bar to prevent the formed aggregates from sedimenting in the cuvette. At time zero, purified unfolded HrcA (8 μM in 8 M urea) was diluted 20-fold to a final concentration of 0.4 μM HrcA and 0.4 M urea in 10 mM Tris/100 mM Na phosphate, pH 8.0, in the absence or presence of GroEL at 15°C. HrcA and GroEL were added at the same time.

Gel retardation assay

The 120 bp [α-^{32}P]dATP-labelled fragment used was obtained by cutting plasmid pAM22 with *Bam*HI and *Kpn*I and subsequently labelling the smaller fragment by fill-in. DNA–protein binding reactions were carried out as described (Yuan and Wong, 1995a). The DNA–protein complexes were loaded on a 4% native polyacrylamide gel and run for 2.5 h at 10 V/cm in gel shift buffer (10 mM Tris–HCl, pH 8.0, 10 mM $MgCl_2$, 100 mM Na phosphate, 2 mM dithiothreitol, 1% glycerol). After electrophoresis, gels were exposed to X-ray film for 3–48 h at –70°C. Quantitation of free DNA and of retarded bands was carried out using a phospho-imager (Instant Imager 2024, Packard).

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