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Nonnative Proteins Induce Expression of the *Bacillus subtilis* CIRCE Regulon

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The chaperone-encoding *groESL* and *dnaK* operons constitute the CIRCE regulon of *Bacillus subtilis*. Both operons are under negative control of the repressor protein HrcA, which interacts with the CIRCE operator and whose activity is modulated by the GroESL chaperone machine. In this report, we demonstrate that induction of the CIRCE regulon can also be accomplished by ethanol stress and puromycin. Introduction of the *hrcA* gene and a transcriptional fusion under the control of the CIRCE operator into *Escherichia coli* allowed induction of this fusion by heat shock, ethanol stress, and overproduction of GroESL substrates. The expression level of this *hrcA-bgaB* fusion inversely correlated with the amount of GroE machinery present in the cells. Therefore, all inducing conditions seem to lead to induction via titration of the GroE chaperonins by the increased level of nonnative proteins formed. Puromycin treatment failed to induce the σ^B-dependent general stress regulon, indicating that nonnative proteins in general do not trigger this response. Reconstitution of HrcA-dependent heat shock regulation of *B. subtilis* in *E. coli* and complementation of *E. coli groESL* mutants by *B. subtilis groESL* indicate that the GroE chaperonin systems of the two bacterial species are functionally exchangeable.

Molecular chaperones such as those represented by the DnaK and GroE machines are induced by heat shock in all organisms from bacteria to humans. Regulation of this heat shock response has been extensively analyzed in *Escherichia coli*, where induction of one class of heat shock proteins including the molecular chaperones is triggered by the accumulation of nonnative proteins in the cytoplasm and governed by the alternative sigma factor σ^{32} (4, 22, 33–35). Increasing the level of active σ^{32} due to stabilization of the protein and enhanced translation of its mRNA following thermal upshock is responsible for the induction of this heat shock regulon (8, 35, 37). The activity of σ^{32} is modulated by the DnaK chaperone system which sequesters most of the σ^{32} molecules under physiological conditions and most probably presents them to a protease such as FtsH (7, 16, 38). Upon accumulation of nonnative proteins within the cytoplasm, the DnaK system is titrated by these substrates, and thereby the amount of active σ^{32} increases transiently. Since chaperones perform important functions in preventing aggregation of nonnative proteins, they are induced by not only heat shock but also by other stimuli such as ethanol, puromycin, viral infection, nalidixic acid, heavy metals such as cadmium chloride, glucose starvation, and osmotic or oxidative stress (13, 19, 22). Induction of these conserved heat shock proteins by a variety of different stimuli is common in bacteria and higher organisms (23).

In *Bacillus subtilis*, at least four classes of heat shock genes can be distinguished. Class I, which comprises the *groESL* and *dnaK* operons, also designated the CIRCE regulon, is transcribed from vegetative promoters and subject to negative control by the HrcA repressor, which acts at the transcriptional level by interacting with an operator, the CIRCE element, located immediately downstream of the start point of transcription (30, 42, 43). Since purified HrcA repressor aggregates in vitro, it was postulated that GroE proteins are required to maintain HrcA in an active state (21). Members of class II absolutely require the alternative sigma factor σ^B for their induction by heat and other stresses (10). Class III currently comprises *clpC*, *clpP*, and *clpE*, and heat and stress induction is mediated by the repressor CtsR (class III stress gene repressor [5]). Heat shock genes, such as *trxA*, *lon*, *ftsH*, *htpG*, and *ahpCF*, not belonging to classes I through III are currently grouped in class IV, but the mechanisms which are responsible for heat induction of these genes have not been characterized (2, 10, 26, 31). In contrast to the other classes and the induction profile in other bacteria, class I heat shock genes seem to be subject to a heat-specific induction in *B. subtilis* (10, 11, 39).

We now show that this class of genes is induced not only by heat shock but also by a group of related stimuli including ethanol stress, treatment with puromycin, and production of inclusion bodies which probably all share the increased formation of nonnative proteins.

MATERIALS AND METHODS

Bacterial strains, plasmids, and growth conditions. The bacterial strains and plasmids used during this study are listed in Table 1. The *B. subtilis* and *E. coli* strains were cultivated under vigorous agitation in LB (25) at 37 and 30°C, respectively. Stresses were imposed during exponential growth according to the following scheme: heat shock (transfer of the culture to 50°C [*B. subtilis*] or 42°C [*E. coli*]); salt stress (addition of NaCl to a final concentration of 4% [wt/vol]); ethanol (addition of ethanol to a final concentration of 4 or 5% [vol/vol]); and puromycin (addition of the inhibitor to a final concentration of 20 μg/ml). Cultures were exposed to the different stimuli for the times indicated in the corresponding figures and figure legends.

RNA isolation and analysis of transcription. Total RNA was prepared by a modification (39) of the acid-phenol method of Majumdar et al. (17). For slot blot analysis, serial dilutions of the RNA were transferred onto a positively charged nylon membrane by slot blotting and hybridized with digoxygenin-labeled RNA probes synthesized in vitro from linearized plasmids as instructed by

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TABLE 1. Bacterial strains and plasmids used

Strain or plasmid	Relevant genotype or description	Reference or source
Strains		
Escherichia coli		
A190	Δ*groEL* Km^r Δ(*lac-pro*) *hsdD5* (r_K^- m_K^-) *thi* F′[*traD36 proAB lacI*^{q} *lacZ*ΔM15]	12
DH5α	F$^-$ϕ80d *lacZ*ΔM15 Δ(*lacZYA-argF*)U169 *deoR recA1 endA1 hsdR17* ($r_K^-m_K^+$) *supE44 thi-1 gyrA69*	Bethesda Research Laboratories
DH10B	Str^r F$^-$ *mcrA* Δ(*mrr-hsdRMS-mcrBC*) ϕ80d *lacZ*ΔM15 Δ*lacX74 deoR recA1 araD139* Δ(*ara leu*)*7697 galU galK endA1 nupG*	Bethesda Research Laboratories
Ω425	*groEL100 groES*$^+$::Tn*10*	21
Ω427	*groEL*$^+$ *groES30*::Tn*10*	21
Bacillus subtilis		
PY22	*trpC2*	P. Youngman
1012	*trpC2 leuA8 metB5 hsrM1*	24
Plasmids		
pBluescript II SK/KS$^{+/-}$	Cloning vector, Ap^r	Stratagene
pSPT18	Cloning vector, Ap^r	Boehringer Mannheim
pAM101	*hrcA hrcA*::*bgaB*, Ap^r Km^r	21
pAM103	pAM101 with *tet* instead of *neo*, Ap^r Tc^r	This work
pBAD-EL	pBAD30 containing the *E. coli groEL*, Ap^r	12
pBAD33	Expression vector allowing cloning of target genes downstream of the P_{BAD} promoter, Cm^r	9
pBAD33-*groESL*	pBAD33 containing a 2.0-kb PCR fragment encoding *E. coli groES* and *groEL*, Cm^r	This work
pDS12-P_{lac}wt-9A-*lucI*	pDS12 containing the firefly *lucI*, Ap^r	B. Bukau
pKSM*sigB*	pBluescript II KS with a 2.9-kb PCR fragment encoding *B. subtilis rsbV*, *rsbW*, *sigB*, and *rsbX* inserted into the *Eco*RV site	This work
pKSM*sigBC*	pBluescript II KS containing *B. subtilis rsbV*, *rsbW*, and *sigB*	This work
pKSB52	pBluescript II KS containing a 610-bp *Hin*dIII-*Cla*I fragment of *B. subtilis sigB*	This work
pQE40-*tst*	pQE40 containing rat *tst*	T. Langer
pREP9	*E. coli-B. subtilis* shuttle vector allowing cloning of target genes downstream of an IPTG-inducible promoter, Cm^r Km^r	15
pREP9-*tst*	pREP9 containing a 900-bp *Bam*HI-*Hin*dIII fragment encoding rat *tst*, Cm^r Km^r	This work
pREP9-*lucI*	pREP9 containing a 1,700-bp *Bam*HI-*Hin*dIII fragment encoding firefly *lucI*, Cm^r Km^r	This work
pREP9-*cbbM*	pREP9 containing a 1,500-bp *Sal*I-*Hin*dIII fragment encoding *R. capsulatus cbbM*, Cm^r Km^r	This work
pSEG247	pSPT18 containing a 800-bp *Hin*dIII-*Eco*RI fragment of *B. subtilis groEL*	This work

the manufacturer (Boehringer Mannheim). The fluorescence of the Vistra ECF substrate was quantified with the Storm860 system from Molecular Dynamics, using RNA dilutions with a signal strength in the linear range of the instrument. Induction ratios were calculated by setting the value of the corresponding control (nontreated or exponentially growing culture) to 1. For the preparation of the digoxigenin-labeled RNA probes, a DNA fragment encompassing the four downstream genes of the *sigB* operon was amplified from chromosomal DNA of the wild-type strain 168 by using the synthetic oligonucleotides sigBF (5′-CGCAG GAAATGGTCAAAAAC) and sigBR (5′-AATAAATCAGCCAATCTCCCT C). The PCR fragment was cloned into pBluescript II KS$^-$ digested with *Eco*RV. The resulting plasmid, pKSM*sigB* was digested with *Cla*I and religated, yielding plasmid pKSM*sigBC*. After digestion of pKSM*sigBC* with *Hin*dIII and *Bam*HI, filling in the ends with Klenow polymerase, and religation of the larger fragment, pKSMB52 was obtained. Synthesis of RNA in vitro with T3 RNA polymerase after linearization of pKSMB52 with *Sac*I can be used for the production of a digoxigenin-labeled RNA probe specific for *sigB*. A suitable fragment for the preparation of a *groEL*-specific probe was generated by cloning an 800-bp *Hin*dIII-*Eco*RI fragment from pASG145 (27) encoding *groEL* of *B. subtilis* into pSPT18. The resulting plasmid, pSEG247, can be used for the preparation of digoxigenin-labeled, *groEL*-specific RNA probe with T7 RNA polymerase after linearization with *Hin*dIII.

Construction of plasmids. To allow regulated expression of GroEL substrates, plasmids pREP9-*tst*, pREP9-*lucI*, and pREP9-*cbbM* were constructed. For the construction of pREP9-*tst*, a DNA fragment encoding the rat *tst* gene was isolated by *Bam*HI-*Hin*dIII digestion of pQE40-*tst* and cloned into pREP9 digested with *Bam*HI and *Hin*dIII. To obtain pREP9-*lucI*, the *lucI* gene was amplified from pDS12-P_{lac}wt-9A-*lucI* by using the synthetic oligonucleotides LUCI-1 (5′-GGCCATGGATCCATGGAAGACGCCAAAAACATAAAGA) and LUCI-2 (5′-GGCCATAAGCTTTTACAATTTGGGCTTTCCGCCCTT). The PCR fragment was cloned into pREP9 digested with *Bam*HI and *Hin*dIII. To construct pREP9-*cbbM*, the *cbbM* gene was amplified from chromosomal DNA of *Rhodobacter capsulatus* by using the synthetic oligonucleotides RubisCo-3 (5′-GGCCATGTCGACATGGATCAGTCTAACCGTTACGCCC) and RubisCo-2 (5′-GGCCATAAGCTTTCAGTTCACGCCCAGAGCAACGC G). The PCR product was cloned into pREP9 digested with *Sal*I and *Hin*dIII. To allow overproduction of the GroE chaperone machine from *E. coli* or *B. subtilis*, pBAD33-*groESL* and pREP9-*groESL* were constructed. For the construction of pBAD33-*groESL*, the *groES* and *groEL* genes of *E. coli* were amplified from chromosomal DNA by using the synthetic oligonucleotides ECES (5′-GGCCA TGAGCTCAAAGGAGAGTTATCAATGAATATTCGT) and ECEL (5′-GG CCATAAGCTTTTACATCATGCCGCCCATGCCACC). The PCR fragment was cloned into pBAD33 digested with *Sac*I and *Hin*dIII. To obtain pREP9-*groESL*, the *groES* and *groEL* genes of *B. subtilis* were amplified from chromosomal DNA by using the synthetic oligonucleotides PP1 (5′-GGCCATGGATC CATGTTAAAGCCATTAGGTGATCGC) and EL-B2 5′-GGCCATGGATCC TTACATCATTCCACCCATACCGCC). The PCR fragment was cloned into pREP9 digested with *Bam*HI. To replace the kanamycin resistance gene of pAM101 with a tetracycline resistance gene, *tet* was isolated from pBgaB-*tet* by *Xba*I-*Not*I digestion and cloned into pAM101 digested with *Xba*I and *Not*I, yielding plasmid pAM103.

Fractionation of *E. coli* proteins. Bacterial cultures (10-ml aliquots) were rapidly cooled to 0°C in an ice water bath and harvested by centrifugation (10 min, 4°C, 5,000 × *g*). Pellets were resuspended in 10× lysis buffer (100 mM Tris-Cl [pH 7.5], 100 mM KCl, 2 mM EDTA, 15% [wt/vol] sucrose, 1 mg of lysozyme per ml) according to optical density (50 μl of lysis buffer for a 10-ml culture with an optical density at 600 nm of 1) and frozen at −20°C. After thawing at 0°C, addition of 10 volumes of ice-cold water, and mixing, the viscous, turbid solution was sonicated with a Branson Cell Disruptor B15 (microtip, level 6, 50% duty cycle, eight strokes) while cooling. Insoluble material was pelleted by centrifugation at 25,000 × *g* for 30 min at 4°C. Supernatants were removed and subjected to precipitation with trichloracetic acid (TCA; 10%, final concentration), and pellets were resuspended in sample buffer. Equal aliquots of soluble and insoluble fractions were analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) followed by immunoblotting or staining with Coomassie brilliant blue.

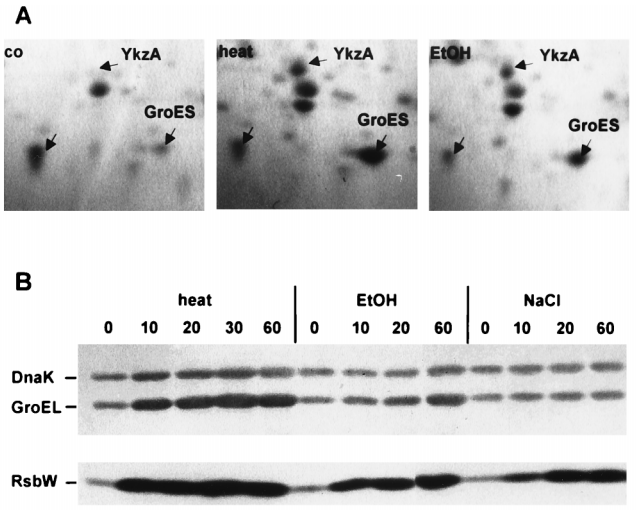

FIG. 1. Influence of heat shock, ethanol, and salt stress on levels of GroEL, DnaK, and RsbW. *B. subtilis* PY22 was grown in LB, and stresses were imposed during exponential growth by transferring the culture from 37 to 48°C or by adding ethanol (EtOH) or NaCl to a final concentration of 4% (vol/vol or wt/vol, respectively). (A) Sections of Coomassie blue R-350-stained two-dimensional protein gels prepared from crude extracts of growing cells (co) or cells harvested 90 min after imposition of stress. Besides GroES, the σ^B-dependent stress protein YkzA and a vegetative protein are labeled. (B) Equal amounts of crude protein extracts (50 μg per lane) prepared from bacteria harvested at the time points (minutes) indicated were separated by SDS-PAGE. After transfer to a nitrocellulose membrane, levels of GroEL, DnaK, and RsbW were determined with specific antibodies raised against the corresponding proteins as described previously (3, 29, 32).

Two-dimensional protein gel electrophoresis. Protein extracts were prepared by passage through a French press after cells had been harvested on ice. Equal amounts of protein (400 μg) were loaded. Proteins were separated with IPG strips (pH 4 to 8) in the first dimension on the Multiphor apparatus supplied by Pharmacia, equilibrated, loaded onto 12.5% polyacrylamide gels, and separated according to molecular mass with the Investigator electrophoresis system of ESA Inc. Proteins were visualized by staining with Phastgel Blue R (Pharmacia Biotech).

General methods. SDS-PAGE and Western blot analysis were performed as described previously (40). *B. subtilis* transformation was carried out as described by Yasbin et al. (41), and transformants were selected on agar containing kanamycin (20 μg/ml) or chloramphenicol (20 μg/ml). All DNA manipulations and *E. coli* transformations were carried out according to standard protocols (25). β-Galactosidase activities were determined as described elsewhere (20).

RESULTS AND DISCUSSION

Ethanol and puromycin trigger induction of the CIRCE regulon in *B. subtilis*. In an effort to identify additional stress proteins of *B. subtilis*, exponentially growing wild-type bacteria were challenged with different stress factors, and their protein patterns were analyzed by two-dimensional protein gel electrophoresis (1). These analyses revealed that the amount of GroES increased not only after heat shock but also after exposure to ethanol stress (Fig. 1A) but not after salt stress or glucose limitation (data not shown). A similar induction pattern was also observed for GroEL and DnaK (data not shown). To quantitate these observations, the kinetics of expression of DnaK and GroEL after heat shock, ethanol, and salt stress were measured by Western blot analysis. Crude extracts were prepared from exponentially growing *B. subtilis* PY22, harvested immediately before (time zero) and at different times after stress treatment; the proteins were separated by SDS-PAGE, transferred to nitrocellulose membranes, and finally probed with antibodies prepared against GroEL, DnaK, and RsbW. Whereas heat shock triggered a rapid increase in the level of GroEL and DnaK as previously reported, ethanol treatment resulted in a slow but continuous accumulation of both proteins (Fig. 1B); salt shock did not influence the level of the chaperones. In contrast, all three stimuli resulted in a rapid increase of the level of RsbW, a class II σ^B-dependent heat shock protein.

To determine whether accumulation of the chaperones was due to an increase in their amount of transcript, the level of *groESL* mRNA was determined by slot blot analysis. Heat shock triggered a rapid increase in the level of *groESL* mRNA as expected, but ethanol caused a slower but continuous increase in the amount of *groESL* mRNA (Fig. 2). To confirm these results by a different approach, *B. subtilis* 1012 carrying an *hrcA-bgaB* transcriptional fusion (20) was subjected to stress treatment. When a strain carrying this fusion was shifted from 37 to 50°C, β-galactosidase activity increased eightfold within 15 min, whereas treatment with 5% (vol/vol) ethanol caused a slow gradual increase, resulting in a fivefold induction after 60 min (Table 2). Addition of 4% (wt/vol) NaCl did not result in any increase in the expression of the *hrcA-bgaB* transcriptional fusion. Significant increases in the *groESL* mRNA level were also observed after treatment with puromycin (20 μg/ml, final concentration) (Fig. 2). For *sigB*, which is itself a member of class II heat shock genes, the slot blot analysis revealed strong induction as quickly as 3 min after heat shock, ethanol, or salt stress (Fig. 2) but no induction after addition of puromycin (Fig. 2). Although class I and class II heat shock genes share some inducers such as heat shock and ethanol, the intracellular signals which trigger induction seem to differ since both classes

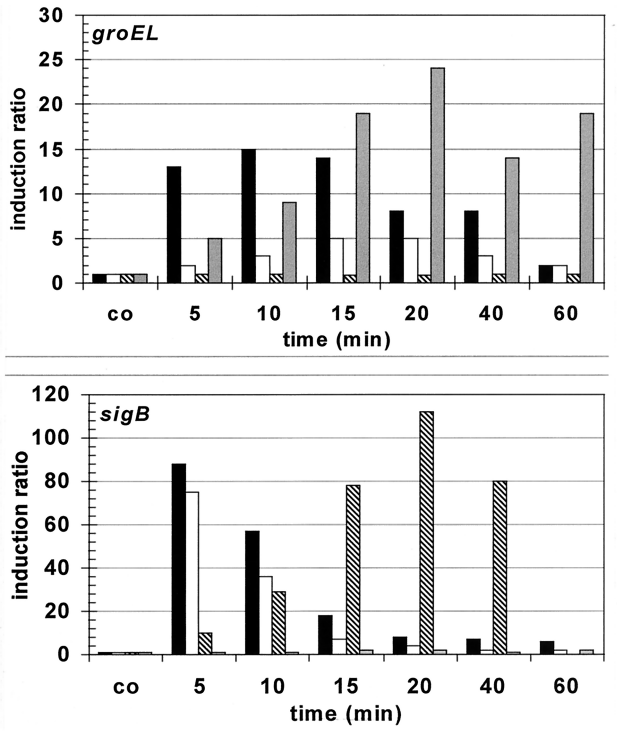

FIG. 2. Levels of *groEL* and *sigB* mRNA in *B. subtilis* after challenge with heat, ethanol, or salt stress and puromycin. Serial dilutions of total RNA prepared from *B. subtilis* PY22 before (co) and at 5, 10, 15, 20, 40, and 60 min after exposure to stress were bound to a positively charged nylon membrane and hybridized with the digoxigenin-labeled antisense RNA probes specific for *groEL* and *sigB* (18, 39). The hybridization signals were quantified with a fluorimager. The mRNA level in the control prior to stress was set to 1, and the induction ratios are shown. Stresses were triggered as described in Materials and Methods. ■, 50°C; □, 4% ethanol; ▧, 4% NaCl; ▨, 20 μg of puromycin per ml.

TABLE 2. Induction of an *hrcA-bgaB* transcriptional fusion in *B. subtilis* 1012 and in *E. coli* DH10B[a]

Species	Stress factor	Relative β-galactosidase activity at indicated time (min) after stress		
		15	30	60
B. subtilis	Heat (37→50°C)	8.6	10.1	7.6
	Ethanol (5%)	1.2	2.3	4.8
	NaCl (4%)	1	1	0.9
E. coli	Heat (30→42°C)	4.3	5.1	4.2
	Ethanol (5%)	3.0	4.9	6.7
	NaCl (4%)	1	1.1	1.1

[a] Cells were grown in LB medium at 37°C (*B. subtilis*) or 30°C (*E. coli*) to mid-exponential phase and then challenged with the stress factors indicated. Samples were withdrawn immediately before (time zero) and 15, 30, and 60 min after exposure to the stress factor, and β-galactosidase activities relative to those at time zero (assigned a value of 1) were determined as described elsewhere (20). The results are the averages of three experiments; standard deviations were less than 10%.

display different induction kinetics and different induction profiles. For example, ethanol treatment results in a fast induction of class II but slow accumulation of class I heat shock proteins, and class II is not induced by puromycin, whereas class I does not respond to salt stress or starvation for glucose.

Induction of an *hrcA-bgaB* transcriptional fusion in *E. coli* by heat, ethanol, and overproduction of GroESL substrates. Recently, the GroE chaperonin machine has been shown to be a major modulator of the regulation of the genes of the CIRCE regulon (21). The heat shock regulation of *hrcA-bgaB* and *groE-bgaB* transcriptional fusions both carrying the CIRCE element could be reconstituted in *E. coli* by supplying the HrcA repressor under control of a constitutive promoter (21, 42). Heat shock, ethanol stress, and treatment with puromycin most probably produce nonnative proteins and thereby titrate the GroE chaperonin system. This in turn may lead to an accumulation of inactive HrcA repressor and induction of the CIRCE regulon (21). We asked whether this mechanism might also trigger induction of an *hrcA-bgaB* fusion in *E. coli*. To test this hypothesis, *E. coli* DH10B harboring plasmid pAM101 (21), which synthesizes HrcA from a constitutive promoter and carries a transcriptional fusion between the CIRCE-controlled *hrcA* promoter and *bgaB*, was challenged with different stress regimens. In agreement with previous data, β-galactosidase activity increased rapidly within 15 min when this strain was grown in LB at 30°C and shifted to 42°C (Table 2 and reference 21). Treatment of DH10B with 5% (vol/vol) ethanol was as effective as the heat shock in induction, whereas 4% NaCl failed to induce expression of *hrcA-bgaB* (Table 2).

If indeed nonnative proteins are the signal for induction of class I genes in *E. coli* as assumed for *B. subtilis*, then overproduction of GroE substrates should induce the *hrcA-bgaB* fusion indirectly by titrating GroEL. *E. coli* DH10B was transformed with plasmid pREP9 (15) or recombinant derivatives carrying either *tst*, *lucI*, or *cbbM* under the control of an isopropyl-β-D-thiogalactopyranoside (IPTG)-inducible promoter. Growth in the presence of IPTG caused overproduction of chloramphenicol acetyltransferase, rhodanese, luciferase, and RubisCO (Fig. 3A). Chloramphenicol acetyltransferase accumulated to levels at least as high as those for the other three proteins (Fig. 3A) and remained almost completely in the soluble fraction (Fig. 3B and C). In contrast, overexpression of rhodanese, luciferase, and RubisCO resulted in the accumulation of considerable amounts of the proteins in the insoluble fraction, with luciferase occurring almost exclusively in this fraction (Fig. 3B and C). This formation of inclusion bodies was accompanied by a low but reproducible induction of the *hrcA-bgaB* fusion in *E. coli* (Table 3). Since overproduction of chloramphenicol acetyltransferase, which is not a substrate of the GroE system (6, 14), did not induce the *hrcA-bgaB* fusion (Table 3), overproduction of a protein per se cannot be the signal for the induction of CIRCE-regulated genes. Rather, overproduction of substrates of the GroESL chaperonin system, such as rhodanese which requires multiple GroE-driven reaction cycles for proper folding (6), might lead to a titration of GroEL and cause the increased expression of the *hrcA-bgaB* fusion.

Variations of the level of GroEL in *E. coli* influence the expression of an *hrcA-bgaB* fusion. Because overproduction of heterologous proteins acts most likely indirectly by sequestering the GroE chaperonin, varying the level of GroESL in *E. coli* should affect expression of class I genes in a way similar to that described for *B. subtilis* (21). To test this assumption, we examined whether depletion of the GroE proteins in *E. coli* would result in induction of an *hrcA-bgaB* transcriptional fu-

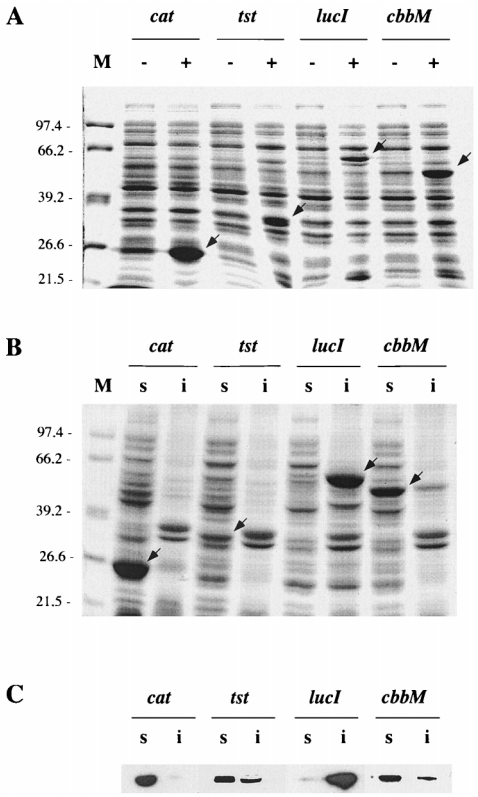

FIG. 3. Overproduction and localization of chloramphenicol acetyltransferase and GroEL substrates in *E. coli*. *E. coli* DH10B transformed with pREP9, pREP9-*tst*, pREP9-*lucI*, or pREP9-*cbbM*, permitting overproduction of chloramphenicol acetyltransferase (*cat*), rhodanese (*tst*), luciferase (*lucI*), or RubisCO (*cbbM*), respectively, were grown to mid-exponential phase and induced with 1 mM IPTG. (A) Whole-cell fractions corresponding to identical amounts of cell culture were collected before (−) or 2 h after (+) the addition of IPTG and resolved by SDS-PAGE. Molecular masses (in kilodaltons) of marker proteins (M) are given; arrowheads indicate localization of the overproduced proteins. (B) Soluble (s) and insoluble (i) fractions of an extract prepared from a culture induced for 2 h with IPTG were prepared as described in Materials and Methods. Aliquots corresponding to identical amounts of cell culture were loaded onto all lanes. (C) Samples identical to those in panel B were resolved by SDS-PAGE and transferred to nitrocellulose. The membranes were probed with anti-chloramphenicol acetyltransferase, antirhodanese, antiluciferase, or anti-RubisCO antibodies and developed by a colorimetric assay.

TABLE 3. Induction of an *hrcA-bgaB* transcriptional fusion by overproduction of GroE substrates in *E. coli* DH10B[a]

Plasmid	Relative β-galactosidase activity at indicated time (h) after addition of IPTG		
	1	2	3
pREP9	1	1	1
pREP9-*tst*	1.6	2.1	2.6
pREP9-*lucI*	1.9	2.6	2.5
pREP9-*cbbM*	1.2	1.7	1.7

[a] *E. coli* DH10B carrying pAM101 (21) and one of the indicated plasmids was grown in LB to mid-exponential phase and then treated with 1 mM IPTG to induce production of the proteins whose genes had been cloned into pREP9. Samples were withdrawn from the cultures at the times indicated. β-Galactosidase activities relative to those at time zero (assigned a value of 1) were determined as described elsewhere (20). All experiments were performed in triplicate; standard deviations were less than 10%.

TABLE 4. Overproduction of *E. coli* GroESL causes reduced heat induction of an *hrcA-bgaB* transcriptional fusion in *E. coli* DH10B[a]

Plasmid	Relative β-galactosidase activity at indicated time (min) after heat shock			
	15	30	60	120
pBAD33[b]	2.8	4.7	4.6	4.4
pBAD33-*groESL*	1.5	2.0	1.9	1.8

[a] Overproduction of GroES and GroEL after addition of arabinose was verified by SDS-PAGE. To analyze the effect of overproduction of the GroE proteins on the expression of an *hrcA-bgaB* operon fusion, pBAD33 or pBAD33-*groESL* was transformed into *E. coli* DH10B carrying pAM101. Both strains were first grown in LB medium at 30°C in the absence of arabinose to mid-exponential phase, then arabinose was added to a final concentration of 0.2%, and 1 h later the culture was shifted from 30 to 42°C. Samples were taken immediately before thermal upshift (time zero) and different times after the heat shock. β-Galactosidase activities relative to those at time zero (assigned a value of 1) were determined as described elsewhere (20). Induction of the two strains was carried out three times with standard deviations less than 10%.

[b] pBAD33 (9) permits expression of target genes from the P_{BAD} promoter, which is under negative control of the AraC repressor.

sion. To this end, *E. coli* A190 in which the chromosomal *groEL* gene has been replaced by a kanamycin resistance cassette and which carries the *groEL* gene on a plasmid under the control of the P_{BAD} promoter (12) was transformed with pAM103, a tetracycline-resistant derivative of pAM101 carrying an *hrcA-bgaB* operon fusion (21). This strain was then grown in the presence of different arabinose concentrations, and β-galactosidase activities were measured. The lowest enzymatic activity was measured in the presence of 0.2% arabinose (Fig. 4). Decreasing the sugar concentration reduced the level of GroEL as determined by immunoblotting (data not shown) and simultaneously increased expression of the *hrcA-bgaB* fusion. Addition of the anti-inducer glucose increased the level of β-galactosidase even further (Fig. 4).

In contrast to the latter results, preloading the cells with *E. coli* GroESL proteins should reduce a later heat shock induction of the *hrcA-bgaB* fusion if the *E. coli* GroE chaperone machine can fully substitute for GroESL of *B. subtilis* in this regulation. Addition of 0.2% arabinose to the growth medium of *E. coli* DH10B carrying pAM101 and pBAD33-*groESL* resulted in overproduction of GroESL proteins during exponential growth (as visualized by SDS-PAGE and Coomassie blue staining [data not shown]) and indeed significantly reduced the heat inducibility of the *hrcA-bgaB* fusion (Table 4). Arabinose had no effect on the heat induction in cells containing only the empty vector pBAD33. To support the notion that the GroESL chaperonins of *E. coli* and *B. subtilis* are functionally exchangeable, the *B. subtilis groESL* operon was inserted into the vector pREP9. This recombinant plasmid, pREP9-*groESL*, complemented *E. coli groES30* and *groEL100* temperature-sensitive mutants for growth at the nonpermissive temperature (data not shown) as previously described for the *groE* operon of *Bacillus stearothermophilus* (28).

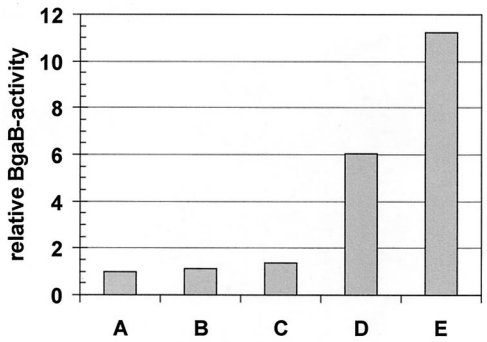

FIG. 4. Depletion of GroEL induces an *hrcA-bgaB* transcriptional fusion in *E. coli*. *E. coli* A190 carrying the two plasmids pAM103 and pBAD33-*groESL* (expressing the *E. coli groESL* genes) was grown in the presence of 0.2% arabinose in LB overnight. The cells were then washed to remove the inducer arabinose and resuspended in LB medium either in the absence or in the presence of the indicated arabinose concentrations or in the presence of 0.5% glucose as anti-inducer. Expression of the *hrcA-bgaB* transcriptional fusion expressed from pAM103 was assayed 4 h after the resuspension. A, 0.2% arabinose; B, 0.06% arabinose; C, 0.02% arabinose; D, no arabinose; E, 0.5% glucose.

In summary, our results clearly show that besides heat shock, other stimuli such as ethanol and puromycin can induce the heat shock genes of the CIRCE regulon of *B. subtilis*. In contrast to class II heat shock genes, which are induced by entirely different signals, induction of the CIRCE regulon remains confined to a group of related stimuli which all most likely produce enhanced amounts of nonnative proteins. The same signal seems to be responsible for the induction of these genes after their transfer into *E. coli.*

There are bacterial species inheriting both the σ^{32} and the HrcA-CIRCE mechanisms (21). In both cases, negative autoregulation is guaranteed. Regulation of chaperone expression by different mechanisms also allows specific induction of the *groE* operon when increased amounts of GroE proteins are specifically needed, e.g., in *R. capsulatus* and in *Synechococcus* spp. RubisCO is a substrate for GroEL, and synthesis of RubisCO is accompanied by an increase in the amount of GroE proteins (36).

We propose that titration of the GroE chaperonin by increased levels of nonnative proteins presumably prevents (re)activation of the HrcA repressor and therefore might permit increased expression of class I heat shock genes. This proposed mechanism of negative autoregulation might ensure a rapid activation and deactivation of the genes of the CIRCE regulon. In addition, this kind of regulation permits precise fine adjustments, to ensure the adequate production of molecular chaperones depending on the growth temperature and other physiological conditions.

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