

Role of HrcA and CIRCE in the Heat Shock Regulatory Network of *Bradyrhizobium japonicum*

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A large number of bacteria regulate chaperone gene expression by the CIRCE-HrcA system in which a DNA element called CIRCE serves as binding site for the repressor protein HrcA under non-heat-shock conditions. We have cloned the two consecutive genes *hrcA* and *grpE* of *Bradyrhizobium japonicum* by using a complementation approach that screened for GrpE function. In vivo and in vitro transcript mapping demonstrated that both genes are transcribed separately from RpoH (σ^{32})-dependent promoters. To investigate the supposed negative regulatory function of HrcA, we compared the expression of putative target genes in the wild type with that in an *hrcA* mutant. Transcription of the CIRCE-associated chaperonin operons *groESL₄* and *groESL₅*, as well as the β -galactosidase activity derived from corresponding *groE-lacZ* fusions, was strongly elevated in the *hrcA* mutant even at physiological temperatures. Expression of other heat shock regulons (RpoH or ROSE dependent) was not affected. To study the activity of HrcA in vitro, we purified a histidine-tagged version of the protein under nondenaturing conditions. Specific binding to the CIRCE element was obtained with a soluble fraction of HrcA in gel retardation experiments.

The survival of a cell depends on its ability to adapt to changing environmental conditions. For a sudden temperature upshift, all organisms cope with the increased amount of misfolded proteins by an enhanced synthesis of so-called heat shock proteins (Hsps). This collection of proteins consists mainly of chaperones and proteases, which are involved in protein (re) folding and protein degradation, respectively (for reviews see references 7 and 18).

Both eukaryotic and prokaryotic cells have established a number of complex regulatory strategies to tightly control the transcription of heat shock genes under any given condition (21, 26, 27). Coordinate expression of heat shock genes in *Escherichia coli* is mediated by alternative sigma factors which direct the RNA polymerase to specific promoter sequences upstream of these genes. An increase in the cellular concentration of σ^{32} (RpoH) after a sudden temperature upshift results in elevated transcription of more than 30 genes of the σ^{32} regulon. The activity and stability of σ^{32} are subject to feedback control by the DnaK machinery, which sequesters the sigma factor under low-temperature conditions. Binding of σ^{32} to the DnaK system prevents association with the RNA polymerase core enzyme and promotes degradation of σ^{32} by the FtsH protease. After a heat shock, denatured proteins presumably titrate the DnaK chaperones away from σ^{32} , leaving the latter stable and competent for complex formation with RNA polymerase (for recent reviews see references 14 and 53).

In contrast to the positive regulation by alternative sigma factors, heat shock expression in a majority of bacteria was found to be controlled by negative regulation (summarized in reference 27). Our knowledge of such systems is far less advanced than that of the *E. coli*-type regulation. The regulatory principle is often based on a specific interaction between a repressor protein and a DNA element that is located in the promoter regions of heat shock genes. Repression is relieved

upon a temperature upshift, thereby allowing transcription of the downstream genes. A widespread negative control mechanism consists of the repressor protein HrcA and a DNA element called CIRCE (for controlling inverted repeat of chaperone expression [54]). The first evidence that this highly conserved inverted repeat might act as a negative *cis* element was obtained by the observation that mutations in one or both arms of the inverted repeat resulted in elevated transcription of the downstream genes even at normal growth temperatures (47, 54). Chromosomal mutations in *Bacillus subtilis* which affected the expression of CIRCE-dependent genes were localized in *orf39*, the first gene of the *dnaK* operon (44, 51). Disruption of *orf39* in *B. subtilis* and of the equivalent gene in *Caulobacter crescentus* confirmed the function of the Orf39 protein as a negative regulator of CIRCE-dependent genes and led to its designation as HrcA (for heat regulation at CIRCE) (38, 43). By analogy with the feedback control of σ^{32} activity and stability by the DnaK machinery in *E. coli*, the activity of *B. subtilis* HrcA was found to be modulated by the GroE chaperonin system (25). A direct correlation between the cellular GroESL level and HrcA activity suggested that the repressor requires chaperonins for proper function in vivo (3, 25). Accordingly, heat stress would render HrcA inactive because GroES and GroEL become engaged in the refolding of denatured proteins.

Unfortunately, attempts to confirm the proposed HrcA-CIRCE and HrcA-GroEL interactions in vitro were hampered by the insolubility of overproduced HrcA, which tends to form inclusion bodies, requiring purification under denaturing conditions. Despite this shortcoming, it has been possible to visualize the binding of purified HrcA of *Staphylococcus aureus* to the CIRCE element by atomic force microscopy (35) and to document CIRCE binding of purified *Bacillus stearothermophilus* HrcA in gel retardation experiments (25).

An unusually complex network of both positive and negative regulatory mechanisms controls transcription of heat shock genes in *Bradyrhizobium japonicum*, the nitrogen-fixing root nodule symbiont of soybeans (27). Three different regulatory systems have been identified so far. One class of heat shock

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genes is transcribed from σ^{32} -dependent promoters. The search for the corresponding sigma factor revealed the existence of three disparately regulated *rpoH* genes whose σ^{32} -like products have different promoter specificities (30, 31). The expression of a second class of heat shock genes is negatively controlled by a highly conserved DNA element called ROSE (for repression of heat shock gene expression) (29). Evidence for a third class of heat shock genes was deduced from the presence of a CIRCE element in the promoter region of the heat shock-inducible chaperonin operons *groESL₄* and *groESL₅* (3). Elevated transcription from the *groESL₄* promoter at normal temperatures, caused either by a deletion of 4 bp within the corresponding CIRCE element or by disruption of *groEL₄*, had clearly demonstrated the regulatory function of CIRCE and, moreover, had indicated the presence of a feedback regulatory mechanism. According to these observations, the existence of a CIRCE-specific repressor protein whose stability or activity or both are modulated by chaperonins was suggested.

Here, we report the cloning of the *B. japonicum* *hrcA* gene and the assignment of its position in the complex heat shock network of this organism. The function of the HrcA protein as a CIRCE-specific repressor protein is demonstrated by a complementary set of in vivo and in vitro experiments.

MATERIALS AND METHODS

Bacterial strains, plasmids, and growth conditions. The bacterial strains and plasmids used in this work are listed in Table 1. *E. coli* cells were grown in Luria-Bertani medium (22) supplemented with ampicillin (200 μ g/ml), chloramphenicol (20 μ g/ml), kanamycin (30 μ g/ml), or tetracycline (10 μ g/ml) if required. The growth temperature for *E. coli* strains was 37°C except for *E. coli* DA259 (*grpE* mutant), which was grown at 30°C. *B. japonicum* strains were propagated aerobically at 30°C in PSY medium (37) supplemented with 0.1% (wt/vol) arabinose. If appropriate, antibiotics were added at the following concentrations: chloramphenicol, 20 μ g/ml (for counterselection against *E. coli* donor strains); kanamycin, 100 μ g/ml; spectinomycin, 100 μ g/ml; and tetracycline, 50 μ g/ml.

DNA manipulations. Recombinant DNA techniques were performed according to standard protocols (40). Construction of *B. japonicum* mutants by cointegrate formation or marker exchange mutagenesis and isolation of chromosomal DNA from *B. japonicum* were performed as described previously (15). Southern blot hybridizations with digoxigenin-11-dUTP-labeled DNA probes were performed according to the manufacturer's instructions (Boehringer Mannheim). DNA was sequenced by the chain termination method (41) with an ABI PRISM 310 genetic analyzer (Applied Biosystems, Foster City, Calif.). The DNA region was sequenced and the deduced proteins were analyzed with the software package of the Genetics Computer Group of the University of Wisconsin, Madison (version 8.0) and the National Center for Biotechnology Information BLAST network server. The CODONPREFERENCE program was applied by using the *B. japonicum* codon usage table (36).

Construction of strains and plasmids. For the construction of *hrcA* deletion mutants the 2.0-kb *NotI* (position 2 in Fig. 1)-*XmnI* (position 2015) fragment of pRJ5530 was ligated into the 6.7-kb *NotI*-*SmaI* vector fragment of pSUP202pol4. In the resulting plasmid the 561-bp *SalI* (position 706)-*XhoI* (position 1267) internal fragment of *hrcA* was replaced by the 1.2-kb *SalI* fragment of pBSL15 containing the neomycin phosphotransferase II cassette (*Km^r*). Both orientations of the resistance cassette relative to the *hrcA* gene were obtained, but for the subsequent work we used plasmid pRJ5549, in which the remaining portion of the *hrcA* gene and the resistance cassette are oriented in opposite directions. Plasmid pRJ5549 was mobilized from *E. coli* S17-1 into *B. japonicum* 110*spe4* for marker replacement mutagenesis, yielding strain 5549 (Fig. 1).

In order to compare the expression of heat shock genes in a wild-type background with that in an *hrcA* mutant background, suitable translational *lacZ* fusions present on pSUP202pol4 derivatives were cointegrated via homologous recombination into the chromosome of wild-type *B. japonicum* and 5549 (Table 1). The correct genomic structure of all mutant strains was confirmed by Southern blot hybridization with appropriate digoxigenin-11-dUTP-labeled DNA probes.

Plasmids used as templates for in vitro transcription experiments were based on pRJ9519, which contains the *B. japonicum* *rrm* terminator and in which different promoter regions were introduced (30). Plasmid pRJ5542 carries a 537-bp *NotI* (position 2 in Fig. 1)-*NruI* (position 538) fragment containing the *B. japonicum* *hrcA* promoter region. The promoter region of *B. japonicum* *grpE* was introduced as a 691-bp *SacI* (position 1109)-*FspI* (position 1796) fragment yielding plasmid pRJ5543.

To construct the overproduction plasmid pRJ5552, the *hrcA* gene was amplified from plasmid pRJ5530 by PCR with *Taq* DNA polymerase. The oligonucleotides used as primers were designed such that they introduced an *NdeI* recognition site overlapping the start codon and a *NotI* site immediately downstream of the stop codon of *hrcA*. The PCR-generated fragment was cut with both *NdeI* and *NotI* and ligated into pET28a(+) digested with the same enzymes. The encoded recombinant protein H₆-HrcA possesses an N-terminal extension of 20 amino acids adding 2.1 kDa to the molecular mass of HrcA (39.2 kDa). The correct nucleotide sequence of the PCR-amplified *hrcA* fragment was confirmed by sequencing plasmid pRJ5552.

Transcript mapping. RNA isolation and primer extension analysis were performed as described previously (3). The following oligonucleotides were used to determine the transcription start sites upstream of *hrcA* and *grpE*: HrcA10 (*hrcA*; position 579), 5'-CAGCCGGGAAATATTGCGTGACGCCACC-3'; HrcA11 (*hrcA*; position 630), 5'-CAGATCGCCATGACGTTGCGAACCAGG-3'; GrpE4 (*grpE*; position 1742), 5'-CTTCCTTCTGCAACAGCTCGACCGAGC-3'; GrpE5 (*grpE*; position 1700), 5'-CGGGCATGATGTAGGGCTTCGACA CCAC-3'. The numbers in parentheses indicate the positions of the 5' ends based on the numbering used in the physical map shown in Fig. 1.

In vitro transcription. Single-round transcription assays with *B. japonicum* RNA polymerase holoenzyme and core enzyme reconstituted with purified RpoH₂ were carried out as described previously (5, 30). Suitable RNA size markers were synthesized in vitro with T7 or T3 RNA polymerase and with linearized pBluescript-based plasmids as templates.

β -Galactosidase assay. *B. japonicum* cells were grown aerobically to exponential phase at 30°C in PSY medium with spectinomycin as the only antibiotic. The β -galactosidase assay was performed as described previously (22).

Overproduction and purification of H₆-HrcA. Freshly transformed *E. coli* BL21/pLysS cells carrying pRJ5552 were used for overproduction of H₆-HrcA. The cells were grown at 30°C in 1 liter of Luria-Bertani LB medium containing chloramphenicol and kanamycin. When the cultures had reached an optical density at 600 nm of 0.5, production of the recombinant protein was induced by addition of 0.1 mM isopropyl- β -D-thiogalactopyranoside (IPTG). After 2 h, the cells were harvested, washed, resuspended in 20 ml of TEPM buffer (50 mM Tris-HCl [pH 8.0], 1 mM EDTA, 1 mM phenylmethylsulfonyl fluoride, 2 mM dithiothreitol, 25 mM MgCl₂, 100 mM KCl), and disrupted in a French pressure cell. The soluble protein fraction was obtained by removing the cell debris and membranes in two subsequent centrifugation steps at 4°C (1.5 h at 30,000 \times g [Sorvall SS-34 rotor] and 1 h at 116,000 \times g [Beckman SW55 Ti rotor]).

All of the following purification steps were performed at 4°C. Binding buffer (8 \times) was added to the supernatant fraction to a final concentration of 500 mM NaCl before loading the fraction onto a 1.5-ml Ni-nitrilotriacetic acid-agarose column (Qiagen, Basel, Switzerland) which had been equilibrated with 1 \times binding buffer (20 mM Tris-HCl [pH 7.9], 500 mM NaCl, 5 mM imidazole). The column was washed with 15 ml of 1 \times binding buffer and then with 4.5 ml of 1 \times binding buffer containing 120 mM imidazole. The H₆-HrcA protein was eluted with 4.5 ml of 1 \times binding buffer containing 300 mM imidazole and collected in fractions of 0.5 ml. The fractions with the highest protein concentrations were pooled, dialyzed for 2 h against storage buffer (20 mM Tris-HCl [pH 8], 200 mM NaCl, 10% glycerol) and centrifuged at 13,000 \times g for 1 min. Aliquots of the supernatant were stored at -80°C. The concentration of purified H₆-HrcA protein was determined by the Bradford method (6).

Gel retardation assay. The DNA-binding activity of H₆-HrcA was tested in gel shift experiments using a 269-bp *PstI*-*KpnI* fragment from pRJ5558 containing the promoter region and the CIRCE element of *groESL₅*, a 168-bp *HpaI*-*KpnI* fragment from pRJ5556 containing the promoter region and the CIRCE element of *groESL₄*, a 104-bp PCR amplification product containing the 5' region of the *groESL₄* promoter region, or a 94-bp PCR-generated fragment containing either the wild-type CIRCE element of *groESL₄* (CTAGCACTCgcccgcacaGACTGC TAA [nucleotides in inverted repeat shown in uppercase letters and nucleotides identical to those in consensus sequence are underlined]) or a mutated DNA fragment in which the consensus sequence had been replaced (AGCTACAGA gcccgcacaAGACATCGA) (see Fig. 6B; CIRCE consensus sequence: TTAGCACTC-N₆-GAGTGCTAA [17]). These DNA fragments were purified from agarose gels and end labeled with [γ -³²P]ATP according to standard protocols. Labeled fragments (25,000 cpm; approximately 10 to 20 fmol of DNA) were mixed with purified H₆-HrcA protein in DNA binding buffer (12 mM HEPES [pH 7.9], 4 mM Tris-HCl [pH 8], 6 mM KCl, 3 mM MgCl₂, 0.5 mM dithiothreitol, 6 mM EDTA; Stratagene, La Jolla, Calif.) in a final volume of 25 μ l. If appropriate, the *E. coli* chaperones DnaK, DnaJ, and GrpE or GroES and GroEL (Epicentre Technologies, Madison, Wis.) were added to H₆-HrcA, and the mixture was preincubated at room temperature for 10 min in DNA binding buffer containing ATP (1.2 mM) and magnesium acetate (23 mM) before the binding reaction was started by the addition of DNA. The DNA-protein mixtures were incubated for 5 or 10 min at room temperature, mixed with 5 μ l of loading dye (30% glycerol, 0.02% bromophenol blue in water), and then loaded onto 6% nondenaturing polyacrylamide gels (cross-linker ratio of 29:1 in 1 \times TBE buffer [pH 8; 89 mM Tris base, 89 mM boric acid, 2.5 mM EDTA]) containing 1 mg of Triton X-100/ml. Gels were run in 1 \times TBE buffer at 4°C, dried under vacuum, and exposed on a phosphorimager screen. Signal intensities of free DNA and retarded bands were quantified with a phosphorimager and the program ImageQuant (version 3.3; Molecular Dynamics, Sunnyvale, Calif.).

TABLE 1. Bacterial strains and plasmids used in this study

Strain or plasmid	Relevant phenotype or genotype	Reference or origin
<i>E. coli</i> strains		
DH5 α	<i>supE44</i> Δ <i>lacU169</i> (ϕ 80 <i>lacZ</i> Δ M15) <i>hsdR17 recA1 gyrA96 thi-1 relA1</i>	Gibco-BRL, Gaithersburg, Md.
S17-1	Sm ^r Sp ^r <i>hdsR</i> RP4-2 <i>kan::Tn7 tet::Mu</i> ; integrated in the chromosome	45
C600	<i>supE44 hsdR thi-1 leuB6 lacY1 tonA21</i>	49
DA258	Cm ^r Km ^r Tc ^r (C600) <i>grpE⁺::Ω-Cm thr::Tn10</i>	2
DA259	Cm ^r Km ^r Tc ^r (C600) Δ <i>grpE::Ω-Cm thr::Tn10</i>	2
BL21/pLysS	Cm ^r F ⁻ <i>ompT</i> ($r_B^- m_B^-$) T7lys	46
<i>B. japonicum</i> strains ^a		
110spc4	Sp ^r (wild type)	37
5549	Sp ^r Km ^r <i>hrcA::kan</i> ; <i>hrcA</i> and <i>kan</i> oriented in opposite directions	This study
Plasmids		
pBluescript II KS+	Ap ^r	Stratagene
pBluescript II SK+	Ap ^r	Stratagene
pBSL15	Ap ^r Km ^r	1
pUC18	Ap ^r	34
pSUP202	Ap ^r Cm ^r Tc ^r <i>oriT</i> from RP4	45
pSUP202pol4	Tc ^r (pSUP202); part of polylinker from pBluescript II KS+ between <i>EcoRI</i> and <i>PstI</i>	10
pNM481	Ap ^r (pUC8) ' <i>lacZ</i>	24
pNM482	Ap ^r (pUC8) ' <i>lacZ</i>	24
pSUP481	Tc ^r (pSUP202pol4) ' <i>lacZ</i> (5.1-kb <i>EcoRI-StuI</i> fragment from pNM481)	H. M. Fischer, unpublished data
pSUP482	Tc ^r (pSUP202pol4) ' <i>lacZ</i> (5.1-kb <i>EcoRI-StuI</i> fragment from pNM482)	H. M. Fischer, unpublished data
pET28a(+)	Km ^r	Novagene, Madison, Wis.
pWSK29	Ap ^r ; low-copy-number vector	48
pBW401	Ap ^r (pWSK29); 1.3-kb <i>SalI-EcoRI</i> fragment containing <i>E. coli grpE</i>	49
pRJ5099	Ap ^r (pRJ9519); 0.9-kb <i>BglII-EcoRI</i> fragment containing the <i>B. japonicum dnaKJ</i> promoter region	30
pRJ5400	Tc ^r (pSUP202pol4) <i>hspA'-lacZ</i>	29
pRJ5523 ^b	Ap ^r (pUC18); 3.9-kb <i>EcoRI</i> fragment containing <i>B. japonicum hrcA</i> , <i>grpE</i> , and ' <i>dnaK</i> opposite to <i>olacZ</i> orientation	This study
pRJ5530 ^b	Ap ^r (pBluescript II SK+); 3.0-kb <i>NotI</i> fragment containing <i>B. japonicum hrcA</i> and <i>grpE</i> opposite to <i>olacZ</i> orientation	This study
pRJ5542	Ap ^r (pRJ9519); 0.5-kb <i>NotI-NruI</i> fragment from pRJ5530 containing the <i>B. japonicum hrcA</i> promoter region	This study
pRJ5543	Ap ^r (pRJ9519); 0.7-kb <i>SacI-FspI</i> fragment from pRJ5530 containing the <i>B. japonicum grpE</i> promoter region	This study
pRJ5549	Tc ^r Km ^r (pSUP202pol4) <i>hrcA::kan</i> ; <i>hrcA</i> and <i>kan</i> oriented in opposite directions	This study
pRJ5552	Km ^r [pET28a(+)]; 1.1-kb <i>NotI-NdeI</i> amplification product containing <i>B. japonicum hrcA</i> gene and plasmid for overexpression of H ₆ -HrcA	This study
pRJ5554	Tc ^r (pSUP482) <i>grpE'-lacZ</i> ; 348-bp <i>MscI-FspI</i> <i>grpE'</i> fragment from pRJ5530	This study
pRJ5556	Ap ^r (pUC18); 198-bp amplification product containing the promoter region of <i>B. japonicum groESL₄</i>	This study
pRJ5558	Ap ^r (pUC18); 265-bp amplification product containing the promoter region of <i>B. japonicum groESL₅</i>	This study
pRJ5559	Tc ^r (pSUP481) <i>hrcA'-lacZ</i> ; 937-bp <i>EcoRI hrcA'</i> fragment from pRJ5530	This study
pRJ7998	Tc ^r (pSUP202pol4) <i>groEL₂'-lacZ</i>	10
pRJ8067	Tc ^r (pSUP202pol4) <i>groEL₁'-lacZ</i>	3
pRJ8092	Tc ^r (pSUP202pol4) <i>groEL₅'-lacZ</i>	3
pRJ8174	Tc ^r (pSUP202pol4) <i>dnaK'-lacZ</i>	23
pRJ8548	Tc ^r (pSUP202pol4) <i>groEL₄'-lacZ</i>	3
pRJ9519	Ap ^r (pBluescript II SK+) containing the <i>B. japonicum rm</i> terminator	30

^a Strains containing chromosomally integrated '*lacZ* fusions are listed in Table 3.

^b The insert of this plasmid is depicted in Fig. 1.

Plant infection test. The symbiotic phenotype of the *B. japonicum hrcA* mutant was determined in a soybean plant infection test as described previously (12, 15).

Nucleotide sequence accession numbers. The nucleotide sequence of the *B. japonicum hrcA* and *dnaK* gene region has been deposited in the GenBank-EMBL database under accession no. Y09633.

RESULTS

Cloning the *B. japonicum hrcA* gene region. In contrast to those of several other organisms, the *B. japonicum hrcA* gene is not part of the *dnaK* operon (23). Our initial attempts to

identify the gene by heterologous hybridization with DNA or oligonucleotide probes derived from *hrcA* homologs failed. The fact that the *C. crescentus hrcA* gene is closely linked to *grpE* (38) prompted us to search first for a *grpE* homolog of *B. japonicum* by a complementation approach. Assuming that *B. japonicum GrpE* would be functional in *E. coli*, we attempted to rescue the heat-sensitive *grpE*-deficient *E. coli* strain DA259 (kindly provided by D. Ang, Geneva, Switzerland) with *B. japonicum* DNA cloned into pUC18. The inability of this strain to grow at 39 or 42°C could be fully complemented by the

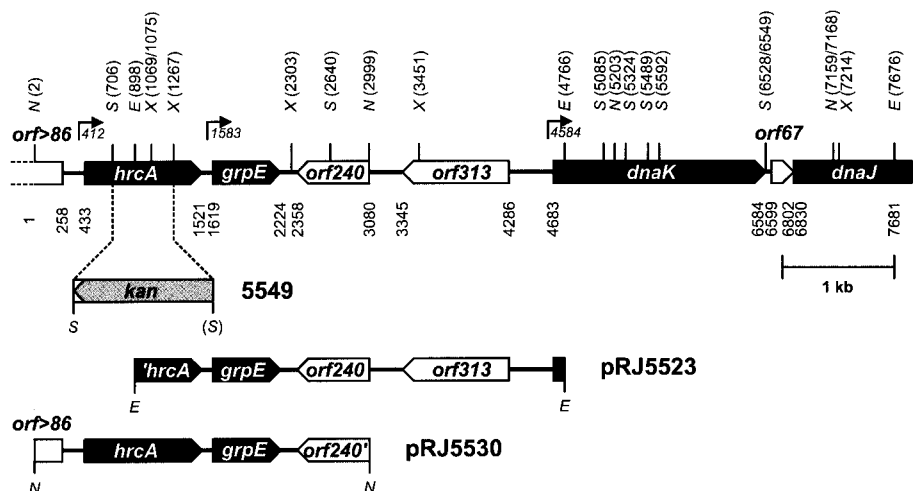


FIG. 1. Physical map of the *B. japonicum* *hrcA* gene region. Numbers indicate start and stop codon positions of open reading frames, the transcription start sites (horizontal arrows above gene map), the beginning (position 1) and the end (position 7681) of the sequenced gene region, and recognition sites of the following restriction enzymes: *E*, *EcoRI*; *N*, *NotI*; *S*, *Sall*; *X*, *XhoI*. The restriction site in parentheses was destroyed during the cloning procedure. The strategy to construct the *B. japonicum* *hrcA* deletion resulting in strain 5549 is indicated. The inserts of plasmids pRJ5523 and pRJ5530 are shown below the physical map.

presence of plasmid pRJ5523, which contains a 3.9-kb *EcoRI* fragment of *B. japonicum* (Table 2; Fig. 1). DNA sequence analysis revealed an open reading frame coding for a protein with a high degree of similarity to all known GrpE proteins. As we had hoped, an open reading frame (3' end) coding for the C terminus of an HrcA-like protein was present upstream of *grpE*. Incidentally, the 5' end of *B. japonicum* *dnaK* was found only 2.4 kb downstream of the *hrcA*-*grpE* cluster (Fig. 1). The complete *B. japonicum* *hrcA* region was subcloned as a 3-kb *NotI* fragment originating from a suitable cosmid, resulting in plasmid pRJ5530 (Fig. 1).

To address the question of whether there is more than one copy of the *hrcA* or *grpE* gene in the *B. japonicum* chromosome, as it is the case for *groESL* (10) and *rpoH* (32), suitable *hrcA* and *grpE* probes were hybridized under low-stringency conditions ($5\times$ SSC [$1\times$ SSC is 0.15 M NaCl plus 0.015 M sodium citrate], 56°C) with *B. japonicum* chromosomal DNA that had been digested with different restriction enzymes. No evidence for an *hrcA* or *grpE* gene family was obtained.

Nucleotide sequence of the *hrcA* gene region. The DNA sequence of the *B. japonicum* *hrcA* gene region, extending from the *NotI* site at position 2 to the *XhoI* site at position 3451, was established (Fig. 1), expanding the previously described sequence of the *dnaKJ* gene region (23). Significant similarities of the deduced amino acid sequences to sequences of *C. crescentus* HrcA (54% identical amino acids) and GrpE (44%) (38) led to the more precise assignment of *B. japonicum*

hrcA (position 433 to 1521) and *grpE* (position 1619 to 2224). The *hrcA* gene begins with the alternative start codon GTG and encodes a predicted protein of 362 amino acids (M_r , 39,165). An alignment of the *B. japonicum* *hrcA* product with 18 HrcA-like proteins deposited in the publicly available databases displayed only limited sequence similarity (overall sequence identity generally around 30% with the exception of HrcA proteins from related organisms, such as *B. japonicum*, *Agrobacterium tumefaciens*, and *C. crescentus*, that have about 50% identical amino acids). The similarity is restricted mainly to three previously described conserved regions (43).

The deduced gene product of *grpE* consists of 201 amino acids and has a molecular weight of 21,655. Further investigation of the DNA region upstream of *dnaK* and *hrcA* revealed three additional open reading frames, all divergently oriented to *hrcA* and *dnaK*. The first open reading frame (*orf>86*) starts at position 258 and codes for the amino-terminal end of a polypeptide of at least 86 amino acids with a high degree of sequence similarity to the putative tRNA nucleotidyltransferase RnpH of *C. crescentus* (67% identical amino acids) encoded by the *rph* gene (38). Moreover, the genetic organization of *orf>86*, *hrcA*, and *grpE* is similar to that of *C. crescentus*. The gene product of *orf240* (M_r , 25,406) shows a high degree of sequence similarity to pyrazin nicotinamidases (e.g., 41% of the amino acids are identical to those of the putative *pncA* gene product of *Aquifex aeolicus* [8]). The *orf313* product (M_r , 33,241) exhibits no significant similarity to any known protein sequence in the database.

Transcriptional analysis of the *hrcA* gene region. The transcription start sites within the *B. japonicum* *hrcA* gene region were determined by primer extension analysis with oligonucleotides complementary to the 5' ends of *hrcA* and *grpE*. A single start site was detected for each gene: 21 nucleotides upstream of the proposed translational start site of *hrcA* (Fig. 2A) and 36 nucleotides upstream of *grpE* (Fig. 2B). Both transcripts were clearly heat inducible, as indicated by 2.3- and 19-fold increases of the reverse transcription products for *hrcA* and *grpE*, respectively, after a 30-min temperature shift of the cells from 30 to 43°C . The deduced promoter regions displayed charac-

TABLE 2. Temperature-dependent growth of *E. coli* DA259 transformed with different plasmids

<i>E. coli</i> strain	Plasmid	Growth at:		
		30°C	39°C	42°C
DA258 (wild type)		+	+	+
DA259 (<i>grpE</i> mutant)		+	-	-
DA259 (<i>grpE</i> mutant)	pUC18	+	-	-
DA259 (<i>grpE</i> mutant)	pBW401 (<i>E. coli</i> <i>grpE</i> ⁺)	+	+	+
DA259 (<i>grpE</i> mutant)	pRJ5523 (<i>B. japonicum</i> <i>grpE</i> ⁺)	+	+	+

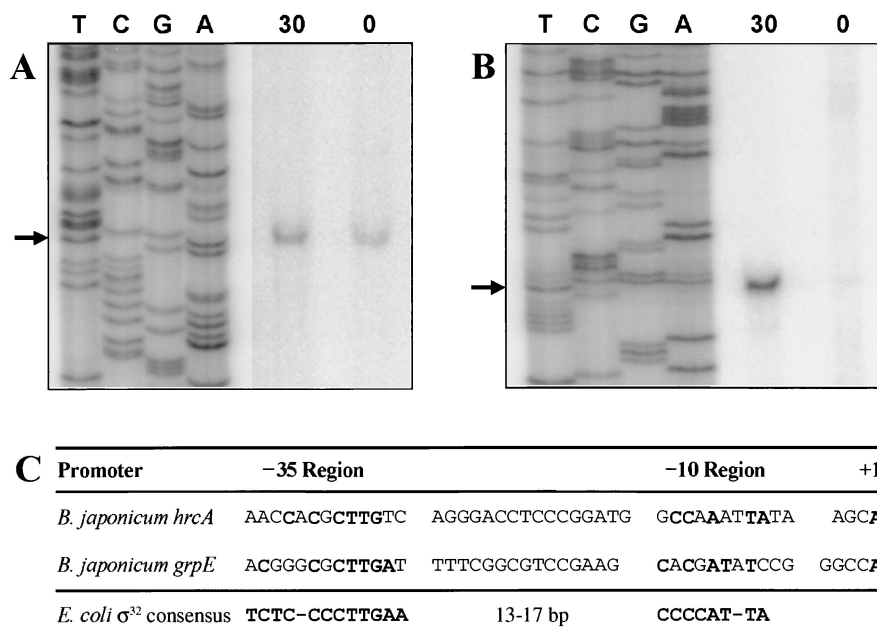


FIG. 2. Determination of the transcription start sites of *hrcA* (A) and *grpE* (B) by primer extension mapping. Total RNA was isolated from *B. japonicum* 110*spc4* cells harvested before (0) and 30 min after a heat shock from 30 to 43°C (30). The extension and sequencing reactions (TCGA) were performed with the primers HrcA11 (A) and GrpE4 (B). The transcription start sites are marked with arrows, and the deduced promoter sequences are shown below (C). Nucleotides matching those of the *E. coli* σ^{32} consensus promoter (52) and transcriptional start sites are in boldface.

teristic sequence motifs of heat-inducible σ^{32} -dependent promoters (Fig. 2C).

The presumed σ^{32} -dependent transcription of *hrcA* and *grpE* was further confirmed by in vitro transcription of both genes with the *B. japonicum* core RNA polymerase reconstituted with purified RpoH₂-H₆ protein, a C-terminally histidine-tagged version of this protein. The RpoH₂ factor is responsible for expression of σ^{32} -dependent genes under normal growth conditions. The resulting *hrcA* and *grpE* transcripts derived from plasmids pRJ5542 and pRJ5543, respectively, are of the expected lengths (286 and 373 nucleotides, respectively; Fig. 3), as judged from a comparison with RNA size markers (not shown) and the *dnaKJ* transcript (358 nucleotides), which was previously shown to be synthesized efficiently by core RNA polymerase reconstituted with RpoH₂-H₆ (Fig. 3A) (30). The core enzyme alone produced only small amounts of transcript (Fig. 3A), most probably due to some residual σ^{32} protein present in the preparation, as described elsewhere (30). Similarly, *hrcA* and *grpE* transcripts were obtained with the *B. japonicum* RNA polymerase holoenzyme, which contains a significant amount of RpoH protein (30). According to these results we conclude that *hrcA* and *grpE* belong to the *rpoH* regulon of *B. japonicum* together with the genes coding for the DnaK machinery.

Heat shock gene expression in an *hrcA* deletion mutant. The *B. japonicum hrcA* mutant 5549 was constructed by replacing an internal *hrcA* fragment with a kanamycin resistance cassette (Fig. 1; see also Materials and Methods). The effect of the *hrcA* deletion on the expression of heat shock genes was determined by both primer extension analysis and β -galactosidase measurements of suitable translational *lacZ* fusions.

The analysis of RNA isolated from wild-type *B. japonicum* and strain 5549 revealed strongly derepressed transcription of the CIRCE-dependent genes *groESL₄* and *groESL₅* under non-heat-shock conditions (Fig. 4). The transcription intensity slightly surpassed even the intensity of heat-induced transcrip-

tion after a 30-min heat shock from 30 to 43°C. By contrast, transcription of σ^{32} -dependent genes (e.g., *groESL₁* and *hrcA*) and heat shock genes regulated by the ROSE element (e.g., *hspA*) was not influenced by the lack of HrcA. In line with the

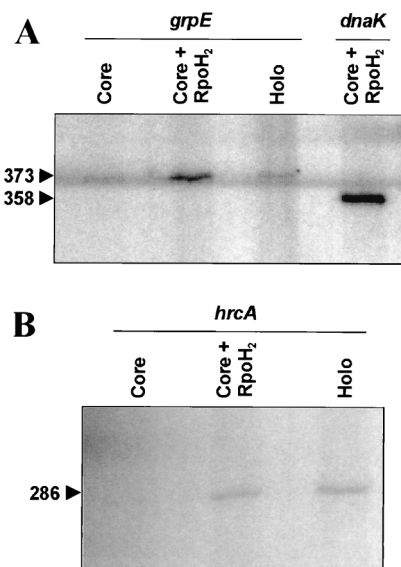


FIG. 3. In vitro transcription from the *B. japonicum grpE* and *dnaK* (A) and *hrcA* (B) promoters. The enzymes used were *B. japonicum* RNA polymerase core enzyme (Core), purified RpoH₂-H₆ protein (RpoH₂), and *B. japonicum* RNA polymerase holoenzyme (Holo). Plasmids pRJ5099, pRJ5542, and pRJ5543 were used as templates to transcribe the 5' ends of *dnaK*, *hrcA*, and *grpE*, respectively. Numbers on the left mark transcript lengths (in nucleotides) of the expected transcripts; lengths were confirmed with suitable in vitro-synthesized RNA size markers (not shown). The signal extending across the entire gel (A) was caused by overloaded RNA size markers on the left.

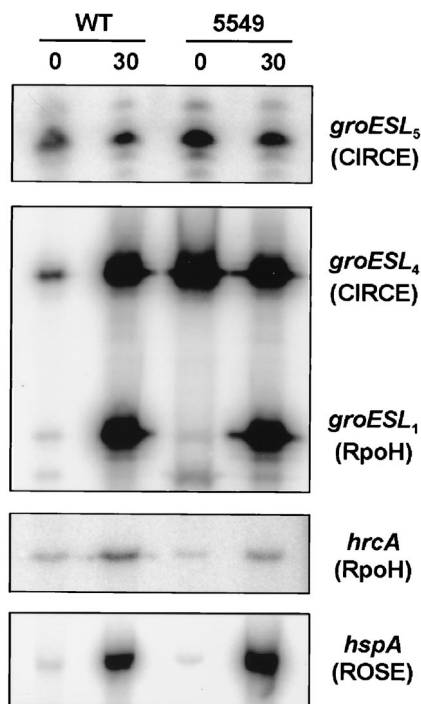


FIG. 4. Effect of an *hrcA* mutation on the transcription of different heat shock genes in *B. japonicum*. Total RNA was isolated from wild-type (WT) *B. japonicum* and 5549 (*hrcA* mutant) cells harvested before (0) and 30 min after a heat shock from 30 to 43°C (30) and analyzed by primer extension experiments. The extension reactions were performed with the primers ESSUP2 (*groESL*₅) (3), 702 (*groESL*₄ and *groESL*₁) (3), HrcA11 (*hrcA*), and Sig107 (*hspA*) (29). The mode of regulation of each gene or operon is indicated.

absence of a CIRCE element upstream of *B. japonicum hrcA*, the gene is not subject to autoregulation as it is in *B. subtilis* (43).

Table 3 shows the expression of different *lacZ* fusions that were integrated into the chromosome of wild-type *B. japonicum* and strain 5549 (see Materials and Methods for the construction of the strains). The two CIRCE-regulated fusions (*groEL*₄'-'*lacZ* and *groEL*₅'-'*lacZ*) exhibited an increased expression in the absence of HrcA, which supports the primer extension results. However, the calculated induction factors (3.2 for *groEL*₄'-'*lacZ* and 2.3 for *groEL*₅'-'*lacZ*) appeared to be lower as compared with the strongly increased transcription of the corresponding genes in strain 5549 (Fig. 4). This is probably due to a posttranscriptional effect of CIRCE, which functions as an mRNA destabilizer promoting the rapid turnover of CIRCE-containing transcripts (19, 50).

The lack of HrcA did not affect the expression of the *lacZ* fusions to *groEL*₁, *grpE* and *dnaK* (σ^{32} regulated), *hspA* (ROSE regulated), and *groEL*₂ (constitutively expressed) (Table 3). The finding for *groEL*₂ supports an earlier notion that the putative element homologous to CIRCE in the promoter region of *groESL*₂ might not be functional (3). Although transcription from the *hrcA* promoter was not influenced by a deletion of the corresponding gene (Fig. 4), the expression of the *hrcA*'-'*lacZ* fusion was increased 2.8-fold in strain H5559 (Table 3). Posttranscriptional events, not further investigated here, might be responsible for this effect.

Complementary evidence that derepression of the *groESL*₄ and *groESL*₅ operons in the *hrcA* mutant raised the cellular GroEL pool was obtained from immunoblots performed with

TABLE 3. Effect of an *hrcA* deletion on the expression of chromosomally integrated '*lacZ* fusions in *B. japonicum*

<i>B. japonicum</i> strains ^a	' <i>lacZ</i> fusion	Induction factor ^b	Mode of regulation
H8092/8092	<i>groEL</i> ₅ '-' <i>lacZ</i>	2.3 ± 0.6	CIRCE
H8548/8548	<i>groEL</i> ₄ '-' <i>lacZ</i>	3.2 ± 0.7	CIRCE
H8067/8067	<i>groEL</i> ₁ '-' <i>lacZ</i>	1.2 ± 0.3	RpoH
H8174/8174	<i>dnaK</i> '-' <i>lacZ</i>	0.9 ± 0.1	RpoH
H5554/5554	<i>grpE</i> '-' <i>lacZ</i>	0.9 ± 0.1	RpoH
H5559/5559	<i>hrcA</i> '-' <i>lacZ</i>	2.8 ± 0.6	RpoH
H5400/5400	<i>hspA</i> '-' <i>lacZ</i>	1.1 ± 0.3	ROSE
H7998/7998	<i>groEL</i> ₂ '-' <i>lacZ</i>	1.3 ± 0.7	Constitutive

^a The indicated '*lacZ* fusion (see also Table 1) was chromosomally integrated into wild-type *B. japonicum* (resulting strains without prefix) and into *hrcA* mutant 5549 (strains with prefix "H").

^b The induction factor is the ratio of the measured β -galactosidase activity in the *hrcA* mutant to that for the wild-type background. Values are means ± standard deviations and are based on at least three independent measurements.

anti-*E. coli* GroEL serum and by two-dimensional gel analysis (data not shown).

Phenotypic characterization of an *hrcA* deletion mutant under symbiotic and heat shock conditions. As it was known that GroEL is critical for nitrogen fixation in *B. japonicum* (11), we tested whether the elevated GroESL₄ and GroESL₅ concentrations in the *hrcA* mutant would affect the performance of *B. japonicum* in root nodule symbiosis and under heat stress conditions. Strain 5549 (*hrcA* mutant) was indistinguishable from the wild-type strain with respect to the ability to nodulate soybean roots and to fix nitrogen. Strain 5549 was further analyzed for its ability to survive a temperature upshift from 30 to 48°C, which is lethal to wild-type *B. japonicum* (33). The survival rate of strain 5549 after exposure to the nonpermissive temperature showed no significant deviation from that for the wild type. Since the elevated GroEL concentration caused by the *hrcA* deletion did not improve the heat tolerance of *B. japonicum*, we conclude that the GroEL pool in wild-type *B. japonicum* cells is not limiting under the conditions tested.

Purification of soluble H₆-HrcA. To obtain in vitro evidence for a physical interaction between CIRCE and HrcA we aimed at performing gel retardation assays with purified components. To this end, an amino-terminally histidine-tagged version of *B. japonicum* HrcA (H₆-HrcA) was expressed in *E. coli*. In contrast to overproduced HrcA from other organisms, a large fraction of *B. japonicum* H₆-HrcA remained in the supernatant even after ultracentrifugation (Fig. 5). This allowed purification under nondenaturing conditions, yielding approximately 4 mg of purified protein per liter of *E. coli* culture.

Specific binding of purified HrcA to CIRCE. Gel retardation experiments with increasing amounts of H₆-HrcA and with radioactively labeled DNA fragments containing CIRCE and the promoter region of *groESL*₄ or *groESL*₅ showed a protein-dependent DNA retardation (Fig. 6A). The *groESL*₄ fragment was used to further delineate the DNA region responsible for HrcA binding (Fig. 6B). Clear DNA binding of H₆-HrcA was observed only with the 94-bp subfragment that contained the -10 promoter region and CIRCE (Fig. 6C), not with a comparable fragment in which the CIRCE element was replaced by a sequence that had no similarity to CIRCE (Fig. 6D; for details see Materials and Methods). The fact that even very high concentrations of the repressor (approximately a 100,000-fold molar excess of protein over DNA) did not result in an appreciable band shift with CIRCE* clearly demonstrates the requirement of a CIRCE sequence for HrcA binding.

In an additional set of gel retardation experiments, we at-

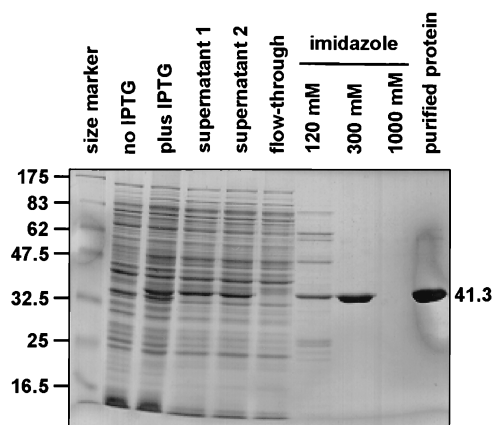


FIG. 5. Overexpression and purification of H_6 -HrcA. Crude extracts of the H_6 -HrcA-overproducing *E. coli* strain harvested before and after induction with IPTG, aliquots from each purification step, and 2 μ l of the dialyzed protein fraction, which was subsequently used for *in vitro* experiments, were separated on an SDS-12% polyacrylamide gel. The proteins were stained with Coomassie blue. Supernatants 1 and 2 mark aliquots of the soluble protein fraction after centrifugation at 30,000 and 116,000 $\times g$, respectively (see Materials and Methods). The apparent molecular masses (in kilodaltons) of H_6 -HrcA (41.3) and the marker proteins are indicated.

tempted to lend support to the concept that the HrcA DNA-binding activity might be chaperonin dependent. H_6 -HrcA was preincubated in binding buffer containing either *E. coli* GroEL and GroES (approximate molar ratio of protomers of HrcA, GroEL, and GroES, 3:10:1 or 3:40:4) or *E. coli* DnaK, DnaJ, and GrpE (approximate molar ratio of protomers of HrcA, DnaK, DnaJ, and GrpE, 10:10:1:1 or 10:40:4:4). Each DNA binding assay was started by the addition of a radiolabeled 168-bp DNA fragment containing the promoter region and the CIRCE element of *groESL*₄. The DNA-binding activity of our soluble HrcA preparation was not improved by the presence of either the DnaK or GroE chaperone machinery. In fact, the addition of GroESL led to a reduction of HrcA-CIRCE complexes (data not shown). In control experiments we confirmed that the GroE preparation had chaperonin activity in the buffer system we used for the gel retardation experiments. This was done by measuring its ability to reactivate heat-denatured malate dehydrogenase (data not shown).

DISCUSSION

This work presents an in-depth *in vivo* and *in vitro* investigation of a representative CIRCE-HrcA system, which is responsible for a widespread heat shock control mechanism in bacteria. It has become evident during the last decade that repressor mechanisms are probably much more abundant among prokaryotes than the *E. coli*-type σ^{32} control of heat shock genes (27). In *B. japonicum*, the CIRCE system is embedded in a complex regulatory network. To elucidate the role of CIRCE in this network, it was imperative to clone the structural gene of HrcA, the putative repressor binding to this DNA element. The amino acid sequences of known HrcA proteins deviate greatly despite the high degree of sequence conservation of the CIRCE elements to which they bind (38, 43). This makes the search for an *hrcA* gene with nucleotide probes rather difficult (13). Here, we made use of the close genetic linkage between *hrcA* and *grpE* that is often observed and established a novel approach that facilitated the isolation of the *B. japonicum hrcA* gene by complementing a temperature-sensitive, *grpE*-deficient *E. coli* strain.

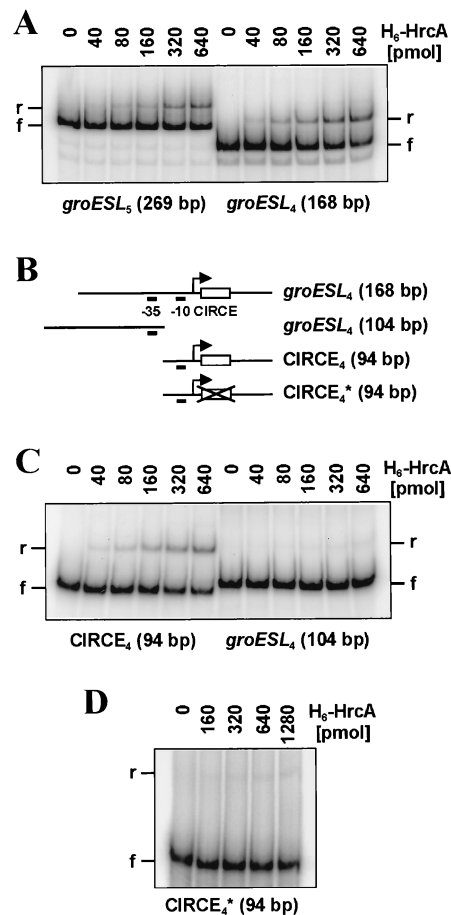


FIG. 6. Gel retardation experiments with purified *B. japonicum* HrcA. DNA-binding reactions were performed with DNA fragments derived from the promoter region of *groESL*₅ (A) and *groESL*₄ (A, C, and D). (B) *groESL*₄ fragments represented schematically. CIRCE₄* represents a DNA fragment in which the CIRCE element was mutated (see Materials and Methods). The amount of H_6 -HrcA used is indicated above each lane in all panels. The positions of radiolabeled free DNA (f) and retarded bands (r) are marked.

Both *hrcA* and *grpE* are transcribed separately from heat-inducible σ^{32} -dependent promoters. The control of the HrcA repressor by a heat shock sigma factor demonstrates that different regulatory mechanisms involved in the *B. japonicum* heat shock response do not act independently but form a complex network. Evidence for a similar network of positive and negative heat shock regulatory mechanisms was also obtained from *C. crescentus*, in which *hrcA* transcription depends on a heat-inducible σ^{32} -like promoter (38). Moreover, its *groESL* operon is subject to dual control by a heat-inducible σ^{32} -dependent promoter and a CIRCE element which is involved in the cell cycle-regulated gene expression (4). Importantly, basal expression of *B. japonicum hrcA* at normal growth temperatures was observed, thus guaranteeing a sufficient amount of HrcA protein to repress the CIRCE regulon. The sigma factor RpoH₂ appears to be responsible for this basal transcription, as it is for a certain threshold level of DnaK (30). Although not proven rigorously, it is reasonable to predict that RpoH₁, the heat shock-induced RpoH factor of *B. japonicum*, is responsible for temperature induction of *hrcA* expression.

Elevated levels of GroESL in an *hrcA* mutant had no influence on free-living growth and symbiotic nitrogen fixation of *B. japonicum*. Similarly, no obvious effect with regard to growth

under normal and stress conditions was observed for *C. crescentus* and *Streptomyces albus hrcA* mutants (13, 38). However, comparable studies of *B. subtilis* showed that the *hrcA* mutant was able to recover from a shift to lethal temperatures whereas the wild type was not (51). A possible reason for this discrepancy between the bacterial species lies in the fact that *B. japonicum*, *C. crescentus*, and *S. albus hrcA* mutants enhance only the concentration of GroE chaperonins whereas the lack of HrcA in *B. subtilis* leads to an overexpression of both chaperone complexes, DnaKJ-GrpE and GroESL. Probably, only increased levels of the complete set of major chaperones improve the capacity of bacterial cells to survive temperature upshifts. This interpretation is also supported by the model in which DnaK and GroE chaperone complexes interact with each other in order to cope with heat shock situations (16).

Previous attempts to perform in vitro studies with HrcA have been impeded by the insolubility of this protein. Gel retardation experiments could only be performed with cell extracts containing HrcA (9, 20) or with renatured HrcA that had been purified under denaturing conditions (25). By contrast, we found that a large fraction of *B. japonicum* H₆-HrcA remained in a soluble form upon overproduction and during purification. The reason for the comparatively high solubility of *B. japonicum* HrcA remains unknown at present. Gel mobility experiments performed with this preparation corroborated the in vivo results in showing a sequence-specific binding of HrcA to CIRCE. However, a large excess of HrcA over DNA was necessary to obtain a band shift. This might be due to an intrinsic tendency of the repressor to aggregate or to reach a nonfunctional conformation. It should be noted that our *B. japonicum* HrcA preparation also tended to precipitate from solution over time. Thus, only a small fraction of the purified protein might really be active. Another, physiologically more relevant reason for the poor retardation activity might be an inherently weak or transient interaction between CIRCE and its repressor. This could explain why both CIRCE-dependent operons in *B. japonicum* are only partially repressed in vivo at normal growth temperatures, resulting in an appreciable basal transcription of the corresponding genes (Fig. 4) (3). Leaky transcription from CIRCE-regulated promoters was also reported for *B. subtilis*, *Clostridium acetobutylicum*, *C. crescentus*, and other organisms (28, 38, 42), indicating that the CIRCE binding site is not permanently occupied by HrcA. It thus appears that the CIRCE-HrcA system is not designed for complete repression of heat shock genes under physiological temperatures because major chaperones are also required for folding processes during normal growth.

There is compelling in vivo evidence that HrcA activity depends on the presence of GroESL (3, 25). The interpretation of these findings was an appealing titration model in which the availability of GroE chaperones controls HrcA activity. Our failure to simulate this feedback control in vitro appears incompatible with this model. While gel retardation of a CIRCE fragment with *B. stearothermophilus* HrcA was significantly improved in the presence of GroEL (25), our gel shift experiments showed that the addition of the complete GroE machinery rather impaired the formation of *B. japonicum* HrcA-CIRCE complexes. One obvious difference between our experiments with *B. japonicum* HrcA and those with *B. stearothermophilus* HrcA is that the latter were performed in the presence of 0.5 M urea after it had been completely denatured during purification (25). It is thus conceivable that GroEL conferred a rather general chaperone activity on this partially denatured HrcA preparation, as was indicated by the prevention of HrcA aggregation in the presence of GroEL. Moreover, it remains unclear why stimulation of gel retardation was ob-

served even in the absence of GroES and ATP (25). The prevention of aggregate formation in the presence of urea might enable partially unfolded HrcA protein to reach an active DNA-binding conformation and, hence, a measurable gel retardation activity with a CIRCE fragment. Experiments with *C. acetobutylicum* HrcA (OrfA), which had been purified under denaturing conditions, revealed that HrcA aggregation was prevented by the addition of DnaK, DnaJ, or the whole DnaK chaperone machinery (39), indicating that urea-denatured HrcA is a substrate not only for the GroE chaperone machinery but also for the DnaK system. While the specific interaction between the CIRCE element and HrcA is well established now, it becomes evident that resolution of the more-debated matters of a physical interaction between HrcA and GroEL and the thermosensing by HrcA must await further studies.

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