

# Differential and Independent Roles of a $\sigma^{32}$ Homolog (RpoH) and an HrcA Repressor in the Heat Shock Response of *Agrobacterium tumefaciens*

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The heat shock response in alpha proteobacteria is unique in that a combination of two regulators is involved: a positive regulator, RpoH ( $\sigma^{32}$  homolog), found in the alpha, beta, and gamma proteobacteria, and a negative regulator, HrcA, widely distributed in eubacteria but not in the gamma proteobacteria. To assess the differential roles of the two regulators in these bacteria, we cloned the *hrcA-grpE* operon of *Agrobacterium tumefaciens*, analyzed its transcription, and constructed deletion mutants lacking RpoH and/or HrcA. The  $\Delta rpoH$  mutant and  $\Delta rpoH \Delta hrcA$  double mutant were unable to grow above 30°C. Whereas the synthesis of heat shock proteins (e.g., DnaK, GroEL, and ClpB) was transiently induced upon temperature upshift from 25 to 37°C in the wild type, such induction was not observed in the  $\Delta rpoH$  mutant, except that GroEL synthesis was still partially induced. By contrast, the  $\Delta hrcA$  mutant grew normally and exhibited essentially normal heat induction except for a higher level of GroEL expression, especially before heat shock. The  $\Delta rpoH \Delta hrcA$  double mutant showed the combined phenotypes of each of the single mutants. The amounts of *groE* and *groE* transcripts before and after heat shock, as determined by primer extension, were consistent with those of the proteins synthesized. The cellular level of RpoH but not HrcA increased significantly upon heat shock. We conclude that RpoH plays a major and global role in the induction of most heat shock proteins, whereas HrcA plays a restricted role in repressing *groE* expression under nonstress conditions.

In the paradigm of bacterial heat shock response studied with *Escherichia coli*,  $\sigma^{32}$  (encoded by *rpoH*) plays a key role in controlling the transcription of all major heat shock proteins (HSPs) in the cytoplasm, including GroE and DnaK chaperone machineries and ATP-dependent proteases, such as Clp and Lon (10, 42). Upon heat shock, the cellular level of  $\sigma^{32}$  increases rapidly both by enhanced translation of *rpoH* mRNA and by transient stabilization of  $\sigma^{32}$ , and this increase in turn activates the transcription of heat shock genes. Homologs of *rpoH* have been identified from some 20 species of eubacteria that belong to the alpha, beta, and gamma subgroups of proteobacteria (1, 13, 15, 20, 21, 24, 30, 31; earlier work cited in reference 21). The *rpoH* homologs from many gamma proteobacteria share common structural features with *E. coli rpoH*, a downstream box, mRNA secondary structure, and highly conserved amino acid sequence of region C, that are important for thermoregulation of *rpoH* translation and  $\sigma^{32}$  stability and activity in *E. coli* (21). In these bacteria, the RpoH homologs indeed exhibit translational induction and stabilization upon heat shock very similar to that found with *E. coli* (22).

In contrast, alpha and beta proteobacteria have diverged from the gamma subgroup in their modes of regulation of *rpoH* expression. Their *rpoH* genes do not contain the downstream box or mRNA secondary structure that are conserved among gamma proteobacteria. Instead, some *rpoH* genes from alpha proteobacteria contain an RpoH-dependent promoter that can

be induced upon heat shock (24, 26, 40). Another important feature found in alpha but not in gamma proteobacteria is the presence of the CIRCE (for controlling inverted repeat of chaperone expression)-HrcA regulatory system. Recent studies of wide groups of eubacteria revealed a variety of heat shock regulatory mechanisms, either positive regulation by alternative  $\sigma$  factors or negative regulation by specific repressor-operator systems (see reviews in references 23 and 28). The most widely distributed system is the CIRCE-HrcA system, extensively characterized in *Bacillus subtilis* as the regulatory mechanism specific for the *groE* and *dnaK* operons (18, 19, 41, 43). CIRCE, with a consensus of TTAGCACTC-N9-GAGTGCTAA, represents a site for binding the HrcA repressor. HrcA could act as a stress sensor whose activity is modulated by the GroEL chaperone. This system has been found in gram-positive bacteria and proteobacteria and implicated in cyanobacteria and several other groups of eubacteria (5, 8, 9, 38, 39). Within proteobacteria, CIRCE and/or *hrcA* were shown to regulate *groE* operons of some alpha proteobacteria, including *Agrobacterium tumefaciens* (3, 27, 37). However a CIRCE-like sequence has not been detected in gamma proteobacteria, except for the *groE* operon of *Chromatium vinosum* (7). It seemed likely that the CIRCE-HrcA system is operative in the alpha and beta proteobacteria but not in most gamma proteobacteria. Thus, alpha and beta proteobacteria appeared to be unique in that they carry both the RpoH system, with regulatory features different from those of gamma proteobacteria, and the CIRCE-HrcA system for regulation of the heat shock response.

In this study, we used *A. tumefaciens* as a model to study the differential roles of the RpoH and CIRCE-HrcA systems in the heat shock response in alpha proteobacteria. The *groE* and *dnaK* operons of *A. tumefaciens* were previously isolated and

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TABLE 1. Bacterial strains used in the study

Strain	Genotype	Source or reference
<i>A. tumefaciens</i>		
GV3101	<i>rpoH<sup>+</sup> hrcA<sup>+</sup></i>	12
KN501	GV3101 $\Delta$ <i>rpoH::tetR hrcA<sup>+</sup></i>	This study
KN613	GV3101 <i>rpoH<sup>+</sup> <math>\Delta</math>hrcA</i>	This study
KN201	GV3101 $\Delta$ <i>rpoH::tetR <math>\Delta</math>hrcA</i>	This study
<i>E. coli</i>		
MC4100	$\Delta$ ( <i>argF-lac</i> )205 <i>ara <math>\Delta</math>139 rpsL150 thiA1 relA1 flb-5301 deoC1 ptsF25</i>	Laboratory stock
KY1454	MC4100 <i>grpE280</i>	14

characterized (34–37), and the CIRCE element was found in the *groE*, but not in the *dnaK*, promoter region. Although the *rpoH* gene was available from our previous study (21), no information on *hrcA* was at hand. Successful isolation of *hrcA* and analysis of the deletion mutants lacking *rpoH* and/or *hrcA* revealed that RpoH plays a major and global role in the induction of HSPs, whereas the role of HrcA is restricted primarily to repressing the *groE* operon under nonstress conditions.

#### MATERIALS AND METHODS

**Bacterial strains, plasmids, media, and growth conditions.** All bacterial strains used are listed in Table 1. The 4-kb *Sma*I fragment containing *hrcA* and *grpE* was inserted into the *Dra*I site of pBBR122 (MoBiTec, Göttingen, Germany) to construct *phrcA-grpE*. For most experiments, *A. tumefaciens* cultures were grown at 25°C in complete medium YEB under constant aeration or on YEB agar (11). For in vivo labeling experiments, Davis minimal medium (6) supplemented with 0.2% glucose, 2  $\mu$ g of thiamine/ml, 50  $\mu$ g of 18 L-amino acids (excluding methionine and cysteine)/ml, and 1/100 volume of YEB were used. Luria-Bertani broth was used for growing *E. coli*.

**Construction and screening of charomid library.** Construction of the charomid library and screening by complementation of the temperature-sensitive *grpE* mutant was done essentially as described previously (21). The DNA inserts of the charomids obtained were subcloned into the pUC-based plasmid vector, and the nucleotide sequence was determined by standard procedures.

**Construction of deletion mutants.** To construct the  $\Delta$ *rpoH* strains, the entire *rpoH* coding sequence with an additional 12 bases of the 5' noncoding region was deleted from the 7-kb *Bam*HI fragment (21) by PCR with primers *rpoH-n1r* (TATCTATGGTCTGGAACGGCACCTCTTTGG) and *rpoH-r1n* (GGCGA CACTCTCTTAAGGAGAGCAGCGCATCC) and replaced by the tetracycline resistance gene from pBR322. The *Bam*HI fragment lacking *rpoH* was inserted into pK18*mobsacB* (32), unable to replicate in *A. tumefaciens*, and introduced into cells of *A. tumefaciens* by electrotransformation with a Gene Pulsar with Pulse Controller (Bio-Rad, Richmond, Calif.). Kanamycin-resistant clones that resulted from homologous recombination between the plasmid and the chromosomal *rpoH* region were selected. To obtain the clones that had lost the inserted plasmid together with the intact copy of *rpoH*, clones that became resistant to 10% sucrose by the loss of *sacB* were isolated from among the initial transformants, and those that were sensitive to kanamycin and resistant to tetracycline were selected. The deletion of *rpoH* was confirmed by Southern blot analysis.

Essentially the same selection procedure was used to construct the  $\Delta$ *hrcA* strains. All of the *hrcA* coding region, except the four N-terminal codons and the termination codon, was deleted by PCR with primers *hrcA-n1r* (CCGCTGAA AAACCCATCTGTCCGTTCTTTATCG) and *hrcA-r1n* (CCGCATAGGAC ACATCAGCAAGATCAGAGAATGCGG). The resulting fragment was used to replace the chromosomal *hrcA* to obtain the *hrcA* deletion mutants. In this case, about 50% of the sucrose-resistant clones from the second selection turned out to be  $\Delta$ *hrcA*, as confirmed by Southern blot analysis.

**Antibodies.** For raising polyclonal antibodies against RpoH and HrcA, coding sequences for each protein were inserted into the expression vector pThioHisA (Invitrogen, Carlsbad, Calif.), and the fusion protein with modified thioredoxin was expressed in *E. coli*, purified with Ni<sup>2+</sup> resin, and used for immunizing rabbits. Rabbit antiserum against *E. coli* GroEL was previously described (17). Antisera against two other *E. coli* HSPs, DnaK and ClpB, were kind gifts from M. Kohiyama (University of Paris 7) and C. Squires (Tufts University), respectively.

**Isolation of RNA and primer extension analysis.** Cells from 50 ml of log-phase culture were quickly chilled, harvested by centrifugation for 1 min at 13,000  $\times$  g, resuspended in 0.25 ml of lysozyme solution (10<sup>4</sup> U/ml)(Ready-Lyse Lysozyme; Epicentre Technologies, Madison, Wis.) with isohypotonic buffer, and mixed with 0.75 ml of ISOGEN-LS (Nippon Gene, Tokyo, Japan). Total RNA was

isolated as described in the manufacturer's manual. The 5' end of primer HrcA-EX1 (GCCTGATCTTTGAAAGCGGTGC; complementary to +13 to 35 of the *hrcA* coding sequence) or GrpE-Ex1 (TCCGCGACGTCGCGTCAGGTCC; complementary to +25 to 47 of the *grpE* coding sequence) was labeled with <sup>32</sup>P and used for primer extension analysis of *hrcA* or *grpE*, respectively. The fluorescent primer GE1 (fluorescein isothiocyanate labeled; TGCCTAATCCC TCGATC; complementary to +70 to 86 relative to the transcription start site of *groE*) or DK1 (fluorescein isothiocyanate labeled; TGAAGCGAGCTGTCTGA ACC; complementary to +58 to 77 relative to the transcription start site of *dnaK*) was used for analysis of *groE* or *dnaK* mRNA, respectively, and 16S2 (FAM labeled; TGCCACTCCCCTTGC GGCGG; complementary to +41 to 61 of the 16S rRNA) was used for analysis of 16S rRNA as an internal control. Primer extension analysis was carried out essentially as described previously (2).

**Nucleotide sequence accession number.** The nucleotide sequence of the entire region around *hrcA* in *A. tumefaciens* has been deposited in GenBank under accession no. AF039940.

#### RESULTS

**Cloning of *hrcA* and the neighboring genes.** Assuming that *hrcA* of *A. tumefaciens* is located adjacent to *grpE* as in *Caulobacter crescentus* (27), we first screened a charomid library of the chromosomal DNA fragments for clones that could complement the temperature-sensitive growth of the *grpE280* mutant of *E. coli* (KY1454). As expected, the resulting clones contained an open reading frame (ORF) which could code for a GrpE homolog. The putative GrpE protein of *A. tumefaciens* is 211 amino acids long and has 28.7 and 34.5% identity with the GrpEs of *E. coli* and *C. crescentus*, respectively. Sequencing of the several clones covering from about 2.5-kbp upstream to 1.5-kbp downstream of *grpE* revealed the presence of four other ORFs that showed homology with the known bacterial genes (Fig. 1). One of them, located directly upstream of *grpE*, separated by 88 bp, showed an appreciable homology with the *hrcA* genes of *C. crescentus* and other bacteria. The predicted amino acid sequence of putative HrcA was 363 amino acids and showed 47.8 and 25.4% identity with those of *C. crescentus* and *B. subtilis*, respectively. The ORF further upstream of *hrcA* on the opposite strand showed high sequence similarity to the *rph* gene encoding RNase PH of *E. coli*. This gene organization is identical with that found in *C. crescentus* (27). On the other hand, two ORFs located downstream on the opposite strand of *grpE*, designated *ptsN* and ORF210 (Fig. 1), had significant homology with *ptsN*, encoding phosphotransferase enzyme IIA, and ORF203, encoding a putative  $\sigma$ <sup>54</sup> modulating protein of *Bradyrhizobium japonicum*, found at the distal end of the *rpoN2* operon (16). A 3' portion of an ORF, homologous to *rpoN*, was also found upstream of ORF210.

**Primer extension analysis of *hrcA* and *grpE* transcripts.** To examine transcripts of the *hrcA* and *grpE* genes, primer extension analysis was carried out with RNAs extracted from wild-type and  $\Delta$ *rpoH* (described below) cells taken before and after a temperature shift from 25 to 37°C, using primers for the 5' end of each gene. Since the transcripts detected were very scarce, strains harboring *hrcA* and *grpE* on a multicopy plasmid (pBBR122) were also used. A single transcript initiated at nucleotide –32 relative to the *hrcA* coding region was detected by using the *hrcA* primer, and its amount increased significantly upon heat shock (Fig. 2a). This transcript was hardly induced

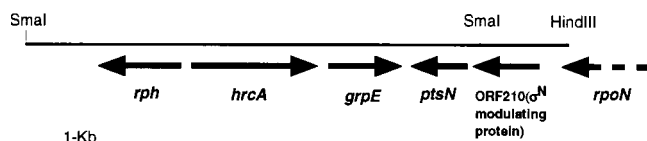


FIG. 1. Gene arrangement around *hrcA* in *A. tumefaciens*. See the text for an explanation of each of the putative genes or ORFs.

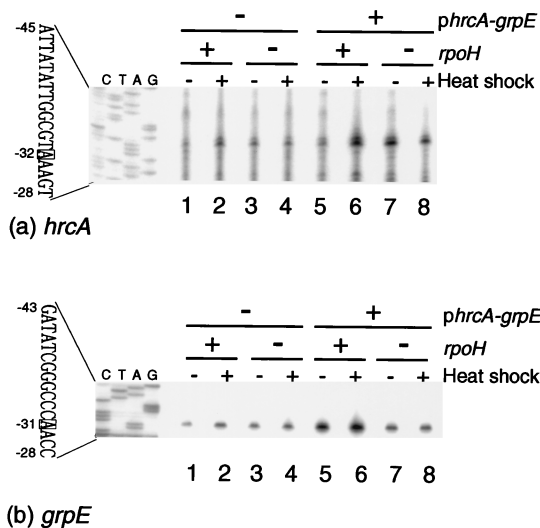


FIG. 2. Transcription analysis of the *hrcA* and *grpE* genes. Cells were grown at 25°C in complete medium to mid-log phase and exposed to 37°C for 15 min. Samples taken before and after heat shock were used for RNA extraction. <sup>32</sup>P-labeled primers for *hrcA* (a) or *grpE* (b) were hybridized with 10 μg of each RNA and subjected to primer extension analysis. The cDNA products were resolved by 6% sequence gels and autoradiographed. Sequence ladders produced by the same respective primers were run as markers, and the positions relative to the initiation codons are indicated. The positions of major signals are marked by boxes. Lanes: 1 and 2, GV3101 (*rpoH*<sup>+</sup>); 3 and 4, KN501 (*ΔrpoH*); 5 and 6, GV3101(*phrcA-grpE*); 7 and 8, KN501(*phrcA-grpE*). +, present; -, absent.

in the *ΔrpoH* mutant, suggesting possible involvement of RpoH in heat induction. Curiously, the basal level found at 25°C was higher in the *ΔrpoH* mutant than in the wild type. A putative promoter corresponding to this transcript showed some similarity to the heat shock promoters of *A. tumefaciens* and also to vegetative promoters of *E. coli* (Fig. 3). An inverted repeat of 12 (6 × 2) bases was found between the -10 and -35 regions of this promoter. This transcript may represent a bicistronic mRNA, since no clear hairpin structure that could implicate a transcription terminator was present between the coding regions of *hrcA* and *grpE*.

Another transcript initiated from between *hrcA* and *grpE*, and induced upon heat shock, was detected by using the *grpE* primer (Fig. 2b). The heat inducibility was lost by the *ΔrpoH* mutation, although appreciable basal levels of this transcript remained, suggesting partial contribution of RpoH to the transcription. Consistently, a putative promoter for this transcript has some sequence similarity to other heat shock promoters

	-35	-10	Start
<i>hrcA</i>	tttccggtTTGccccg	<u>g</u> cgttgcaacg	CatTATaTtgGcgtg
<i>grpE</i>	cgcaagcCTTGatttt	tgcccgtcaagCtcg	ATaTcgGgccca
<i>groES</i>	gcgcggtCTTGactcc	gggagagcgctCct	TATcTcgGctct-(CIRCE)
<i>dnaK</i>	cttgcgCCTTGcagca	ggaaaaatcgctCct	TATaTAcGccgc
<i>rpoH</i>	gcggtcgCTTGaaacg	cggcgggCGcgtaca	TATcTgtcacat
<i>clpP</i>	ttcgtgtCTTGaagtgc	ggggcagggacaCtc	TATgTAgGgagaa
consensus	CTTGa	(15-17)	CnnTATnTngG
<i>E. coli</i> σ <sup>32</sup>	CnCTTGAA	(13-17)	CCCCATnT
<i>E. coli</i> σ <sup>70</sup>	TTGACA	(16-18)	TATAAT

FIG. 3. Promoter sequences of *hrcA*, *grpE*, and other heat shock genes in *A. tumefaciens*. An inverted repeat in the *hrcA* promoter is shown by arrows. The start sites of heat-inducible transcripts are underlined. Bases found in at least five of six possible RpoH-dependent promoters are indicated in upper case. Consensus deduced from these sequences and those of the *E. coli* σ<sup>32</sup> and σ<sup>70</sup> promoters are shown for comparison. References for the sequences are as follows: *groESL*, 34; *dnaKJ*, 35; *rpoH* and *clpP*, 19a; *E. coli* σ<sup>32</sup> consensus, 10; *E. coli* σ<sup>70</sup> consensus, 25.

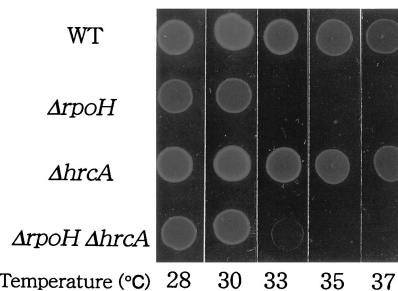


FIG. 4. Growth of the *ΔrpoH* and *ΔhrcA* mutants. Overnight cultures grown in complete medium (YEB) at 25°C were diluted 10-fold, and 5-μl samples were spotted on the YEB plates and incubated overnight at the indicated temperatures. WT, GV3101; *ΔrpoH*, KN501; *ΔhrcA*, KN613; *ΔrpoH ΔhrcA*, KN201.

whose activities depend at least partially on RpoH (Fig. 3) (see below). Thus, at least two transcripts initiated from upstream of *hrcA* and *grpE*, respectively, were shown to be involved in transcription of the two genes that may form an operon.

**Construction of *ΔrpoH*, *ΔhrcA*, and the double-deletion mutants.** To analyze the roles of RpoH and HrcA in the heat shock response, we introduced *ΔrpoH* and *ΔhrcA* deletions into the *A. tumefaciens* chromosome and examined their effects, separately and in combination, on the heat shock response. The entire coding sequence of *A. tumefaciens rpoH* on the *E. coli* plasmid was replaced by the tetracycline resistance gene (*tet*), and *ΔrpoH* strains were constructed by homologous recombination between the *ΔrpoH::tet* fragment and *rpoH* on the host chromosome at 25°C. Similarly, most of the coding region of *hrcA* was deleted from the wild type or the *ΔrpoH* mutant to construct *ΔhrcA* or the *ΔrpoH ΔhrcA* double mutant, respectively. All these deletions were confirmed by Southern blotting. Immunoblotting analysis with the specific antisera revealed that the band for RpoH or HrcA, found in the wild-type extract, was not detected in the corresponding mutant extracts (data not shown). These results suggested that both the *rpoH* and *hrcA* genes exist in single copies and are expressed in the wild-type cells. The *ΔrpoH* mutant was able to grow at 30°C but not at higher temperatures, and growth was significantly slower even at permissive temperatures (e.g., 25°C) (Fig. 4). In contrast, the *ΔhrcA* mutant grew normally or slightly faster than the wild type at physiological temperatures. The double mutant lacking both RpoH and HrcA exhibited temperature-sensitive growth similar to that of the *ΔrpoH* mutant but grew slightly faster at the permissive temperature than the *ΔrpoH* single mutant.

**Effects of *ΔrpoH* and *ΔhrcA* mutations on HSP synthesis.** The set of mutants described above were examined for the synthesis of HSPs before and after temperature upshift. When wild-type cells grown at 25°C were shifted to 37°C, the synthesis of several HSPs was rapidly and transiently induced (Fig. 5a). Among them, the most abundant proteins, of about 90, 70, and 60 kDa, were identified as ClpB, DnaK, and GroEL homolog, respectively, by immunoprecipitation with specific antisera for the respective proteins of *E. coli* (data not shown). In the *ΔrpoH* mutant lacking RpoH, none of these HSPs except GroEL appeared to be induced significantly; GroEL was synthesized apparently normally at 25°C but was only modestly enhanced upon heat shock (Fig. 5b). These results suggested that RpoH is responsible for the heat induction of most, if not all, HSPs in *A. tumefaciens*. On the other hand, the *ΔhrcA* deletion hardly affected the synthesis of HSP, except that GroEL synthesis was slightly higher than in the wild type both before and after temperature upshift (Fig. 5c), indicating the role of HrcA in repressing GroE expression. The synthesis patterns in

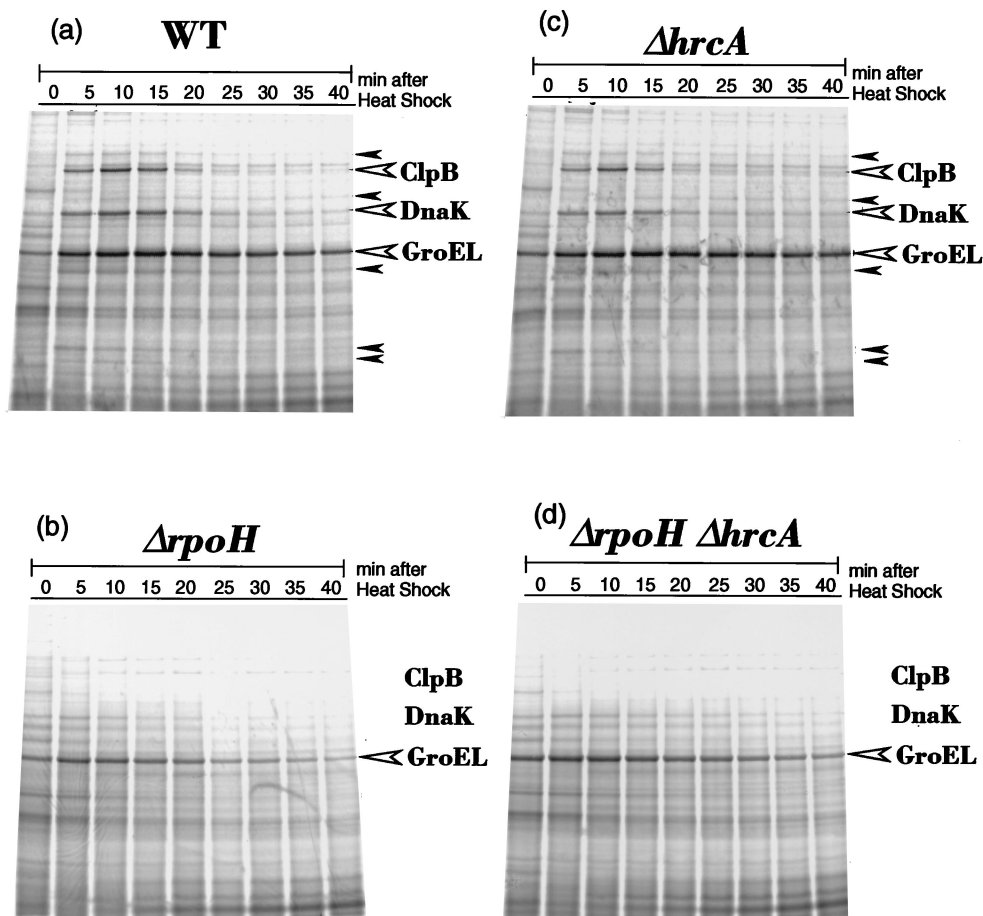


FIG. 5. Sodium dodecyl sulfate (SDS)-polyacrylamide gel patterns of HSP synthesis in mutants. The cells were grown in enriched minimal medium at 25°C and shifted to 37°C at time zero. Samples were taken at intervals, pulse labeled with [<sup>35</sup>S]methionine for 2 min, and treated with trichloroacetic acid. Whole cell proteins were analyzed by SDS-polyacrylamide gel (7.5% gel) electrophoresis and visualized with a phosphorimager. The bands of the most abundantly synthesized HSPs (GroEL, DnaK, and ClpB) are marked with open arrowheads, and other possible HSPs are marked with closed arrowheads. (a) WT, GV3101; (b)  $\Delta rpoH$ , KN501; (c)  $\Delta hrcA$ , KN613; (d)  $\Delta hrcA \Delta rpoH$ , KN201.

the double mutant were very similar to those in the  $\Delta rpoH$  single mutant except for the higher rates of GroEL expression throughout (Fig. 5d); thus, the  $\Delta rpoH$  and  $\Delta hrcA$  mutations exhibited additive effects on the synthesis of these proteins.

The synthesis rates of three major HSPs were quantified to further evaluate the effects of the deletions on the kinetics and extent of heat shock response. The GroEL synthesis was indeed higher in the  $\Delta hrcA$  mutant than in the wild type at all periods tested, but especially higher rates of derepression were observed before heat shock and during the shutoff periods, not at the peak of heat induction (Fig. 6a). A slight but significant induction of GroEL which peaks at about 10 min was observed in the  $\Delta rpoH$  mutant and even in the  $\Delta hrcA \Delta rpoH$  mutant. This induction in the double-deletion mutant may be due to specific processing and stabilization of the *groEL* mRNA (36), since the amount of mRNA immediately downstream of the 5' end of the *groE* operon did not change significantly upon heat shock (see below). On the other hand, induction of DnaK and ClpB in the  $\Delta hrcA$  mutant was similar to that in the wild type, except for the slightly lower extents of induction for both proteins (Fig. 6b and c). Synthesis of DnaK and ClpB in the  $\Delta rpoH$  mutant, which could not be accurately determined due to the background, was found by immunoprecipitation to be less than

6 or 4%, respectively, of the maximum synthesis rates in the wild type (data not shown).

**Analysis of the *dnaK* and *groE* transcripts with the mutants.** To clarify the roles of RpoH and HrcA in the transcriptional induction of HSP genes, we chose *groE* and *dnaK* operons that had been analyzed previously and examined the amounts of the respective mRNAs by primer extension (Fig. 7). The *groE* major transcript markedly increased upon heat shock in the wild type, as reported previously (36). This induction was strikingly affected by the  $\Delta rpoH$  mutation, the mRNA level after heat shock being less than 40% that of the wild type (Fig. 7a, compare lanes 2 and 6). However, the basal-level expression before heat shock was hardly affected by the lack of RpoH (compare lanes 1 and 5). A similar effect was observed in the  $\Delta hrcA$  background (compare lanes 4 and 8 and lanes 3 and 7). These results indicated that RpoH contributes greatly to the heat induction but not to the basal-level transcription of *groE* mRNA. Apparently, the basal transcription depends mostly on a  $\sigma$  factor(s) other than RpoH.

On the other hand, the  $\Delta hrcA$  deletion caused about a two-fold increase in the *groE* transcript under nonstress conditions in both the *rpoH*<sup>+</sup> and  $\Delta rpoH$  strains (Fig. 7a, compare lanes 1 and 3 and lanes 5 and 7) but had little effect on the transcript

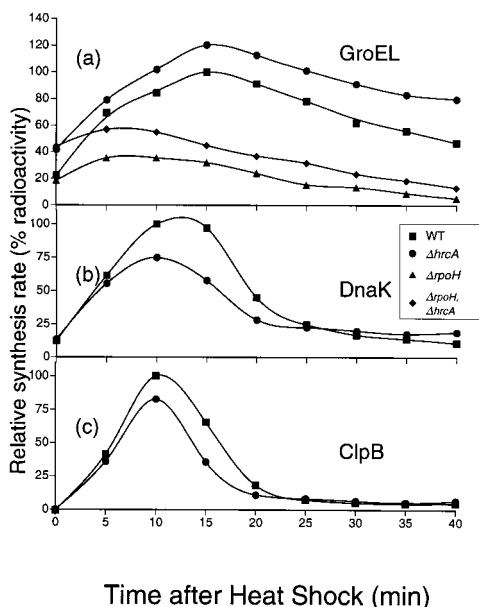


FIG. 6. Kinetics of HSP induction upon temperature upshift. Radioactivity associated with the protein bands of the three major HSPs shown in Fig. 3 were quantified to analyze the kinetics of induction. (a) GroEL; (b) DnaK; (c) ClpB. The radioactivity of each band was plotted after normalization to the maximum synthesis rate in the wild type, set at 100. DnaK and ClpB in the  $\Delta rpoH$  and  $\Delta hrcA$   $\Delta rpoH$  mutants were not plotted, as they were below the background levels. ■, GV3101 (wild type); ▲, KN501 ( $\Delta rpoH$ ); ●, KN613 ( $\Delta hrcA$ ); ◆, KN201 ( $\Delta hrcA$   $\Delta rpoH$ ).

level obtained after heat shock (compare lanes 2 and 4 and lanes 6 and 8). Thus, the repression by HrcA is exerted efficiently during steady-state growth but much less so upon temperature upshift. This was consistent with the previous Northern analyses of transcripts from the *groE* operon with deletions or mutations in the CIRCE sequence (37). These results are also in good agreement with findings with other bacteria (27, 33, 41).

When both *rpoH* and *hrcA* were deleted, no induction of *groE* transcription was observed upon heat shock, in contrast to the partial induction observed with either of the single mutants. It appears, therefore, that the two regulatory factors, RpoH and HrcA, work independently and that their activities

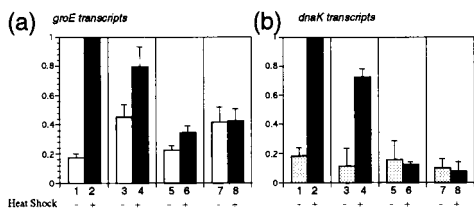


FIG. 7. Transcription analyses of the *groE* and *dnaK* genes. Cells were grown at 25°C in complete medium to mid-log phase and exposed to 37°C for 15 min. Samples taken before and after heat shock were used for RNA extraction. The amount of heat shock transcripts was determined by primer extension analysis. The extension reaction was carried out with each of the *groE* (a) or *dnaK* (b) primers complementary to the region downstream of the known transcription start sites of each operon and with 16S rRNA primer labeled with different fluorescent dyes. The cDNA products were analyzed on 8% sequence gels. Four experiments with two separate RNA samples were carried out. The transcripts were quantified by using 16S rRNA as an internal reference and then normalized to the value for the heat-shocked wild-type cells, and average values are presented with standard errors. Open and shaded bars, RNA from nonstressed cells; solid bars, RNA from heat-shocked cells.

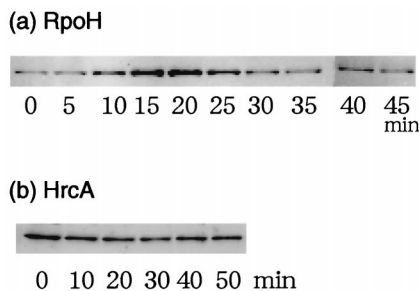


FIG. 8. The cellular levels of RpoH and HrcA during the heat shock response. Cells of wild-type *A. tumefaciens* (GV3101) were grown to mid-log phase in complete medium at 25°C and shifted to 37°C at time zero. Samples were taken at the indicated times, mixed with an equal volume of 2× sodium dodecyl sulfate (SDS) loading buffer, and boiled for 5 min. Equal amounts of protein adjusted by the optical density (in Klett units) of the culture were loaded on the SDS-polyacrylamide gels (12.5% gels), blotted onto a nitrocellulose membrane (Hybond ECL; Amersham Life Science), and detected with rabbit antiserum against RpoH (a) or HrcA (b) by chemiluminescence techniques.

are mostly responsible for heat induction of *groE* transcription in *A. tumefaciens*.

Transcriptional regulation of *dnaK* which does not contain CIRCE appeared simpler. The major transcript, initiated from the single known start site, markedly increased upon heat shock in the wild type and in the  $\Delta hrcA$  mutant, whereas no induction was detected in the  $\Delta rpoH$  mutant or the  $\Delta rpoH$   $\Delta hrcA$  double mutant (Fig. 7b), indicating that *dnaK* transcriptional induction upon heat shock depends solely on RpoH. On the other hand, the  $\Delta hrcA$  mutation caused significant reduction in *dnaK* transcript both before and after heat shock. This reduction, consistent with the reduced rate of DnaK synthesis (Fig. 6b), may be attributed to the increased level of GroE which might somehow reduce the activity of RpoH.

**Increased expression of RpoH but not HrcA during heat shock response.** To analyze the expression of RpoH and HrcA in *A. tumefaciens*, we raised antisera against recombinant RpoH and HrcA and used them to determine the changes in protein levels during the heat shock response by immunoblotting. A significant level of RpoH protein was detected in cells grown at 25°C, and its level was markedly enhanced (by four- to fivefold) upon the shift to 37°C. After reaching the maximum at about 15 min, the RpoH level gradually decreased to near the preshift level after about 30 min (Fig. 8a). In spite of the increase of *hrcA* transcript observed, the HrcA protein was not induced by the heat shock and the HrcA level did not change significantly for at least 50 min after the shift to 37°C (Fig. 8b).

## DISCUSSION

Previous work indicated the involvement of the CIRCE-HrcA system in regulation of the *groE* operons in alpha proteobacteria, including *A. tumefaciens* (3, 27, 37). These studies showed that the repression of *groE* transcription is mediated by the CIRCE-HrcA system, particularly under nonstress conditions. However, the role of this system in the heat induction of *groE* could not be directly examined, because heat shock induction occurred even when *hrcA* or CIRCE had been deleted, presumably by contribution of the heat shock  $\sigma$  factor, RpoH. Here we have shown that both HrcA and RpoH contribute independently to the heat shock response of *groE* transcription in *A. tumefaciens*, since the single-deletion mutants lacking either *hrcA* or *rpoH* could still respond to heat shock, albeit weakly, and enhanced *groE* transcripts significantly whereas

the double deletions virtually abolished the ability to respond to high temperature.

In *B. subtilis* and perhaps other low-G+C gram-positive bacteria, the CIRCE-HrcA system plays a key regulatory role in expression of both the *groE* and *dnaK* operons encoding major chaperones (33, 41). In contrast, CIRCE is found in many species of alpha proteobacteria but only in the *groE* operons (3, 4, 37, 38), implying that *groE* is probably the only target regulated by the CIRCE-HrcA system in this group. The present study revealed that HrcA is indeed involved in the regulation of *groE* transcription of *A. tumefaciens* by repressing it under nonstress conditions and releasing the repression upon heat stress. However, HrcA contributes only modestly to the temperature regulation of *groE* transcription, which is also controlled by RpoH. In addition, the heat shock regulation of GroE in this bacterium is not confined to transcriptional control but also involves specific processing and stabilization of *groEL* mRNA (36): thus, the contribution of HrcA to the heat shock response of GroE is further limited. The specific stabilization of *groEL* mRNA may explain the slight induction of GroEL synthesis in the  $\Delta rpoH \Delta hrcA$  double mutant and the higher rate of GroEL synthesis in the  $\Delta hrcA$  mutant upon heat shock (Fig. 6a), neither of which was observed at the transcript level as determined for the *groES* segment (Fig. 7a).

As for the heat induction of other HSPs, no appreciable effect was observed from the lack of HrcA, although induction of both ClpB and DnaK was slightly reduced (Fig. 6b and c). Since no direct effect of HrcA on *dnaK* transcription is expected, this may be an indirect effect of the increased GroE level in the  $\Delta hrcA$  mutant, which might diminish the stress caused by heat shock and reduce the RpoH-mediated heat shock response.

The *hrcA* transcript of *A. tumefaciens*, determined by semi-quantitative primer extension, was significantly induced after heat shock. Since the putative promoter has some similarity to other heat shock promoters, contribution of RpoH to this promoter was expected, as in the case of *C. crescentus* (27). Indeed, the possible contribution of RpoH to this transcription was suggested, because heat induction observed with the wild type was not found in the  $\Delta rpoH$  mutant. In addition, the level of this transcript seen under nonstress condition was higher in the  $\Delta rpoH$  mutant than in the wild type, suggesting possible involvement of another regulatory mechanism for the transcription. Currently, the nature of the mechanism remains unknown; an inverted repeat found between the  $-35$  and  $-10$  regions might play a role in this or other regulation of HrcA expression. In this connection, an interesting paradox is that the heat-induced transcription of *hrcA* did not result in significant enhancement of HrcA protein upon heat shock. The transcriptional induction might produce untranslatable RNA, or HrcA might be destabilized at high temperature. Further work is needed to solve these problems.

In contrast to the restricted role of HrcA, RpoH appears to be responsible for heat induction of most HSPs, acting as a global regulator of the heat shock response, like  $\sigma^{32}$  in *E. coli*. When produced in *E. coli*, RpoH of *A. tumefaciens* can correctly recognize the *dnaK* and *groE* heat shock promoters of *E. coli* (20). Since most of the HSP genes so far examined in *A. tumefaciens*, *groESL*, *dnaKJ*, *grpE*, *rpoH*, and *clpP*, contain heat-inducible promoters similar to the heat shock promoters of *E. coli* (Fig. 3), RpoH was anticipated to be responsible for transcription of these promoters. As expected, the deletion of *rpoH* resulted in a complete or partial loss of heat-induced transcription from the *dnaK* or *groE* promoter, respectively (Fig. 7). Furthermore, all the heat shock proteins detected by pulse labeling were markedly affected in the  $\Delta rpoH$  mutant,

clearly implying that induction of most if not all HSPs in *A. tumefaciens* is primarily controlled by RpoH.

In spite of a major role of RpoH in the synthesis of HSP, the  $\Delta rpoH$  mutant of *A. tumefaciens* showed a relatively mild temperature sensitivity, the inability to grow only above 30°C, compared with the *E. coli*  $\Delta rpoH$  mutant, which cannot grow above 20°C. This may be related to the relatively high basal expression of GroEL in *A. tumefaciens* in the absence of RpoH (Fig. 5 to 7), in view of the findings with the *E. coli*  $\Delta rpoH$  mutant and its temperature-resistant revertants that the cellular level of GroE primarily determines the upper limit of growth temperature (17). The expression of GroEL in the  $\Delta rpoH$  mutant depends on the transcript initiated apparently from the same start site used in the *rpoH*<sup>+</sup> strain. We don't know which sigma factor(s) is responsible for this transcription in the  $\Delta rpoH$  mutant, but it seems likely that the same sigma is responsible for basal level transcription of *groE*, *grpE*, and other heat shock genes in the *rpoH*<sup>+</sup> strain. Such a sigma factor would not be activated by heat shock, as judged by the *groE* transcription in the  $\Delta rpoH \Delta hrcA$  double mutant or by *dnaK* transcription in the  $\Delta rpoH$  mutants. The existence of a multiple RpoH-like  $\sigma$  factor(s), as was found in *B. japonicum*, seems unlikely, because neither proteins that can cross-react with anti-RpoH polyclonal antibodies nor a DNA fragment that hybridizes with an *rpoH* fragment even with very low stringencies was found in cell extracts or genomic DNA of  $\Delta rpoH$  cells, respectively. However a very distant relative of RpoH might exist and be responsible for basal transcription of heat shock genes, including *groE*. Alternatively, the vegetative sigma factor SigA, a  $\sigma^{70}$  homolog, could recognize the *groE* promoter, which has some similarity to  $\sigma^{70}$  promoters, especially at its  $-35$  region (34). Also, the region upstream of  $-35$  might be important for the high basal activity of the *groE* promoter, since deletion of bases  $-37$  to  $-53$  greatly reduces transcription from this promoter (37); an AT stretch present in this region may act like an UP element (29). In any event, the potentially high basal activity specifically seen with the *groE* promoter may provide a basis for negative control by the CIRCE-HrcA system even in the absence of activation by RpoH, leading to efficient and versatile regulation by the combination of two systems.

We found that the amount of RpoH increases markedly upon heat shock and reaches a maximum at about 15 min. This induction seems to be autoregulated primarily at the level of transcription initiated from an RpoH-dependent promoter (19a), as reported in *C. crescentus* (26, 40). If that is the case, an additional mechanism(s) which not only triggers but also terminates the positive-feedback circuit should exist for modulating RpoH induction. Indeed, the induction of DnaK and ClpB peaks within 10 min after temperature upshift, when the cellular level of RpoH still continues to increase, suggesting that a certain mechanism controlling RpoH activity is involved. Further work on the regulation of RpoH in *A. tumefaciens* should reveal the nature of both conserved and divergent strategies in the regulation of RpoH and of the heat shock response in proteobacteria.

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#### REFERENCES

- Andersson, S. G., A. Zomorodipour, J. O. Andersson, T. Sicheritz-Ponten, U. C. Alsmark, R. M. Podowski, A. K. Naslund, A. S. Eriksson, H. H. Winkler, and C. G. Kurland. 1998. The genome sequence of *Rickettsia prowazekii* and the origin of mitochondria. *Nature* **396**:133-140.

2. Ausubel, F. M., R. Brent, R. E. Kingston, D. D. Moore, J. G. Seidman, J. A. Smith, and K. Struhl (ed.). 1987. Current protocols in molecular biology. Wiley, New York, N.Y.
3. Babst, M., H. Hennecke, and H. M. Fischer. 1996. Two different mechanisms are involved in the heat-shock regulation of chaperonin gene expression in *Bradyrhizobium japonicum*. Mol. Microbiol. **19**:827–839.
4. Baldini, R. L., M. Avedissian, and S. L. Gomes. 1998. The CIRCE element and its putative repressor control cell cycle expression of the *Caulobacter crescentus* *groESL* operon. J. Bacteriol. **180**:1632–1641.
5. Ballard, S. A., M. Go, R. Segers, and B. Adler. 1998. Molecular analysis of the *dnaK* locus of *Leptospira interrogans* serovar copenhageni. Gene **216**: 21–29.
6. Davis, B. D. 1949. Isolation of biochemically deficient mutants of bacteria by means of penicillin. Proc. Natl. Acad. Sci. USA **35**:1–10.
7. Ferreyra, R. G., F. C. Soncini, and A. M. Viale. 1993. Cloning, characterization, and functional expression in *Escherichia coli* of chaperonin (*groESL*) genes from the phototrophic sulfur bacterium *Chromatium vinosum*. J. Bacteriol. **175**:1514–1523.
8. Glatz, A., I. Horvath, V. Varvasovszki, E. Kovacs, Z. Torok, and L. Vigh. 1997. Chaperonin genes of the *Synechocystis* PCC 6803 are differentially regulated under light-dark transition during heat stress. Biochem. Biophys. Res. Commun. **239**:291–297.
9. Grandvalet, C., G. Rapoport, and P. Mazodier. 1998. *hrcA*, encoding the repressor of the *groEL* genes in *Streptomyces albus* G, is associated with a second *dnaJ* gene. J. Bacteriol. **180**:5129–5134.
10. Gross, C. A. 1996. Function and regulation of the heat shock proteins, p. 1382–1399. In F. C. Neidhardt, R. Curtiss III, J. L. Ingraham, E. C. C. Lin, K. B. Low, B. Magasanik, W. S. Reznikoff, M. Riley, M. Schaechter, and H. E. Umberger (ed.), *Escherichia coli* and *Salmonella*: cellular and molecular biology. ASM Press, Washington, D.C.
11. Hirayama, T., T. Muranaka, H. Ohkawa, and A. Oka. 1988. Organization and characterization of the *virCD* genes from *Agrobacterium rhizogenes*. Mol. Gen. Genet. **213**:229–237.
12. Holsters, M., B. Silva, F. Van Vliet, C. Genetello, M. De Block, P. Dhaese, A. Depicker, D. Inze, G. Engler, and R. Villarroel. 1980. The functional organization of the nopaline *A. tumefaciens* plasmid pTiC58. Plasmid **3**:212–230.
13. Huang, L. H., Y. H. Tseng, and M. T. Yang. 1998. Isolation and characterization of the *Xanthomonas campestris* *rpoH* gene coding for a 32-kDa heat shock sigma factor. Biochem. Biophys. Res. Commun. **244**:854–860.
14. Ishiai, M., C. Wada, Y. Kawasaki, and T. Yura. 1992. Mini-F plasmid mutants able to replicate in *Escherichia coli* deficient in the DnaJ heat shock protein. J. Bacteriol. **174**:5597–5603.
15. Karls, R. K., J. Brooks, P. Rossmeissl, J. Luedke, and T. J. Donohue. 1998. Metabolic roles of a *Rhodobacter sphaeroides* member of the  $\sigma^{32}$  family. J. Bacteriol. **180**:10–19.
16. Kullik, I., S. Fritsche, H. Knobel, J. Sanjuan, H. Hennecke, and H. M. Fischer. 1991. *Bradyrhizobium japonicum* has two differentially regulated, functional homologs of the  $\sigma^{54}$  gene (*rpoN*). J. Bacteriol. **173**:1125–1138.
17. Kusukawa, N., and T. Yura. 1988. Heat shock protein GroE of *Escherichia coli*: key protective roles against thermal stress. Genes Dev. **2**:874–882.
18. Mogk, A., G. Homuth, C. Scholz, L. Kim, F. X. Schmid, and W. Schumann. 1997. The GroE chaperonin machine is a major modulator of the CIRCE heat shock regulon of *Bacillus subtilis*. EMBO J. **16**:4579–4590.
19. Mogk, A., A. Volker, S. Engelmann, M. Hecker, W. Schumann, and U. Volker. 1998. Nonnative proteins induce expression of the *Bacillus subtilis* CIRCE regulon. J. Bacteriol. **180**:2895–2900.
- 19a. Nakahigashi, K. Unpublished results.
20. Nakahigashi, K., M. Kanemori, M. Morita, H. Yanagi, and T. Yura. 1998. Conserved function and regulation of  $\sigma^{32}$  homologues in Gram-negative bacteria. J. Biosci. **23**:407–414.
21. Nakahigashi, K., H. Yanagi, and T. Yura. 1995. Isolation and sequence analysis of *rpoH* genes encoding  $\sigma^{32}$  homologs from gram negative bacteria: conserved mRNA and protein segments for heat shock regulation. Nucleic Acids Res. **23**:4383–4390.
22. Nakahigashi, K., H. Yanagi, and T. Yura. 1998. Regulatory conservation and divergence of  $\sigma^{32}$  homologs from Gram-negative bacteria: *Serratia marcescens*, *Proteus mirabilis*, *Pseudomonas aeruginosa*, and *Agrobacterium tumefaciens*. J. Bacteriol. **180**:2402–2408.
23. Narberhaus, F. 1999. Negative regulation of bacterial heat shock genes. Mol. Microbiol. **31**:1–8.
24. Narberhaus, F., P. Krummenacher, H. M. Fischer, and H. Hennecke. 1997. Three disparately regulated genes for  $\sigma^{32}$ -like transcription factors in *Bradyrhizobium japonicum*. Mol. Microbiol. **24**:93–104.
25. Record, M. T., Jr., W. S. Reznikoff, M. L. Craig, K. L. McQuade, and P. J. Cshlax. 1996. *Escherichia coli* RNA polymerase ( $E\sigma^{70}$ ), promoters, and the kinetics of the steps of transcription initiation, p. 792–821. In F. C. Neidhardt, R. Curtiss III, J. L. Ingraham, E. C. C. Lin, K. B. Low, B. Magasanik, W. S. Reznikoff, M. Riley, M. Schaechter, and H. E. Umberger (ed.), *Escherichia coli* and *Salmonella*: cellular and molecular biology. ASM Press, Washington, D.C.
26. Reisenauer, A., C. D. Mohr, and L. Shapiro. 1996. Regulation of a heat shock  $\sigma^{32}$  homolog in *Caulobacter crescentus*. J. Bacteriol. **178**:1919–1927.
27. Roberts, R. C., C. Toochinda, M. Avedissian, R. L. Baldini, S. L. Gomes, and L. Shapiro. 1996. Identification of a *Caulobacter crescentus* operon encoding *hrcA*, involved in negatively regulating heat-inducible transcription, and the chaperone gene *grpE*. J. Bacteriol. **178**:1829–1841.
28. Ron, E. Z., G. Segal, M. Robinson, and D. Graur. 1998. Control elements in the regulation of bacterial heat shock response, p. 143–152. In E. Rosenberg (ed.), *Microbial ecology and infectious disease*. ASM Press, Washington, D.C.
29. Ross, W., K. K. Gosink, J. Salomon, K. Igarashi, C. Zou, A. Ishihama, K. Severinov, and R. L. Gourse. 1993. A third recognition element in bacterial promoters: DNA binding by the  $\alpha$  subunit of RNA polymerase. Science **262**: 1407–1413.
30. Sahu, G. K., R. Chowdhury, and J. Das. 1997. The *rpoH* gene encoding  $\sigma^{32}$  homolog of *Vibrio cholerae*. Gene **189**:203–207.
31. Sato, S., and H. Ishikawa. 1997. Expression and control of an operon from an intracellular symbiont which is homologous to the *groE* operon. J. Bacteriol. **179**:2300–2304.
32. Schafer, A., A. Tauch, W. Jager, J. Kalinowski, G. Thierbach, and A. Puhler. 1994. Small mobilizable multi-purpose cloning vectors derived from the *Escherichia coli* plasmids pK18 and pK19: selection of defined deletions in the chromosome of *Corynebacterium glutamicum*. Gene **145**:69–73.
33. Schulz, A., and W. Schumann. 1996. *hrcA*, the first gene of the *Bacillus subtilis* *dnaK* operon, encodes a negative regulator of class I heat shock genes. J. Bacteriol. **178**:1088–1093.
34. Segal, G., and E. Z. Ron. 1993. Heat shock transcription of the *groESL* operon of *Agrobacterium tumefaciens* may involve a hairpin-loop structure. J. Bacteriol. **175**:3083–3088.
35. Segal, G., and E. Z. Ron. 1995. The *dnaKJ* operon of *Agrobacterium tumefaciens*: transcriptional analysis and evidence for a new heat shock promoter. J. Bacteriol. **177**:5952–5958.
36. Segal, G., and E. Z. Ron. 1995. The *groESL* operon of *Agrobacterium tumefaciens*: evidence for heat shock-dependent mRNA cleavage. J. Bacteriol. **177**:750–757.
37. Segal, G., and E. Z. Ron. 1996. Heat shock activation of the *groESL* operon of *Agrobacterium tumefaciens* and the regulatory roles of the inverted repeat. J. Bacteriol. **178**:3634–3640.
38. Segal, G., and E. Z. Ron. 1996. Regulation and organization of the *groE* and *dnaK* operons in Eubacteria. FEMS Microbiol. Lett. **138**:1–10.
39. Tan, M., B. Wong, and J. N. Engel. 1996. Transcriptional organization and regulation of the *dnaK* and *groE* operons of *Chlamydia trachomatis*. J. Bacteriol. **178**:6983–6990.
40. Wu, J., and A. Newton. 1997. The *Caulobacter* heat shock sigma factor gene *rpoH* is positively autoregulated from a  $\sigma^{32}$ -dependent promoter. J. Bacteriol. **179**:514–521.
41. Yuan, G., and S. L. Wong. 1995. Isolation and characterization of *Bacillus subtilis* *groE* regulatory mutants: evidence for *orf39* in the *dnaK* operon as a repressor gene in regulating the expression of both *groE* and *dnaK*. J. Bacteriol. **177**:6462–6468.
42. Yura, T., H. Nagai, and H. Mori. 1993. Regulation of the *Escherichia coli* heat-shock response. Annu. Rev. Microbiol. **47**:321–350.
43. Zuber, U., and W. Schumann. 1994. CIRCE, a novel heat shock element involved in regulation of heat shock operon *dnaK* of *Bacillus subtilis*. J. Bacteriol. **176**:1359–1363.