Replicon-Specific Regulation of Small Heat Shock Genes in *Agrobacterium tumefaciens*

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Four genes coding for small heat shock proteins (sHsps) were identified in the genome sequence of *Agrobacterium tumefaciens*, one on the circular chromosome (*hspC*), one on the linear chromosome (*hspL*), and two on the pAT plasmid (*hspAT1* and *hspAT2*). Induction of sHsps at elevated temperatures was revealed by immunoblot analyses. Primer extension experiments and translational *lacZ* fusions demonstrated that expression of the pAT-derived genes and *hspL* is controlled by temperature in a regulon-specific manner. While the sHsp gene on the linear chromosome turned out to be regulated by RpoH (σ^{32}), both copies on pAT were under the control of highly conserved ROSE (named for repression of heat shock gene expression) sequences in their 5' untranslated region. Secondary structure predictions of the corresponding mRNA strongly suggest that it represses translation at low temperatures by masking the Shine-Dalgarno sequence. The *hspC* gene was barely expressed (if at all) and not temperature responsive.

The virulence of pathogenic microorganisms is strongly affected by temperature (11, 16, 41). Whereas a temperature of 37°C induces expression of virulence genes in mammalian pathogens, much lower temperatures are typically favorable for the infection process of plant pathogens. Many virulence genes in *Agrobacterium tumefaciens*, *Pseudomonas syringae*, and different *Erwinia* species are induced at temperatures between 18 and 28°C and repressed at higher temperatures (41). Various reasons account for the reduced infectivity of *A. tumefaciens* at temperatures above 28°C. (i) The signal transduction pathway mediated by the VirAG two-component regulatory system is functional only below this temperature (15). (ii) Assembly of T-pilus components required for DNA transfer to plant cells is compromised (3, 4, 8, 9, 17). (iii) Degradation of a subset of virulence proteins might occur at elevated temperatures (4).

A puzzling observation was recently made during studies on the temperature stability of the *A. tumefaciens* type IV secretion system, which translocates DNA and protein into plant cells. Concomitant with the accumulation of plant secondary metabolite-induced virulence proteins at low temperatures, small heat shock proteins (sHsps) were induced (see Discussion of reference 4). Synthesis of sHsps was also observed during symbiosis of *Sinorhizobium meliloti* with the legume *Melilotus alba* (30), and root exudates of the symbiotic host *Alnus glutinosa* induced sHsp synthesis in the actinomycete *Frankia* sp. strain ACN12a-ts_r (12), suggesting a functional importance of sHsps during plant-microbe interaction. Generally, sHsps belong to a large and heterogeneous family of molecular chaperones, also called α -crystallin-type heat shock

proteins (α -Hsps) (6, 26). As the name indicates, sHsps are small (around 20 kDa) and usually induced under heat stress conditions. The *Escherichia coli* Ibp proteins (inclusion body binding proteins IbpA and IbpB) were named Ibp, because they were found associated with inclusion bodies derived from massively overproduced recombinant protein (1). It has been established that sHsps exhibit chaperone-like activity (13, 14). They stably bind to unfolded proteins, maintain them in a folding-competent state, and thus prevent formation of large insoluble aggregates (7, 18). In contrast to most other bacteria, rhizobia produce multiple sHsps after heat shock (23). Two distinct classes could be distinguished on the basis of their primary amino acid sequences. Members of both classes have chaperone activity and assemble into large class-specific oligomers (42).

At least three different mechanisms are known to confer temperature regulation to sHsp-encoding genes in bacteria (26). In many organisms, alternative sigma factors, such as RpoH (σ^{32}), induce heat shock gene transcription under stress conditions (46). RhoA, a repressor protein, inhibits transcription of Hsp18 in *Streptomyces albus* (40). Posttranscriptional control by ROSE (named for repression of heat shock gene expression), a conserved sequence in the 5' untranslated regions (5'-UTRs) of sHsp genes, in rhizobia has been reported (27, 32). The sequence is predicted to fold into a secondary structure that masks the Shine-Dalgarno (SD) sequence at low temperatures (31). Local melting of that structure at higher temperatures liberates the SD sequence and allows translation (5).

Regulation of the heat shock response in *A. tumefaciens* is only partially understood. The *dnaKJ* operon, *clpB*, and several other heat shock genes are under control of RpoH (19, 24, 38). The *groESL* operon was found to be under dual control of RpoH and the HrcA repressor (24, 39). Analysis of the heat shock proteome by two-dimensional protein gel electrophoresis confirmed these results but also raised additional questions,

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TABLE 1. Strains, plasmids and oligonucleotides used in this study

Strain, plasmid, or oligonucleotide	Relevant characteristic(s) or sequence ^a	Source or reference
Strains		
<i>E. coli</i> DH5 α	Cloning host	Gibco-BRL
<i>E. coli</i> BL21 (λ DE3)	Expression host	43
<i>A. tumefaciens</i> C58	Wild type	C. Baron
Plasmids		
pUC18	Cloning vector, Ap ^r	33
pSUP482	Translational <i>lacZ</i> fusion vector, Tc ^r	21
pT7-7StrepII	Strep tag vector carrying T7 promoter	This study
pVS-BADNco	<i>A. tumefaciens</i> expression vector, Sp ^r St ^r	45
pAT5277	<i>hspC-lacZ</i> fusion in pVS-BADNco	This study
pAT5278	<i>hspAT2-lacZ</i> fusion in pVS-BADNco	This study
pAT5279	<i>hspL-lacZ</i> fusion in pVS-BADNco	This study
Oligonucleotides		
Sig212	AAACTGCAGATTGAAGACCCGATCGAACC (PE of <i>hspAT1</i>)	
Sig213	AAACCCGGGAGCTCGGCTGAGACAGTCAT (construction of pAT5278)	
Sig214	AAACTGCAGATTGAGCGCCGATCGAAAC (construction of pAT5278, PE of <i>hspAT2</i>)	
Sig215	AAACCCGGGCGTTTCGATGATGAAATCGAC (construction of pAT5277)	
Sig216	AAACTGCAGATCAAAACCCAGCAAAAGAGG (construction of pAT5277, PE of <i>hspC</i>)	
Sig217	AAACCCGGGGGCTTTTTCAGGAGCCTTTC (construction of pAT5279)	
Sig218	AAACTGCAGCATCGAGAAAAGACGGTCGAA (construction of pAT5279, PE of <i>hspL</i>)	
Sig225	AAAATCAACGTGACGCATGG (PE of <i>hspL</i>)	
Sig228	CAAAAGAGGGTGGGTGAATG (PE of <i>hspC</i>)	
ibpA5	GGCCGGTACCGATGCGTCACGTTGATTTTTCC (construction of plasmid encoding Strep-tagged HspL)	
ibpA3	GGCCCTGCAGTTTAGTTGACCTGAGCCGG (construction of plasmid encoding Strep-tagged HspL)	
StrepII5	CATGGCTAGCTGGAGCCACCCGAGTTCGAAAAATCGAAGGGCGCGCGGTACG (construction of pT7-7StrepII)	
StrepII3	CATGGGTACCGCGCGCCCTTCGATTTTTTCGAACTGCGGGTGGCTCCAGCTAGC (construction of pT7-7StrepII)	

^a Introduced restriction sites are underlined. PE, primer extension.

since 32 of 56 heat shock proteins were induced independently of RpoH and HrcA (34). The existence of at least one additional control mechanism was proposed. Our present study on regulation of the four *A. tumefaciens* sHsps revealed that ROSE-mediated control is one of these mechanisms.

MATERIALS AND METHODS

Strains and growth conditions. *E. coli* cells were grown at 37°C in Luria-Bertani (LB) medium supplemented with ampicillin (AMP) (200 μ g/ml), tetracycline (10 μ g/ml), streptomycin (50 μ g/ml), or spectinomycin (50 μ g/ml) where applicable. *A. tumefaciens* was routinely grown at 30°C in YEB complex medium at 25°C in AB minimal medium with 1% glucose (36).

Plasmid constructions. Recombinant DNA work was performed by standard protocols (35). Plasmids pAT5277, pAT5278, and pAT5279 (Table 1) were constructed as follows. PCR-generated fragments were digested with SmaI and PstI before they were ligated into pUC18 treated with the same enzymes. The correct nucleotide sequence was confirmed by automated sequencing. Subsequently, the fragments were transferred into pSUP482 to construct in-frame fusions to the *lacZ* gene. The entire *hsp-lacZ* fusions were then cloned via the SmaI and XbaI sites into pVS-BADNco. Reporter plasmids were transferred into *E. coli* via transformation and into *A. tumefaciens* via electroporation.

To produce proteins with an N-terminal StrepII tag for affinity purification, pT7-7Nco (37) was linearized with NcoI, followed by ligation of the two 5'-phosphorylated and annealed oligonucleotides StrepII5 and StrepII3, which encode the tag sequence. The correct orientation of the ligated oligonucleotides in the resulting vector pT7-7StrepII was confirmed by DNA sequencing.

Immunoblot analyses and generation of antisera. Cells from liquid cultures or from plates were washed with 0.9% NaCl prior to use. Protein concentrations were determined with the Bradford assay (Bio-Rad). Equal amounts (20 μ g of protein) of each extract were separated by sodium dodecyl sulfate–12% polyacrylamide gel electrophoresis and blotted onto Hybond-C extra nitrocellulose filters (Amersham). Antisera were used in 1/10,000 dilutions. Bound peroxidase-

coupled secondary anti-rabbit immunoglobulin G was detected with the Pierce SuperSignal kit.

Rabbit serum against streptavidin-tagged HspL was generated as follows. The *hspL* gene was PCR amplified from *A. tumefaciens* C58 chromosomal DNA with oligonucleotides ibpA5 and ibpA3. The fragment was cleaved with Acc65I and PstI and ligated into similarly cleaved pT7-7StrepII. Transformed cells were plated on LB medium supplemented with AMP (50 μ g/ml), and the desired clones were identified by restriction analysis and DNA sequencing. The T7 promoter-carrying plasmid was introduced into *E. coli* strain BL21(λ DE3) for protein overproduction. Cells in 800-ml culture (4x 200 ml in 500-ml flasks) in LB medium containing AMP (100 μ g/ml) were induced for 4 h at 37°C with 0.5 mM isopropyl- β -D-thiogalactopyranoside (IPTG) for overproduction of the protein fused to the N-terminal affinity (StrepII) tag (sequence was WSHPOFEK). The overexpressed StrepII tag fusion protein was purified from cell lysates in one step by affinity chromatography on a streptactin Sepharose column (IBA, Göttingen, Germany) according to the manufacturer's instructions. The purified protein (700 μ g) was lyophilized and injected into rabbits for the generation of a specific antiserum (BioGenex, Berlin, Germany).

RNA isolation and primer extension. Total RNA was prepared using hot acid phenol as described previously (2). The oligonucleotides used to map the transcription start sites of *hspAT1*, *hspAT2*, *hspL*, and *hspC* are listed in Table 1.

β -Galactosidase assays. The β -galactosidase activity in exponentially growing *E. coli* and *A. tumefaciens* cells was measured by standard protocols (20).

RESULTS AND DISCUSSION

Four small heat shock genes in *A. tumefaciens*. The genome of *A. tumefaciens* is composed of four replicons, a circular chromosome (2.84 Mb), a linear chromosome (2.07 Mb), the pAT plasmid (0.54 Mb), and the Ti plasmid (0.21 Mb) (10, 44). A search of the complete genome sequences revealed the presence of four genes coding for sHsps on three different repli-

FIG. 1. Overview of the four small heat shock genes encoded by *A. tumefaciens*. The genomic locations (pAT plasmid, linear chromosome, and circular chromosome) are indicated in the gene designations. Potential transcription start sites are represented by arrows. Presumed regulatory mechanisms are listed, and ROSE-type sequences are shown as grey boxes.

cons. The pAT plasmid encodes two sHsp genes, whereas each chromosome contains one such gene (Fig. 1). The genes were designated *hspAT1*, *hspAT2*, *hspL*, and *hspC* according to their chromosomal origin.

Many rhizobia have been shown to produce multiple heat shock genes which often can be divided into two distinct classes, class A and B (23, 26). All four *Agrobacterium* sHsps clearly fall into class A whose members closely resemble the *E. coli* Ibp proteins. Pairwise identity between the deduced agrobacterial sHsps and *E. coli* IbpA ranges from 46 to 55%. Homology to class B proteins is only marginal, e.g., HspAT2 barely shares 20% identical amino acids with *Bradyrhizobium japonicum* HspC or *Methanococcus jannaschii* Hsp16.5. Identical residues are restricted to highly conserved motifs, such as the AxxxNGxL motif towards the end of the α -crystallin domain and the IxI motif in the C-terminal extension (data not shown). Agrobacterial sHsps from different replicons exhibit around 45% identity, whereas both proteins derived from the pAT plasmid have 74% of their amino acids in common.

Temperature-dependent production of α -Hsps in *A. tumefaciens* was monitored by immunoblot analysis using antisera raised against HspL. sHsps were not detected during exponential growth at 20 or 30°C or after a shift from 20 to 30°C (Fig. 2). Continuous growth at 37°C and shifts from 20 to 37 or 42°C triggered sHsp synthesis. In line with the severity of the heat shock, an upshift from 30 to 37°C for 30 min induced much smaller amounts of sHsps than a shift from 30 to 42°C. The results demonstrate that *A. tumefaciens* precisely records the

FIG. 2. Heat-induced production of sHsps in *A. tumefaciens*. Cells were grown in YEB medium at the temperatures indicated. For heat treatment, aliquots of the cells were shifted to higher temperatures for 30 min. Harvested cells were subjected to Western blot analysis as described in Materials and Methods using an anti-HspL serum. The detected band migrates at 18 kDa.

ambient temperature and adjusts the cellular level of sHsps accordingly.

By using a proteomic approach, 52 proteins were found to be induced by a temperature upshift in *A. tumefaciens* (34). Among the proteins identified by MALDI-TOF (matrix-assisted laser desorption ionization–time of flight) mass spectrometry, only one sHsp was found. However, several other heat-induced spots in the low-molecular-mass range were not identified. They could potentially represent the remaining sHsps. As all four sHsps have similar sequences and molecular masses, only one heat-induced band of 18 kDa was detected by our Western blot analysis. Thus, it remained unclear whether one or more of the four proteins were induced under heat stress conditions. In order to further dissect the mechanisms controlling small heat shock gene expression in *A. tumefaciens*, all four genes were studied individually.

ROSE-controlled expression of pAT-encoded sHsps. Both *hspAT1* and *hspAT2* are preceded by a sequence strongly resembling the ROSE motif of many other rhizobial small heat shock genes (31, 32). To assign the precise transcription start sites, primer extension experiments were performed with RNA from normally grown cells and heat-shocked cells. As in *B. japonicum*, *Rhizobium* sp. strain NGR234, and other species (29, 32), corresponding transcripts were detected only in RNA preparations from heat-treated *A. tumefaciens* cells (Fig. 3A and B). The deduced –10 and –35 regions of both *hspAT* genes are identical and conform to the consensus sequence of a σ^{70} -type promoter. Both approximately 80-nucleotide-long 5'-UTRs downstream of the transcription start sites are highly similar, in particular towards the 3' end where more than 30 residues including the SD sequences are completely identical (Fig. 3C). Computer-assisted secondary structure predictions of the 5'-UTRs suggest in each case an extended secondary structure resembling ROSE-like elements found in other rhizobia (shown for *hspAT2* in Fig. 3D). The characteristic features include the SD sequence and AUG start codon being masked by base pairing as well as a strictly conserved bulged G residue opposite the SD sequence. The corresponding bulged residue in ROSE₁ of *B. japonicum* (G83) was shown to be instrumental in translation initiation at elevated temperatures in vivo (31). Accordingly, thermal melting of an in vitro-synthesized transcript lacking that residue started to melt only at temperatures above 45°C (5).

The absence of transcript at 30°C implies transcriptional control. However, both transcripts originate from a classical housekeeping promoter which should allow significant transcription at low temperatures. Although transcriptional regulation by an unknown mechanism cannot be excluded, the potential RNA structures masking the SD sequence strongly suggests translational control of the pAT-encoded small heat shock genes. In analogy to ROSE-mediated control in other members of the family *Rhizobiaceae*, we propose that the folded RNA produced at low temperatures is rapidly degraded (31). The melted RNA might either display no RNase recognition sites or might be protected from degradation by the physical presence of ribosomes.

The *hspL* gene belongs to the RpoH regulon. Primer extension experiments with oligonucleotides complementary to the 5' end of *hspL* revealed transcripts of *hspL* only under heat shock conditions (Fig. 4A). The deduced transcription start

FIG. 5. Temperature-dependent expression of *A. tumefaciens* small heat shock genes. Cultures were grown to exponential phase in AB medium with glucose at the temperatures indicated before β -galactosidase activity (in Miller units [MU]) was measured.

sions to *hspAT2*, *hspL*, and *hspC* (Materials and Methods and Table 1). All constructs led to basal expression when *A. tumefaciens* was grown at 25°C (Fig. 5). Expression of *hspAT2-lacZ* was elevated during growth at 30°C and induced more than 10-fold at 37°C. The *hspL-lacZ* fusion was induced fivefold during growth at 37°C. Both results confirm that expression of the ROSE- and RpoH-controlled sHsp genes is temperature regulated. In contrast, the expression of *hspC* increased only slightly with temperature, indicating again that it is not significantly expressed and not temperature controlled.

Likewise, expression of the *hspC-lacZ* fusion was not significantly altered after a shift from 25 to 37°C in *A. tumefaciens* or *E. coli* (data not shown). On the other hand, a temperature shift resulted in clear induction of *hspAT2* and *hspL* expression in *A. tumefaciens* (Fig. 6). An equivalent shift induced only *hspAT2* expression in *E. coli* (Fig. 6), which suggests that ROSE-mediated control is retained in this organism. Similar results were observed with ROSE elements from *B. japonicum* (5, 31). Although the RpoH-dependent promoter of the *hspL* gene resembles the consensus sequence for σ^{32} (Fig. 4B), expression of this gene was not induced by increasing the temperature in *E. coli* (Fig. 6). It has been observed previously that promoter sequences from α -proteobacteria were not recognized by *E. coli* RNA polymerase. *E. coli* σ^{32} -containing RNA polymerase was unable to transcribe the *B. japonicum dnaK* gene (22), and the σ^{70} -containing holoenzyme of *E. coli* did not recognize the housekeeping-type *hspA-rpoH₁* promoter of *B. japonicum* (31). The *A. tumefaciens hspL* gene appears to be another example of this phenomenon. On the other hand, the *E. coli dnaK* promoter was heat activated in *A. tumefaciens* (38), and the *E. coli groESL* promoter was transcribed by the *B. japonicum* RNA polymerase carrying sigma factor RpoH₁ or RpoH₂ (28). These data suggest that promoter recognition by RNA polymerases from α -proteobacteria is less strict than transcription initiation by the *E. coli* enzyme.

To date, *A. tumefaciens* is the only known organism in which heat-induced synthesis of sHsps is controlled by two different strategies. The different mechanisms correlate with the chromosomal location, i.e., pAT-encoded genes are regulated by ROSE, and the sHsp gene on the linear chromosome is regulated by RpoH. It is unclear whether both regulons respond to

FIG. 6. Expression of *lacZ* fusions to *hspAT2* and *hspL* after a temperature upshift in *A. tumefaciens* and *E. coli*. *A. tumefaciens* cells grown in AB medium with glucose and *E. coli* grown in LB medium were shifted from 20 to 37°C before β -galactosidase activity was measured at the time points indicated. The activities at timepoint zero were set to 1.

different stimuli. The ROSE sequence presumably measures the ambient temperature, since it was shown to be an RNA thermometer (5). The RpoH system most likely responds to the cellular level of unfolded protein, since the activity of RpoH in *A. tumefaciens* is tightly controlled by the chaperone DnaK (25), which binds to unfolded substrates under stress conditions.

Our results identified the ROSE element in *A. tumefaciens* as one of the additional heat shock control systems proposed by Rosen et al. (34). However, only two genes, *hspAT1* and *hspAT2*, are regulated by this translation mechanism. A search for additional ROSE-type sequences in the genome of *A. tumefaciens* demonstrated that no other genes bear such an RNA thermometer. Since 32 of 56 heat shock proteins were shown to be induced independently of RpoH and HrcA (34), induction of a substantial number of proteins still remains to be elucidated.

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