Heat Shock Proteome of Agrobacterium tumefaciens: Evidence for New Control Systems

Ran Rosen,1 Knut Büttner,2 Dörte Becher,2 Kenji Nakahigashi,3 Takashi Yura,3 Michael Hecker,2 and Eliora Z. Ron1*

Department of Molecular Microbiology and Biotechnology, George S. Wise Faculty of Life Sciences, Tel Aviv University, Ramat Aviv, Israel; Institut für Mikrobiologie und Molekularbiologie, Ernst-Moritz-Arndt-Universität-Greifswald, D-17487 Greifswald, Germany; and HSP Research Institute, Kyoto Research Park, Shimogyo-ku, Kyoto 600-8813, Japan

Received 28 September 2001/Accepted 20 December 2001

The regulation of Agrobacterium tumefaciens heat shock genes involves a transcriptional activator (RpoH) and repressor elements (HrcA-CIRCE). Using proteome analysis and mutants in these control elements, we show that the heat shock induction of 32 (out of 56) heat shock proteins is independent of RpoH and HrcA. These results indicate the existence of additional regulatory factors in the A. tumefaciens heat shock response.

The heat shock response is characterized by the induction of several proteins, some of which are highly conserved. In Escherichia coli the heat shock regulon is controlled by alternative sigma factors, mainly σ32 (encoded by rpoH), which regulates transcription of all major cytoplasmic heat shock proteins (6, 35) and has been identified in more than 20 species of gram-negative eubacteria (1, 11, 12, 16, 17, 19, 20, 26). The heat shock proteins of the gram-positive Bacillus subtilis are divided into at least three regulatory classes (7). The chaperones encoded by the orf39-grpE-dnaK-dnaJ operon and by the groESL operon are transcribed by the vegetative sigma factor even during heat shock (4) and regulated by the HrcA repressor, which binds to CIRCE (controlling inverted repeat of chaperone expression) (9, 13–15, 37). A second class of heat shock proteins is regulated by σ32 (7–9), and the others are HrcA and σB independent (7, 9). In Agrobacterium tumefaciens and other α-proteobacteria, two heat shock control elements were identified, the RpoH homologue and the CIRCE-HrcA regulatory system (16, 24, 29, 30, 32). In contrast to the gram-positive bacteria the CIRCE-HrcA system is found only in the groESL operon and heat shock transcription of the major chaperone genes is controlled by RpoH (16, 18, 27–32). To further study the complex heat shock response in A. tumefaciens, we used two-dimensional (2-D) gel electrophoresis. We found 56 heat shock proteins, 5 of which are newly identified heat shock proteins. The heat shock proteins can be divided into at least three regulatory groups. The first group is RpoH dependent (24 proteins); among these, GroEL and GroES may be considered as a separate group since they are also repressed by HrcA in non-heat shock conditions. The expression of genes of the third group (32 proteins) is regulated independently of the known control elements (σ32 and HrcA) and indicates the existence of additional regulatory factors or controls.

Effects of RpoH on the heat shock response. Proteome analysis of A. tumefaciens indicated the heat shock induction of 56 proteins (25). It should be noted that several groups of proteins were not studied in our experiments, i.e., membrane proteins and proteins with very high or very low pI values. We examined the involvement of RpoH in a wild-type strain and an ΔrpoH mutant (16) (Table 1). Using 2-D gel analysis (3, 25), we found that, in all strains, the majority of the non-heat shock proteins were strongly down-regulated during heat shock (Fig. 1 and 2), as represented by the periplasmic binding protein (Fig. 3a). Out of the 56 proteins induced by heat shock in wild-type A. tumefaciens (Fig. 1, top and middle), 32 were induced in the ΔrpoH mutant (Fig. 1, bottom), indicating the existence of one or more heat shock control elements yet to be discovered.

Effects of HrcA on the heat shock response. HrcA is a repressor of groESL transcription in non-heat shock conditions (16, 29). To search for other proteins similarly regulated, we separated proteins from hrcA mutants on 2-D gels. As expected, a significant overexpression of GroEL and GroES was found in the mutants under non-heat shock conditions, but no additional proteins repressed by HrcA were found. Only 52 proteins were induced upon heat shock in the hrcA mutant, 50 of them also induced in the wild type and 2 not induced by heat shock in the wild type (NWH). These differences in heat shock induction cannot be explained by the known role of HrcA and probably reflect a secondary effect of the high levels of GroEL and GroES in the mutants. The high level of chaperones can shift the balance between refolding and degradation of defective proteins at the early stages of the heat shock response and can also affect specific regulatory proteins. One such protein is the vegetative sigma factor σB, whose concentration in E. coli is influenced by the level of chaperones (33, 35, 36).

Heat shock response in an rpoH hrcA double mutant. Exposure of the rpoH hrcA double mutant to a temperature of 42°C...
resulted in induction of 40 proteins (Fig. 2, lower panel), 37 of them heat shock proteins detected in the wild type and 3 (NWH) induced only in hrcA mutants. A comparison between the double mutant and the wild type indicates a pleiotropic effect that was not predicted from the examination of each mutant by itself. During exponential growth the only difference is a significant overexpression of GroEL and GroES in the double mutant (Fig. 1, top, and 2, top). However, during heat shock there are several proteins that are induced in the double mutant but not in single mutants. This result may be explained by the combination of two major imbalances in the double mutant: an imbalance in protein folding and degradation due to excess of the chaperones GroEL and GroES and reduced competition between the vegetative and other sigma factors due to the lack of σ^32.

Regulation patterns of heat shock proteins. Several regulation patterns were found.

(i) Typical σ^32-dependent expression: DnaK (Fig. 3b). Heat shock induction is almost abolished in rpoH mutants. The results are compatible with those of transcription analysis (16) indicating that DnaK expression is regulated exclusively at the transcription level by σ^32. Expression of these proteins during exponential growth in the rpoH mutant and the double mutant is lower than that in the wild type, suggesting that even at 25°C the promoter is recognized by more than one sigma factor.

(ii) Regulation by Hrca and σ^32: GroESL (Fig. 3c and d). The operon is induced by σ^32 under heat shock conditions and repressed by Hrca under non-heat shock conditions. Here also, the results are compatible with previous analysis of transcripts, and apparently more than one sigma factor is involved in transcription. The quantitative differences between the GroES and GroEL proteins probably result from the different transcript stabilities of these two genes (28).

(iii) Heat shock induction independent of σ^32 and Hrca: PstB (Fig. 3e). The pattern of induction independent of σ^32 and Hrca is exemplified by protein H35, the high-affinity phosphate transport protein, newly identified as a heat shock protein in this work. This protein is involved in the phosphate flux into cells, which affects the survival of Lactococcus lactis during heat shock and the quality of the heat shock response (5).

PstB represents a group of 20 proteins that have low expression at 25°C and are induced at 42°C even in ΔrpoH and hrcA mutants. The heat shock level in the ΔrpoH mutant was higher than that in the wild type, probably due to the lack of competition for RNA polymerase core enzyme by σ^2, enabling better recruitment of the RNA polymerase by the sigma factor required for the transcription of the genes in this group.

H26-G, a general stress protein. Among the proteins whose expression is RpoH and Hrca independent (Fig. 3f), we identified a general stress protein (H26-G) which is a low-specificity L-threonine aldolase catalyzing the interconversion of serine and glycine, both of which are major sources of one-carbon units necessary for the synthesis of key compounds such as purine, thymidylate, and methionine (21). This protein is also induced upon exposure to 2 mM H2O2, by a shift from pH 7.2 to 5.5, and by heat shock (25).

Identification of heat shock proteins. Sixteen heat shock proteins were identified by trypsin digestion and matrix-assisted laser desorption ionization–time of flight measurements of the peptide spectra (Table 2, which also summarizes the regulation of the identified proteins). Among the identified proteins there are two forms of periplasmic mannitol binding protein, SmoM (H44 and H45). Interestingly, the heat shock induction of H44 is σ^32 independent and the induction of H45 is σ^32 dependent. Moreover, there is an additional SmoM protein that is not induced by heat shock.

The present study deals with expression of heat shock proteins, as seen on 2-D gels. We studied the heat shock proteomes of wild-type A. tumefaciens and strains carrying deletions in the genes encoding the known regulators of the heat shock response of this bacterium (i.e., RpoH and Hrca [16]). Less than half of the heat shock proteins are under the control of RpoH, and only one operon is under the control of Hrca, implying the existence of at least one unknown additional control element. The fraction of RpoH-independent heat shock proteins (32 out of 56) is much larger than expected, since in E. coli 38 heat shock proteins, out of 48, are regulated by heat shock-specific sigma factors (34). In E. coli an additional heat shock sigma factor, called σ^32 (encoded by rpoE), regulates the induction of periplasmic heat shock proteins (6, 22, 23, 36). A putative homologue of rpoE is present in the recently published genomes of some α-proteobacteria (A. tumefaciens, Sinorhizobium meliloti, and Mesorhizobium loti). Therefore, it is conceivable that some of the 32 RpoH-independent heat shock proteins in A. tumefaciens are transcribed by the σ^32 homologue. Sequence analysis of the genes coding for the RpoH-independent proteins did not identify a common promoter sequence or a specific σ^32 promoter, and this issue will be clarified when σ^32 mutants are available. Yet, the number of RpoH-independent heat shock genes is substantial, suggesting the existence of an additional alternative sigma factor or a transcriptional regulator that functions together with the vegetative transcription factor σ^70.

The results presented here illustrate the complexity of the regulation of the heat shock response in A. tumefaciens. It should be noted that we studied only one level of gene expression, the proteome, from which it is not possible to determine whether the regulation is at the level of transcription, posttranscription, or protein turnover. Indeed, one posttranscriptional control system was already identified in the heat shock response of A. tumefaciens (28). In addition to evidence for the existence of new control regulators, we also identified several heat shock genes that were not previously shown to be induced by shifts to higher temperature. The new heat shock proteins identified in the present study include SmoM (periplasmic mannitol binding protein), ketol acid reductoisomerase, PstB, a hypothetical transcriptional regulator with a molecular mass of 18.7 kDa, and ribosomal protein L7/L12, previously regarded as a cold shock protein. An additional new heat shock protein is the low-specificity L-threonine aldolase, which appears to be induced by other types of stress also (oxidative

<table>
<thead>
<tr>
<th>Strain</th>
<th>Genotype</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>GV3101</td>
<td>rpoH^- hrcA^-</td>
<td>10</td>
</tr>
<tr>
<td>KN501</td>
<td>GV3101 ΔrpoH::tetR hrcA^-</td>
<td>16</td>
</tr>
<tr>
<td>KN613</td>
<td>GV3101 rpoH^- hrcA^-</td>
<td>16</td>
</tr>
<tr>
<td>KN201</td>
<td>GV3101 ΔrpoH::tetR hrcA^-</td>
<td>16</td>
</tr>
</tbody>
</table>
FIG. 1. Dual-channel images of A. tumefaciens. Proteins were obtained from cultures of wild-type A. tumefaciens growing exponentially at 25°C (top gel) or heat shocked for 15 min at 42°C (middle gel) and from cultures of heat-shocked ∆rpoH mutants (bottom gel). The experiment was performed as described in the work of Rosen et al. (25). The silver-stained gels were scanned and computer stained in green (first channel). The gels were then dried and subjected to autoradiography. The autoradiograms obtained were computer stained in red (second channel). The two color channels were superimposed to give the dual-channel image: proteins that remained the same during heat shock show as yellow, proteins that were up-regulated during heat shock appear red, and these down-regulated during heat shock are green (2). All picture manipulations were performed using Adobe Photoshop. Spots numbered with H (H1, H2, etc.) are heat shock-specific proteins. These include the red spots in the middle panel that are missing in the top panel and spots that changed their color from yellow to red (e.g., H1 and H2). Spots designated H x-A are heat shock proteins also induced by mild acid stress, and spots designated H x-O are heat shock proteins also induced by H2O2 oxidative stress. H26-G is a protein induced by all three types of stress (25).
FIG. 2. Dual-channel images of proteins in an ΔrpoH ΔhrcA double mutant. Proteins were extracted from cultures of the A. tumefaciens ΔrpoH ΔhrcA strain growing exponentially (top gel) or heat shocked (bottom gel) as described in the Fig. 1 legend. NWH represents proteins that are induced during heat shock only in the mutant and not in the wild type.
stress and mild acidic stress). This is the only protein induced by all three types of stress (25) and could constitute part of a general stress response.

\( \text{A. tumefaciens} \) belongs to the \( \alpha \)-proteobacteria, a group that includes many types of bacteria whose physiology involves close bacterium-host relationships. These relationships involve symbiosis (as in the case of nitrogen-fixing \( \text{Rhizobiaceae} \) family members) or pathogenicity for plants (\( \text{A. tumefaciens} \) or \( \text{Bacteroidaceae} \) family members). Analysis of the control of the stress response in \( \text{A. tumefaciens} \) will further our understanding of the physiology of members of this group and their interaction with the environment. Furthermore, \( \text{A. tumefaciens} \) is closely related to some human pathogens which are difficult to cultivate (\( \text{Brucellaceae} \) and \( \text{Rickettsiaceae} \)) and for which molecular experiments are difficult to implement. Therefore, this experimental system offers possibilities for performing genetic

---

**FIG. 3. Expression patterns of proteins.** Levels of several proteins were monitored in the wild type, \( \Delta \text{rpoH} \) and \( \Delta \text{hrcA} \) mutants, and the \( \Delta \text{rpoH} \Delta \text{hrcA} \) double mutant during exponential growth (−) and under heat shock conditions (+). The expression is presented as spot percentage volume, as calculated with ImageMaster 2D Elite from a virtual gel, averaging several gels. As a typical non-heat shock protein, we chose the periplasmic binding protein (a); the typical RpoH-dependent heat shock protein is DnaK (H1) (b); the RpoH- and HrcA-dependent proteins are GroEL (H2) (c) and GroES (H51) (d), respectively; the typical RpoH-independent protein is PstB (H35) (e); and the general stress protein is H26-G (f). WT, wild type.
<table>
<thead>
<tr>
<th>Spot no.</th>
<th>Protein</th>
<th>Mol wt</th>
<th>pI</th>
<th>Matched peptide (m/z)</th>
<th>Regulator(s)</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>H1</td>
<td>DnaK</td>
<td>68,106.3</td>
<td>4.87</td>
<td>1,015.5992, 1,136.5508, 1,168.6049, 1,320.6810, 1,512.6548, 1,519.8212, 1,564.8047, 1,608.9074, 1,659.8809, 1,761.7504, 1,854.8913, 1,989.0032, 2,004.9997, 2,546.3035</td>
<td>RpoH</td>
<td>This study</td>
</tr>
<tr>
<td>H2</td>
<td>GroEL</td>
<td>57,547.4</td>
<td>5.04</td>
<td>914.6100, 1,000.5792, 1,259.6464, 1,327.6715, 1,344.6933, 1,360.6897, 1,373.7016, 1,499.7443, 1,514.7573, 1,606.9714, 1,759.9212, 1,775.9455, 1,853.8398, 2,020.9912, 2,088.1259, 2,275.2950, 3,397.7592</td>
<td>RpoH, HrcA</td>
<td>25</td>
</tr>
<tr>
<td>H9-O</td>
<td>ClpB</td>
<td>97,543.7</td>
<td>5.56</td>
<td>1,416.6388, 1,302.6890, 1,343.6445, 1,460.8005, 1,488.8022, 1,557.8037, 1,573.8026, 1,635.8794, 1,654.9022, 1,713.9059, 1,729.8989, 1,782.9952, 1,908.0147, 2,184.1529, 2,219.1377, 2,341.2645, 2,827.4029</td>
<td>RpoH</td>
<td>This study</td>
</tr>
<tr>
<td>H13</td>
<td>ClpB</td>
<td>97,543.7</td>
<td>5.56</td>
<td>1,014.6050, 1,141.6317, 1,302.6580, 1,343.6276, 1,488.7825, 1,557.7739, 1,635.7401, 1,654.8602, 1,713.8579, 1,782.9506, 1,907.9720, 2,184.1330, 2,219.0910, 2,341.2567, 2,827.4440</td>
<td>RpoH</td>
<td>This study</td>
</tr>
<tr>
<td>H15</td>
<td>HspD</td>
<td>17,918.3</td>
<td>5.44</td>
<td>1,133.5760, 1,841.9782, 1,968.9528, 2,175.3368</td>
<td>RpoH</td>
<td>This study</td>
</tr>
<tr>
<td>H16</td>
<td>HspD</td>
<td>17,918.3</td>
<td>5.44</td>
<td>1,055.5656, 1,071.5593, 1,115.5383, 1,133.5730, 1,420.7135, 1,436.7104, 1,685.8877, 1,841.9833, 1,968.9618, 1,984.9676, 2,753.3905</td>
<td>RpoH</td>
<td>This study</td>
</tr>
<tr>
<td>H19-A</td>
<td>Hypothetical transcriptional regulator</td>
<td>18,750.0</td>
<td>5.97</td>
<td>920.5519, 948.4223, 1,168.5723, 1,405.7803, 1,726.8976, 1,925.9580, 1,962.9384, 2,119.0387</td>
<td>RpoH</td>
<td>This study</td>
</tr>
<tr>
<td>H22-A</td>
<td>Ribosomal protein L7/L12</td>
<td>12,735.7</td>
<td>4.79</td>
<td>Identified by N-terminal sequencing</td>
<td>Unknown</td>
<td>25</td>
</tr>
<tr>
<td>H26-G</td>
<td>Low-specificity t-threonine aldolase</td>
<td>38,110.5</td>
<td>5.14</td>
<td>954.4624, 979.4919, 1,079.5684, 1,110.5565, 1,114.5920, 1,125.5338, 1,179.5926, 1,235.6749, 1,309.6209, 1,614.8120, 1,714.8618, 2,019.9065, 2,035.9184</td>
<td>Unknown</td>
<td>This study</td>
</tr>
<tr>
<td>H35</td>
<td>PstB</td>
<td>30,428.0</td>
<td>5.74</td>
<td>1,176.6386, 1,488.8141, 1,504.7740, 1,530.7893, 1,932.9748, 2,264.1320</td>
<td>Unknown</td>
<td>This study</td>
</tr>
<tr>
<td>H37</td>
<td>GrpE</td>
<td>22,830.6</td>
<td>4.66</td>
<td>936,4875, 955.5046, 1,032.5263, 1,133.5719, 1,140.7033, 1,156.6955, 1,188.6252, 1,204.6206, 1,254.7196, 1,289.6674</td>
<td>RpoH</td>
<td>This study</td>
</tr>
<tr>
<td>H43</td>
<td>Ketol acid reductoisomerase</td>
<td>36,710.4</td>
<td>6.02</td>
<td>919,4560, 935,4887, 1,143.6393, 1,248.6740, 1,302.5858, 1,378.5791, 1,597.8369, 2,177.1501, 2,226.0525, 2,693.3102</td>
<td>RpoH</td>
<td>This study</td>
</tr>
<tr>
<td>H44</td>
<td>SmoM</td>
<td>40,257.1</td>
<td>7.01</td>
<td>1,752.9705, 1,765.9097, 1,916.0265, 2,659.3562</td>
<td>Unknown</td>
<td>This study</td>
</tr>
<tr>
<td>H45</td>
<td>SmoM</td>
<td>40,215.3</td>
<td>7.60</td>
<td>1,703.9364, 1,968.0862, 2,197.0174, 2,212.9993, 2,229.0145, 2,665.2962</td>
<td>RpoH</td>
<td>This study</td>
</tr>
<tr>
<td>H51</td>
<td>GroES</td>
<td>10,493.9</td>
<td>5.14</td>
<td>Identified by N-terminal sequencing</td>
<td>RpoH, HrcA</td>
<td>25</td>
</tr>
</tbody>
</table>
and physiological manipulations that will help us to understand the molecular biology of these pathogens.

We thank Dvora Biran for valuable help and discussions. We thank Jean-François Tomb, Forrest G. Chumley, and Robert A. Larossa of DuPont for their permission to search unpublished databases.

We thank S. Colton and J. Colton for a generous scholarship (R.R.). This work was partially supported by an EMBO fellowship (R.R.) and the Manja and Morris Leigh Chair for Biophysics and Biotechnology (E.Z.R.).

REFERENCES


