Coping with High Temperature: A Unique Regulation in *A. tumefaciens*

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Contents

Abstract Elevation of temperature is a frequent and considerable stress for mesophilic bacteria. Therefore, several molecular mechanisms have evolved to cope with high temperature. We have been studying the response of *Agrobacterium tumefaciens* to temperature stress, focusing on two aspects: the heat-shock response and the temperature-dependent regulation of methionine biosynthesis. The results indicate that the molecular mechanisms involved in *A. tumefaciens* control of growth at high temperature are unique and we are still missing important information essential for understanding how these bacteria cope with temperature stress.

1 Introduction

Most bacteria are exposed to frequent changes in the environment, especially changes in temperature. Exposure to elevated temperatures results in major physiological changes, including decreased activity of enzymes due to structural changes of the proteins. Therefore, immediate and precise adaptation to temperature changes is essential. The reaction to elevated temperatures involves two different processes—one is a quick response to the change, a rescue response aimed at repairing damaged processes and preventing further injury. The second process

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involves adaptation to the new temperature by regulating the biochemical and physiological processes for maintaining balanced growth at the new temperature. In the two processes, the level of response to the elevated temperature is proportional to the severity of the change.

The adaptation to the change in temperature is regulated by a global regulatory network that controls the simultaneous expression of a large number of stress-related genes—the heat-shock response (Arsene et al. 2000; Craig 1985; Ghazaei 2017; Guan et al. 2017; Lindquist 1986; Mathew and Morimoto 1998; Mathew et al. 2000; Ron 2009; Schumann 2016; Segal and Ron 1998; Yura and Nakahigashi 1999).

The first attempts to study the temperature-dependent regulatory networks were performed in *Escherichia coli* using O'Farrell two-dimensional gels to compare proteomes in bacteria exposed to elevated temperatures. These experiments resulted in the identification of a large group of proteins, the heat-shock proteins, which are induced following the increase in temperature (Neidhardt et al. 1981; O'Farrell 1975). These data indicated the existence of global regulatory systems that control the expression of a large regulon, the heat-shock regulon. This regulated heat-shock response protects against the increase in temperature and provides thermo-tolerance as well as tolerance to additional stress conditions (Inbar and Ron 1993; Ramsay 1988).

The heat-shock response is the first discovered global regulatory system (Craig 1985), and is found in all living cells examined: bacteria, yeast, insects (*Drosophila melanogaster*) (Michaud et al. 1997), worms (*Caenorhabditis elegans*) (Rose and Rankin 2001), and mammals (Christians et al. 2002; Li et al. 2002; Srivastava 2002). The heat-shock response is characterized by the induction of a large set of proteins (heat-shock proteins; HSPs) as a result of a rapid increase in the environmental temperature. The proteins involved in the response to elevated temperature include mostly components of protein quality control, such as chaperons (e.g., GroEL, GroES, DnaK, and DnaJ) and ATP-dependent proteases (e.g., ClpP, Lon (La) and HslVU). Although many of the HSPs are highly conserved, their regulatory elements differ considerably between organisms, as well as the conditions under which the response systems are activated. Clearly, mesophiles respond to much lower temperatures than the thermophiles.

The complementary process, maintenance of balanced growth as a function of temperature, was studied in *Enterobacteriaceae* (gamma proteobacteria) and in *Agrobacterium tumefaciens* (alpha proteobacterium). These findings indicate that the maintenance of balanced growth at elevated temperatures is regulated by the availability of methionine (Biran et al. 1995; Gur et al. 2002; Ron 1975; Ron et al. 1990; Ron and Davis 1971; Ron and Shani 1971; Rotem et al. 2013).

Here, we discuss the two response mechanisms to elevated temperature, maintaining balanced growth and the heat-shock response, in *A. tumefaciens*.

Growth at elevated temperature is limited by the availability of methionine

When grown in minimal medium (Chilton et al. 1974), growth of *A. tumefaciens* is severely inhibited at 39 °C (Fig. 1a). However, the inhibition is relieved by the

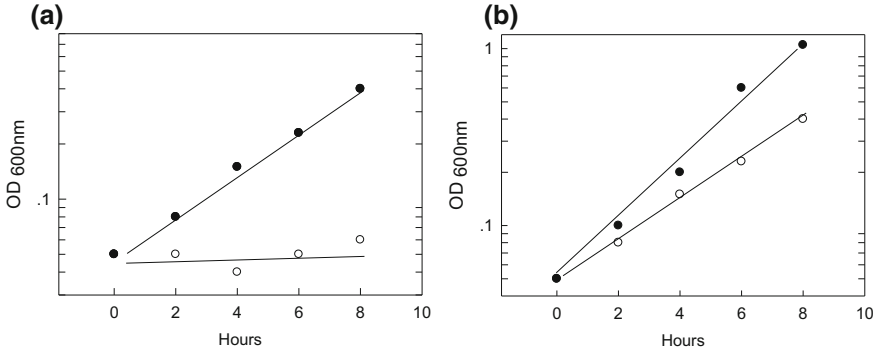

Fig. 1 Growth of *A. tumefaciens* at elevated temperatures. Bacteria were grown in minimal medium (Chilton et al. 1974) **a** or LB broth (Difco) **b** at 30 °C (filled circles) or 39 °C (empty circles). Growth was monitored by turbidity at OD 600 nm

addition of nutrients found in LB medium (Difco) (Fig. 1b). These results are similar to results obtained with bacteria belonging to the *Enterobacteriaceae,* where growth at elevated temperatures is limited by the availability of methionine (Ron 1975). *E. coli*, for example, depends on exogenous methionine for growth above 43 °C because the activity of the first enzyme in the methionine biosynthetic pathway, homoserine trans-succinylase, is extremely temperature-sensitive (Gur et al. 2002).

The first step in methionine biosynthesis is the activation of homoserine. In *E. coli*, this activation uses succinyl-coA. However, in *A. tumefaciens*, the first enzyme for methionine synthesis is not a trans-succinylase, but a trans-acetylase (acetyl transferase), using acetyl-CoA for the activation of homoserine (Rotem et al. 2013). *A. tumefaciens* MetA has severely reduced activity at 39 °C, a temperature at which it undergoes proteolysis. Proteolysis of the first enzyme in the methionine biosynthetic pathway was also shown in *E. coli* (Biran et al. 2000; Gur et al. 2011; Katz et al. 2009). It therefore appears that *A. tumefaciens* maintains balanced growth at elevated temperatures by regulating the availability and activity of the MetA protein, homoserine acetyl transferase.

The similarity of this regulatory mechanism between *A. tumefaciens* and the *Enterobacteriaceae* is not trivial, as they are phylogenetically quite distant (α and γ proteobacteria, respectively) and the enzymes have different substrates and temperature-dependent activity. It was also unexpected that a biosynthetic enzyme is not stable but has a short half-life. These findings can therefore be explained by assuming that regulation of growth rate as a function of elevated temperature is a critical necessity for mesophilic bacteria, and that such regulation is best performed via the availability of methionine. Methionine is a key biochemical compound because, in addition to its structural role as a component of proteins, it is involved in the initiation of protein synthesis and in many other biochemical processes such as the biosynthesis of S-adenosyl-methionine, purines, pyrimidines, fatty acids, and polyamines.

2 The Heat-Shock Response

The heat-shock response involves the induction of a large group of proteins, the heat-shock proteins, by an increase in temperature. The heat-shock response is a universal regulatory network involving highly conserved heat-shock proteins. These include the chaperones Hsp60 (GroEL) and Hsp70 (DnaK), and ATP-dependent proteases. Although the heat-shock proteins are highly conserved, the regulatory mechanisms responsible for the induction of these proteins are variable. The best studied regulation of the heat-shock response is in *E. coli* where the heat-shock genes are transcribed by a special sigma factor, σ32, from specific promoters (Fig. 2) (Erickson et al. 1987; Grossman et al. 1984; Taylor et al. 1984; Yura et al. 1990; Zhou et al. 1988). The transcriptional activator σ32 is unstable at low temperatures, as it is degraded by a specific protease FtsH (HflB), but it is stabilized as the temperature increases (Herman et al. 1995; Shenhar et al. 2009; Tomoyasu et al. 1995). This mechanism ensures the selective transcription of heat-shock promoters only at elevated temperatures. However, the induced transcription of the heat-shock operon is transient, and after an initial burst, its rate eventually returns to a basal level typical for the new temperature. This decrease in transcription of heat-shock genes is explained by the "titration model" which assumes that the level of HSPs is regulated in correlation with the amount of their substrates, unfolded proteins (Guisbert et al. 2004; Straus et al. 1990; Tomoyasu et al. 1998).

In *Bacillus subtilis*, there is no heat-shock-specific sigma factor and the heat-shock genes use regular σ70 promoters. However, upstream of the promoter of heat-shock genes there is an inverted repeat, CIRCE (Conserved Inverted Repeat Control Element), which binds the repressor HrcA. At elevated temperatures, the

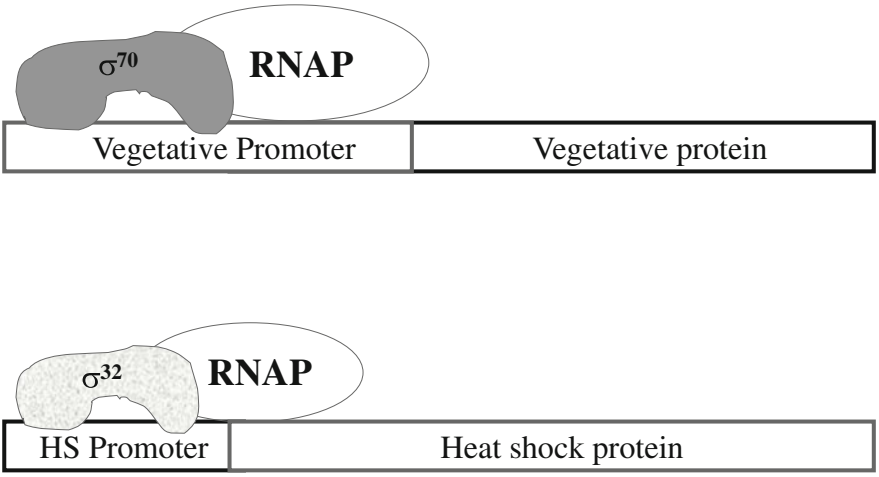

Fig. 2 The *E. coli* Heat-Shock Response. RNA polymerase (RNAP) binds with σ70 to promoters of vegetative genes (top) and with σ32 to promoters of heat-shock genes (bottom)

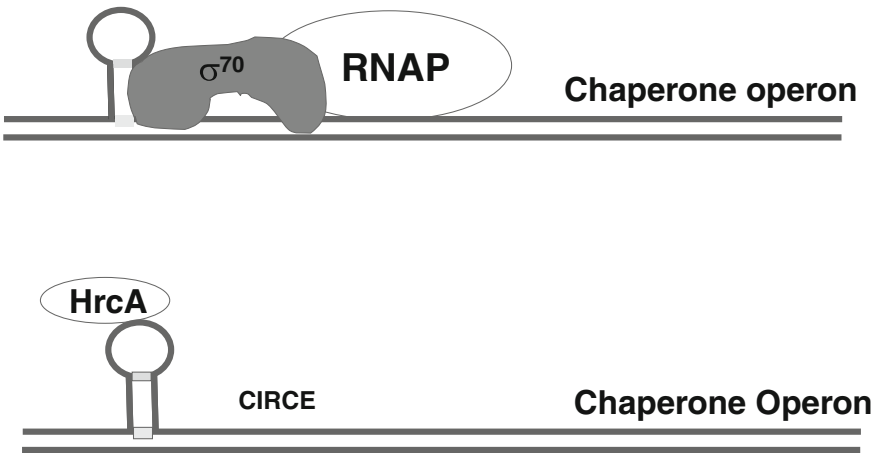

Fig. 3 The *B. subtilis* Heat-Shock Response.RNA polymerase (RNAP) binds with σ70 to promoters of heat-shock chaperone genes at elevated temperatures (top) but not at low temperatures when transcription is inhibited by a CIRCE element repressed by an HcrA repressor (bottom)

repressor is released, thus enabling transcription (Fig. 3) (Hecker et al. 1996; Schumann 2003).

Proteomic studies of *A. tumefaciens* (Rosen and Ron 2011) indicated that there are at least 40 heat-shock-induced proteins, 16 of which are also induced by other stress conditions. These include the set of heat-shock proteins which are conserved in bacteria, such as the Lon and Clp proteases and the DnaK and GroESL chaperones (Boshoff et al. 2008; Rosen et al. 2001, 2002; Rosen and Ron 2002). At least two of the heat-shock proteins, Lon and HspL, are important for virulence (Hwang et al. 2015; Su et al. 2006; Tsai et al. 2009, 2010, 2012). The regulation of the heat-shock response in *A. tumefaciens* is interesting, as there is a combination of the two regulatory systems (Segal and Ron 1996b). The *groESL* operon, encoding Hsp60 and Hsp10, is regulated by the CIRCE inverted repeat and the HrcA protein (Segal and Ron 1993, 1995b, 1996a). However, there is also a heat-shock-specific sigma factor which recognizes a specific heat-shock promoter upstream of heat-shock genes such as *dnaK* (Fig. 4) (Nakahigashi et al. 1998, 1999; Segal and Ron 1995a). These promoters are not similar to the heat-shock promoters of *E. coli*, in contrast to the σ70 promoters which are highly conserved (Fig. 5) (Segal and Ron 1995a). It is interesting to note that *metA* expression is regulated by a σ32 promoter and an S-adenosyl-methionine riboswitch and is poorly transcribed in mutants deleted for σ32 (Rotem et al. 2013). This transcription control directly links the heat-shock response and the growth balancing process under elevated temperature, probably facilitating propagation only after physiological adaptation has been attained.

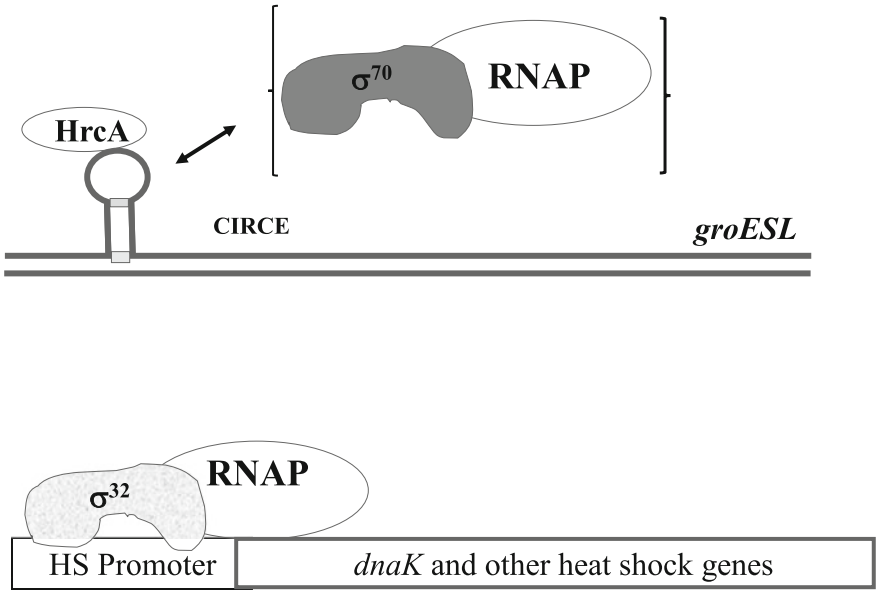

Fig. 4 Heat-Shock Response in *A. tumefaciens*. Transcription of the *groESL* chaperon is regulated by a CIRCE-HcrA element (top) while transcription of other heat-shock genes, such as *dnaK*, is regulated by RNAP bound to σ32 (bottom)

Consensus promoter σ70	TTGACA	‹17›	TATAAT
A. tumefaciens σ32 promoter	CTTG	‹17/18›	CYTAT-T—G
E. coli σ32 promoter	TCTC-CCCTTGAA	‹13/14›	CCCCAT-AT

Fig. 5 Consensus promoters. The σ70 promoter DNA sequence is conserved in bacteria (*E. coli, B. subtilis*, and *A. tumefaciens*) whereas the heat-shock promoters of *E. coli* and *A. tumefaciens* are different

Thus, we already know the basic facts, but there are still numerous unanswered questions:

1. HrcA-CIRCE regulation involves release of the repressor as the temperature increases. However, the heat-shock response is gradual and gets stronger with an increase in temperature. The release of a repressor is an on-off event. So how can the strength of the response depend on the increase of temperature? What are the quantitative aspects of HrcA-CIRCE concentrations as a function of temperature? How is the cellular concentration of HrcA regulated?
2. In *E. coli* σ32 has a stronger affinity for the RNA polymerase than does σ70. However, σ32 is an unstable protein which is stabilized at high temperatures. This observation explains how heat-shock genes are transcribed preferentially upon temperature elevation. However, the σ32 of *A. tumefaciens* is stable at low temperatures (Nakahigashi et al. 1995, 1999), so how can we explain the fact that it transcribes the heat-shock genes only when the temperature is elevated?

Are there additional regulatory elements involved, such as temperature-dependent anti-sigma factors?

3. Is there an evolutionary advantage to having two independent heat-shock control systems, the HcrA-CIRCE and the σ32 (Nakahigashi et al. 1999)?
4. Last but not least: a large fraction of the heat-shock proteins are still induced at high temperatures in mutants deleted for σ32 (Rosen et al. 2002). This finding means that there are additional factors that regulate the heat-shock response in *A. tumefaciens*. One can think of additional sigma factors, yet to be discovered. Alternatively, there may exist posttranscriptional mechanisms which stabilize the transcripts of heat-shock genes. For example, the transcript of *yedU* (*hchA*) in *E. coli* is stabilized at elevated temperatures, and there are RNA chaperones which stabilize transcripts of stress genes during the stress (Mujacic and Baneyx 2006; Rasouly et al. 2007).

3 Conclusions

The heat-shock response is a regulatory network which is critical for the maintenance of life at elevated temperatures. Coping with elevated temperatures involves regulatory mechanisms that control the growth rate as a function of temperature. In many Gram-negative bacteria, including *A. tumefaciens*, regulation of the growth rate is achieved by regulating the availability of methionine via temperature-dependent changes in the first biosynthetic enzyme. In addition to maintenance of regulated growth rate as a function of temperature, there is also a response to the change, the heat-shock response. This response involves the induction of many chaperones and proteases which consist of protein quality control and assure the availability of functional proteins during the change in temperature. *A. tumefaciens* has evolved a complicated control system for the heat-shock response, which includes repressor binding to conserved stem-loop structures upstream of heat-shock genes, as well as a specialized sigma factor which activates specific promoters of heat-shock genes. On top of these two regulatory systems, there are likely additional control systems which are still undiscovered, that are responsible for the temperature-dependent induction of about 20 genes, which are not controlled by either σ32 or the CIRCE-HrcA apparatus.

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