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CHAPTER 1.32

Bacterial Stress Response

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Introduction

Most bacteria live in a dynamic environment where temperature, availability of nutrients, and presence of various chemicals vary. Quick adaptation to these environmental changes is carried out by a series of global regulatory networks that control the simultaneous expression of a large number of genes. There are global regulatory systems that respond to change of temperature, pH, nutrients, salts and oxidation. The level of response by these regulatory networks is proportional to the extent of the change. Since the response level is highest under changes that constitute a stress condition, the control networks are labeled "stress response" systems.

The stress response systems show a high degree of similarity in prokaryotes, and some (e.g., the heat shock response) are also conserved in eukaryotes and archaea. However, the conditions under which the response systems are activated differ significantly from one organism to another. Clearly, the temperatures in which the heat shock response is activated will be much lower for a mesophile than for a thermophile, or the response to salt stress will be completely different in halophiles.

Global Regulatory Networks in Bacteria

The first attempts to study the extent of such regulatory networks were based on proteomic analysis, using O'Farrell two-dimensional (2D) gels, and resulted in the identification of the large group of *Escherichia coli* heat-shock proteins (O'Farrell, 1975; Neidhardt et al., 1981). Later, proteomic-based experiments followed by microarray studies of gene transcription (Hatfield et al., 2003) revealed the size and composition of the various-stress induced stimulons of *E. coli* (VanBogelen et al., 1987b). This induction of large groups of genes in response to a specific environment suggested the existence of global regulatory systems that control the expression of large regulons.

Gene expression can be regulated at the level of transcription or posttranscription. The level of transcription can be regulated by positive control elements—activators—or by negative control elements—repressors. Some of these control elements are specific for one gene, whereas others control a large group of genes, thus creating a regulon. In addition to transcriptional regulation, many posttranscriptional regulatory systems evolved affecting different steps along the way from the gene to the active protein. The posttranscriptional regulatory systems control the stability of the mRNA and the rate of translation initiation. In addition, they can determine the stability of the protein and its activity by carrying out posttranslational modifications. The existence of all of the control elements described here was demonstrated in the global regulatory systems that control the response to heat shock and other environmental and physiological conditions.

Transcriptional regulation is the primary mechanism that regulates gene expression. The process of RNA synthesis and its control was extensively studied in bacteria, especially in *E. coli* and *Bacillus subtilis* (Burgess and Anthony, 2001). The *E. coli* DNA-dependent RNA polymerase is the enzyme responsible for all cellular RNA synthesis. This enzyme consists of a core (subunits $a_2bb'w$) that is capable of elongation and termination of transcription, and an additional subunit (σ), which binds to the RNA polymerase to form the holoenzyme, increases the efficiency of transcription initiation, and determines specific promoter recognition (Burgess et al., 1969). In *E. coli* there are seven known sigma factors: σ^{70} and the vegetative sigma factors, σ^{S}, σ^{32}, σ^{F}, σ^{E}, σ*fecI* and σ^{54} (Helmann and Chamberlin, 1988; Lonetto et al., 1992; Burgess and Anthony, 2001). The sigma factors serve as master regulators mainly by competition for the core RNA polymerase, which is the limiting component of the transcription machinery (Ishihama, 2000). Additional regulation of transcription is exerted by repressors, transcriptional activators, sigma-binding anti-sigma factors, and even by small RNAs (Hughes and Mathee, 1998; Helmann, 1999; Vicente et al.,

1999; Ishihama, 2000; Severinov, 2000; Wassarman and Storz, 2000).

These various control elements regulate the expression of genes during environmental conditions such as starvation, sporulation and additional stress conditions. For example, the *E. coli* stationary phase is regulated by the master regulator σ^S (Lange and Hengge-Aronis, 1991). The levels of σ^S itself are affected by *cis* and *trans* elements—small molecules such as guanosine 5′-diphosphate 3′-diphosphate (ppGpp) and homoserine lactone, and the proteins that react to them, such as cAMP receptor protein (CRP)-cAMP (Hengge-Aronis, 2000). Sigma factor S regulates the induction of more than 50 genes (Hengge-Aronis, 2000). All of these elements create a complex regulatory network that enables the bacterial cell to adapt to the changing environment.

Stress Response, Stimulons and Regulons

In bacteria the stress responses are regulated by several control patterns: 1) Transcriptional control by alternative sigma factors is the most prevalent control pattern. Basically, genes or operons that belong to a specific response regulon contain a promoter that is recognized by a specific, alternative, sigma factor. The function of this sigma factor correlates with the conditions that bring about the response. As an example, in Gram-negative bacteria, the response to elevated temperatures is mediated by two alternative sigma factors (σ32 and σE) whose activities are temperature-dependent. 2) Transcription is controlled by repressor binding to a DNA control element. An example of this control is the HrcA repressor that binds to a conserved inverted repeat control element known as "CIRCE" (for "controlling inverted repeat of chaperone expression") present upstream of operons that code for heat shock proteins in Gram-positive and some Gram-negative bacteria. 3) Transcription is controlled by proteolysis. Well defined is the salt overly sensitive (SOS) response to genotoxic effects, which is mediated by a series of autoregulated proteases. Recently, control by proteolysis has emerged as one of the major systems regulating the availability of alternative sigma factors and other stress-related global processes (Hengge and Bukau, 2003). And 4) transcription is controlled by small RNAs. Recent findings indicate that small RNAs, about 50 of which are present in the *Escherichia coli* genome, control the cellular concentration of RpoS (sigma38), the alternative sigma factor of the starvation (or stationary) response. Small RNAs also control the response to oxidative stress.

A regulon is defined as all genes regulated by the same control pattern, while a stimulon is defined as all the genes whose expression responds to the same conditions. Stimulons are easily delineated by monitoring gene expression in a micro-array or on two-dimensional protein gels. Regulons can only be established following characterization of the molecular basis for the change in gene expression. Clearly, level of overlap between the various regulons and stimulons is high. Thus, the stimulon that responds to shifts to higher temperatures contains genes from at least two regulons (i.e., σ32 and σE). Yet, some of the genes of the σ32 regulon may also be controlled by the HrcA repressor, and so on.

Here, the focus is on two stress response networks—one responding to shifts to higher temperatures (heat shock response) and the other to limitation of carbon source and stationary phase (general stress response).

The Heat Shock Response

The heat shock response was the first global regulatory system to be discovered and is one of the most fundamental. This response is general, found in all living cells examined (Craig, 1985), and is a protective and homeostatic cellular process that increases thermotolerance. It has been studied in many cellular systems such as 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. Many of the HSPs are molecular chaperones (e.g., GroEL, GroES, DnaK and DnaJ) and ATP-dependent proteases (e.g., ClpP, Lon (La) and HslVU) that play a critical role in the restoration of protein folding and in protein degradation under normal and stress conditions. Proteins such as GroEL (the bacterial homolog of Hsp 60) and DnaK (the bacterial homolog of Hsp 70) are highly conserved in evolution all the way from bacteria to humans (Boorstein et al., 1994; Gupta, 1995). Although the major proteins in the heat shock response are highly conserved, the regulation of the response varies between different organisms and different bacterial species. Several regulatory systems evolved in bacteria and will be discussed here.

The Hsps are important for protection against environmental stress, and they produce tolerance against high temperature, high salt and

Table 1. Major heat-shock proteins of *Escherichia coli*.

Protein	Function	Molecular weight (kDa)	Theoretical pI	Reference(s)
ClpB	Chaperone	96	5.37	Kitagawa et al., 1991
DnaJ	Chaperone	39	7.98	Bardwell et al., 1986
DnaK	Chaperone	69	4.83	Bardwell and Craig, 1984
GroEL	Chaperone	57	4.85	Neidhardt et al., 1981
GroES	Chaperone	10	5.15	Tilly et al., 1983b
HslR (Hsp 15)	Chaperone	15	9.94	Chuang and Blattner, 1993
Hsp33 (HslO)	Chaperone	33	4.65	Chuang and Blattner, 1993
HtpG	Chaperone	71	5.09	Bardwell and Craig, 1987
IbpA (HtpN, HslT)	Chaperone	16	5.57	Allen et al., 1992
IbpB (HtpE, HslS)	Chaperone	16	5.19	Allen et al., 1992
ClpP	Protease	24	5.52	Maurizi et al., 1990
ClpX	Protease	46	5.24	Gottesman et al., 1984
DegP (HtrA)	Protease	50	8.65	Lipinska et al., 1988
FtsH (HflB)	Protease	71	8.91	Ishihama, 2000
HslU (Clp Y, HtpI)	Protease	49	5.24	Chuang et al., 1993
HslV (Clp Q, HtpO)	Protease	19	5.96	Chuang et al., 1993
Lon (La)	Protease	87	6.01	Gayda et al., 1985
σ^{32} (RpoH, HtpR, Hin, Fam)	Sigma factor	32	5.64	Landick et al., 1991
σ^{20} (RpoD, Alt)	Sigma factor	70	4.69	Burton et al., 1981
σ^{E} (σ^{24}, RpoE)	Sigma factor	22	5.38	Raina et al., 1995
PrpA (PphA)	Phosphatase	25	6.94	Morita et al., 2000
Htp X	Unknown	32	6.60	Kornitzer et al., 1991
Htp Y (HtgA)	Unknown	21	9.44	Missiakas and Raina, 1997
HtrC	Unknown	21	9.33	Raina and Georgopoulos, 1990
PspA	Unknown	25	5.39	Jovanovic et al., 1996
FtsJ	Unknown	23	9.44	Herman et al., 1995

heavy metals (VanBogelen et al., 1987a; Inbar et al., 1993; Hecker and Volker, 1998). Heat-shock proteins also play critical roles in bacterial virulence and in protective systems such as the human immune system (Christians et al., 2002; Li et al., 2002). Several Hsps were found to protect against damage induced by temperature upshifts. Among the characterized proteins are the main cellular chaperone machineries GroE and DnaK, the ATP-dependent proteases Lon (La), HslVU, ClpP, DegP and FtsH (FhlB), and other proteins involved in protein folding, refolding, quality control, and degradation. GroE and DnaK are both multimeric complexes that have ATP-dependent activity (Sherman and Goldberg, 1992; Sherman and Goldberg, 1996; Kandror et al., 1994). The GroE catalytic complex involves GroEL and GroES in a ratio of 1 : 2, creating a football-shape molecular structure (Sparrer et al., 1997). This complex catalyzes protein refolding, and is involved in protein degradation by the ATP-dependent proteases (Sherman and Goldberg, 1992; Sherman and Goldberg, 1996; Kandror et al., 1994). These ATP-dependent proteases degrade abnormal proteins under stress and nonstress conditions, and in addition play major regulatory functions by controlling the degradation of specific proteins (Goldberg, 1972; Maurizi, 1992; Gottesman, 1996; Deuerling et al., 1997; Zhou et al., 2001). The role of these and other *E. coli* Hsps in protection against temperature-induced damage is summarized in Table 1.

Heat shock—a rapid up-shift in the environmental temperature—results in various physical and chemical changes in bacterial proteins and membranes. Presumably, these changes, such as protein unfolding, are detected by cellular systems, which induce the large set of heat shock proteins to cope with the changes and the potential damage. This heat shock response is regulated by several control elements, thus dividing the major stimulon of heat shock proteins into several regulatory groups (regulons).

The heat shock proteins are highly conserved, whereas the control of their expression is highly variable between organisms and even between various bacteria. One of the control elements found in Gram-negative bacteria is a heat shock σ factor that regulates the transcription of the major Hsps. The Gram-negative *E. coli* is a good example of this system because the synthesis of the major Hsps is regulated by the alternative sigma factor called "σ^{32}." In addition, there is a group of proteins induced under conditions of elevated temperature that is regulated by another heat shock sigma factor, σ^{E} (encoded by *rpoE*). In other Gram-negative bacteria, such as the *Agrobacterium tumefaciens* of the Alphaproteobacteria, the control systems are more

complicated. For example, the transcription of GroESL synthesis is stimulated during heat shock by a σ^{32}-like activator, but in non-heat shock conditions, transcription is repressed by the HrcA protein that binds to the CIRCE sequence upstream of the promoter region (Segal and Ron, 1993; Nakahigashi et al., 1999). The control system of HrcA-CIRCE was first described in the Gram-positive *Bacillus subtilis* (Zuber and Schumann, 1994).

The following sections will describe the specific control mechanisms in various bacterial groups. In short, the heat shock response in bacteria is controlled by one or a combination of both of the following control systems: 1) The first system involves alternative sigma factors that act as transcriptional activators by recognizing specific heat shock promoters upstream of heat shock genes. Among these are σ^{32} and σ^{E} of the Gram-negative bacteria, and σ^{B} of the Gram-positive bacteria. 2) The second system utilizes transcriptional repressors. The most conserved and the most ubiquitous among these repressors is HrcA (heat regulation at CIRCE), which binds to a conserved CIRCE present upstream of the heat shock operons. Heat shock operons controlled by HrcA-CIRCE are transcribed by the vegetative sigma factor σ^{A} (= σ^{70}) in Gram-positive bacteria and by the heat shock sigma factor s^{32} in Gram-negative bacteria.

Heat Shock Control Elements in Gram-Negative Bacteria

The first model organism for studying the heat shock response in Gram-negative bacteria was *E. coli*. Most of the heat shock genes of this bacterium are regulated by transcriptional activators, the alternative sigma factors (s^{32} or s^{E}).

Sigma-32-Controlled Genes

The heat shock response of Gram-negative bacteria is regulated mainly by the alternative sigma factors σ^{32} and σ^{E} (Morita et al., 2000). Sigma 32 is a master regulator encoded by the *rpoH* (*htpR* or *hin*) gene that was the first of a group of minor sigma factors discovered in *E. coli* (Grossman et al., 1984; Landick et al., 1984; Yura et al., 1984). This discovery of minor sigma factors led to the general concept of gene regulation by specific sigma factor-dependent transcription. In *E. coli*, at least 34 heat shock genes (out of 51 heat-shock induced loci) are regulated by σ^{32} (Richmond et al., 1999; Morita et al., 2000). The genes were identified by transcription analysis of specific genes, an examination of the synthesis rates of individual proteins, or proteomics and genomics approaches. This regulon includes all the major cytoplasmic Hsps of *E. coli*.

The σ^{32} regulon includes most of the proteins involved in protein folding, repair, and degradation. Such proteins are the heat-shock-induced molecular chaperones ClpB, DnaK, DnaJ, GroEL and GroES, which are involved in protein folding and prevention of protein aggregation (Neidhardt et al., 1981; Tilly et al., 1983b; Bardwell and Craig, 1984; Bardwell and Craig, 1986; Kitagawa et al., 1991; Tomoyasu et al., 2001). The regulon comprises also all of the important cytosolic proteases Lon (La), ClpP, ClpX, HslV (ClpY), and HslU (ClpQ; Goldberg, 1972; Gayda et al., 1985; Maurizi et al., 1990; Chuang et al., 1993b), and the membranal metaloprotease FtsH (HflB; Herman et al., 1995; Tomoyasu et al., 1995). Other important σ^{32}-regulated proteins are HTS (homoserine transsuccinylase), which is a key enzyme in methionine biosynthesis (Biran et al., 1995), proteins involved in protein isomerization (PpiD; Dartigalongue et al., 1998) and HtrM (Raina and Georgopoulos. 1991), and the vegetative sigma factor (σ^{70-}; Burton et al., 1981).

Homologs of *rpoH* were identified in more than twenty species of eubacteria from the alpha, beta and gamma subgroups of proteobacteria (Sahu et al., 1997; Andersson et al., 1998; Emetz et al., 1998; Huang et al., 1998; Karls et al., 1998; Nakahigashi et al., 1998; Nakahigashi et al., 1999; Nakahigashi et al., 2001). In some of these bacteria, the *rpoH* homologs demonstrates translational induction and stabilization upon heat shock, which are very similar to those found in *E. coli* (Nakahigashi et al., 1998).

The general function of the σ^{32} regulon was studied in several bacterial species by analysis of *rpoH* mutants. These mutants were usually found to be temperature sensitive (Zhou et al., 1988; Huang et al., 1998; Nakahigashi et al., 1999). As expected from their temperature-sensitive phenotype, some of the heat-shock proteins are essential at elevated temperature.

The levels of σ^{32} and its activity are temperature-regulated at several levels. At low temperature (30°C), when low amounts of heat-shock proteins are required, the intracellular concentration of σ^{32} is fewer than 50 molecules per cell (Straus et al., 1987; Craig et al., 1991). These low levels are maintained due to transcriptional repression and protein instability. Upon a rapid shift to 42°C, the level increases 15–20-fold within 5 min, and then declines to a new steady state level, 2–3-fold higher than the pre-shift level (Straus et al., 1987). The levels and the time-course of σ^{32} induction are sufficient for the necessary induction of heat-shock-gene expression upon temperature upshift. A relatively modest heat shock activates the translation of *rpoH* transcripts, and transiently stabilizes σ^{32} (Straus et al., 1987; Nagai et al., 1991), whereas a severe

heat shock (a rapid shift from 30 to 50°C) can also activate *rpoH* transcription (Morita et al., 2000). The decrease in the synthesis of heat-shock proteins upon temperature downshift is primarily a result of the decrease in σ^{32} activity (rather than its levels) caused mainly by an excess of the DnaK chaperone machinery (Straus et al., 1989; Taura et al., 1989).

The transcriptional regulation of the *rpoH* gene is very complex. It can be transcribed from at least four promoters, three of them (P1, P4 and P5) are recognized by the vegetative σ^{70}, and the fourth (P3) is recognized by σ^{E} (Erickson et al., 1987; Nagai et al., 1990). P3- and P4-transcription of *rpoH* is negatively regulated by DnaA (Wang and Kaguni, 1989), and P4-and P5-transcription is controlled by an additional negative control system—the cAMP-CRP/CytR nucleoprotein complex (Kallipolitis et al., 1998).

Several findings indicate that the heat shock induced σ^{32} levels are also regulated at the translational level. Expression of *rpoH-lacZ* translational fusion but not transcriptional fusion can be induced. Furthermore, heat induction of the fusion protein occurs even when RNA synthesis is inhibited (Nagai et al., 1991). Recent results based on extensive in vivo and in vitro experiments related to the secondary RNA structure have shown that the translation regulation of RpoH is mediated by the rpoH mRNA's secondary structure (Morita et al., 1999; Morita et al., 2000).

Sigma-32 level is regulated by not only its expression level but also the turnover of the protein. Although this protein is unstable during normal growth at 30°C (or even at 42°C), significant stabilization occurs immediately upon temperature upshift from 30°C to 42°C and continues for 4–5 minutes (Straus et al., 1987). The protein instability involves the DnaK chaperone machinery. Mutants in DnaK, DnaJ, or GrpE markedly stabilize σ^{32} under nonstress conditions (Tilly et al., 1983a; Tilly et al., 1989; Straus et al., 1990), indicating this involvement of these proteins in σ^{32} turnover. The initial studies suggested that the membrane-associated metalloprotease FtsH (HflB) is responsible for σ^{32} degradation (Herman et al., 1995; Tomoyasu et al., 1995). However, later studies were able to show that the cytosolic proteases Lon (La), HslVU and ClpP are also involved in σ^{32} degradation (Wawrzynow et al., 1995; Kanemori et al., 1997; Kanemori et al., 1999; Morita et al., 2000). Although the relative significance of each protease is difficult to determine in σ^{32} degradation, the latter three proteases seem to play a significant role in the degradation, possibly even equivalent to that of FtsH (Kanemori et al., 1997). Presumably during heat shock the DnaK machinery and the proteases become occupied by the misfolded and unfolded proteins that accumulate because of the denaturing effect of temperature increase. Consequently, levels of the proteolytic machinery are insufficient to bring about σ^{32} degradation and it accumulates and activates the transcription of the heat shock genes. Since the DnaK chaperones and the proteases have σ^{32} promoters, their synthesis is increased and therefore a few minutes after the temperature upshift, the level of the proteases and chaperones is high enough to destabilize σ^{32}, bringing the level of the heat shock proteins to a new steady state.

The final level of σ^{32} regulation is activity regulation (Morita et al., 2000). This regulation operates mainly by creating ternary complexes of (DnaK-ADP)-DnaJ-σ^{32} that sequester the σ^{32} that competes with the RNA polymerase core enzyme (Gamer et al., 1992; Gamer et al., 1996; Liberek et al., 1992; Liberek and Georgopoulos, 1993). Then, GrpE binds to the ternary complex and stimulates ADP release and complex dissociation by triphosphate (TP) binding. This cycle of binding and release appears to play an important role in σ^{32} activity (and possibly stability) in vivo (Gamer et al., 1992; Morita et al., 2000).

The σ^{32} control system has been well characterized in *E. coli* and other Gammaprotebacteria. However, σ^{32}-like heat shock transcriptional activators have recently been demonstrated in other bacteria, such as *Agrobacterium tumefaciens* of the Alphaprotebacteria (Nakahigashi et al., 1995; Nakahigashi et al., 1998; Nakahigashi et al., 1999; Segal and Ron, 1995a). The σ^{32} of the Alphaprotebacteria is different from that of *E. coli*, and the heat shock promoters are also different in the two groups of Gram-negative bacteria (Nakahigashi et al., 1999; Segal and Ron, 1995a; Fig. 1). The physiological difference of the two sigma factors may be more important: while the *E. coli* σ^{32} is unstable and tightly controlled by proteolysis carried out by the FtsH protease, the alphaprotebacterial σ^{32} is a stable protein, whose activity is affected mainly by a DnaK-mediated control (Nakahigashi et al., 2001).

Genes Controlled by σ^{E}

Another alternative sigma factor involved in the heat-shock response is σ^{E} (σ^{24}), which was found to be an essential gene in *E. coli* at all temperatures (De Las Penas et al., 1997). Presumably the σ^{E} regulon protects cells against extracytoplasmic stress-derived damage. Genes belonging to the σ^{E} regulon are important for bacterial pathogenesis: the mucoid phenotype of *Pseudomonas aeruginosa* in cystic fibrosis infections is controlled by AlgU, an analogue of σ^{E} (Yu et al., 1995), and *rpoE* mutants of *Salmonella typhimu-*

Putative promoters

Heat shock promoter in α-subdivision	CTTG	<17/18>	CYTAT-T
Heat shock promoter in γ-subdivison	TCTC-CCTTGAA	<13/14>	CCCAT-AT
Vegetative promoter in γ-and α-subdivsions	TTGACA	<17>	TATAAT

Promoter recognition domains

Sigma factor domain		2.4	4.2
α-Subdiviaion	σ-32	IKA**S**I**Q**EYILRSWS**L**VK**MG**TT	YGVS**R**ERVRQ**I**EK**R**AMKKLR
γ-Subdivision	σ-32	IKA**E**I**H**EYVLR**N**WRIVK**VA**TT	YGVS**A**ERVRQ**L**EK**N**AMKKLR
		* * * * * *	* * *
α-Subdiviaion	σ-70	IRQAITRSIADQARTIRIPVHM	F**S**VTRERIRQIEAKALRNV**K**
γ-Subdivision	σ-70	IRQAITRSIADQARTIRIPVHM	F**D**VTRERIRQIEAKALRNV**R**
			* *

Fig. 1. Putative heat shock promoters and promoter recognition domains of σ-32 and σ-70 in alpha-purple and gamma-purple proteobacteria. (From Segal and Ron [1995a] and Nakahigashi et al. [1999].)

rium are defective in growth inside cells (Humphreys, 1999).

The *E. coli* σ^E controls the expression of genes encoding periplasmic folding catalysts, proteases, biosynthetic enzymes for the lipopolysaccharide component lipid A, and other proteins whose functions are involved with the cell envelope. Members of this regulon include periplasmic proteins that are involved in protein metabolism: the protease DegP (HtrA) and the periplasmic peptidyl prolyl isomerase FkpA (Erickson and Gross, 1989; Strauch et al., 1989; Dartigalongue and Raina, 1998).

As mentioned above, σ^E activates transcription of *rpoH* under conditions of severe heat shock, and because it has a σ^E promoter, it also regulates itself. The response is regulated by RseA (an inner membrane antagonist of σ^E), RseB (a periplasmic protein that binds to the periplasmic face of RseA), and the proteases DegS and YaeL. Envelope stress promotes RseA degradation, which occurs by a proteolytic cascade initiated by DegS. There is evidence that one σ^E-inducing stress (OmpC overexpression) directly activates DegS to cleave RseA (Alba and Gross, 2004).

HrcA-CIRCE-Controlled Genes

The HrcA-CIRCE repression system is the major system regulating the operons coding for chaperones in Gram-positive bacteria. This system is comprised of an inverted repeat *cis* element and a *trans* protein-repressor encoded by the *hrcA* gene. The first reported inverted repeat upstream to the *groE* operon was found in *Mycobacterium tuberculosis* in 1989 (Baird et al., 1989). Recognition of this element as a widespread heat-shock control element for the *groE* and *dnaK* operons took several years. Several lines of direct evidence for the role of CIRCE as a negative *cis* element were obtained (Narberhaus, 1999): 1) deletion of the inverted repeat relieved the repression of a reporter gene fusion (*amyS*; Van Asseldonk et al., 1993); 2) placement of CIRCE behind a foreign promoter reduced the expression of the downstream gene (Zuber and Schumann, 1994); and 3) site-directed mutation, or the removal of three or four nucleotides in one arm of the inverted repeat, resulted in an elevated transcription of the downstream genes at normal growth temperature (Zuber and Schumann, 1994; Babst et al., 1996). Transcription remained derepressed when the inverted repeat was restored by compensating mutations in the second arm of the inverted repeat. Therefore, the CIRCE is not only a potential stem and loop structure (because its sequence by itself is required for repression), but also a binding site for a sequence-specific repressor protein that binds to the CIRCE.

The elucidation of CIRCE as a potential repressor-binding site initiated a search for the

counterpart repressor. Major steps towards tracking the repressor were accomplished by two observations (Narberhaus, 1999): 1) a deletion of *orf39*—the first gene of the *dnaK* operon of *B. subtilis* resulted in an elevated levels of *groE* transcript (Schulz et al., 1995); and 2) *B. subtilis* mutants affected in the regulation of *groE* and *dnaK* operons were mapped to *orf39* (Yuan and Wong, 1995a). Moreover, production of Orf39 from a plasmid that carries a functional copy of *orf39* restored the repression activity in one of the mutants (Yuan and Wong, 1995a). The binding of Orf39 to CIRCE was shown by gel retardation (Narberhaus, 1999), and the name "HrcA" ("heat regulation at CIRCE") was given to this protein after disruption of the equivalent gene in *Caulobacter crescentus* (Roberts et al., 1996).

For several years, the HrcA-CIRCE system was found only in Gram-positive bacteria and was considered as a Gram-positive heat-shock control element. However, since the first discovery of the CIRCE element in the Gram-negative Alphaproteobacterium *A. tumefaciens* (Segal and Ron, 1993), many CIRCE elements were identified in other Gram-negative bacteria. The inverted repeat was detected in a large number of phylogenetically distant bacteria, including Gram-negative bacteria of the Alpha-, Beta-, and $Gamma_1$-purple proteobacteria. The only groups where it is probably not present at all are the $Gamma_2$ and $Gamma_3$ purple bacteria, which also include the Gram-negative model organism *E. coli* (Segal and Ron, 1998; Ron et al., 1999). The inverted repeat (TTAGCACTC-N9-GAGTGCTAA) is highly conserved in all of the studied genes (R. Segal and Ron, 1996; Segal and Ron, 1998).

In contrast to Gram-positive bacteria where CIRCE-regulated genes are transcribed with the vegetative sigma factor (σ^A), in *A. tumefaciens* the *groEL* operon is HrcA-CIRCE controlled, but is transcribed mainly by σ^{32} (Nakahigashi et al., 1999). In *A. tumefaciens*, it was possible to show, using 2D gels, that GroE proteins are the only proteins whose synthesis is repressed by the HrcA-CIRCE system (Rosen et al., 2002b). In *Bradyrhizobium japonicum*, two *groESL* operons were found: $groESL_1$ is σ^{32} regulated while $groESL_2$ is CIRCE-HrcA-σ^{96} dependent (σ^{96} recognizes the housekeeping promoter of *B. japonicum*; Munchbach et al., 1999a). The control of chaperone expression by the HrcA-CIRCE system seems to be more ancient than the σ^{32}-dependent transcription of heat-shock genes because it is found in all the bacteria except two small groups that lost it during evolution, whereas σ^{32}-dependent transcription is found only in Gram-negative bacteria (Ron et al., 1999).

Minor Regulatory Elements

Expression of at least ten genes in *B. japonicum*, seven of which code for small Hsps, is under the control of ROSE (repression of heat-shock gene expression; Narberhaus et al., 1998; Munchbach et al., 1999b). This negatively *cis*-acting DNA element confers temperature control to a s^{70}-type promoter. ROSE elements are not restricted to *B. japonicum* but are also present in *Bradyrhizobium* sp. (*Parasponia*), *Rhizobium* sp. strain NGR234, and *Mesorhizobium loti* (Nocker et al., 2001). The latest model for ROSE activity suggests that ROSE controls heat-shock protein expression by a temperature-dependent secondary structure of ROSE mRNA that controls the access of the ribosome to the ribosome binding site (Nocker et al., 2001).

Proteome analysis of *A. tumefaciens* and in its mutants deleted for *rpoH*, *hrcA* or in both, showed 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 (Rosen et al., 2001; Rosen et al., 2002b). These uncharacterized regulatory elements may also involve ROSE because *A. tumefaciens* belongs to the Rhizobiaceae group.

An additional unique posttranscriptional control mechanism demonstrated in *A. tumefaciens* involved a specific cleavage of the *groESL* operon transcript. The resulting *groES* transcript is rapidly degraded, whereas the *groEL* transcript is stable, leading to a differential expression of the two genes of the operon—as could be detected by quantitative analysis of the protein expression, using 2D-gels (Segal and Ron, 1995b; Rosen et al., 2002b). This mRNA processing is temperature dependent and constitutes the first example of a controlled processing of transcripts in bacteria.

The General Stress Response in *E. coli*

The "general stress response" is induced during carbon starvation or entry into stationary phase. In *E. coli* these conditions result in a variety of physiological and morphological changes that, presumably, ensure survival during periods of prolonged starvation. Although this general stress response was believed to involve the induction of 30–50 proteins (Lange and Hengge-Aronis, 1991), this stimulon now appears to be much larger and involve almost 500 genes, most of which are induced by osmotic shock. About half are induced by stationary phase or acidic stress, and many are induced by more than one, or all of

these stresses (R. Hengge, personal communication). The general stress response is also important in quorum sensing (Schuster et al., 2004).

The genes coding for the general stress response in *E. coli* are transcribed by an alternative sigma factor, σS (RpoS), which recognizes a consensus promoter upstream of the general stress genes. The promoter specificity of σS has been difficult to determine, as the promoter it recognizes appears quite similar to those recognized by the vegetative σ70. The specific σS promoter elements were recently characterized (Becker and Hengge-Aronis, 2001; Gaal et al., 2001; Hengge-Aronis, 2002; Lee and Gralla, 2002; A. Typas and R. Hengge-Aronis, personal communication) and the results suggest that the selectivity is provided by the K173 (the lysine in position 173 of the amino acid sequence) in σS (which is glutamate in σ70). σS binds to the C(-13) and the distal upstream (UP) element –35 of the promoter.

The *E. coli* RpoS is a highly unstable protein, whose degradation is inhibited by various stress signals, such as carbon starvation, high osmolarity and heat shock. As a consequence, these stresses result in the induction of σS-regulated stress-protective proteins (Bouche et al., 1998). Proteolysis of σS requires the response regulator RssB (a direct recognition factor with phosphorylation-dependent affinity for σS, which targets σS to the ClpXP protease; Zhou et al., 2001; Pruteanu and Hengge-Aronis, 2002). Recognition of σS by the RssB/ClpXP system involves two distinct regions—region 2.5 of RpoS is a long α-helix which binds phosphorylated RssB. This binding exposes a second region of RpoS, located in the N-terminal part, which is a binding site for the hexameric ring of the ClpX chaperone (Studemann et al., 2003).

Recent studies demonstrate the involvement of small, noncoding RNAs (Vogel et al., 2003) in the proteolysis of σS. These small noncoding RNA sequences are abundant—around 50 such sRNAs were described in *E. coli*. The levels of many of these sRNAs vary with changing environmental conditions, suggesting a potential regulatory function. At least three sRNAs were found to affect the regulation of RpoS translation (Repoila et al., 2003). DsrA and RprA stimulate RpoS translation in response to low temperature and cell surface stress, respectively, whereas OxyS represses RpoS translation in response to oxidative shock. However, in addition to regulating RpoS translation, DsrA represses the translation of HNS (a global regulator of gene expression), whereas OxyS represses the translation of FhlA (a transcriptional activator), allowing the cell to coordinate different pathways involved in cell adaptation.

Control of the Heat Shock Response and the General Stress Response in Gram-Positive Bacteria

Although the stress gene and proteins in Gram-negative and Gram-positive bacteria are highly conserved, regulation of these genes is very variable. The presence of HrcA-CIRCE control elements has been noted in only some Gram-negative bacteria, and a comparison of Gram-negative with Gram-positive bacteria reveals major differences. Table 2 shows the factors affecting regulation of major stress genes in Gram-positive bacteria and in two Gram-negative bacteria belonging to the Alphaproteobacteria and Gammaproteobacteria. The data indicate that the expression of a stress protein can be under the control of different regulons, and also show difference in control elements between the various bacteria.

Many of the genes that in Gram-negative bacteria belong to the heat shock regulon (as their expression is controlled by the heat shock tran-

Table 2. Regulation of major stress genes.

Gene	Function of gene product	Bacteria	Regulon	Transcription during stress	Control element	Stability of gene product
DnaK	Chaperone	Gram positive	Heat shock	σA (σ70)	CIRCE	
		Alphaproteobacteria	Heat shock	σ32		
		Gammaproteobacteria	Heat shock	σ32		
GroEL	Chaperone	Gram positive	Heat shock	σ70	CIRCE	
		Alphaproteobacteria	Heat shock	σ32	CIRCE	
		Gammaproteobacteria	Heat shock	σ32		
rpoH	Activator—σ32	Alphaproteobacteria	Heat shock	σ32		Stable
		Gammaproteobacteria	Heat shock	σ32, σE		Unstable
lon, clpP	Proteases	Gram negative	Heat shock	σ32		
		Gram positive	General stress	σB		

Abbreviation: CIRCE, a conserved inverted repeat control element or "controlling inverted repeat of chaperone expression."

scriptional activator σ32) constitute part of the general stress response in Gram-positive bacteria. The only genes that are truly "heat shock genes" in Gram-positive bacteria are the genes coding for the major chaperones—Hsp10 and Hsp60 (GroES and GroEL) and the Hsp70 group (DnaK, DnaJ and GrpE).

Heat Shock Response

The model organism for studying the heat shock response in Gram-positive bacteria is *B. subtilis*. In contrast to *E. coli*, where most heat shock proteins are exclusively under the control of the alternative sigma factor σ32, Gram-positive bacteria have no heat-shock specific sigma factor. Rather, the heat-shock response of these bacteria involves the induction of the major chaperones, which is regulated by the HrcA-CIRCE control elements (Zuber and Schumann, 1994; Hecker and Volker, 1998), and several groups of proteins regulated by specific control elements, all of which are discussed below. Another major difference is that some of the proteins, which are part of the heat shock regulon in *E. coli* (such as the Clp proteases), are part of general stress proteins (GSPs) in *B. subtilis*, whose induction is regulated by the alternative sigma factor σ^B.

HRCA-CIRCE CONTROLLED GENES. This system, consisting of the HrcA repressor which binds to the CIRCE inverted repeat, was already described in the section The Heat Shock Response and General Stress Response in Gram-Negative Bacteria. Though in Gram-negative bacteria this system controls only the *groESL* operon, its role in Gram-positive bacteria is much more central. In the Gram-positive bacteria, the genes coding for Hsp70 (DnaK) and the proteins functionally associated with it are also under the control of HrcA-CIRCE. Thus, this control element regulates the expression of the genes coding for all the major chaperones. Notably, in these bacteria, the genes coding for the group of Hsp70 chaperones are usually organized in one operon: *grpE-dnaK-dnaJ*. In the group of low G+C Gram-positive bacteria, such as *B. subtilis*, this operon also contains the gene coding for the HrcA repressor and is *hrcA-grpE-dnaK-dnaJ*(R. Segal and Ron, 1996).

In *B. subtilis*, the operons regulated by the HrcA-CIRCE system (*groESL* and *dnaK* operons) are always transcribed during heat shock by the vegetative sigma factor σ^A (Yuan and Wong, 1995b). This situation is different from the Gram-negative bacteria, in which all the heat shock operons, including the *groESL* operon (which contains the CIRCE element) are transcribed by the specific heat shock σ32. Recently GroE itself has been shown to autogenously regulate the transcription of the *groE* and *dnaK* operons by the finding that the GroE chaperonin machine modulates the activity of the HrcA repressor (Mogk et al., 1997).

GENES CONTROLLED BY ADDITIONAL REPRESSORS. In *Streptomyces coelicolor* and *Streptomyces albus*, the $groESL_1$ operon and the $groEL_2$ gene are regulated by tandem CIRCE elements, whereas the *dnaK* operon encodes its own autoregulatory repressor (Bucca et al., 1995; Bucca et al., 1997). Heat-inducible transcription of the *dnaK* operon (*dnaK*, *grpE*, *dnaJ* and *hspR*) initiates from the vegetative promoter. Disruption of *hspR* led to high and constitutive transcription levels of the *dnaK* operon but had no effect on the *groE* expression level (Bucca et al., 1997). Similar to the GroE modulation of HrcA activity, DnaK protein forms a specific ATP-independent complex with the *Streptomyces* HspR repressor, and this interaction is necessary for HspR to bind a dnaKp fragment in gel-shift assays (Bucca et al., 2000). The *dnaK* heat-induction model suggested by Bucca et al. suggests a decrease in the availability of DnaK because of the accumulation of heat-damaged proteins (Bucca et al., 2000). This model has many similarities to the heat induction of the σ^{32}-dependent transcription in *E. coli*, a model that will be discussed in detail below.

Another heat-shock control element found in *S. albus* is the RheA, which represses the transcription of *hsp18* (encoding a small heat-shock protein) by binding specifically to the *hsp18* promoter (Servant and Mazodier, 1996; Servant et al., 1999). Transcription analysis of *rheA* in the *S. albus* wildtype and in *rheA* mutant strains suggested that RheA represses transcription not only of *hsp18* but also of *rheA* itself (Servant et al., 1999).

The General Stress Response

SIGMA B-CONTROLLED GENES. Sigma B was found to control a stress-starvation regulon that comprises a very large set of general stress genes (for reviews, see Hecker et al. [1996] and Hecker and Volker [1998]). These σ^B-dependent genes are strongly induced by heat, ethanol, acid or salt stress, as well as by starvation for a carbon source, phosphate and oxygen (Bernhardt et al., 1997; Bernhardt et al., 1999; Hecker and Volker, 1998; Buttner et al., 2001). Recent experiments (Petersohn et al., 2001) using gene arrays containing all currently known open reading frames of *B. subtilis* suggest that as many as 125 genes are under the control of σ^B. At least 24 of these also seem to be subject to a second, σ^B-independent stress induction mechanism. Most of the σ^B-dependent general stress proteins are probably located in the cytoplasm, but 25 contain at least one membrane-spanning domain, and at

least 6 proteins appear to be secreted. This very large stress regulon seems to give a basal level of protection against a large variety of stress conditions.

Two groups of signals were found to trigger the induction of *sigB*, the gene that codes for σ^B. The first group contains extracellular signals (i.e., glucose, oxygen, or phosphate, but not amino acid, starvation) that result in a drop of the ATP level (Maul et al., 1995). (Amino acids trigger the induction of ppGpp and keep the ATP pool constant.) The second group of stimuli includes physical stress-factors, such as heat, salt and acid stress, but not oxidative stress (Hecker and Volker, 1998). This group of stimuli induces the synthesis of σ^B via a two-component system (RsbS and RsbT) that changes the balance of a complex network of anti-sigma (RsbW) factor and its agonist (nonphosphorylated RsbV) to activate σ^{B-} (Akbar and Price, 1996; Yang et al., 1996). For the expression of some genes, the involvement of σ^B is essential, whereas for others it seems to be nonessential because it can be replaced by alternative stress-induction mechanisms (Hecker and Volker, 1998).

Not much is known about many of the 125 GSP genes (Petersohn et al., 2001), and their physiological role in the complex general stress response is not understood. The identified GSPs can be assigned to five main groups (Hecker and Volker, 1998): 1) Group 1 is the σ^B-dependent genes that encode subunits of stress-inducible proteases. ClpP, ClpC, and ClpX are probably essential for the renaturation or degradation of misfolded or denatured proteins that accumulate in the cell upon exposure to stress conditions (Gottesman, 1996; Gerth et al., 1998). Null mutants of *clpC*, *clpP* and *clpX* are extremely sensitive to heat, salt or ethanol stress, and much more sensitive than mutants of *sigB* (Kruger et al., 1994; Msadek et al., 1994; Gerth et al., 1998). 2) Group 2 is the σ^B-dependent genes that encode general oxidative stress-protective proteins (such as *katE*, which encodes catalase; Engelmann et al., 1995) and the DNA-protecting protein Dps (Antelmann et al., 1997b). Other σ^B-dependent proteins (such as thioredoxin ClpC, ClpP and the fifth and sixth gene products of the *clpC* operon [*sms* and *yacK*]; Kaan et al., 1999) may also be involved in adaptation to oxidative stress (Hecker and Volker, 1998). 3) The third group is proteins with a putative role in the adaptation to salt or water stress. A proline-uptake system encoded by a functional copy of *opuE* is required by *B. subtilis* for the use of external proline as an osmoprotectant (Hecker and Volker, 1998). However, the physiological role of σ^B in the expression of *opuE* is still unclear because exogenously provided proline was used as an osmoprotectant in a *sigB* mutant (Von Blohn et al., 1997). YtxH and GsiB are homologous to plant-desiccation proteins, which are involved in water-stress protection, and YkzA is a homolog of the *E. coli* OsmC, which is involved in osmo-adaptation (Mueller et al., 1992; Volker et al., 1994; Maul et al., 1995; Varon et al., 1996). 4) Group 4 is a heterogeneous group of proteins: their role in adaptation to stress is yet to be determined. One of these proteins, GspA (Antelmann et al., 1995), is also induced upon amino acids starvation (Eymann and Hecker, 2001) and seems to be involved in the expression of *hag*, which encodes flagellin, or UDP-glucose pyrophosphorylase, which participates in cell-wall metabolism (Varon et al., 1993). Some proteins seem to participate in nicotinamide adenine dinucleotide (NAD) synthesis (e.g., *nadC* and *nadE* gene products) or might catalyze reduced NAD phosphate (NADP[H])-dependent reactions (Antelmann et al., 1997a; Antelmann et al., 1997b; Hecker and Volker, 1998; Scharf et al., 1998). And 5), the fifth group consists of a large number of proteins that, so far, show no significant similarity to known proteins (Petersohn et al., 2001).

Several of the general stress operons in Gram-positive bacteria were found to be regulated by more than one control element. The *B. subtilis clpC*, *clpP* and *trxA* operons are under the control of the vegetative sigma factor σ^A and the stress sigma factor σ^B (Kruger et al., 1996; Gerth et al., 1998; Scharf et al., 1998). Although both promoters were used under a number of stress conditions, the induction pattern of the genes varied for the different genes and the particular stress condition. A *cis* element that contains a heptameric tandem consensus sequence was found upstream of the *clpC*, *clpE*, and *clpP B. subtilis* operons and was shown to be the binding site of the CtsR repressor (Kruger and Hecker, 1998; Derre et al., 1999a; Derre et al., 1999b). CstR was lately found also in *Listeria monocytogenes* (Nair et al., 2000).

Complexity of the Stress Response Networks

Regulation of bacterial stress response involves various positive and negative control elements that often interact with each other. Some heat shock proteins are directly regulated by only one control element, but other genes and operons are regulated by several control elements (e.g., *E. coli pspABCE* [Jovanovic, 1996], *A. tumefaciens groESL* [Segal and Ron, 1995b; G. Segal and Ron, 1996; Nakahigashi et al., 1999], and *B. subtilis clpC* [Kruger et al., 1996; Gerth et al., 1998; Scharf et al., 1998]). However, the stress

response is always a complex response that regulates itself. As an example, the heat-shock response is induced by damaged proteins, whose cellular concentration increases with temperature. Yet, since the heat shock stimulon contains the genes coding for proteases and chaperones, their induction at increased temperatures reduces the concentration of the damage proteins, thus reducing the level of induction of the heat shock response.

Because the regulatory elements of these complex stress response networks are associated with each other, any impairment of the cellular steady state at one point may affect the whole network, directly or indirectly. Therefore, the study of these global regulatory networks requires global analysis methods (Rosen and Ron, 2002a). Such methods for transcriptome and proteome analysis are now available and have been implemented in this field. For comprehensive understanding, more than one method should be used. Analysis of mRNA levels is required to define all the genes whose transcription is affected by environmental conditions or regulatory genes. This analysis, however, is insufficient because the expression and activity of the stress genes are controlled at posttranscriptional, higher regulatory levels. Thus, global analysis at the protein level (i.e., proteomics studies) also must be performed. These studies define the final cellular level of the various proteins, as well as their modifications, some of which may be controlled by stress conditions. One important protein modification shown to play a role in global regulatory networks is protein phosphorylation, usually at one or a few amino acids. Recently, a new group of highly phosphorylated proteins has been identified. These proteins accumulated during several stress conditions and may be involved in the degradation process (Rosen et al., 2004). In eukaryotic systems, protein phosphorylations are known to be involved in protein labeling and in many signal transduction pathways. In bacteria, the number of known phosphorylated proteins is much lower. However, several phosphorylated proteins are involved in the heat shock response of various bacteria, as will be shown in the following examples. The heat shock transcriptional activation of the *lonD* gene of *Myxococcus xantus* is controlled by a two-component histidine-aspartate phosphorylation system (Ueki and Inouye, 2002). The general-stress sigma factor of *B. subtilis* (σ^B) is regulated by several regulatory kinases and phosphatases (the Rsb proteins), which catalyze the release of σ^B from an anti-σ^B factor (Akbar and Price, 1996; Yang et al., 1996; Akbar et al., 2001; Zhang et al., 2001). Another heat shock protein (Hsp70 of *Mycobacterium leprae*) was found to be phosphorylated at threonine-175 (Peake et al., 1998), which results in an increased affinity for a model polypeptide substrate. One of the best-studied examples of stress-controlled protein modification was already discussed above, in the section The General Stress Response of E. coli. The phosphorylated form of RssB (a stationary phase response regulator) targets the alternative transcriptional activator σ^S for degradation by ClpXP (Bouche et al., 1998; Zhou et al., 2001). In view of these examples, protein modification will probably be demonstrated as one of the important control elements in global regulatory networks.

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