FEMS Microbiology Reviews, fux015, 41, 2017, 549–574

doi: 10.1093/femsre/fux015
Advance Access Publication Date: 11 April 2017
Review Article

REVIEW ARTICLE

Regulation of heat-shock genes in bacteria: from signal sensing to gene expression output

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One sentence summary: The authors provide an updated review of the strategies employed by many important bacteria to finely regulate transcription of heat-shock genes, including a comprehensive description of the several ways in which environmental cues are perceived and processed.

Editor: Franz Narberhaus

ABSTRACT

The heat-shock response is a mechanism of cellular protection against sudden adverse environmental growth conditions and results in the prompt production of various heat-shock proteins. In bacteria, specific sensory biomolecules sense temperature fluctuations and transduce intercellular signals that coordinate gene expression outputs. Sensory biomolecules, also known as thermosensors, include nucleic acids (DNA or RNA) and proteins. Once a stress signal is perceived, it is transduced to invoke specific molecular mechanisms controlling transcription of genes coding for heat-shock proteins. Transcriptional regulation of heat-shock genes can be under either positive or negative control mediated by dedicated regulatory proteins. Positive regulation exploits specific alternative sigma factors to redirect the RNA polymerase enzyme to a subset of selected promoters, while negative regulation is mediated by transcriptional repressors. Interestingly, while various bacteria adopt either exclusively positive or negative mechanisms, in some microorganisms these two opposite strategies coexist, establishing complex networks regulating heat-shock genes. Here, we comprehensively summarize molecular mechanisms that microorganisms have adopted to finely control transcription of heat-shock genes.

Keywords: heat-shock response; transcriptional regulation; alternative σ factors; HspR repressor; HrcA repressor; CtsR repressor

INTRODUCTION

The heat-shock response is a protective mechanism that is crucial for bacterial survival and adaptation to hostile environmental conditions. This response is apparently universal as it has been observed in every bacterial species investigated. It consists of a set of well-coordinated responses and processes, mostly involving the strictly regulated production of various heat-shock proteins. These, in turn, mainly include molecular chaperones and proteases, whose intracellular abundance rapidly increases upon exposure to a variety of environmental stresses. The principal function of heat-shock proteins is to assist protein folding. They are also involved in rescue or degradation of denatured proteins and deleterious misfolded aggregates. Upon sudden temperature increase, heat-shock proteins transiently accumulate in the cell. Once the organism has adapted to the new temperature, the amount of heat-shock proteins decreases to a steady-state level that is frequently greater than the initial basal level in order to assist bacterial growth under non-optimal environmental conditions.

Besides their roles in protecting cellular proteins from environmental insults and in maintaining cellular homeostasis, some heat-shock proteins are important virulence factors, while others appear to affect pathogenesis indirectly. Although the heat-shock response is universally conserved in both prokaryotes and eukaryotes, the basic molecular mechanisms governing the regulation of heat-shock genes differ considerably among

Received: 30 November 2016; **Accepted:** 14 March 2017

bacterial species. In particular, bacteria have evolved different regulatory strategies that combine transcriptional and posttranscriptional mechanisms to rapidly induce heat-shock protein synthesis only when required.

In order to rapidly respond to environmental cues and to ensure expression of heat-shock proteins only when appropriate, bacteria employ specific sensory biomolecules that are able to perceive temperature fluctuations and translate them into coordinated gene expression outputs. In this respect, a wide repertoire of thermal sensors has evolved among microorganisms. Specifically, bacteria can sense temperature changes using nucleic acids (DNA or RNA) or proteins.

In the present review article, we provide an updated, comprehensive description of the strategies employed by many important microorganisms to finely regulate transcription of genes coding for heat-shock proteins. In the first part of the article, we survey the molecular mechanisms of regulation (e.g. positive, negative, simple and complex) that guarantee the proper transcriptional output once stress signals are detected. In the second part, we provide a comprehensive description of the several ways in which environmental cues are perceived and processed.

The heat-shock response: role and principal mediators

The heat-shock response was first described by Ritossa as a phenomenon observed in the polytene chromosomes of salivary glands of *Drosophila melanogaster* flies when moved from their normal growth temperature to 37°C (Ritossa 1962). Triggered by environmental stress insults, the heat-shock response consists of the induction or upregulation of synthesis of a group of proteins that help the cell to survive under conditions that would normally be lethal. Actually, several stress conditions are able to elicit heat-shock response, such as temperature variations, osmotic changes, desiccation, antibiotics, solvents or heavy metals. However, the heat-shock response triggered by a sudden temperature increase has been widely used as a model system for studying the impact of stress on biological systems. Environmental stress conditions primarily affect cellular protein homeostasis (maintenance of an overall balance of folded proteins), posing a serious threat to their integrity. Heat-shock proteins are involved in several processes in bacterial cells, including assisting the folding of newly synthesized proteins, preventing aggregation of proteins under stress conditions and recovering proteins that have been partially or completely unfolded by stresses such as a sudden temperature increase. During stress, in fact, spontaneous folding of newly synthetized proteins is inefficient and error-prone, and a predominant fraction of nascent polypeptides undergoes a chaperone-mediated folding process (Mogk *et al*. 1999). Also, a large fraction of already folded proteins gets partially or completely denatured and becomes prone to form deleterious aggregates. Moreover, the rates of transcription and translation decrease because the stability of the RNA polymerase subunits and of the translation factor EF-G are sensitive to temperature increase (Mogk *et al*. 1999).

Some heat-shock proteins are also required during normal growth condition and they are abundant under all metabolic conditions. GroEL and DnaK, the bacterial representatives of the two major chaperone families Hsp60 and Hsp70, respectively, play a key role in protein folding even during non-stressed growth conditions, although their action becomes more important during stress. They bind hydrophobic surfaces of unfolded proteins and, together with their co-chaperones (GroES and DnaJ-GrpE) and ATP hydrolysis, they promote the acquisition of proper folding by the substrate polypeptides through different mechanisms that reflect their different architectures. While GroEL monomers assemble into a cylindrical complex to form two heptameric rings that enclose the entire substrate protein inside a large cavity, giving the polypeptide a chance to fold without interacting with any other protein, the DnaK chaperone is mostly monomeric, exerting its action by binding short surface-exposed hydrophobic amino-acid sequences (Xu, Horwich and Sigler 1997; Mogk *et al*. 1999).

It is worth mentioning that the names *groEL* and *groES* should be used for *Escherichia coli* genes only, because this nomenclature was adopted following the observation that mutation of these genes prevented the plating of several bacteriophages (Georgopoulos *et al*. 1972; Takano and Kakefuda 1972). An alternative used nomenclature has been proposed, in which homologs of GroEL are called Cpn60, and those of GroES are named Cpn10 (Coates, Shinnick and Ellis 1993). As both nomenclatures are still widely used, in this review we decided to adopt the nomenclature used for *E. coli*, GroEL and GroES.

Another group of heat-shock proteins expressed by the bacterial cell and upregulated during stress is composed by proteases. These proteins are responsible essentially for the removal of damaged polypeptides from stressed cells. Some proteases are multicomponent systems, in which a catalytic subunit (e.g. ClpP and HslV) associates to substrate recognition subunits (ClpA or ClpX for ClpP and HslU for HslV), which are co-chaperones able to remodel substrate polypeptides upon ATP hydrolysis and deliver them to proteolytic degradation (Missiakas *et al*. 1996; Wawrzynow, Banecki and Zylicz 1996). While these proteases assemble into complex ring-shaped structures, other members of this group of heat-shock proteins combine on a single polypeptide both chaperone and protease activities (e.g. Lon, FtsH and the periplasmic serine protease DegP).

The small heat-shock proteins constitute a highly heterogeneous group of proteins whose expression is induced upon stress challenges. Their principal role is to bind and protect unfolded proteins, by holding them in a conformation not subjected to degradation, until they will be efficiently refolded by ATP-driven chaperones (Matuszewska *et al*. 2005).

Finally, some heat-shock proteins have been proposed as important virulence factors, while others appear to affect pathogenesis indirectly. In this respect, there are several examples in which molecular chaperones seem to have taken on diverse functions other than just protein folding. For example, GroEL1 of *Mycobacterium smegmatis*, a GroEL paralog with no heat-shock-related functions, is involved in mycolic acid synthesis and biofilm formation (Ojha *et al*. 2005). Moreover, the GroES homolog of *Helicobacter pylori*, besides its prototypical role as co-chaperonin, plays a role in the storage and trafficking of Ni^{2+} ions, a crucial virulence determinant for this human gastric pathogen (de Reuse, Vinella and Cavazza 2013). On the other hand, there is evidence that molecular chaperones in many cases might act as direct virulence factors. Even though it still remains a controversial concept, it has been proposed that several bacterial species use cell surface GroEL and DnaK chaperones as adhesins during the interaction with host cells (reviewed in Henderson and Martin 2011). Moreover, chaperones proteins have been shown to have cell-to-cell signaling properties and, in several cases, are able to induce the synthesis of proinflammatory cytokines and promote apoptosis (Henderson and Martin 2011).

REGULATORY MECHANISMS OF HEAT-SHOCK GENES

In bacteria, regulation of heat-shock genes can be positive or negative. Positive regulation exploits alternative sigma factors to specifically direct the transcriptional machinery to a subset of selected promoters, while negative regulation is orchestrated by transcriptional repressors. Interestingly, while several bacteria adopt exclusively positive or negative mechanisms, in some microorganisms these opposite strategies coexist, establishing a complex regulatory network of heat-shock genes.

Positive transcriptional regulation of heat-shock genes

Positive transcriptional regulation of heat-shock genes relies on the use of a dedicated alternative σ factor, the subunit of the RNA polymerase that confers promoter recognition specificity to the transcription enzyme. In general, this transcriptional control mechanism exploits the ability of condition-specific alternative σ factors to outcompete the housekeeping σ subunit normally associated with RNA polymerase and redirects the enzyme to a selected subset of heat-shock gene promoters, thereby reprogramming cellular transcription. The strategy of transiently increasing transcription of heat-shock genes being dependent upon temperature stress through a dedicated σ factor is widely used among bacteria, although the model organism *Escherichia coli* has long served as the paradigm for this kind of regulation.

Escherichia coli heat-shock sigma-32 (σ^{32})

The heat-shock response in *E. coli* is governed by the *htpR* gene product, whose high amino acid sequence similarity with the housekeeping σ^{70} was previously described (Landick *et al.* 1984). The 32-kDa HtpR polypeptide was purified from crude extracts and combined *in vitro* with RNA polymerase 'core' enzyme, consisting of the four subunits α_2, β and β'only (a preparation of the RNA polymerase enzyme that retains catalytic activity but is unable to initiate *de novo* transcription *in vitro*). This *in vitro* transcription assay showed that HtpR was able to confer specificity to RNA polymerase in recognizing heat-shock promoters, without requiring the vegetative sigma factor, σ^{70}. Following the demonstration that HtpR was a σ factor able to promote transcription initiation at heat-shock promoters, its gene was renamed *rpoH* (in analogy to the already characterized *rpoD* gene, coding for the vegetative σ^{70} factor) and the gene product was called sigma-32 (σ^{32}, sometimes also called σ^{H}) (Grossman, Erickson and Gross 1984). As stated above, to exert its function, σ^{32} interacts with RNA polymerase core enzyme and directs the transcription machinery to specific promoters. The vegetative σ factor and almost all alternative σ factors constitute a homologous set of proteins (except σ^{54}, which belongs to a separate family), the σ^{70} family of proteins, with several regions of sequence conservation. The extensive σ-core interaction in the formation of the RNA polymerase holoenzyme (the enzymatic form with $\alpha_2\beta\beta'\sigma$ subunit composition) involves the same regions and even equivalent residues in σ^{32} and σ^{70} (Sharp *et al.* 1999). What specifically targets the transcription machinery to heat-shock genes is the recognition of particular promoter elements by σ^{32} DNA-binding domains (DBD) (Kumar *et al.* 1995). Several genome-wide studies that addressed the definition of heat-shock promoter elements confirmed that those recognized by σ^{32} are different from those bound by RNA polymerase associated with the housekeeping σ^{70}. Specifically, while the –35 box consensus sequence for σ^{32} binding, TTGAAA, is highly similar to the σ^{70} TTGACA consensus, the σ^{32} specific CCCCATNT -10 box shows no similarities to the σ^{70} TATAAT -10 consensus hexamer (Nonaka *et al.* 2006; Wade *et al.* 2006; Koo *et al.* 2009). Accordingly, a mutational analysis of residues belonging to σ^{32} regions 2.4 and 4.2 (conserved regions among the various σ factors belonging to the σ^{70} family, which are involved in the recognition of –10 and –35 core promoter elements, respectively) showed that the recognition of the –35 box is mediated by similar amino acids clustered in regions 4.2 of σ^{32} and σ^{70}, while the interaction with the downstream –10 element involves different residues (Kourennaia, Tsujikawa and Dehaseth 2005). The rapid, transient transcriptional increase of heat-shock genes upon shift to higher temperature is directly linked to the amount of active σ^{32} in the cell. Normally present in very small amounts and with a half-life of just a few minutes at 37°C, the σ^{32} level rapidly and transiently increases during heat-shock as a result of changes in its rate of synthesis and stability (Grossman *et al* 1987; Lesley, Thompson and Burgess 1987; Straus, Walter and Gross 1987). Even though the cellular level of σ^{32} depends mostly on post-transcriptional regulatory mechanisms, the control of *rpoH* transcription appears to be quite complex. The region upstream of the *rpoH* coding sequence harbors five promoters (named P1 and P3 through P6) recognized by different σ factors and responsive to several stimuli (Fig. 1, left panel). Specifically, P4 and P5 are transcribed by σ^{70}-associated RNA polymerase and P3 and P6 are bound by σ^{24} and σ^{54}, respectively, while P1 can be transcribed by σ^{70} or σ^{S} depending on the growth phase (Erickson *et al.* 1987; Erickson and Gross 1989; Wang and Kaguni 1989a; Nagai *et al.* 1990; Missiakas and Raina 1998; Pallen 1999; Janaszak *et al.* 2009). In addition, the P6 promoter is catabolite sensitive and its transcription is dependent on cAMP levels and on the CRP activator (Nagai *et al.* 1990), while cAMP-CRP together with CytR anti-activator form a repression complex overlapping P3, P4 and P5 (Kallipolitis and Valentin-Hansen 1998). Also DnaA, the DNA-binding protein involved in initiation of chromosomal DNA replication, takes part in the regulation of *rpoH* by repressing the P3 and P4 promoters (Wang and Kaguni 1989b) (Fig. 1, left panel). This complex regulatory network is responsive to various environmental signals and conditions, providing the proper amount of *rpoH* transcript and, accordingly, an extensive adaptability of the stress response. The hypothesis that the transient increase of cellular σ^{32} amount following heat stress occurs at the translational level has been proposed according to independent observations. First, the striking increase of the rate of synthesis of σ^{32} upon heat-shock does not correlate with a significant increase of *rpoH* transcription (Erickson *et al.* 1987). Second, to understand the contribution of transcriptional and/or post-transcriptional mechanisms to the expression of σ^{32}, the activity of a reporter gene (*lacZ* encoding the β-galactosidase enzyme) was monitored using transcriptional and translational fusions with the 5′ region of *rpoH*. Results from these analyses showed that temperature regulated increase of σ^{32} was not dependent on the *rpoH* promoter, but was controlled by the translational signals of RpoH mRNA, suggesting a mechanism of translational control for σ^{32} during heat-shock (Straus, Walter and Gross 1987). Several studies have identified the regions of *rpoH* mRNA important for proper σ^{32} regulation, suggesting a mechanism involving the mRNA secondary structure formed by the 5′ coding sequence of *rpoH* close to the translation start codon and, thereby, affecting translation efficiency (Kamath-Loeb and Gross 1991; Nagai, Yuzawa and Yura 1991; Yuzawa *et al.* 1993). All these findings converged in the current model of temperature-mediated translational regulation of σ^{32}. Specifically, during normal growth

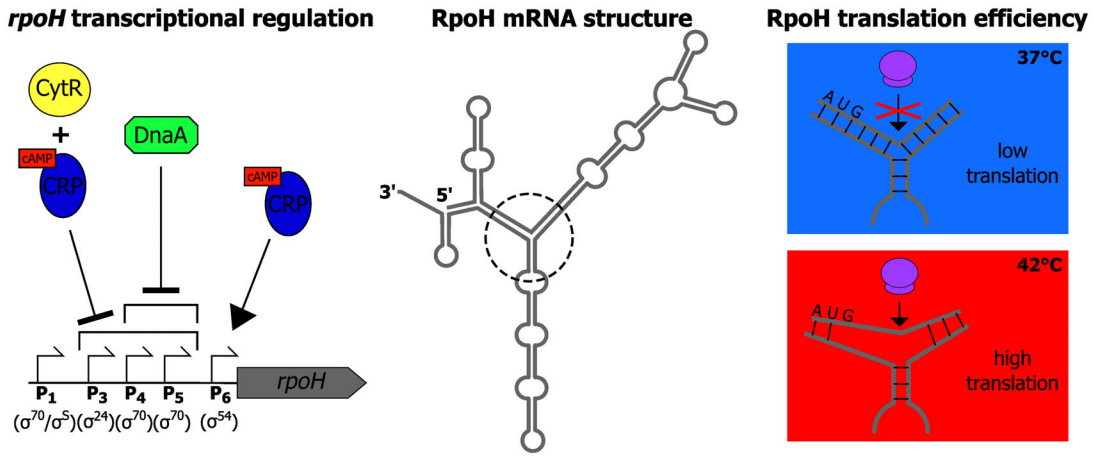

Figure 1. Transcriptional and translational control of σ^{32} expression. Left panel: schematic representation of the region upstream of the *rpoH* coding sequence (depicted by a gray block arrow) containing five promoters (indicated by bent arrows and named P1 and P3 to P6) recognized by different σ factors (indicated underneath each promoter name). Transcription from some of these promoters is also regulated by transcriptional regulators: the arrowhead denotes positive regulation, while hammerheads indicate negative regulation. Central panel: schematic representation of the RpoH mRNA secondary structure at physiological temperature. Transcript sequences of crucial importance for the efficient initiation of translation close to the ribosome-binding site and the AUG start codon are sequestered in double-stranded structures, which leads to inefficient ribosome binding (gray dashed circle indicates the portion of the transcript containing these translation initiation sequences). Right panel: enlarged view of the RpoH mRNA region involved in the initiation of translation. While during normal growth conditions ribosome binding is hindered by secondary structure formation, at high temperature the partial melting of the mRNA secondary structure enhances ribosome entry and translational initiation. Black lines indicate Watson–Crick base pairing.

conditions, the *rpoH* mRNA folds in a complex secondary structure (schematically represented in Fig. 1, central panel) that hides sequences crucial for the efficient initiation of translation surrounding the ribosome-binding site and the AUG start codon. Conversely (Fig. 1, right panel), at high temperature the partial melting of the mRNA secondary structure enhances ribosome entry and translational initiation without the involvement of additional cellular components (Morita *et al.* 1999). *rpoH* represents the first described example of a class of temperature-sensing RNA sequences known as RNA thermometers (Kortmann and Narberhaus 2012), which provide a rapid post-transcriptional mechanism to control the synthesis of heat-shock proteins (discussed below). The temperature-mediated positive regulation of σ^{32} synthesis through structured mRNA combines with the complex control of the heat-shock σ factor activity and stability, mediated by the concerted action of chaperones and proteases. The finding that *E. coli* strains carrying mutations in the *dnaK*, *dnaJ* and *grpE* genes were characterized by enhanced expression of heat-shock proteins at low temperature and by a prolonged transcriptional response upon heat challenge suggested a role for the DnaK-DnaJ-GrpE machinery as a negative regulator of heat-shock response (Straus, Walter and Gross 1990). The key role of the DnaK system in the regulation of heat-shock gene expression was further characterized, and a direct physical ATP-dependent interaction between σ^{32} and DnaK, DnaJ and GrpE chaperones was demonstrated both *in vitro* and *in vivo* (Gamer, Bujard and Bukau 1992; Liberek *et al.* 1992). In addition, the σ^{32} functionality is modulated by another major heat-shock protein, the chaperonin GroEL, as its depletion determined enhanced transcription of heat-shock genes, while its overexpression established the opposite effect (Kanemori, Mori and Yura 1994; Guisbert *et al.* 2004; Patra *et al.* 2015). The activity of σ^{32} appears to be dependent on its direct interaction with free chaperones (Guisbert *et al.* 2004). When unfolded proteins accumulate in the cell, they bind to and sequester chaperones, allowing σ^{32} to dissociate as a stable protein. In other words, it is widely accepted that the degree of sequestration of the DnaKJE/GroESL chaperones through binding to misfolded proteins determines the level of transcription of heat-shock genes, linking in this way the transcriptional response to the cellular folding state of proteins (Tomoyasu *et al.* 1998). The effect of chaperones on σ^{32} stability is directly linked to specific cellular proteases involved in its degradation. In particular, it was shown that an *E. coli* strain depleted of the membrane-bound ATP-dependent FtsH metalloprotease (formerly named HflB) accumulated σ^{32} and induced the heat-shock response. Furthermore, the half-life of the highly unstable σ factor increased by a factor of up to 12-fold in mutants with reduced FtsH function (Herman *et al.* 1995). Moreover, *in vitro* assays with purified components confirmed the direct association and the FtsH-mediated processing of σ^{32} (Tomoyasu *et al.* 1995). Also, additional cellular proteases, such as the ATP-dependent protease HslVU (also known as ClpQY), seem to play a role in the heat-shock response by modulating *in vivo* the turnover of σ^{32} (Kanemori *et al.* 1997). All the experimental data converged to a model for the homeostatic control of σ^{32} concentration in the cell in response to environmental stimuli. Normally present in low amounts and highly unstable, upon temperature upshift, σ^{32} levels rapidly increase as a consequence of enhanced mRNA translation and transient stabilization (positive feedforward mechanism). This induction phase is followed by a recovery/shut-off phase, mediated by chaperones and proteases (known as negative feedback loops), in which the activity and the stability of σ^{32} rapidly decrease to a new steady-state level. Even though the role of the DnaK and GroE chaperone systems in modulating σ^{32} activity and in promoting protease-mediated degradation was well documented *in vivo*, some contradictory observations had for many years hampered its recapitulation *in vitro* and the detailed mechanism remained elusive. Specifically, several studies, focused on the identification and the characterization of mutants with altered homeostatic regulation (known as 'dysregulation mutants'), led to the definition of a short region of σ^{32} (named homeostatic control region) that appeared to be important for both stability and activity of σ^{32} (Obrist and Narberhaus 2005; Yura *et al.* 2007). *Escherichia coli* cells expressing heat-shock σ factor with mutations in the homeostatic control region showed increased σ^{32} activity and stability,

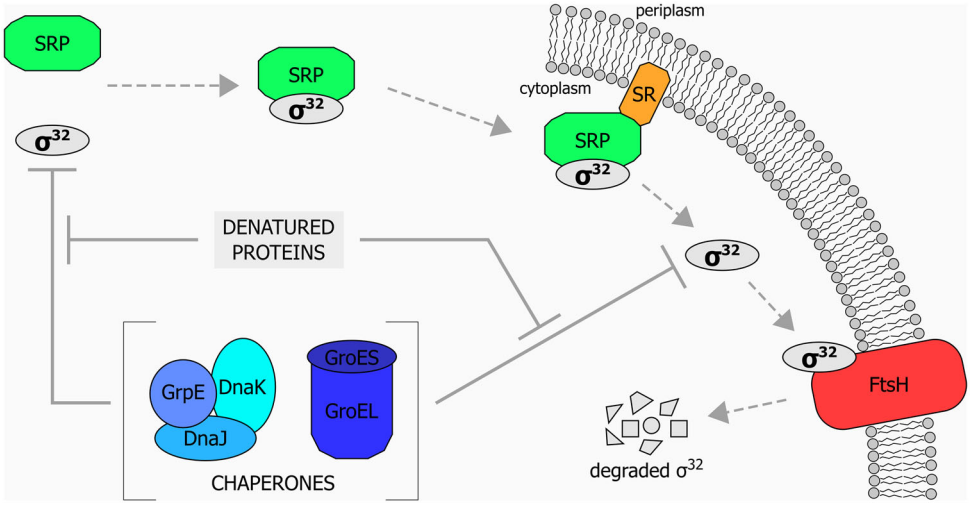

Figure 2. Representation of the current model for activity and stability control of σ^{32}, depicting its interaction with chaperones, its transit to the inner membrane and its FtsH protease-mediated degradation. Gray arrowheads denote negative modulation.

consistent with its inferred role in the feedback control mechanism (Obrist and Narberhaus 2005; Yura *et al.* 2007). Surprisingly, purified dysregulation mutant σ^{32} isoforms showed normal binding to chaperones in *in vitro* interaction assays and were not inhibited by chaperones when assayed by *in vitro* transcription experiments (Yura *et al.* 2007; Suzuki *et al.* 2012). These discrepancies suggested that the negative feedback control of σ^{32} mediated by DnaK and GroE chaperones must be more complex than previously believed and that there must be at least an additional step involving some other cellular components besides chaperone binding. This missing step was recently characterized by Lim *et al.* (2013). In particular, they showed that σ^{32} is associated to the inner membrane instead of being distributed throughout the cytoplasm, as previously thought. This membrane association is mediated by the signal recognition particle (SRP) in combination with the SRP receptor (SR). This cotranslational protein targeting machinery (SRP-SR), normally acting only on periplasmic and membrane proteins, specifically targets σ^{32} to the inner membrane, which is crucial for proper homeostatic control upon heat-shock. The homeostatic control region of σ^{32} is directly involved in this process. Using *in vivo* crosslinking assays, it was recently shown that the homeostatic control region of σ^{32} encompasses the binding site recognized by a subunit of SRP (Miyazaki *et al.* 2016). These latter findings allowed for revision of the model for activity and stability control of σ^{32} as shown in Fig. 2. It is worth noting that membrane localization of σ^{32} may allow the heat-shock regulator to monitor protein folding status both in the cytoplasm and in the membrane, linking cytoplasmic and membrane protein damage to the heat-shock response.

The regulation of gene expression through the use of alternative sigma factors guarantees a global transcriptional reprogramming of the bacterial transcriptome in response to changing environmental conditions. In this light, it is not surprising that σ^{32} has a broad regulon, controlling the transcription of hundreds of genes upon temperature increase. Several genome-wide studies focusing on the characterization of the σ^{32} regulon monitored gene expression changes upon inducible overexpression of σ^{32} using microarrays (Zhao, Liu and Burgess 2005; Nonaka *et al.* 2006). Besides known genes encoding proteases and chaperones involved in protein homeostasis, these analyses allowed the identification of σ^{32}-dependent targets involved in other key cellular processes. These processes include protection of both DNA and RNA, enhancement of transcription and translation at high temperature, as well as genes coding for other transcriptional regulators, thus propagating the response. Moreover, the finding that several σ^{32}-dependent genes have important roles in membrane protection and functionality suggested for the first time an intimate link between σ^{32}-regulation and membrane homeostasis (Nonaka *et al.* 2006). Combining chromatin immunoprecipitation with microarrays (ChIP-to-chip), Wade *et al.* (2006) identified *in vivo* several new σ^{32} promoters and compared their locations with the positions of σ^{70} promoters determined in a separate study. Notably, they demonstrated that the majority of σ^{32} promoters overlaps with σ^{70} promoters, with promoter elements typical of one σ factor interspersed within the specific elements of the other σ, and that they can be transcribed, both *in vivo* and *in vitro*, by the RNA polymerase associated with σ^{32} or σ^{70} (Wade *et al.* 2006). Even though these data confirmed previous observations on *rrnBP1* and *gapA* promoters (Newlands *et al.* 1993; Charpentier and Branlant 1994), the functional overlap between σ^{32} and σ^{70} was found to be much more extensive than expected. This promoter organization allows basal σ^{70}-dependent expression of many genes under normal conditions of growth to be boosted by σ^{32} upon temperature challenge.

Considering the huge reprogramming of *E. coli* transcriptome imposed by σ^{32}, a strategy that directly or indirectly targets this pleiotropic regulator might be exploited by any invading organism to manipulate host response. In fact, the first J-domain protein (i.e. a protein that contain a J-domain able to stimulate DnaK activity) encoded by a bacteriophage has recently been isolated (Perrody *et al.* 2012). Notably, this protein, named Rki, is expressed by the phage RB43 early after infection of *E. coli* and, by specifically interacting and interfering with the DnaK chaperone function, can stabilize σ^{32}, thereby promoting accumulation of heat-shock proteins. The authors proposed that σ^{32} stabilization by Rki could help phage growth, likely promoting middle- and late-gene transcription and/or facilitating the proper folding of phage proteins as a consequence of the GroESL chaperonin accumulation (Perrody *et al.* 2012).

Escherichia coli heat-shock sigma-E (σ^E)

Besides σ^{32}, the master regulator of heat-shock response, in *E. coli* a second heat-shock regulon is governed by another

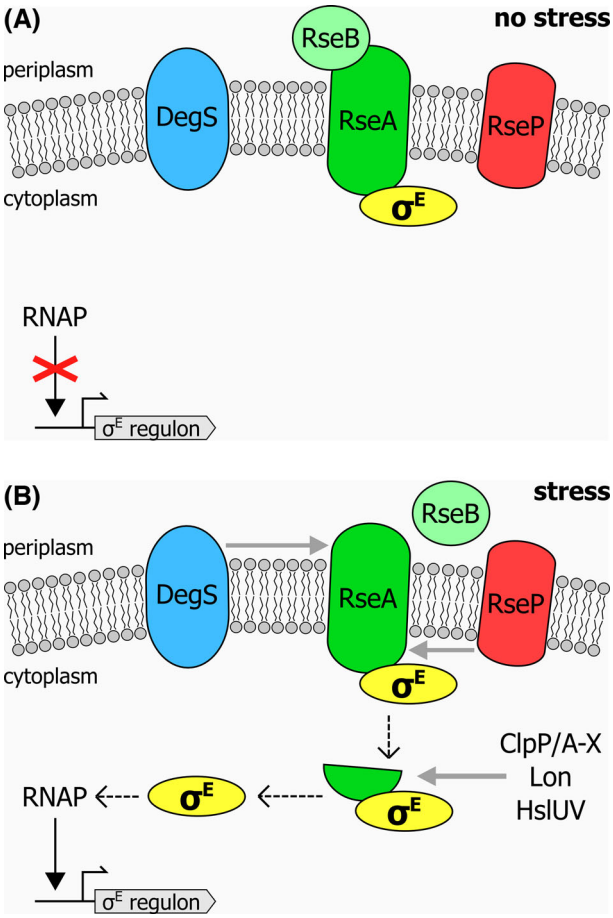

Figure 3. Schematic representation of the mechanism of regulation of σ^E activity. During normal growth conditions (**A**), σ^E is sequestered to the inner membrane by the concerted action of the antisigma factor RseA and the periplasmic co-antisigma factor RseB. As a consequence, genes belonging to the σ^E regulon are transcriptionally inactive. Upon stress (**B**), RseA is completely degraded by a cascade of regulated proteolytic events in which DegS and RseP membrane-associated proteases are primarily involved. Following the complete degradation of RseA by cytoplasmic proteases, σ^E is available for interaction with the RNA polymerase enzyme and the transcription of σ^E-specific promoters is rapidly activated.

alternative sigma factor named σ^E (also known as σ^{24}). This 24-kDa alternative σ factor, encoded by the leading gene of the tetracistronic *rpoE-rseA-rseB-rseC* operon, was initially isolated as the factor responsible for the transcription of *rpoH* P3 promoter (as reported in Fig. 1, left panel) with distinct promoter specificity from the known sigma factors (Mecsas *et al.* 1993). It is a member of the extracytoplasmic function subfamily of sigma factors that function as effector molecules responding to extracytoplasmic stimuli such as cell wall and membrane stress and the oxidation state (Missiakas and Raina 1998; Helmann 2002). Specifically, *E. coli* σ^E is heat inducible and responsive to stress in the extracytoplasmic compartment of the bacterial cell (like, for example, accumulation of misfolded proteins translocated across the cytoplasmic membrane into the periplasmic space) and its activity is governed by a complex mechanism based on regulated proteolysis (Mecsas *et al.* 1993). Under normal growth conditions, σ^E is tethered to the membrane and kept inactive by the antisigma factor RseA (Fig. 3A). RseB and RseC, co-expressed in the same operon together with σ^E and RseA, also take part in σ^E regulation. Specifically, while the mechanism of positive regulation exerted by the inner-membrane-associated RseC protein on σ^E activity still remains to be fully characterized (Missiakas *et al.* 1997), it was demonstrated that RseB is located in the periplasmic space and acts as a co-antisigma factor when a direct interaction with RseA is established (Cezairliyan and Sauer 2007). Upon stress, RseA is completely degraded by the sequential action of different proteases and σ^E is free to interact with the RNA polymerase core enzyme to direct transcription of a specific subset of promoters characterized by peculiar –10 and –35 consensus elements (Rhodius *et al.* 2006; Ades 2008) (Fig. 3B). In detail, upon stress signal perception, the first event of the regulatory cascade is the activation of DegS, a membrane-associated protease that cleaves the periplasmic domain of RseA only upon dissociation of RseB from the antisigma factor (Walsh *et al.* 2003; Grigorova *et al.* 2004; Lima *et al.* 2013). A second membrane-associated protease named RseP further digests RseA in its transmembrane region, releasing in the cytoplasm a truncated C-terminal portion of the antisigma factor still associated to σ^E (Kanehara, Ito and Akiyama 2002, 2003). The last step of the σ^E activation process involves other cellular proteases such as ClpP/X-A, Lon and/or HslUV that completely degrade the remaining undigested part of RseA, freeing σ^E in the cytoplasm (Chaba *et al.* 2007)(Fig. 3B). This membrane localization of σ^E, a strategy also employed to maintain other alternative σ factors in their inactive form when their activity is not necessary, differs from the membrane-localized state of σ^{32} described above, a mechanism used for the dynamic regulation of the principal *E. coli* regulator. The σ^E regulon consists of tens of genes, whose function is almost completely devoted to folding of proteins in the cell envelope and to biosynthesis/transport of lipopolysaccharide (LPS), a component of the outer membrane of Gram-negative bacteria (Dartigalongue, Missiakas and Raina 2001; Rhodius *et al.* 2006). In fact, in addition to several genes involved in LPS biosynthesis, some σ^E-regulated genes encode periplasm-localized proteins that act on misfolded polypeptides (such as, for example, the periplasmic protease HtrA/DegP), while other regulon members code for cytoplasmic proteins involved in coordinating environmental stimuli with expression of the σ^E regulon (Dartigalongue, Missiakas and Raina 2001; Rhodius *et al.* 2006). However, as in the case of σ^{32} described above, an extensive functional overlap has been observed between σ^E and the housekeeping σ^{70}. In fact, a substantial fraction of σ^E-transcribed promoters can also be bound by σ^{70}, demonstrating that in *E. coli* the wide overlap of alternative σ factors with σ^{70} is not limited to σ^{32} (Wade *et al.* 2006).

Negative transcriptional regulation of heat-shock genes

In addition to positive regulation by means of specialized σ factors, another strategy to control heat-shock genes transcription relies on dedicated repressors. Under normal laboratory growth conditions, these DNA-binding regulators bind specific operators and repress transcription of heat-shock genes, while upon heat stress, the transcription of these genes is rapidly induced. Promoters driving transcription of genes controlled by heat-shock repressors harbor –10 and –35 elements recognized and bound by RNA polymerase associated with the vegetative σ factor. The first indications of the existence of alternative mechanisms of transcriptional regulation that are different from heat-shock σ factors came from studies of *Bacillus subtilis* stress response. In this bacterium, it was initially thought that the σ^{28}-containing RNA polymerase controlled the transcription of heat-shock genes because of its overlapping promoter specificity with

E. coli σ^{32}-containing RNA polymerase (Briat, Gilman and Chamberlin 1985). Subsequent studies demonstrated that σ^{28} was dedicated to transcription of flagellar genes and to some genes coding for chemotaxis effectors and that different *B. subtilis* chaperone genes, *groESL* and *dnaK* among others, were preceded by vegetative promoter sequences (Li and Wang 1992; Wetzstein *et al.* 1992). Afterwards, Chang *et al.* (1994) demonstrated that transcription of *groESL* was dependent on the vegetative sigma factor, σ^{43}, indicating that other mechanisms of heat-shock gene regulation, different from positive strategies based on alternative σ factors, must exist. In the subsequent years, several heat-shock repressor proteins were identified and their mechanisms of regulation via interaction with conserved DNA elements located in the promoters of heat-shock genes were characterized. A common feature of heat-shock repressors is, besides the negative regulation of target genes, that they repress their own promoter. This regulatory scheme, known as negative autoregulation, is widely found among transcriptional repressors (negative autoregulation has been observed for about 50% of *E. coli* repressors) and guarantees fast response kinetics upon signal perception (Alon 2007). Consequently, upon heat stress and subsequent rapid induction of the controlled promoters, repressor concentration rapidly increases and reaches a threshold amount necessary for the re-establishment of the repressed state. In this way, the induction phase that rapidly appears upon heat stress is immediately followed by a shut-off phase of transcription.

Currently, it appears well established that negative transcriptional regulation through dedicated repressors constitutes an important and widespread mechanism employed by bacteria to control heat-shock gene expression, not necessarily as an alternative to positive regulation described above.

HrcA repressor

The observation that a conserved sequence element is embodied in the promoter regions controlling the transcription of class I heat-shock genes of *B. subtilis* (consisting of two operons, the bicistronic *groES-groEL* and the heptacistronic *dnaK* operon) paved the way to the identification and characterization of the HrcA repressor, one of the most widespread heat-shock negative regulators in bacteria. Specifically, an inverted repeat (IR) consisting of 9 bp separated by a 9-bp spacer was identified in the DNA region close to the transcription start site of *dnaK* and *groESL* genes (Schmidt *et al.* 1992; Wetzstein *et al.* 1992). The role of this IR as a negative *cis*-element in the control of chaperone expression was demonstrated by introducing mutational changes within the upstream, downstream or both halves of the IR preceding *dnaK* and observing increased expression at low temperature and a reduction in the stimulation of the operon after heat-shock (Zuber and Schumann 1994). Considering that the conserved IR was observed in many different bacterial species, always within promoters controlling chaperone genes, it was named CIRCE for controlling inverted repeat of chaperone expression. Even though the possibility was initially considered that the CIRCE sequence could act alone through the formation of a temperature-dependent secondary structure, the demonstration of involvement of a DNA-binding protein in CIRCE-dependent negative regulation followed soon after. In particular, the inactivation of *orf39*, the first gene of the *B. subtilis dnaK* operon, led to the up-regulation of *dnaK* and *groESL* even at physiological growth temperature (Schulz, Tzschaschel and Schumann 1995). Moreover, the characterization of regulatory mutants constitutively overexpressing *groE* and *dnaK* at low temperature revealed that all mutations map within *orf39*. The finding that cell extracts of *E. coli* overproducing Orf39 were able to specifically retard DNA probes harboring a CIRCE sequence in electrophoretic mobility shift assays (EMSA) clearly suggested that Orf39 was the negative regulator of chaperones expression in *B. subtilis*, likely acting as a CIRCE-specific DNA-binding repressor (Yuan and Wong 1995b). Similar observations were made for *Caulobacter crescentus*, where disruption of an *orf39* homologous gene led to the conclusion that it encoded a negative regulator and, thus, it was named HrcA for *heat-shock* regulation at CIRCE elements (Roberts *et al.* 1996). Direct interaction between HrcA protein and the CIRCE element was demonstrated in *B. subtilis* by showing that a recombinant purified HrcA was able to retard a labeled probe containing a CIRCE element in the EMSA assay (Mogk *et al.* 1997). Detailed biochemical studies aimed at the molecular level dissection of the HrcA-CIRCE interaction have been hampered in many cases by its instability and strong tendency to form insoluble aggregates. For this reason, HrcA binding to target promoters has been characterized in detail only in a limited number of cases. For example, in *Helicobacter pylori* the development of a dedicated expression and purification protocol allowed demonstration by DNase I footprinting that HrcA binds operator elements similar to the CIRCE sequences overlapping both *groESL* and *dnaK* core promoters (Roncarati *et al.* 2007a,b). On both target promoters, the protected regions cover about 30 bp, suggesting that a dimer of HrcA interacts with the DNA. A similar situation was also observed for *Chlamydia trachomatis*, where HrcA showed full protection of the CIRCE element and neighboring nucleotides on the *dnaK* promoter, covering about 40 bp of the DNA probe (Wilson and Tan 2004). In this latter microorganism, *in vitro* transcription assays of selected promoters carried out in the presence of different concentrations of purified HrcA demonstrated that it was able to specifically repress *dnaK* transcription in a concentration-dependent manner (Wilson and Tan 2002). The position of the CIRCE element is almost always in a region close to the transcription start site, overlapping the portion of the DNA contacted by the RNA polymerase during initiation of transcription. The mechanism of repression exerted by HrcA upon binding to the CIRCE element relies on the physical occupancy of the core promoter with a consequent impediment of RNA polymerase binding under physiological growth conditions (Fig. 4A). Consistent with this model, when the position of the CIRCE element of the *B. subtilis groE* promoter was shifted from the original position to + 4 further downstream to position + 25 with respect to the transcription start site, the negative regulatory effect mediated by this cis-acting element was abolished (Yuan and Wong 1995a). HrcA-mediated repression of heat-shock genes is generally responsive to different kinds of environmental stresses. In many cases, when bacterial cells are exposed to stressful conditions such as high temperature, high salt concentration or a condition that provokes accumulation of misfolded proteins in the cytoplasm, the transcription from HrcA-controlled promoters is rapidly derepressed (Schmidt, Hertel and Hammes 1999; Laport *et al.* 2004). Even though it is well established that CIRCE acts as a specific HrcA-binding site at the DNA level, it has also been observed that the IR affects the stability of the RNA transcript. Specifically, in *Rhodobacter capsulatus*, the secondary structure imposed by the CIRCE element on the *groESL* mRNA appears to differentially affect transcript stability at various temperatures (Jäger, Jäger and Klug 2004). To date, the crystal structure of the HrcA repressor of the hyperthermophile *Thermotoga maritima* provides the only detailed structural information for this heat-shock regulator. The 2.2 Å resolution crystal structure shows that *Tm*HrcA forms a dimeric structure, and each monomer is composed of a winged helix-turn-helix (HTH)

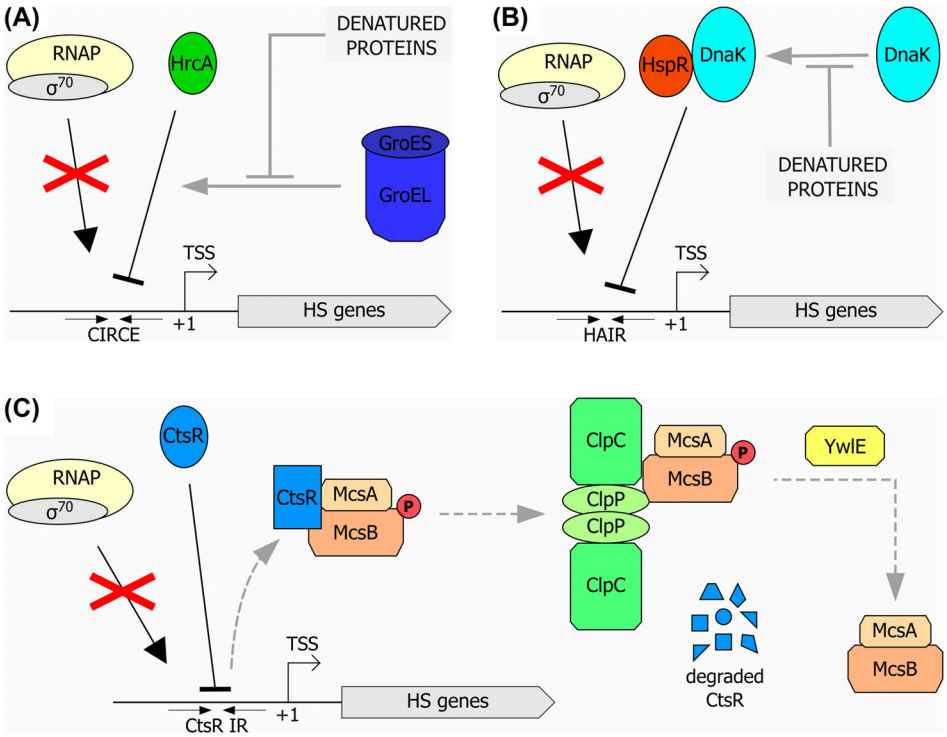

Figure 4. Negative regulation of heat-shock gene transcription by HrcA, HspR and CtsR repressors. (**A**) Under physiological growth conditions, HrcA binds the CIRCE conserved IR and represses transcription by hindering RNA polymerase promoter binding (depicted by black arrowhead). GroES-GroEL chaperonin interacts with HrcA and stimulates its DNA-binding activity. This GroE-mediated feedback control of HrcA activity is negatively modulated by accumulation of unfolded proteins during stress. (**B**) HspR-mediated repression of heat-shock genes at normal growth temperature is due to binding of the regulator to conserved HAIR sequences close to the core promoter, thereby interfering with RNA polymerase binding (indicated by a black arrowhead). In *S. coelicolor*, DnaK chaperone interacts with HspR and positively modulates its DNA-binding activity. Upon direct interaction between HspR and DnaK, the repressor becomes competent for promoter binding, while DnaK acts as a corepressor of HspR, with no direct contact to DNA. The HspR-DnaK functional interaction is negatively modulated by the accumulation of misfolded proteins in the cytoplasm that titrate away the chaperone. (**C**) Current model for the mechanism of repression and for the heat-dependent regulation of CtsR repressor activity. During control conditions, CtsR binds to conserved IR sequences and represses transcription of heat-shock genes (depicted by a black arrowhead). Following a sudden increase of temperature, CtsR undergoes a heat-induced structural alteration and loses affinity for DNA (indicated by a gray dashed line). During environmental stress conditions, upon temperature-dependent autophosphorylation, McsB becomes active and, assisted by its co-activator McsA, functions as an adaptor to target CtsR to ClpP/C-mediated proteolytic degradation. Upon dephosphorylation by the cognate phosphatase YwlE, McsB becomes deactivated.

N-terminal DBD, a central GAF-like domain (probably involved in dimerization rather than in cGMP binding) and a C-terminal inserted dimerizing domain. However, the conformation of both DBDs in the dimer suggests that the *Tm*HrcA is incompatible with DNA binding and may represent an inactive form of this protein (Liu *et al.* 2005). The detailed structure of the DBD, combined with results of mutagenesis studies of conserved residues (Hitomi *et al.* 2003; Wiegert and Schumann 2003), suggests that HrcA binding to DNA should involve the interaction of the recognition helix of the HTH domain with the DNA major groove and the interaction of a loop connecting two α-helices with the DNA backbone. It is worth noting that the amino acid sequence similarity of HrcA proteins from different species is surprisingly low. The overall sequence identity is often <30% with the exception of HrcA proteins from related organisms (Narberhaus 1999). Hence, the molecular features derived from the solved structure described above may be different among the various HrcA regulators. In order to identify additional genes regulated by HrcA besides the typical *groE* and *dnaK* genes, genome-wide expression studies have been combined with the search of conserved CIRCE elements and with *in vitro* DNA-binding studies. What generally emerges from such approaches is that HrcA is involved, directly or indirectly, in the regulation of several genes linked to various cellular processes. For example, a microarray-based transcriptome analysis performed in *Listeria monocytogenes* revealed that HrcA regulates, albeit indirectly, genes involved in stress response, metabolism, translation and DNA replication (Yuewei *et al.* 2007). Similar pleiotropic effects of *hrcA* mutation were observed with analogous approaches in other microorganisms, including *H. pylori* and *Lactobacillus plantarum* (Roncarati *et al.* 2007a; Van Bokhorst-van de Veen *et al.* 2013). However, the number of genes directly regulated by HrcA appears to be restricted and limited to genes involved in stress response. For example, in the human pathogen *Mycoplasma genitalium*, the genes encoding the stress proteases Lon and ClpB possess a conserved CIRCE element within their promoter regions, suggesting a HrcA-mediated negative regulation (Musatovova, Dhandayuthapani and Baseman 2006). Moreover, various lines of evidence indicate that the expression of *clp* genes in several *Lactobacillus* species is regulated by HrcA, instead of CtsR, the conserved *clp* gene regulator of low G+C Gram-positive bacteria (Suokko *et al.* 2005, 2008), while in *Streptococcus salivarus* these two heat-shock repressors are cooperative in controlling *clpP* gene transcription (Chastanet and Msadek 2003).

HspR repressor

The heat-shock transcriptional repressor HspR was first discovered and characterized in the *Streptomyces* genus. Specifically, it was shown that, in *Streptomyces coelicolor*, the distal gene of

the heat-inducible *dnaK* operon encoded a novel heat-shock protein, similar to the GlnR repressors of *Bacillus* spp. and to the MerR family of transcriptional regulators. *In vitro* DNA-binding assays were carried out to demonstrate that HspR was able to bind to three inverted repeat sequences (IR1, IR2 and IR3) in the *dnaK* promoter region, centred at positions –75, –49 and +4 with respect to the transcriptional start site. This strongly suggests a direct role for HspR in heat-shock gene regulation (Bucca *et al.* 1995). Genetic and biochemical evidence of HspR regulatory function followed soon after. In particular, it was shown that disruption of the *hspR* gene led to high-level constitutive transcription of *dnaK* operon. Moreover, the addition of an *E. coli* purified recombinant his-tagged HspR to *in vitro* transcription assays clearly demonstrated repression of *dnaK* transcription, providing additional evidence for direct involvement of HspR in *dnaK* regulation (Bucca, Hindle and Smith 1997). Similar observations were made in the same year in *S. albus* (Grandvalet, Servant and Mazodier 1997), where HspR was shown to bind an IR identical to IR3 of *S. coelicolor* in the promoter region of the protease gene *clpB* (Grandvalet, de Crécy-Lagard and Mazodier 1999). Genes similar to *hspR* were observed in several other bacterial species, and HspR-binding sites were detected upstream of various heat-shock genes of these microorganisms. The HspR-binding site was named HAIR (for *H*spR *A*ssociated *I*nverted *R*epeats) and a consensus sequence was proposed (Grandvalet, de Crécy-Lagard and Mazodier 1999). So far, HspR repressors have been found and characterized, to various extents, in several bacterial species and it is now well established that this represents, in addition to the widespread HrcA repressor, a widely employed system to negatively control heat-shock genes transcription. The HAIR sequences have been found, in most cases, in the vicinity of the core promoter region, often overlapping the –10 and/or –35 boxes (Bucca *et al.* 1995; Grandvalet, de Crécy-Lagard and Mazodier 1999; Stewart *et al.* 2002). This observation suggests that HspR prevents RNA polymerase binding at physiological temperature by a steric hindrance mechanism at the promoter (Fig. 4B). In some cases, however, HspR-HAIR interaction takes place far upstream of the core promoter region, in an atypical position for a transcriptional repressor (Spohn *et al.* 2004; Schmid *et al.* 2005). The long distance between the HAIR sequence and the core promoter –10/–35 boxes supports the hypothesis of a complex mechanism for HspR-mediated transcriptional repression, which encompasses more than simple steric occlusion of the promoter. In this respect, dual repression of some heat-shock genes mediated by HspR and HrcA in *H. pylori* represents a clear example of this mechanism. The distal binding sites of HspR on *groESL* and *dnaK* promoters (between positions –43/–120 and –78/–149 with respect to the transcriptional start site, respectively) combine with proximal HrcA/CIRCE interactions, occluding core promoter regions (Roncarati *et al.* 2007a, discussed in detail below). Another intriguing example of complex HspR-mediated regulation has been observed in *Mycobacterium tuberculosis*. In this intracellular human pathogen, the *acr2* gene, encoding a member of the widespread α-crystallin family of molecular chaperones, is transcriptionally controlled by HspR in combination with the response regulator PhoP (Singh *et al.* 2014). In this case, the heat-shock repressor HspR binds to the HAIR sequence located in the core promoter region (Stewart *et al.* 2002), while PhoP-binding region maps downstream from the transcriptional start site. Moreover, a direct HspR-PhoP interaction has been demonstrated, as well as the crucial importance of the simultaneous presence of both regulators for heat-shock-dependent *in vivo* regulation of *acr2* (Singh *et al.* 2014). Accordingly, the current model for *acr2* regulation postulates that under normal growth condition both the PhoP and HspR, bound to their target sites, form a higher-order DNA-protein structure that prevents RNA polymerase binding and the direct HspR-PhoP interaction provides additional stability to this complex.

Initially identified as the regulator of major chaperone genes such as *dnaK* in *Streptomycetes* spp., the identification of additional genes controlled by HspR in several different bacterial species was enabled by the advent of microarray techniques. In *S. coelicolor*, DNA microarray-based analysis of gene expression in wild-type and *hspR*-disruption mutant strains led to the identification of 17 genes controlled, directly or indirectly, by HspR (Bucca *et al.* 2003). This analysis was refined in a subsequent work by the same group, by implementation of a high-density microarray (Bucca *et al.* 2009). Specifically, gene expression analysis was combined with immunoprecipitation of *in vivo* HspR-binding sites and hybridization of the recovered DNA fragments on a DNA microarray chip (ChIP-on-chip). From this analysis, it emerged that, in *S. coelicolor*, HspR directly represses transcription of genes encoding chaperones (*dnaK-grpE-dnaJ-hspR* operon) and heat-shock proteases (*lon* and *clpB*), as well as one ribosomal RNA gene and two tRNA genes, thus broadening, for the first time, the regulatory role of HspR in this microorganism. In *H. pylori*, to investigate if the regulatory function of HspR is restricted to promoters of chaperone genes or if it is directly involved in controlling additional genes, an *in vitro* selection of genomic DNA fragments bound by purified HspR protein was developed (Delany *et al.* 2002). Besides the three previously characterized binding sites in the promoter regions of the multicistronic operons coding for the components of the DnaK and GroE machineries (Spohn and Scarlato 1999), two novel binding sites were identified, located in the 3′ region of both *speA* and *tlpB* genes, which encode an arginine decarboxylase and a methyl-accepting chemotaxis protein, respectively. Moreover, sequence alignment of novel HspR-binding sites highlighted HAIR-like sequences as well as conserved nucleotides extending outside the previously proposed consensus binding sequence. However, parallel macroarray hybridization of cDNA probes deriving from *H. pylori* wild-type and *hspR*-mutant strains failed to observe deregulation of these novel target genes, suggesting the existence of a minority of non-canonical binding sites, apparently not associated with regulatory functions (Roncarati *et al.* 2007a). In the last 15 years, the identification of members of the HspR regulon was also pursued through array-based whole transcriptome analyses in several other unrelated bacterial species including *Campylobacter jejuni*, *M. tubercolosis* and *Deinococcus radiodurans* (Stewart *et al.* 2002; Andersen *et al.* 2005; Schmid *et al.* 2005; Holmes, Penn and Lund 2010). Overall, these studies suggested that HspR directly represses, alone or in combination with other transcriptional regulators, the transcription of a limited set of genes that encode the major cellular chaperones and heat-shock proteases. However, indirect effects of *hspR* disruption affecting transcript abundance of genes involved in diverse cellular processes not strictly related to heat-shock have been observed. For example, in *H. pylori* and in the closely related *C. jejuni*, *hspR* deletion led to substantially lower expression of many genes coding for proteins involved in cell motility, and accordingly, *hspR*-mutant strain showed reduced motility (Andersen *et al.* 2005; Roncarati *et al.* 2007a). One hypothesis for the intersection of HspR-regulated heat-shock response and flagellar assembly may be that accumulation of chaperone proteins in the mutant strain alters the assembly of the flagellar apparatus and/or increases the activity of dedicated flagellar transcriptional regulators, which in turn establishes negative feedback for the programmed transcription of motility genes. Another

interesting example of HspR-mediated interplay between the heat-shock response and an unrelated central cellular process was observed in *Bifidobacterium breve* UCC2003 (Zomer *et al.* 2009). In this microorganism, an SOS response is induced by oxidative stress as well as by heat-shock. Repression of DNA damage repair genes is mediated by the LexA regulator, whose self-cleavage and consequent activation is promoted by the RecA protein. Induction of RecA expression is probably under the control of HspR under heat-shock conditions, an observation that directly links the repressor to a process employed by *Bifidobacterium* to respond to an excess of DNA damage (Zomer and van Sinderen 2010).

CtsR repressor

The characterization of *B. subtilis* heat-shock response revealed the existence of a subgroup of stress-responsive genes whose transcription is governed by two alternative promoters: one recognized by the RNA polymerase complexed with the vegetative sigma factor σ^A and the other is dependent on the general stress sigma factor σ^B. The transcription from both the vegetative σ^A- and σ^B-dependent promoters of this class III subgroup of genes (consisting of *trxA*, *clpC* and *clpP* operons) was induced by various stress signals, even though the induction pattern differed with respect to the particular gene and stress conditions (Krüger, Msadek and Hecker 1996; Gerth *et al.* 1998; Scharf *et al.* 1998). The observation that vegetative promoters of the above-mentioned genes remained stress inducible even on a σ^B-mutant background, together with the lack of a CIRCE consensus sequence typical of *B. subtilis* HrcA-regulated class I genes, prompted scientists to search for a novel heat-shock repressor responsible for this regulation. The first gene of the *clpC* operon, named *orf1*, was shown to encode a product with a predicted HTH DBD, suggesting a regulatory role for the protein (Krüger *et al.* 1997). This hypothesis was confirmed by the observation that transcript amounts of the class III *clpC* and *clpP* genes were significantly upregulated in a *orf1*-mutant strain compared to the wild-type under normal growth conditions, while class I (*dnaK*) and class II (*ctc*) genes were similarly expressed between the two strains (Krüger and Hecker 1998). The experimental confirmation of Orf1 being a direct transcriptional repressor came from EMSA assays carried out on a radiolabeled *clpC* operon promoter (Krüger and Hecker 1998). Subsequent studies by Derré, Rapoport and Msadek (1999) expanded the analysis of targets directly bound by the repressor, thereafter renamed CtsR for Class three stress gene repressor. Specifically, it was shown that CtsR binds a directly repeated heptanucleotide sequence positioned in close proximity of the transcription start site of *clpP* and *clpC* operons (Derré, Rapoport and Msadek 1999). Moreover, CtsR binding was shown to take place on five distinct binding sites nearby the *clpE* transcription start point, also postulating the existence of complex promoter architectures (Derré *et al.* 1999). Both CtsR homologs and target sequences were found upstream of *clp* and other heat-shock genes of several Gram-positive bacteria, an observation that suggested CtsR as a widespread transcriptional repressor involved in heat-shock regulation. In this respect, it was subsequently shown that, in *L. monocytogenes*, CtsR regulation is strikingly similar to that in *B. subtilis*, describing for the first time a stress response regulatory gene in a human pathogen (Nair *et al.* 2000). Specifically, it was demonstrated that *L. monocytogenes* CtsR represses the transcription of *clpP*, *clpC*, *clpE* and *clpB* genes by directly binding to their respective vegetative promoters (Nair *et al.* 2000; Chastanet *et al.* 2004). Considering that the positions of the binding sites characterized so far on several promoters of various bacterial species overlap the core promoter region, CtsR should exert its repressive function by sterically occluding RNA polymerase binding to DNA (Fig. 4C). The first indications that the prototypical *B. subtilis* CtsR could bind DNA as a dimer, evinced from analysis of the binding sites and from some biochemical studies (Derré *et al.* 2000), were further confirmed by the determination of the crystal structure of the repressor bound to a 26 bp DNA derivative of the *clpC* promoter (Fuhrmann *et al.* 2009). The crystal structure revealed that each protomer is characterized by an N-terminal winged HTH DBD and a C-terminal dimerization domain composed by four α-helices organized as a four-helix bundle. DNA recognition is based on the insertion of the HTH domain in the major groove and a concomitant interaction of the extended β-hairpin wing with the minor groove. Some crucial residues, located within the HTH domain and involved in DNA binding, were identified and found to be highly conserved in the CtsR protein family (Fuhrmann *et al.* 2009). The CtsR repressor is historically considered the master regulator of the cellular protein quality control genes of low-GC Gram-positive bacteria. This definition is based on the observation that CtsR typically controls the expression of genes encoding stress proteases mainly involved in degradation of misfolded/denatured polypeptides as well as of deleterious protein aggregates. For example, in the model organism *B. subtilis*, but also in the human pathogen *L. monocytogenes* and in the probiotic bacterium *Lactococcus lactis*, CtsR essentially controls the transcription of several components of the Clp machinery (Derré *et al.* 1999; Nair *et al.* 2000; Varmanen, Ingmer and Vogensen 2000; Chastanet *et al.* 2004), while the regulation of the classical molecular chaperones, such as DnaK and GroE, is HrcA dependent. In some cases, however, CtsR is also able to regulate the expression of HrcA-dependent genes. In *Staphylococcus aureus*, for example, *dnaK* and *groE* operons are coregulated by both CtsR and HrcA. These regulators contact adjacent binding sites and act synergistically to maintain low basal levels of expression of both operons in the absence of stress (Chastanet, Fert and Msadek 2003). In some other instances, as in *Oenococcus oeni*, CtsR solely regulates *clp* machinery genes and also GroE/DnaK molecular chaperones. This lactic acid bacterium lacks *hrcA* in its genome and CtsR has taken over the regulation of typical HrcA targets (Grandvalet *et al.* 2005). Interestingly, some studies have revealed new non-canonical genes regulated by CtsR. These include genes encoding small heat-shock proteins, such as Hsp18 in *O. oeni* and Hsp1 in *Lactobacillus plantarum*, the membrane-bound FtsH protease in *L. plantarum* and the *tatAC* genes that code for two minimal translocases responsible for the twin arginine translocation (Tat) pathway in *L. monocytogenes* (Grandvalet *et al.* 2005; Hu *et al.* 2007; Fiocco *et al.* 2009, 2010). The CtsR master regulator binds the promoters of target genes and represses their transcription under physiological growth conditions. Intriguingly, upon heat-shock, CtsR loses DNA-binding activity and the non-functional protein undergoes regulated proteolytic degradation mediated by the ClpCP protease (Elsholz *et al.* 2010) (Fig. 4C). The first indications that CtsR is a specific target of ClpPC under stress conditions came from *in vivo* stability assays performed in *B. subtilis* wild-type and in *clpP* or *clpC* mutant strains (Krüger *et al.* 2001). Specifically, Krüger and colleagues demonstrated that, after inhibiting translation with tetracycline, CtsR was much more labile in the wild-type strain, compared to mutant strains. It also became clear that the products of two genes belonging to the CtsR operon, McsA and McsB, play a crucial role in the proteolytic turnover of the repressor. In particular, *mcsB* encodes a heat-activated kinase that targets nonfunctional heat-inactivated CtsR, while *mcsA* codes for an activator of McsB

(Kirstein *et al*. 2005, 2007). The current model for CtsR proteolytic degradation, represented in Fig. 4C, postulates that, under normal growth conditions, McsB is kept inactive by interaction with ClpC. Upon heat exposure, McsB is titrated away from ClpC and becomes activated by temperature-dependent autophosphorylation and by interaction with McsA activator. Following this sophisticated dual activation step, McsB acts as an adaptor, targeting non-functional CtsR species and efficiently driving them to degradation. Active McsB adaptor is downregulated by either dephosphorylation by the cognate phosphatase YwlE or by rapid degradation of the phosphorylated form (Elsholz *et al*. 2010).

RheA repressor

RheA represents an example of heat-shock transcriptional repressor with a restricted distribution among bacteria, being found only in *S. albus*. The first indications that *S. albus* possessed an additional heat-shock transcriptional regulator, in addition to HrcA and HspR, came from the analysis of a DNA region upstream of the *hsp18* gene, encoding a heat-inducible protein belonging to the small heat-shock protein family. This open reading frame, named *orfY* and coding for a 225-amino acid polypeptide, is transcribed from a vegetative promoter in the opposite orientation to *hsp18*. Moreover, disruption of *orfY* led to the accumulation of *hsp18* transcript, even at low temperature, suggesting a direct or indirect involvement of OrfY in the transcriptional regulation of *hsp18* (Servant and Mazodier 1996). Additional biochemical and genetic studies provided evidence that OrfY was the direct repressor of *hsp18*. Thereafter, OrfY was named RheA for repressor of *h*eat-shock protein *e*ighteen. In particular, expression of *S. albus* RheA in *E. coli* determined repression of transcription from the *hsp18* promoter fused to *lacZ* as reporter gene. Moreover, a DNA probe encompassing the *hsp18* promoter was specifically retarded by a crude cell extract containing RheA in EMSA assays (Servant, Rapoport and Mazodier 1999). Considering that the transcription start sites of the divergent *hsp18* and *rheA* genes are separated only by 25 nucleotides and that the –10 boxes of the two promoters partially overlap, it was not surprising to discover that RheA also negatively autoregulates its own transcription (Servant, Rapoport and Mazodier 1999). The RheA repressor binds two IRs (a perfect IR TGTCATC-N_5-GATGACA, flanked by an imperfect IR GTCATC-N_5-GACGAC) located in the intergenic region of the divergent *rheA* and *hsp18* core promoters, thereby occluding the access to RNA polymerase and repressing both genes' transcription under normal growth conditions (Servant, Grandvalet and Mazodier 2000). RheA represents the first described transcriptional repressor of small heat-shock proteins, anticipating the similar scenario confirmed in *O. oeni* and *Clostridium acetobutylicum*, where *hsp18* is transcriptionally repressed by CtsR (Derré, Rapoport and Msadek 1999).

Feedback regulatory circuits modulate the activity of heat-shock repressors

Similar to the complex homeostatic control of σ^{32} in *E. coli* described above, feedback regulatory circuits of heat-shock transcriptional repressor activity mediated by chaperones have been observed in several bacterial species.

Soon after the identification and characterization of the HrcA repressor as a negative regulator of class I heat-shock genes in *B. subtilis*, experimental evidence pointed out the role of the GroE chaperonin as a modulator of HrcA regulon (Mogk *et al*. 1997). In particular, depletion of the intracellular level of GroESL was associated with high expression of the HrcA-controlled *dnaK* operon at all temperatures. By contrast, GroESL overexpression led to the hyper-repression of transcription from the target promoter. Moreover, EMSA assays showed that the affinity of HrcA binding to a DNA probe containing a CIRCE operator is increased in the presence of GroESL. It is worth noting that the shifted fragment migrated to the same position in the absence or presence of GroEL, indicating a role for the chaperonin in the modulation of HrcA DNA-binding activity rather than functioning as a co-repressor (Mogk *et al*. 1997). Following the formal demonstration of the direct interaction between HrcA and GroE *in vitro* (Reischl, Wiegert and Schumann 2002), a model was proposed for their functional interplay (Fig. 4A). This model, known as the 'titration model', postulates that HrcA is kept in an active conformation by GroESL, competent for binding to CIRCE elements. Upon stress insult, the accumulation of misfolded polypeptides in the cell sequesters and titrates away the chaperonin, which can no longer interact with the HrcA repressor. Without the presence of bound chaperonin, HrcA loses DNA-binding affinity and repression of class I heat-shock genes is relieved. This model is also supported by the finding that addition of ethanol, treatment with puromycin (that causes accumulation of truncated polypeptides in the cytoplasm) and overexpression of substrates of GroESL derepress the HrcA regulon (Mogk *et al*. 1998). The same feedback mechanism operates in *C. trachomatis* (Wilson *et al*. 2005), where the interaction between HrcA and GroE was investigated in more detail. It is worth noting that *C. trachomatis* harbors in its genome three genes coding for GroEL homologs: *groEL1*, *groEL2* and *groEL3*. These genes are expressed constitutively throughout the developmental cycle of the bacterium (Karunakaran *et al*. 2003) and differentially regulated in response to heat-shock. In fact, while transcription of *groEL1* is negatively regulated by the HrcA-CIRCE system and induced upon temperature increase, transcription of *groEL2* and *groEL3* is not responsive to heat-shock nor to HrcA regulation (Karunakaran *et al*. 2003; Hanson and Tan 2015). In this system, the characteristic additional C-terminal tail of chlamydial HrcA interfered with repressor binding to the CIRCE element *in vitro* and also with HrcA-mediated transcriptional repression *in vitro* and *in vivo* (Chen, Wilson and Tan 2011). Intriguingly, the negative effect exerted by the HrcA C-terminal inhibitory region could be counteracted by GroE. Specifically, it was shown that recombinant GroEL was able to enhance HrcA activity (HrcA-binding activity to CIRCE elements *in vitro* as well as HrcA-mediated repression *in vivo*) and that this effect was more pronounced on the full-length HrcA rather than on the truncated version (without the C-terminal tail) of the repressor. Intriguingly, the positive effect of recombinant GroEL on HrcA-binding activity to its operator was shown to be ATP independent, suggesting a non-canonical mechanism of action of the chaperonin (Chen, Wilson and Tan 2011). The characterization of the GroESL-mediated feedback control of HrcA repressor activity in several distant bacterial species, like for example in *C. crescentus* and *H. pylori* (Susin *et al*. 2004; Roncarati *et al*. 2007a), suggests that it may represent a general mechanism to post-transcriptionally control repressor functionality in response to environmental stimuli.

The hypothesis that homeostatic control of transcriptional regulators by chaperones might represent a novel common theme in heat-shock gene regulation is confirmed by the characterization of the feedback control of the heat-shock master repressor HspR by DnaK. The DnaK-HspR functional interaction was initially characterized in *S. coelicolor*. It was shown that the *in vitro* formation of a stable complex between HspR and its DNA target was DnaK dependent, suggesting that DnaK stimulates HspR DNA-binding activity (Bucca *et al*. 2000). Surprisingly, the

DnaK-mediated modulation of HspR was shown to be independent from co-chaperones DnaJ and GrpE and required no addition of ATP, thereby excluding a mechanism based on DnaK chaperoning function. *In vitro* EMSA assays showed the formation of a ternary complex composed of DnaK interacting with HspR bound to DNA. Thus, *S. coelicolor* DnaK acts as a corepressor of HspR, with no direct contact with DNA (Bucca *et al.* 2000). This role of DnaK in heat-shock gene regulation was further confirmed both *in vitro* and *in vivo*. In particular, it was shown that DnaK was able to stimulate HspR-mediated repression in *in vitro* transcription assays (Bucca *et al.* 2000). Moreover, *in vivo* depletion of cellular DnaK led to high-level expression of HspR-repressed promoters at normal growth temperature (Bucca *et al.* 2003). All these findings converged in a model of DnaK feedback control of HspR activity that resembles, with some differences, the titration model valid for GroE-HrcA (Fig. 4B). That is, under normal environmental conditions DnaK interacts with HspR and the ternary complex binds to target DNA, resulting in tight transcriptional repression. During heat-shock, DnaK is sequestered by unfolded proteins that accumulate in the cytoplasm and HspR is less efficiently able to repress the transcription of target genes whose expression results induced. More recently, a similar DnaK-mediated feedback loop controlling HspR activity has been investigated and characterized in *M. tubercolosis*, although with some important differences. In this bacterium, DnaK interacts directly with the HspR-HAIR complex and stimulates repressor DNA-binding activity in an ATP-independent manner (Parijat and Batra 2015). However, in this pathogen HspR modulation does not depend solely on DnaK as in *S. coelicolor*. Evidence supports an important role played also by the GroE chaperonins GroEL1 and GroEL2 in the HspR activation process (Das Gupta, Bandyopadhyay and Das Gupta 2008; Bandyopadhyay *et al.* 2012).

In the context of feedback regulation of heat-shock repressors, *H. pylori* represents a peculiar example in which two chaperones modulate the activity of two distinct transcriptional regulators. As described above, *H. pylori* uses both HrcA and HspR repressors for the regulation of heat-shock response. However, while HrcA activity is feedback regulated by the GroESL chaperonin according to the typical 'titration model' proposed for *B. subtilis*, the DNA-binding activity of HspR is negatively modulated by the heat-shock protein CbpA, rather than by DnaK (Fig. 5). In analogy with the homologous protein of *E. coli* (Chae *et al.* 2004; Bird *et al.* 2006), the HspR-repressed *cbpA* gene is thought to encode a dual-function protein, working both as a DnaJ-like co-chaperone of DnaK and as a nucleoid-associated protein involved in nucleoid structuring function. In order to characterize a possible post-transcriptional control over HspR by the DnaK chaperone system, surprisingly it was found that, upon direct protein–protein interaction, CbpA alone was able to negatively modulate HspR binding to target promoters *in vitro* without contacting the DNA, but only when the repressor was not bound to its operators. In addition, overexpression of CbpA led to deregulation of heat-shock response *in vivo* (Roncarati, Danielli and Scarlato 2011). These findings suggest important considerations and add new perspectives in heat-shock gene regulation. First of all, the effect of CbpA on HspR DNA binding (negative effect) is opposite to the modulation typically exerted by DnaK or GroE on HspR and HrcA (positive effect), implying that the accepted model for chaperone feedback regulation of transcriptional repressors is insufficient to explain CbpA-HspR functional interaction in *H. pylori*. A possible explanation is that CbpA regulation of HspR-binding activity is adopted by *H. pylori* to fine-tune the regulatory network shutoff response governing heat-shock response (Roncarati, Danielli and Scarlato 2011). Second, considering the putative functions of CbpA as both a co-chaperone and a nucleoid-associated protein (preliminary results suggest that CbpA of *H. pylori* possesses both functions; D. Roncarati, personal communication), it would be informative to characterize the functional interplay between HspR and CbpA. In other words, an intriguing hypothesis is that the heat-shock regulator HspR itself, aside from its role in the repression of transcription regulated by CbpA, might influence the co-chaperone and/or nucleoid-associated activity of CbpA by direct protein–protein interaction (Fig. 5). This would be a novel example in which heat-shock gene regulation intersects with distinct cellular functions, as for example the maintenance and regulation of the bacterial nucleoid.

Cross-comparison of heat-shock transcriptional repressors

Experimental data available so far allow to highlight similarities as well as differences among the major heat-shock transcriptional repressors described in the previous sections. Starting from the mechanism of regulation, they all repress

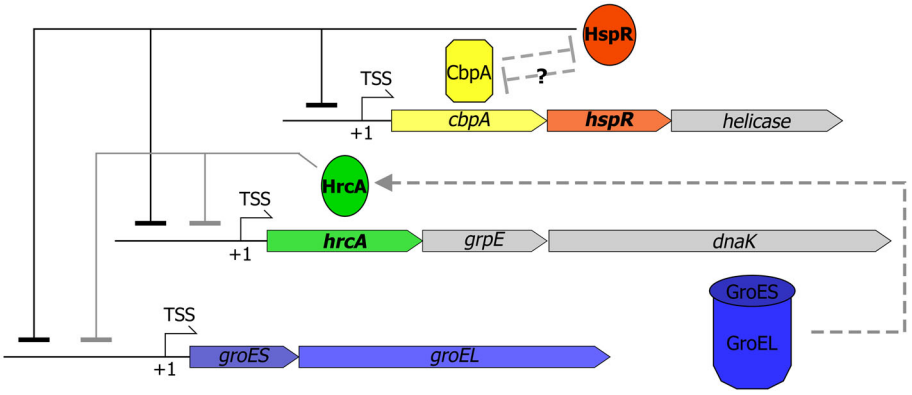

Figure 5. Model for HrcA and HspR-dependent heat-shock gene regulation in *H. pylori*. Two dedicated transcriptional repressors, HrcA and HspR, directly repress three multicistronic operons containing the major chaperone genes of *H. pylori*. Specifically, the *cbp* operon encodes a homolog of the *E. coli* curved DNA-binding protein A (CbpA), the heat-shock transcriptional repressor HspR and a protein with putative helicase function; the *dnaK* operon encodes the heat-shock transcriptional repressor HrcA, the co-chaperone GrpE and the DnaK chaperone; the *gro* operon encodes the chaperonin system GroES-GroEL. Positive regulation of HrcA activity by the GroESL chaperonin in depicted by a gray dashed arrow, while negative modulation of HspR by CbpA is highlighted by a grey dashed hammerhead. The hypothetical negative effect of HspR on CbpA activity is indicated by a grey dashed hammerhead marked with a question mark.

transcription, under normal growth conditions, by sterically occluding RNA polymerase binding to target promoters. In almost all cases, in fact, these repressors interact with the target promoters by binding conserved sequences that overlap core promoter elements, such as –10 and –35 boxes. A closer inspection of their DNA-binding architecture reveals some differences. While HrcA-regulated promoters typically contain a single CIRCE IR and are bound by a dimeric form of the repressor, other promoters are controlled in a more complex manner by HspR and CtsR repressors. For instance, *B. subtilis clpE* promoter, but also *S. coelicolor* and *H. pylori dnaK* promoters harbor multiple repressors' binding sites spread all over the 5′ control region, suggesting the existence of complex mechanisms of transcriptional regulation. Another common feature shared by all heat-shock repressors is their negative autoregulation. Negative autoregulation is a recurrent regulatory pattern, also called network motif, found in ∼40% of the known transcriptional regulators in *E. coli* (Thieffry *et al.* 1998). This network motif has been suggested to increase the homeostasis of the autoregulated gene product in the context of stochastic gene expression noise, to speed up the response kinetics upon signal perception and to provide more linear dose responses (Becskei and Serrano 2000; Rosenfeld, Elowitz and Alon 2002; Nevozhay *et al.* 2009). In the context of heat-shock gene regulation, negative autoregulation ensures tight control of steady-state repressors amount under normal environmental conditions, followed by a prompt induction upon heat-shock of regulators' gene transcription. Many more peculiarities emerge when considering the post-transcriptional regulation of repressors activity and stability/turnover mediated by chaperones and proteases, respectively. As shown in Fig. 4, while CtsR and RheA activity does not appear to be feedback modulated by molecular chaperones, both HrcA and HspR interplay with distinct and specific chaperone systems, whose actions add an additional level of regulation. On the other hand, the repressors' turnover that takes place following heat stress has been deeply characterized for CtsR (shown in Fig. 4C), while detailed information for the other heat-shock repressors is still missing. In this respect, Jastrab *et al.* (2015) have recently identified a novel pathway responsible for the degradation of the heat-shock repressor HspR in *M. tubercolosis*. Specifically, they showed that the heat-shock repressor is actively degraded by the prokaryotic proteasome encoded by this human pathogen, and that degradation is enhanced by an ATP-independent proteasome activator, called PafE, and by HspR denaturation.

Combinations of heat-shock transcriptional regulators

The transcriptional regulation of heat-shock genes displays varying degrees of complexity among various microorganisms, reflecting the extreme diversity of genetic regulatory mechanisms in bacteria.

In several bacteria, heat-shock transcriptional regulation is managed exclusively by a dedicated σ factor that enables the RNA polymerase enzyme to transcribe specific genes important for the response to temperature stress (Fig. 6A, left panel, and Table 1). In such simple cases, all the thermoresponsive genes are under the control of a single regulator. For example, in *Pseudomonas aeruginosa* and in *Vibrio cholerae*, transcriptional regulation of heat-shock genes is governed by an alternative sigma factor specific for heat stress, similar in sequence and function to *E. coli* σ^{32} (Allan *et al.* 1988; Slamti, Livny and Waldor 2007). As described above, the model organism *E. coli* also adopts exclusively positive regulators for the heat-shock response. Here, two alternative σ factors (σ^{32} and σ^{E}) are involved in heat-shock regulation, with σ^{32} playing a major role in sensing cytoplasmic and inner-membrane stimuli, while σ^{E} is committed to extracytoplasmic (envelope) stress response (Fig. 6A, left panel, and Table 1).

In other bacterial species, the coexistence of positive and negative control mechanisms, together regulating the expression of distinct sets of heat-shock genes, has been observed. For example, in the Gram-positive pathogen *L. monocytogenes*, there are three groups of heat-shock genes, each one controlled by a different strategy (Fig. 6A, central panel). In detail, genes belonging to class I (encoding members of the major chaperone systems DnaK and GroE) and to class III (encoding chaperones and ATP-dependent Clp proteases) are negatively regulated by HrcA and by the CtsR heat-shock repressor, respectively. In contrast, genes belonging to class II (coding for general stress proteins) are positively controlled by the alternative sigma factor σ^{B} (Table 1) (van der Veen *et al.* 2007). A similar situation, in which positive and negative mechanisms are both employed to control separate sets of genes, has been described in other bacteria such as *B. subtilis* and *Agrobacterium tumefaciens* (Table 1) (Nakahigashi *et al.* 1999; Schumann 2003). Intriguingly, in cyanobacteria, both positive and negative strategies of transcriptional regulation combine to control expression of some heat-shock genes (reviewed by Rajaram, Chaurasia and Apte 2014). For instance, in *Synechocystis* PCC6803, the *groESL* operon is negatively regulated by the HrcA/CIRCE system and positively regulated by the alternative sigma factors SigE and SigB (Nakamoto, Suzuki and Kojima 2003; Singh *et al.* 2006; Kojima and Nakamoto 2007). Furthermore, the histidine kinase Hik34 is involved in the negative regulation of the chaperonin gene (Slabas *et al.* 2006; Červeny *et al.* 2015). In addition to transcriptional mechanisms, RNA-based regulation of heat-shock genes has also been reported in cyanobacteria. Expression of the *hsp17* gene, coding for a small heat-shock protein also known as HspA, is transcriptionally dependent on the alternative sigma factors SigE and SigB (Singh *et al.* 2006; Tuominen *et al.* 2006, 2008), and post-transcriptionally controlled by a temperature-sensitive RNA structure in the 5′-UTR portion of the gene (Table 1) (Kortmann *et al.* 2011).

The regulatory schemes found in bacteria that adopt only negative mechanisms of transcriptional regulation could be very simple. However, combinations of repressors and overlaps of their regulons are not rare and sometimes lead to complex regulatory networks. One of the simplest regulatory scheme is employed, for example, by the human pathogen *M. genitalium* (Fig. 6A, right panel). This Gram-positive bacterium, which has the smallest genome among self-replicating free living organisms, possesses a single housekeeping σ factor and manages heat-shock gene regulation exclusively through the HrcA repressor (Table 1) (Musatovova, Dhandayuthapani and Baseman 2006). Similarly, a single transcriptional repressor, CtsR, controls heat-shock response in the lactic acid bacterium *O. oeni* (Grandvalet *et al.* 2005) (Fig. 6A, right panel, and Table 1). A common manner of combining negative regulators is based on the exploitation of several transcriptional repressors, each specifically controlling a particular set of genes. In *B. subtilis* and closely related species, the HrcA regulator is in charge of keeping *groESL* and *dnaK* genes repressed under normal growth conditions, while Clp protease-encoding genes are regulated solely by the master repressor CtsR (Table 1) (Chastanet *et al.* 2001). An extreme example is represented by *S. albus*, a soil bacterium that uses an arsenal of three different repressors to specifically regulate distinct sets of heat-shock genes without any cross-regulation and regulons overlap

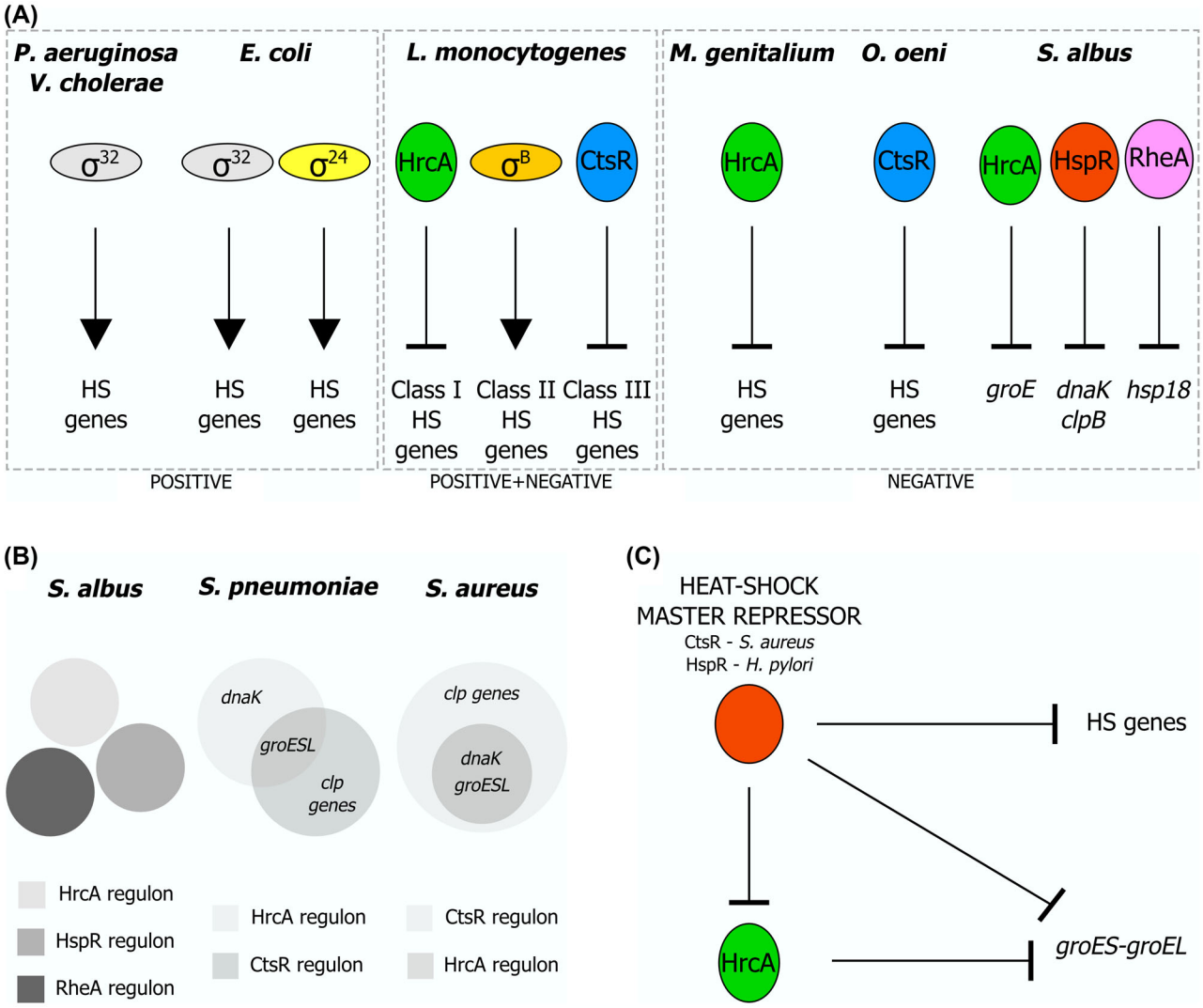

Figure 6. Combinations of heat-shock transcriptional regulators and complex regulatory circuits. (**A**) While in some bacterial species heat-shock transcription is controlled exclusively by positive (left panel) or negative (right panel) mechanisms of regulation, in several cases positive and negative control strategies coexist (central panel). Black arrows indicate positive regulation, while black hammerheads show negative regulation. (**B**) Examples in which distinct transcriptional repressors are employed to control heat-shock gene expression. In *S. albus* HrcA, HspR and RheA repressors regulate separate sets of genes, whereas in *S. pneumoniae* HrcA and CtsR regulons partially overlap. In *S. aureus*, the HrcA regulon is completely embedded within the CtsR regulon. (**C**) Schematic representation of the incoherent feed-forward loop governing heat-shock gene regulation in *S. aureus* and *H. pylori*. A master regulator, shown in red (HspR for *H. pylori* or CtsR for *S. aureus*), represses several heat-shock genes. Among these genes, the *groESL* operon is also repressed by the HrcA regulator, which is, in turn, under the control of the master repressor.

(Fig. 6A, right panel, and Fig. 6B). Specifically, while *groEL* regulation involves HrcA (Grandvalet, Rapoport and Mazodier 1998), *dnaK* and *clpB* genes are repressed by HspR (Bucca, Hindle and Smith 1997; Grandvalet, Servant and Mazodier 1997) and the *hsp18* gene is transcriptionally controlled by RheA (Table 1) (Servant, Rapoport and Mazodier 1999). However, in several other bacterial species, the regulons of two different heat-shock repressors partially overlap and this results in some genes being simultaneously controlled by more than one regulatory protein, rendering these systems more complicated than the examples described above. HrcA is most commonly involved in those situations, partnering with CtsR in some bacteria, while in some other cases it interacts directly with HspR. A typical example is the dual regulation of *groESL* by CtsR and HrcA in *S. pneumoniae* (Fig. 6B). In this microorganism, the *groESL* 5′ region harbors a highly conserved CIRCE operator, which is the specific recognition sequence for HrcA-binding mapping immediately downstream from the transcription start site. Also, there is an upstream CtsR-binding site overlapping the –10 and –35 hexamers (Chastanet *et al.* 2001). The two binding sites are in close proximity, separated by only 16 bp. This tandem operator arrangement, involving a crucial region for transcription initiation, results in the *S. pneumoniae groESL* operon being under dual repression by both HrcA and CtsR (Fig. 6B). Accordingly, it was demonstrated that, in a mutant strain lacking CtsR, transcription of *groESL* was just slightly increased at 37°C, consistent with functional HrcA repression (Chastanet *et al.* 2001). This non-redundant dual repression mechanism, also employed very similarly by *S. salivarus* for the regulation of *groESL* and *clpP* (Table 1) (Chastanet and Msadek 2003), allows transcription of these genes to be maintained at very low levels in the absence of stress by the synergistic action of both CtsR and HrcA and to be fully induced by both regulators upon signal perception. Also, in *S. aureus*, CtsR and HrcA combine to control the expression of *groESL* and *dnaK*,

Table 1. Regulatory mechanisms controlling heat-shock genes' transcription in a panel of bacteria.

Organism	Transcriptional regulator	Regulated genes
Agrobacterium tumefaciens	RpoH	*groESL*, *dnaK-dnaJ*, *grpE*, *clpP* and others
	HrcA	*groESL*
Bacillus subtilis	HrcA	Class I: *groESL*, *dnaK-dnaJ*, *grpE*, and others
	σ^B	Class II: genes coding for general stress proteins
	CtsR	Class III: *clpP*, *clpE*, *ctsR-mcsA-mcsB-clpC* and others
	Unknown	
	CssS/CssR	Class IV: *htpG*
	Unknown	Class V: *htrA*, *htrB* and others
		Class VI: *ftsH*, *clpX* and others
Escherichia coli	σ^{32} (σ^H, RpoH)	*groESL*, *dnaK-dnaJ*, *grpE*, *ibpA* and others
	σ^E (σ^{24})	*degP*, *clpX*, *lon* and others
Helicobacter pylori	HspR	*cbpA-hspR-helicase*, *groESL*, *hrcA-grpE-dnaK*
	HrcA	*groESL*, *hrcA-grpE-dnaK*
Listeria monocytogenes	HrcA	Class I: *groESL*, *dnaK*, and others
	σ^B	Class II: genes coding for general stress proteins
	CtsR	Class III: *clpP*, *clpB*, *hslU* and others
Mycoplasma genitalium	HrcA	*dnaK*, *lon*, *clpB*
Oenococcus oeni	HrcA	*groESL*, *dnaK*, *clpP*, *hsp18* and others
Pseudomonas aeruginosa	σ^H (RpoH)	*groESL*, *dnaK*, and others
Staphylococcus aureus	HrcA	*hrcA-dnaK*, *groESL*
	CtsR	*clpP*, *clpC*, *clpB*, *hrcA-dnaK*, *groESL*
	σ^B	genes coding for general stress proteins
Streptococcus pneumoniae	HrcA	*groESL*, *dnaK-dnaJ*, *grpE*
	CtsR	*groESL*, *clpP*, *clpC*, *clpE*
Streptococcus salivarus	HrcA	*clpP*, *groESL*, *dnaK*
	CtsR	*clpP*, *groESL*, other clp genes
Streptomyces albus	HrcA	*groESL1*, *groEL2*, *dnaJ2*
	HspR	*dnaK*, *clpB*
	RheA	*hsp18*
Synechocystis PCC6803	HrcA	*groESL*, *groEL-2*, *dnaK2* and others
	SigB	*groESL*, *groEL-2*, *dnaK2*, *hspA*, *htpG* and others
	SigE	*groESL*, *groEL-2*, *dnaK2*, *hspA*, *htpG* and others
	HiK34	*groESL*, *dnaK2*, *htpG*
Vibrio cholerae	σ^{32} (RpoH)	*groESL*, *dnaK-dnaJ*, *lon*, *clpB*, and others

but they interact in an even more peculiar and complex way (Fig. 6B). In particular, *S. aureus* CtsR alone represses the transcription of several *clp* genes, but also combines with HrcA to control expression mediated by the *dnaK* and the *groESL* operons. Moreover, since *hrcA* is the first gene of the *dnaK* operon, this results in the HrcA regulon being entirely embedded within the CtsR regulon (Table 1) (Chastanet, Fert and Msadek 2003). As in *S. pneumoniae*, CtsR- and HrcA-specific operators are organized in tandem and overlap the region adjacent to the transcription start site, allowing non-redundant dual repression during normal growth. Strikingly, an almost identical regulatory network has been described in the distantly related Gram-negative human pathogen *H. pylori*, where HrcA combines with the HspR repressor (Fig. 5). Here, HspR alone represses transcription of its own tricistronic operon (Spohn and Scarlato 1999), while, in combination with HrcA, it controls the expression of *groESL* and *hrcA-grpE-dnaK* operons (Spohn *et al.* 2004). Hence, similarly as for *S. aureus*, this results in the *H. pylori* HrcA regulon being embedded within the regulon of a second master heat-shock repressor (i.e. HspR instead of CtsR). A detailed analysis of *in vitro* DNA binding by HrcA and HspR revealed that their operators are arranged in tandem, consistent with the typical architecture for dually regulated promoters, but that the position of the HspR operator maps to far upstream from the core promoter to an atypical position for a repressor (Roncarati *et al.* 2007a). Moreover, even though both repressors are required for regulation (a single *hspR* or *hrcA* mutation leads to complete derepression of the dually controlled promoters), binding *in vitro* to their respective adjacent operators occurs in an independent, non-cooperative manner. An interesting explanation for the peculiar interaction of heat-shock repressors found in *H. pylori* and *S. aureus* can be inferred by considering a logical scheme involving both regulators (Fig. 6C). In both organisms, there is a master regulator (HspR or CtsR) which directly regulates another regulator (HrcA) and a target gene (*groESL*), which, in turn, is regulated by both HrcA and the master regulator (HspR or CtsR). Moreover, considering that all three regulatory interactions are repressive, these circuits seem to represent rare examples of incoherent type-2 feed forward loops (Alon 2007; Danielli, Amore and Scarlato 2010). This peculiar network motif is employed to modulate the dynamic behavior of the circuit, greatly speeding up the transcriptional response of target genes upon input stress signals. Some experimental observations of *H. pylori* sustain this hypothesis. In fact, transcription of *groESL* was rapidly induced (2 min) upon heat-shock in a wild-type genetic background, while in a mutant strain in which the binding site of HspR on the *groESL* promoter was deleted (and, so, the direct connection between HspR and *groESL* was interrupted), the derepression of *groESL* transcription was observed only 60 min after temperature challenge (Spohn *et al.* 2004). Even though further experimental

characterizations are needed to dissect the dynamic properties of regulatory circuits such as those described above, they could reflect specific evolutionary adaptations to particular needs encountered by bacteria in their specific niches.

The examples described above provide a complex picture of heat-shock genes' regulation and raise interesting questions about their role and evolution. Even though, in many cases, clear experimental evidences are still missing, some speculations can be put forward. For instance, the presence in the same organism of diverse regulatory mechanisms involved in the control of distinct as well as overlapping sets of genes might have evolved to allow bacteria a proper response to distinct stresses. Alternatively, such regulatory systems might account for a 'differential response' in terms of both stress severity and differential pattern of gene expression in response to a stress insult. The latter aspect has been observed in *S. albus*, where different sets of heat-shock genes are controlled by different repressors (Fig. 6A). Specifically, two different regulatory patterns have been observed, consisting of constitutive synthesis of GroEL and Hsp18 (regulated by HrcA and RheA, respectively) at high temperature and transient heat-shock-induced synthesis of HspR-regulated ClpB and DnaK proteins (Mazodier *et al.* 1991). A heat-shock intensity-dependent response (differential response) of some regulatory systems or individual genes has been documented in different bacterial species, including *M. tuberculosis* and *Corynebacterium glutamicum* (Young and Garbe 1991; Engels *et al.* 2004). The use of diverse regulatory systems by the same organism to control different heat-shock genes becomes particularly relevant and interesting when it involves the differential regulation of multiple copies of genes coding for the GroEL chaperonins. It has been observed that a significant proportion of bacterial genomes contains two or more chaperonin genes and, in several instances, these genes appear to be differentially regulated (Lund 2009). The current notion is that, where present, these multiple chaperonins encoded by duplicated *groE* genes have evolved a degree of subfunctionalization and the differential regulation observed may reflect the various contexts in which they are needed or the ecological niche they have to cope with. For example, in *Bradyrhizobium japonicum*, a nitrogen-fixing and roots-nodulating bacterium belonging to the Alphaproteobacteria group, the seven chaperonin genes present in its genome show complex pattern of transcriptional regulation, not limited to heat-shock response. At least one of these chaperonin genes has been shown to be transcriptionally regulated by the NifA activator and by σ^{54}, which are both involved in the regulation of nitrogen fixation genes under limited oxygen conditions (Fischer 1994). Genetic analyses provide additional evidences for a link between the regulation of some chaperonins, root nodulation and nitrogen fixation, even though the specificity of chaperonin novel functions appeared not absolute (Lund 2009). A similar scenario of differential regulation of multiple chaperonin genes linked to the evolution of chaperonins' specialized functions has also been proposed for other bacterial groups, including Cyanobacteria and Chlamydia (Lund 2009).

MECHANISMS OF HEAT SENSING

The ability of bacteria to rapidly respond to sudden temperature increase depends on heat-sensing mechanisms that integrate environmental cues to activate appropriate response pathways. To date, various mechanisms of thermoregulation have been described in bacteria and they involve nearly all classes of biomolecules including lipids, proteins and nucleic acids (i.e. both DNA and RNA). All of these classes can act as thermosensors that detect changes in the environmental temperature and initiate relevant cellular responses. Heat-sensing mechanisms can be direct, by which the temperature directly affects the activity of the sensing biomolecule, or can be indirect, by which the consequences of a sudden temperature increase (for example, the accumulation of misfolded proteins in the cytoplasm) are detected. Even though temperature is a ubiquitous signal that influences several cellular pathways, this chapter focuses mainly on sensing mechanisms that trigger heat-shock gene expression, and just a few examples of thermosensors involved in the regulation of virulence determinants will be described (extensively reviewed by Klinkert and Narberhaus 2009; Shapiro and Cowen 2012).

Temperature sensing through RNA

The most rapid way of changing gene expression in response to temperature variations is based on a *cis*-regulatory element that is part of the mRNA encoding the heat-shock protein to be regulated. This mechanism of thermoregulation guarantees a very fast response upon signal perception because temperature affects the translation efficiency of both the intracellular pool of mRNA molecules as well as transcription already in progress. The general principle is based on the formation of zipper-like, temperature-sensitive secondary structures that characterize the mRNAs subjected to this kind of regulation (Fig. 7A). In particular, at physiological temperature the 5′ region of this category of mRNA forms a structure that hinders sequence elements crucial for the initiation of translation, such as the ribosome-binding site (also known as the Shine-Dalgarno sequence) and the translation start codon. When such sequence elements are involved in a secondary structure, the recognition and binding of the transcript by the ribosome is hampered, thereby negatively affecting translation of the mRNA. Upon a temperature increase, the secondary structure goes through a rearrangement or partial melting. As a consequence, ribosomes can easily gain access to the 5′ mRNA region and translation is enhanced. Several temperature-sensing RNA sequences, also known as RNA thermometers, have been discovered and characterized in some details in the last two decades and were recently reviewed in a comprehensive and detailed article by Kortmann and Narberhaus (2012). The first RNA thermometer was discovered in *E. coli* and regulates the expression of the heat-shock alternative sigma factor σ^{32}, already discussed above. It represents one of the most complex RNA thermometers known so far, with an extended secondary structure that is not confined only to the RpoH mRNA 5′ region (Fig. 1A). Specifically, although in the characterized secondary structure the Shine-Dalgarno sequence is partially exposed, translation of RpoH mRNA is hindered at low temperature by an intramolecular pairing between the 5′ untranslated region and a portion of the coding sequence immediately downstream the AUG start codon (Kortmann and Narberhaus 2012). However, much simpler RNA thermometers exist, as exemplified by the one controlling the expression of the *Bradyrhizobium japonicum hspA* gene. This *cis*-regulatory sequence constitutes the founding member of the most abundant class of RNA thermometers called ROSE for *r*epression *o*f heat-shock genes *e*xpression. ROSE elements are typically involved in the regulation of small heat-shock proteins and they have a length ranging from 60 to about 120 nucleotides. ROSE elements were noticed as a conserved DNA element preceding several heat-shock genes in various rhizobia. Initially, it was speculated that they act at the DNA level and their regulatory mechanism was

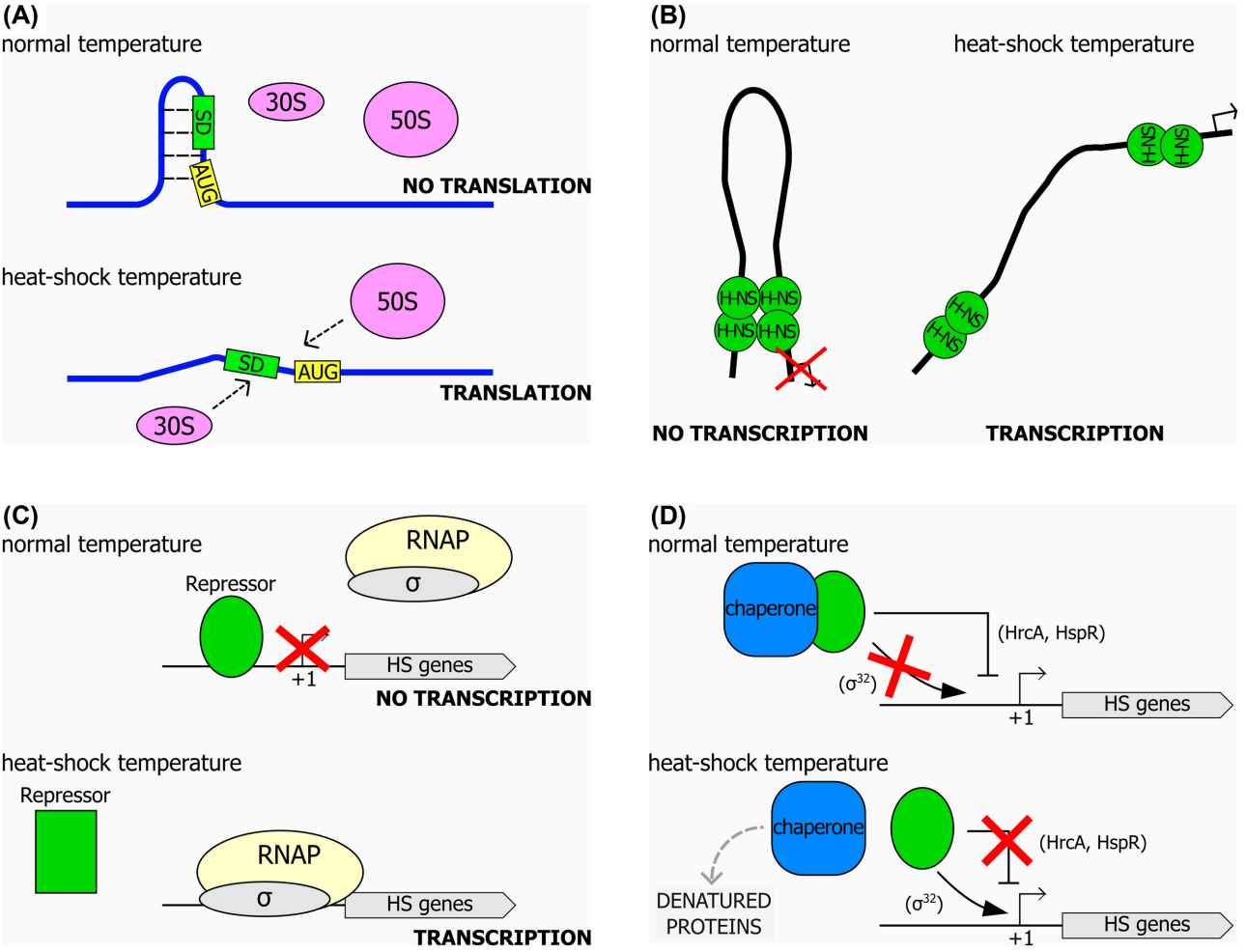

Figure 7. Mechanisms of heat sensing. (**A**) Sensing through RNA involves temperature-sensitive secondary structures in the 5′ region of the mRNA subjected to this kind of regulation. During normal growth conditions, a secondary structure forms and sequence elements crucial for efficient initiation of translation are masked and poorly accessible for ribosome binding. Upon temperature increase, a structural rearrangement or resolution of the structure exposes such sequence elements and translation is enhanced. Symbols: SD, Shine-Dalgarno sequence element; AUG, translation start codon. (**B**) Temperature sensing through DNA: model of temperature-dependent regulation of *virF* transcription in *S. flexneri*. The DNA region flanked by the H-NS binding sites assumes a curvature at low temperature that allows contacts between H-NS dimers bound to separate binding sites and the formation of a repressive nucleoprotein complex. At higher temperature, this curvature weakens and the promoter is more accessible to RNA polymerase for *virF* gene transcription. (**C**) Transcriptional regulators as intrinsic heat sensors. Intrinsic heat-sensing repressors are competent for DNA binding at permissive temperature (green oval), and transcription of heat-shock genes is repressed. Upon heat challenge, a temperature-induced structural transition (shown by a green rectangle) lead to a decrease of repressor DNA-binding activity and transcription is derepressed. (**D**) Indirect heat-sensing mechanism mediated by chaperones. The DNA-binding activity of transcriptional regulators is modulated by chaperones. During normal growth conditions, the chaperone interacts with the heat-shock regulator and exerts its regulatory function. Several positively modulated repressors (such as HrcA and HspR in some bacterial species) gain DNA-binding activity upon chaperone interaction, while in other instances the interaction with the chaperone results in the sequestration of the activator (for example, *E. coli* σ^{32}). Following heat stress, chaperones are sequestered by misfolded proteins that accumulate in the cytoplasm and transcriptional regulators are released and their DNA-binding activity results are positively or negatively affected.

thought to be dependent on the binding of a repressor protein (Narberhaus *et al.* 1998). Noting that it was possible to predict a similar secondary structure for all 15 known ROSE elements, a post-transcriptional mechanism was proposed and verified by a detailed mutational analysis of a ROSE-*hspA*-*lacZ* translational fusion (Nocker *et al.* 2001). Subsequent extensive molecular and structural characterizations revealed some key features of such kinds of RNA thermometers (Chowdhury *et al.* 2003, 2006). Interestingly, it was shown that ROSE-based RNA thermometers comprise two to four stem loops and that they can gradually respond to temperature variation and provide differential levels of expression regulation according to the severity of heat stress. Moreover, the dissection of the crucial intramolecular interactions involved in secondary structure formation revealed that, even though ROSE elements are characterized by a conserved short stretch of nucleotides (U(U/C)GCU), non-canonical base pairs also seem to be crucial for fine differential temperature sensing. Additional examples of RNA thermometers containing ROSE or ROSE-like elements have also been found in other bacterial species such as *Caulobacter*, *Salmonella* and *Escherichia coli*. In the latter species, a ROSE-like RNA thermometer controls the temperature-dependent expression of the small heat-shock protein IbpA (coding for an inclusion body binding protein), whose transcription is σ^{32} dependent (Nocker *et al.* 2001; Waldminghaus *et al.* 2009). Another class of RNA thermometers is based on a short stretch of four conserved uridines that base pair with the AGGA nucleotide sequence constituting the Shine-Dalgarno sequence. This element, called fourU, was initially

characterized in *Salmonella enterica*, where it controls the temperature-dependent expression of the *agsA* gene that encodes for a small heat-shock protein (Waldminghaus *et al.* 2007). Contrary to ROSE elements, the formation of the double-stranded RNA secondary structure in fourU elements is governed solely by canonical base pairing (Kortmann and Narberhaus 2012). Moreover, a peculiar feature of the fourU elements, derived during a detailed structural investigation of the *agsA* thermometer, is that the melting process in response to temperature fluctuation is dependent on the concentration of Mg^{2+} ions, due to the presence of an Mg^{2+}-binding site placed directly on the fourU element (Rinnenthal *et al.* 2011). It is worth noting that thermoregulation based on RNA-sensing structures has not evolved exclusively with respect to heat-shock genes. Several pathogenic bacteria, which are subjected to shifts in temperature during their life cycle (from environmental temperature of 28°C–30°C to body temperature of 37°C or even higher during infection), can modulate the expression of some virulence-associated genes in response to temperature variations using RNA thermometers (Grosso-Becera, Servín-González and Soberón-Chávez 2015). For example, in the obligate commensal of the human nasopharynx *Neisseria meningitidis*, RNA thermometers were found in the 5′ region of three genes involved in the meningococcal resistance against immune killing. Specifically, *cssR*, *fHbp* and *lst* genes (the first coding for an enzyme involved in the exopolysaccharide biosynthesis, the second coding for the host complement regulator factor H-binding protein and the third encoding a protein necessary for LPS sialylation) harbor RNA thermometers adjacent to the Shine-Dalgarno sequence that enhance their expression at 42°C, a temperature that is reached in the nasopharynx during *Neisseria* and viral co-infections (Loh *et al.* 2013; Barnwal *et al.* 2016).

Temperature sensing through DNA

In some cases, temperature variations can be directly sensed by the DNA of the bacterial cell. Several physiological metabolic pathways of a microorganism are influenced by the external conditions experienced in its ecological niche. In turn, the metabolic state of the cell is influenced, including the ADP to ATP ratio. As a consequence, enzymes that require ATP as a co-factor, such as DNA gyrase, will be affected. This enzyme, whose activity is strictly linked to the topological state of the DNA, is dependent on ATP and inhibited by ADP (so change in the ATP:ADP ratio influences DNA gyrase activity). For this reason, external fluctuating conditions that affect metabolic processes, including osmotic and heat-shock, can ultimately influence the global level of DNA supercoiling (Hsieh, Burger and Drlica 1991; Dorman and Corcoran 2009). Considering that DNA supercoiling can influence gene transcription, DNA can be considered as a thermosensor of environmental temperature change acting through variations of the global topological state of the chromosome in response to external stimuli. One of the primary parameters of DNA topology that responds to temperature changes is plasmid supercoiling. In particular, it was demonstrated both in mesophilic and hyperthermophilic bacteria that sudden temperature variations lead to transient changes in plasmid DNA topology and this effect, in turn, has a significant impact on transcription efficiency (López-García and Forterre 1997). In some other cases, however, local DNA structures mediate temperature sensing and affect transcription of neighboring genes. Some DNA sequences identified in *E. coli* and in several other bacteria are characterized by a sequence specific topological conformation that is able to assume various conformations in response to temperature variation. When these DNA regions are proximal to promoters, the local conformational variation induced by heat-shock is transduced into a modulation of RNA polymerase binding efficiency, thereby affecting the transcription of the downstream genes (Nickerson and Achberger 1995). An example is represented by the region upstream of the *plc* gene, encoding the phospholipase C (PLC) in *Clostridium perfrigens* (Katayama *et al.* 1999). In this case, the three phased A-tracts that precede the PLC encoding gene confer a strongly bent conformation to this DNA sequence. By using *in vitro* transcription assays, it was demonstrated that the stimulatory effect on the promoter activity of the A-tract sequence was temperature dependent, probably due to changes in the bending angle upon temperature fluctuations. Besides the direct activation of transcription mediated by bent DNA through the facilitation of RNA polymerase binding, temperature-dependent local DNA curvatures can indirectly regulate the efficiency of transcription by affecting the interaction of proteins with a regulatory role on gene expression. In this respect, nucleoid-associated proteins such as IHF, Fis, HU and H-NS have been shown to be involved in this kind of process. One of the best studied examples concerns the temperature-dependent regulation of *virF* transcription in *Shigella flexneri*, a pathogenic bacterium able to invade human intestinal epithelium (Fig. 7B). The AraC-like VirF regulator, a protein that triggers the activation of several genes with invasive functions, must be expressed only after the shift from the outside environment to the host. It has been demonstrated that the transcription of the *virF* promoter is kept repressed at non-permissive temperatures (below 32°C) by the nucleoid-associated protein H-NS (Colonna *et al.* 1995). Moreover, it was shown that the *virF* promoter harbors two H-NS binding sites separated by an intrinsically bent region, whose existence was predicted *in silico* and demonstrated by *in vitro* experimental results (Falconi *et al.* 1998; Prosseda *et al.* 2004). Intriguingly, upon temperature increase, this DNA region undergoes an abrupt structural transition (at ~32°C) that affects H-NS accessibility to target DNA sites and represses the *virF* promoter (Prosseda *et al.* 2004). A similar interplay between temperature-dependent DNA bending and H-NS binding was demonstrated to modulate hemolysin gene expression in the model organism *E. coli* (Madrid *et al.* 2002) and to enhance the expression of a type III secretion system above 30°C in *S. enterica* (Duong *et al.* 2007).

To summarize, even though several examples of temperature-dependent regulation of gene expression point to the role of DNA as a sensor biomolecule, it appears that this mechanism of heat sensing is much more pertinent to regulating the expression of virulence genes rather than in the heat-shock response.

Sensing through proteins

Bacteria can also sense and respond to temperature fluctuations in their ecological niches by using proteins as heat sensors. Different classes of proteins, including kinases, heat-shock transcriptional repressors and chaperones, have been characterized as sensors of temperature changes (Klinkert and Narberhaus 2009). However, the two latter categories of proteins are primarily employed to transduce heat stress signals that trigger a transcriptional heat-shock response. Intrinsic heat-sensing transcriptional repressors are able to directly modulate the transcription of target genes in response to temperature fluctuation. Specifically, they are competent for promoter binding and repress gene transcription only at physiological temperature. Upon heat-shock, these thermosensing repressors undergo

a conformational change that lowers relative binding affinity for their operators. As a consequence, target gene transcription becomes derepressed (Fig. 7C). With respect to the previous point, CtsR, the global repressor of heat-shock genes of *Bacillus subtilis* and other low-GC Gram-positive bacteria, is one of the best characterized examples (Elsholz *et al.* 2010). *In vitro* DNA-binding assays carried out at different temperatures showed that CtsR-binding activity to the *clpC* promoter drastically drops under heat-shock conditions (50°C), compared to normal growth temperature at 37°C. Interestingly, the temperature-dependent loss of DNA-binding activity of CtsR was demonstrated to be reversible. In fact, when CtsR was first incubated at a non-permissive temperature and then at 37°C, the regulator regained DNA-binding activity. In the case of CtsR, the temperature-dependent conformational change responsible for the loss of DNA-binding activity was shown to be limited to a short glycine-rich loop region within the DBD, constituting the precise functional site for heat sensing (Elsholz *et al.* 2010). The *Streptomyces albus* heat-shock repressor RheA described above was shown to be capable of sensing temperature variations in the absence of other factors, behaving similarly to CtsR. Upon heat challenge, RheA loses DNA-binding activity and the transition from active to inactive form was related to a temperature-induced reversible conformational change (Servant, Grandvalet and Mazodier 2000). A similar paradigm of temperature-dependent DNA-binding capacity was also clearly demonstrated for the virulence-associated RovA regulator of *Yersinia pestis* and for the TlpA regulator of *S. enterica* serovar Typhimurium (Hurme *et al.* 1997; Herbst *et al.* 2009). In the latter example, in particular, temperature reversibly affects the DNA-binding competent coiled-coil domains of the oligomerized state of the protein, thereby affecting DNA-binding ability. Another widespread heat-shock repressor, HrcA, can act, in some cases, as an intrinsic protein thermometer. The observation that the thermal denaturation profiles of *B. subtilis* and of *B. thermoglucosidasius* HrcA-CIRCE complexes (assayed by light scattering) were highly consistent with the different growth temperatures of the two microorganisms led to the proposal that the HrcA repressor might have a role as a thermosensor (Hitomi *et al.* 2003). A direct role as thermosensor was recently demonstrated for the HrcA repressor of *Helicobacter pylori*. Specifically, *in vitro* DNaseI footprinting assay results showed that HrcA-mediated DNA binding is strictly temperature dependent (Roncarati, Danielli and Scarlato 2014). When *H. pylori* HrcA was exposed to temperatures above 37°C (physiological growth temperature), it dramatically lowered binding affinity to CIRCE operators and became essentially inactive. Intriguingly, the loss of binding activity upon heat-shock treatment appeared to be irreversible *in vitro*, likely as a consequence of the major structural change as the temperature exceeded 40°C. However, the situation is different *in vivo*, where HrcA is able to recover its repressive function after the temperature challenge (Roncarati, Danielli and Scarlato 2014). Furthermore, it was demonstrated that the GroESL chaperonin plays a key role and that it is able to restore HrcA-binding activity lost upon heat challenge. The working model for HrcA-mediated heat sensing in *H. pylori* postulates that, after a sudden increase of temperature, heat-mediated inactivation of the regulator leads to promoter derepression and increased transcription of the heat-shock gene promoters. Then, following the induction step, at least a fraction of the denatured repressor is refolded by GroESL and can take part, together with the newly synthetized HrcA, in transcriptional regulation of the target genes. Considering the key role of GroESL in regulating HrcA-binding activity upon heat sensing, this functional interaction could be exploited by *H. pylori* to adjust the transcriptional response under various stress conditions. That is to say that, depending on the severity of a particular stress stimulus, the amount of GroE chaperonin available for the functional interaction with HrcA will change, influencing the restoration of target gene regulation. A different situation was observed for *Chlamydia trachomatis* HrcA-dependent heat-shock gene regulation. In this obligate intracellular human pathogen, HrcA binds to CIRCE operators within *groESL* and *dnaK* promoters and represses their transcription under normal growth conditions. Heat-shock gene transcription is then induced upon a sudden temperature increase. However, it was shown that HrcA-binding activity to CIRCE was not affected by elevated temperature *in vitro* (Wilson and Tan 2004). Recently, Hanson and Tan (2015) developed a method based on ChIP to study heat-shock gene regulation during an intracellular infection, and they were able to monitor *in vivo* HrcA binding to target promoters at various temperatures. This approach allowed demonstration that, upon a sudden temperature increase, the *in vivo* induction of heat-shock gene transcription was due to a decrease of HrcA binding to *groESL* and *dnaK* promoters. The discrepancy between the *in vitro* and *in vivo* results for HrcA temperature sensitivity suggests that elevated temperature is not sufficient *per se* to modulate chlamydial HrcA-binding capacity and implies that additional *in vivo* factors must be involved in the regulation of the HrcA-mediated heat-shock response. Based on previous observations of a functional interaction between HrcA and GroE (Wilson *et al.* 2005), it has been proposed that the chaperonin is the actual heat sensor of the regulatory circuit. The latter example represents a well-characterized mechanism employed in several cases that link environmental stress signals to a corresponding transcriptional response. This signal-sensing system does not directly detect the temperature increase; rather, the consequences of heat-shock on cellular proteins are sensed. Specifically, this strategy, represented in Fig. 7D, is based on the direct interaction between heat-shock transcriptional regulators and the major cellular chaperones. DNA-binding activity of transcriptional regulators is positively or negatively regulated by chaperones that are available for interaction during normal growth conditions. Upon sudden temperature upshift, chaperones are titrated away by denatured proteins that accumulate in the cytoplasm and transcriptional regulators are released. As described above (Figs 2 and Fig. 4A and B), the activity of HrcA and HspR repressors of several bacterial species, but also of the heat-shock σ^{32} of *E. coli*, is regulated in this way by the principal cellular chaperones such as the GroE chaperonin and DnaK-DnaJ-GrpE system (Straus, Walter and Gross 1990; Babst, Hennecke and Fischer 1996; Mogk *et al.* 1997; Bucca *et al.* 2000; Reischl, Wiegert and Schumann 2002; Wilson *et al.* 2005). According to this indirect heat-sensing mechanism, chaperones, and not transcriptional regulators, are the actual 'stress sensors'.

An interesting aspect emerging from the examples of the sensing mechanisms described above is that, in several cases, the regulation of the heat-shock response involves both direct temperature sensing (through RNA thermometers and intrinsic heat-sensing transcriptional regulators) and indirect chaperone-mediated sensing of unfolded proteins. These composed systems allow bacteria to respond differentially, depending on severity and type of environmental stresses. For example, RNA thermometers can detect minor temperature variations allowing the system to immediately respond, before cellular processes are altered. In this respect, *E. coli* σ^{32} regulation represents a perfect example. Here temperature-regulated translation of RpoH mRNA combines with two feedback loops that

control σ^{32} activity and stability. An interesting implication of these combined systems of signal sensing is that cells might discriminate heat stress from any other type of stress. Consequently, bacteria might differentially induce the expression of specific set of genes, thus increasing specificity of stress response.

CONCLUSIONS

This review highlights the importance of a sudden response of various bacterial species to temperature changes and summarizes the key mechanisms that diverse bacteria adopt to counteract imminent cellular damage. This highly conserved defense mechanism against environmental stress, known as the heat-shock response, appears to be controlled by a plethora of different strategies in bacteria. Control of heat-shock gene transcription is successfully and efficiently achieved by the use of specialized regulatory proteins exerting their positive or negative action on the initiation of transcription by the RNA polymerase enzyme. Strikingly, positive and negative mechanisms of regulation, alone or in combination, are employed by microorganisms to rapidly reprogram gene transcription upon stress perception. Additionally, control of gene transcription can be combined with posttranscriptional mechanisms of protein expression.

The model organism *Escherichia coli* has long served as the central paradigm for understanding the mechanism of stress induced activation of heat-shock gene transcription based on the use of alternative sigma factors. After decades of intense research on the heat-shock sigma factor σ^{32}, it appears that a complex multilayered regulatory cascade orchestrates σ^{32} homeostasis, in which transcriptional and translational control combines with chaperone mediated modulation of σ^{32} stability. Moreover, the recent discovery of inner membrane localization of the heat-shock sigma factor mediated by the SRP-SR co-translational targeting system adds another key part to this intricate story. However, further efforts are needed to understand several mechanistic aspects of the transit of σ^{32} to the membrane.

Negative regulation of heat-shock gene transcription through dedicated repressors has been studied in detail in several bacterial species. Different aspects of the major heat-shock repressors have been characterized, such as their DNA-binding mechanism, the characterization of their regulon and their biochemical properties (such as, for example, their heat-sensing capabilities). In this respect, it is worth noting that the way in which the master heat-shock repressor HspR senses fluctuations of environmental temperature remains elusive. In fact, while for HrcA, CtsR and RheA several studies pinpointed the direct or indirect (chaperone-mediated) mechanisms of heat sensing, the HspR repressor appears to be a heat stable protein and its interplay with the DnaK chaperone complex could be experimentally demonstrated in only a few cases. Further studies should be carried out to understand how temperature can modulate the DNA-binding activity of HspR.

In several cases, heat-shock regulatory circuits appear to be built on very complex structures in which mechanisms of transcriptional control combine with several feedback loops, whose effects on the properties of the regulatory systems are hardly predictable. In this respect, the question arises as to whether the use of such complex regulation strategies in the heat-shock systems is the result of evolutionary accidents or it derives from specifically designed solutions to different requirements in the field of heat-shock response. Rigorous analyses have been carried out with systems biology approaches and computational simulations of *E. coli* heat-shock response (El-Samad *et al.* 2005; Kurata *et al.* 2006). As described above, the *E. coli* heat-shock response involves complicated interactions, where both the activity and stability of the alternative sigma factor σ^{32} are feedback controlled by chaperones and proteases, respectively. From these studies, it emerges that the use of feedback loops gives valuable properties to the system, in terms *in primis* of robustness, defined as the capability of a system to operate reliably when its physical parameters vary within their expected ranges. Furthermore, the use of FtsH protease-mediated degradation feedback appears to provoke a faster response to a heat stimulation and to limit the effects of biochemical noise (El-Samad *et al.* 2005; Kurata *et al.* 2006). Further similar studies will undoubtedly be crucial for the investigation and characterization of other complex heat-shock regulatory schemes.

Another intriguing aspect of heat-shock gene regulation that deserves further characterization concerns the combination of different transcriptional regulators that establish highly complex regulatory schemes. In particular, the cases of *Staphylococcus aureus* and *Helicobacter pylori*, for which two repressors are employed and the regulon of one repressor is embedded within the regulon of the other repressor, represent rare examples of regulatory networks whose functional implications remain to be defined.

The recent development and application of the 'omics' techniques will allow further detailed characterization and understanding of the heat-shock regulons specifically governed by various heat-shock regulators. Functional genomics studies are expected to deepen and expand our knowledge of the impact of heat stress on the bacterial transcriptome and of the regulatory mechanisms that control such responses.

FUNDING

This work was supported by Grants from the Italian Ministry of Education, University and Research (2010P3S8BR_003) and by a grant from the University of Bologna.

Conflict of interest. None declared.

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