Part One
From Model Systems to Crop Improvement

1
General Stress Response of a Model Bacterium

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1.1
Introduction

Microorganisms have evolved to perform optimally in their normal habitat and they can attain very high growth rates under ideal conditions. Owing to their adaptational skills microorganisms set out the boundaries of the biosphere, and microbial habitats can include extreme environments such as hot water springs, cold water lakes, oceanic trenches, salt lakes, extreme acidic or alkaline locations, and so on [1]. Growth of most known microorganisms is, however, restricted to more moderate conditions and a shift to unfavorable surroundings inflicts a cellular stress that, depending on the severity, can kill them. In fact, the confrontation with stressful situations is quite common in nature and these nonextremophiles have acquired many different strategies to respond to a number of stresses [2, 3]. Dedicated stress responses exist that allow mesophilic bacteria such as *Escherichia coli* to cope with specific stress conditions for a particular period by repairing the stress-induced damage. A typical example of such a response is the induction of the SOS regulon triggered by DNA damage. This SOS response governs the expression of a variety of genes encoding repair functions, error-prone polymerases, and a cell division inhibitor, which all cooperate to repair the incurred DNA damage and restore growth after repair [4]. By contrast, the ''general stress response,'' which will be the focus of this chapter, is triggered by a wide variety of stresses and renders bacteria resistant to a broad variety of environmental insults. In fact, this response is rather preventive than reparative [5]. Over the last 20 years, the general stress response of the model bacterium *E. coli* has been the subject of intense and continuous study, and serves as a paradigm for the level of systemic complexity that can be reached in prokaryotic cells.

1.2
General Stress Response

In their natural environment bacteria are usually faced with a limited availability of nutrients and, as a consequence, starvation is one of the most prevailing stresses

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encountered [6, 7]. Under nutrient starvation, bacteria arrest growth and enter a stationary phase during which the cells reprogram their gene expression, change their metabolism, and start to exhibit a distinctive resistance toward a whole range of adverse environmental conditions, including low pH, high osmolarity, and low temperature [6, 8, 9]. At the molecular and genetic level, these physiological changes are established by an alternative sigma factor, σ^S or RpoS, which is the master regulator of the general stress response.

Sigma factors are able to direct the specificity of the transcription machinery to a dedicated subset of promoters and changing the sigma factor associated with the RNA polymerase (RNAP) can correspondingly bring about a drastic reprogramming of the cell's expression profile. During the stationary phase, σ^S is able to hijack the RNAP from the regular housekeeping σ^{70} factor that predominates during steady-state growth and to direct expression of about 500 genes, some of which indirectly [10–12].

1.2.1 The σ^S Regulatory Network

Since the recent genome-wide expression analysis of Weber *et al.* [10] revealed that up to 10% of the *E. coli* genes are under direct or indirect control of σ^S, it is becoming clear that the general stress response constitutes a global regulatory network rather than a regulon [5, 13]. In fact, multiple connections exist between the σ^S network and other global regulons such as the cAMP/cAMP receptor protein (CRP) global regulon. Indeed, more than half of the σ^S-controlled genes contain a putative cAMP/CRP-binding site in their promoter regions and even *rpoS* expression itself is under the cAMP/CRP control (see Section 1.3.1). Moreover, a large number of σ^S-controlled genes in turn encode regulatory proteins that increase the possibility of interconnectivity and a hierarchical structure between various regulatory networks [10].

The σ^S positively regulated genes can be divided into a core set of genes that are controlled by most σ^S-inducing conditions and different subsets or modules that are controlled by more specific σ^S-inducing conditions [10]. The expression of the core set of genes is thought to change directly in parallel with the σ^S level, implying that their expression follows the induction of σ^S by multiple stresses. However, most of the σ^S positively controlled genes (>70%) fall in a ''stress-specific'' category, indicating that certain modules of the σ^S-dependent general stress response can be temporarily recruited by more stress-specific regulons (see Section 1.2.3). In general, the σ^S-controlled genes belong to various functional categories besides stress management, which actually accounts for only 11% of the total σ^S-controlled genes. A fair amount of genes coding for metabolic enzymes (19%), membrane transporters (14%), and regulatory proteins (8%) are under σ^S control, while a surprising 43% are of yet unknown function [10].

Several studies have highlighted the importance of σ^S in metabolic regulation during the stationary phase [10, 11]. The σ^S positively controls the expression of genes involved notably in glycogenesis, anaerobic respiration, and the pentose

phosphate shunt, as well as membrane trafficking [10]. The tricarboxylic acid (TCA) cycle and acetate-utilizing pathway are also affected [11]. Together, these metabolic changes might lead to an increase of the internalization of nutritional resources, and redirect the central metabolism to fermentation and anaerobic respiration.

Another striking feature that was evidenced recently is the fairly large amount of genes that are actually negatively regulated by σ^S [10, 14]. This group includes genes required for flagella synthesis, the TCA cycle, transport, and Rac prophage-encoded genes [10, 11, 14]. This negative regulation might be the result of an indirect mechanism such as σ factor competition for RNAP (see Section 1.3.4) or alternatively through direct repression by a σ^S-controlled repressor.

σ^S also plays a role in the control of several pathways during logarithmic and early stationary phases in spite of its very low levels and activity at these growth stages [11, 14, 15]. Indeed, there are indications that σ^S is required during logarithmic growth for the protection against osmotic shock [16] and acid stress in certain culture media [17]. In fact, using an *rpoS* mutant of *E. coli*, Dong *et al.* [15] demonstrated that the modulation of gene expression by σ^S during the logarithmic phase is still quite extensive, with more than 250 genes found to be positively controlled by σ^S and 24 genes found to be negatively controlled. Genes coding for chaperones and for the utilization of iron and carbon sources appear to be part of the σ^S exponential regulatory network, and the Crl regulator is important for the transcription of some of these genes [15].

In what follows, we will discuss the role of the σ^S network in osmotic and acid shock resistance in more detail, thereby focusing on the function of σ^S-dependent genes.

1.2.2
E. coli Osmotic Shock Resistance

Microorganisms cope with osmotic challenges by controlling the level of intracellular osmolytes, thereby allowing the water content to be adjusted by osmosis. Osmolytes comprise notably amino acids (e.g., glutamate, proline), amino acid derivatives (e.g., ectoine, proline betaine), methylamines (glycine betaine), and sugars (trehalose). These solutes might accumulate through uptake or synthesis to high intracellular levels, without disturbing bacterial physiology [18]. Expression of enzymes and active channels involved in osmolyte production and uptake is tightly controlled at the transcriptional level, some of their genes being under the control of σ^S [16]. Here, we will focus on trehalose synthesis and proline and glycine betaine uptake in *E. coli*.

The sugar trehalose is an important osmoprotectant in *E. coli* that is synthesized *de novo* since it cannot be taken up from the environment. The *otsAB* operon is responsible for trehalose production. The *otsA* gene encodes the trehalose-6-phosphate synthase that is responsible for the condensation of glucose-6-phosphate and UDP-glucose to generate trehalose-6-phosphate. This intermediate is then rapidly dephosphorylated by the trehalose-6-phosphate phosphatase enzyme encoded by the *otsB* gene [19]. The *otsAB* operon is under the control of σ^S and is strongly induced

upon osmotic shock, together with 420 other σ^S-dependent genes [10, 20]. Stationary-phase and carbon-starved *E. coli* cells are also highly osmotolerant [21, 22]. When trehalose is present in the extracellular medium, the TreA periplasmic trehalase hydrolyzes it into two glucose molecules that are taken up by glucose-specific phosphotransferase system (PTS) [23]. The *treA* gene is also under σ^S control and induced upon osmotic upshift [19].

Proline and glycine betaine play an important role in protecting cells from osmotic stress. The ProP transport system is responsible for the uptake of a wide variety of osmoprotectants, among them proline and glycine betaine. ProP is an H^+ symporter located in the inner membrane. The *proP* gene transcription is driven by two different promoters P1 and P2. The P2 promoter is controlled by σ^S and the σ^S-dependent transcription is enhanced by the nucleoid-associated protein FIS [24].

1.2.3 *E. coli* Acid Resistance: An Example of a Differentially Controlled σ^S Module

Acid resistance is the ability to sustain very low pH conditions. Due to its lifestyle in the mammalian digestive tract, *E. coli* has a remarkable ability to adapt to pH stress. This capacity enables *E. coli* to survive gastric acidity and volatile fatty acids produced by fermentation in the intestine. Numerous acid survival mechanisms have been identified, depending on the culture medium composition and the pH range examined [25]. Here, we will illustrate that depending on the stress conditions, the acid resistance genes will be governed by the σ^S regulatory control or not.

The *gadA* and *gadBC* genes as well as their regulators *gadE*, *gadX*, and *gadW* are essential for acid resistance [26–29]. *gadA* and *gadB* encode glutamate decarboxylases, and *gadC* encodes a putative glutamate γ-aminobutyric antiporter. Amino acid decarboxylase systems are thought to confer acid resistance by consuming intracellular protons. Under acid stress, glutamate is taken up by the cell using the GadC antiporter, and decarboxylation of glutamate by GadA and GadB produces γ-aminobutyric acid that will expel through GadC. This results in alkalinization of the cytoplasm. Interestingly, these genes (except *gadBC*) and others involved in acid resistance are located in a cluster of σ^S-dependent genes called the ''fitness island for acid adaptation'' [27]. Expression of these genes is strongly induced in the stationary phase in a σ^S-dependent manner giving a molecular explanation for the acid-resistant phenotype displayed by stationary-phase cells [30]. In addition, this cluster is under the control of another global regulator, the H-NS protein, which downregulates its expression [27].

Although σ^S expression is strongly induced upon acid stress and about 200 genes are expressed in a σ^S-dependent fashion, most of them appear to belong to the nonspecific core gene set [10]. Interestingly, however, the expression of the *gad* genes themselves upon an acid shift is mostly σ^S-independent, indicating a switch in the genetic control of these genes has occurred under such conditions. This underscores the existence of modules within the σ^S regulatory network that might

be controlled by multiple regulators depending of the environmental signal [10]. The GadE regulator has been proposed to control this switch, by integrating the stationary-phase signal through the GadX regulator and the ''acid'' signal most likely through the EvgSA two-component system and the YdeO pathway [10, 26]. Moreover, the GadW and GadY positive regulators might act as H-NS counter-silencers by displacing H-NS off the promoter regions of the *gad* genes [31].

1.3 Regulation of σ^S

It is clear that given the profound physiological rearrangements caused by σ^S [32, 33], the expression and availability of this sigma factor must be tightly regulated and allowed only in times of stress. σ^S is barely detectable in rapidly growing cells in laboratory conditions and *rpoS* defective mutants show a growth rate comparable to that of wild-type cells [21, 34, 35]. Under stress or starvation conditions, however, the amount of σ^S rapidly rises up to 30% to that of σ^{70}, allowing for the formation of σ^S-associated RNAP that in turn activates σ^S-dependent genes. Therefore, the expression, stability, and activity of σ^S in the cell must be strongly regulated and controlled at the transcriptional, translational, and post-translational levels [36–38]. Moreover, all of these regulatory mechanisms allow the integration of different environmental cues and, consequently, the fine-tuning of the response. The intricate regulation that is imposed on the general stress response counts as a true hallmark of bacterial complexity.

1.3.1 Transcriptional Regulation of σ^S

Although transcriptional regulation of *rpoS* has not been studied extensively and in depth, it is at least known to be controlled by several trans-acting factors [5, 13, 39]. The *nlpD* gene is located immediately upstream of the *rpoS* gene and harbors the main *rpoS* promoter, although some background expression stems from *nlpD* promoter itself [40]. The main *rpoS* promoter is σ^{70}-dependent and gives rise to a monocistronic mRNA transcript comprising a 567-bp untranslated region. Interestingly, the *rpoS* promoter contains two putative cAMP/CRP-binding sites, and several studies using mutants in *cya* (encoding adenylate cyclase) and *crp* have indicated that cAMP/CRP is a negative regulator of *rpoS* transcription in the exponential phase [21, 40]. It consequently follows that modulators of adenylate cyclase activity, like the Crr protein, in turn also affect *rpoS* transcription [41]. Recently, it has been established that not only *rpoS* itself is regulated by cAMP/CRP, but that also quite a number of σ^S-controlled genes contain putative cAMP/CRP-binding sites, indicating a strong overlap between the σ^S and cAMP/CRP regulons [10].

It was found that polyphosphate indirectly enhances *rpoS* expression, although the actual molecular mechanism still remains to be identified [42]. Inorganic polyphosphate is a linear polymer of hundreds of phosphate residues that can

accumulate in bacteria under stressful conditions [43]. The polymer is synthesized by polyphosphate kinase by polymerization of the terminal phosphate group of ATP to a phosphate chain [44], while degradation of polyphosphate is catalyzed by exopolyphosphatase [45]. Overexpression of exopolyphosphatase correspondingly inhibits the increase of σ^S levels upon entry into the stationary phase [42]. Interestingly, exopolyphosphatase activity is inhibited by the alarmone (p)ppGpp [46] – an effector of the stringent response that is produced when levels of amino acids, carbon, phosphate, or nitrogen become limited [47, 48]. This link between (p)ppGpp and polyphosphate is likely to explain earlier reports observing a positive effect of (p)ppGpp on *rpoS* transcription [49, 50]. In cells lacking (p)ppGpp, however, *rpoS* transcription was compromised at the level of elongation rather than the initiation of transcription [50].

Aside from the effects of polyphosphate or (p)ppGpp, it appears that both *rpoS* mRNA and RpoS protein levels are reduced in an *E. coli barA* mutant [51]. As BarA is a sensor kinase, its positive effect on *rpoS* transcription is probably mediated by a yet unknown cognate response regulator.

1.3.2 Translational Regulation of σ^S

E. coli produces a fair amount of *rpoS* mRNA even under conditions where σ^S protein is barely detectable [52]. It is assumed that the rate of translation is heavily controlled by the mRNA secondary structure, with base-pairing in the translational initiation region being responsible for the occlusion of the ribosome-binding site and the corresponding inhibition of translation under noninducing conditions. Several proteins and small regulatory RNAs (sRNAs) are involved in translational control, which makes the analysis of translational regulation a very complex endeavor [5, 13].

The Hfq protein is an RNA-binding protein [53] that is required for efficient *rpoS* translation [54]. It has been suggested that binding of Hfq to *rpoS* mRNA occurs to U-rich sequences [55] and could either directly stabilize specific secondary structures in the *rpoS* transcript or facilitate its interactions with sRNAs. So far, three such sRNA species have been found to be involved in *rpoS* translation: DsrA and RprA promoting translation, and OxyS inhibiting it.

DsrA has been described as an inhibitor of *rpoS* mRNA intramolecular base-pairing using an anti-antisense mechanism in which DsrA pairs with the translational initiation region, thereby making the ribosome binding site fully accessible [56–59]. Hfq has also been reported to cooperate with DsrA [60]. Binding of Hfq to the noncoding DsrA sRNA accelerates the binding of DsrA to the *rpoS* mRNA [59]. DsrA further stimulates *rpoS* translation by binding to *hns* mRNA (see below) and inhibiting its translation [57, 58, 61]. DsrA itself is repressed by LeuO. The other sRNA that positively influences *rpoS* translation is RprA, but the *rprA* promoter is active only at temperatures below 30 °C [62]. Like DsrA, RprA stimulates *rpoS* translation by pairing with the *rpoS* mRNA, negatively regulates *hns*, and is repressed by LeuO [63].

The negative regulation of *rpoS* translation by OxyS sRNA is not yet understood [63], but may be due to binding of OxyS with Hfq, thereby inhibiting interaction between Hfq and *rpoS* mRNA [64]. OxyS is a member of the OxyR regulon and is induced by oxidative stress [65]. The repression of σ^S during oxidative stress makes sense, since certain overlaps exist between genes expressed by OxyR and σ^S. Repression would avoid the pointless drain on cellular resources [65]. Thus, sRNAs represent different signal transduction pathways that converge to regulate the amount of σ^S protein.

In addition to Hfq, several other protein factors are involved in *rpoS* translation. HU, for example, is essentially a DNA-binding protein with binding preference for secondary structures such as bends or kinks [66]. However, it was shown to specifically bind *rpoS* mRNA and enhance its expression [67]. Another nucleoid structuring protein, H-NS, is a global regulator that preferentially binds to bended DNA and reduces the transcription of over 100 genes [68, 69]. However, it has been revealed that H-NS also negatively affects the translation of some gene transcripts, including *rpoS* [70]. This could explain why H-NS$^-$ mutants exhibit dramatically raised σ^S levels in the exponential phase, similar to those observed normally in stationary-phase cells [71].

Interestingly, the alarmone (p)ppGpp not only seems to play an important role in *rpoS* transcription, but also stimulates translational efficiency of *rpoS* mRNA. Brown *et al.* [72] found that rather than interacting directly with ribosomes, (p)ppGpp affects activity of the DksA protein, which was shown earlier to play a role in the translational regulation of *rpoS*. Other molecules that play a role in *rpoS* translation include DnaK, a heat shock chaperone, as well as the cold shock proteins CspC and CspE, EIIa(Glc), and UDP-glucose [5, 13].

All these regulatory factors contribute to a very complex and highly intertwined network that is characterized by positive and negative feedback mechanisms allowing a high degree of fine-tuning. Therefore, the output of this network may be difficult to predict under changing environmental conditions [5, 13].

1.3.3
Post-Translational Regulation of σ^S

Although the *rpoS* gene is moderately expressed during the exponential phase of growth [7], cellular levels of the σ^S protein remain low. This is partly due to a high instability of this sigma factor, with a half-life of only 2 min. Interestingly, this half-life rises to more than 30 min on entry into the stationary phase or when a stress is inflicted upon the cell [73]. The identification of cellular factors involved in this dramatic decrease in σ^S turnover, as well as how they are steered by environmental cues, has received much attention.

The instability of σ^S in the exponential phase is caused by its rapid degradation by the ClpXP protease [74]. However, the increased stability of σ^S in the stationary phase could not be linked to a reduction in ClpXP concentration. In fact, Western analysis showed that the ClpXP concentration in stationary phase even increased by 50 % compared to that of exponentially growing cells [74]. Pratt and Silhavy [75]

showed that another important factor was involved in the regulation of σ^S turnover – the adaptor protein RssB (SprE) that binds directly to σ^S and targets it to the ClpXP protease [38]. Accordingly, a null mutation in *rssB* leads to stabilization of σ^S and elevated levels in the exponential phase [75]. Interestingly, RssB contains a conserved CheY response regulator domain and therefore it has been speculated that RssB activity is adjusted by phosphorylation [5, 13]. In the phosphorylated state it would bind to σ^S, thereby labeling the latter for degradation by the ClpXP complex. However, Peterson *et al.* [76] showed that an *E. coli* strain expressing a mutant RssB protein only missing the phosphorylation site resembled a wild-type strain rather than an *rssB* null mutant in its ability to control σ^S levels. They concluded that although phosphorylation might contribute to maximal RssB activity, it is not indispensable and other regulatory mechanisms, independent of (de)phosphorylation, must be involved.

Recently, an antiadaptor protein has been discovered in *E. coli*, IraP (YaiB), that interferes with RssB functioning through direct protein–protein interactions and is independent of the phosphorylation status of the latter [77]. Interestingly, deletion of *iraP* only interferes with σ^S stabilization during phosphate starvation, but not during carbon starvation, and only partly during the stationary phase or nitrogen starvation. IraP synthesis itself is induced by phosphate starvation in a (p)ppGpp-dependent manner [78].

After the discovery of IraP, other proteins have been sought that could regulate RssB under the starvation conditions where IraP played no role. As such, two new antiadaptors were discovered, IraM and IraD, that can counteract RssB activity and stabilize σ^S. The IraM protein proved essential for stabilization of σ^S during magnesium starvation, while IraD proved important for its response to DNA damage [79].

Another part of the mechanism that can profoundly affect σ^S degradation by ClpXP is in fact the level of occupation of this protease by other proteins. It was shown [80] that inducing translational errors by specific mutations or drugs elevated σ^S stability. Indeed, the increase in erroneous and misfolded proteins that result from reduced ribosomal fidelity saturate the ClpXP machinery and allow σ^S to accumulate. Correspondingly, artificially increasing translational fidelity or ClpXP production attenuated σ^S stability.

1.3.4 Competition for RNAP and Promoters

When σ^S is finally formed and stabilized, it can only instigate the general stress response when it effectively associates with the RNAP core enzyme to reprogram gene expression. However, this association is by no means gratuitous, as it is believed that *in vivo* the availability of the RNAP core enzyme is limited so that different sigma factors are in fierce competition for its acquisition. This phenomenon was nicely demonstrated by the fact that compromising σ^S function not only attenuated expression of σ^S-dependent genes, but also caused superinduction of several σ^{70}-dependent genes [81]. Therefore, σ^S needs to be able to compete

with the overabundant vegetative σ^{70} factor to occupy the RNAP core enzyme [82]. However, as σ^{70} naturally displays the highest affinity for RNAP *in vitro* [83, 84], it can be expected that the mere availability of σ^S itself is not sufficient.

Interestingly, again a pivotal role is reserved for (p)ppGpp to bring about an effective shift in RNAP core sequestration *in vivo*. Although, as discussed earlier in this section, (p)ppGpp has a number of activities, it is well documented that it associates with the RNAP core enzyme [85, 86], where it seems to influence the differential binding abilities of sigma factors to core RNAP. As such, in the presence of (p)ppGpp, σ^S is able to sequester part of the available RNAP core enzyme and instigate the general stress response [47].

When σ^S is associated with the RNAP it recognizes promoters with a common sequence pattern and favors their expression. However, σ^S- and σ^{70}-dependent promoters bear similarity, so that sometimes additional factors will decide whether a promoter will be transcribed by RNAP-σ^S or RNAP-σ^{70}. The *dps* gene, for example, can be transcribed by RNAP-σ^S in the stationary phase, or by RNAP-σ^{70} when it cooperates with OxyR that has been activated by H_2O_2 [87]. Another, more global, discriminator between RNAP-σ^S and RNAP-σ^{70} at the same promoter seems to be the Lrp protein. Lrp is a nucleoid associated global regulator that can affect DNA structure [88] and such changes in DNA topology could shift σ^S/σ^{70} selectivity [10].

1.4 Conclusions

The σ^S network drives a systemic defense that integrates a great number of intra- and extracellular cues, and that truly differentiates stationary phase from logarithmic-phase cells. In general, the competition between σ^{70} and σ^S represents the bacterial tradeoff between growth and reproduction, on the one hand, and maintenance and repair, on the other. In this context, the massively imposed regulation serves to adequately synchronize the allocation of resources between these opposing states of proliferation and survival with the quality and demands of the surrounding environment [82].

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