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Stress induced remodeling of the bacterial proteome

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Abstract

Microorganisms live in fluctuating environments, requiring stress response pathways to resist environmental insults and stress. These pathways dynamically monitor cellular status, and mediate adaptive changes by remodeling the proteome, largely accomplished by remodeling transcriptional networks and protein degradation. The complementarity of fast, specific proteolytic degradation and slower, broad transcriptomic changes gives cells the mechanistic repertoire to dynamically adjust cellular processes and optimize response behavior. Together, this enables cells to minimize the "cost" of the response while maximizing the ability to survive environmental stress. Here we highlight recent progress in the understanding of transcriptional networks and proteolysis that illustrates design principles used by bacteria to generate the complex behaviors required to resist stress.

1. Introduction

Bacteria and other single-celled organisms have evolved to survive in variable and at times extreme conditions, and must sense and mount effective responses to environmental challenges as diverse as heat, oxidative damage, anti-microbial agents, and nutritional limitation. While bacteria have a number of programs that they can use to combat these environmental challenges, mounting a costly response in the absence of stress is detrimental, as resources that could be utilized for growth are wastefully funneled into unneeded adaptations [1]. Since bacteria are in constant competition with other species in their environment, organisms with more efficient stress responses have a competitive advantage. Thus, stress responses are carefully regulated so that they are activated only when required and to the extent necessary.

This review will describe emerging stories in bacterial stress responses that highlight design principles used by bacteria to mount stress responses that are fast, accurate, cost efficient,

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and successful. We focus on two complementary mechanisms that remodel the proteome to oppose stress: rewiring the transcriptome and modulating proteolysis. While transcription can activate broad swathes of genes in concert, proteolysis is best suited to quickly adjust the availability of specific cellular proteins to favor required processes. Together, these mechanisms allow cells to maintain a dynamic equilibrium, continually re-optimizing processes in response to changing environmental cues.

2. Transcriptional remodeling in response to stress

The first step in a transcriptional response is to convert the signals from the environment into transcriptional change, leading to production of new proteins and adaptation. Regulators can sense stress through two general mechanisms: 1) Consequence sensing (e.g., sensing heat by the accumulation of unfolded proteins); 2) Direct sensing (e.g., a regulatory RNA whose structure is melted by heat), also called “feed-forward” sensing [2] (note that this is distinct from the “feed-forward loop” regulatory motif [3, 4]). Notably, these stress signals often control transcription factors post-transcriptionally (e.g., by protein degradation or regulation of activity). This decreases the lag time of transcriptional responses, enabling both a rapid initial response and rapid adaptation. As stresses are alleviated, the activity of stress-responsive transcription factors then decreases to reach a new homeostasis.

In this section, we review emerging stories about bacterial stress-responsive transcription factors, focusing on two large families, two-component systems and alternative sigma factors (σs). Two-component systems are comprised of a sensor histidine kinase and a cognate response regulator [5, 6]. When activated, a histidine kinase auto-phosphorylates and then transfers the phosphate group to the response regulator, which modulates gene expression [5–11]. σs are subunits of RNA polymerase holoenzyme that mediate promoter recognition; alternative, non-housekeeping σs are widely used in stress responsive signal-transduction pathways [12–14]. Typically, every bacterial species contains multiple members of each of these families. We discuss how these transcription factors sense and relieve the deleterious effects of stress as quickly and accurately as possible, and how stress systems limit spurious cross-activation between pathways to ensure an accurate and specific response.

Stress sensory domains in two-component systems

How do two-component systems sense stress signals? For histidine kinases, which auto-phosphorylate on a specific histidine residue, the current model is that ligand binding induces conformational changes that properly position the catalytic domain and facilitate phosphorylation of the target histidine, activating the response [10, 15–21]. Indeed, this is the mechanism proposed for the *Escherichia coli* histidine kinase EnvZ, which regulates the membrane porins OmpC and OmpF with its response regulator, OmpR [22–24]. EnvZ crosses the inner membrane and monitors a variety of signals (osmolarity, pH, temperature, procaine), though the location of the primary signal (periplasm vs cytoplasm) is unknown [22, 23].

Recent work has demonstrated that high osmolarity directly alters the conformation of the cytoplasmic fragment of EnvZ (EnvZ-C) [24], an example of feed-forward sensing. High

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osmolarity drives EnvZ-C to adopt a more compact structure, properly positioning the catalytic and auto-phosphorylation sites, and activating OmpR [24]. While EnvZ-C may be sufficient for osmo-sensing [24, 25], the other domains of EnvZ may still play a role in response, as substitutions in the EnvZ transmembrane domains are known to affect EnvZ activity [26]. Like many histidine kinases, EnvZ contains inner membrane proximal HAMP domains, which mediate transduction of periplasmic or transmembrane stimuli into conformational changes in the cytoplasm [20, 21, 27, 28]. Thus, the periplasmic portions of EnvZ may sense other types of signals, play a role in EnvZ dimerization, or sense osmolarity in a concerted fashion with EnvZ-C by mediating conformational change of EnvZ-C [22]. Lastly, the periplasmic portion of EnvZ interacts with MzrA, which modulates EnvZ activity, but does not preclude EnvZ signal sensing [29]. While the direct signals that modulate MzrA activity are unknown, MzrA may be regulated by both CpxA/CpxR and σ^E, two sensors of membrane status [29, 30]. While EnvZ-C may be a feed-forward sensor for osmolarity, the EnvZ periplasmic domains may have a role in sensing other EnvZ signals and properly modulating the activity of EnvZ.

Other two-component systems often contain Per-ARNT-Sim (PAS) domains, a structural motif found across all kingdoms of life that can be feed-forward sensors for signals as varied as light, redox potential, and metabolites, though the mechanisms that activate most PAS domains remain unknown [31–33]. As PAS domains are highly modular, they can be exchanged or conjugated to alternative proteins to reprogram signaling and response [34–38]. This allows development of various genetic tools; for example, a light activated histine kinase, by switching the oxygen sensitive PAS domain of *Bradyrhizobium japonicum* FixL for the light sensitive PAS domain of *Bacillus subtilis* YtvA [36], or a chimeric histidine kinase that cooperatively responds to both light and oxygen, by fusing the YtvA light sensitive PAS domain to the FixL oxygen sensing domain [35].

Unfortunately, the activating signals and the mechanism of activation are unknown for most signaling pathways. We have excellent tools (e.g., microarrays, proteomics, ChIP) to identify the downstream targets of regulatory systems, but the methods for identifying the signals that activate the relevant regulators and the mechanisms for this activation have yet to mature. This is an important area of investigation, as identification of these signals is critical for both understanding the organism and systems biology.

How σ^{32} maintains protein-folding homeostasis

Maintaining protein-folding homeostasis is a critical task for all cells. It is especially important for cells living in environments with variable temperature, as heat alters protein folding. The highly regulated universal heat shock response controls expression of a core set of chaperones in all organisms, as well as many additional organism-specific proteins, including a set of conserved proteases in bacteria [39–42]. In *E. coli* and other proteobacteria, the HSR is controlled by σ^{32}, the master regulator of ~100 genes [42, 43]. Recent progress in understanding *E. coli* σ^{32} illustrates the complexity of control that allows σ^{32} to monitor protein folding in the cytoplasm and inner membrane.

σ^{32} is controlled by two mechanisms that enable a rapid response: σ^{32} translation is regulated by a feed-forward sensing mechanism, as heat directly melts an inhibitory mRNA

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structure that dampens σ^{32} translation [44, 45]; σ^{32} activity and stability is controlled by two feedback loops that sense protein folding status [42]. σ^{32} activity is regulated by the cytoplasmic chaperones (e.g. DnaK/DnaJ), which bind directly to σ^{32} to inhibit its activity, and σ^{32} protein level is mediated by the inner membrane localized FtsH protease, which degrades σ^{32} (Fig. 1) [46–49]. When stresses induce protein unfolding, the chaperones and proteases are titrated away from σ^{32}, activating the heat shock response (Fig. 1) [42]. These regulators are also themselves transcriptionally activated by σ^{32}, forming a negative feedback loop [42]. Thus, regulation of σ^{32} is responsive both to heat and to cellular protein folding status.

Despite this complexity, the known circuitry could not explain two key features of σ^{32} response: a) mutations in a small region of σ^{32} (a “homeostatic control region”) disrupt inhibition of σ^{32} by chaperones and FtsH *in vivo*, leading to hyperactive σ^{32}, but do not alter σ^{32} regulation by these factors *in vitro* [50–53]; b) σ^{32} is thought to monitor the folding status of inner membrane proteins, but the mechanism for this was unknown [42, 54]. These observations suggest that σ^{32} may monitor the inner membrane through a key regulator that had not been found.

This missing regulator was recently identified as the Signal Recognition Particle (SRP; Ffh + 4.5S RNA) [54]. SRP is part of the co-translational membrane trafficking system that mediates inner membrane protein biogenesis. SRP binds to and targets ribosomes with nascent proteins that contain hydrophobic N-terminal signal sequences to the inner membrane for cotranslational insertion and folding (Fig. 1) [55–57]. Surprisingly, although σ^{32} does not have a signal sequence, SRP also traffics σ^{32} to the inner membrane; membrane localization of σ^{32} is essential for proper regulation by chaperones and FtsH (Fig. 1) [54]. In fact, mutants in the σ^{32} homeostatic control region are hyperactive because they reduce binding to SRP, therefore they are not membrane localized by SRP and cannot be inhibited by chaperones and FtsH [54]. Thus, membrane localization is vital for proper σ^{32} regulation.

SRP allows σ^{32} to sense the protein folding status of the inner membrane. Since SRP is substoichiometric relative to ribosome (~1:100 SRP:ribosome), free SRP levels depend on efficient SRP recycling [58, 59]. As almost all inner membrane proteins are trafficked by SRP, defects in trafficking may alter SRP recycling or lead to accumulation of ribosomes with signal sequence proteins, preventing SRP from interacting with σ^{32} or localizing σ^{32} to the membrane [54, 55]. Thus, the amount and activity of σ^{32} will dynamically adjust in response to flux of proteins through the inner membrane (Fig. 1).

Why would σ^{32} sense inner membrane protein folding? The σ^{32} regulon contains SRP, FtsH, and is additionally enriched in proteins that are involved in or reside in the inner membrane [42, 43]. Furthermore, FtsH not only degrades σ^{32} but also is the main protease that mediates quality control of membrane proteins [60, 61]. Active σ^{32} will reduce inner membrane dysfunction by increasing levels of SRP (to ameliorate trafficking) and FtsH (to reduce unfolded protein load). As σ^{32} activity is further regulated by cytoplasmic chaperones, this allows σ^{32} to integrate the folding status of both inner membrane and cytosolic proteins, a significant advantage as inner membrane proteins comprise 20–30% of total cellular protein [55, 62].

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How σ^E maintains homeostasis of the outer membrane

The first line of defense for gram-negative bacteria is the outer membrane, which presents a formidable permeability barrier to protect against antibiotics and other stresses [63, 64]. The outer membrane is an asymmetric lipid bilayer: its outer leaflet is composed of lipopolysaccharides (LPS) and its inner leaflet of phospholipids [63, 65]. The outer membrane additionally contains proteins, including the outer membrane proteins (OMPs) that allow access to selected solutes [63, 64, 66, 67]. Both LPS and OMPs rely on complex machines for their transport and assembly into the outer membrane [65, 68–72]. As outer membrane integrity depends on proper balance of its components [63, 64, 71], maintaining appropriate levels of assembly machines and substrates is vital.

To monitor stress in this compartment, *E. coli* and other γ-proteobacteria employ σ^E, which regulates genes required for assembly of all major components of the outer membrane [73, 74]. The major challenge for the σ^E system is how to convey the information about the status of the outer membrane into the cytoplasm to mediate transcriptional change. We discuss the current model for how each σ^E regulator senses assembly of a different outer membrane component to generate an integrated portrait of envelope status (Fig. 2).

σ^E monitors outer membrane protein folding through the rate of cleavage of its negative regulator RseA [75–77]. RseA, an inner membrane protein that sequesters σ^E in an inactive conformation, can be cleaved by the protease DegS, which permits secondary cleavage by RseP and subsequent degradation of RseA, freeing σ^E to activate transcription [78–83]. DegS is activated only when it binds to unfolded OMP C-termini in the periplasm [77, 84–86]. As these unfolded species are thought to accumulate when OMPs are inefficiently assembled into the outer membrane, activation of DegS is a reflection of outer membrane dysfunction [87, 88].

How is the status of outer membrane LPS sensed? σ^E has a second negative regulator, RseB, which binds to RseA and protects it from cleavage by DegS [89–92]. Recent studies have shown that LPS can bind to and dissociate RseB from RseA [93]. *In vitro*, RseA degradation in the presence of RseB requires both OMPs and LPS: OMPs activate DegS, and LPS dissociates the RseA/RseB complex [93]. Similarly, *in vivo*, perturbations that lead to accumulation of off-pathway LPS (e.g., mutations that partially inactivate the LPS assembly machinery or alter LPS structure), in combination with activated DegS, lead to dramatic activation of σ^E [93, 94]. Thus, maximal activation of σ^E *in vivo* requires two signals of outer membrane stress (Fig. 2).

Why do cells integrate these two signals of outer membrane assembly? OMPs and LPS are the major unique components of the bacterial outer membrane, and thus excellent indicators of outer membrane status [65]. Requiring concomitant defects in the assembly of both OMPs and LPS reduces the chances for spurious activation, ensuring that a large and costly response is not provoked by normal variation in the flux of proteins or LPS through the periplasm. For this mechanism to be an effective response, sustained defects in either OMP or LPS assembly must provoke defects in assembly of the other, ensuring σ^E activation. Indeed, certain LPS species have been shown to reduce the efficiency of OMP assembly, as the altered outer membrane environment may be less conducive to proper OMP assembly

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[63, 95, 96]. Similarly, the major component of the LPS assembly machine is inserted into the outer membrane by the same mechanism as is used for other OMPs, thus defects in OMP assembly will eventually lead to defects in LPS assembly [97, 98]. Thus, sensing assembly intermediates for multiple outer membrane components allows bacteria to monitor outer membrane status more accurately and comprehensively.

OMPs are among the most abundant proteins in the cell and there is tremendous OMP flux to the outer membrane [63]. For this reason, increasing the production of OMP chaperones, proteases and assembly factors may be insufficient to rapidly restore proper folding. Thus, σ^E also reduces OMP synthesis by inducing two small RNAs (sRNAs), MicA and RybB, that target OMP mRNA for degradation, thereby dramatically decreasing the flow of OMP precursors to the envelope [99–103]. The vital role of these sRNAs is demonstrated by the fact that overexpression of either sRNA can protect the cell from the deleterious effects of depleting σ^E, which normally leads to lysis and cell death [104, 105]. Interestingly, the strategy employed by bacteria to address OMP folding is reminiscent of the intercompartmental eukaryotic unfolded protein response (UPR). Upon sensing stress in the endoplasmic reticulum, the UPR opposes folding stress both by upregulating folding factors and by downregulating the flow of precursors to the endoplasmic reticulum [106–108].

Dynamic responses in σ^B activation

To optimize stress responses, cells must tailor the timing, amplitude, and dynamics of the response to each stress. Indeed, many responses contain entwined positive and negative feedback loops that can generate distinct, sophisticated behaviors like bistability or oscillation [4, 109]. Furthermore, while many systems have regulators that suppress stochastic fluctuations (noise) to prevent spurious activation (e.g., σ^E system, see section 2.3), noise can also be utilized to generate sophisticated response behaviors [4, 92, 110–113]. Indeed, recent studies have demonstrated that noise is used in the *Bacillus subtilis* σ^B system to generate vastly different dynamic behaviors depending on the inducing stress (Fig. 3) [114, 115].

In *B. subtilis* and related gram-positives, σ^B is a “general stress factor”, which induces a core stress regulon that is expressed in concert with stress-specific responses [116, 117]. In steady state, σ^B is bound and inhibited by its anti-σ, RsbW [117–119]. Under stress, the antagonist RsbV binds RsbW and frees σ^B, activating response [119–121]. This partner-switching mechanism is regulated by the phosphorylation state of RsbV: unphosphorylated RsbV binds RsbW, but RsbV~P cannot [119, 120]. Notably, RsbW is the kinase that phosphorylates RsbV [119]. Therefore RsbW keeps σ^B activity in check both by binding σ^B and phosphorylating RsbV [119, 120]. To activate σ^B, two different phosphatase systems can dephosphorylate RsbV~P: 1) RsbP, activated by nutrient limitation (e.g., limiting NTPs), and 2) RsbU, activated by environmental stress (e.g., ethanol). Environmental stress induces the highly conserved 1.8 MDa supermolecular “stressosome” complex to release RsbT, which, in turn, activates RsbU phosphatase activity (Fig. 3) [121–125].

Although both energy and environmental stress modulate σ^B activity through RsbV dephosphorylation, each stress leads to different dynamics in the σ^B response [114, 115]. Nucleotide limitation (via mycophenoic acid treatment) leads to continuous stochastic pulses

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of σ^B activity that vary in timing but not in intensity from cell-to-cell (Fig. 3) [114, 115]. In contrast, ethanol induces a single pulse of σ^B activity that is synchronous across the population (Fig. 3) [114, 115]. How does the circuitry governing σ^B generate these diverse responses? Opposing kinase (RsbW) and phosphatase (RsbP or RsbU) activities leads to an ultrasensitive response, so that σ^B is activated in a sharp, switch-like manner [114]. Cell-to-cell variability in either the initial level phosphatase or RsbW would mean that different cells would require different levels of phosphatase to oppose RsbW and cross the threshold of activation. Thus, during energy stress, small fluctuations (noise) in RsbW/RsbP ratio per cell could lead single-cell variability in the timing of σ^B activation [114]. In contrast, environmental stress induces mass release of RsbT from the stressosome, enabling RsbT to activate RsbU and overwhelm inhibition by RsbW, thus activating σ^B in a synchronous manner in all cells [115].

What could be the advantage in responding differently to these stresses? This is an important question that requires investigation. As *Bacillus* devotes up to 40% of its translational capacity to the σ^B regulon during stress, misregulation of σ^B is an enormous metabolic cost [126]. One posibility is that different patterns of σ^B activity are optimizations that minimize the cost of response for each stress. Another possibility could be that σ^B pulsing is a bet-hedging mechanism, as the cell may anticipate that nutrient limitation is a precursor for other stresses that may require σ^B [115]. This is supported by the fact that σ^B pulses indefinitely during nucleotide limitation, suggesting that σ^B activity does not lead to adaptation in this condition [114]. Testing these types of hypotheses is a difficult but important challenge, as these different dynamic response behaviors will be present in other systems, particularly those that sense multiple types of stress. These studies suggest that single-cell analysis of stress systems will continue to reveal novel behaviors not previously appreciated in bulk studies.

Cross-talk in signaling systems

Most bacteria have dozens if not hundreds of paralogous two-component systems, each recognizing their own signals [127, 128]. Since these systems evolved and proliferated via genomic duplication events, they often share considerable sequence and structural similarity, creating significant potential for spurious cross-activation, or “cross-talk” [128, 129]. Indeed, histidine kinases have been observed to activate non-cognate response regulators when their cognate regulator is lost [130, 131]. As cross-talk may activate non-beneficial responses, networks that are prone to cross-talk will evolve mechanisms to insulate responses [128, 129].

The current model is that the co-evolution of residues in the interaction surfaces of histidine kinases and their cognate response regulators is a major molecular basis for preventing cross-talk in two-component systems [128, 132–134]. As these residues (called “specificity residues”) determine the histidine kinase/response regulator interaction, amino acid substitutions on either the histidine kinase or response regulator can lead to recognition of non-cognate histidine kinases or response regulators [132, 134–136]. For example, a single amino acid change to the specificity residues of the histidine kinase EnvZ is sufficient to allow phosphorylation of the non-cognate response regulator RstA [132]. Global approaches

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have demonstrated that orthogonality (lack of cross-talk) is the norm for nearly all histidine kinases within a genome, ensuring proper insulation of signaling [133, 137].

How is orthogonality maintained during genome evolution? This was examined for the broadly conserved PhoB/PhoR two-component system, which is present in α, β, and γ-proteobacteria. Interestingly, while the specificity residues of PhoR are highly similar in β- and γ-proteobacteria, there is lower conservation between γ- and α-proteobacteria [133]. Analysis revealed this to be an evolutionary adaptation in α–proteobacteria to insulate PhoB/R from an α-specific paralog, NtrY/NtrX, preventing cross-talk between these two systems. Indeed, *E. coli* PhoR (γ) can phosphorylate both *Caulobacter* PhoB and NtrX (α), whereas *Caulobacter* PhoR is specific for PhoB [133]. Furthermore, a mutant PhoR that cross-activates NtrX is detrimental to growth under PhoR-inducing conditions; this growth defect is almost fully suppressed by deletion of *ntrX* [133]. Thus, cross-talk can produce selective pressure that drives newly acquired signaling pathways to diverge and insulate themselves against paralogous systems.

These principles are also observed for the extra-cytoplasmic σ-factors, a highly diverse group of alternative σs, which comprise 43 phyolgenetically distinct subgroups [14, 138]. Recent work with 40 σs from 20 different subgroups indicates that, in general, σs are inhibited only by their cognate anti-σ, and recognize only promoters within their subgroup [139]. However, questions remain as to whether σs within a subgroup are as well insulated. For example, the soil bacterium *Streptomyces coelicolor* contains an astonishing 63 σs, with 4 σs derived from subgroup 39 [138]. Do these σs initiate cooperative response, or are they well-insulated from each other? Furthermore, the evolutionary trajectories that mediate σ orthogonality are not well understood. Such analyses are key to understanding the design of signaling systems and the selective pressures that drive their evolution.

3. Regulatory proteolysis in stress response

As a counterpoint to transcriptional remodeling, regulatory proteolysis represents an alternative way of altering the protein content of the cell in response to stress. In all organisms, failure to degrade proteins that are unfolded or damaged by stress leads to protein aggregation and deleterious consequences such as cell death in bacteria and disease and aging in eukaryotes [39, 40, 140]. Proteolytic control is particularly important in bacteria, as most proteins are otherwise stable and diluted only by cell division [141]. Recently, the role of proteolytic machines in regulating transcriptional response as well as being direct sensors and effectors for stress has emerged. We have already described how proteolysis controls the amount of σ^{32} (see section 2.2), and the activity of σ^{E} (see section 2.3). In this section, we focus on recent stories about how the major cytoplasmic proteases ClpXP and Lon directly sense stress and modulate their proteolytic activity in response.

The AAA+ proteases ClpXP and Lon, are members of a large, well-conserved family of proteins that assemble into heptameric or hexameric rings [142–144]. Proteolysis occurs in a central pore that acts as a degradation chamber [142, 143]. Cycles of ATP hydrolysis drive conformational changes that promote target protein unfolding and translocation into this chamber [142]. It is estimated that together, Clp and Lon are responsible for ~75% of ATP-

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dependent proteolysis in bacteria [141, 145]. Importantly, as degradation is irreversible, these proteases utilize adapter proteins to specifically recognize intended targets and thus avoid spurious degradation [142, 143, 146].

The intimate role of proteolysis in controlling the general stress response

In *E. coli* and related gram-negatives, the "general stress response" is mediated by σ^S, which is induced by many different conditions, including DNA damage, low Mg^{2+} or PO_4, and low nutrients/stationary phase [147]. While σ^S is controlled in every possible way, σ^S protein level is regulated by proteolysis [147, 148]. In unstressed cells, the adaptor protein RssB targets σ^S to ClpXP for degradation [149–151]. In appropriately stressed cells, σ^S is stabilized, activating its regulon. Recent studies demonstrate that σ^S is stabilized by two discrete mechanisms (Fig. 4).

Firstly, a suite of stress responsive "anti-adaptor" proteins (IraD, IraM, IraP) bind to RssB and prevent it from targeting σ^S to ClpXP for degradation [147, 152, 153]. Each Ira is induced by a different stress condition (in *E. coli*: IraD – nutrient limitation/stationary phase or DNA-damage, IraM – low Mg^{2+} or Ca^{2+}, IraP – low PO_4) [147, 152–155], thereby communicating each discrete stress to ClpXP by interfering with RssB function (Fig. 4). Although Ira proteins all bind to RssB, they are not members of the same protein family, do not have sequence similarity, and interact with different residues of RssB [147, 156], indicating that they have arisen independently to tune σ^S proteolysis. How Ira proteins are themselves inhibited to turn off the σ^S response remains unclear. An additional question is how σ^S responds to stresses that activate multiple Ira proteins. Most laboratory experiments focus on examining effects of a single stress, but in the environment, multiple stresses may occur simultaneously. These stresses may have combinatorial effects, encouraging bacteria to evolve systems that process information from multiple stresses in an integrated way.

Secondly, ClpXP tunes its own proteolytic capacity to alter σ^S degradation in response to ATP limitation [157]. ClpXP is ATP-dependent, creating the potential for ATP availability to affect rates of substrate degradation [158]. ClpXP degradation of σ^S is in fact exceptionally sensitive to intracellular ATP concentration: at low levels of ATP, many canonical ClpXP substrates are degraded normally, but degradation of σ^S is blocked (Fig. 4) [157]. Though the mechanism for this ATP dependence is unknown, it is thought that reducing ATP levels slows ClpXP translocation and may cause accumulation of partially folded substrates that interfere with further unfolding or degradation [157–159]. Interestingly, since low ATP is an indicator of nutrient stress, nutrient limitation regulates ClpXP degradation of σ^S both directly by ATP and indirectly by the nutrient responsive anti-adaptor IraD [155]. What differentiates these two mechanisms? Direct ATP control of σ^S proteolysis may be a feed-forward response that couples σ^S activity directly and dynamically to cellular metabolism. In contrast, while accumulation of IraD during transition to stationary phase may be slower, once made, IraD can constitutively block σ^S degradation, since it is not degraded with σ^S [153]. These complementary mechanisms may allow σ^S to be highly responsive to nutrient state, leading to both rapid and sustained activation of σ^S.

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Proteolysis can generate alternative forms of proteins required during stress

The proteome can also be altered by programmed ribosomal frameshifting that generates alternative forms of proteins, such as the two forms of DnaX in *E. coli*. DnaX is a subunit of the complex that loads the DNA replication sliding clamp, which is required for processive replication in all organisms [160]. Both forms of DnaX protein (shorter γ and full-length τ) are present in the cell, with full-length τ generated by a frameshifting event [161–163]. However, the significance of these two forms of DnaX and whether and how bacteria other than *E. coli* and *Salmonella* produced these forms was unclear.

Recent work has shown that *Caulobacter* produces the shorter γ form of DnaX from full-length τ by ClpXP proteolysis, rather than by ribosomal frameshifting [164]. *Caulobacter* DnaX contains a glycine-rich, “slippery” tract adjacent to a stably folded domain that promotes release of partially degraded DnaX γ from ClpXP [164]. *In vitro,* ClpXP had been observed to release degradation intermediates of specific artificial substrates, but native substrates with this property had not been previously identified [165–168].

Both long and short form of DnaX are required for growth in *Caulobacter* [164]. Importantly, processing of τ DnaX to the γ form is required for proper recovery from DNA damage, as cells that constitutively express γ and a form of τ that cannot be processed are sensitive to DNA damaging agents [164]. Processing to γ may be required for efficient exchange to alternative, mutagenic DNA polymerases, which are employed during DNA damage [164]. Indeed, loss of τ processing leads to a reduced level of UV-induced mutagenesis, suggesting that proper usage of the alternative DNA polymerases has been inhibited [164]. This suggests that there is a stress-related rationale for generating two variants of the clamp loader. Interestingly, there are several known eukaryotic examples of partial proteolysis by the ubiquitin-proteosome system [169–171]. Ci, a regulator of hedgehog signaling, and NFκB, a mammalian transcription factor involved in inflammatory response, are both released when the proteosome encounters a low complexity sequence (e.g., glycine tracts) adjacent to a stably folded domain [169–171]. As this is the same mechanism that causes relese of DnaX, this conservation suggests that there are likely more examples of partial proteolysis in other organisms.

Proteome Remodeling by Lon

Lon, the first protease to be discovered, is thought to be the most widely conserved of all energy-dependent proteases [172]. Its housekeeping function is degradation of unfolded and abnormally folded proteins [144, 172]. This model is supported by the recent realization that the recognition tags for Lon comprise aromatic and hydrophobic residues that are buried in folded proteins [142, 173, 174]. Like ClpXP, Lon also participates in regulating stress responses. Indeed, the first phenotype determined for a deletion of *lon* was extreme UV-sensitivity [144, 172]. New studies highlight two further activities for Lon and additionally suggest that Lon may target additional proteins when stimulated by stress.

Recently, it was realized that Lon could act as a chaperone as well as a protease (Fig. 5). Lon variants that neither hydrolyze ATP nor degrade substrates can suppress severe unfolded protein stress, by binding to target proteins [175]. This chaperone activity is

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proposed to arise from ATP-independent conformational changes that are coupled to protein remodeling [175, 176]. Thus, chaperone activity may be a dominant function when ATP is limiting. Lon can also use its ATPase activity to inactivate the cell-division inhibitor, SulA (Fig. 5) [177–179]. As Lon mutants that are defective in chaperone activity can still inhibit SulA, this suggests that chaperone activity and SulA inhibition are distinct mechanisms for Lon [175].

Additionally, Lon can remodel its substrate specificity by altering its quaternary structure. Normally hexameric, Lon can also exist in a dodecameric state that closes off the entryway to its degradation chamber (Fig. 5) [180]. This may gate this chamber so that large substrates (>12–25 kDa) can no longer enter and be proteolyzed [180]. Importantly, the cellular concentration of Lon is high enough to support dodecamer formation, and constitutively dodecameric Lon mutants can complement many *lon* deletion phenotypes *in vivo* [180, 181]. As dodecamers cannot recognize large protein aggregates, dodecamer formation can realign the powerful degradation capacity of Lon to focus on important small regulatory proteins during times of high protein unfolding and aggregation [180].

Although it is an open question whether specific conditions or stresses promote chaperone activity or dodecamer formation, it is already known that heat can alter the substrate specificity of Lon. Under conditions of severe heat shock (shift to 45°C), replication is arrested in *C. crescentus* as a result of degradation of the DNA replication initiation protein DnaA [182, 183]. DnaA is stable in rich media, but rapidly depleted during severe temperature upshift and in several other stress conditions, leading to growth arrest [183–186]. This effect was recently traced to Lon-mediated degradation of DnaA [187].

Intriguingly, while DnaA is not a normal substrate for Lon *in vitro*, addition of a model unfolded protein substrate stimulated specific, robust degradation of DnaA (Fig. 5) [187]. Folded substrates of Lon could not stimulate degradation of DnaA, nor did unfolded protein significantly increase the degradation rate of other known Lon substrates [187]. In normal *in vivo* conditions, unfolded proteins are continually removed by cytoplasmic chaperones (e.g., Hsp70), and thus unavailable to activate Lon degradation of DnaA. However, following sudden onset of severe stress (shift to 45°C), unfolded pr oteins exceed the capacity of the protein refolding machinery, activating DnaA degradation and arresting replication [187]. As Lon and the chaperone machinery are widely distributed among bacteria, regulated DnaA degradation by Lon may be a broad mechanism for inducing growth arrest during stress. Intriguingly, there is an additional example of Lon targeting proliferation proteins for degradation: In *E. coli* that have lost the Hsp70 chaperone machine (Δ*dnaKJ*), Hsp33 (HslO) can interact with the ribosomal elongation factor Tu (Ef-Tu) and target it for degradation by Lon, thereby inhibiting translation of proteins and leading to growth arrest [188].

Why would cells want to target proliferation factors for degradation? During times of severe stress, if cells cannot maintain genome integrity or ensure survival during growth, it may become better for them to assume a nonproliferative (persister) state [189–193]. In fact, persister cells are highly resistant to stresses and antibiotics [189–192]. By stopping replication and reducing protein synthesis, the cell can focus on stress response while waiting for a more opportune condition to resume growth. Such behavior would be a form of

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bet-hedging, trading current fitness for future benefits [194, 195]. These may be examples of general mechanism, whereby stress exposes vital proliferation factors as proteolytic targets to induce growth arrest.

4.0 Perspective

Stress responses are not disconnected pathways, but are closely integrated into bacterial physiology. As there is no limit in the variety of ways that stress can alter cellular pathways, responses have evolved to be equally complex, monitoring and maintaining every cellular process. Since stress responses are so intimately connected to cellular state, studying them has provided an elegant window into the mechanisms that regulate the homeostasis of the cell. We have started to develop an understanding of the mechanisms that sense stress, the molecular tools that comprise responses, the logic of how responses are constructed and linked, and the dynamic outcomes that can result. However, many questions remain. For many responses, we still do not know the inducing signal, all the players, how the players fit together, or the behaviors that can result. As there are only a finite number of sensors and regulators to face an infinite variety of stresses, not all responses may be perfectly adaptive [196]. Additionally, we do not know how bacteria integrate the combinatorial stresses they are likely to have faced in the environment. This is especially important for pathogens, as they experience a characteristic set of stresses in a defined temporal order, and responses may be optimized to reflect this [197, 198]. Lastly, we are only beginning to grasp at the variability that may occur on the single-cell level. How pervasive are these behaviors in stress? Why have particular response behaviors been selected over others? Answering these and other questions will be crucial for understanding bacterial physiology and engineering.

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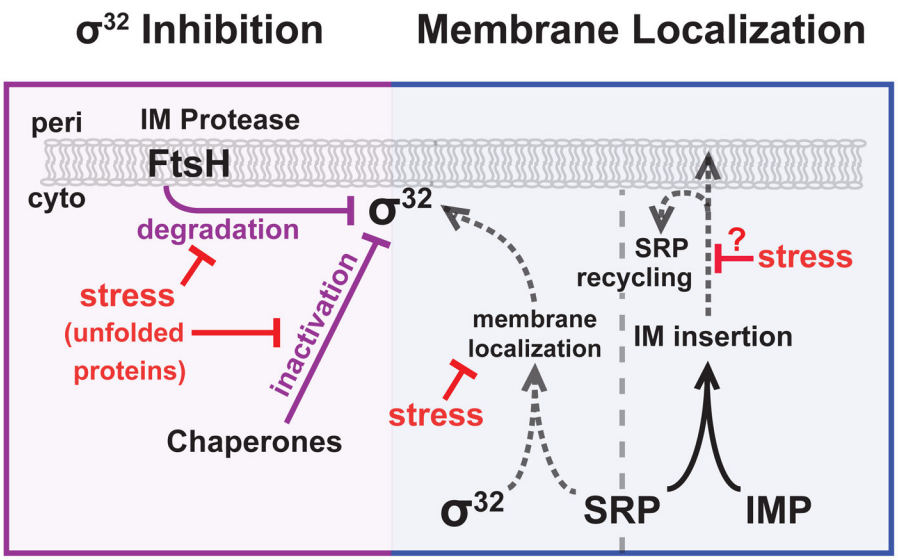

Figure 1. Regulation of σ^{32} at the inner membrane

(Left) σ^{32} inhibition. Membrane localized σ^{32} is inhibited via degradation by FtsH and inactivation by the cytoplasmic chaperones. Unfolded proteins relieve inhibition by competing for FtsH and titrating chaperones away from σ^{32}. (Right) σ^{32} membrane localization. σ^{32} is brought to the membrane by the signal recognition particle (SRP), which also traffics inner membrane proteins (inner membranePs) to the membrane. When stress stalls or prevents proper SRP-dependent inner membraneP insertion, this may prevent σ^{32} from being trafficked to the membrane for inactivation.

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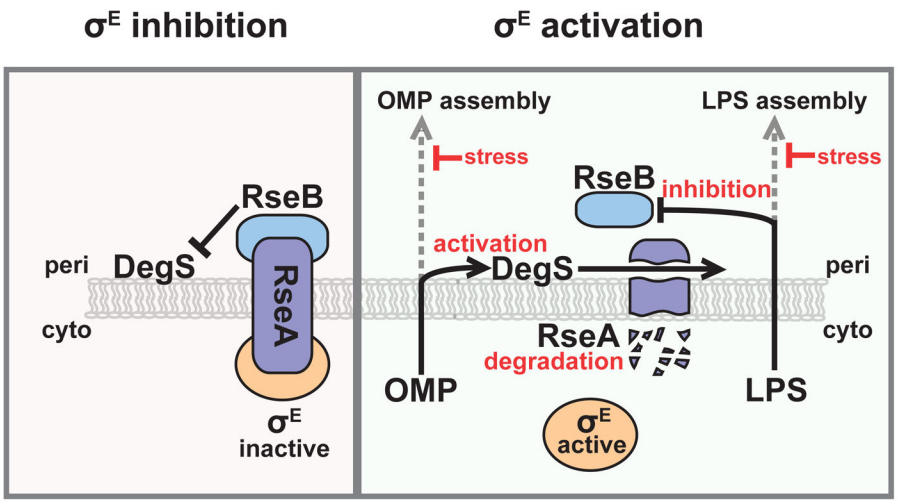

Figure 2. Two signals are required for σ^E activation

(Left) σ^E inhibition. σ^E is held inactive by RseA in the inner membrane. DegS, a protease, can cleave RseA if activated, but RseA cleavage is prevented by RseB. (Right) σ^E activation. When concomitant defects in OMP assembly and LPS assembly occur, σ^E is activated. Periplasmic LPS dissociates RseB from RseA, and periplasmic OMPs activate DegS to cleave RseA. This leads to a proteolytic cascade that degrades RseA, releasing and activating σ^E.

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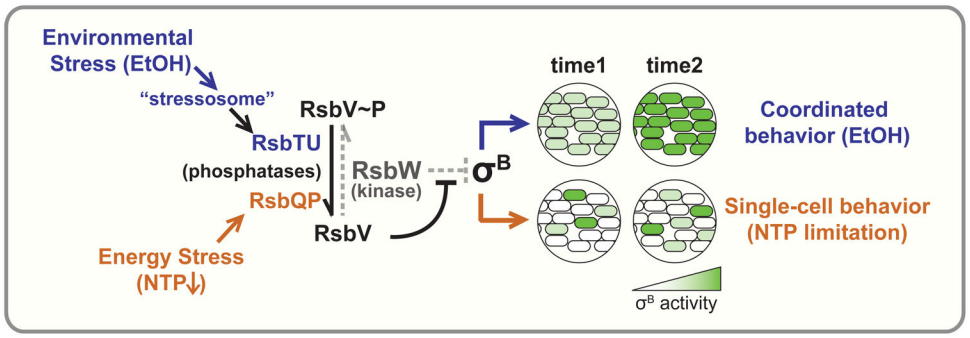

Figure 3. Activation of σ^B by different stresses leads to distinct responses

Activation of σ^B by environmental stress (e.g., EtOH, blue) and energy stress (decreased NTP, orange) lead to two different σ^B behaviors: EtOH induces a coordinated response, while nucleotide limitation induces stochastic pulses (same maximum amplitude) on a single-cell level. σ^B is inhibited by RsbW, which is in turn inhibited by RsbV. RsbW, a kinase, phosphorylates RsbV to relieve its own inhibition. Countering this, stress-specific phosphatases such as RsbTU (activated by EtOH, blue) and RsbQP (activated by NTP limitation, orange) dephosphorylate RsbV~P, allowing RsbV to bind to and inhibit RsbW and thereby promoting release and activation of σ^B.

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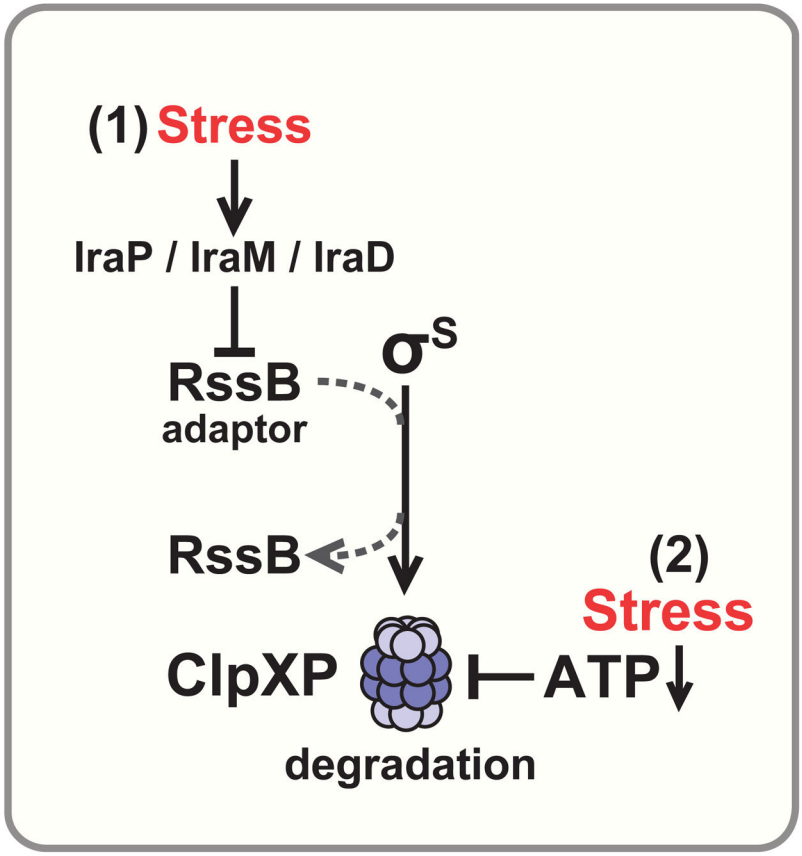

Figure 4. ClpXP degradation of σ^S is regulated via two mechanisms

The adaptor RssB targets σ^S to ClpXP for degradation. Stress prevents σ^S degradation via two mechanisms: (1) Specific stresses induce expression of corresponding anti-adaptor proteins (low PO_4, IraP; low Mg^{2+}, IraM; stationary phase/DNA damage, IraD), which prevent RssB from interacting with σ^S. (2) ClpXP degradation of σ^S is particularly sensitive to ATP levels; low ATP (nutrient limitation) thus specifically prevents ClpXP degradation of σ^S.

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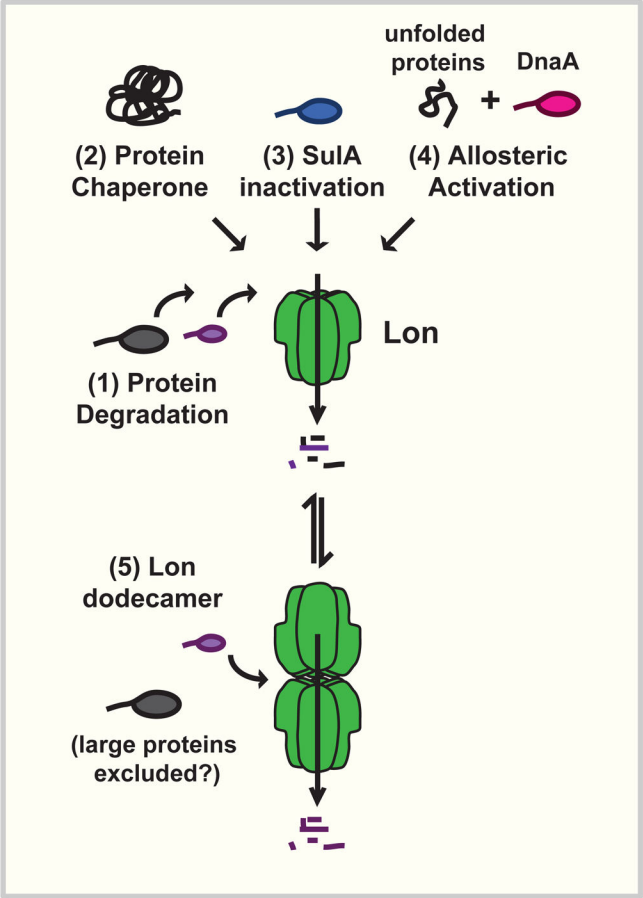

Figure 5. Many cellular functions of Lon

(1) Lon is responsible for degradation of many cellular proteins. (2) Lon can act as a chaperone to prevent protein aggregation. (3) Lon inactivates the cell division inhibitor SulA. (4) Lon is allosterically activated by unfolded proteins to degrade the initiation protein DnaA. (5) Lon exists as a hexamer and a dodecamer. Large proteins are thought to be excluded from entering the pore of the dodecamer and being degraded by Lon. It is not known if the dodecamer may also have chaperone activity, mediate SulA inhibition, or degrade DnaA.