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Regulation of the extracytoplasmic stress response in Escherichia coli

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REGULATION OF THE EXTRACYTOPLASMIC STRESS RESPONSE IN ESCHERICHIA COLI

by

LYNN ELIZABETH CONNOLLY

DISSERTATION

Submitted in partial satisfaction of the requirements for the degree of

DOCTOR OF PHILOSOPHY

in

CELL BIOLOGY

in the

GRADUATE DIVISION

of the

UNIVERSITY OF CALIFORNIA SAN FRANCISCO

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This thesis is dedicated to my mother,

Karen Rae Connolly.

Acknowledgements

Several people have contributed to the completion of this thesis, either directly or indirectly, and I owe each of them a debt of gratitude for their help and support over the years. There probably does not exist sufficient paper or time on this earth for me to do justice to the contributions of all these supporters, but I would particularly like to thank the people mentioned here.

My parents, Karen and Alex Connolly, have each inspired and supported me in their unique ways. My mother was my first role model as a biologist, and has provided me with invaluable opportunities throughout my scientific journey. Through a colleague, she negotiated a job in the laboratory of Larry Shapiro for her then very green, but enthusiastic, daughter and is now helping to raise my own daughter so than I can pursue the interests she helped to set in motion. Perhaps much to the dismay of others in the family, my father passed on his love of all things rigorous and exacting to me, and, despite the seeming lunacy of many of my life decisions, has provided me with unlimited and unquestioning support.

In my time at UCSF, I have been blessed with three wonderful bay mates who have also proven to be exceptional mentors. The best thing to come out of my time spent at the Gladstone Institute of Virology and Immunology in John Young's lab was my relationship with Kurt Zingler. I can only hope that a fraction of his organizational skills and efficiency rubbed off on me, and will always be grateful to him for acting as a substitute advisor when John was unable to carry out these duties. Alejandro De Las Peñas (affectionately known as Cano) warmly welcomed me into Carol's lab at a time in which I most needed to be felt welcome, and taught me to think about *E. coli* as more than just a means of producing plasmids. Lastly, Christophe Herman's amazing ability to think creatively and make connections between seemingly divergent processes helped to guide my project down

new paths, and his excellent pre- and post-natal advice helped to convince me that even I could be a good parent.

I would also like to thank my thesis committee, Joanne Engel, Peter Walter, and Keith Yamamoto for their helpful advice and support. My only regret in regards to my committee was that I didn't take advantage of their expertise more often. I owe Keith a further debt of gratitude for the excellent advice he gave me when my original advisor left UCSF. He prompted me to candidly examine my expectations and needs as a graduate student, and this discussion helped me come to the decision that Carol's lab would provide me with the environment I needed to flourish. I would also like to thank Elizabeth Blackburn and Tris Parslow for the support they showed me during this difficult time.

As I have eluded to, the decision to join Carol's lab proved to be a turning point in my graduate career. Although I originally decided to join her lab based on a project that interested me, it was Carol's exceptional skills as both a scientist and a mentor that helped to re-ignite my passion for science. She not only provided me with a fantastic project, but helped to rebuild my confidence in both my abilities as a scientist and in science as a worthwhile pursuit. I will always treasure the time spent working so closely with Carol, and will never forget the lessons she taught me about being both a rigorous and a compassionate mentor.

Despite her amazing abilities, Carol did not create such a supportive environment on her own, and I would like to extend my thanks to the many co-workers that helped to enrich my experience in the Gross lab. In particular, I would like to thank Chi Zen Lu whose warm smile always made me feel at home and whose hard work keeps the lab running smoothly. I thank the original Wisconsin lab members--Pierre Rouvière, Mike Lonetto, and Alejandro De Las Peñas--both for welcoming me into the lab and teaching me all I know about bacterial physiology. Jon Tupy and Meghan Sharp came to Carol's lab at the same time as me, and have been a constant source of friendship and support as we have together watched other lab members leave. With their arrival, Sarah Ades, Benjamin Alba,

and Brian Young injected some much needed enthusiasm into the lab and I only wish they could have arrived a little earlier. Lastly, I thank Sarah, Ben, Jon and Chris Onufryk for carrying on with various aspects of the σ^E project, thus giving me the satisfaction of knowing that my work provided new paths for others to follow.

I have established numerous wonderful friendships while at UCSF, but would especially like to thank Jody Rosenblatt and Michael Redd for their enduring friendship. Jody and I both fled a horrific life as industry technicians to find ourselves together again at UCSF. She has been a constant source of support, gossip, fun and inspiration over these many years and I would have often been lost without her. In the last few years, Jody and Mike have shown me that it is possible to be both a good scientist and a good parent, and I look forward to watching their dual careers flourish over the years to come.

Finally, I would like to thank my partner José de la Torre for sticking by me through both the soaring highs and crashing lows that have composed our time together at UCSF. His unwavering belief in me gave me the strength to persevere through my darkest moments, and his generous and warm spirit ensured that even the smallest victories were celebrated.

Statement Regarding Previously Published Material with Multiple Authors

A version of Chapter One is currently in press as (Connolly, L., T. Yura, and C. A. Gross. (1998) Autoregulation of the heat shock response in procaryotes. In B. Bukau, ed., *Molecular Chaperones and Folding Catalysts: Regulation, Cellular Functions and Mechanisms,* Harwood Academic Publishers, Amsterdam), and is reprinted here with permission from Harwood Academic Publishers (see page ix-xi) who retains all copyright privileges. I contributed all of the material concerning the extracytoplasmic stress response to this chapter.

Chapter Two has previously been published as (De Las Peñas, A., L. Connolly, and C. A. Gross (1997) The σ^E-mediated response to extracytoplasmic stress in *Escherichia coli* is transduced by RseA and RseB, two negative regulators of σ^E. Mol Microbiol 24:373-385), and is reprinted here with permission from Blackwell Science, Ltd. (see page xii) who retains all copyright privileges. This is a co-first author paper and my contributions to the work were the determination and analysis of the *rpoE* operon sequence (Fig. 2-1), the purification and *in vitro* characterization of a soluble form of the cytoplasmic domain of RseA (Fig. 2-2b and 2-3b), the co-purification of RseB with the periplasmic domain of RseA (Fig. 2-5b), and the development of the model of signal transduction to σ^E (Fig. 2-6). In addition, the manuscript was written by me.

Chapter Three has previously been published as (De Las Peñas, A., L. Connolly, and C. A. Gross (1997) σ^E is an essential sigma factor in *Escherichia coli.* J Bact 179:6862-6864), and is reprinted here with permission from American Society for Microbiology Press (see page xiii) who retains all copyright privileges. The work in this chapter was initiated by Alejandro De Las Peñas and I conceived of and carried out the experiment described in Table 3-3.

Chapter Four has previously been published as (Connolly, L., De Las Peñas, A., Alba, B.M., and Gross, C.A. (1997) The response to extracytoplasmic stress in *Escherichia coli* is controlled by partially overlapping pathways. Genes & Development 11:2012-2021), and is reprinted here with the permission of Cold Spring Harbor Laboratory Press (see page xiv) who retains all copyright privileges. This work began as a summer project for Benjamin Alba which was initially conceived by Alejandro De Las Peñas, Carol Gross, and me. The selection for multicopy suppressors was carried out by Ben with help from Alejandro and initial deletion analysis and sequencing of the suppressor plasmid were carried out by Ben in conjunction with me. The remainder of the work was carried out by me.

The work in Chapter Five represents work in progress. Although this project was initially conceived by me, the experiment in Table 5-2 (cotransduction of *argR*:Tn*5* and *ΔdegS1*) was conceived of and carried out by Benjamin Alba, and the data presented in Figure 5-1b (Cpx activity in strains carrying mutations in *degP*, *prc*, or *ompT*) and Figure 5-4a (σ^E activity in strains carrying the *Δ(degQ-degS)1* allele and mutations in *cpxR*, *degP*, or *prc*) was also collected by Ben.

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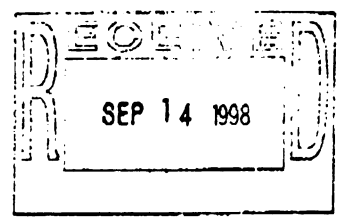

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Regulation of the Extracytoplasmic Stress Response in *Escherichia coli*

by

Lynn Elizabeth Connolly

Carol A. Gross

Carol A. Gross, Ph.D.
Thesis Advisor
Chairperson, Thesis Committee

Abstract:

The cellular response to misfolded or unfolded protein is one of the most highly conserved regulatory responses amongst all organisms. In addition, this response is compartmentalized into separate pathways that regulate protein folding processes in different subcellular compartments. In the Gram-negative bacterium *Escherichia coli* the response to unfolded protein is compartmentalized into cytoplasmic and extractyoplasmic responses. In this thesis, I present evidence that the extracytoplasmic response is controlled by two partially overlapping pathways, the two-component Cpx system and the essential alternate sigma factor, σ^E. While the Cpx pathway can compensate for a loss of σ^E under some conditions, activation of σ^E does not appear to compensate for a loss of the Cpx pathway and the two systems appear to respond to distinct types of stressors.

σ^E activity is induced in response to the accumulation of outer membrane protein precursors in the periplasmic space. In this thesis, I show that σ^E activity is under the control of three genes, *rseABC* (for regulator of sigmaE), encoded immediately downstream of the sigma factor. RseA is an inner membrane protein whose cytoplasmic

domain acts as a σ^E-specific anti-sigma factor. RseB binds to the periplasmic domain of RseA and appears to modulate the activity of RseA. Finally, I present evidence that DegS/HhoB is an essential inner membrane protease and that this protease plays a role in the signal transduction cascasde leading to activation of σ^E, perhaps by regulating the stability of RseA.

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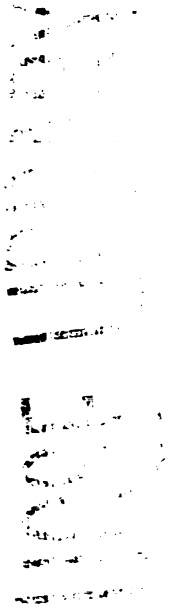

Chapter One

General Introduction

The hallmark of the Gram-negative bacterial cell is the existence of two membrane layers, the inner or cytoplasmic membrane and the outer membrane which in turn form the boundaries of two aqueous subcellular compartments, the cytoplasm and the periplasm. The conditions within each of these compartments differ markedly. The cytoplasm is an energy rich, highly regulated, reducing environment in which basic cellular processes such as transcription, DNA replication, and translation are carried out. In contrast, the extracytoplasmic compartment is a relatively energy poor, oxidizing environment whose conditions vary with those of the external environment due to the existence of pores in the outer membrane which allow the free exchange of small molecules and some specific substrates (Nikaido, 1994). Optimal cellular growth depends upon the ability of the cell to sense and respond to potentially harmful changes in these disparate subcellular compartments brought about by exposure to deleterious environmental conditions such as thermal stress.

When cells of any type are shifted to high temperature, the heat shock response (hsr) ensues and the synthesis of a small number of proteins, called the heat shock proteins (hsps), is rapidly induced. In the Gram-negative bacterium *Escherichia coli*, the hsr was discovered independently by the Neidhardt and Yura groups, who monitored the rate of synthesis of individual proteins after a temperature upshift using either 1D or 2D gels (Lemaux et al., 1978; Yamamori et al., 1978). A group of about 20 proteins exhibited a large (10 to 20-fold) but transient increase in synthetic rate upon temperature upshift and a corresponding decrease in synthetic rate upon temperature downshift (Lemaux et al., 1978; Yamamori et al., 1978; Neidhardt and VanBogelen, 1987; Straus et al., 1989; Taura et al., 1989). This group of proteins comprises the *E. coli* hsps. Their expression is regulated at the transcriptional level (Yamamori and Yura, 1980; Taylor et al., 1984; Cowing et al., 1985) by the amount and/or activity of the alternative sigma factor, σ^{32}, which directs RNA polymerase to transcribe this set of genes (Lesley et al., 1987; Skelly et al., 1987; Straus et al., 1987). These hsps, including the chaperones DnaK-DnaJ and GroEL-GroES,

are required for growth at physiological temperatures. Whereas *E. coli* normally grows at temperatures between 25°C and 40°C, deletion of the gene encoding σ^{32} restricts growth to temperatures below 20°C (Zhou et al., 1988). Overexpression of the GroEL-GroES and DnaK-DnaJ chaperone machines restores high temperature growth, suggesting that these chaperones play a crucial role in adaptation to high temperature.

E. coli also has a second heat-controlled regulon, controlled by σ^E (σ^{24}), another alternative sigma factor (Erickson and Gross, 1989; Wang and Kaguni, 1989). Most members of this regulon have yet to be identified. The two responses are intertwined because holoenzyme containing σ^E ($E\sigma^E$) transcribes σ^{32} at extreme temperature. However, each response also has a distinct role in the cell: σ^{32} controlled genes respond to conditions in the cytoplasm of the cell whereas σ^E controlled genes respond to the extracytoplasmic state. The σ^E regulon also plays a key role in temperature adaptation as σ^E is an essential sigma factor, at least at temperatures above 18°C. Strains lacking σ^E also exhibit defects in the cell envelope, emphasizing the dual role played by members of this regulon.

The heat induction of several additional genes may occur by other mechanisms. σ^S controls genes involved in adaptation to stationary phase and is also somewhat induced upon shift to high temperature, suggesting that genes in the σ^S regulon exhibit temperature regulation (Hengge-Aronis, 1996) . Finally, the psp operon is controlled by a dedicated activator protein that promotes *psp* transcription by $E\sigma^{54}$ following shift to very high temperatures (Brissette et al., 1990).

Two global approaches, one monitoring protein synthesis and the other monitoring RNA synthesis, have been used to identify most of the hsps. In the protein based approach, spots on 2D gels have been correlated with known genes (Neidhardt et al., 1981; Georgopoulos et al., 1982; Tilly et al., 1983). In the RNA based transcriptional mapping approach, radioactively labeled cDNA, made to total *E. coli* RNA, is hybridized to membrane filters containing an ordered *E. coli* genomic library carried in λ clones (the

Kohara library) and λ clones whose transcription increases are identified (Chuang and Blattner, 1993; Chuang et al., 1993b). A compendium of the proteins whose rates of synthesis increase upon temperature upshift is presented in Table 1-1.

Regulation of the σ^{32} heat shock response

Discovery of σ^{32}

The gene encoding σ^{32} was discovered in 1975 as a nonsense mutation that affected the synthesis of the GroEL hsp. The mutation was initially thought to be located in the structural gene for GroEL (Cooper and Ruettinger, 1975). Subsequently, it was found that mutant cells had a global defect in the hsr, suggesting instead that the gene encoded a regulator of the hsr (Neidhardt and VanBogelen, 1981; Yamamori and Yura, 1982). The sequence of the gene revealed strong homology to σ^{70} (Landick et al., 1984; Yura et al., 1984) and the regulator was shown to be σ^{32}, the first alternative sigma factor identified in *E. coli* (Grossman et al., 1984). σ^{32} directs core RNA polymerase to promoters that differ considerably from those recognized by RNA polymerase containing σ^{70}, the housekeeping sigma (Cowing et al., 1985). The fact that expression of the hsps is uniquely responsive to the amount or activity of σ^{32} provides a means to regulate their expression separately from other cellular proteins.

How does σ^{32} regulate the response to temperature shift?

When cells experience a temperature upshift, for example after shift from 30°C to 42°C, the rate of synthesis of the hsps increases 10 to 20-fold by 5 minutes after upshift and thereafter declines to a new steady state rate of synthesis. Interestingly, at steady state, the amount of hsps at 42° is only 2-fold greater than that at 30°. The large increase in rate of hsp synthesis immediately after temperature upshift allows cells to rapidly accumulate the

new steady state level of hsps (Lemaux et al., 1978; Yamamori et al., 1978; Straus et al., 1987).

The response of hsps to heat induction is controlled at the transcriptional level, primarily by the amount of σ^{32} in the cell. At low temperature, cells contain very little σ^{32}, on the order of 10 to 50 molecules per cell. By 5 minutes after temperature upshift, the amount of σ^{32} increases about 15-fold and thereafter declines to a new steady state level (Lesley et al., 1987; Straus et al., 1987). Changes in the amount of σ^{32} following temperature upshift result from changes in both the stability and synthesis of σ^{32} (Lesley et al., 1987; Straus et al., 1987). During steady state growth, σ^{32} is translated at a very low rate. In addition, σ^{32} is very unstable, with a T 1/2 for degradation of about 1 minute. As a result, little σ^{32} accumulates in the cell. However, for the first 5 minutes following temperature upshift, the rate of translation of σ^{32} increases about 5-fold and σ^{32} is stabilized against degradation. Following this time, the rate of translation decreases and rapid degradation resumes. Together, these two regulatory changes permit the transient accumulation of σ^{32}. To a first approximation, changes in the rate of hsp synthesis after temperature upshift primarily mirror changes in the amount of σ^{32} (Lesley et al., 1987; Skelly et al., 1987; Straus et al., 1987). However, careful examination of the kinetics suggest that shut-off of hsp synthesis in the adaptation phase of the hsp response may slightly precede the decrease in the amount of σ^{32}. Regulation of σ^{32} activity (see below) may be involved in this phenomenon.

When cells experience a temperature downshift, for example after shift from 42°C to 30°C, the rate of synthesis of hsps declines 10 to 20-fold within 5 minutes after downshift. This rate of hsp synthesis is considerably lower that normally exhibited by 30°C cells (Straus et al., 1989; Taura et al., 1989). By one to two doublings after downshift, the cell gradually resumes the 30° rate of synthesis. Presumably, existing hsps are diluted out during the long shut-off period. Hsp synthesis resumes when their amounts approximate that characteristic of the low temperature cell. The rapid drop in transcription

of heat shock genes upon temperature downshift results from a decrease in σ^{32} activity, rather than from a decrease in the amount of σ^{32}. Temperature downshift is not the only condition that promotes inactivation of σ^{32}. Overexpression of hsps at constant temperature also reduces σ^{32} activity, suggesting that cells can sense the amount of hsps and adjust the activity of σ^{32} accordingly (Straus et al., 1989; Craig and Gross, 1991).

These studies indicate that the translation, stability and activity of σ^{32} are all regulated by the cell in response to temperature. The extent to which temperature regulation of each of these processes is understood at a mechanistic level is discussed below, and a speculative model of the regulation of σ^{32} activity is presented in Figure 1.

Translational regulation of σ^{32}

Translational regulation includes both translational induction, which occurs immediately following temperature upshift, and translational repression, which occurs subsequently during the adaptation phase of the hsr. The cis-elements and the trans-acting factors required for induction and repression differ, suggesting that these two processes are mechanistically distinct.

The mechanism of translational induction has been probed by both deletion and point mutational analysis of a σ^{32} - β -galactosidase fusion protein (Kamath-Loeb and Gross, 1991; Nagai et al., 1991; Yuzawa et al., 1993). These studies indicate that two regions within σ^{32}, termed A and B, are required for translational induction (Fig. 1-1). Region A, located near the start of translation initiation (nucleotide 6 -20), has homology to the "downstream box", which is required for high rates of translation in several prokaryotic systems. Deletion of the downstream box leads to very low, uninducible synthesis of σ^{32}. Region B is a grossly defined, internal region extending from nucleotide 110 - 210, part of which has the capacity to base pair with a portion of Region A. Deletion of Region B, as well as some point mutations in the region, leads to high constitutive synthesis of σ^{32}. Initial speculation that thermal induction might simply be explained by disruption of base-

pairing potential between the two regions, led to an analysis of compensating mutational changes between putative base-pairing partners. These studies indicated that recovery of base pairing is not always sufficient for regulation, leading to the suggestion that sequence, as well as structure, is important for regulation (Yuzawa et al., 1993; Yura, 1996). The current view is that an unknown transacting factor is involved in this regulatory event.

The mechanism of translational repression is distinct from that of translational induction. Translational repression requires Region C of σ^{32} (nucleotide 364-433; amino acid 122-144) and the DnaK, DnaJ, GrpE chaperone machine (Straus et al., 1990; Nagai et al., 1994). Deletion analysis indicates that lack of region C prevents repression, and analysis of a frameshift of Region C indicated that polypeptide rather than nucleotide sequence was involved in the response. Interestingly, a peptide scan of σ^{32} using a library of overlapping 13 amino acid-long peptides identified Region C as the site of two high affinity DnaK binding sites within σ^{32}, leading to speculation that the function of Region C may be to bind DnaK (McCarty et al., 1996). Further support for this notion comes from comparative analysis of the sigma family of polypeptides. Whereas this region of sigma is highly conserved among σ^{32} homologues from diverse bacteria, it is poorly conserved among sigma factors in general (Nakahigashi et al., 1995). It is certainly plausible that a non-conserved region within the sigma family of proteins has become specialized for a regulatory function specific to σ^{32} homologues. Co-translational binding of DnaK to Region C may then mediate translational repression by an unknown mechanism.

Regulation of σ^{32} stability

The instability of σ^{32} is a key feature of the response to temperature upshift. Because σ^{32} is so unstable (T1/2=1 min.) during steady state growth, increases in its rate of synthesis are immediately reflected in commensurate increases in the level of σ^{32} available to promote transcription of the heat shock genes. Great advances in understanding this process have recently been reported. Both *in vivo* and *in vitro* studies indicate that σ^{32} is

proteolysed by HflB, an ATP dependent protease located in the inner membrane (Tomoyasu et al., 1993; Herman et al., 1995; Tomoyasu et al., 1995). Depleting cells of HflB (FtsH), or inactivating mutant HflB by shift to high temperature stabilizes σ^{32} about 10-fold indicating that HflB is a major protease responsible for σ^{32} degradation. Moreover, HflB can degrade σ^{32} *in vitro.* Interestingly, HflB is a member of the σ^{32} regulon and the only essential protease thus far reported in *E. coli.*

There are still important, unresolved questions concerning the physiology of σ^{32} degradation. Currently, the rate of degradation of σ^{32} *in vitro* (T 1/2= 18 mins.) is much slower than the *in vivo T 1/2* of 1 min. *In vivo,* the DnaK-DnaJ-GrpE chaperone machine is required for degradation of σ^{32}, and mutations in *dnaK, dnaJ* or *grpE* decrease the rate of σ^{32} degradation as much as 10-fold (Tilly et al., 1989; Straus et al., 1990). Region C of σ^{32}, described above as a possible DnaK binding site, may couple these chaperones to the process of degradation. In support of this idea, the Region C frameshift mutant inhibits degradation of σ^{32} *in vivo* (Nagai et al., 1994). However, the *in vitro* degradation system currently in use exhibits no requirement for these hsps (Tomoyasu et al., 1995). Moreover, the presence of core RNA polymerase inhibits the *in vitro* degradation of σ^{32} by HflB, and this inhibition is not reversed by the DnaK-DnaJ-GrpE chaperone machine. Thus, the *in vitro* system is not yet a faithful mimic of *in vivo* degradation, either because of missing components or altered conditions.

Regulation of σ^{32} activity

Inactivation of σ^{32} appears to be a primary mode of regulation whenever σ^{32} is present in excess in the cell (Straus et al., 1989; Taura et al., 1989; Straus et al., 1990). This regulatory mode features most prominently on temperature downshift, but also most likely sharpens the shut-off phase of the heat shock response. The DnaK-DnaJ-GrpE chaperone machine is involved in σ^{32} inactivation, as cells carrying mutations in these genes are defective in this process (Straus et al., 1989 and unpublished experiments). Inactivation is

reversible as σ^{32} regains activity after extraction from the cell (Straus et al., 1989). These characteristics led to the proposal that the DnaK-DnaJ-GrpE chaperone machine reversibly binds to σ^{32} to inhibit its function (Straus et al., 1989) (Fig. 1-2).

Elegant *in vitro* studies from the Bukau and Georgopoulos laboratories are beginning to establish the molecular basis for inactivation of σ^{32}. Both DnaK and DnaJ can bind independently to σ^{32} (Gamer et al., 1992; Liberek et al., 1992; Liberek and Georgopoulos, 1993; Gamer et al., 1996) . In addition, all three also form an ATP-dependent ternary complex with distinct properties from each of the binary complexes (Liberek and Georgopoulos, 1993; Gamer et al., 1996) It is only this ternary complex that shows decreased activity with core RNA polymerase (Liberek and Georgopoulos, 1993; Gamer et al., 1996). Thus, together DnaK and DnaJ function as an anti-sigma factor. When bound to σ^{32}, they inhibit the formation of the σ^{32}-core RNA polymerase complex (Gamer et al., 1996).

Understanding the mechanistic details of the interactions of DnaK and DnaJ with σ^{32} is in its infancy. Indeed, further study of this interaction is likely to yield important insights concerning the regulatory loop governing σ^{32} activity, and also into the nature of chaperone interaction with native substrates. The DnaK-σ^{32} binary complex is relatively weak (K_d=5 μM), and this binding is considerably decreased by ATP (Gamer et al., 1992; Liberek et al., 1992; Liberek and Georgopoulos, 1993; Gamer et al., 1996). Interestingly, the low binding constant reflects a very slow on rate, as the DnaK-σ^{32} complex is quite stable once formed (t 1/2 >30 minutes) (Gamer et al., 1996). In contrast, the stronger DnaJ-σ^{32} binary complex (K_d=20nM; measured in the Biacore), actually dissociates more rapidly than the DnaK-σ^{32} complex (Gamer et al., 1996). The ternary complex, which requires ATP for its formation, somehow stabilizes the σ^{32}-DnaK interaction and effectively competes with σ^{32} for binding to core RNA polymerase. It is currently unknown how DnaJ promotes formation of this ternary complex. However, DnaJ binding to substrate may not be necessary for its effect. Some DnaJ mutants that do not bind σ^{32}

still promote an ATP-resistant σ^{32}-DnaK interaction, and may do so catalytically (Liberek et al., 1995). It is not known, however, whether these σ^{32}-DnaK binary complexes inhibit σ^{32} mediated transcription.

What are the signals governing expression of the σ^{32} heat shock regulon? The challenge of the cell is to integrate diverse environmental information to program the level of hsp expression that is appropriate for the perceived cumulative stress level. Exactly how this is accomplished is still a matter of speculation. We have a great deal of information about initial inputs--expression of the regulon is triggered by heat, ethanol and other diverse insults. Likewise, we are fairly knowledgeable about the final outputs--regulation of both the activity and amount of σ^{32} lead to a defined rate of transcription of the heat shock genes. However, the nature of the signal-transduction pathway(s) that couple(s) the two ends of this regulatory loop remains an area of active investigation.

There are at least two distinct signal-transduction pathways governing expression of the hsps. The first pathway controls translation of σ^{32} mRNA in a positive way: increased environmental stress leads to increased translation. This pathway is induced by exposure to heat and ethanol, but not by accumulation of unfolded proteins. To date, the only identified player in this pathway is cis-acting σ^{32} mRNA sequences. Neither the trans-acting factors, nor the signaling molecule(s) have been identified. Our understanding of the remainder of the regulatory events governing the amount of active σ^{32} is somewhat more advanced. Regulating σ^{32} stability, activity and translational repression have in common the involvement of the DnaK, DnaJ and GrpE chaperone machine in the signal transduction pathway. Regulation of these diverse processes may be controlled either by a single pathway, or by multiple, interconnected pathways.

A homeostatic mechanism coupling the occupancy of the DnaK, DnaJ, GrpE chaperone machine to the amount and activity of σ^{32} has been proposed (Straus et al., 1990; Craig and Gross, 1991; Bukau, 1993; Liberek et al., 1995). Cellular stress is

monitored by how well σ^{32} can compete with all other unfolded or misfolded proteins for binding to the DnaK, DnaJ, GrpE chaperone machine. Inducing signals increase unfolded or misfolded proteins, thus titrating DnaK, DnaJ and GrpE away from σ^{32} and relieving their negative regulatory effects on stability and translation. As a consequence, the amount of σ^{32} will rise. Conversely, repressing signals will decrease unfolded or misfolded proteins, thus freeing DnaK, DnaJ and GrpE to inactivate σ^{32}. This response is self limiting because under or over production of DnaK, DnaJ and GrpE will restore the free pool of these chaperones to an appropriate level. Thus, the amount of free DnaK, DnaJ, and GrpE is a "cellular thermometer" that measures the "folding state" of the cell. There is some evidence in favor of this model, however, critical experiments to test the proposition that the DnaK, DnaJ and GrpE chaperones play a regulatory role have yet to be carried out.

Regulation of the σ^E (σ^{24}) heat shock response

Discovery of σ^E

σ^E was originally discovered as the sigma factor responsible for maintaining transcription of *rpoH* at extreme temperatures. *rpoH* has four promoters, three of which are transcribed by $E\sigma^{70}$ (Fig. 1-1a). The fourth promoter, *rpoHp3,* is recognized by $E\sigma^E$. *rpoHp3* accounts for only 2% of total *rpoH* transcription at 30°C, but drives over 90% at the lethal temperature of 50°C (Erickson et al., 1987). The continued production of σ^{32} at 50°C is critical to cellular survival, as the σ^{32} dependent hsps represent the majority of proteins expressed under these extreme conditions (Neidhardt et al., 1984; Pack and Walker, 1986). σ^E was purified based on its ability to direct transcription from *rpoHp3* (Erickson and Gross, 1989; Wang and Kaguni, 1989), and the structural gene encoding σ^E was recently identified (Raina et al., 1995; Rouvière et al., 1995).

What is the nature of the signal inducing σ^E activity?

In addition to being induced by the general stresses of heat and solvents, the σ^E pathway is uniquely induced in response to alterations in the expression or maturation of outer membrane proteins (OMPs) (Mecsas et al., 1993). Overexpression of OMPs induces σ^E activity, and underexpression of OMPs decreases σ^E activity. The inducing signal arises either during or after translocation because cytoplasmic accumulation of OMP precursors does not induce σ^E activity. Although σ^E activity is induced by overexpression of some periplasmic proteins with known folding defects (Missiakas et al., 1996a), overexpression of most periplasmic proteins does not induce σ^E, indicating that the signal is probably not arising due to titration of the translocation machinery. Expression of a mutant OMP that is properly translocated but fails to be inserted into the outer membrane also induces σ^E activity. Taken together, these results suggest that the signal arises in the periplasmic space, after translocation but prior to insertion into the outer membrane.

Outer membrane proteins undergo a complex series of folding events during their maturation into trimeric porins. Blocking this pathway at a step after the signal intermediate is generated should cause an increase in σ^E activity. Using this and related strategies, several putative periplasmic folding agents have been identified, including the peptidyl prolyl isomerases SurA and FkpA, and the Skp protein (Missiakas et al., 1996a; Rouvière and Gross, 1996). Loss of function mutations in each of these genes induce σ^E activity. The role of SurA in maturation of the trimeric porin LamB has been investigated (Lazar and Kolter, 1996; Rouvière and Gross, 1996). SurA appears to catalyze the formation of a folded monomeric species from unfolded monomer. Cells lacking SurA and cells overexpressing LamB both accumulate the unfolded monomer form at the expense of folded monomer. The observation that two different inducing conditions result in accumulation of unfolded monomer suggests that the signal for σ^E induction occurs somewhere prior to the formation of the folded monomer species (Rouvière and Gross, 1996).

Regulation of σ^E

The activity of σ^E is regulated, in part, at the level of transcription. σ^E is transcribed from a σ^E-dependent promoter and transcription from this promoter reflects the level of σ^E activity in the cell under steady state conditions (Raina et al., 1995; Rouvière et al., 1995). However, both the observation that transcription of σ^E is low under steady state conditions and that σ^E activity increases rapidly in response to induction suggest additional regulatory controls.

Homology arguments suggested that σ^E is under the control of negative regulators likely to be encoded in the same operon as *rpoE*, and this turns out to be the case. σ^E belongs to the ECF subclass of the σ^{70} family of proteins, most of which regulate extracytoplasmic functions (Lonetto et al., 1994; Rouvière et al., 1995). Operons encoding other ECF sigmas have previously been shown to also encode regulators of the sigma factor activity. In particular, the operon encoding the closely related *algU/T* sigma factor required for alginate biosynthesis in *P. aeruginosa*, includes two negative regulators of AlgU/T activity, MucA and MucB (Martin et al., 1993c). MucA inhibits AlgU/T activity *in vivo* and *in vitro* (Schurr et al., 1996; Xie et al., 1996), and previous work had identified an open reading frame encoded immediately downstream of *rpoE*, termed *mclA*, that showed significant homology to *mucA* (Raina et al., 1995; Rouvière et al., 1995; Yu et al., 1995). Three genes, *rseABC* (for regulator of sigmaE), are encoded immediately downstream of *rpoE*, and genetic experiments reveal that *rseA* (formerly *mclA*) and *rseB* negatively regulate σ^E activity (De Las Peñas et al., 1997b; Missiakas et al., 1997). Deletion of *rseA* leads to a 25-fold induction of σ^E activity, whereas deletion of *rseB* gives only 2.5-fold induction, indicating that RseA is the major negative regulator of σ^E. RseA is an inner membrane protein, whose cytoplasmic domain binds directly to σ^E and inhibits σ^E-directed transcription *in vivo* and *in vitro*. Thus, the cytoplasmic domain of RseA acts as an anti-sigma factor. The periplasmic domain of RseA interacts with RseB, which is located in the periplasm. Although genetic experiments indicate that RseC has a slight

positive effect on σ^E activity, a clear role for this protein in the signal transduction cascade leading to activation of σ^E has not been established.

How is the extracytoplasmic signal transduced to σ^E?

RseA is the central regulatory molecule in the signal transduction cascade to σ^E. Cells lacking RseA are unresponsive to induction because they are already maximally induced. Moreover, cells containing only RseA modulate σ^E activity in response to inducer, indicating that RseA alone or in conjunction with unknown molecules responds to the inducing signal. Several mechanisms of RseA inactivation by the inducer can be envisioned including modification, degradation, or oligomerization of the anti-sigma factor.

RseB may act to fine-tune this RseA-based signal transduction pathway. Binding of RseB to the periplasmic domain of RseA might shift RseA to a conformation where it is most effective as an anti-sigma (Fig. 1-3a). If RseB binding to RseA were competitive with binding to a signal molecule, RseB would be titrated away from RseA as the concentration of the signal increases (Fig. 1-3b). This would leave RseA in a conformational state where it is a less effective anti-sigma, and lead to a small increase in σ^E activity. At still higher concentrations, the signal molecule would interact either with an intermediate factor or with RseA itself to further increase σ^E activity (Fig. 1-3c).

The direct induction signal and how it affects RseA is currently unknown. σ^E is induced by the build up of early intermediates in the maturation pathway of outer membrane porins, the accumulation of a few periplasmic proteins, and a deficit of any of several periplasmic folding agents (DsbA, FkpA, Skp and SurA) (Mecsas et al., 1993; Missiakas et al., 1996a; Rouvière and Gross, 1996). The Rse proteins may detect the levels of misfolded protein directly. Alternatively, RseA and/or RseB may monitor the levels of free periplasmic folding agents, including SurA, FkpA, and the Dsb proteins. Decreases in the free levels of each of these proteins in response to the accumulation of unfolded or misfolded species in the periplasmic space may additively induce the σ^E pathway.

Upon generation of a signal, σ^E is released from the complex with RseA, leading to a positive feedback loop. The newly active σ^E transcribes its own promoter to generate more σ^E and RseA. As long as the signal is present, RseA will be unable to interact with σ^E, but when the signal is removed or reduced, RseA, possibly in concert with RseB, will again repress σ^E, achieving a new steady state level. Although this model bears a superficial resemblance to the regulation of σ^{32}, it is unlikely that RseA targets σ^E for degradation, or that RseA interacts with the signal in the same manner as it interacts with σ^E.

The cellular role of σ^E

σ^E is an essential sigma factor, at least at temperatures above 18°C, and cells lacking σ^E rapidly accumulate a suppresor of this lethality (De Las Peñas et al., 1997a). Cells lacking σ^E, and containing this suppressor mutation, form colonies at 42°C to 43°C with greatly reduced efficiency (10^{-3} to 10^{-5}), and die more rapidly than wild type cells after exposure to lethal temperatures (Hiratsu et al., 1995; Raina et al., 1995; Rouvière et al., 1995), while cells containing the suppressor alone are temperature resistant (Connolly and Gross, unpublished observations). These phenotypes confirm the importance of the σ^E regulon for resistance to thermal stress.

Overexpression of σ^E leads to the induction of at least 10 proteins (Raina et al., 1995; Rouvière et al., 1995). However, only four members of the regulon have been identified (Table 1-1). In addition to *rpoH*, Eσ^E transcribes the periplasmic protease *degP*, the periplasmic peptidyl-prolyl isomerase *fkpA* (Danese and Silhavy, 1997), and one of the two promoters upstream of *rpoE* itself.

Why does *E. coli* need two heat-inducible regulons? Part of the answer might be that the two regulons respond to stress in different cellular compartments. Some inducers, such as heat and solvents, affect all cellular compartments and thus induce both regulons. Other inducers specifically alter protein folding in either the cytoplasmic or

extracytoplasmic environments, and uniquely induce σ^{32} or σ^{E} activity, respectively. Just as the σ^{32} response has a close parallel in the eukaryotic heat shock response, the σ^{E} pathway also has a eukaryotic counterpart. Accumulation of unfolded proteins in the endoplasmic reticulum (ER) leads to the transcriptional induction of several ER resident folding agents (Cox et al., 1993; Mori et al., 1993). Like the σ^{E} pathway, the ER response, known as the unfolded protein response (UPR), is controlled separately from the cytoplasmic heat shock response. Although the central regulator of the UPR shares no common features with RseA, it remains to be seen whether the two systems share common mechanisms of sensing the initial signal.

E. coli has a second signal transduction pathway, the Cpx two-component system, capable of relieving extracytoplasmic stress. Although the Cpx system is not required for growth at high temperature (Connolly and Gross, unpublished observations), activation of the pathway suppresses the envelope-associated toxicity conferred by certain LamB mutant proteins by inducing the expression of DegP (Cosma et al., 1995; Danese et al., 1995; Snyder et al., 1995). Interestingly, activation of the Cpx pathway also restores the ability to grow at high temperature to cells lacking σ^{E}, in a *degP*-dependent manner (Connolly et al., 1997). Overexpression of *degP* alone does not suppress the *rpoE*$^{-}$ temperature sensitive phenotype, indicating that other Cpx-controlled genes are required (see Table 1-1 for a list of Cpx-controlled genes). Future work aimed at elucidating the relationship between the Cpx pathway and the σ^{E}-mediated response should help to clarify the roles of each system in responding to protein misfolding outside of the cytoplasm.

Work on the σ^{E} pathway is just beginning. The next few years should provide us with exciting insights into the members of the regulon, the nature of the signal, and the regulatory network that links the cellular compartments. In addition, σ^{E} has already proven to be an invaluable tool in the search for periplasmic folding agents and rapid progress in the understanding of folding processes in this cellular compartment is likely to follow.

Heat shock regulation in other prokaryotic organisms

Study of the heat shock response in a number of different bacteria indicates that the basic *E. coli* regulatory paradigm is not universal. Although σ^{32} homologues are widespread among gram negative bacteria, additional regulatory mechanisms also affect the primary heat shock response in some of these organisms. Moreover, the gram positive organisms examined to date do not have σ^{32} homologues.

σ^{32} homologues have been isolated from a number of Gram negative bacteria (Garvin and Hardies, 1989; Benvenisti et al., 1995; Fleischmann et al., 1995; Naczynski et al., 1995; Nakahigashi et al., 1995; Yura, 1996). All of these homologues can restore growth to *E. coli* cells lacking functional σ^{32}, indicating that the transcriptional function of the protein is conserved. However, sequence analysis suggests that only some of the regulatory inputs are conserved. All σ^{32} homologues identified to date contain Region C, which binds DnaK with high affinity and is required for control of σ^{32} stability. In contrast, the regions of σ^{32} mRNA implicated in translational control are conserved in γ but not α proteobacteria. If translational control of σ^{32} exists in a proteobacteria, it must be mechanistically distinct from the *E. coli* model. These observations suggest that diverse mechanisms may control the amount and/or activity of σ^{32} in different gram negative species.

Our knowledge about the heat shock response in gram positive organisms comes from studies of *Bacillus subtilis* and *Clostridium acetobutylicum* (Narberhaus and Bahl, 1992; Narberhaus et al., 1992; Schmidt et al., 1992; Wetzstein et al., 1992; Zuber and Schumann, 1994; Yura, 1996). In these organisms, the major chaperone genes are transcribed by the housekeeping sigma and are preceded by a conserved inverted repeat sequence. This inverted repeat, named CIRCE for controlling inverted repeat for chaperone expression, is the binding site for a putative repressor (Yuan and Wong, 1995). The mechanism of thermal induction of genes regulated by the CIRCE element has not yet been elucidated. CIRCE has also been detected in some gram negative bacteria suggesting

that it is rather widely involved in the heat shock response. In *Bradyrhizobium japonicum*, σ^{32} and CIRCE together control expression of heat shock genes (Babst et al., 1996), suggesting that parallel regulatory strategies may exist in some organisms.

In contrast to σ^{32}, the degree of conservation of σ^{E} has not been determined. Although several sigma factors belonging to the ECF family have been described in both Gram-negative and positive bacteria (Lonetto et al., 1994; Rouvière et al., 1995), their possible role in the heat shock response of these organisms has not been widely studied. Only one of the ECF sigmas in addition to σ^{E} has been implicated in the resistance to thermal stress. *Pseudomonas aeruginosa* cells lacking the σ^{E} homologue AlgU, show increased killing at 50°C compared to AlgU^{+} strains (Martin et al., 1994), and the activity of AlgU is induced in response to heat shock (Schurr et al., 1995a). However, AlgU carries out additional cellular functions not mediated by σ^{E}. For example, AlgU- cells show increased sensitivity to superoxide-generating compounds (Martin et al., 1994), and AlgU plays a key role in the production of the exopolysaccharide alginate (Deretic et al., 1994). One possibility is that the σ^{E}-mediated response has been co-opted by other signaling systems in *P. aeruginosa*, and it will be interesting to determine how AlgU and σ^{E} utilize similar signaling molecules to respond to diverse extracellular signals.

Summary and prospects

Although recent studies have given us insight into the mechanisms responsible for the regulation of both σ^{32} and σ^{E}, several basic questions concerning the response to thermal stress in *E. coli* remain unresolved. For example, the exact nature of the initial signal and sensing mechanism have not been elucidated. Further dissection of the response loops of each sigma factor should provide us with a greater understanding of not only the heat shock response but also of the process of protein folding in each cellular compartment. We have only begun to understand the *in vivo* role of the chaperones, and to identify periplasmic

protein folding agents. The next few years should prove to be an exciting time in the dual fields of thermal stress response and protein folding.

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Table 1-1. Heat and Stress Inducible Proteins in *Escherichia coli*

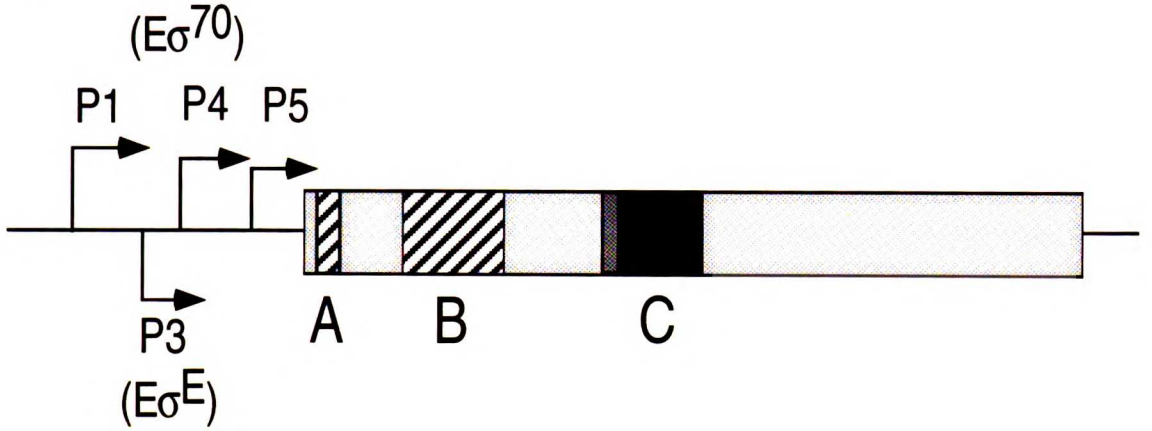
Min	Protein	Molecular Weight	Function	Reference(s)
σ^{32} Regulon				
0.3	HtpY	21	?	(Missiakas et al., 1993)
0.3	DnaK	69	chaperone	(Bardwell and Craig, 1984)
0.3	DnaJ	39	chaperone	(Bardwell et al., 1986)
10.0	Lon	89	protease	(Gayda et al., 1985)
10.0	ClpP	24 (22)	protease	(Maurizi et al., 1990)
10.0	ClpX	46	chaperone	(Gottesman et al., 1993)
10.0	HslA	65	?	(Chuang and Blattner, 1993)
10.8	HtpG	70	chaperone	(Bardwell and Craig, 1987)
19.2	HslC	80	?	(Chuang and Blattner, 1993)
39.3	GapA	35.5	dehydrogenase	(Charpentier and Branlant, 1994)
39.8	HslK	49	?	(Chuang and Blattner, 1993)
40.3	HtpX	32	?	(Kornitzer et al., 1991)
56.0	ClpB	84	chaperone	(Kitagawa et al., 1991; Squires et al., 1991)
56.8	GrpE	26	nucleotide exchange factor	(Lipinska et al., 1988a)
67.0	σ^{70}	70	sigma factor	(Burton et al., 1981)
69.2	FtsJ	26		(Tomoyasu et al., 1993; Herman et al., 1995)
69.2	HflB	70	protease	(Tomoyasu et al., 1993; Herman et al., 1995)
75.0	HslO	33	?	(Chuang and Blattner, 1993)
75.0	HslP	30	?	(Chuang and Blattner, 1993)
81.2	HtrM (RfaD)	34	epimerase	(Raina and Georgopoulos, 1991)
83.0	IbpB (HtpE, HslS)	16.3	chaperone	(Allen et al., 1992; Chuang and Blattner, 1993)
83.0	IbpA (HtpN, HslT)	15.8	chaperone	(Allen et al., 1992; Chuang and Blattner, 1993)
89.0	ClpY (HtpI, HslU)	49	protease	(Chuang et al., 1993a; Missiakas et al., 1996b; Kanemori et al., 1997)
89.0	HslV (HtpO, ClpQ)	21	chaperone	(Chuang et al., 1993a; Missiakas et al., 1996b; Kanemori et al., 1997)
90.0	HtrC	21	?	(Raina and Georgopoulos, 1990)

Table 1-1, cont. Heat and Stress Inducible Proteins in *Escherichia coli*

Min	Protein	Molecular Weight	Function	Reference(s)
94.2	GroEL	60	chaperone	(Hemmingsen et al., 1988)
94.2	GroES	16	chaperone	(Hemmingsen et al., 1988)
94.2	HslW	22	?	(Chuang and Blattner, 1993)
94.8	HslX	51	?	(Chuang and Blattner, 1993)
94.8	HslY	45		(Chuang and Blattner, 1993)
94.8	HslZ	37		(Chuang and Blattner, 1993)
σ^E Regulon:				
3.9	DegP (HtrA)	50	protease	(Lipinska et al., 1988b; Strauch et al., 1989)
55.5	σ^E	24	sigma factor	(Lonetto et al., 1994; Raina et al., 1995; Rouvière et al., 1995)
77.5	σ^{32}	32	sigma factor	(Landick et al., 1984; Yura et al., 1984)
74.9	FkpA	29	peptidyl-prolyl isomerase	(Danese and Silhavy, 1997)
CpxR regulon:				
3.9	DegP (HtrA)	50	protease	(Danese et al., 1995)
87.0	DsbA	23	disulfide bond isomerase	(Danese and Silhavy, 1997; Pogliano et al., 1997)
75.2	PpiA	20	peptidyl-prolyl isomerase	(Pogliano et al., 1997)
88.0	CpxP	19	?	(Danese and Silhavy, 1998)
9.5	PpiD	70	peptidyl-prolyl isomerase	(Dartigalongue and Raina, 1998)
Others:				
29.4	PspA	28		(Yamamori and Yura, 1982)
29.7	HslE	60	?	(Chuang and Blattner, 1993)
29.7	HslF	51	?	(Chuang and Blattner, 1993)
29.7	HslG	41	?	(Chuang and Blattner, 1993)
30.6	HslI (HtpH)	36	?	(Chuang and Blattner, 1993)
30.6	HslJ	14	?	(Chuang and Blattner, 1993)
69.2	HslM	31	?	(Chuang and Blattner, 1993)
75.0	HslQ	24	?	(Chuang and Blattner, 1993)
75.0	HslR	18	?	(Chuang and Blattner, 1993)
93.5	LysU	60	Lysyl-tRNA synthetase	(Chuang and Blattner, 1993)

Figure 1-1. The promoters and translational regulatory regions of *E. coli rpoH*. (a) Regions A and B of the mRNA are involved in translational induction by modulating the secondary structure shown in (b), whereas region C of σ^{32} is involved in chaperone mediated translational repression and protein stability (see text). (b) A possible secondary structure of the mRNA formed under nonstress conditions. (Reproduced with permission from Yura, 1996).

a


(Eσ70)
P1
P4
P5
P3
(EσE)
A
B
C

b

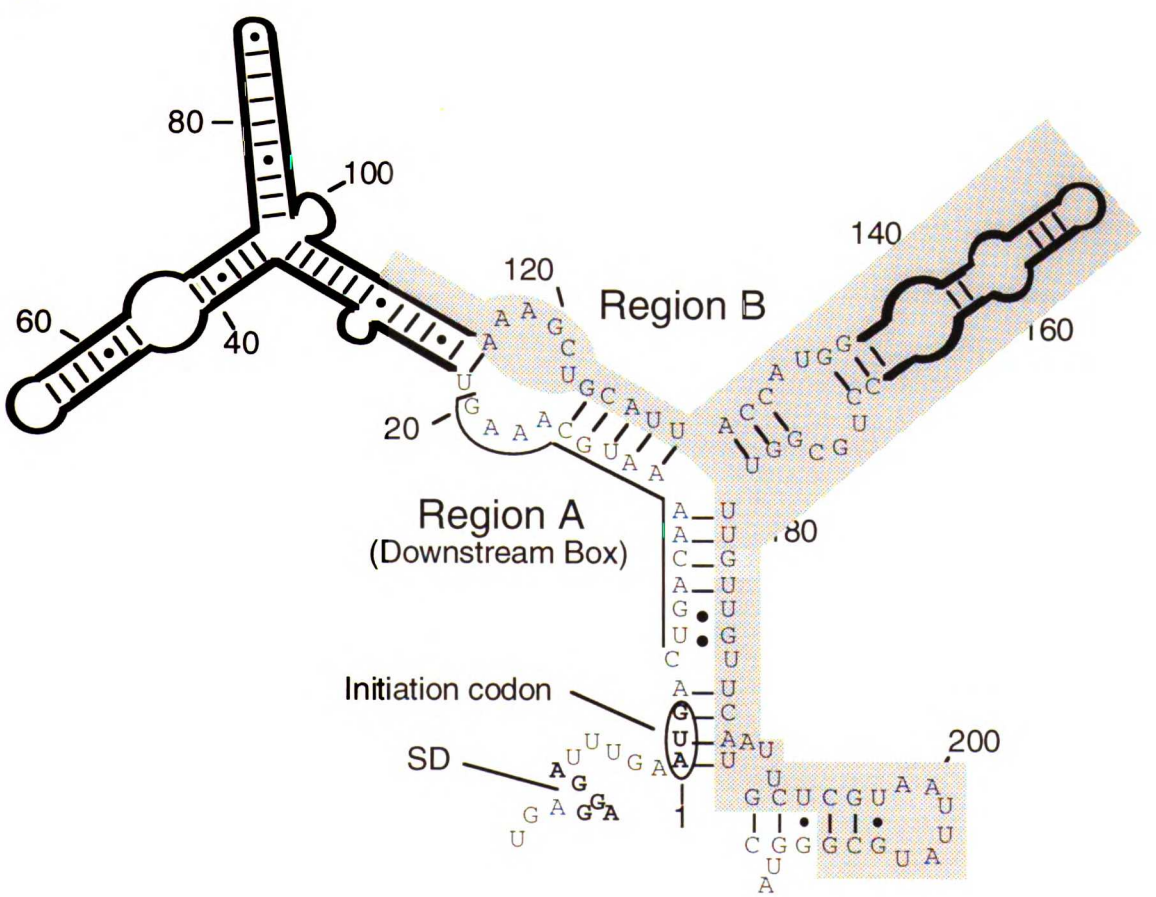

80
100
60
40
120
140
160
Region B
20
Region A
(Downstream Box)
180
Initiation codon
SD
200

Figure 1-2. Speculative model for the mechanism by which DnaK, DnaJ, and GrpE regulate expression of hps by controlling σ^{32} activity. Upon temperature upshift, the increase in misfolded protein substrates leads to a decrease in the free levels of DnaK, DnaJ, and GrpE resulting in increased σ^{32} stability. Upon temperature downshift, the increase in the free pool of these chaperones leads to inactivation of σ^{32}. In addition to these effects, a role for DnaK, DnaJ, and GrpE in negatively regulating the increase in translation of σ^{32} observed upon temperature upshift has been proposed (see text). (Figure adapted from Gross, 1996).

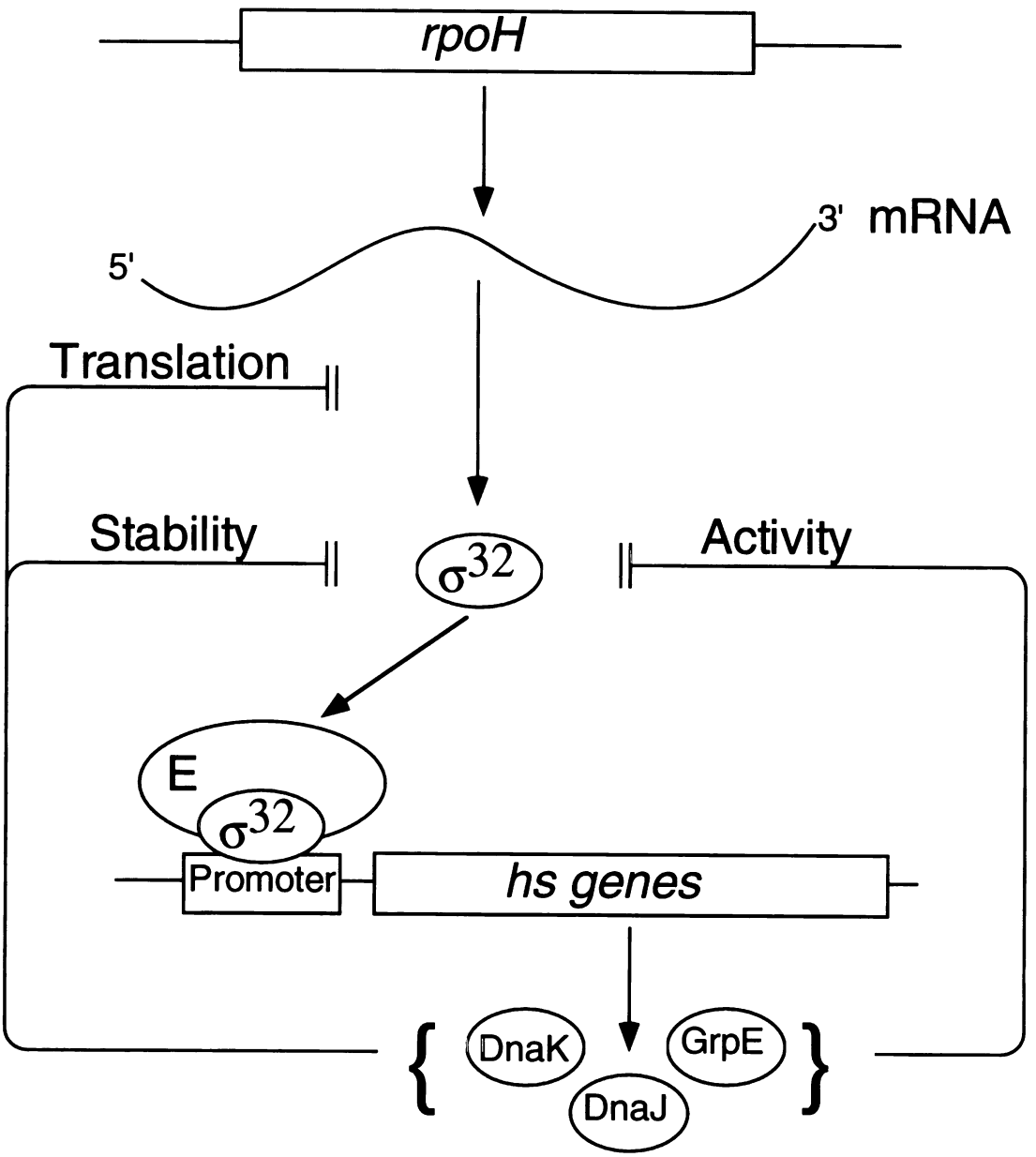
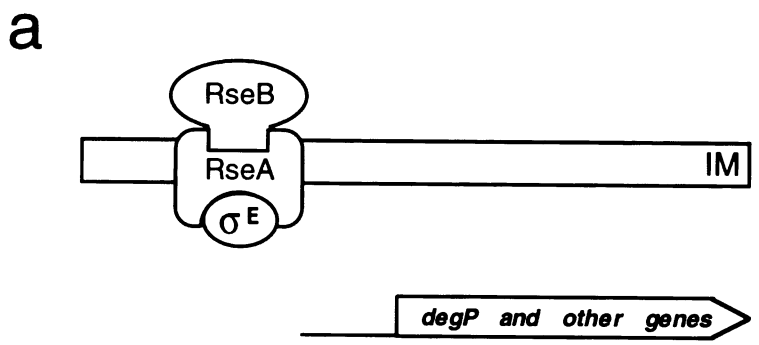
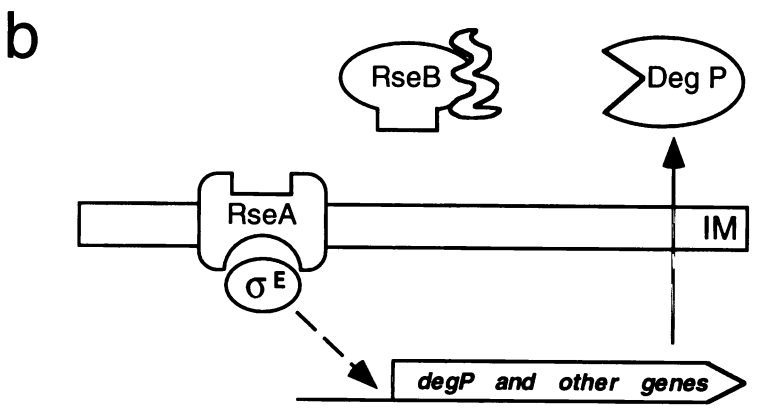
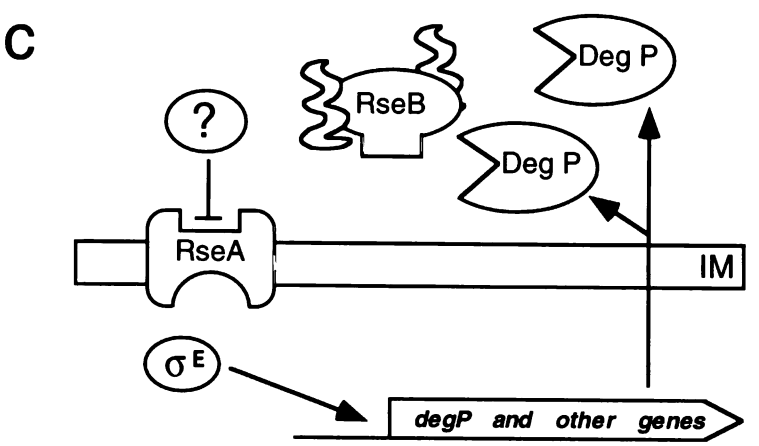

rpoH
3' mRNA
5'
Translation
Stability
σ32
Activity
E
σ32
Promoter
hs genes
DnaK
GrpE
DnaJ

Figure 1-3. Speculative model of the signal transduction cascade leading to activation of σ^E. (a) In the presence of low levels of signal, σ^E is sequestered to the membrane by a protein complex consisting of RseA and RseB, leaving σ^E activity low. (b) Under conditions of low level signal, RseB is titrated off of RseA, leaving RseA in a conformation that is less active as an anti-sigma factor, resulting in a small increase in σ^E activity. (c) When the signal is high, RseA is further inactivated either by interaction with the signal molecule itself or some intermediate factor, resulting in a large induction of σ^E activity.


a
RseB
RseA
σ E
IM
degP and other genes


b
RseB
Deg P
RseA
σ E
IM
degP and other genes


c
?
RseB
Deg P
Deg P
RseA
IM
σ E
degP and other genes

Chapter Two

The σ^E-mediated response to extracytoplasmic stress in *Escherichia coli* is transduced by RseA and RseB, two negative regulators of σ^E

Summary

The extracytoplasmic stress response in *E. coli* is controlled by the alternate sigma factor, σ^E. σ^E activity is uniquely induced by the accumulation of outer membrane protein precursors in the periplasmic space, and leads to the expression of several proteins, including the periplasmic protease DegP, that are thought to be required for maintaining cellular integrity under stress conditions. Genetic and biochemical experiments show that σ^E activity is under the control of three genes, *rseABC* (for regulator of sigmaE), encoded immediately downstream of the sigma factor. Deletion of *rseA* leads to a 25-fold induction of σ^E activity. RseA is predicted to be an inner membrane protein, and the purified cytoplasmic domain binds to and inhibits σ^E-directed transcription *in vitro*, indicating that RseA acts as an anti-sigma factor. Deletion of *rseB* leads to a slight induction of σ^E, indicating that RseB is also a negative regulator of σ^E. RseB is a periplasmic protein and was found to co-purify with the periplasmic domain of RseA, indicating that RseB probably exerts negative activity on σ^E through RseA. Deletion of *rseC*, in contrast, has no effect on σ^E activity under steady state conditions. Under induction conditions, strains lacking RseB and/or C show wild type induction of σ^E activity, indicating either the presence of multiple pathways regulating σ^E activity, or the ability of RseA alone to both sense and transmit information to σ^E.

Introduction

The stress response is one of the most highly conserved regulatory responses amongst all organisms. Upon exposure to high temperature or other stressors leading to protein denaturation, both prokaryotic and eukaryotic cells synthesize a conserved set of proteins, the heat shock proteins (hsps), that function to combat the accumulation of denatured protein (Bukau, 1993; Yura et al., 1993; Becker and Craig, 1994; Georgopoulos et al., 1994; Gross, 1996). Most hsps are either chaperones or proteases, and function to stabilize (prevent aggregation), refold, or degrade misfolded or unfolded proteins (Bukau, 1993; Yura et al., 1993; Becker and Craig, 1994; Georgopoulos et al., 1994; Gross, 1996).

The stress response in the gram negative bacterium *Escherichia coli* is compartmentalized into cytoplasmic and extracytoplasmic responses that are controlled by distinct alternate sigma factors. The well-characterized cytoplasmic response is co-ordinated by σ^{32} (Grossman et al., 1984; Landick et al., 1984; Yura et al., 1984) which responds to the accumulation of misfolded protein by directing the transcription of a well-characterized set of genes including those encoding the chaperones DnaK and DnaJ (Bukau, 1993; Yura et al., 1993; Becker and Craig, 1994; Georgopoulos et al., 1994; Gross, 1996). These chaperones, in turn, act to downregulate σ^{32} activity upon relief of cytoplasmic stress (Straus et al., 1989; Straus et al., 1990; Gamer et al., 1992; Liberek et al., 1992; Liberek and Georgopoulos, 1993; Gamer et al., 1996).

The extracytoplasmic response, in contrast, is less well defined and is controlled by at least two partially overlapping signal transduction systems, the Cpx two-component system (Danese et al., 1995; Raina et al., 1995) and the σ^{E}-mediated system (Mecsas et al., 1993). Activation of the Cpx pathway by either overexpression of the outer membrane lipoprotein NlpE (Gupta et al., 1995; Snyder et al., 1995) or mutational activation suppresses the envelope-associated toxicity of LamB mutant proteins by inducing synthesis

of the periplasmic protease DegP (Cosma et al., 1995; Danese et al., 1995; Snyder et al., 1995). Because the effects of the Cpx pathway have thus far been described only in relation to mutational activation or activation by genes in multicopy, the physiological role of this system in responding to extracytoplasmic stress is unclear.

The σ^E-mediated response is uniquely signaled by the accumulation of immature outer membrane protein (OMP) precursors in the periplasmic space in addition to induction by the generalized stressors of heat and ethanol (Mecsas et al., 1993; Rouvière et al., 1995) For example, overexpression of a class of OMPs called the porins, or deletion of proteins involved in porin maturation similarly induce σ^E (Raina et al., 1995; Missiakas et al., 1996a; Rouvière and Gross, 1996). Overexpression of σ^E, in turn, leads to the induction of at least ten different proteins including the periplasmic protease DegP (Raina et al., 1995; Rouvière et al., 1995). The mechanism by which periplasmic stress is signaled to σ^E in the cytoplasm is unknown. We report on the identification and mechanism of action of members of the signal transduction cascade responsible for transmitting extracytoplasmic stress to σ^E.

Results

Identification of three open reading frames downstream of *rpoE*

During the original cloning and analysis of *rpoE*, it became evident that the activity of σ^E is under negative control. σ^E transcribes its own gene, establishing a positive feedback loop, but in the absence of induction, σ^E activity is low, indicating negative regulation (Raina et al., 1995; Rouvière et al., 1995). Homology arguments suggested that these negative regulators might be encoded in the same operon (Raina et al., 1995; Rouvière et al., 1995). σ^E belongs to a subclass of the σ^{70} family of proteins known as the ECF sigmas that are involved in regulating extracytoplasmic functions (Lonetto et al., 1994). Operons encoding other ECF sigmas have previously been shown to also encode regulators of the sigma factor activity. For example, the operon encoding AlgU/T an alternate sigma factor required for alginate biosynthesis in *P. aeruginosa* (Hershberger et al., 1995; Schurr et al., 1995a) encodes four downstream genes, *mucAmucB/algNmucCmucD*, involved in regulating AlgU/T activity (Flynn and Ohman, 1988; Goldberg et al., 1993; Martin et al., 1993a; Martin et al., 1993b; Martin et al., 1993c). MucA inhibits AlgU/T activity *in vitro* (Schurr et al., 1996; Xie et al., 1996) and previous work in *Escherichia coli* had identified a partial open reading frame immediately downstream *rpoE*, termed *mclA* (*mucA*-like), that showed significant homology to *mucA* (Yu et al., 1995).

Our sequence analysis of the region immediately downstream of *rpoE* revealed the existence of three open reading frames termed *rseABC* for regulator of sigma E (Fig. 2-1). The previously mapped promoter for the *lepA* operon lies 120 bp downstream of the stop codon of *rseC* (March and Inouye, 1985), indicating that the *rpoE* operon most likely ends with *rseC*. Sequence alignment reveals that this operon structure is conserved amongst many ECF sigma factors including those from *E. coli, Photobacterium SS9, H. influenzae, and P. aeruginosa.* For example, there is 66%, 30%, 26%, and 31% identity at the amino acid level between the σ^E and RseABC proteins of *E. coli* and the AlgU and MucABC

proteins of *Pseudomonas aeruginosa* (Boucher et al., 1996) . The conservation of these sequences suggests that each regulator may play a similar role in modulating sigma factor activity in response to variations in the extracytoplasmic environment.

Roles of RseA, RseB and RseC in σ^E regulation

To determine whether any of the *rse* genes encode regulators of σ^E, we constructed chromosomal mutations in each putative regulator and then tested the effect of these mutations on σ^E activity. To avoid polarity, the *rseA* and *rseB* mutations were constructed as internal deletions. *rseC* was disrupted by a chloramphenicol resistance cassette (Ω) inserted 67 bases into the coding region. The *rseBC* double mutant was a deletion extending from base pair 212 of *rseB* to base pair 223 of *rseC* followed by insertion of the Ω cassette. σ^E activity was determined by monitoring β-galactosidase expression of a chromosomal σ^E-dependent *lacZ* reporter gene in a $\Phi\lambda$[*rpoH*P3::*lacZ*] fusion (Fig. 2-2). Deletion of *rseA* leads to a 25 fold increase in σ^E activity, indicating that *rseA* encodes a negative regulator of σ^E. Deletion of *rseB* also causes an induction in σ^E activity, but the 2.3-fold induction observed indicates that RseB plays a minor role in σ^E regulation. In contrast, deletion of *rseC* has no effect on σ^E activity. However, the loss of *rseC* function in an *rseB* mutant abrogates the slight induction seen in the *rseB* single mutant, indicating that *rseC* might play a minor positive role in modulating σ^E activity.

To determine whether any of the *rse* genes play a role in signal transduction to σ^E, the effects of each *rse* mutation on σ^E activity was tested under conditions known to induce σ^E. σ^E was induced by overexpression of the outer membrane protein, OmpX (Mecsas et al., 1993; Mecsas et al., 1995) (Fig. 2-2). The wild type strain showed a 7.4 fold of induction of σ^E activity in the presence of OmpX. In a Δ*rseA* strain, the basal level of σ^E activity is already very high (25-fold higher than the wt), and showed no further induction in the presence of OmpX. Surprisingly, σ^E activity is still induced to wild type levels in

strains lacking RseB and RseC, indicating that neither is absolutely required for signal transduction.

We conclude that RseA plays a pivotal role in signal transduction to σ^E, whereas RseB and RseC are dispensable, at least under the conditions tested. In uninduced conditions, deletion of RseB has only a 2.3-fold effect on σ^E activity and the effect of RseC is detectable only in the absence of RseB. These results raise the possibility that RseA alone is capable of both sensing and transmitting information to σ^E. Alternatively, other unidentified pathways or molecules may be involved in modulating RseA activity.

Mechanism of action of RseA

These genetic experiments indicate that RseA is a negative regulator of σ^E, raising the possibility that RseA is an anti-sigma factor. Anti-sigma factors inhibit transcriptional activity by binding directly to their cognate sigma (Brown and Hughes, 1995). To determine whether RseA interacts directly with σ^E, we tested whether RseA could co-immunoprecipitate with σ^E using an anti-σ^E antibody (Fig.2-3a). The anti-σ^E antibody immunoprecipitated RseA in the presence of σ^E (lane 4), but not in its absence (lane 2), indicating a direct interaction between RseA and σ^E. Neither RseB nor RseC co-immunoprecipitated with σ^E (data not shown), indicating that RseA may be the sole end-point regulator of σ^E.

Unlike most previously described anti-sigmas, RseA is predicted to be an inner membrane protein, with a single transmembrane spanning segment and approximately 100 amino acid cytoplasmic and periplasmic domains. The cytoplasmic domain would be predicted to interact with σ^E. To test this prediction, the N-terminal 100 amino acids of RseA were fused to a C-terminal histidine tag, giving rise to protein RseA-cyto. Purified RseA-cyto and σ^E were incubated either alone or together and run on native polyacrylamide gels. As a control, RseA-cyto was also incubated with σ^{70}. As shown in figure 3B (lane 3), a new complex appears in samples containing both σ^E and RseA-cyto at

the expense of each individual protein. In contrast, no new complexes are visible in samples containing RseA-cyto and σ^{70} (lane 5). To unambiguously identify proteins in the complex, each band was excised from the gel and run on denaturing SDS-PAGE. This analysis revealed that both σ^{E} and RseA-cyto were present in the complex (lane 8), indicating that the N-terminal 100 amino acids of RseA interact directly with σ^{E}. Excision of bands corresponding to σ^{70} and RseA-cyto confirmed that neither band contained σ^{70}-RseA-cyto complexes, at least at the limit of detection used in this experiment (data not shown).

Both the full length and the cytoplasmic domain of RseA bind to σ^{E} *in vitro*, suggesting that RseA binding to σ^{E} results in inhibition of σ^{E} activity. To determine whether the cytoplasmic domain of RseA inhibited σ^{E}-directed transcriptional activity *in vivo*, we expressed RseA-cyto under the control of an IPTG-inducible promoter and assayed σ^{E} activity by monitoring β-galactosidase expression of a chromosomal σ^{E}-dependent *lacZ* reporter gene at different times after induction at 30°C (Fig. 2-4a). The addition of IPTG to the strain carrying the *rseA-cyto* plasmid resulted in an almost immediate reduction in σ^{E} activity and arrested cell growth after 2 doublings. In a similar experiment, overexpression of full length RseA inhibited σ^{E} activity to the same extent as the cytoplasmic domain alone (data not shown). σ^{E} activity in the parental strain carrying vector was unaffected by IPTG, and grew to saturation under both conditions. These results indicate that the cytoplasmic domain of RseA is able to inhibit σ^{E} activity *in vivo*. Interestingly, although cells lacking σ^{E} appear wild type at 30°C, overexpression of either RseA or RseA-cyto is toxic to the cell, as indicated by the growth arrest observed after 2 doublings. This toxicity could be due to accumulation of the RseA-σ^{E} complex, or RseA could have another, essential target in the cell.

To determine whether RseA-cyto inhibits σ^{E} activity directly, we added increasing amounts of purified RseA-cyto to *in vitro* transcription reactions containing a constant amount of either σ^{E} or σ^{70} containing holoenzyme. The cytoplasmic domain of RseA

inhibited σ^E directed transcription at ~1:1 ratio of RseA to sigma factor, but had no effect on σ^{70}-directed transcription, even at ratios of RseA-cyto:sigma as high as 10:1 (Fig. 2-4b). Coupled with the *in vivo* results, these experiments show that RseA is a σ^E-specific anti-sigma factor.

Mechanism of action of RseB

Topology predictions indicated that RseB is potentially a periplasmic protein, with a predicted signal sequence cleavage at A24, leaving a mature protein of 229 amino acids. Cellular fractionation experiments confirmed the predominantly periplasmic (P) localization of RseB (Fig. 2-5a, lanes 7, 8), as well as ß-lactamase used as a control for a periplasmic protein (lanes 5,6). The predicted cleavage site at residue 24 was confirmed by N-terminal amino acid sequencing of RseB purified from periplasmic extracts as described in Materials and Methods.

Due to its periplasmic localization, it is unlikely that RseB interacts directly with σ^E, raising the possibility that RseB might be exerting its negative effect on σ^E through RseA. Indeed, RseB co-purifies with N-terminally his_6-tagged RseA over a nickel chelating column (data not shown). It is most likely that RseB interacts with the periplasmic domain of RseA, and we asked whether RseB would co-purifiy with a his-tagged version of the predicted periplasmic domain of RseA (RseA-peri). RseB purifies over the nickel chelating column when his-tagged RseA-peri is present (Fig. 2-5b, lane 8), but not in its absence (Fig. 2-5b, lane 7). These results indicate that RseB interacts with RseA via the periplasmic domain of RseA, and suggest that RseB exerts its negative effects on σ^E through interaction with a second negative regulator, RseA.

Discussion

σ^E is a heat inducible sigma factor involved in the regulatory response to the accumulation of misfolded or unfolded proteins in the extracytoplasmic space. Because the signal for σ^E induction is generated outside of the cytoplasm, a pathway for transmitting extracytoplasmic information to σ^E must exist. Here we describe the Rse proteins, which are the first characterized members of this signal transduction pathway. RseA is a negative regulator of σ^E and is predicted to be an integral membrane protein with a single transmembrane domain. This assignment is supported by alkaline phosphatase fusion experiments reported in the accompanying manuscript (Missiakas et al., 1997). The cytoplasmic face interacts with σ^E, whereas the periplasmic face interacts with RseB, a second negative regulator located in the periplasm. RseC, a putative membrane bound protein, has a positive effect on σ^E only in the absence of RseB.

Based on the data presented here, we propose that under steady state conditions, the cytoplasmic domain of RseA acts to negatively regulate σ^E activity by binding directly to the sigma factor and inhibiting transcriptional activity. Binding of RseB to the periplasmic domain of RseA, might shift RseA to a conformation where it is most effective as an anti-sigma factor, thus keeping σ^E activity low in the absence of signal (Fig. 2-6a). If RseB binding to RseA were competitive with binding to a signal molecule, RseB would be titrated away from RseA as the concentration of the signal increases (Fig. 2-6b). This would leave RseA in a conformational state where it is a less effective anti-sigma, and lead to a small increase in σ^E activity. At still higher concentrations, the signal molecule would interact either with an intermediate factor or with RseA itself to further increase σ^E activity (Fig. 2-6c). The newly active σ^E transcribes its own promoter to generate more σ^E and RseA. As long as the signal is present, RseA will be unable to interact with σ^E, but when the signal removed or reduced, RseA will be able to again repress σ^E, achieving a new steady state level.

RseA is the central regulatory molecule in this signal transduction cascade, whereas RseB fine-tunes this RseA-based signal transduction pathway. In the absence of RseA, σ^E is unresponsive to inducing signals because it is maximally induced. Thus, RseA is the sole endpoint regulator of the response. Moreover, cells containing only RseA modulate σ^E activity in response to inducer, indicating that RseA alone or in conjunction with unknown molecules responds to the inducing signal. Several mechanisms of RseA inactivation by the inducer can be envisioned including modification, degradation, or oligomerization of the anti-sigma factor.

This model is reminiscent of the regulation of the other heat shock sigma factor of *E. coli*, σ^{32} (Bukau, 1993; Yura et al., 1993; Becker and Craig, 1994; Georgopoulos et al., 1994; Gross, 1996). σ^{32} activity is regulated by the chaperones DnaK and DnaJ, which bind to σ^{32} leading to both inactivation and degradation. Upon the accumulation of misfolded or unfolded proteins in the cytoplasm, DnaK and DnaJ preferentially interact with these substrates, allowing σ^{32} to transcribe the heat shock genes, which include *dnaK* and *dnaJ*. Upon removal of the signal, the newly generated DnaK and DnaJ proteins again bind to σ^{32}, achieving a new steady state level of sigma activity (Bukau, 1993; Yura et al., 1993; Becker and Craig, 1994; Georgopoulos et al., 1994; Gross, 1996).

The direct induction signal and how it affects RseA is currently unknown. σ^E is induced by the build up of early intermediates in the maturation pathway of outer membrane porins, the accumulation of a few mutant periplasmic proteins, and a deficit of several periplasmic folding agents (DsbA, FkpA, Skp and SurA) (Mecsas et al., 1993; Raina et al., 1995; Missiakas et al., 1996a; Rouvière and Gross, 1996). The levels of several different periplasmic proteins, including putative folding agents, may be monitored either directly or indirectly by RseA and/or RseB, and decreases in the free levels of each of these proteins may additively induce the σ^E pathway. In support of this model, overexpression of SurA or FkpA in cells activated for the σ^E response reduces σ^E activity (Missiakas et al., 1996a). Alternatively, RseA and/or RseB may interact directly with the signal or some

intermediate factor that itself is responsible for monitoring levels of protein folding occurring in the extracytoplasmic space.

Comparison to the AlgU/T signal transduction pathway

The most highly studied of the ECF sigmas is AlgU/T of *P. aeruginosa*. Available evidence suggests that the AlgU/T pathway for transducing information to the cytoplasm is similar to that of σ^E. Like RseA and B of *E. coli*, MucA and B are negative regulators of sigma factor activity (Goldberg et al., 1993; Martin et al., 1993a; Martin et al., 1993c) and MucA has been shown to act as an anti-sigma factor *in vitro* (Schurr et al., 1996; Xie et al., 1996). MucB was shown to be a periplasmic protein, and has been proposed to modulate AlgU/T activity by some unknown intermediate factor (Schurr et al., 1996). In light of our data showing that RseB binds to the periplasmic domain of RseA, it is highly likely that MucB exerts its effects on AlgU/T via interaction with MucA. Based on homology to RseA, MucA is likely to be an inner membrane protein. Like *rseC*, deletion of *mucC* has no effect on AlgU/T activity (Schurr et al., 1996). However, the environmental conditions inducing these two pathways are likely to differ. Cells lacking AlgU/T show increased sensitivity to superoxide-generating compounds (Martin et al., 1994). In contrast, *rpoE*$^-$ cells exhibit a normal response to oxidative stress (Rouvière et al., 1995). In addition, the *algU/T* operon encodes a fifth protein, MucD (Boucher et al., 1996), that shows homology to the periplasmic protease DegP of *E. coli* (Lipinska et al., 1988b; Strauch et al., 1989). Deletion of *mucD* leads to activation of AlgU/T and a mucoid phenotype (Boucher et al., 1996), whereas deletion of *degP* has no effect on σ^E activity (Raina et al., 1995; Boucher et al., 1996). Finally, the requirement for several two-component systems in the generation of the mucoid phenotype suggests that the induction of mucoidy involves the integration of several extracellular signals (Deretic et al., 1989; Wozniak and Ohman, 1994). It will be interesting to determine how AlgU/T and σ^E utilize similar signaling molecules to respond to diverse extracellular signals.

Comparison to the unfolded protein response (UPR)

Like the periplasm, the endoplasmic reticulum (ER) represents a more oxidizing environment than the cytoplasmic compartment, and eukaryotic cells monitor unfolded proteins in the lumen of the ER, just as *E. coli* monitors unfolded proteins in the periplasm. In both cases, accumulation of unfolded proteins leads to the transcriptional induction of several proteins involved in promoting protein folding and oligomerization (Lee, 1987). In eukaryotes the molecule involved in the initial sensing and transmission of the unfolded protein response (UPR) is a transmembrane serine/threonine kinase, IRE1, thought to be localized to the ER membrane (Nikawa and Yamashita, 1992; Cox et al., 1993; Mori et al., 1993). Oligomerization of the kinase leads to activation of the UPR, and several models explaining the mechanism of oligomerization have been proposed (Shamu et al., 1994). One hypothesis posits that IRE1 monitors the free levels of an hsp70 homologue, BiP/KAR2, in the ER lumen. Under conditions of low levels of unfolded protein, free BiP levels are high, and the UPR is low. Upon accumulation of unfolded protein, BiP preferentially binds to unfolded substrate, allowing IRE1 oligomerization and activation. Both responses (UPR and σ^E response) utilize a membrane transducer (RseA/IRE1), and each pathway is thought to utilize an auxiliary protein (RseB/KAR2) that potentially interacts both with the signal and the transmembrane regulator. Whereas KAR2 appears to be an obligatory part of the signal transduction cascade, RseB functions to modulate the response.

Role of RseC

Our current studies uncover only a minor role for RseC in the regulation of σ^E. RseC may modulate σ^E activity in response to specific signals not tested in this study. Alternatively, or in addition, RseC may have other cellular functions. *S. typhimurium* cells lacking *rseC* are unable to synthesize thiamine via the alternative pyrimidine biosynthetic pathway (APB), and this defect is independent of *rpoE* (B. Beck *et al.*, in preparation).

Transposon insertions in *rseB* of *Photobacterium* SS9 lead to a loss of pressure and cold-temperature adaptation, and this phenotype may be due to the loss of *rseC* (Chi and Bartlett, 1995). Primer extension analysis of *E. coli rseC* revealed the existence of possible σ^E-independent promoters upstream of *rseC* (A. De Las Peñas, unpublished data), suggesting its regulation may be distinct from the rest of the σ^E-operon. In addition, *rseC* homologues that are not encoded in an operon with an ECF sigma have been described in several organisms. For example, *Haemophilus influenzae* has two *rseC* homologues (Fleischmann et al., 1995), neither of which is linked to an ECF sigma, and the first 177 amino acids of the *Rhodobacter capsulatus* RnfF protein, involved in nitrogen fixation, exhibits 52% similarity and 28% identity to RseC (Schmehl et al., 1993).

Other pathways involved in signaling extracytoplasmic stress

In addition to the σ^E pathway, *E. coli* has a second signal transduction pathway involved in combating periplasmic stress, the CpxAR two-component pathway (Danese et al., 1995; Raina et al., 1995). Activation of the Cpx pathway leads to increased expression of the periplasmic protease, DegP (Cosma et al., 1995; Danese et al., 1995; Snyder et al., 1995), which is also under the control of σ^E. Although maximal induction of *degP* expression by the Cpx pathway requires σ^E, activation of the Cpx pathway in *rpoE*- cells still induces DegP expression approximately three-fold, indicating that the two systems are independent (Danese et al., 1995). Interestingly, activation of the Cpx pathway suppresses some phenotypes of *rpoE*$^-$ cells (L. Connolly *et al.*, in preparation). Overexpression of *degP* alone does not suppress these *rpoE*$^-$ phenotypes, indicating that Cpx controlled genes other than *degP* are required for full suppression (L. Connolly *et al.*, in preparation). It will be interesting to determine to what extent these two regulatory systems overlap in terms of both the types of signals they respond to and the gene products they regulate.

Experimental Procedures

Media and Chemicals

Luria-Bertani (LB) and M9 minimal medium were prepared as described (Sambrook et al., 1989). M9 was supplemented with 0.2% glucose, 1mM Mg_2SO_4, 2μg/ml thiamine and all amino acids (40μg/ml) except for cysteine and methionine for labeling experiments. Where needed media was supplemented with ampicillin 100μg/ml (Ap100), kanamycin 30μg/ml (Km30), chloramphenicol 15μg/ml (Cm15), rifampicin 150μg/ml (Rif150) and tetracycline 10μg/ml (Tc10). Isopropyl-thio-ß-galactoside (IPTG) was added to a final concentration of 1mM.

Bacterial strains

Bacterial strains used in this study are listed in Table 1.

Plasmids

To create pAP28, which carries an internal deletion of *rseA*, a 1.7 kb Hind III *rpoE* operon fragment deleted for the first 323 bp of *rseA*, was introduced into pBIP3 (Slater and Maurer, 1993). To create pAP115, which carries an internal deletion in *rseB*, a 2.9 kb Sph I-Pst I *rpoE* operon fragment with a 717 bp deletion of *rseB* spanning nucleotides 213-930 was cloned into pUCD4121 (Kamoun et al., 1992). To disrupt *rseC*, a 3.8 kb Ω cassette carrying chloramphenicol resistance (Ω-Cm^R) (Fellay et al., 1987) was introduced into the HindIII site at position 66 of *rseC*, giving rise to pAP114. To create a combined deletion/insertion in *rseB* and *rseC*, a 961 bp Mfe I-Blp I fragment extending from bp 212 of *rseB* to bp 223 of *rseC* was deleted and replaced with the Ω-Cm^R cassette, creating pAP86.

To create plasmids for overexpressing His_6-tagged proteins, PCR generated fragments cut at restriction sites (indicated by underlining) in the primers described below

were cloned into the corresponding sites of the T7 overexpression vector pET28b (Novagen, Madison, WI). pLC216, which encodes the N-terminal 100 residues of RseA (RseA-cyto) carrying a C-terminal His_6-tag, was constructed by amplifying primers RSEA15 (5'GGGTATTAGCCATGGAGAAAGAAC) and RSE16 (5'CGTCCGTGGGCGGC ACAGAAGCTTCAAATG). The Nco I site at the 5' end of the gene introduced a single point mutation into the coding sequence, changing the Q at position two to E. pLC235, which encodes the C-terminal 96 amino acids of RseA (RseA-peri) carrying an N-terminal His_6-tag, was constructed by amplifying primers RSEA23B (5'GTCCATATGTATAATGGACAATCTGAAACG) and RSEA24 (5'CGGGATCCTTACTGCGATTGCGTTCC).

Plasmids for cell fractionation and co-immunoprecipitation experiments were generated as T7 transcriptional or translational fusions. pAP67, a transcriptional fusion of *rseA*, has a 1 kb Hind III-Msc I fragment cloned into pET24. pAP77, a transcriptional fusion of *rseB,* has a 1.0 kb EcoRI/ Hind III fragment cloned into pET24. pKPL3 is a derivative of pPER76 (Rouvière et al., 1995), *rpoE* subcloned in pET11c, a translational fusion without tags.

All pET derived plasmids were introduced into strain BL21 (DE3, P*lacUV5*::T7 polymerase) for expression purposes.

Sequencing the *rpoE* operon

Sequence extending from the last 31 nucleotides of *rseA* through the entire coding region of *rseC* was obtained. Sequence was gathered from both strands by the dideoxy sequencing technique using Sequenase (US Biochemical Corp., Cleveland, OH) or Taq polymerase (Gibco-BRL, Gaithersburg, MD) sequencing kits according to manufacturer's directions. Sequence fragments were assembled and edited using the Fragment Assembly suite of programs in the Genetics Computer Group (GCG) Sequence Analysis Software Package.

The final sequence was entered into Genbank (Accession #U37455), and agrees completely with independent entries (Accession #'s U37089 and D64044).

Disruption of chromosomal *rseA*, *rseB*, *rseC* and *rseBC*

An internal deletion of *rseA* was introduced into the chromosome, using pAP28 as described (Slater and Maurer, 1993). An internal deletion of *rseB* was introduced into the chromosome by a two step process. First, CAG22941 [MC1061 *polA1 nadB-3140*::Tn*10*Km] transformants carrying pAP115 were selected on Cm15, and the cointegrate was verified by colony PCR . Second, sucrose counterselection (Kamoun et al., 1992) identified colonies that had lost plasmid sequences. *rseB* deletion strains were identified by colony PCR. Δ*rseB* was moved into wild type strain CAG16037 [MC1061 lysogenized with Φλ(*rpoH* P3::*lacZ*)] by P1 transduction selecting for the closely linked *nadB-3140*::Tn*10* and screened for the *rseB* deletion by colony PCR. A deletion-insertion of *rseBrseC* and an insertion in *rseC* were introduced into the chromosome using the *recBCsbcB* protocol (Winans et al., 1985). The insertions were transduced out by P1 to strain CAG16037 [MC1061 lysogenized with Φλ(*rpoH* P3::*lacZ*)] by selecting for *nadB-3140*::Tn*10* on Km30 and screening for the insertion in either *rseBC* or *rseC* on Cm15 with the expected linkage of 90%. Colony PCR was performed on these candidates to confirm the presence of the insertion.

β-galactosidase assays

σ^E activity was assayed by monitoring β-galactosidase activity from a chromosomal σ^E-dependent *lacZ* reporter gene in Φλ[*rpoH*P3::*lacZ*] as described (Miller, 1972; Mecsas et al., 1993). Cells to be assayed were grown at 30°C in M9 defined medium and duplicate samples harvested at $O.D._{450nm}$ of 0.25. Data presented represents the average of 3 independent experiments. For differential plots, single point determinations were made at the indicated times.

Labeling of cell proteins and cell fractionation

BL21(DE3) strain derivatives were grown at 30°C in M9 defined medium without methionine and cysteine to an $O.D._{450nm}$ of 0.25. IPTG was added to induce synthesis of T7 polymerase and labeled with [^{35}S]trans-Met as previously described (Rouvière et al., 1995) with the following changes: to uniquely labeled the proteins under the control of T7 polymerase, 150 μg/ml of rifampicin was added to the cultures prior to labeling to decrease endogenous transcription, and the labeled cells were rapidly frozen in a dry-ice ethanol bath prior to processing. Periplasmic (P) and membrane [inner and outer]/cytoplasmic (M/C) fractions were isolated under native conditions by osmotic lysis (Randall and Hardy, 1986) from the labeled strains.

Co-immunoprecipitation

Anti-His_6-tagged-σ^E antibody was used to co-immunoprecipitate σ^E along with RseA. Each protein was uniquely labeled and fractionated into Periplasmic (P) or Membrane/Cytoplasmic (M/C) fractions (Randall and Hardy, 1986). 10^5 cpm of M/C fractions containing RseA, σ^E, or both proteins were co-immunoprecipitated. Samples were run on a 12% SDS-polyacrylamide gel. The gels were dried and analyzed on a PhosphorImager (Molecular Dynamics, Sunnyvale, CA).

Overexpression and purification of RseA-cyto

To overexpress and purify RseA-cyto, a 100 ml culture of strain CAG33077 (pLC216) was grown in LB Km30 at 30°C to an $O.D._{600nm}$ of 0.5. Expression of the T7 polymerase was induced by adding IPTG, and the cells were harvested after one hour incubation at 30°C in the presence of IPTG. The cells were pelleted at 4,000 x g for 10 minutes, washed once with 10 mM Tris-Cl pH 8.0, pelleted again and resuspended in 4 mls of 1x BB (5 mM imidazole, 250 mM NaCl, 20 mM Tris-Cl pH 8.0). Lysozyme was added to 20 μg/ml and the cells incubated on ice for 15 minutes. The cells were lysed by

sonication (5 x 10" bursts). Insoluble material was pelleted by centrifugation at 39,000 x g for 30 minutes. The soluble material was bound to a 0.5 ml Ni-NTA column (Qiagen) pre-equilibrated with 1x BB. The column was then washed with 5 mls of 1xBB, and 5 mls of wash buffer (15 mM imidazole, 250 mM NaCl, 20 mM Tris-Cl pH 8.0). Proteins were then step eluted with 10 mls of 500 mM imidazole, 250 mM NaCl, 20 mM Tris-Cl pH 8.0. The protein was concentrated and exchanged into storage buffer (30 mM Tris-Cl pH 8.0, 100 mM NaCl, 1 mM EDTA, 1 mM DTT, 40% glycerol) to a final volume of 0.8 mls, and a final imidazole concentration of 0.0125 mM using a Centriprep-10 concentrator (Amicon, Inc. Beverly, MA). RseA-cyto concentration was determined in relation to σ^E standards run on a 15% SDS-PAGE gel and quantitated using the spot quantitation function of an IS-1000 gel documentation system (Alpha Innotech Corporation, Oakland, CA).

Analysis of the σ^E-RseA-cyto complex

100 pmoles of purified His_6-σ^E (Rouvière et al., 1995) or His_6-σ^{70} (Chan and Landick, 1993) were incubated either alone or with 100 pmoles of RseA-cyto at 37°C in transcription buffer (40 mM Tris-Cl pH 7.9, 150 mM NaCl, 10 mM MgOAc, 1 mM DTT, and 10 µg/ml BSA) for 10 minutes in a total volume of 20 µl. 20 µl of 2X-native gel buffer (100 mM Tris-Cl pH 6.8, 20% glycerol, 0.2% bromophenol blue) was added, and 20 µl of the sample was run on duplicate 12% native Tris-glycine polyacrylamide gels at a constant voltage of 150 volts. Complexes were visualized by staining with Coomassie brilliant blue and excised from one of the duplicated gels. The gel fragments were then equilibrated in 500 µl of SDS gel loading buffer minus glycerol and bromophenol blue (50 mM Tris-Cl pH 6.8, 100 mM DTT, 2% SDS) at RT for 15 minutes and loaded into the wells of a 15% SDS-polyacrylamide gel. The gel was run at a constant voltage of 160V and proteins visualized by Coomassie brilliant blue staining.

In vitro transcription assays

Linear templates for transcription were constructed by PCR. A 340 bp σ^E-dependent template containing the *degP* promoter was amplified from plasmid pSK17 as previously described (Rouvière et al., 1995). The σ^{70}-dependent template derived from pCL185 containing the T7A1 promoter has been previously described (Chan and Landick, 1993).

Dilutions corresponding to 5X final concentrations of RseA-cyto were made in 1X transcription buffer (40 mM Tris-Cl pH 7.9, 150 mM NaCl, 10 mM MgOAc, 1 mM DTT, and 10 µg/ml BSA), and 4 µl of the appropriate dilution was added to duplicate tubes. Control reactions not containing RseA-cyto were set up by adding 4 µl of 1X transcription buffer to duplicate tubes. 15.5 µl of σ^E or σ^{70} transcription mixes containing 1X transcription buffer, 250 µM each GTP, ATP, UTP, 50 µM CTP, 5 µCi [α-^{32}P]CTP (3,000 mCi/mmol, Dupont-NEN, Boston, MA), 20 nM DNA template, 40 nM σ^E or σ^{70}, and 8 units of RNasin (Boehringer Mannheim, Indianapolis, IN) were added to each tube on ice. Core RNA polymerase (gift of Cathleen Chan) was added to a final concentration of 40 nM to start the reactions. Control reactions lacking sigma factor were set up separately. The reactions were incubated at 37°C for 20 minutes and terminated by the addition of one volume of 2x stop buffer [1X Tris-borate buffer (pH 8.3), 7 M urea, 1% SDS, 10 mM EDTA, 0.05% each bromophenol blue and xylene cyanol]. 5 µl of each transcription reaction was run in duplicate on 6% polyacrylamide gels (acrylamide:bisacrylamide 19:1) containing 7M urea at constant power of 50 W. The gels were dried and analyzed on a PhosphorImager (Molecular Dynamics, Sunnyvale, CA) using ImagQuant software (Molecular Dynamics, Sunnyvale, CA).

Co-purification of RseB and the periplasmic domain of RseA (RseA-peri)

To determine whether RseB co-purifies with RseA-peri, strains CAG33149 and CAG22491 were grown to an O.D.$_{600nm}$ of 0.5 at 30°C in 150 mls of LB Km30. The cells were then induced with IPTG and grown for one hour at 30°C. After one hour,

CAG33149 was harvested by spinning at 4,000 x g for 10mins. The cell pellet was washed with 10 mls of 10 mM Tris-Cl pH 8.0 and re-pelleted. After one hour of induction with IPTG, CAG22491 was treated with 150 μg/ml rifampicin at 30°C for one hour. The cells were then harvested and processed identically to CAG33149. Cell pellets were resuspended in 6 mls of 1XBB (5 mM imidazole, 100 mM NaCl, 20 mM Tris-Cl pH 8.0), and 2 mls of each culture were combined to create three 4 ml samples: 33149, 22491, and 33149 + 22491. The cells were then treated with 200 μg/ml lysozyme on ice for 15 minutes and lysed by sonication. The lysates were then spun at 39,000 x g for 30 minutes to pellet insoluble material. Soluble material was then loaded onto 0.5 ml Ni-NTA columns (Qiagen, Chatsworth, CA) pre-equilibrated with 1XBB. Columns were washed with 5 mls of 1XBB, and 5 mls of 1XWB (25 mM imidazole, 100 mM NaCl, 20 mM Tris-Cl pH 8.0). Proteins were eluted with 5 mls of 1XEB (500 mM imidazole, 100 mM NaCl, 20 mM Tris-Cl pH 8.0), and 0.5 ml fractions were collected. Samples representing approximately 10^7 cells were run on 15% SDS-PAGE gels, and proteins visualized by Coomassie brilliant blue staining.

Purification of periplasmic RseB for microsequencing

Mature RseB was purified over an RseA affinity column using the same conditions as the co-purification of RseB with RseA-peri with the following exceptions. Periplasmic lysates containing RseB were prepared from overexpressing cells by osmotic lysis (see Materials and Methods for cellular fractionation), and extracts containing full length histidine-tagged RseA were used. The eluted fraction containing histidine-tagged RseA and periplasmic RseB was analyzed by 10% SDS-PAGE, and the proteins transferred onto PVDF membrane (Millipore, Bedford, MA). Proteins were visualized by Ponceau-S staining, and the band corresponding to mature RseB was excised for automated microsequencing.

Acknowledgements

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Table 2-1. Strains and plasmids used in this work

Strain/ Plasmid	Relevant Genotype	Source/Reference
Strain		
BL21(DE3)	*hsdS gal ompT* $r_B^- m_B^-$ Φλ(DE3 P*lacUV5*::T7 polymerase)	(Studier and Moffatt, 1986)
JC7623	*argE3 his4 leu6 proA2 thr1 thi1 rpsL31 galK2 lacY1 ara14 xyl15 mtl1 supE44 kdgK51 recB15 recC22 sbcB15*	(Oishi and Cosloy, 1972)
MC1061	*araD* Δ(*ara-leu*)*7697* Δ*lacX74 galU galK hsr hsn strA*	(Casadaban and Cohen, 1980)
CAG16037	MC1061 (Φλ *rpoH* P3::*lacZ*)	(Mecsas et al., 1993)
CAG16141	16037 pJE100, Ap^R	(Mecsas et al., 1993)
CAG22156	JC7623 λ(Kohara 435)/ λ^+ *nadB-3140*::Tn*10*, Km^R	(Rouvière et al., 1995)
CAG22293	BL21(DE3) pET11c, Ap^R	This work
CAG22344	BL21(DE3) pKPL3, Ap^R	This work
CAG22372	BL21(DE3) pET24, Km^R	This work
CAG22467	BL21(DE3) pAP67, Km^R	This work
CAG22491	BL21(DE3) pAP77, Km^R	This work
CAG22850	16037 *rseBC*::Ω *nadB-3140*::Tn*10* pJE100, Cm^R Km^R Ap^R	This work
CAG22935	16037 pAP9, Ap^R	This work
CAG22941	MC1061 *polA1 nadB-3140*::Tn*10*, Km^R	This work
CAG22963	16037 Δ*rseB nadB-3140*::Tn*10* pAP9, Km^R Ap^R	This work
CAG22970	16037 *rseBC*::Ω *nadB-3140*::Tn*10* pAP9, Cm^R Km^R Ap^R	This work
CAG22972	16037 *rseC*::Ω *nadB-3140*::Tn*10* pAP9, Cm^R Km^R Ap^R	This work
CAG22973	16037 *rseC*::Ω *nadB-3140*::Tn*10* pJE100, Cm^R Km^R Ap^R	This work
CAG22974	16037 Δ*rseB nadB-3140*::Tn*10* pJE100, Km^R Ap^R	This work
CAG22976	16037 Δ*rseA nadB-3140*::Tn*10* pAP9, Tc^R Ap^R	This work
CAG22977	16037 Δ*rseA nadB-3140*::Tn*10* pJE100, Tc^R Ap^R	This work
CAG22988	16037 pPLT13 pAP9 pSU19, Km^R Ap^R Cm^R	This work
CAG22993	16037 pPLT13 pAP9 pLC227, Km^R Ap^R Cm^R	This work
CAG33077	BL21(DE3) pLC216, Km^R	This work
CAG33149	BL21(DE3) pLC234, Km^R	This work
Plasmid		
pBIP3	cloning vector for sucrose counterselection, Km^R	(Slater and Maurer, 1993)

Table 2-1, cont. Strains and plasmids used in this work

Strain/ Plasmid	Relevant Genotype	Source/Reference
Plasmid		
pBR322	cloning vector, Tc^R Ap^R	(Bolivar et al., 1977)
pET11c	T7 expression vector for translational fusions, Ap^R	Novagen
pET24	T7 expression vector for transcriptional fusions, Km^R	Novagen
pET24b	T7 expression vector for translational fusions, Km^R	Novagen
pET28b	T7 expression vector for C- or N- terminal 6 his fusions, Km^R	Novagen
pHP45W	Plasmid carrying the Ω Cm^R interposon, Cm^R Ap^R	(Fellay et al., 1987)
pSU19	cloning vector, white/blue screen, P15A ori, Cm^R	(Bartolomé et al., 1991)
p*Trc* 99A	IPTG-inducible expression vector, Ap^R	Pharmacia
pUCD4121	cloning vector for sucrose counterselection, Cm^R	(Kamoun et al., 1992)
pJE100	*ompX* cloned in pBR322, Ap^R	(Mecsas et al., 1993)
pKS17	*degP* cloned into pACYC184, Cm^R	(Strauch et al., 1989)
pPLT13	MiniF carrying *lacI*q, Km^R	(Tavormina et al., 1996)
pAP9	pBR322 ΔEcoR V/Msc I, Ap^R , Tc^S	This work
pAP28	Δ*rseA* cloned in pBIP3, Km^R	This work
pAP67	*rseA* in pET24, Km^R	This work
pAP77	*rseB* in pET24, Km^R	This work
pAP86	*rseBC*::Ω in pBR322, Cm^R Tc^R	This work
pAP114	*rseC*::Ω in pBR322, Cm^R Tc^R	This work
pAP115	Δ*rseB* cloned in pUCD4121, Cm^R	This work
pKPL3	*rpoE* in pET11c, Ap^R	This work
pLC216	0.3 kb fragment encoding the predicted cytoplasmic domain of *rseA* fused to a C-terminal histidine tag in pET28b, Km^R	This work
pLC227	0.512 kb fragment encoding the predicted cytoplasmic domain of *rseA* fused to the *lac* promoter in pSU19, Cm^R	This work
pLC234	0.3 kb fragment encoding the predicted periplasmic domain of *rseA* fused to an N-terminal histidine tag in pET28b, Km^R	This work

Figure 2-1. Structure of the *rpoE* operon. Three open reading frames downstream of *rpoE* were discovered by sequence analysis. *rseA* encodes a 27 kDa protein predicted to reside in the inner membrane, *rseB* a potential periplasmic protein of 35 kDa, and *rseC* a putative inner membrane protein of 17 kDa. Interestingly, *rseA* and *rseB*, and *rseB* and *rseC* appear to be translationally coupled, as the stop codon of the first open reading frame overlaps the start codon of the next. The conservation of this operon structure in other bacteria, including *Photobacterium* SS9, implies that this arrangement may play a role in regulating the expression of the Rse proteins.

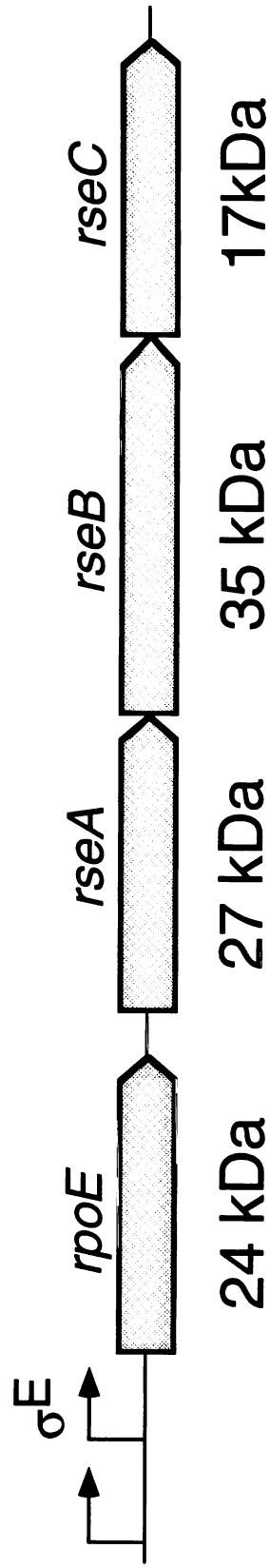

σE
rpoE
24 kDa
rseA
27 kDa
rseB
35 kDa
rseC
17kDa

Figure 2-2. Changes in σ^E activity under steady state and induction conditions resulting from mutation of the *rse* genes. σ^E activity was assayed by monitoring β-galactosidase activity as described in Materials and Methods from a single copy Φλ[*rpoH*P3::*lacZ*] fusion in either wild type (CAG16037) or isogenic mutant strains carrying Δ*rseB*, *rseC*::Ω, *rseBC*::Ω and Δ*rseA*. Each strain was assayed in duplicate with either vector alone (white bars; pAP9) or p*ompX* (grey bars; pJE100) to induce σ^E activity. The data presented represents the average of 3 independent experiments and is reported in Miller Units. Note that the Δ*rseA* data is plotted on a different scale. Small standard deviations do not show in the graph.

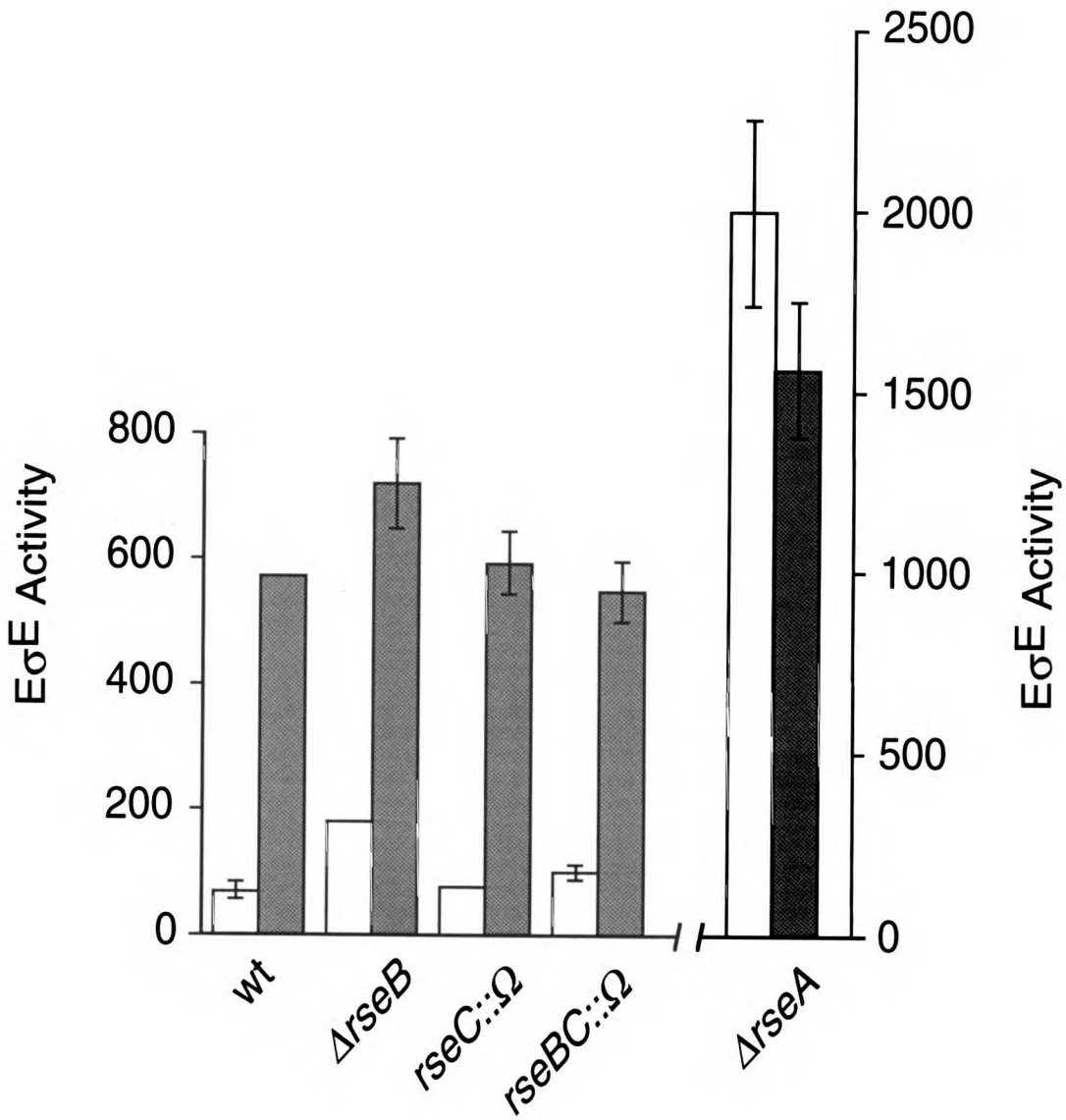
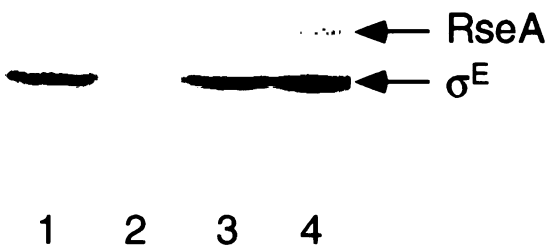

EσE Activity
800
600
400
200
0
2500
2000
1500
1000
500
0
EσE Activity
wt
ΔrseB
rseC::Ω
rseBC::Ω
ΔrseA

Figure 2-3. Analysis of the σ^E-RseA interaction (a) *In vivo* analysis. Strain BL21(DE3) carrying either pT7::*rseA* (pAP67) or pT7::*rpoE* (pKPL3) was grown and the T7-transcriptionally controlled proteins uniquely labeled as described in Materials and Methods. 10^5 cpm of Membrane/Cytoplasmic (M/C) fractions (see Materials and Methods) containing RseA (lane 2), σ^E (lane 3), or RseA plus σ^E (lane 4) were immunoprecipitated (IP) with anti-σ^E antibody (Ab) and analyzed by SDS-PAGE. The starting material (SM) is shown in lane 1. (b) *In vitro* analysis. RseA-cyto was pre-incubated with equimolar amounts of either σ^E or σ^{70} at 37°C and run on a native polyacrylamide gel along with each protein alone. Proteins were visualized by Coomassie brilliant blue staining, and bands corresponding to σ^E, RseA-cyto, and the complex were excised from the gel and analyzed by denaturing SDS-PAGE to assign identity to proteins in each band. (Lanes 1,6) σ^E alone; (lanes 2,7) RseA-cyto alone; (lanes 3,8) Complex; (lane 4) σ^{70} alone; (lane 5) σ^{70} plus RseA-cyto.

a

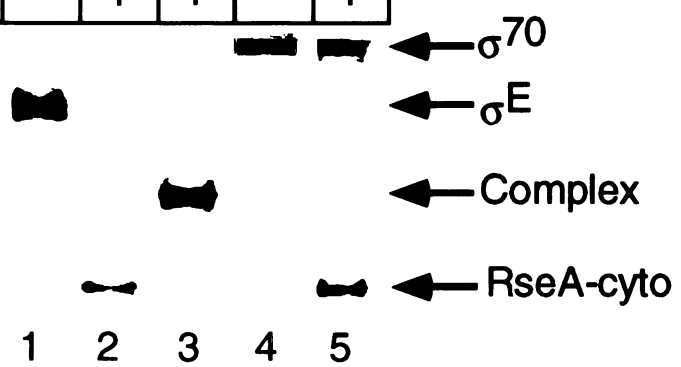
	SM	IP		
RseA	+	+	-	+
σ^E	+	-	+	+
Anti-σ^E Ab	-	+	+	+



b

Native Gel

σ^E	+	-	+	-	-
σ^{70}	-	-	-	+	+
RseA-cyto	-	+	+	-	+



Denaturing Gel

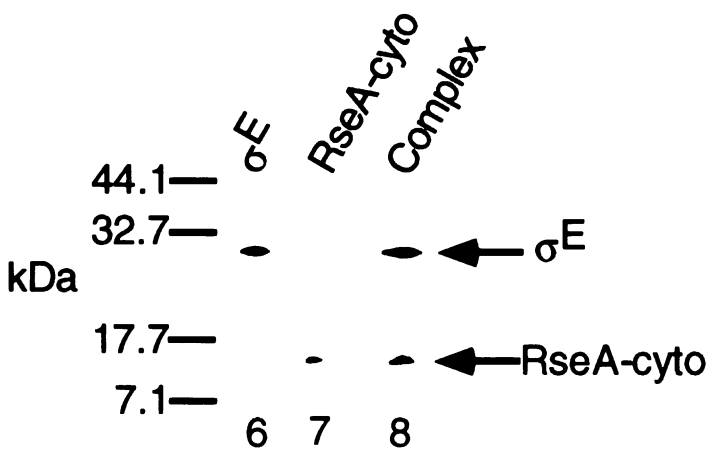


Figure 2-4. Analysis of RseA-cyto activity *in vivo* and *in vitro* (a) $E\sigma^E$ activity *in vivo*. $E\sigma^E$ activity was assayed by monitoring β-galactosidase activity from a single copy Φλ[*rpoH*P3::*lacZ*] fusion in strains carrying either vector alone or a plasmid overexpressing the cytoplasmic domain of RseA (*rseA*-cyto) under the control of an IPTG-inducible promoter at 30°C. The cells were grown for 5 generations and the cultures were divided into two at an O.D. $_{450nm}$ of 0.1. IPTG was added to one culture to induce *rseA-cyto*, and both cultures were sampled for β-galactosidase activity at various times. A plot of β-galactosidase activity per 0.5 ml cells versus O.D.$_{450nm}$ of vector alone without (❑) and with (◆) IPTG and the isogenic strain overexpressing RseA-cyto (*rseA*-cyto) without (○) and with (Δ) IPTG is shown. (b) $E\sigma^E$ activity *in vitro*. *In vitro* transcription reactions containing a constant amount of either σ^E (❑) or σ^{70} (■) and core RNA polymerase were carried out in the presence of increasing amounts of purified RseA-cyto (see Materials and Methods). The transcription reactions were run in duplicate on urea containing polyacrylamide gels, and quantitated by PhosphorImager analysis. The average and standard deviation of three independent experiments is shown.

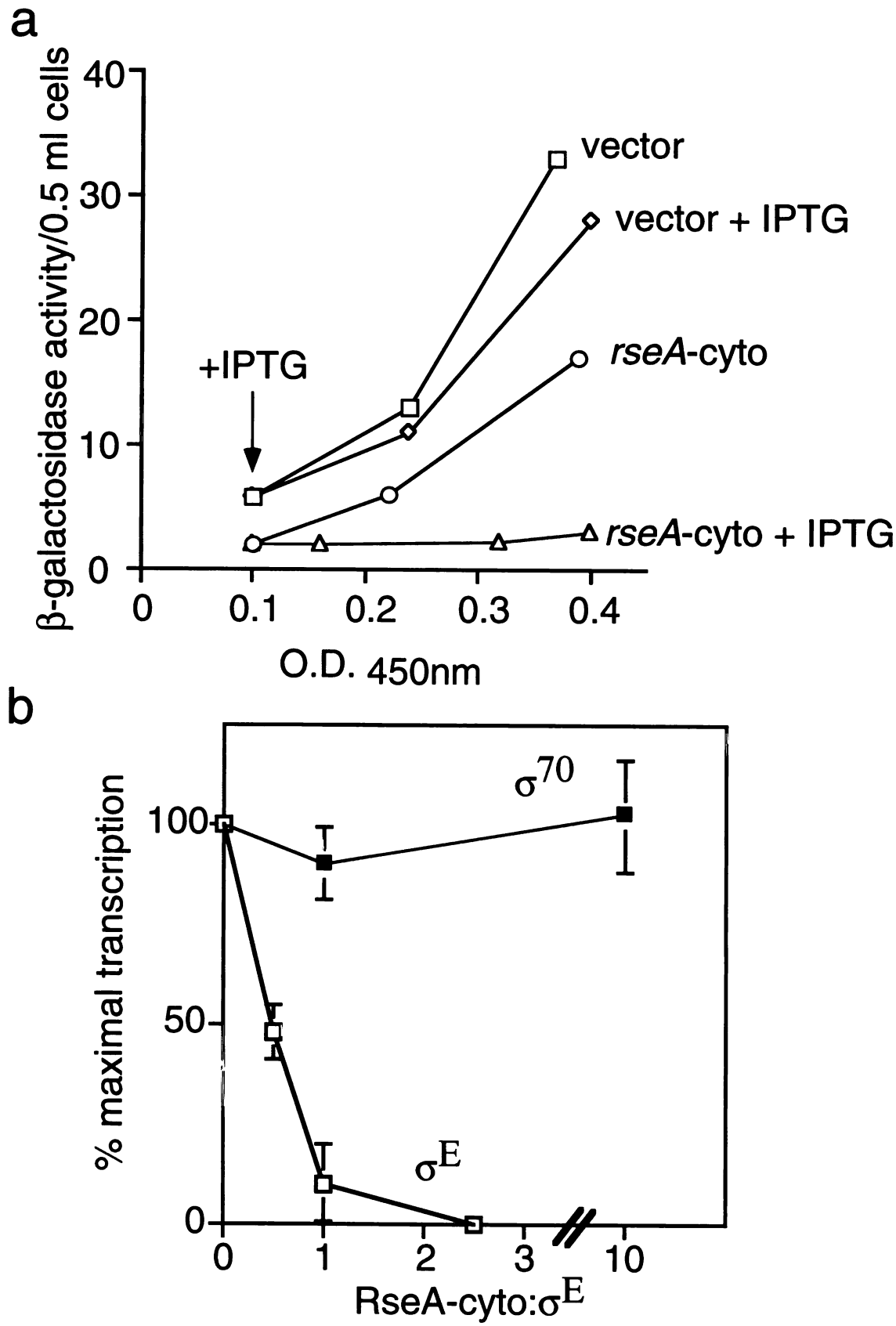
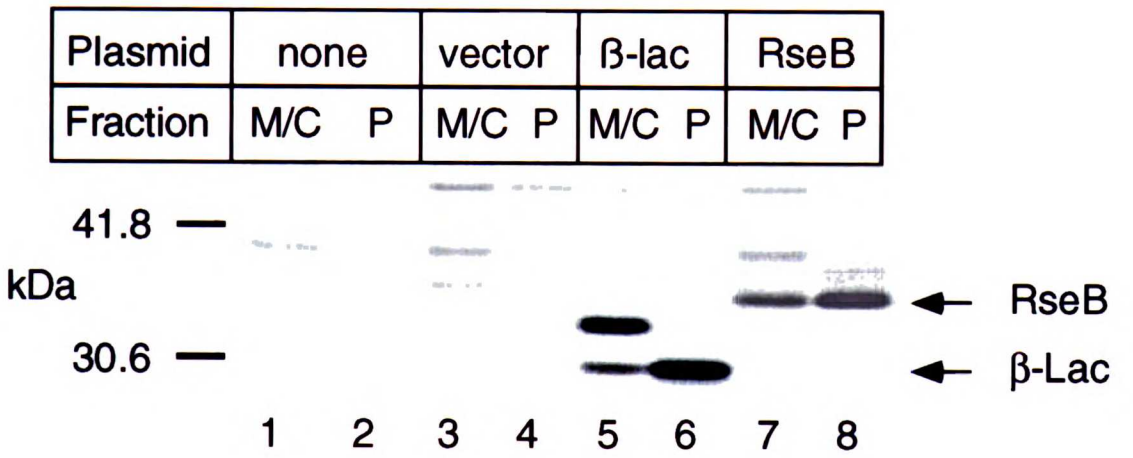

a
β-galactosidase activity/0.5 ml cells
40
30
20
10
0
0
0.1
0.2
0.3
0.4
O.D. 450nm
+IPTG
vector
vector + IPTG
rseA-cyto
rseA-cyto + IPTG
b
% maximal transcription
100
50
0
σ70
σE
0
1
2
3
10
RseA-cyto:σE

Figure 2-5. Analysis of RseB localization and interaction with RseA (a) RseB localization. Strain BL21(DE3) carrying no plasmid, vector alone, pT7::β-lactamase, or pT7::*rseB* was grown and the proteins uniquely labeled as described in Materials and Methods. Cells were subjected to osmotic shock and fractionated into Membrane/Cytoplasmic (M/C) and Periplasmic (P) fractions as described in Materials and Methods. 10^4 cpm of each fraction was analyzed by SDS-PAGE. A scanned image of an autoradiograph is shown. (Lanes 1,2) no plasmid; (lanes 3,4) vector; (lanes 5,6) ß-lactamase as control for a periplasmic protein; (lanes 7,8) RseB. (b) RseB interaction with RseA. Cellular extracts containing histidine tagged RseA-peri, RseB, or both proteins were fractionated over Ni-NTA columns. Onput (lanes 1-5) and eluate (lanes 6-8) fractions were analyzed by SDS-PAGE and proteins visualized by Coomassie brilliant blue staining. (Lanes 1,2) vector; (lane 3,6) RseA-peri; (lane 4, 7) RseB; (lane 5, 8) RseA-peri plus RseB.

a


Plasmid
none
vector
β-lac
RseB
Fraction
M/C P
M/C P
M/C P
M/C P
41.8
kDa
30.6
RseB
β-Lac
1 2 3 4 5 6 7 8

b

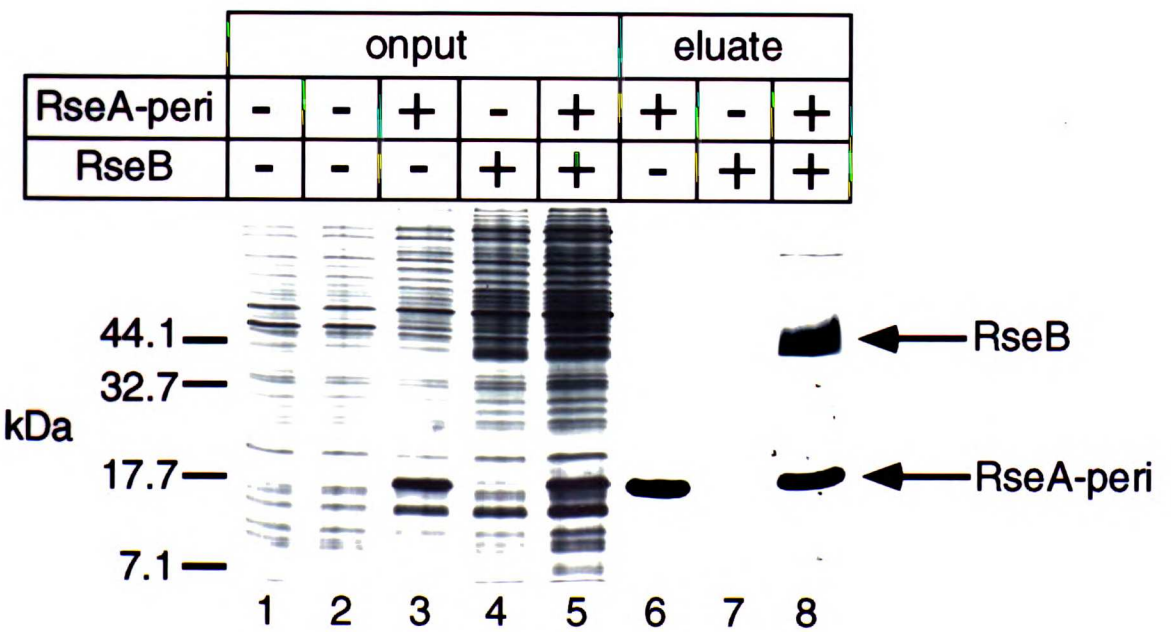
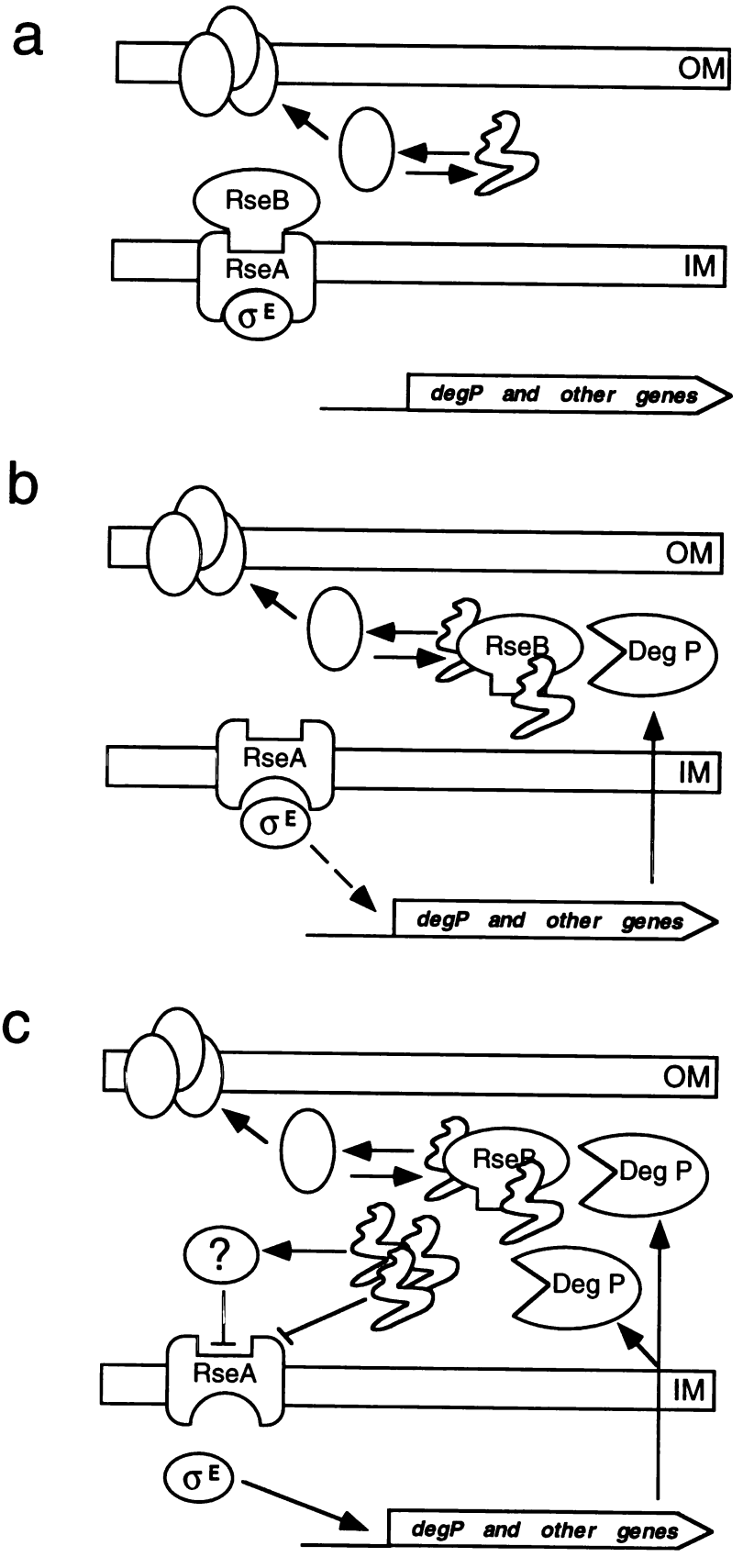

onput
eluate
RseA-peri
- - + - + + - +
RseB
- - - + + - + +
44.1
32.7
kDa
17.7
7.1
RseB
RseA-peri
1 2 3 4 5 6 7 8

Figure 2-6. Model of signal transduction to σ^E (a) Under conditions of low signal, σ^E is sequestered to the membrane by a complex consisting of RseA and B, resulting in low σ^E activity. (b) At low levels of extracytoplasmic stress, RseB is titrated off of RseA by an undefined signal molecule generated as a consequence of protein denaturation. Release of RseB from RseA leads to a conformational shift in RseA resulting in a diminution of RseA activity. (c) At higher levels of signal, RseA is more completely inactivated either via direct interaction with the signal molecule, or interaction with an intermediate protein that itself directly monitors the level of signal.


a
OM
RseB
RseA
IM
σ E
degP and other genes
b
OM
RseB
Deg P
RseA
IM
σ E
degP and other genes
c
OM
RseB
Deg P
?
Deg P
RseA
IM
σ E
degP and other genes

Chapter Three

σ^E is an essential sigma factor in *Escherichia coli*

Summary

σ^E is an alternative sigma factor that controls the extracytoplasmic stress response in *Escherichia coli*. σ^E is essential at high temperatures, but was previously thought to be non-essential at temperatures below 37°C. We present evidence that σ^E is an essential sigma factor at all temperatures. Cells lacking σ^E are able to grow at low temperature because of the presence of a frequently arising, unlinked suppressor mutation.

Introduction

σ^E, encoded by the *rpoE* gene, is an alternative sigma factor (Erickson and Gross, 1989; Wang and Kaguni, 1989; Raina et al., 1995; Rouvière et al., 1995) that controls the extracytoplasmic stress response in *Escherichia coli* (Mecsas et al., 1993; Rouvière and Gross, 1996). The activity of σ^E is induced not only by heat or ethanol but also, and uniquely, by disruption of protein folding in the periplasm (Mecsas et al., 1993; Missiakas et al., 1996a). σ^E is negatively regulated by a membrane bound anti-sigma factor, RseA, and a periplasmic protein, RseB, which binds to RseA (De Las Peñas et al., 1997b; Missiakas et al., 1997). σ^E directs transcription of at least 10 genes (Raina et al., 1995; Rouvière et al., 1995) of which only 4 have been identified: *rpoH,* encoding σ^{32}, which coordinates the cytoplasmic stress response (Erickson et al., 1987; Raina et al., 1995; Rouvière et al., 1995), *rpoE,* encoding σ^E (Raina et al., 1995; Rouvière et al., 1995), *fkpA*, encoding a periplasmic peptidyl prolyl isomerase (Danese and Silhavy, 1997) , and *degP*, encoding a periplasmic protease (Lipinska et al., 1988b; Erickson and Gross, 1989; Lipinska et al., 1989; Strauch et al., 1989). Because σ^E is responsible for the majority of *rpoH* transcription at extreme temperatures, σ^E is also required for the cytoplasmic stress response at lethal temperatures (Erickson et al., 1987).

To further characterize the cellular role of σ^E, three different groups have constructed null mutations in the *rpoE* gene in different strain backgrounds (Hiratsu et al., 1995; Raina et al., 1995; Rouvière et al., 1995). In each case, the resultant cells exhibited a temperature sensitive (Ts) phenotype, but were viable at 30°C, indicating that *rpoE* was not essential at low temperature. However, there were growth differences between these strains. Whereas MC1061 *rpoE*::Ω chloramphenicol (Cm) grows at wild type rates and forms uniform colonies on plates (Rouvière et al., 1995), MC4100 *rpoE*::Ω Kanamycin (Km) grows very slowly and forms heterogeneous colonies (Danese et al., 1995; Hiratsu et al., 1995), suggesting that MC1061 may contain a suppressor. We have investigated this

possibility and present evidence indicating that σ^E is essential for cell growth at all temperatures.

Results and Discussion

rpoE::ΩCm cannot be cotransduced with a linked marker

All strains used in this report are derivatives of MC1061 (Mecsas et al., 1993; Rouvière et al., 1995). Our first indication that *rpoE* was essential came from attempts to cotransduce *rpoE*::ΩCm with a linked marker. The *nadB* locus is 90-100% linked to *rpoE*. We compared cotransduction frequencies of *rpoE*::ΩCm with *nadB* in haploid recipients and in recipients carrying a plasmid encoding the *rpoE* operon (*rpoE, rseA, rseB, rseC*), hereafter referred to as the p*rpoE* strain. If *rpoE* is not essential, both strains should show equivalent, high cotransduction of *rpoE*::ΩCm with *nadB3140*::Tn*10*Km. On the other hand, if *rpoE* is essential, and the *rpoE*::ΩCm parent strain carries an unlinked suppressor mutation, only the p*rpoE* strain, which covers the missing locus with a plasmid, should give proper linkage. In fact, we see the latter result (Table 3-1). Whereas the p*rpoE* cells exhibited appropriate linkage between the two markers, (Table 3-1, line 2), the haploid cells gave very few Km^R transductants, with little or no linkage to Cm (Table 3-1, line 1), even when the plates were incubated for as long as 48 hours. The reduced number of transductants in haploid cells is not due to reduced transduction competence. A control transduction using P1 carrying only the *nadB3140*::Tn*10*Km marker gave approximately comparable numbers of transductants in both haploid and p*rpoE* strains (Table 3-1, last column). Moreover, the inability to cotransduce *rpoE*::ΩCm and *nadB3140*::Tn*10*Km is not peculiar to initial selection for kanamycin, as cotransduction experiments using *nadB51*::Tn*10*Tc (tetracycline) gave similar results (data not shown). Likewise, a deletion of the entire *rpoE* operon did not cotransduce with the *nadB* linked marker, indicating that the residual portion of *rpoE* present in the *rpoE*::ΩCm strain does not encode a toxic product (data not shown). Finally, we were also unable to obtain cotransduction of *rpoE*::ΩCm with *nadB3140*::Tn*10*Km at 18°C (data not shown). The most plausible

explanation for these results is that *rpoE* is essential for cell growth, at least at temperatures of 18°C or above.

rpoE mutant strains contain a suppressor

We originally obtained our *rpoE::*ΩCm strain by selecting directly on Cm in a haploid strain (Rouvière et al., 1995). In view of the above results, we decided to repeat the cotransduction, but selecting first for *rpoE*::Ω by plating on Cm and then scoring linkage to *nadB3140*::Tn*10*Km by patching on Km. Remarkably, transduction into both the haploid recipient and the p*rpoE* strain gave similar numbers of transductants, with high linkage of the two markers (Table 3-2, compare lines 1 and 2). However, the haploid strain required 48 hours for colony formation, whereas the p*rpoE* strain formed colonies after 16 hours (Table 3-2).

The observation that *rpoE*::ΩCm transductants derived from a haploid strain require 48 hours to grow versus 16 hrs for the p*rpoE* strain suggests that the haploid cells must also acquire a suppressor mutation in order to grow. If the suppressor were unlinked to *rpoE*, it should be easy to demonstrate its existence by the procedure outlined in Figure 3-1. *rpoE*::ΩCm *nadB3140*::Tn*10*Km cells can be transduced to *rpoE*$^+$, giving rise to a strain containing the suppressor alone. Because this strain has the putative suppressor, *rpoE*::ΩCm should show appropriate cotransduction with *nadB3140*::Tn*10*Km following initial selection on kanamycin. Indeed, when we carried out this experiment, haploid recipients gave high numbers of transductants on kanamycin plates, and *rpoE*::ΩCm exhibited appropriate cotransduction frequency (98%, 49 out of 50 transductants). Moreover, these transductants were observed at 16 hours, rather than at the 48 hours required in the original strain. Haploid MC1061 cells can be transduced to *rpoE*::ΩCm with high efficiency because almost 100% of the recipients acquire an unlinked suppressor permitting growth at 30°C. It is unclear why direct selection for the *rpoE*::ΩCm allele allows for the accumulation of the suppressor mutation while selection for the linked

nadB3140::Tn*10*Km or *nadB51*::Tn*10*Tc markers does not. One possibility may be that loss of σ^E activity interferes with the establishment of Km or Tc resistance in the cotransduction experiment. Alternatively, the mutation frequency may be enhanced in the presence of Cm, allowing for increased accumulation of the suppressor upon direct selection of the *rpoE*::ΩCm allele.

Inhibition of σ^E prevents cell growth

If σ^E activity is essential for cell growth, then inactivation of σ^E should lead to growth inhibition. σ^E is negatively regulated by the inner membrane anti-sigma factor RseA, and the periplasmic protein RseB. In previous work, we found that overexpression of RseA was deleterious, with growth ceasing by two doublings after induction of the protein (De Las Peñas et al., 1997b; Missiakas et al., 1997). There are three possible explanations for the observed toxicity of RseA overexpression: (1) inhibition of σ^E, (2) inhibition of another cellular protein essential for cell growth, (3) other toxic effects, such as alterations in the membrane due to increased amounts of RseA itself or the RseA-σ^E complex. To distinguish these possibilities, we determined whether overexpression of RseA and B was toxic only when active σ^E is required for viability. The efficiency of plating of wild type, *rpoE*::Ω *sup*, or *rpoE*$^+$ *sup* strains containing either p*Trc* or p*Trc*::*rseA rseB* was determined in the presence of IPTG, which induces expression of the plasmid-borne *rse* genes. We find that overexpression of RseA and B is toxic only in *rpoE*$^+$ cells lacking the suppressor (Table 3-3, lines 2 and 6). Although *rpoE*$^+$*sup* cells overexpressing RseA and B are somewhat smaller than their *rpoE*::Ω counterparts, they plate with similar efficiencies on IPTG (Table 3-3, lines 4 and 6), arguing that toxicity in the wild type strain is not due to deleterious effects of the σ^E-RseAB complex, but rather to loss of σ^E activity. Moreover, the lack of toxicity of RseA and B in cells lacking σ^E (Table 3-3, line 4) argues that these proteins do not target a second, essential factor. Similar results were obtained at 30°C and 18°C, supporting the idea that *rpoE* is essential for cell growth at temperatures above 18°C.

Why is σ^E essential?

The σ^E transcribed genes identified so far do not account for the essentiality of σ^E. Expression of the DegP protease is essential for viability only at temperatures of 42°C or higher (Lipinska et al., 1988b; Strauch and Beckwith, 1988; Lipinska et al., 1989; Strauch et al., 1989), and FkpA is not essential to cell growth (Missiakas et al., 1996a). One or more unidentified members of the σ^E regulon may be essential for cell growth. Alternatively, σ^E may coordinate transcription of several redundant functions and, when expression of all are eliminated, cell growth may cease. Given our current ideas about the role of σ^E in the cell, we imagine that this function might be related to extracytoplasmic protein folding, catalysis, or degradation. We are currently mapping the suppressor, and characterization of this mutation should provide us with further insight into the cellular role of the σ^E regulon.

Acknowledgments

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Table 3-1. Cotransduction of *rpoE*::ΩCm with *nadB3184*::Tn*10*Km

	# Colonies P1(*nadB3184*::Tn*10*Km *rpoE*::ΩCm)			# Colonies P1(*nadB3184*::Tn*10*Km)
Recipient	Km^R	Cm^R	% linkage	Km^R
Haploid	2	0	0	100
p*rpoE*	50	50	100	150

Table 3-1. Haploid or p*rpoE* recipient cells were crossed with the P1 donor (*rpoE*::ΩCm *nadB3140*::Tn*10*Km), and *nadB3140::*Tn*10*Km transductants were selected on Luria Bertani (LB) plates plus 20 μg/ml Km at 30°C. Km^R transductants were then screened on LB plus 12 μg/ml Cm at 30°C to identify *rpoE*::ΩCm cotransductants. % linkage represents the number of Km^RCm^R colonies divided by the total number of Km^R colonies screened. The P1 donor (*nadB3140*::Tn*10*Km) was used to control for the transduction competence of each strain. Transductions were made as previously described (Miller, 1972).

Table 3-2. Cotransduction of *nadB3180*::Tn*10* with *rpoE*::ΩCm

	# colonies			
Recipient	Cm^R	Km^R	% linkage	Time (h) to colony formation
Haploid	50	47	94	48
p*rpoE*	50	49	98	16

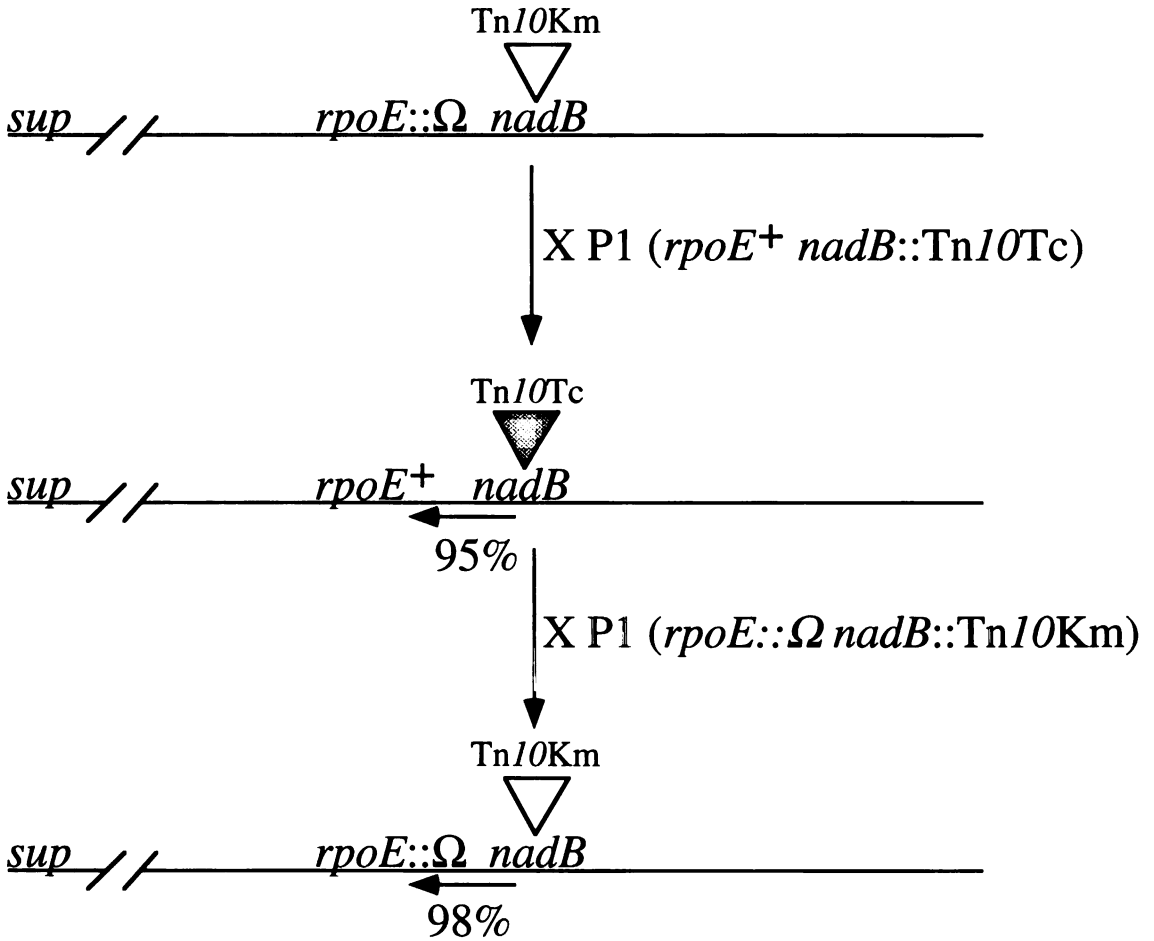
Table 3-2. Haploid or p*rpoE* recipient cells were crossed with the P1 donor (*rpoE*::ΩCm *nadB3140*::Tn*10*Km), and *rpoE*::ΩCm transductants were selected on LB plus 12 μg/ml Cm at 30°C. Cm^R colonies were then screened on LB plus 20 μg/ml Km at 30°C to identify *nadB3140*::Tn*10*Km cotransductants. % linkage represents the number of Km^RCm^R colonies divided by the total number of Cm^R colonies screened. Transductions were made as previously described (Miller, 1972).

Table 3-3. Overexpression of RseAB

Strain	Plasmid	Efficiency of Plating at:	
		18°C	30°C
wild type	p*Trc*	1.0	1.0
wild type	p*Trc*::*rseA rseB*	1.5×10^{-4}	4.2×10^{-4}
rpoE::Ω *sup*	p*Trc*	1.0	1.0
rpoE::Ω *sup*	p*Trc*::*rseA rseB*	0.7	0.9
rpoE$^+$ *sup*	p*Trc*	1.0	1.0
rpoE$^+$ *sup*	p*Trc*::*rseA rseB*	0.2	0.8

Table 3-3. Wild type, *rpoE*::Ω, or *rpoE*$^+$ *sup* strains containing either p*Trc* or p*Trc*::*rseA rseB* were grown in LB plus 100 µg/ml Ampicillin (Ap100) and 0.4% glucose until saturation. Dilutions were plated on LB Ap100 plus 0.4% glucose for viable count, and on LB Ap100 plus 1mM IPTG to induce the *Trc* promoter. The cells were grown at either 30°C or 18°C, and the efficiency of plating determined by dividing the number of cells arising on IPTG by the number of cells arising on glucose. The values shown represent the average of three independent experiments.

Figure 3-1. Demonstration of a suppressor mutation in *rpoE::Ω* cells. *rpoE*::ΩCm *nadB3140*::Tn*10*Km recipient cells were crossed with the P1 donor (*rpoE*$^+$ *nadB51*:Tn*10* Tc), and *nadB51*:Tn*10*Tc transductants were selected on LB plus 10 μg/ml Tc at 30°C. Tc^R colonies were then screened on LB plus 20 μg/ml Cm at 30°C to identify *rpoE*$^+$ cotransductants. % linkage represents the number of Tc^{R}Cm^S colonies divided by the total number of Tc^R colonies screened. The resulting Tc^{R}Cm^S cells were then crossed with P1 donor (*rpoE*::ΩCm *nadB3140*::Tn*10*Km), and *nadB3140*::Tn*10*Km transductants identified by selection on 20 μg/ml Km at 30°C. Km^R transductants were then screened on LB plus 12 μg/ml Cm at 30°C to identify *rpoE*::ΩCm cotransductants. Transductions were made as previously described (Miller, 1972).


Tn10Km
sup
rpoE::Ω nadB
X P1 (rpoE+ nadB::Tn10Tc)
Tn10Tc
sup
rpoE+ nadB
95%
X P1 (rpoE::Ω nadB::Tn10Km)
Tn10Km
sup
rpoE::Ω nadB
98%

Chapter Four

The response to extracytoplasmic stress in *Escherichia coli* is controlled by partially overlapping pathways

Summary

The activity of the alternate sigma factor σ^E of *Escherichia coli* is induced by several stressors that lead to the extracytoplasmic accumulation of misfolded or unfolded protein. The σ^E regulon contains several genes, including that encoding the periplasmic protease DegP, whose products are thought to be required for maintaining the integrity of the cell envelope because cells lacking σ^E are sensitive to elevated temperature and hydrophobic agents. Selection of multicopy suppressors of the temperature sensitive (Ts) phenotype of cells lacking σ^E revealed that overexpression of the lipoprotein, NlpE, restored high temperature growth to these cells. Overexpression of NlpE has previously been shown to induce DegP synthesis by activating the Cpx two-component signal transduction pathway, and suppression of the Ts phenotype by NlpE was found to be dependent on the Cpx proteins. In addition, a constitutively active form of the CpxA sensor/kinase also fully suppressed the Ts defect of cells lacking σ^E. DegP was found to be necessary, but not sufficient, for suppression. Activation of the Cpx pathway has also been shown to alleviate the toxicity of several LamB mutant proteins. Together, these results reveal the existence of two partially overlapping regulatory systems involved in the response to extracytoplasmic stress in *E. coli*.

Introduction

The response to misfolded or unfolded protein is one of the most highly conserved regulatory responses amongst all organisms. When exposed to stressors such as heat or ethanol, all cells undergo the transcriptional induction of a conserved set of genes known as the heat shock genes that are required to combat cellular stress (Bukau, 1993; Yura et al., 1993; Becker and Craig, 1994; Georgopoulos et al., 1994; Gross, 1996). The majority of these genes encode proteins such as chaperones or proteases that act to either refold, prevent aggregation, or degrade misfolded protein (Bukau, 1993; Yura et al., 1993; Becker and Craig, 1994; Georgopoulos et al., 1994; Gross, 1996).

In addition to being highly conserved, the stress response is compartmentalized into separate pathways that regulate protein folding processes in different subcellular compartments (McMillan et al., 1994). For example, the cytoplasmic stress response in eukaryotes is co-ordinated by a family of transcriptional activators known as the heat shock transcription factors (HSFs) (Wu, 1995; Morimoto et al., 1996). A separate pathway for the response to the accumulation of misfolded protein in the endoplasmic reticulum, known as the unfolded protein response (UPR) (McMillan et al., 1994; Shamu et al., 1994) is controlled by a novel signal transduction pathway (Cox et al., 1993; Mori et al., 1993; Cox and Walter, 1996; Sidrauski et al., 1996). In this way, cells can respond to insults that affect only a single subcellular compartment without inducing a cell-wide response.

In *Escherichia coli*, the stress response is compartmentalized into cytoplasmic and extracytoplasmic responses. The cytoplasmic response is co-ordinated by the alternate sigma factor σ^{32} (Grossman et al., 1984; Landick et al., 1984; Yura et al., 1984), which responds to the accumulation of misfolded protein by directing the transcription of a well-characterized set of genes including those encoding the DnaK/DnaJ chaperone complex (Bukau, 1993; Yura et al., 1993; Becker and Craig, 1994; Georgopoulos et al., 1994; Gross, 1996). These chaperones, in turn, are thought to downregulate σ^{32} activity upon

relief of cytoplasmic stress (Straus et al., 1989; Straus et al., 1990; Gamer et al., 1992; Liberek et al., 1992; Liberek and Georgopoulos, 1993; Gamer et al., 1996).

The extracytoplasmic response, in contrast, is less well defined and is believed to be controlled by at least two signal transduction systems, the Cpx two-component system and the σ^E-mediated system. The Cpx system, composed of an inner membrane sensor kinase encoded by *cpxA*, and a response regulator encoded by *cpxR*, is activated by either overexpression of the outer membrane lipoprotein NlpE (Gupta et al., 1995; Snyder et al., 1995) or mutational activation of CpxA (Cosma et al., 1995; Danese et al., 1995; Snyder et al., 1995). Upon activation of the Cpx system, the envelope-associated toxicity of certain LamB mutant proteins is suppressed by inducing synthesis of the periplasmic protease DegP (Cosma et al., 1995; Snyder et al., 1995). The physiological signal inducing this pathway remains to be definitively identified. Because cells lacking the Cpx pathway have no obvious phenotype (Danese et al., 1995; Connolly, unpublished observations) the cellular role of this signal transduction system remains obscure.

The second periplasmic stress response system is co-ordinated by the heat and ethanol responsive alternate sigma factor, σ^E. In addition to being induced by these general stresses, the σ^E pathway is uniquely induced in response to alterations in the expression or maturation of a class outer membrane proteins (OMPs) called the porins (Mecsas et al., 1993). The porins undergo a complex series of folding and oligomerization steps prior to being inserted into the outer membrane (Pugsley, 1993), and alterations in this pathway lead to a buildup of folding intermediates that are believed to be sensed by the σ^E pathway (Mecsas et al., 1993; Rouvière and Gross, 1996). This signal is transduced to σ^E by RseA, a σ^E specific anti-sigma factor located in the inner membrane, and a periplasmic protein, RseB (Mecsas et al., 1993; Rouvière and Gross, 1996; De Las Peñas et al., 1997b; Missiakas et al., 1997). Activation of σ^E, in turn, leads to the induction of at least ten different proteins, four of which have been definitively identified: the periplasmic protease DegP, the second heat shock sigma factor, σ^{32}, the periplasmic peptidyl prolyl

isomerase, FkpA, and σ^E itself (Erickson et al., 1987; Lipinska et al., 1988b; Erickson and Gross, 1989; Wang and Kaguni, 1989; Raina et al., 1995; Rouvière et al., 1995; Danese and Silhavy, 1997). Genes under the control of σ^E are believed to encode functions required for the maintenance of envelope integrity under stress conditions because cells lacking σ^E are sensitive to elevated temperature, SDS/EDTA, and crystal violet (Hiratsu et al., 1995; Raina et al., 1995; Rouvière et al., 1995). In an attempt to isolate either genes downstream of σ^E, or components of other signal transduction cascades capable of relieving periplasmic stress, we isolated genes that when overexpressed were capable of restoring growth at high temperature to cells lacking σ^E.

Results

Selection and Identification of a Suppressor Plasmid

To isolate genes capable of restoring growth at high temperature to cells lacking σ^E, *rpoE*$^-$ cells were transformed with a genomic library containing DNA prepared from the prototypic wild type *E. coli* strain, MG1655. Following phenotypic expression at 30°C, one tenth of the cells were plated at 30°C for viable count, and the remainder at 42°C for the selection of temperature resistant (Tr) candidates. Out of a total of 3.7×10^5 possible transformants, 423 Tr suppressor candidates were identified. Because our recipient strain contains a *lacZ* reporter gene which is exclusively transcribed by holoenzyme containing σ^E (Mecsas et al., 1993), we were able to identify and discard transformants containing wild type *rpoE* sequences by screening on X-gal. Plasmid DNA was then purified from white candidate colonies, and re-transformed into the original *rpoE*$^-$ strain to ensure that the Tr phenotype was plasmid dependent. After re-transformation, one candidate plasmid, pBA25, retained suppressor ability. Remarkably, pBA25 raised the plating efficiency of the parental strain four orders of magnitude, from 4.2×10^{-5} for cells containing vector alone to 0.6 (Fig. 4-1).

Mapping and Sequence Analysis

To determine the region of the genome located on the suppressor plasmid, sequence was obtained from either end of the insert using vector-based primers. The sequence was then subjected to a BLAST search (Altschul et al., 1990). This analysis revealed that the plasmid contained three full open reading frames and a partial open reading frame mapping to minute 4.7 of the *E. coli* chromosome. The insert size, restriction map (data not shown), and identity of sequences at either end of the insert indicated that only one insert was contained in the plasmid. The insert encodes the open reading frames *yaeJ*, *yaeF*, and

the recently described gene, *nlpE* in addition to a partial copy of the *drpA/proS* gene (Fig. 4-1).

Deletion of *yaeF* and *drpA/proS* by removal the AflII-XbaI fragment revealed that the segment containing *yaeJ* and *nlpE* was responsible for suppression of the temperature sensitive defect (Fig. 4-1). Overexpression of *nlpE* has recently been shown to induce expression of the periplasmic protease DegP by activating the Cpx two-component signal transduction pathway (Danese et al., 1995). *degP* is also a member of the σ^E regulon, and we reasoned that *nlpE* might be suppressing *rpoE*$^-$ cells by activating this pathway, leading to the induction of *degP*.

To determine whether *nlpE* alone was capable of restoring growth to *rpoE*$^-$ cells at high temperature in the absence of *yaeJ*, *nlpE* was PCR amplified from the original suppressor plasmid and placed under the control of the *lac* promoter. *nlpE* alone retained the ability to rescue the Ts phenotype of the parental *rpoE*$^-$ strain (Fig. 4-1), indicating that overexpression of *nlpE* is responsible for suppression.

During the course of characterizing pBA25, we discovered a single restriction digest change in *nlpE* that could potentially change the coding sequence of the protein. To ensure that any mutations in the *nlpE* allele we isolated were not responsible for suppressor activity, we introduced plasmid pLD404 (Snyder et al., 1995), which carries a known wild-type allele of *nlpE*, into the original *rpoE*$^-$ strain. *rpoE*$^-$ cells containing pLD404 plated with similar efficiency at 42°C as cells containing plasmids derived from the original suppressor plasmid (Fig. 4-1). This result indicates that wild-type *nlpE* suppresses the temperature sensitive phenotype of *rpoE*$^-$ cells, and that any mutations present within the allele of *nlpE* originally isolated are not responsible for suppression.

Mechanism of Suppression

To determine whether *nlpE* requires the Cpx pathway for suppression, we constructed *rpoE*$^-$ *cpxA*$^-$ mutant strains and asked whether *nlpE* containing plasmids could restore

growth at high temperature to these double mutant cells. As shown in Table 2, although cells lacking both σ^E and the Cpx pathway plate with equal efficiency at 42°C as cells lacking σ^E alone (1.9×10^{-5} versus 1.1×10^{-5}), plasmids containing *nlpE* were no longer able to suppress the temperature sensitive phenotype. These results demonstrate that NlpE requires the Cpx pathway for suppression of the Ts phenotype of cells lacking σ^E. One caveat to these experiments is that the vector alone decreased the plating efficiency of the double mutant strain 1000-fold. β-lactamase expression from these Ap^R plasmids may constitute a second periplasmic stressor which might interfere with any residual suppression by *nlpE* in the Cpx^- background.

The requirement of the Cpx pathway for suppression by NlpE indicated that NlpE was probably acting via the Cpx proteins to suppress the *rpoE*⁻ Ts phenotype. If this were true, then other conditions that activate this pathway should similarly suppress the *rpoE*⁻ Ts phenotype. To test this hypothesis, we introduced a gain-of-function allele of *cpxA* (*cpxA**) (Cosma et al., 1995; Danese et al., 1995) into the original *rpoE*⁻ strain and asked whether mutational activation of the Cpx pathway would suppress the *rpoE*⁻ cells to the same extent as overexpression of *nlpE*. *rpoE*⁻ cells containing the *cpxA** allele plated with the same efficiency at 42°C as cells overexpressing *nlpE* (Fig. 4-2, lanes 2 and 4), indicating that activation of the Cpx pathway suppresses the temperature sensitive phenotype.

DegP is required, but not sufficient, for suppression

Cpx and σ^E both regulate the expression of the periplasmic protease DegP. We reasoned that activation of the Cpx pathway might be suppressing the *rpoE*⁻ phenotype by simply restoring expression of a single gene in the *rpoE* regulon, *degP*, and decided to test whether *degP* was both required and sufficient for suppression.

To test whether *degP* was required for suppression of the *rpoE*⁻ phenotype by the Cpx pathway, we introduced a *degP* null allele into *rpoE*⁻ cells containing the *cpxA** allele.

Introduction of a *degP* null allele into *rpoE*$^-$ cells reduced the plating efficiency 1500$^-$fold, and subsequent introduction of the *cpxA** allele did not restore growth at high temperature (Fig. 4-3, lanes 2 and 4). In addition, the plating efficiency of *rpoE*$^-$ *degP*$^-$ double mutant strains was not restored by overexpression of NlpE, as double mutant cells containing vector alone or a plasmid overexpressing NlpE plated with virtually the same efficiency (<1 .8 x 10^{-7} versus < 5.0 x 10^{-7}). These results indicate that *degP* is as essential component of suppression of the *rpoE*$^-$ Ts phenotype by the Cpx pathway.

To determine whether overexpression or restoration of *degP* expression was sufficient to rescue *rpoE*$^-$ cells, we introduce a plasmid encoding *degP* into the original *rpoE*$^-$ strain. Introduction of the *degP* plasmid partially restores growth at high temperature to *rpoE*$^-$ cells, as cells containing this plasmid exhibit a 10^3-fold higher plating efficiency than a similar strain containing vector alone (Fig. 4-4a, lanes 2 and 1). However, *degP* did not restore plating efficiency to the same extent as full activation of the Cpx pathway (Fig. 4-4a, lanes 2 and 4).

To ensure that *degP* expression in cells carrying the plasmid was at least as high as cells fully suppressed by activation of the Cpx pathway, Western blot analysis of DegP expression was carried out (Fig. 4-4b). The steady state level of DegP in cells grown at 30°C or 42°C was determined, and these studies revealed that *rpoE*$^-$ cells containing the *degP* plasmid (Fig. 4-4b, lanes 4 and 9) expressed at least as much, if not more, DegP as cells carrying the *cpxA** allele (Fig. 4-4b, lanes 3 and 8) at both temperatures. Together, these analyses show that overexpression of DegP alone does not restore growth to the same levels as does activation of the Cpx pathway, indicating that other Cpx-controlled genes are required for full suppression of the *rpoE*$^-$ Ts defect.

Discussion

The activity of the alternate sigma factor σ^E of *E. coli* is induced by several stressors, including elevated temperature, ethanol, and alterations in the expression and maturation of outer membrane proteins, that lead to the periplasmic accumulation of misfolded or unfolded protein species (Erickson et al., 1987; Erickson and Gross, 1989; Raina et al., 1995; Rouvière et al., 1995; Missiakas et al., 1996a; Rouvière and Gross, 1996). The σ^E regulon includes several genes whose products are likely to be required for envelope integrity because cells lacking σ^E are sensitive to several conditions that potentially disrupt outer membrane function (Hiratsu et al., 1995; Raina et al., 1995; Rouvière et al., 1995). Here we show that activation of a second signal transduction cascade, the Cpx pathway, can restore viability to *rpoE*$^-$ cells at elevated temperature. Suppression of the Ts phenotype requires the expression of at least two Cpx-dependent genes, one of which encodes for the periplasmic protease, DegP, whose expression is also under σ^E control. These results suggest that *E. coli* has at least two, partially overlapping signal transduction cascades capable of relieving extracytoplasmic stress.

Cellular role of the Cpx pathway

Although the *cpx* genes were first identified over a decade ago (McEwen and Silverman, 1980b), a clear cut role for the Cpx pathway in *E. coli* physiology has proven more elusive. The Cpx pathway has been described in relation to pleiotropic phenotypes including defective conjugative plasmid transfer (McEwen and Silverman, 1980c; McEwen and Silverman, 1980a), low level resistance to aminoglycoside antibiotics (Thorbjarnardottir et al., 1978) and alterations in the protein composition of the outer membrane (McEwen and Silverman, 1982; McEwen et al., 1983), that result from constitutive activation of this pathway. It is unclear whether these phenotypes reflect the normal function of the Cpx pathway or result from aberrant activation (Danese et al.,

1995). In addition, cells lacking the Cpx pathway show no obvious phenotypes (Danese et al., 1995; Connolly, unpublished observations).

The observation that the Cpx pathway modulates expression of the periplasmic protease DegP (Danese et al., 1995) led to the idea that this pathway is involved in combating extracytoplasmic stress, and activation of the Cpx pathway by either mutational induction or overexpression of NlpE has been shown to relieve the toxicity of several periplasmic LamB mutant proteins (Cosma et al., 1995; Snyder et al., 1995). The recent finding that the Cpx pathway modulates the expression of other periplasmic proteins involved in folding supports the idea that this pathway is involved in regulating protein folding and turnover in the extracytoplasmic compartment (Danese and Silhavy, 1997; Pogliano et al., 1997).

What does the Cpx pathway sense?

The ability of the Cpx pathway to relieve extracytoplasmic stress due to protein misfolding has been described only in relation to mutational activation of this pathway (Cosma et al., 1995) or activation by *nlpE* in multicopy (Snyder et al., 1995), suggesting that CpxA may not directly sense the folding state of this compartment. The mechanism of induction by overexpression of NlpE remains unclear. Overexpression of several other lipoproteins did not activate the Cpx pathway (Danese et al., 1995), indicating that NlpE is a specific activator. In addition, the fast kinetics of activation by NlpE (Pogliano et al., 1997) suggest that this activator acts directly on the Cpx pathway and not through slowly altering some physical or biochemical property of the envelope. NlpE has a putative serine protease inhibitor motif, and it has been proposed that NlpE may modulate or monitor protease activity in periplasm (Snyder et al., 1995). For instance, NlpE may use the serine protease inhibitor to monitor free levels of DegP activity in the periplasm. Upon an increase in substrates, DegP may be titrated off of NlpE, and an increase in the levels of free NlpE may signal the need for increased transcription of *degP* to the Cpx pathway.

In this regard, it is interesting to note that the temperature sensitive phenotype of cells lacking *degP* can be suppressed by null mutations in the gene encoding a second envelope-associated protease, OmpT (Nancy McFarland, unpublished observations). An imbalance in the protease activities of the envelope may lead to a similar imbalance of key substrates, which in turn might alter envelope physiology. For example, if OmpT were a substrate of DegP, then deletion of DegP would lead to an increase in OmpT activity and a subsequent decrease in OmpT substrates. Indeed, it has been proposed that several of the phenotypes of cells containing constitutive alleles of *cpxA* result from such an imbalance of proteolytic activity that leads to alterations in the protein composition of the outer membrane (Danese et al., 1995).

The Cpx pathway has also been shown to be required for the pH-dependent activation of the *Shigella sonnei virF* gene (Nakayama and Watanabe, 1995) which encodes a positive regulator of the *ipaBCD* invasion genes. This observation suggests that the Cpx system may be responsive to changes in pH and play a role in bacterial pathogenesis. It will be interesting to determine whether known Cpx-dependent genes are also modulated in response to pH alterations, and whether any of these genes encode functions required for survival under such conditions.

Lastly, the Cpx pathway is activated in cells lacking phosphatidyl-ethanolamine (Milekovskaya and Dowhan, 1997), suggesting that this pathway may also sense alterations in the structural or physical integrity of the cell envelope. Deletion of *nlpE* from these strains did not alter Cpx activation, suggesting that the Cpx pathway may directly sense these envelope changes (Milekovskaya and Dowhan, 1997). It remains to be seen whether each of the conditions described above generate a single, common signal that is sensed by CpxA, or if this pathway is capable of responding to diverse signals.

What is the relationship between the Cpx and σ^E pathways?

Several lines of evidence suggest that the Cpx and σ^E pathways represent distinct stress-response systems that do not sense or regulate redundant functions (Fig. 4-5). Deletion of either the Cpx pathway or *nlpE* leads to no obvious phenotype (Danese et al., 1995; Snyder et al., 1995; Connolly, unpublished observations), suggesting that this pathway may contribute to envelope homeostasis under a specific set of conditions not normally achieved in the laboratory or that Cpx-controlled genes exhibit a basal level of transcription that is sufficient for cell growth. In contrast, the σ^E pathway appears to be involved in envelope homeostasis under most growth conditions. Cells lacking σ^E are sensitive to membrane disrupting agents and fail to grow at high temperature (Hiratsu et al., 1995; Raina et al., 1995; Rouvière et al., 1995). In fact, recent work suggests that σ^E directed functions are required at all temperatures as we have discovered that our strains lacking σ^E contain an unidentified suppressor that is required for low temperature growth (De Las Peñas et al., 1997a). The presence of a *cpxA** allele does not alleviate the requirement for this suppressor (Connolly, unpublished observations), indicating that the Cpx pathway cannot substitute for σ^E under all conditions. In addition, the suppressor alone does not confer a Ts phenotype (Connolly, unpublished observations), indicating that the Ts phenotype observed in *rpoE*$^-$ strains is due to a loss of σ^E-dependent gene products. Activation of the Cpx pathway compensates for this loss of σ^E-dependent gene expression normally required at high temperature as evidenced by the observation that restoration of expression of a single σ^E-dependent gene product, DegP, by activation of the Cpx pathway accounts for the majority of suppression (Fig. 4-4a).

In addition to exhibiting distinct phenotypes, each system appears to respond to different signals. Overexpression of NlpE does not induce σ^E activity in general, as measured from a minimal σ^E-dependent promoter (Danese et al., 1995), and several types of stressors that induce σ^E activity do not induce the Cpx pathway (Danese et al., 1995). For example, alterations in the maturation of outer membrane proteins (OMPs), either by

overexpression or the titration or periplasmic folding agents, uniquely induce σ^E (Mecsas et al., 1993; Danese et al., 1995; Missiakas et al., 1996a; Rouvière and Gross, 1996). Alterations in the lipid composition of the envelope have recently been shown to induce the Cpx pathway (Milekovskaya and Dowhan, 1997), and it remains to be seen whether these conditions similarly alter σ^E activity.

The Cpx and σ^E regulons overlap at *degP*, however, several observations suggest that the two regulons are not identical. First, the Cpx system does not exclusively rely on holoenzyme containing σ^E for transcriptional activation. Although CpxR and σ^E appear to act together at the *degP* promoter, CpxR is capable of inducing *degP* transcription approximately three-fold in the absence of σ^E (Danese et al., 1995). In addition, new members unique to either the Cpx or σ^E regulons have been described (Fig. 4-5) (Danese and Silhavy, 1997; Pogliano et al., 1997). The Cpx proteins regulate expression of the disulfide bond isomerase, *dsbA* (Danese and Silhavy, 1997; Pogliano et al., 1997), and the peptidyl prolyl isomerase (PPIase), *ppiA* (Pogliano et al., 1997), while σ^E induces the expression of a second PPIase, FkpA (Danese and Silhavy, 1997). Although each regulon encodes similar biochemical activities, and activation of the Cpx pathway can rescue the Ts defect of *rpoE*$^-$ cells, activation of the σ^E pathway can not substitute for the Cpx system under some conditions. For example, activation of σ^E by overexpression of OMPs does not relieve the toxicity of LamB mutant proteins (Snyder et al., 1995). These results suggest that the two pathways can be induced independently in response to unique stressors, and that they may integrate these disparate signals arising in the face of multi-faceted insults. A complete understanding of the relationship between these two systems awaits further molecular characterization of their regulon members and inducing signals.

Regulation of DegP

From these experiments and those of others, it is clear that DegP plays a key role in combating envelope stress (Lipinska et al., 1989; Strauch et al., 1989; Cosma et al., 1995;

Snyder et al., 1995). During the original characterization of the *degP* promoter, it became evident that *degP* was a heat shock gene (Lipinska et al., 1988b). This heat induction appeared to be independent of the main heat shock sigma factor, σ^{32}, and was instead found to depend upon a second heat shock sigma, σ^{E} (Lipinska et al., 1988b; Erickson and Gross, 1989). Indeed, these experiments provided some of the first evidence that σ^{E} controlled a second heat shock regulon. However, it became clear during the course of our experiments that the expression of DegP is still induced by heat shock in the absence of σ^{E} (Fig. 4-4b). The heat shock regulation of *degP* has recently been shown to be independent of the Cpx pathway in cells containing σ^{E} (Pogliano et al., 1997) and we have observed heat shock induction of DegP in cells lacking both of these systems (Connolly, unpublished observations). These results raise the possibility that a third regulatory cascade modulates DegP expression, and help to explain the seemingly contradictory observation that cells lacking σ^{E}, and, by extension the entire σ^{E} regulon, plate 100-fold better than cells lacking DegP alone (10^{-3} versus 10^{-5}; Connolly, unpublished observations). It is also clear that this residual expression contributes substantially to the survival of *rpoE*$^{-}$ cells at high temperature (Fig. 4-3), underscoring the key role that DegP plays in maintaining envelope integrity. From these experiments, it is unclear at what level this σ^{E}-independent induction of DegP is occurring, and it will be interesting to determine how DegP levels are regulated in the absence of the σ^{E} and Cpx systems.

Experimental Procedures

Media, reagents, and enzymes

Luria-Bertani (LB) and M9 minimal media was prepared as described (Sambrook et al., 1989). Where needed, media was supplemented with ampicillin 100μg/ml (Ap100), amikacin 3 ug/ml, spectinomycin 50 ug/ml (Sp50), tetracycline 10 ug/ml (Tc10), kanamycin 30 ug/ml (Km30), chloramphenicol 12 ug/ml (Cm12), or glucose 0.4%.

Strains

Bacterial strains used in this study are described in Table 4-1.

Plasmids

Plasmids used in this study are listed in Table 4-1. The *E. coli* genomic library was a generous gift of Alan Derman, and contains PstI digested chromosomal DNA from MG1655 cloned into the PstI site of pUC19 (Norrander et al., 1983) as described (Neumann et al., 1992). pBA25 was isolated directly from this library. During the course of subcloning *nlpE* from this plasmid, we noticed a difference in a single restriction site versus a previously reported sequence derived from MC4100 (Snyder et al., 1995), suggesting that sequence heterogeneity of this gene may exist amongst different *E. coli* isolates. pBA26 was constructed by deleting a 2.1 kb AflII-XbaI fragment from pBA25. pLC224 was constructed by PCR amplification of *nlpE* from pBA25 using primers NLPE28 (5' CAGCGGTCGGGAATTCAAAGAAGGAATG) and NLPE29 (5' GGGGGGAAGCTTACGCCTTATCCGGCCTAC) . The resulting product was then cloned into the EcoRI to HindIII sites of pLC222 {an Ap^R version of pSU18 (Bartolomé et al., 1991), containing the *bla* gene from pBS SK+ cloned into the SspI sites of pSU18}. pAP87 was constructed by subcloning a 3.0 kb DraI-BamHI fragment from pKS17 into pACYC177 (Rose, 1988).

DNA Sequencing

Sequence was obtained from either end of the insert in pBA25 using vector derived primers (M13 Sequencing Primer and M13 Reverse Sequencing Primer) and a cycle sequencing kit according to manufacturer's directions (Gibco-BRL, Gaithersburg, MD).

Plating Efficiencies

recA56 versions of all strains were used to determine plating efficiencies with the exception of the data in Fig. 4-3 (CAG22216, CAG33104, 33067, CAG33245).

Ten-fold dilutions of the desired strain were made in 1 ml of LB from fresh overnight cultures grown at 30°C in LB plus the appropriate antibiotic and 0.4% glucose for *rpoE*::ΩCm *cpxR*::ΩSp strains containing the following plasmids: pUC18, pBA25, pLC222, and pLC224. 100 ul of each dilution was plated in duplicate on LB or Ap100 for plasmid containing strains. One plate was incubated at 30°C, the other at 42°C for 24-48 hours, and the resulting number of colonies counted. Plating efficiency was scored as the number of colonies arising at 42°C divided by the number at 30°C, and the values reported represent the average of at least three independent determinations.

Detection of DegP and MBP by Western Blot

15 mls of LB were inoculated with 150 ul of a fresh overnight of the desired strains. Cells were grown at 30 °C to an OD_{600} of 0.5, and 0.9 mls of culture were sampled directly into 0.1 ml of 50% TCA on ice, vortexed, and placed at -20°C overnight. The remainder of the culture was shifted to 42°C, and grown for 20 minutes. After 20 minutes of growth, the OD_{600} of the culture was determined and 0.9 mls sampled directly into TCA as above. The next day, TCA precipitates were pelleted at 17,500 x g for 30 minutes at 4°C. The pellets were washed twice with 100% acetone, air-dried, and resuspended in 1X protein sample buffer (50 mM Tris-Cl pH 6.8, 100 mM DTT, 2% SDS, 0.2% bromophenol blue) to a concentration of 10^7 cells/ul. 2.5 x 10^7 cells were run on 10% SDS-PAGE and

transferred to a nitrocellulose membrane. The membrane was blocked overnight at room temperature with 3% non-fat dry milk in TBST (10 mM Tris pH 8.0, 150 mM NaCl, 0.2 % Tween-20). A mixture of anti-DegP and MBP primary antibodies (provided by Jon Beckwith) were used at 1:10,000 concentration in TBST plus 1% milk for one hour at room temperature. The blot was washed three times for five minutes each with TBST, then incubated with 1:10,000 dilution of sheep α-rabbit POD (Boehringer Mannheim, Indianapolis, IN) for one hour at room temperature, and washed again as before. It was then developed with enhanced chemiluminescence (ECL) reagents (Amersham, Arlington Heights, IL) and Hyperfilm ECL (Amersham, Arlington Heights, IL) and exposed to film from five to thirty seconds.

Acknowledgments

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Table 4-1. Strains and plasmids used in this study

Strain/Plasmid	Relevant Genotype	Source/Reference
Strain		
CAG16037	MC1061 ϕ(λ *rpoH P3::lacZ)*	(Mecsas et al., 1993)
CAG37193	CAG16037 *srl-300::Tn10 recA56*	This work
CAG22216	CAG16037 *rpoE::Ω*Cm	(Rouvière et al., 1995)
CAG22700	CAG22216 *srl-300::Tn10 recA56*	This work
CAG33126	CAG22700, pUC18	This work
CAG33127	CAG22700, pBA25	This work
CAG33183	CAG22700, pBA26	This work
CAG33267	CAG22700, pBR322	This work
CAG33268	CAG22700, pLD404	This work
CAG33190	CAG22700, pLC222	This work
CAG33191	CAG22700, pLC224	This work
CAG33113	CAG22700, pAP87	This work
CAG33114	CAG22700, pACYC177	This work
PND325	MC4100 λRS88(*degP-lacZ*) *cpxR::ΩSp*	(Danese et al., 1995)
CAG18636	MG1655 *zii-3088::Tn10Km*	(Singer et al., 1989)
CAG33064	PND325 *zii-3088::Tn10Km*	This work
CAG33069	CAG22216 *zii-3088::Tn10Km cpxR::ΩSp*	This work
CAG33091	CAG33069 *srl-300::Tn10 recA56*	This work
CAG33239	CAG33091, pUC18	This work
CAG33240	CAG33091, pBA25	This work
CAG33241	CAG33091, pLC222	This work
CAG33242	CAG33091, pLC224	This work
CAG16237	CAG16037 *degP::Km*	(Mecsas et al., 1993)
CAG33245	CAG33104 *degP::Km*	This work
CAG33067	CAG22216 *degP::Km*	This work
CAG33070	CAG33067 *srl-300::Tn10 recA56*	This work
CAG33163	CAG33070, pUC18	This work
CAG33164	CAG33070, pBA25	This work
CLC145	MC4100 *cpx103 lamBA23D*	(Cosma et al., 1995)
CAG33104	CAG22216 *cpx103*	This work
CAG33131	CAG33104 *srl-300::Tn10 recA56*	This work
Plasmid		
pUC18	cloning vector, ColE1 ori, Ap	(Norrander et al., 1983)
pBA25	*yaeJ*, *nlpE*, *yaeF*, *drpA/proS* in pUC, Ap	This work
pBA26	*yaeJ* and *nlpE* in pUC, Ap	This work
pSU18	cloning vector, p15A ori, Cm	(Bartolomé et al., 1991)
pLC222	*bla* gene from pUC cloned into pSU18, Ap	This work
pLC224	*nlpE* in pLC2222, Ap	This work
pBR322	coloning vector, ColE1 ori, Ap, Tc	(Bolivar et al., 1977)
pLD404	*yaeJ, nlpE, and yaeF* in pBR322	(Snyder et al., 1995)
pACYC177	cloning vector, p15A ori, Km, Ap	(Rose, 1988)
pAP87	*degP* in pACYC177, Km	This work

Table 4-2. Plating efficiency of *rpoE*$^-$ strains lacking the Cpx pathway

Strain	Genotype	Plasmid	Plating Efficiency (# colonies 42°C/30°C)
CAG22700	*rpoE::Ω*Cm *recA56*	none	1.1 x 10^{-5}
CAG33126	CAG22700	vector	4.2 x 10^{-5}
CAG33127	CAG22700	p*nlpE*	0.6
CAG33091	*rpoE::Ω*Cm *cpxR::ΩSp recA56*	none	1.9 x 10^{-5}
CAG33239	CAG33091	vector	< 1.6 x 10^{-8}
CAG33240	CAG33091	p*nlpE*	< 3.9 x 10^{-8}

The plating efficiency of each strain was determined as described in Experimental Procedures. Vector containing cells carry pUC18, and p*nlpE* represents pBA25.

Figure 4-1. Maps and suppressor activity of plasmid subclones. pBA25 is the original suppressor plasmid. pBA26 was created by the removal of the represented sequences and religating, pLD404 has been previously described (Snyder et al., 1995), and pLC224 was generated by PCR amplification and subcloning of a fragment containing *nlpE* alone from pBA25. Restriction enzyme site abbreviations: P, PstI; A, AflII; X, XbaI; E, EcoRI; H, HindIII; S, StyI. Note that the plating efficiency of cells containing vector alone was 4.2 x 10^{-5} (pUC18 for pBA25 and pBA26), 4.0 x 10^{-6} (pBR322 for pLD404), and 7.1 x 10^{-7} (pLC222 for pLC224).

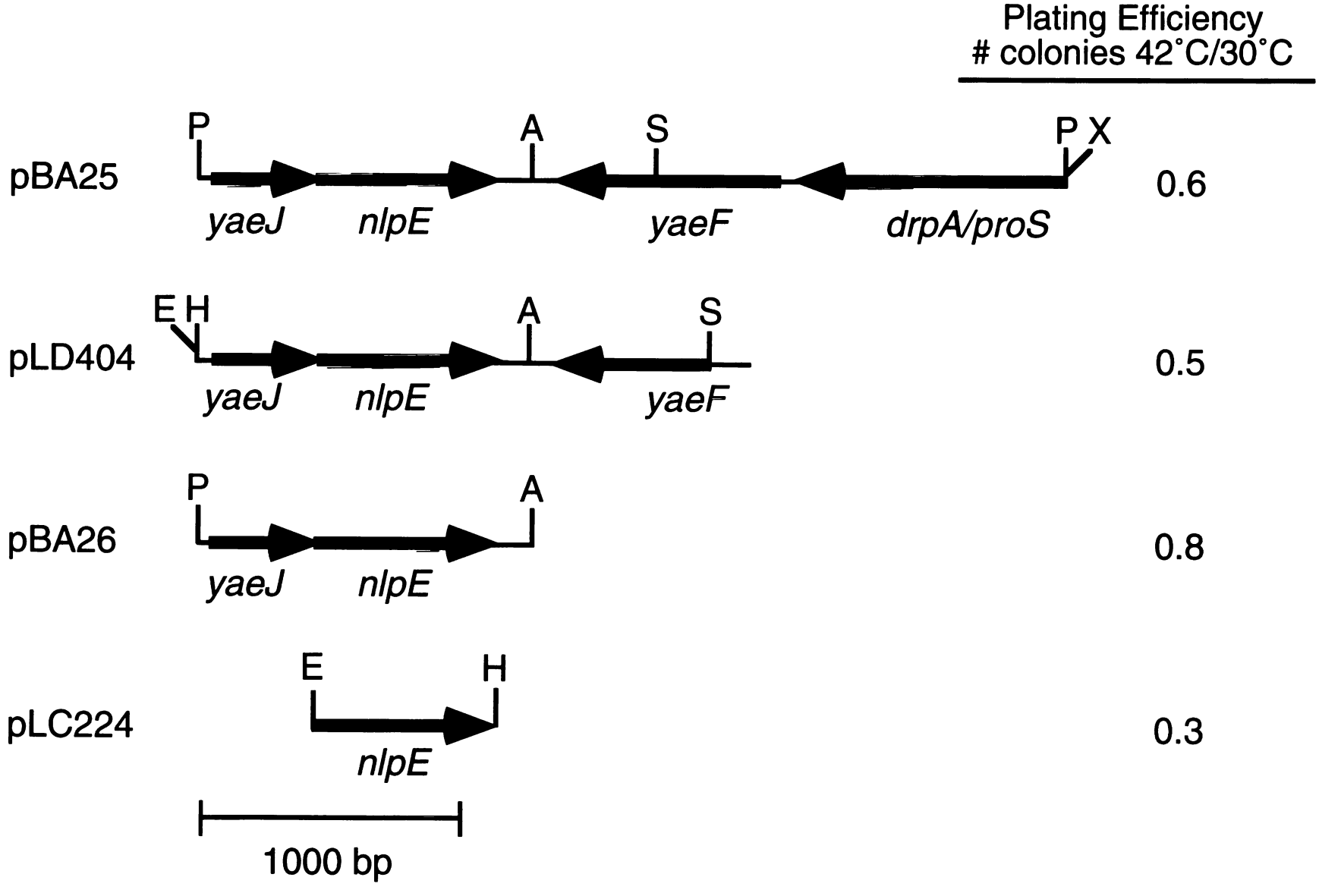

Plating Efficiency
colonies 42°C/30°C
pBA25
P
A
S
P X
yaeJ
nlpE
yaeF
drpA/proS
0.6
pLD404
E H
A
S
yaeJ
nlpE
yaeF
0.5
pBA26
P
A
yaeJ
nlpE
0.8
pLC224
E
H
nlpE
0.3
1000 bp

Figure 4-2. Plating efficiency of *rpoE::Ω*Cm cells activated for the Cpx pathway. The efficiency of plating of *rpoE::Ω*Cm cells containing vector alone (lane 1) or pBA25 (lane 2), or no plasmid and a wild-type (lane 3) or constitutively activated (lane 4) *cpxA* allele was determined as described in Experimental Procedures. The numerical values of the plating efficiency of each strain were vector alone, 4.2×10^{-5}; pBA25, 0.6; *cpxA*$^{+}$, 1.1×10^{-5}; *cpxA**, 0.8.

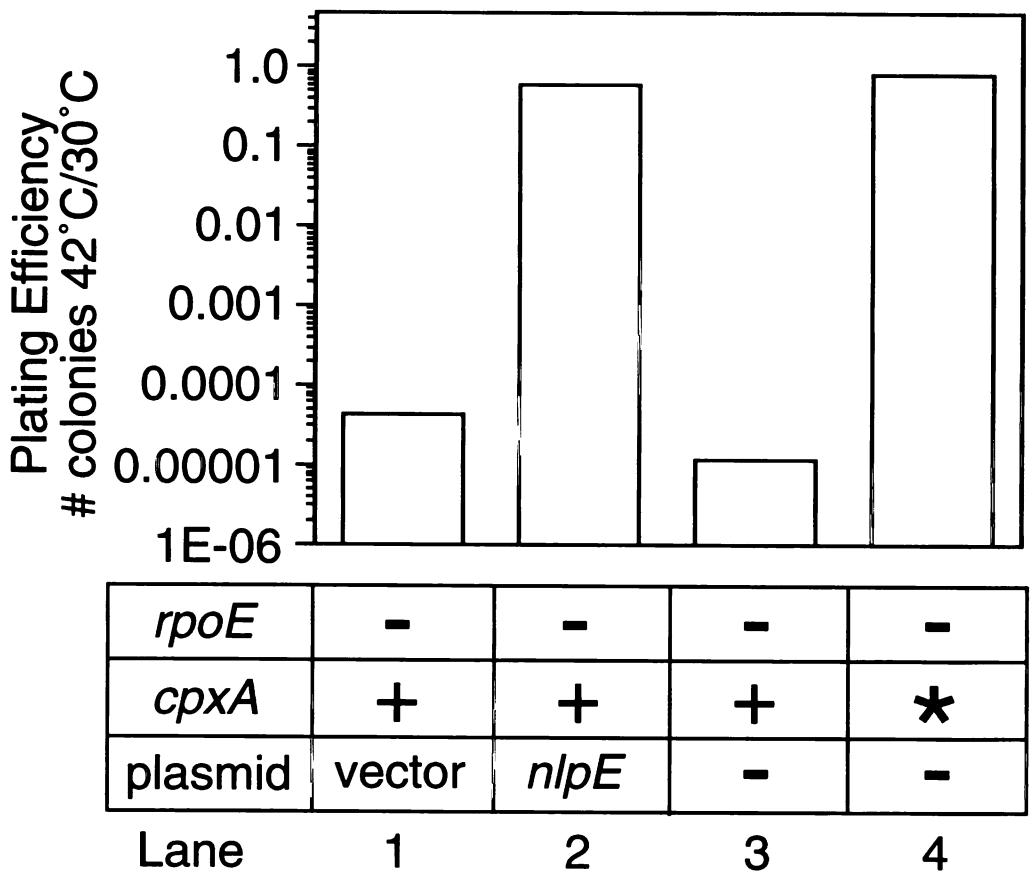

Plating Efficiency
colonies 42°C/30°C
1.0
0.1
0.01
0.001
0.0001
0.00001
1E-06
rpoE - - - -
cpxA + + + *
plasmid vector nlpE - -
Lane 1 2 3 4

Figure 4-3. Plating efficiency of *rpoE::Ω*Cm cells lacking *degP*. The plating efficiency of *rpoE::Ω*Cm *cpxA*$^+$ (lane 1), *rpoE::Ω*Cm *cpxA*$^+$ *degP*::Km (lane 2), *rpoE::Ω*Cm *cpxA** (lane 3), and *rpoE::Ω*Cm *cpxA* degP*::Km (lane 4) strains was determined as described in Experimental Procedures. The numerical values of each plating efficiency were *rpoE::Ω*Cm *cpxA*$^+$, 1.0 x 10^{-3}; *rpoE::Ω*Cm *cpxA*$^+$ *degP*::Km, 6.7 x 10^{-7}; *rpoE::Ω*Cm *cpxA**, 0.6; and *rpoE::Ω*Cm *cpxA* degP*::Km, 1.2 x 10^{-6}. Note that all strains used in this experiment were *recA*$^+$.

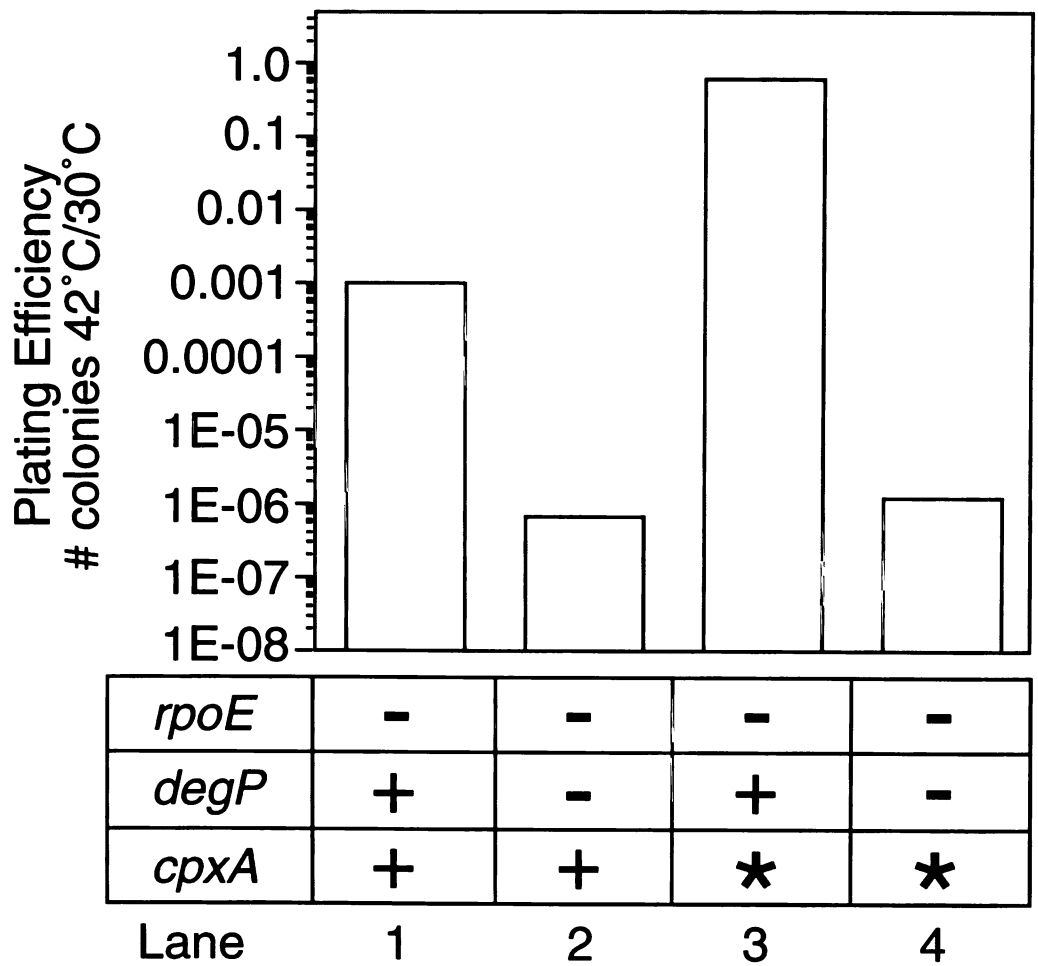
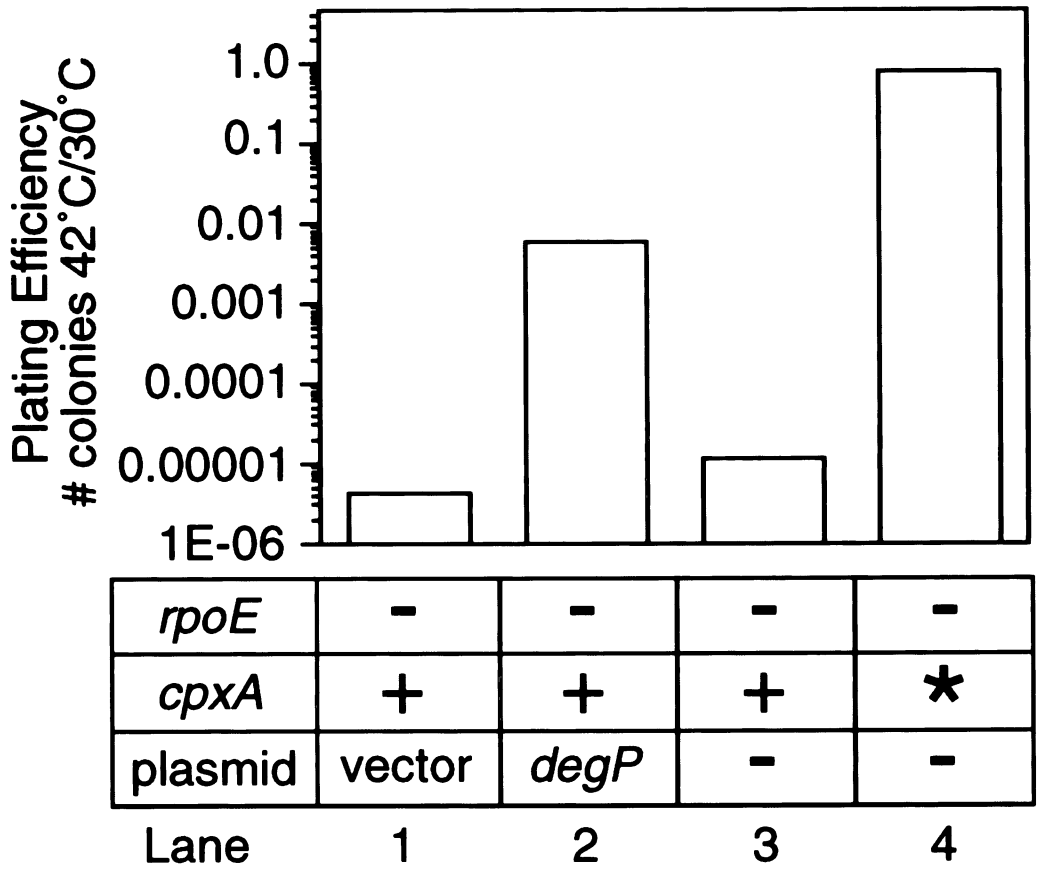

Plating Efficiency
colonies 42°C/30°C
1.0
0.1
0.01
0.001
0.0001
1E-05
1E-06
1E-07
1E-08
rpoE - - - -
degP + - + -
cpxA + + * *
Lane 1 2 3 4

Figure 4-4. Phenotype of *rpoE::*Ω**Cm cells overexpressing DegP. (a) Plating efficiency. The efficiency of plating at 42°C of *rpoE::*Ω**Cm cells containing vector alone (lane 1) or a plasmid overexpressing *degP* (lane 2) was determined. For comparison, the plating efficiencies of *rpoE*$^-$ cells containing a wild-type (lane 3) or fully activated (lane 4) *cpxA* allele are also shown. The numerical values of each plating efficiency were vector alone, 4.1 x 10^{-6}; p*degP*, 6.0 x 10^{-3}; *cpxA*$^+$, 1.1 x 10^{-5}; and *cpxA**, 0.8. (b) Western blot analysis of DegP expression. Wild-type cells (lanes 1 and 6), *rpoE::*Ω**Cm cells containing wild-type (*cpxA*$^+$, lanes 2 and 7) or activated (*cpxA**, lanes 3 and 8) *cpxA* alleles, or *rpoE::*Ω**Cm cells containing either a plasmid overexpressing *degP* (p*degP*, lanes 4 and 9) or vector alone (lanes 5 and 10) were grown at 30°C to mid-log phase and then shifted to 42°C and grown for 20 minutes. The steady state level of DegP expression in cells grown before and after heat shock was then determined by Western Blot analysis as described in Experimental Procedures. As a loading control, the blot was also probed with antibodies against maltose binding protein (MBP).

a


Plating Efficiency
colonies 42°C/30°C
1.0
0.1
0.01
0.001
0.0001
0.00001
1E-06
rpoE - - - -
cpxA + + + *
plasmid vector degP - -
Lane 1 2 3 4

b

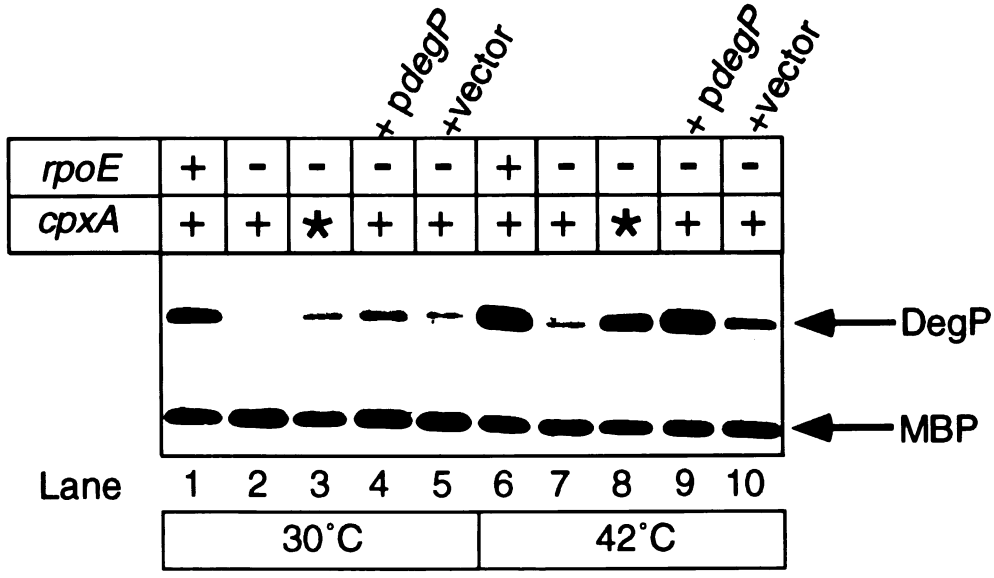
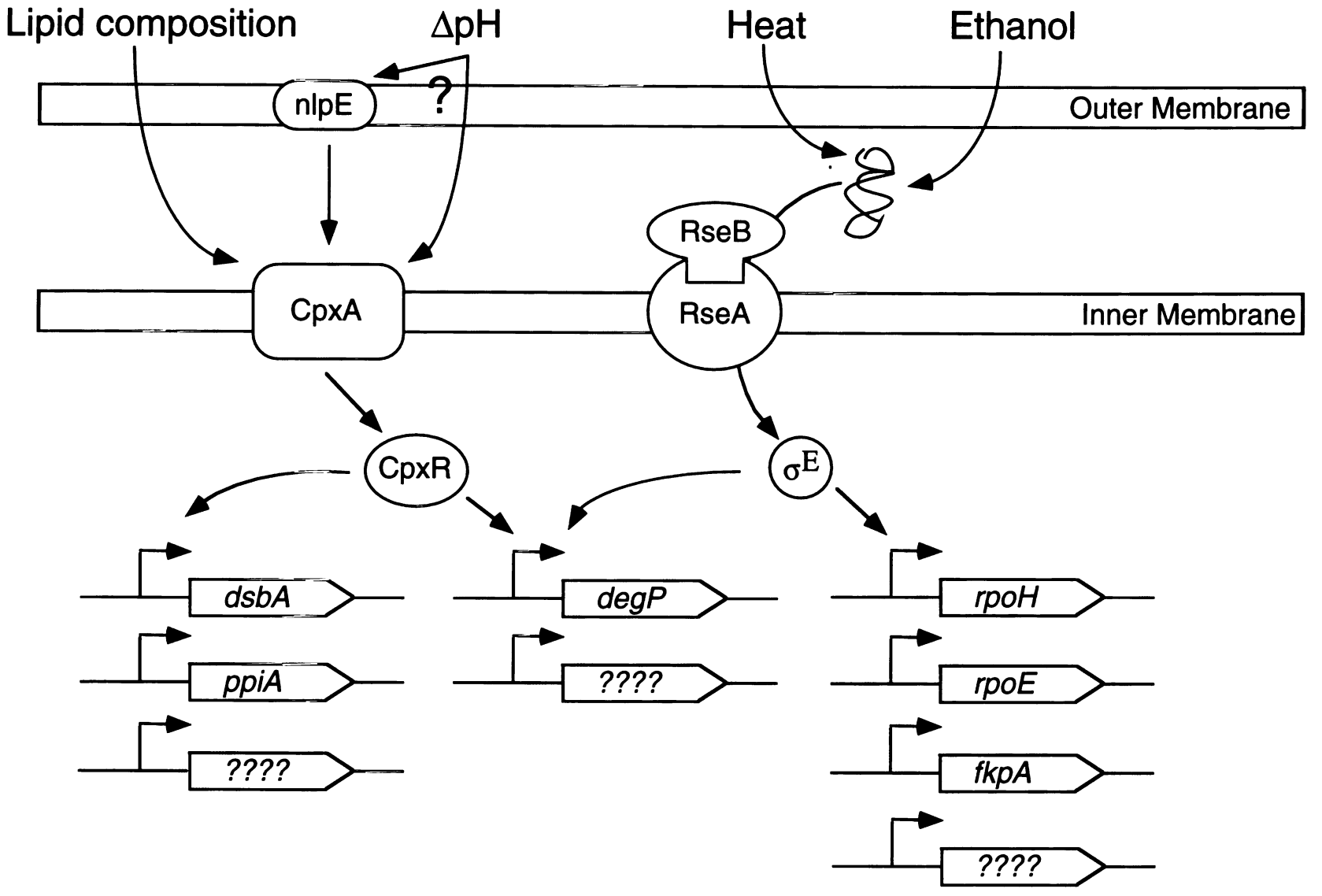

+ pdegP
+vector
+ pdegP
+vector
rpoE + - - - - + - - - -
cpxA + + * + + + + * + +
DegP
MBP
Lane 1 2 3 4 5 6 7 8 9 10
30°C
42°C

Figure 4-5. The extracytoplasmic stress response is controlled by partially overlapping pathways. The Cpx pathway controls the expression of several resident periplasmic folding proteins (DegP, PpiA, and DsbA) in response to overproduction of the outer membrane lipoprotein NlpE, and perhaps structural or physical alterations in the envelope induced by changes in pH or lipid composition. The alternate sigma factor, σ^E, controls the expression of *degP*, *rpoH*, *rpoE*, *fkpA*, and several other unidentified genes in response to the accumulation of misfolded outer membrane protein precursors in the periplasmic space. See Discussion for further details.


Lipid composition
ΔpH
Heat
Ethanol
nlpE
?
Outer Membrane
RseB
CpxA
RseA
Inner Membrane
CpxR
σE
dsbA
ppiA
????
degP
????
rpoH
rpoE
fkpA
????

Chapter Five

DegS/HhoB is an essential protease in *Escherichia coli* and plays a role in the σ^E-mediated response to extracytoplasmic stress

Summary

The activity of the stress-responsive sigma factor σ^E of *Escherichia coli* is induced by the extracytoplasmic accumulation of misfolded or unfolded protein. This stress signal is transduced to σ^E by the Rse proteins but it is not understood how the activity of these regulators is modulated in respose to the accumulation of misfolded protein It has been proposed that such stress-responsive pathways monitor protein folding by incorporating chaperones and/or proteases into the signal transduction cascade. Here we explore whether the extracytoplasmic proteases DegP, OmpT, Tsp/Prc, DegQ/HhoA or DegS/HhoB play a role in periplasmic folding and signal transduction to σ^E by examining the effects of deletion of the genes encoding these proteases on σ^E activity. Our results show that the inner membrane protease DegS/HhoB is an essential protease whose activity is required for σ^E function, suggesting that DegS/HhoB may play a direct role in the σ^E signal transduction cascade.

Introduction

The cellular response to environmental stress is one of the most highly conserved regulatory responses amongst all organisms. Exposure to increased temperature or other potentially deleterious environmental conditions leads to the expression of a conserved set of proteins, the heat shock proteins (hsps) (Bukau, 1993; Yura et al., 1993; Becker and Craig, 1994; Georgopoulos et al., 1994; Gross, 1996). Most hsps are either chaperones or proteases, and act to stabilize, prevent aggregation, refold, or degrade misfolded or unfolded proteins (Bukau, 1993; Yura et al., 1993; Becker and Craig, 1994; Georgopoulos et al., 1994; Gross, 1996).

The stress response is composed of separate pathways that monitor different subcellular compartments (McMillan et al., 1994). This allows the cell to respond to insults affecting a single compartment without inducing a cell-wide response. In the Gram-negative bacterium *Escherichia coli* the stress response is compartmentalized into cytoplasmic and extracytoplasmic responses. The cytoplasmic response is governed by the alternate sigma factor σ^{32} (Grossman et al., 1984; Landick et al., 1984; Yura et al., 1984) while the extracytoplasmic response is controlled by two partially overlapping signal transduction cascades, the σ^{E} and Cpx two-component systems (Erickson and Gross, 1989; Wang and Kaguni, 1989; Mecsas et al., 1993; Danese et al., 1995; Connolly et al., 1997).

These stress-responsive signal transduction cascades are believed to sense stress by monitoring the protein folding pathways in various cellular compartments. This is achieved by incorporating chaperones and/or proteases directly into the signal transduction cascade (McMillan et al., 1994). For example, the activity of σ^{32} is thought to be controlled both by chaperone-mediated inactivation and regulated proteolysis. The DnaK-DnaJ-GrpE chaperone machine binds reversibly to σ^{32} to inhibit its function (Straus et al., 1989) and cellular stress is thought to be monitored by how well σ^{32} can compete with misfolded or unfolded proteins for binding to this chaperone complex (Straus et al., 1990; Craig and

Gross, 1991; Bukau, 1993). In addition, the regulated degradation of σ^{32} plays a key role in the response to temperature upshift. The main protease responsible for σ^{32} degradation, the inner membrane protease HflB, is the first essential protease described in *E. coli* and it is thought that the interaction with chaperones may be responsible for targeting σ^{32} for degradation by this protease (Tomoyasu et al., 1993; Herman et al., 1995; Tomoyasu et al., 1995).

The activity of the second stress-responsive sigma factor, σ^{E} is negatively controlled by an inner membrane anti-sigma, RseA whose activity is in turn modulated by the periplasmic protein RseB (De Las Peñas et al., 1997b; Missiakas et al., 1997). Activation of σ^{E} under stress conditions requires an alleviation of this negative regulation, but the mechanism by which RseA and B are inactivated in response to the accumulation of misfolded protein is unknown. The Rse proteins may directly detect misfolded proteins and this interaction may lead to modification, degradation or conformational change of the Rse proteins. Alternatively, these sensors may monitor the levels of free periplasmic folding agents and proteases. Similar to the competition between σ^{32} and misfolded protein for the DnaK-DnaJ-GrpE chaperone complex, the Rse proteins may compete with misfolded proteins in the extracytoplasmic compartment for periplasmic folding agents, and changes in the conformational state of the Rse proteins caused by disruption of this interaction may render them inactive or susceptible to modification or degradation.

In addition to the regulatory role proteolysis plays in the cytoplasmic stress response, proteolysis plays a role in maintaining cellular integrity by ridding the cell of abnormal proteins produced under both steady state and stress conditions (Gottesman and Maurizi, 1992; Gottesman, 1996; Miller, 1996). Substrates arising as a result of normal cellular processes include misfolded proteins, proteins synthesized with abnormal amino acids and proteins synthesized from truncated mRNAs, and these are all rapidly degraded in growing cells (Gottesman and Maurizi, 1992; Gottesman, 1996; Miller, 1996). Exposure to high temperature leads to an increase in misfolded protein, signalling the need

for increased proteolysis under these conditions, and, not surprisingly, the expression of several of the cytoplasmic proteases is under the control of σ^{32} (Gross, 1996). Often, a single protease can play both general degradative and regulatory roles (Gottesman, 1996). For example, in addition to its regulatory role described above, HlfB plays a role in maintaining the integrity of the inner membrane by degrading uncomplexed inner membrane proteins present in excess of their oligomeric binding partners (Kihara et al., 1995; Akiyama et al., 1996).

In contrast, the cellular roles of extracytoplasmic proteases and how they interact with the stress response are less well understood. Like several of their cytoplasmic counterparts, some of the extracytoplasmic proteases such as DegP, Tsp/Prc, OmpT, DegQ/HhoA and DegS/HhoB may possess dual regulatory and general degradative functions. DegP participates in the degradation of abnormal periplasmic proteins and is required for survival at high temperature (Strauch and Beckwith, 1988; Lipinska et al., 1989; Strauch et al., 1989). In addition, *degP* expression is controlled by both the σ^{E} and Cpx responses (Lipinska et al., 1988b; Erickson and Gross, 1989; Danese et al., 1995), underscoring the central role this protease plays in ridding the cell of abnormal protein. Tsp/Prc is involved in the degradation of periplasmic proteins synthesized from truncated mRNAs (Keiler et al., 1996), and cells lacking this protease fail to grow at high temperature on hypotonic media (Hara et al., 1991), demonstrate increased sensitivity to some antibiotics (Seoane et al., 1992) and leak periplasmic proteins (Hara et al., 1991), suggesting that Tsp may also play a role in maintaining outer membrane integrity. OmpT plays a role in maintaining the correct balance between extracytoplasmic proteins normally found in oligomeric complexes (Skare et al., 1993) and shows increased expression at high temperature (Rupprecht et al., 1983). Overexpression of DegQ/HhoA is capable of suppressing the temperature sensitivity of cells lacking either Prc/Tsp or DegP (Bass et al., 1996; Waller and Sauer, 1996), suggesting that the substrate specificities, but not cellular roles, of these proteases overlaps. Cells lacking the inner membrane protease DegS/HhoB

grow slowly and rapidly accumulate suppressors (Waller and Sauer, 1996), suggesting that DegS/HhoB may play a unique and essential cellular role. In this work, we explore the potential roles of these proteases in the extracytoplasmic stress response by assaying σ^E and Cpx activity in cells lacking these proteases. While our results support general and overlapping roles for most of these proteases in the removal of abnormal extracytoplasmic proteins, they also support a regulatory role for DegS/HhoB in the σ^E-mediated signal transduction cascade.

Results

Deletion of *prc, degP,* or *ompT* does not alter the extracytoplasmic stress response

Potential roles for the extracytoplasmic proteases in the stress response include both the degradation of misfolded proteins occurring as result of stress and/or controlling the activity of signal transduction molecules (Gottesman and Maurizi, 1992; Gottesman, 1996). Deletion of proteases responsible for the degradation of abnormal envelope proteins may cause an induction of the stress response under steady state conditions by leading to an accumulation of misfolded protein. Alternatively, and similar to the cytoplasmic degradation of abnormal protein, several proteases may be responsible for the degradation of misfolded substrates and deletion of a single protease may not greatly alter σ^E or Cpx activity. Loss of a regulatory protease, on the other hand, may be expected to affect the stress response either positively or negatively depending on the level of interaction of the protease with the signal transduction cascade. For example, such a protease may determine the stability of the negative regulators of σ^E and loss of this protease would stabilize these regulators, leading to a decrease in σ^E activity. Alternatively, the protease may be responsible for controlling the stability of a putative signal molecule generated by the accumulation of misfolded protein and sensed by the Rse proteins. Deletion of such a protease would lead to an increase in the signal molecule and σ^E activity.

To determine whether any of the extracytoplasmic proteases discussed above are involved in either the σ^E or Cpx-mediated stress response, we asked whether deletions of these proteases altered σ^E or Cpx activity under steady state conditions as measured from single copy *lacZ* reporter genes [Mecsas, 1993 #206][Jones, 1997 #259]. Deletion of the extracytoplasmic proteases DegP, Tsp/Prc, and OmpT showed little or no effect on either σ^E or Cpx activity (Fig. 5-1), suggesting that these proteases do not play unique regulatory roles in signal transduction and that their housekeeping functions may be redundant.

A *ΔdegQ1*, but not a *degQ2*::Km, allele induces both the σ^E and Cpx pathways

In contrast to deletion of *prc*, *degP* or *ompT*, the introduction of a *ΔdegQ1* allele led to a 3-fold induction of both the σ^E and Cpx pathways (Fig. 5-2). A second *degQ* allele, a *degQ2*::Km insertion, showed no effect on the σ^E pathway and less than a two-fold effect on the Cpx pathway (Fig. 5-2). One possible explanation for the differing effects of these two alleles lies in the putative protein products of each.

DegQ/HhoA is a periplasmic serine protease of 455 residues, including conserved Ser, His, and Asp residues of the putative catalytic triad (Kolmar et al., 1996; Waller and Sauer, 1996). In addition, the C-terminal half of DegQ/HhoA contains two putative PDZ domains which are thought to mediate protein-protein interaction and recognition of substrates (Pallen and Ponting, 1997; Pallen and Wren, 1997). The *ΔdegQ1* allele was constructed by creating an in-frame deletion resulting in the removal of 155 amino acids of the mature protein, including the active site Ser (Waller and Sauer, 1996). This internal deletion leaves a protein containing a signal sequence, two of the active site residues, most of the first PDZ domain, and the entire second PDZ domain intact. In contrast, the *degQ2*::Km allele could potentially express only the first portion of the protein, including the signal sequence and two of the active site residues but lacking the PDZ domains (Waller and Sauer, 1996). This raises the possibility that the *ΔdegQ1* allele is expressing a protein with altered function while the *degQ2*::Km allele expresses no protein or a nonfunctional segment of protein. The altered protein produced in the *ΔdegQ1* cells could be capable of binding to substrates or forming complexes with other proteases via its intact PDZ domain, but be incapable of carrying out proteolysis. Substrates normally degraded by DegQ/HhoA would accumulate, leading to possible induction of the stress response. Such substrates might not be expected to accumulate in cells truly lacking DegQ/HhoA as other proteases which show similar substrate specificity may substitute for DegQ/HhoA under these conditions.

DegS/HhoB is an essential protease

Cells lacking DegS/HhoB show an obvious slow-growth phenotype and appear to accumulate rapidly-growing suppressors at a high frequency (Waller and Sauer, 1996). In fact, one group was unable to construct strains lacking this protease (Bass et al., 1996). In addition, curing a pseudo-diploid *ΔdegS1* strain of a plasmid encoding wild type *degS* leads to a mixture of cells showing widely divergent phenotypes (Alba, Connolly and Gross, unpublished observations). These observations suggest that DegS/HhoB may be an essential protease.

To determine whether *degS* is an essential gene, we compared transduction frequencies of *ΔdegS1* with a tightly linked marker, *argR*::Tn*5* [Tian, 1994 #322], in haploid recipients and in recipients carrying a plasmid containing a wild type copy of *degS*, hereafter referred to as the p*degS* strain. *argR*::Tn*5* is predicted to be > 90% linked to *degS*. If *degS* is not essential, then both strains should show equivalent, high cotransduction of *ΔdegS1* and *argR*::Tn*5*. If *degS* is essential, then only the p*degS* strain which covers the missing locus with a plasmid, should show the appropriate linkage. We observe the latter result. p*degS* cells exhibit a 91% linkage between the two markers (Table 5-2, line 2), whereas the haploid cells gave rise to very few Arg- cells with no linkage to *ΔdegS1* (Table 5-2, line 1). The reduced number of kanamycin-resistant, Arg- transductants in haploid cells is not due to a reduction in transduction competence. A control transduction using the P1 donor *zhd3171*::Tn*10*Km (Singer et al., 1989) gave rise to similar numbers of kanamycin-resistant transductants in both strains (Table 5-2). The most probable explanation for these results is that *degS* is essential for cellular growth.

Deletion of *degS* leads to a decrease in σ^E activity

To determine the effect of the *ΔdegS1*1 allele on σ^E and Cpx activity, we created *ΔdegS1* strains by curing a *ΔdegS1* strain of a plasmid encoding wild type *degS* (see Experimental Procedures). To minimize the possibly misleading effects of suppressor mutations, two

independent stable isolates of each strain were chosen for further study. This analysis revealed a 5-fold decrease in σ^E activity in strains lacking DegS/HhoB (Fig. 5-3a). Analysis of similar strains constructed with the Cpx reporter showed that deletion of *degS* had no effect on Cpx activity (Fig. 5-3b), suggesting that DegS/HhoB may play a unique role in the σ^E-mediated response.

The small amount of σ^E activity observed in the *ΔdegS1* strains also shows a loss of growth rate regulation normally observed in wt cells. To determine whether the alteration in σ^E activity observed was due to a suppressor mutation rather than a loss of *degS* function, we asked whether a plasmid encoding DegS/HhoB could restore wild type σ^E activity to these cells. The *degS* plasmid was capable of fully complementing both the decrease in σ^E activity and the loss of growth rate regulation of this activity, indicating that the loss of *degS* is responsible for the observed effects on σ^E activity (Fig. 5-3c).

Is the protease activity of DegS/HhoB required for wt σ^E activity?

To determine whether the protease activity of DegS/HhoB is required for σ^E function, we asked whether an active site mutant could complement the σ^E defect seen in *ΔdegS1* cells. Introduction of an Ala residue at the putative active site Ser of DegS/HhoB on a plasmid revealed that this mutant, *degS* S201A, failed to complement the loss of σ^E activity observed in *ΔdegS1* cells (Fig. 5-3c). These results indicate that the protease activity of DegS/HhoB is required for wild type σ^E activity, but do not rule out the possibility that DegS/HhoB may possess other important biochemical activities.

Deletion of both *degQ* and *degS* strongly induces the σ^E and Cpx pathways

degQ and *degS* are located next to each other at minute 72 of the *E. coli* chromosome (Bass et al., 1996; Waller and Sauer, 1996). Although several lines of evidence suggest that the two genes are transcribed and translated separately (Waller and Sauer, 1996), the close proximity of these genes and the existence of a single transcript encoding both proteases

suggests that their expression may be coordinately regulated under some conditions. Deletion of *degQ* and *degS* led to strong induction of both the σ^E and Cpx pathways, the σ^E pathway showing an 8.5-fold induction and the Cpx pathway an astounding 230-fold induction (Fig. 5-4). Although these results should be interpreted with caution due to the high likelihood that the double mutant strains contain suppressors of *degS*$^-$ lethality, they raise the possibility that σ^E and Cpx pathways could be sensing the same signal under these conditions or that deletion of both proteases generates multiple signals. Alternatively, one pathway could be activated as a consequence of activation of the other. For example, the extreme level of activation of the Cpx pathway in these double mutant cells could lead to a folding imbalance in the periplasm that in turn would activate the σ^E pathway. Consistent with the latter hypothesis is the observation that the levels of the regulators of σ^E, RseA and B, become drastically reduced in the *Δ(degQ-deg)1* strain, leading to an alteration in the Rse:σ^E ratio and a large induction of σ^E activity (Fig. 5-5a).

One possibility is that induction of the Cpx pathway and/or deletion of the two Deg proteases leads to activation of other proteolytic activities in the periplasm, causing degradation of the Rse proteins and induction of σ^E. Similar decreases in the levels of the Rse proteins are not observed under other induction conditions such as overexpression of OmpX in which the Cpx pathway is not induced (Fig. 5-5b). To ask whether activation of the Cpx pathway in the *Δ(degQ-degS)1* strain is leading to activation of the σ^E pathway, we introduced a *cpxR*::Ω allele into this strain and assayed the effects on σ^E activity . Deletion of the Cpx pathway in *Δ(degQ-degS)1* cells had no effect on σ^E activity, indicating that induction of σ^E is not occurring as a result of activation of the Cpx pathway under these conditions (Fig. 5-4a). Similarly, introduction of a *Δprc3*::Km allele did not alter σ^E activity indicating that Tsp/Prc is not responsible for degrading the Rse proteins and inducing the σ^E pathway (Fig. 5-4a). Not surprisingly, deletion of the third Deg protease, DegP, led to a further increase in σ^E activity (Fig. 5-4a). These results suggest that DegP is also not degrading the Rse proteins under these conditions.

Discussion

In this work, we attempt to clarify the potential roles of several extracytoplasmic proteases in the σ^E and Cpx mediated stress responses. Of the five proteases studied, our data suggests that only the inner membrane protease DegS/HhoB plays a unique regulatory role in signal transduction to σ^E and we present evidence that this protease is essential for viability. DegS/HhoB is only the second protease, and the first extracytoplasmic protease, found to be essential for cellular growth in *E. coli.*

The role of proteolysis in the regulation of extracytoplasmic stress

Proteases play two essential cellular roles. They are involved in the removal of misfolded or nonfunctional proteins under both steady state and stress conditions, and also play a role in controlling the activity of key regulatory molecules in the cell (Gottesman and Maurizi, 1992; Gottesman, 1996). Several proteases contribute to the turnover of abnormal cytoplasmic proteins (Gottesman and Maurizi, 1992; Gottesman, 1996; Miller, 1996), and not surprisingly, our results support the idea that multiple proteases also play a role in ridding the cell of abnormal extracytoplasmic proteins. Deletions of single proteases (DegP, Prc/Tsp, OmpT, DegQ/HhoA) do not greatly alter the stress response, indicating that their ability to degrade nonfunctional or misfolded protein probably overlaps, and only upon deletion of several proteases will these substrates accumulate. In fact, the substrate specificity of DegP and DegQ/HhoA, at least *in vitro*, are almost identical (Kolmar et al., 1996) and overexpression of DegQ/HhoA suppresses the temperature sensitive phenotype of cells lacking either DegP or Prc/Tsp (Bass et al., 1996; Waller and Sauer, 1996). These findings do not suggest that these proteases are equivalent under all growth conditions. For example, the extreme temperature sensitivity of cells lacking DegP underscore the importance of this protease in ridding the cell of abnormal protein generated at high temperature (Lipinska et al., 1989; Strauch et al., 1989).

In addition to their role in degrading abnormal proteins, several proteases can also act as regulatory molecules by determining the stability of key regulatory proteins (Gottesman and Maurizi, 1992; Gottesman, 1996). For example, the essential inner membrane protease HflB rids the cell of uncomplexed inner membrane proteins (Kihara et al., 1995; Akiyama et al., 1996) and is also the main protease responsible for the regulated degradation of the heat shock factor, σ^{32} (Herman et al., 1995; Tomoyasu et al., 1995). Our results support both a housekeeping and a regulatory role for the second essential inner membrane protease, DegS/HhoB. Deletion of DegS/HhoB alone leads to a decrease in σ^E activity, without affecting the Cpx pathway, suggesting a regulatory role for this protease in signal transduction to σ^E. Deletion of DegQ/HhoA leads to a slight induction of the Cpx pathway, and removal of DegS/HhoB from these cells leads both to further induction of Cpx activity and induction of σ^E. Although these results should be interpreted with caution as the effects of possible suppressor mutations in the double mutant strains have not been assessed, they suggest that DegQ/HhoA and DegS/HhoB might normally be involved in the turnover of an overlapping set of abnormal substrate molecules whose accumulation in the double mutant leads to the activation of both signal transduction pathways.

Why is DegS/HhoB essential?

DegS/HhoB is an inner membrane serine protease of 355 residues including conserved His, Ser and Asp residues of the classical catalytic triad and a single PDZ domain (Waller and Sauer, 1996; Pallen and Ponting, 1997; Pallen and Wren, 1997). Both the catalytic core and PDZ domain are predicted to reside in the periplasmic space. Here we show that DegS/HhoB is an essential protease and that DegS/HhoB activity is required for the normal steady state activity of σ^E.

σ^E itself is an essential sigma factor and cells lacking this sigma rapidly accumulate an unlinked suppressor mutation or mutations that allow the cell to grow (De Las Peñas et

al., 1997a). The essential role of DegS/HhoB may simply be to maintain adequate levels of σ^E activity. The recent observation that the *ΔdegS1* allele cotransduces with *argR*::Tn*5* with higher frequency into cells containing the suppressor of *rpoE* lethality versus wild type cells (~40% vs. 0%; Alba and Gross, unpublished data) supports this idea.

In addition to the σ^E phenotype, cells lacking DegS/HhoB fail to grow in minimal media (M9) (Connolly and Gross, unpublished observation). This phenotype can be complemented by plasmids encoding *degS* indicating that it is due to a loss of DegS/HhoB activity (Connolly and Gross, unpublished data). The osmolarity of M9 is double that of LB, a media in which *ΔdegS1* strains grow, and such strains have recently been cultured successfully in 0.5X M9 (Alba and Gross, unpublished results). This result suggests that cells lacking DegS/HhoB are salt sensitive. Introduction of a plasmid encoding σ^E into *ΔdegS1* cells does not restore the ability to grow in minimal media (Connolly and Gross, unpublished data), suggesting that the σ^E and salt-sensitive phenotypes are unrelated. This observation indicates that in addition to its role in the maintenance of σ^E activity, DegS/HhoB plays a role in the cellular response to salt and it is unclear which, if either or both, of these roles is essential.

The role of DegS/HhoB in signal transduction to σ^E

Several lines of evidence suggest that DegS/HhoB may play a direct role in signal transduction to σ^E. The activity of the central regulator of σ^E, RseA, has recently been shown to be regulated by stability (Ades and Gross, unpublished data). RseA is slightly unstable under steady state growth conditions, and the half-life of RseA decreases dramatically immediately upon exposure to elevated temperature or outer membrane protein overproduction. In addition, RseA stability is somewhat decreased in cells showing high steady state levels of σ^E activity suggesting that, at least in part, the half-life of RseA determines the levels of σ^E activity in the cell even under steady state conditions.

Deletion of the protease responsible for degrading RseA is predicted to stabilize RseA and lead to a decrease in σ^E activity. This phenotype is observed in cells lacking DegS/HhoB and not in cells lacking other extracytoplasmic proteases. Cells lacking the protease responsible for RseA degradation should be uninducible for σ^E and conditions normally inducing this response may prove lethal to such cells. Introduction of a plasmid encoding the outer membrane protein OmpX is lethal to *ΔdegS1* cells (Connolly and Gross, unpublished observation). While these findings support the notion that DegS/HhoB is responsible for the regulated degradation of RseA, direct studies such as the analysis of RseA stability in cells lacking or overproducing DegS/HhoB and the demonstration of a physical interaction between the two proteins need to be done.

Data presented in Figures 5-4 and 5-5 suggest that even if DegS is the main protease responsible for the degradation of RseA, it may not be the only one capable of degrading this regulator. Cells doubly mutant for DegQ/HhoA and DegS/HhoB show dramatically reduced levels of RseA and RseB while maintaining approximately normal σ^E levels. Because the Rse proteins are encoded within the same operon as σ^E (De Las Peñas et al., 1997b; Missiakas et al., 1997), it is unlikely their decrease observed in *Δ(degQ-degS)1* cells is ocurring at the transcriptional level. Although we cannot rule out the possibility that the decrease in RseA and B is occurring at the level of transcript processing or translation, it seems likely that the two proteins are being degraded. Such dramatic degradation of RseA and B is not observed under other steady state induction conditions leading to similar levels of σ^E activity (Fig. 5-5b), suggesting either that the normal signal transduction cascade is not operational in cells lacking DegS/HhoB and DegQ/HhoA or that more than one mechanism for activating this pathway exists. Attempts to complement the σ^E phenotype with plasmids encoding various combinations of DegQ/HhoA and DegS/HhoB will help to determine whether the observed effects on σ^E and its regulators are truly due to the loss of these proteases or are due to undefined suppressor mutations in these strains.

Rather than being responsible for RseA degradation, DegS/HhoB may play an indirect role in regulating σ^E activity by regulating the level of extracytoplasmic signal. σ^E activity is reduced in cells with decreased OMP content (Mecsas et al., 1993), and deletion of DegS/HhoB may decrease OMP levels by altering the activity of positive regulators of OMP expression or may allow the escape of OMP precursors into the extracellular media by altering the permeability of the outer membrane. Characterization of OMP expression and outer membrane permeability in cells lacking DegS/HhoB may help to clarify the role this protease plays in modulating σ^E activity.

Experimental Procedures

Media, reagents, and enzymes

Luria-Bertani (LB) and M9 minimal medium were prepared as described (Sambrook et al., 1989). M9 was supplemented with 0.2% glucose, 1 mM $MgSO_4$, 2 ug/ml thiamine, and all amino acids (40 ug/ml). Where needed, media was supplemented with 30 ug/ml kanamycin (Km), 20 ug/ml chloramphenicol (Cm), 100 ug/ml ampicillin (Ap), or 100 ug/ml L-canavanine.

Strains

Bacterial strains used in this study are described in Table 1. Their construction is briefly described below.

ompT::Km, Δ*prc3*::Km, *degP*::Km, and *cpxR*::Ω alleles were moved by P1 transduction as previously described (Miller, 1972).

Δ*degQ11*, *degQ2*::Km and Δ(*degQ-degS)1* strains carrying σ^E or Cpx dependent reporter genes were created by lysogenizing PW147, PW152, and PW148 with ϕλ[*rpoHP3-lacZ*] (Mecsas et al., 1993) or ϕλRS88[*cpxP-lacZ*] (Jones et al., 1997a).

Δ*degS11* strains carrying the σ^E or Cpx dependent reporter genes ϕλ[*rpoHP3-lacZ*] and ϕλRS88[*cpxP-lacZ*], respectively (CAG33315 and CAG33403) were constructed in two steps. PW149was first lysogenized with each reporter, creating CAG33304 and CAG33400. CAG33304 and CAG33400 were then cured of the *degS*-encoding plasmid pPW142 which contains a temperature-sensitve origin of replication by the following procedure: CAG33304 or CAG33400 was grown overnight at 37°C in LB and the resulting culture plated on LB at 30°C for single colonies. Single colonies were then screened for plasmid loss (Cm-sensitivity) at 30°C on LB Cm plates. Cm sensitve colonies were then screened by PCR to confirm loss of the wild type allele of *degS*. Two independent isolates of each strain were retained for further study.

Plasmids

Plasmids used in this study are listed in Table 5-1. pLC259 was created by polymerase chain reaction (PCR) amplification of the *degS* gene and its promoter from MC1061 chromosomal DNA using Pfu polymerase and the primers DEGS74 (5' GCTCTAGATGT CGTAAACCGGGCATCAGG) and DEGS75 (5' GGGGTACCGAGCGCACGACTT AATTGGTTG). The resulting fragments were then cut at restriction sites (indicated by underlining) and cloned into the corresponding sites of the general cloning vector pSU21 (Bartolomé et al., 1991). pLC261 was constructed by creating a point mutation at codon 201 of *degS*, altering Ser 201 to Ala. Primer pairs DEGS78 (5' CCACGGTAACGCTGG CGGCGC)/DEGS69 (5' CAGTTGATCTATACCACCGC) and DEGS79 (5' GCGCC GCCAGCGTTACCGTGG)/DEGS68 (5' TTGACAGTACCGATGAGACG) were amplified from pLC259, creating two separate PCR fragments containing the point mutation. These fragments were then joined by PCR using primers DEGS69 and DEGS68. A 370 bp BamHI-BssHII fragment containing the point mutation was then subcloned from the joined PCR fragment back into pLC259 to create pLC261. The point putation creates a MwoI restriction site, and the presence of the mutation was confirmed by restriction digest. LC239 was created by removing a 1.5 kb BglI fragment encoding *lepA* from pAP131.

β-galactosidase assays

σ^E activity was assayed by monitoring β-galactosidase activity from a chromosomal σ^E-dependent *lacZ* reporter gene in Φλ[*rpoHP3-lacZ*] (Mecsas et al., 1993)and Cpx activity was assayed by monitoring β-galactosidase activity from a chromosomal CpxR-dependent *lacZ* reporter gene in ΦλRS88[*cpxP::lacZ*] (Jones et al., 1997a) as previously described (Miller, 1972; Mecsas et al., 1993). Cells to be assayed were grown at 30°C in M9 or LB media and single point determinations were made at the indicated times. Fold effects were

determined by comparing the initial slopes of curves derived from wild-type or mutant cells.

Detection of σ^E, RseA, and RseB by Western Blot

15 mls of LB were inoculated with 150 ul of a fresh overnight of the desired strains. Cells were grown at 30 °C to an OD_{600} of 0.5-0.6, and 0.9 mls of culture were sampled directly into 0.1 ml of 50% TCA on ice, vortexed, and placed on ice for 30 minutes. TCA precipitates were pelleted at 17,500 x g for 30 minutes at 4°C. The pellets were resuspended in 1X protein sample buffer (50 mM Tris-Cl pH 6.8, 100 mM DTT, 2% SDS, 0.2% bromophenol blue) to a concentration of 10^7 cells/ul. 10^8 cells were run on 10% SDS-PAGE in duplicate and transferred to a nitrocellulose membrane. The membrane was blocked overnight at room temperature with 3% non-fat dry milk in TBST (10 mM Tris pH 8.0, 150 mM NaCl, 0.2 % Tween-20) and probed with a mixture of anti-σ^E, anti-RseA, anti-RseB and anti-MBP (for loading control purposes) polyclonal antisera at 1:10,000 dilutions in TBST plus 1% milk for one hour at room temperature. The blot was washed three times for five minutes each with TBST, then incubated with 1:10,000 dilution of sheep anti-rabbit POD (Boehringer Mannheim, Indianapolis, IN) for one hour at room temperature, and washed again as before. It was then developed with enhanced chemiluminescence (ECL) reagents (Amersham, Arlington Heights, IL) and Hyperfilm ECL (Amersham, Arlington Heights, IL) and exposed to film from five to thirty seconds.

Acknowledgements

We thank Eric Roche and Bob Sauer for sharing strains and plasmids and communicating unpublished results. This work was supported by the Public Service Grant GM36278 from the National Institute of Health. LC was also supported by the UCSF MSTP GM07618.

Table 5-1. Strains and plasmids used in this work

Strain/Plasmid	Relevant Genotype	Source/Reference
Strain		
MC1061	*araD* Δ(*ara-leu*)*7697* Δ*lacX74 galU galK hsr hsn strA*	(Casadaban and Cohen, 1980)
CAG16037	MC1061 Φλ[*rpoHP3-lacZ*]	(Mecsas et al., 1993)
CAG16237	CAG16037 *degP*::Km	(Mecsas et al., 1993)
EK1181	MC4100 *ompT*::Km Δ*ftsH*	(Kihara et al., 1996)
CAG33263	CAG16037 *ompT*::Km	This work
KS1000	X90 Δ*prc3*::Km *eda-51*::Tn*10*	(Silber and Sauer, 1994)
CAG33314	CAG16037 Δ*prc3*::Km	This work
PW147	MC1061 Δ*degQ1*	(Waller and Sauer, 1996)
CAG33303	PW147 Φλ[*rpoHP3-lacZ*]	This work
PW149	MC1061 Δ*degS1*, pPW142	(Waller and Sauer, 1996)
CAG33304	Δ*degS1*	This work
CAG33315	CAG33304 Φλ[*rpoHP3-lacZ*]	This work
PW148	MC1061 Δ(*degQ-degS*)*1*	(Waller and Sauer, 1996)
CAG33336	PW148 Φλ[*rpoHP3-lacZ*]	This work
PW152	MC1061 *degQ2*::Km	(Waller and Sauer, 1996)
CAG33397	PW152 Φλ[*rpoHP3-lacZ*]	This work
SP594	MC4100 ΦλRS88[*cpxP*::*lacZ*]	(Jones et al., 1997a)
CAG33398	MC1061 ΦλRS88[*cpxP*::*lacZ*]	This work
CAG43024	CAG33398 *degP*::Km	This work
CAG43026	CAG33398 *ompT*::Km	This work
CAG43027	CAG33398 Δ*prc3*::Km	This work
CAG33399	CAG33398 Δ*degQ1*	This work
CAG33401	CAG33398 Δ(*degQ-degS*)*1*	This work
CAG33402	CAG33398 *degQ2*::Km	This work
CAG43020	CAG33336 *cpxR*::ΩSp	This work
CAG43037	CAG33336 *degP*::Km	This work
CAG43039	CAG33336 Δ*prc3*::Km	This work
CAG22935	CAG16037, pAP9	(De Las Peñas et al., 1997b)
CAG16141	CAG16037, pJE100	(Mecsas et al., 1993)
CAG22309	CAG16037, pACYC177	This work
CAG33285	CAG16037, pLC239	This work
CAG33330	CAG16037, pSU21	This work
CAG33333	CAG16037, pLC259	This work
CAG33391	CAG33315, pPLT13, pSU21	This work
CAG33387	CAG33315, pPLT13, pLC259	This work
CAG33393	CAG33315, pPLT13, pLC261	This work
CAG33385	CAG16037, pPLT13, pSU21	This work

Table 5-1, cont. Strains and plasmids used in this work

Strain/Plasmid	Relevant Genotype	Source/Reference
Plasmid		
pAP9	pBR322 ΔEcoRV/MscI, Ap	(De Las Peñas et al., 1997b)
pJE100	*ompX* in pBR322, Ap	(Mecsas et al., 1993)
pACYC177	cloning vector, p15A ori, Km, Ap	(Rose, 1988)
pLC239	*rpoE* operon in pACYC184, Ap	This work
pSU21	cloning vector, p15A ori, Cm	(Bartolomé et al., 1991)
pPLT13	MiniF carrying *lacI*q, Km	(Tavormina et al., 1996)
pLC259	*degS* in pSU21, Cm	This work
pLC261	*degS* S201A in pSU21, Cm	This work
pPW142	*degS* in pMAK705, Ts pSC101 ori, Cm	(Waller and Sauer, 1996)

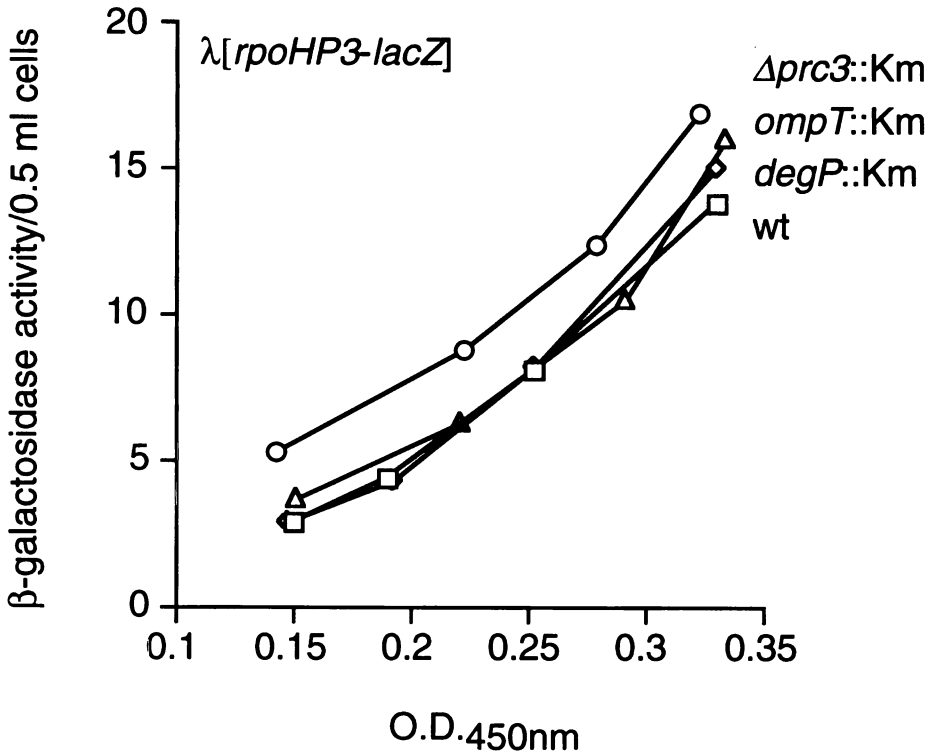
Table 5-2. Cotransduction of Δ*degS* with *argR*::Tn*5*

	# Colonies P1 (*argR*::Tn*5* Δ*degS*)				# Colonies P1 (*zhd3171*::Tn*10*Km)
Recipient	Km^R	L-canavanine^R	Δ*degS1*	% linkage	Km^R
Haploid	20	7	0	0	83
p*degS*	40	35	32	91	62

Table 5-2. Haploid or p*degS* recipient cells were crossed with the P1 donor (*ΔdegS1 argR::*Tn*5)*, and *argR::* Tn*5* transductants were selected on 30 μg/ml Km at 30°C. Km^R colonies represent a mixture of *argR*::Tn*5* transductants and random Tn*5* hops, and true Arg- cells were identified by screening on 100 μg/ml L-canavanine (Tian and Maas, 1994). L-canavanine resistant colonies were then screened by PCR to identify Δ*degS1* cotransductants. % linkage represents the number of Δ*degS1* colonies identified by PCR divided by the total number of L-canavanine resistant colonies screened. The P1 donor *zhd*3171::Tn*10*Km was used to control for the transduction competence of each strain. Transductions were performed as previously described (Miller, 1972).

Figure 5-1. σ^E and Cpx activity in strains lacking the extracytoplasmic proteases DegP, Prc/Tsp or OmpT. (a) σ^E activity was assayed by monitoring β-galactosidase activity produced from a single copy Φλ[*rpoHP3-lacZ*] fusion in either wild type (□) or isogenic mutant strains carrying *degP*::Km (◊), *Δprc3*::Km (○) or *ompT*::Km (Δ). Cell density was measured at O.D.$_{450}$ because the cells were grown in M9 media. (b) Cpx activity was assayed by monitoring β-galactosidase activity produced from the CpxR-dependent reporter ΦλRS88[*cpxP-lacZ*] in either wild type (□) or isogenic mutant strains carrying *degP*::Km (◊), *Δprc3*::Km (○) or *ompT*::Km (Δ). Cell density was measured at O.D.$_{600}$ because the cells were grown in LB media.

a


λ[rpoHP3-lacZ]
Δprc3::Km
ompT::Km
degP::Km
wt
β-galactosidase activity/0.5 ml cells
20
15
10
5
0
0.1
0.15
0.2
0.25
0.3
0.35
O.D.450nm

b

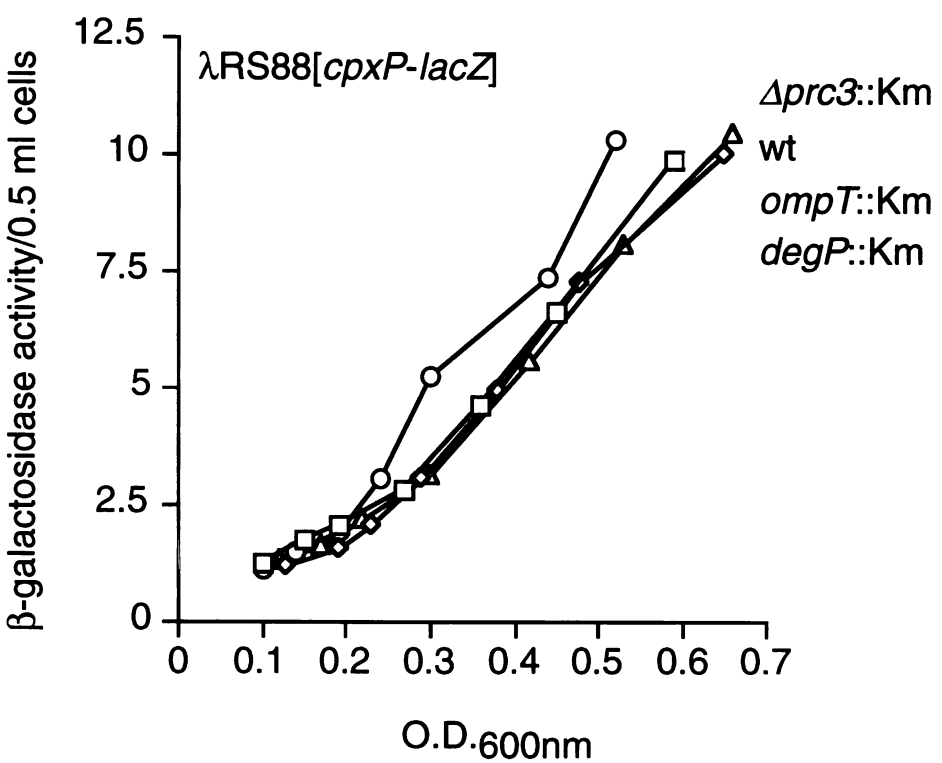
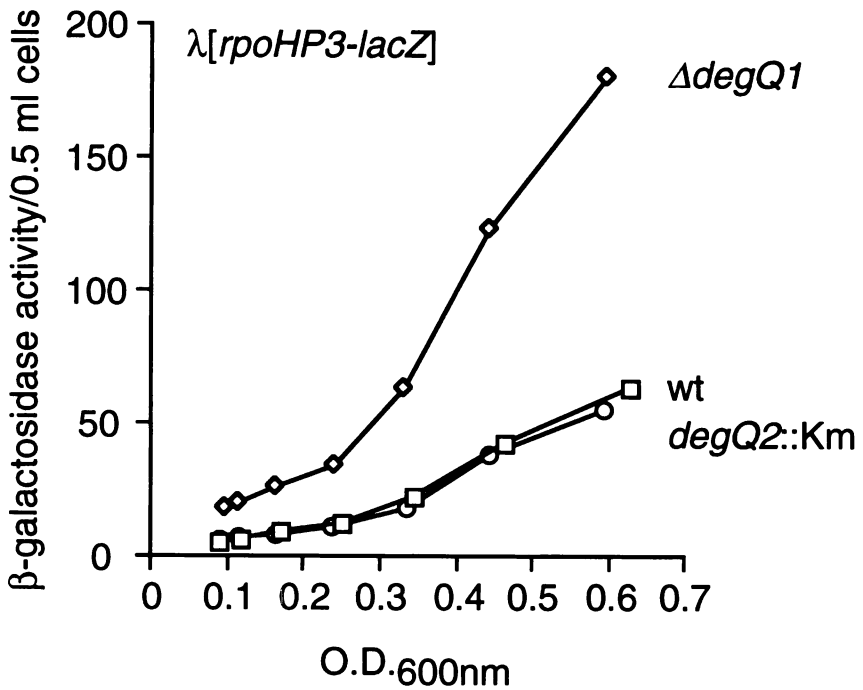

λRS88[cpxP-lacZ]
Δprc3::Km
wt
ompT::Km
degP::Km
β-galactosidase activity/0.5 ml cells
12.5
10
7.5
5
2.5
0
0
0.1
0.2
0.3
0.4
0.5
0.6
0.7
O.D.600nm

Figure 5-2. σ^E and Cpx activity in strains carrying mutations in *degQ*. (a) σ^E activity was assayed by monitoring β-galactosidase activity produced from a single copy Φλ[*rpoHP3-lacZ*] fusion in either wild type (□) or mutant strains carrying a *ΔdegQ1* (◊) or *degQ2*::Km (○) allele. (b) Cpx activity was assayed by monitoring β-galactosidase activity produced from a single copy ΦλRS88[*cpxP-lacZ*] fusion in either wild type (□) or mutant strains carrying a *ΔdegQ1* (◊) or *degQ2*::Km (○) allele.

a


λ[rpoHP3-lacZ]
ΔdegQ1
wt
degQ2::Km
β-galactosidase activity/0.5 ml cells
200
150
100
50
0
0 0.1 0.2 0.3 0.4 0.5 0.6 0.7
O.D.600nm

b

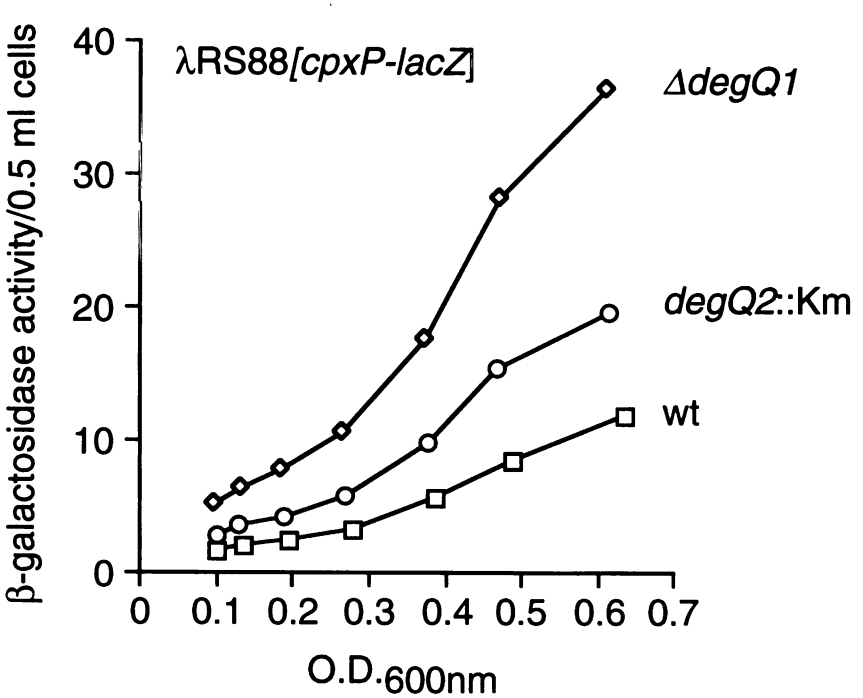

λRS88[cpxP-lacZ]
ΔdegQ1
degQ2::Km
wt
β-galactosidase activity/0.5 ml cells
40
30
20
10
0
0 0.1 0.2 0.3 0.4 0.5 0.6 0.7
O.D.600nm

Figure 5-3. σ^E and Cpx activity in strains carrying the *ΔdegS1* mutation. (a) σ^E activity was assayed by monitoring β-galactosidase activity produced from a single copy Φλ[*rpoHP3-lacZ*] fusion in either wild type (□) or two independent isolates of a *ΔdegS1* strain (○ and ◊). (b) Cpx activity was assayed by monitoring β-galactosidase activity produced from a single copy ΦλRS88[*cpxP-lacZ*] fusion in either wild type (□) or two independent isolates of a *ΔdegS1* strain (○ and ◊). (c) σ^E activity was assayed by monitoring β-galactosidase activity produced from the σ^E-dependent reporter Φλ[*rpoHP3-lacZ*] in wild type cells carrying vector alone (□), *ΔdegS1* cells carrying vector alone (◊), *ΔdegS1* cells carrying a plasmid encoding wild type *degS* (○) and *ΔdegS1* cells carrying a plasmid encoding the *degS* mutant S201A (Δ).

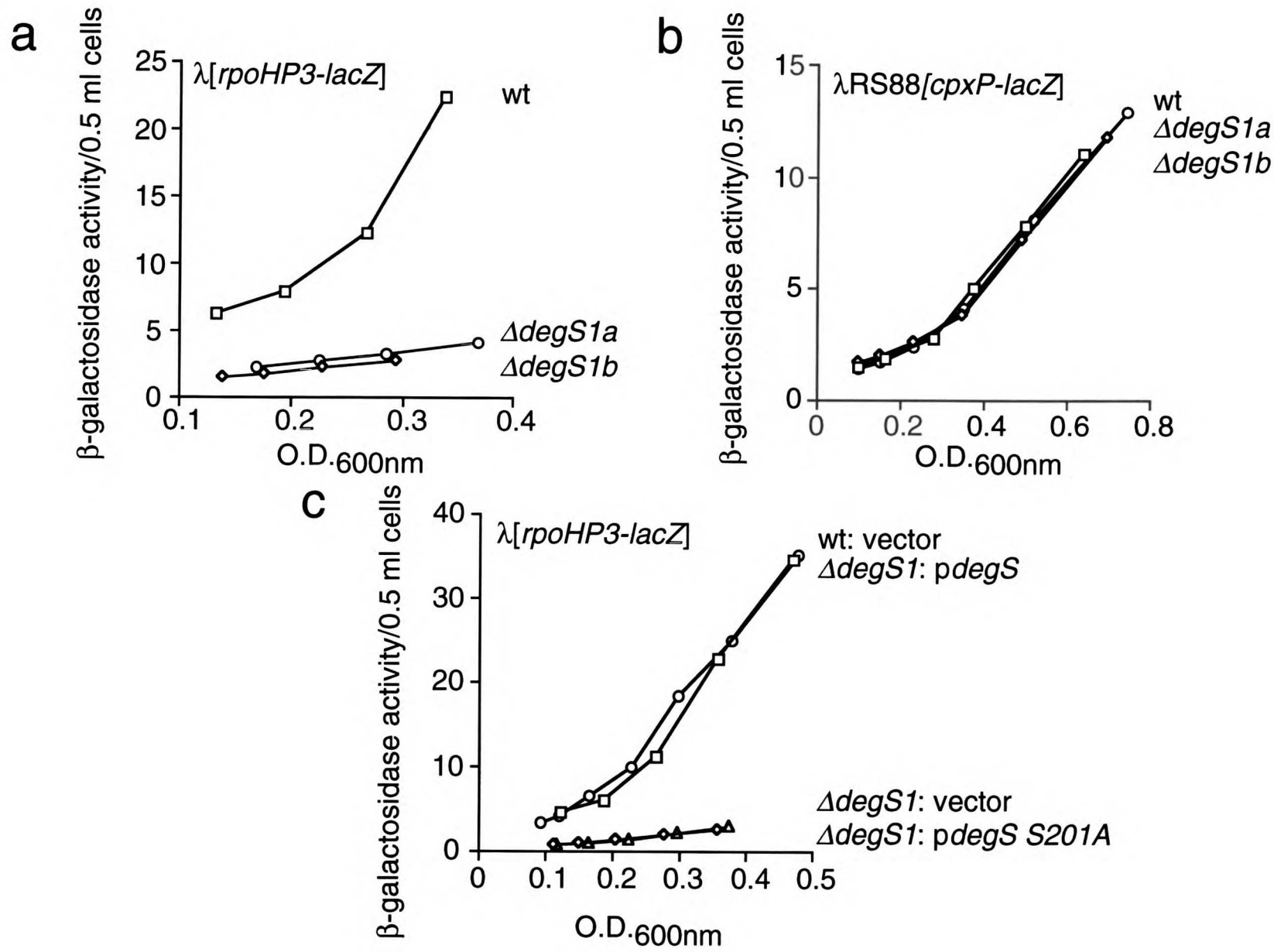
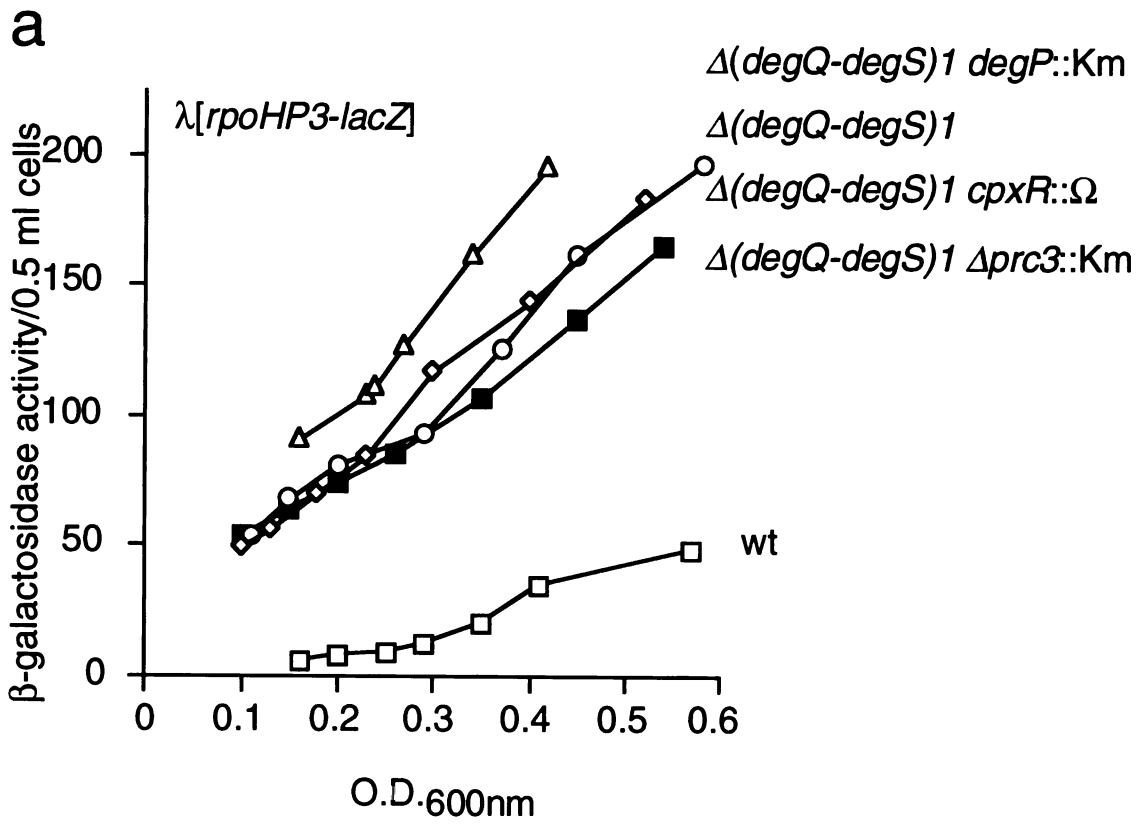

a
β-galactosidase activity/0.5 ml cells
λ[rpoHP3-lacZ]
wt
ΔdegS1a
ΔdegS1b
O.D.600nm
b
β-galactosidase activity/0.5 ml cells
λRS88[cpxP-lacZ]
wt
ΔdegS1a
ΔdegS1b
O.D.600nm
c
β-galactosidase activity/0.5 ml cells
λ[rpoHP3-lacZ]
wt: vector
ΔdegS1: pdegS
ΔdegS1: vector
ΔdegS1: pdegS S201A
O.D.600nm

Figure 5-4. σ^E and Cpx activity in cells carrying a *Δ(degQ-degS)1* double mutation. (a) σ^E activity was assayed by monitoring β-galactosidase activity produced from the σ^E-dependent reporter Φλ[*rpoHP3-lacZ*] in wild type (□), *Δ(degQ-degS)1* (◊), *Δ(degQ-degS)1 cpxR*::Ω (○), *Δ(degQ-degS)1 degP*::Km (Δ) and *Δ(degQ-degS)1 Δprc3*::Km (■) strains. (b) Cpx activity was assayed by monitoring β-galactosidase activity produced from a single copy ΦλRS88[*cpxP-lacZ*] fusion in wild type (□) and *Δ(degQ-degS)1* (◊) strains. Note that due to the extreme activation of the Cpx pathway in *Δ(degQ-degS)1* cells the data is plotted on a log scale in panel (b).


a
λ[rpoHP3-lacZ]
Δ(degQ-degS)1 degP::Km
Δ(degQ-degS)1
Δ(degQ-degS)1 cpxR::Ω
Δ(degQ-degS)1 Δprc3::Km
wt
β-galactosidase activity/0.5 ml cells
0
50
100
150
200
0
0.1
0.2
0.3
0.4
0.5
0.6
O.D.600nm

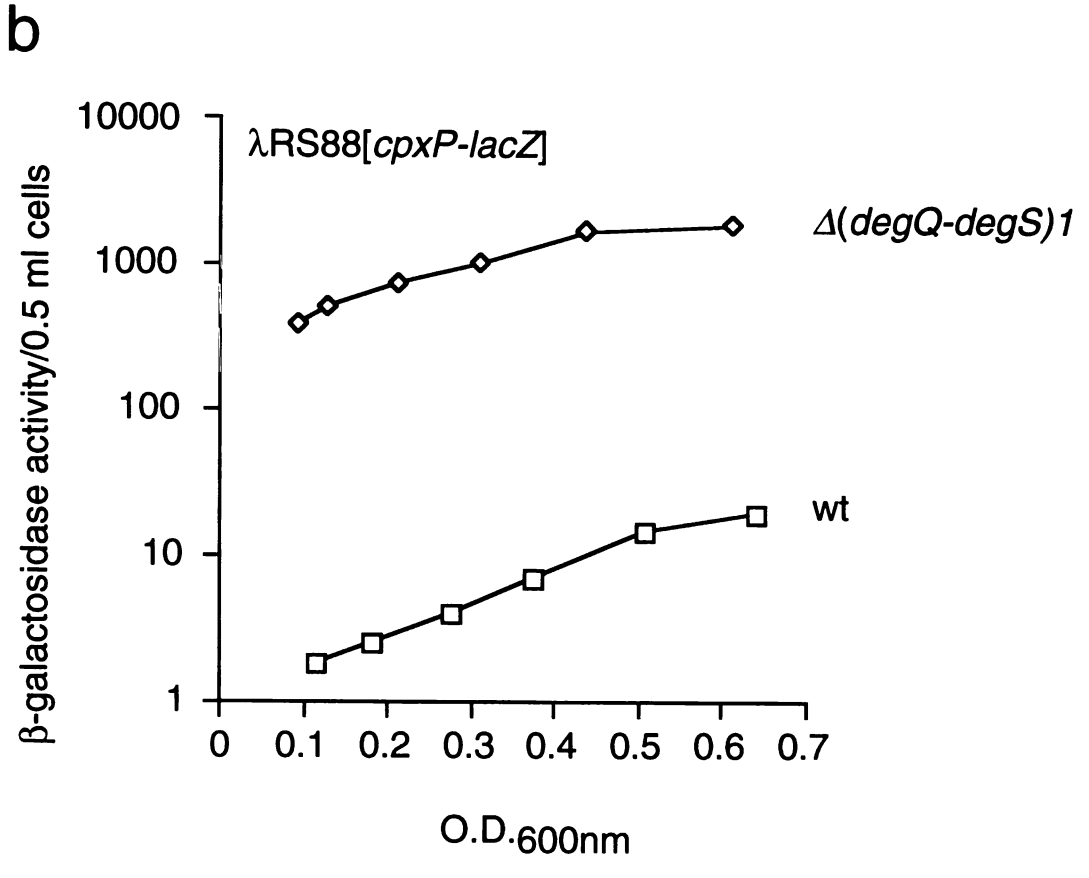
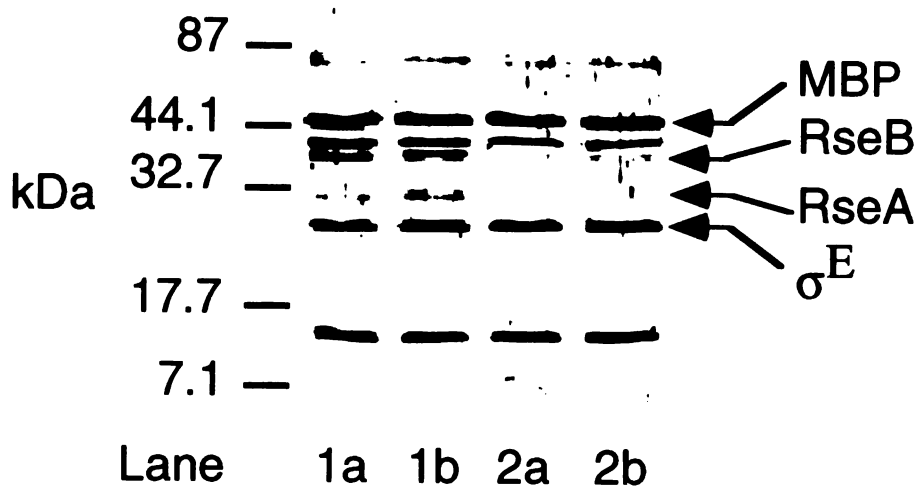
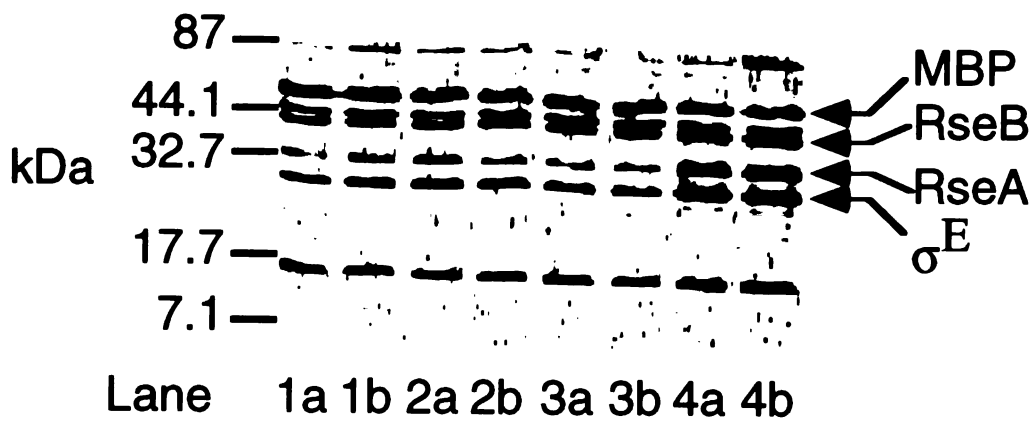

b
λRS88[cpxP-lacZ]
Δ(degQ-degS)1
wt
β-galactosidase activity/0.5 ml cells
1
10
100
1000
10000
0
0.1
0.2
0.3
0.4
0.5
0.6
0.7
O.D.600nm

Figure 5-5. Western blot analysis of σ^E, RseA and RseB levels in wild type, *Δ(degQ-degS)1* and p*ompX* cells. (a) σ^E, RseA, and RseB levels in wild type and *Δ(degQ-degS)1* cells. Wild type (lane 1) and *Δ(degQ-degS)1* (lane 2) cells were grown in LB at 30°C to mid-log phase and sampled. The steady state levels of σ^E, RseA, and RseB were determined in duplicate by Western blot analysis as described in Experimental Procedures. As a loading control, the blot was also probed with antibodies against maltose-binding protein (MBP). (b) σ^E, RseA, and RseB levels in cells induced for σ^E by overexpression of OmpX. Cells containing vector alone (pBR322; lanes 1a and 1b) or a plasmid encoding *ompX* (lanes 2a and 2b) were grown in LB at 30°C to mid-log phase and sampled. To ensure that antibody levels were saturating, cells containing vector alone (pACYC177; lanes 3a nd 3b) or an *rpoE* operon plasmid (lanes 4a and 4b) were also analyzed. The steady state levels of σ^E, RseA, and RseB were determined by Western blot analysis as described in Experimental Procedures. As a loading control, the blot was also probed with antibodies against maltose-binding protein (MBP).

a


87
44.1
32.7
17.7
7.1
kDa
MBP
RseB
RseA
σE
Lane 1a 1b 2a 2b

b


87
44.1
32.7
17.7
7.1
kDa
MBP
RseB
RseA
σE
Lane 1a 1b 2a 2b 3a 3b 4a 4b

Chapter Six

Summary and Conclusions

Exposure of both prokaryotic and eukaryotic cells to environmental conditions that lead to the accumulation of misfolded or unfolded protein induces the expression of a highly conserved set of proteins that act to combat the accumulation of denatured proteins by stabilizing, refolding or degrading them (Bukau, 1993; Yura et al., 1993; Becker and Craig, 1994; Georgopoulos et al., 1994; Gross, 1996). In the Gram-negative bacterium *Escherichia coli* this regulatory response is compartmentalized into cytoplasmic and extracytoplasmic responses. The extracytoplasmic stress response is controlled by two partially overlapping pathways, the σ^E-mediated response and the two-component Cpx system (Erickson and Gross, 1989; Mecsas et al., 1993; Danese et al., 1995; Connolly et al., 1997). In this thesis, I have presented evidence that the σ^E-mediated pathway is essential not only in times of stress but also under steady state growth conditions (Chapter 3). I have described the discovery of several proteins involved in signal transduction to σ^E, and have begun to elucidate the mechanism by which the periplasmic accumulation of misfolded protein is signaled to σ^E (Chapters 2 and 5). I have detailed the relationship between σ^E and the second extracytoplasmic stress response pathway, the Cpx two-component system (Chapter 4), and explored the potential roles of several extracytoplasmic proteases in these stress-response systems (Chapter 5). These studies suggest that the inner membrane protease DegS/HhoB is essential for viability and plays a role in signal transduction to σ^E (Chapter 5).

The cellular role of σ^E

σ^E is an essential sigma factor, at least at temperatures above 18°C, and cells lacking σ^E rapidly accumulate a suppresor of this lethality (Chapter 3). Cells lacking σ^E, and containing this suppressor mutation, form colonies at 42°C to 43°C with greatly reduced efficiency (10^{-3} to 10^{-5}), and die more rapidly than wild type cells after exposure to lethal temperatures (Hiratsu et al., 1995; Raina et al., 1995; Rouvière et al., 1995) while cells containing the suppressor alone are temperature resistant (Connolly and Gross,

unpublished observations). These phenotypes confirm the importance of the σ^E regulon both for the resistance to thermal stress and the maintenance of periplasmic folding homeostasis under steady state growth conditions. Because σ^E is responsible for most of *rpoH* transcription at lethal temperatures above 45°C (Erickson et al., 1987) and the σ^{32} dependent hsps represent the majority of proteins expressed under these conditions (Neidhardt et al., 1984; Pack and Walker, 1986), σ^E also plays a role in maintaining the cytoplasmic stress response under extreme conditions.

Our current understanding of the σ^E regulon does not explain the essentiality of this sigma factor. Overexpression of σ^E leads to the induction of at least 10 proteins (Raina et al., 1995; Rouvière et al., 1995), 8 of which have been identified. In addition to *rpoH*, $E\sigma^E$ transcribes the periplasmic protease *degP* (Erickson and Gross, 1989; Lipinska et al., 1989), the periplasmic peptidyl-prolyl isomerase *fkpA* (Danese and Silhavy, 1997), and one of the two promoters upstream of the *rpoE* operon (Raina et al., 1995; Rouvière et al., 1995), encoding σ^E itself as well as the regulatory proteins RseA, RseB, and RseC (De Las Peñas et al., 1997; Missiakas et al., 1997). Although σ^{32} is required for viability at temperatures above 20 °C (Zhou et al., 1988), σ^E accounts for only a small percentage of *rpoH* transcription at temperatures below 42°C (Erickson et al., 1987) and the loss of σ^E-dependent *rpoH* transcription is unlikely to account for the essentiality of σ^E. DegP is only essential at temperatures above 42°C (Lipinska et al., 1989; Strauch et al., 1989), and FkpA is not essential for viability (Missiakas et al., 1996). A new member of the σ^E regulon, *ompK*, has recently been reported (Missiakas and Raina, 1998) and may prove to be the first essential gene under the control of σ^E. Alternatively, σ^E may control the expression of several genes whose functions are redundant and it is only upon the complete loss of expression of all of these genes by the removal of σ^E that cell growth ceases.

Although cells containing the suppressor of *rpoE*$^-$ lethality are temperature resistant, they show several phenotypes, including sensitivity to SDS/EDTA and the antibiotic novobiocin, that suggest that outer membrane integrity is altered in these mutants

(Connolly, Onufryk, and Gross, unpublished observations). Several mechanisms of suppression can be envisioned. The suppressor may reduce the level of expression of a signal molecule or molecules that normally require the activities of the σ^E regulon for their removal. For example, conditions that lead to a decrease in outer membrane protein (OMP) levels lead to a similar decrease in steady state σ^E activity (Mecsas et al., 1993). Alternatively, the suppressor could activate a second signal transduction pathway controlling the expression of periplasmic folding agents, thus restoring the functions lost in *rpoE*$^-$ cells. In this regard, it is of interest to note that although activation of the Cpx pathway suppresses the temperature sensitivity of *rpoE*$^-$ cells, it does not obviate the requirement for the suppressor of *rpoE*$^-$ lethality. Finally, the suppressor may confer σ^E-independent expression of an essential member of the σ^E regulon, or raise the basal level of transcription of several regulon members. The mechanism of suppression may turn out to be more complex than any of these scenarios. Indeed, initial mapping of the suppressor suggests that more than one locus, and more than one mechanism, might be involved (Onufryk and Gross, unpublished data). Further characterization of the suppressor of *rpoE*$^-$ lethality as well as suppressors of other *rpoE*$^-$ phenotypes may provide us with key insights into the cellular role of σ^E.

Signal transduction to σ^E

In this thesis, I show that σ^E activity is under the control of three genes, *rseABC* (for regulator of sigmaE), encoded immediately downstream of the sigma factor (De Las Peñas et al., 1997; Missiakas et al., 1997). RseA is an inner membrane protein whose cytoplasmic domain acts as a σ^E-specific anti-sigma factor. RseB binds to the periplasmic domain of RseA and modulates the activity of RseA. Deletion of *rseC* has minimal effects on σ^E activity, and several lines of evidence suggest that *rseC* may play a role or roles in the cell unrelated to σ^E (see Chapter 2 for further information). Cells lacking both RseB

and RseC are capable of responding to extracytoplasmic stress in a wild type manner, indicating that RseA is the central regulatory molecule of this cascade.

The use of anti-sigma factors to control sigma factor activity is a common theme in prokaryotic regulation (Brown and Hughes, 1995). Anti-sigmas were originally thought to act by competitively inhibiting sigma binding to core RNA polymerase. Recent studies suggest that this model may be simplistic. The anti-sigma factor FlgM of *S. typhimurium* binds tightly to its cognate sigma, FliA, and has also been detected in holoenzyme complexes containing FliA (Hughes, unpublished data). This data suggests that FlgM may act as an anti-holoenzyme specifically for polymerase molecules bound to FliA. The phage encoded anti-σ^{70} factor, AsiA, does not inhibit σ^{70} activity by preventing core binding, but rather forms a ternary complex with holoenzyme containing σ^{70} and interferes with sigma-mediated recognition of the -35 region of the promoter (Colland et al., 1998; Severinova et al., 1998). The majority of anti-sigmas characterized to date are soluble molecules, and it will be interesting to determine if the mechanism of action of RseA, an inner membrane protein, differs from the models currently being developed for these soluble anti-sigmas.

In order to induce σ^E activity, RseA activity must be abolished or reduced. This could be achieved by several means, including the degradation of RseA, covalent modification of RseA, dimerization or RseA, or conformational change in RseA that disrupts the interaction with σ^E. Initial pulse chase experiments suggested that RseA might be an unstable molecule (Connolly and Gross, unpublished data), and a more careful analysis has revealed that the stability of RseA is regulated in response to cellular stress (Ades and Gross, unpublished data). During steady state growth, the half life of RseA is 26 minutes and drops to about 2 minutes immediately following induction of σ^E by shift to high temperature or overexpression of the outer membrane protein OmpC. The signal is generated within minutes following induction as RseA labeled prior to induction is degraded without a detectable lag. Under steady state induction conditions (i.e. *surA*$^-$

cells) in which σ^E activity is uniformly high, RseA is slightly unstable, showing a 3-fold decrease in half life (8-10 minutes). In addition, RseA is slightly destabilized in cells lacking RseB (~2-fold), accounting for the 2-fold induction of σ^E activity seen in such strains.

These results suggest that the initial increase in σ^E activity observed immediately after induction is due to the regulated degradation of RseA, leading to a decrease in the RseA:σ^E ratio and an increase in the free pool of σ^E (Fig. 6-1). This free σ^E then transcribes its regulon, including its own promoter, leading to increases in the levels of σ^E regulon members, σ^E itself and the Rse proteins. Existent and newly synthesized regulon members act to reduce the level of stress signal in the periplasm, which in turn modulates the half life of RseA, and, ultimately, the activity of σ^E. RseB binding may act to protect RseA from degradation, and the disruption of this interaction in response to extracytoplasmic stress may initiate the degradation of RseA and transmission of the signal to σ^E. It is currently unknown whether additional regulatory mechanisms play a role in controlling σ^E activity during induction or adaptation, and detailed analysis of σ^E and RseA expression as well as σ^E stability under these conditions will lead to a clearer picture of the mechanisms by which σ^E activity is modulated in response to stress.

Current data suggests that at least one signal leading to induction of σ^E is an intermediate in the porin folding pathway occurring just prior to formation of the folded monomer (Mecsas et al., 1993; Rouvière and Gross, 1996). The mechanism by which this signal is detected remains unknown. The signal may be detected directly by RseB and/or RseA, altering the conformation of RseA and rendering it susceptible to proteolysis. How well this signal molecule competes with RseA binding to RseB may determine the level of signal at which signal transduction is initiated. Alternatively, the protease responsible for RseA degradation may become activated in response to the signal. In addition to or instead of detecting the signal molecule directly, RseB and/or RseA may sense the signal indirectly by monitoring the free levels of protein folding catalysts and proteases in the periplasm.

The identity of the protease responsible for the degradation of RseA has not been determined, but several lines of evidence suggest that the essential inner membrane protease DegS/HhoB may play this role. The observation that RseA is slightly unstable even under steady state conditions suggests that the activity of the protease is not restricted to induction conditions and that σ^E activity under all growth conditions is determined, at least in part, by the activity of this protease. This model predicts that cells lacking the protease should show a steady state decrease in σ^E activity. Complete stabilization of RseA by deletion of the protease may reduce σ^E activity to a level incompatible with survival, suggesting that the protease may itself be essential. In this thesis, I have presented evidence that DegS/HhoB is an essential protease and strains lacking DegS/HhoB show a 5-fold decrease in σ^E activity (Chapter 5). Similar deletions of several other extracytoplasmic proteases (DegP, DegQ/HhoA, Prc/Tsp) have no effect on σ^E activity. In addition, cells lacking the protease responsible for RseA degradation are predicted to be uninducible for σ^E activity. In fact, the introduction of plasmids encoding outer membrane proteins into Δ*degS1* strains is lethal (Connolly and Gross, unpublished observations). Although these observations support the idea that DegS/HhoB is responsible for the regulated degradation of RseA, definitive identification of the protease responsible for determining RseA stability awaits direct evidence, such as stabilization of RseA in a protease deficient strain, demonstrating an interaction between this protease and RseA.

The ECF sigmas

σ^E belongs to a family of σ^{70}-like factors whose cellular functions are related to extracytoplasmic processes, and have thus been grouped into a subset of sigma factors known as the extracytoplasmic function or ECF sigmas (Lonetto et al., 1994). Sigmas belonging to the ECF subfamily have been described in both Gram-negative and Gram-positive bacteria as well as in mycobacteria, and have been shown to be involved in such disparate cellular functions as the regulation of iron uptake, the light-induced production of

protective pigments, the production of antibiotics, survival at high temperature, and the production of extracellular polysaccharides (see Table 6-1 for a partial listing of known ECF sigmas). The promoter sequences recognized by the ECF sigmas show a surprisingly high degree of conservation, and many ECF sigmas utilize a similar regulatory scheme in which an inner membrane protein binds to and negatively regulates sigma factor activity (Missiakas and Raina, 1998). This inner membrane anti-sigma is thought to act as both a sensor and signaling molecule, allowing the sigma factor activity to respond to environmental change. Studies of *M. xanthus* CarQ and its cognate regulator CarR provide evidence that like *E. coli* RseA, CarR is degraded in response to signal (Gorham et al., 1996), suggesting that even the mode of regulation of the anti-sigma factor is conserved amongst ECF family members.

The relationship between σ^E and AlgU of *Pseudomonas aeruginosa*

Aside from *E. coli* σ^E, the most well characterized of the ECF sigmas is AlgU of *Pseudomonas aeruginosa*. *P. aeruginosa* is a ubiquitous water and soil bacterium that often causes disease in humans with abnormal host defenses. Chronic lung infections caused by this organism represent a major source of both morbidity and mortality in patients with the inherited disorder cystic fibrosis (CF) (Deretic et al., 1994; Deretic et al., 1995). Although initial isolates obtained from CF patients are often nonmucoid, persistent colonization of the lungs in these patients is associated with the development of a mucoid phenotype due to the production of the exopolysaccharide alginate (Deretic et al., 1994; Deretic et al., 1995). Studies in animal models suggest that the mucoid phenotype inhibits bacterial clearance from the lung (Boucher et al., 1997b; Yu et al., 1998), helping to explain the persistent nature of *Pseudomonas* infections in CF patients. Genetic studies of the control of alginate production revealed that AlgU is required for the transcription of the biosynthetic operons responsible for alginate synthesis (Martin et al., 1993a; Hershberger et al., 1995), emphasizing the central role this sigma factor plays in the conversion to mucoidy.

The *rpoE* and *algU* operons show a high degree of similarity and evidence to date suggests that their regulation is similar. AlgU activity is controlled by four proteins encoded downstream of the sigma factor; MucA, MucB, MucC and MucD (Martin et al., 1993c; Schurr et al., 1994; Boucher et al., 1996; Schurr et al., 1996; Xie et al., 1996). MucA and B show 30 and 26% identity at the amino acid level to RseA and B, respectively, and act as negative regulators of AlgU activity (Goldberg et al., 1993; Martin et al., 1993b; Martin et al., 1993c). Like RseA, MucA has been shown to be an inner membrane protein that binds directly to the sigma factor to negatively regulate its activity (Schurr et al., 1996; Xie et al., 1996). MucB, in turn, is a periplasmic protein (Schurr et al., 1996; Mathee et al., 1997), and based on similarity to RseB, probably exerts its negative effects on AlgU via direct interaction with MucA. One significant difference between these two systems is the magnitude of the effect of RseB and MucB. Deletions in *rseB* only partially induce σ^E activity (Chapter 2) whereas inactivation of *mucB* leads to de-repression of AlgU to levels similar to those observed in strains lacking MucA (Martin et al., 1993b). One possible explanation for this difference is that the *mucB* allele used in these studies contains a Tn*10* insertion which is predicted to be polar on downstream genes. Some of the induction observed in *mucB*::Tn*10* strains may in fact be due to loss of *mucD* expression (see below for further explanation). Similar to *rseC*, effects of *mucC* deletions on AlgU activity are only observed in cells containing mutations in other *muc* genes, but in contrast to *rseC*, these data suggest a negative regulatory role for *mucC* (Boucher et al., 1997a) .

Two recent reports have suggested that the anti-sigma activity of MucA only partially accounts for the post-translational regulation of AlgU (Schurr et al., 1995; Mathee et al., 1997). 2D gel analysis of purified AlgU produced in *E. coli* reveals several AlgU isoforms (Schurr et al., 1995). Treatment of this purified protein with phosphatases shifted these forms to the basic side of the gel, suggesting that AlgU is phosphorylated. Further, underphosphorylated species of AlgU copurified with RNA polymerase,

suggesting that the activity of this sigma factor is controlled by phosphorylation. 2D gel analysis of σ^E has not revealed similar isoforms of this sigma factor, and a definitive role for phosphorylation in the regulation of either sigma factor awaits analysis of phosphorylation *in vivo* and the demonstration of differential activity of such isoforms under physiological conditions.

It has also been suggested that MucA and B act to control the stability of AlgU (Mathee et al., 1997). In this scenario, reminiscent of σ^{32} regulation, AlgU is targeted for degradation by the Muc proteins under steady state conditions and becomes stabilized upon induction. However, no direct evidence of AlgU instability such as a pulse chase analysis has been carried out and these indirect studies are complicated by several experimental difficulties that render the data difficult to interpret. The effects of a *mucA* single and a *mucA mucB* double mutation on *algU* activity and levels are analyzed in multicopy in cells carrying a wild type chromosomal copy of the *algU* operon. The Muc gene products are translationally coupled, suggesting that the stoichiometry of these proteins is important for their function and stoichiometry is undoubtedly altered in these "pseudo-diploid" cells. Furthermore, the plasmids used encode only two of the regulators, MucA and B. In addition, the *mucA* allele, *mucA22*, used in these experiments presents several problems. This allele is one of the most common mutations leading to mucoidy in patient isolates (Boucher et al., 1997b). However, cells used in these studies which carry a wild type chromosomal copy of the *algU* operon and *mucA22* along with wild type *algU* on a plasmid are not mucoid and no explanation is given for this discrepancy. This observation suggests that the protein produced by this allele retains partial function which is somehow strengthened in the presence of the wild type chromosomal copy of *mucA*. In contrast to these data, no evidence for the instability of σ^E has been found. Pulse-chase analysis and translational inhibition studies suggest that σ^E is stable under steady state conditions (Connolly and Gross, unpublished data). Direct determination of AlgU stability in haploid

cells of defined genetic background would provide definitive evidence that the activity of this sigma factor is regulated by degradation.

Although most *P. aeruginosa* isolates have the capacity to synthesize alginate, mucoid strains are rarely identified in environmental samples, leading to the suggestion that conditions unique to the environment of the CF lung induce a signal transduction cascade leading to activation of AlgU and alginate production (Deretic et al., 1994). In fact, mutations in the regulatory genes responsible for alginate production are often identified in patient isolates (Boucher et al., 1997b). Notably, the vast majority of mucoid *P. aeruginosa* patient isolates contain mutations in *mucA* (Boucher et al., 1997b), underscoring the central role of this gene in regulating AlgU activity. Intriguingly, very few of these mutations map to the cytoplasmic domain, suggesting either that disruption of the sigma/anti-sigma interface requires multiple mutations or that disruption of this interaction is toxic to the cell. In fact, greater than 77% of the mutations map to the extracytoplasmic domain, and often encode proteins with truncations in the periplasmic domain. The observation that a higher level of expression of plasmid-encoded MucA is required to complement a true null mutation than to complement two different truncations suggests that these truncated proteins retain partial function. By analogy to RseA and RseB, some of these truncations may disrupt the MucA/B interaction, leading to a slight destabilization of MucA in the absence of signal which is sufficient to induce mucoidy without completely deregulating AlgU. Further study of these naturally occurring *mucA* mutations and the construction of similar mutations in *rseA* will help to identify functional regions of these proteins and aid in the dissection of their signal transduction cascades.

Despite the similarities in the regulation and promoters of σ^E and AlgU, their cellular roles and the environmental conditions leading to their activation do not appear to be identical. Although both σ^E and AlgU are involved in the resistance to thermal stress (Martin et al., 1994; Hiratsu et al., 1995; Raina et al., 1995; Rouvière et al., 1995; Yu et al., 1995), cells lacking AlgU show increased sensitivity to superoxide generating

compounds (Martin et al., 1994; Yu et al., 1995) while *rpoE*$^-$ cells exhibit a normal response to oxidative stress (Rouvière et al., 1995). In addition, the *algU* operon encodes a fifth protein, MucD (Boucher et al., 1996) which shows homology to *E. coli* DegP. Null mutations in *mucD* or the MucD homolog *algW* induce AlgU activity (Boucher et al., 1996) whereas similar mutations in the Deg proteases of *E. coli* have no effect on or actually decrease σ^E activity (Raina et al., 1995)(Raina et al., 1995; Chapter 5). It is not known whether AlgU activity is responsive to the extracytoplasmic protein-folding state and the requirement for several two-component systems in the production of alginate suggests that the induction of mucoidy involves the integration of several extracellular signals (Deretic et al., 1989; Wozniak and Ohman, 1994). Identification of additional members of the *algU* and σ^E regulons may help to clarify their cellular roles. The observation that overexpression of MucA and B in *E. coli* reduces the expression of a chromosomal *algU*-dependent *lacZ* reporter well below basal levels detected in the absence of AlgU (Schurr et al., 1994) suggests both that σ^E is capable of transcribing this *algU*-dependent reporter and that the Muc proteins are capable of regulating σ^E activity to some degree . Careful study of the signals inducing AlgU and the study of chimeric RseA/MucA and RseB/MucB regulatory proteins may help to elucidate how AlgU and σ^E utilize similar signaling molecules to respond to diverse extracellular signals.

The Cpx pathway

In addition to the σ^E pathway, *E. coli* utilizes a second signal transduction pathway, the Cpx two-component system, to monitor extracytoplasmic stress. This system is composed of CpxA, an inner membrane sensor/kinase and CpxR, the response regulator. Although the *cpx* genes were first identified nearly two decades ago (McEwen and Silverman, 1980b), a role for the Cpx pathway in *E. coli* physiology has only recently been elucidated. The Cpx pathway was originally described in relation to pleiotropic phenotypes that result from constitutive activation of this pathway (Thorbjarnardottir et al., 1978; McEwen and

Silverman, 1980a; McEwen and Silverman, 1980c; McEwen and Silverman, 1982; McEwen et al., 1983). It is now clear that these phenotypes do not reflect the normal function of the Cpx pathway but rather result from either overexpression of Cpx-regulated genes (Danese et al., 1995) or the aberrant activation of non-cognate response regulators by hyperactivated forms of CpxA (Pogliano et al., 1998).

The finding that activation of the Cpx pathway by either mutational induction or overexpression of NlpE relieves the toxicity of several periplasmic LamB mutant proteins (Cosma et al., 1995; Snyder et al., 1995) led to the proposal that the Cpx pathway is involved in regulating periplasmic folding events. The observation that the Cpx pathway is induced in cells expressing the pilin protein PapG in the absence of its dedicated chaperone and the recent finding that the Cpx pathway modulates the expression of periplasmic folding agents further support the idea that this pathway is involved in regulating protein folding and turnover in the extracytoplasmic compartment (Danese and Silhavy, 1997; Pogliano et al., 1997). More recently, cells lacking the Cpx pathway have been shown to be sensitive to high extracellular pH and the expression of Cpx regulon members is induced under these conditions (Danese and Silhavy, 1998; Nakayama and Watanabe, 1998), suggesting a role for this pathway in the cellular response to alkaline pH. In addition, pH-regulated expression of the *Shigella sonnei* global virulence factor VirF was found to mediated by the Cpx pathway (Nakayama and Watanabe, 1995; Nakayama and Watanabe, 1998), implicating this regulatory system in bacterial pathogenesis.

What does the Cpx pathway sense?

The Cpx pathway is activated under several conditions that might lead to or reflect the accumulation of misfolded protein in the extracytoplasmic space. Expression of the pilin subunit PapG in the absence of its dedicated chaperone PapD induces the Cpx pathway presumably via the accumulation of misfolded, unfolded, or aggregated PapG subunits (Jones et al., 1997). Overexpression of the lipoprotein NlpE (Snyder et al., 1995) and

growth in alkaline pH (Danese and Silhavy, 1998) also specifically induce this pathway. Lastly, the Cpx pathway is activated in cells lacking phosphatidyl-ethanolamine (Milekovskaya and Dowhan, 1997), suggesting that CpxA may sense alterations in the structural or physical integrity of the cell envelope. Deletion of *nlpE* from these strains did not alter Cpx activation, suggesting that this pathway may directly sense these envelope changes (Milekovskaya and Dowhan, 1997). It remains to be seen whether each of the conditions described above generate a single, common signal that is sensed by CpxA, or if this pathway is capable of responding to diverse signals.

Mechanism of signal transduction by the Cpx pathway

Recent studies characterizing several gain of function mutations in *cpxA* have shown that the Cpx pathway appears to function as a typical two-component signal transduction system (Raivio and Silhavy, 1997). CpxA is the inner membrane sensor-kinase which modulates the activity of the transcriptional regulator CpxR by altering its phosphorylation state in response to signal. Phosphorylated CpxR binds to several sites upstream of Cpx-responsive genes to activate their transcription. Gain of function mutations which map to the cytoplasmic domain of CpxA alter phosphotransfer to CpxR, mainly by decreasing the phosphatase activity of CpxA, leaving an increased concentration of active, phosphorylated regulator. A second class of gain of function mutations, including a 32 amino acid deletion, map to the periplasmic region of CpxA. Proteins carrying alterations in the periplasmic domain are incapable of responding to signals that normally induce the Cpx pathway, suggesting that they define a region of the protein which is responsible for interaction with a signal molecule or molecules. It has been proposed that under steady state conditions a negatively acting effector binds to this region of CpxA. Upon induction, this factor is titrated off of CpxA, leading to an alteration in CpxA conformation and activity. The genetic identification of second site suppressors of these mutations or

biochemical characterization of proteins that interact with this region of CpxA will help to clarify the mechanism by which the Cpx pathway senses envelope stress.

Relationship between the Cpx and σ^E pathways

Several lines of evidence suggest that the Cpx and σ^E pathways represent distinct stress-response systems that do not sense or regulate redundant functions (Figure 6-2). Cells lacking the Cpx pathway show sensitivity to alkaline pH but no other obvious defects have been detected (Danese et al., 1995; Danese and Silhavy, 1998). In contrast, the σ^E pathway appears to be involved in envelope homeostasis under most, if not all, growth conditions. σ^E is as essential sigma factor (Chapter 3), and cells lacking σ^E are sensitive to membrane disrupting agents and fail to grow at high temperature (Hiratsu et al., 1995; Raina et al., 1995; Rouvière et al., 1995).

Although both the Cpx and σ^E pathways respond to the extracytoplasmic accumulation of misfolded protein, they appear to be monitoring different protein folding pathways . Overexpression of NlpE does not induce σ^E activity in general, as measured from a minimal σ^E-dependent promoter (Danese et al., 1995), and expression of PapG in the absence of PapD induces Cpx activity far more than σ^E activity (Jones et al., 1997). Alterations in the maturation of OMPs, either by overexpression or the titration or periplasmic folding agents, uniquely induce σ^E (Mecsas et al., 1993; Danese et al., 1995; Missiakas et al., 1996; Rouvière and Gross, 1996). Alterations in the lipid composition of the envelope have recently been shown to induce both pathways (Milekovskaya and Dowhan, 1997) and these conditions are likely to alter the folding pathways of several different classes of envelope proteins.

Although the Cpx and σ^E regulons overlap at *degP*, they are not identical. The Cpx system does not exclusively rely on holoenzyme containing σ^E for transcriptional activation. Although CpxR and σ^E appear to act together at the *degP* promoter, CpxR is capable of inducing *degP* transcription approximately three-fold in the absence of σ^E

(Danese et al., 1995). In addition, new members unique to either the Cpx or σ^E regulons have been described (Figure 6-2)(Danese and Silhavy, 1997; Pogliano et al., 1997; Danese and Silhavy, 1998; Dartigalongue and Raina, 1998; Missiakas and Raina, 1998). The Cpx proteins regulate expression of the disulfide bond isomerase, *dsbA* (Danese and Silhavy, 1997; Pogliano et al., 1997), the peptidyl prolyl isomerases (PPIases), *ppiA* (Pogliano et al., 1997) and *ppiD* (Dartigalongue and Raina, 1998) and the periplasmic protein *cpxP* (Danese and Silhavy, 1998), while σ^E uniquely induces the expression of a second PPIase, *fkpA* (Danese and Silhavy, 1997) and the newly described *ompK* (Missiakas and Raina, 1998). Although each regulon encodes similar biochemical activities, and activation of the Cpx pathway can rescue the Ts phenotype of *rpoE*$^-$ cells (Chapter 4), activation of the σ^E pathway cannot substitute for the Cpx system. For example, activation of σ^E by overexpression of OMPs does not relieve the toxicity of LamB mutant proteins (Snyder et al., 1995). These results suggest that the two pathways can be induced independently in response to unique stressors, and that they may integrate these disparate signals arising in the face of multi-faceted insults. A complete understanding of the relationship between these two systems awaits further molecular characterization of their regulon members and inducing signals.

Novel pathways involved in the response to extracytoplasmic stress

Several observations suggest that in addition to the σ^E and Cpx pathways, at least one additional regulatory system exists that is capable of responding to extracytoplasmic stress. Overexpression of a truncated form of TetA (TetA'), a polytopic inner membrane protein, is capable of suppressing the toxicity associated with several LamB mutant proteins possessing defects in signal sequence processing (Cosma et al., 1998). Impressively, the mechanism of suppression does not lead to the degradation of the defective LamB proteins, but rather allows them to become fully folded and inserted into the outer membrane with their signal sequences intact. Suppression by TetA' does not require the σ^E or Cpx

pathways, and, considering the mechanism, is unlikely to be direct. In fact, TetA' may induce a third stress-responsive pathway that specifically monitors the folding state of the inner membrane.

Evidence presented in Chapter 4 shows that DegP expression is still induced in response to heat shock in the absence of σ^E. Heat-shock regulation of *degP* has been shown to be independent of the Cpx pathway (Pogliano et al., 1997) and we have observed heat-shock induction in the absence of both these systems (Connolly and Gross, unpublished data). These results raise the possibility that a third regulatory system modulates *degP* expression and explain the seemingly contradictory observation that cells lacking σ^E, and by extension the entire σ^E regulon, plate 100-fold better at high temperature than cells lacking DegP alone (10^{-3} vs. 10^{-5}; Connolly and Gross, unpublished data). This third regulatory system may not have been detected due to its relatively small contribution to *degP* expression in the presence of the σ^E and Cpx pathways. The finding that σ^{32} can transcribe *degP in vitro* (C. Chan, personal communication) suggests that this sigma factor may account for the increased level of DegP observed at high temperature in the absence of σ^E and Cpx. The identification of additional cellular factors required for both TetA' mediated suppression and heat-shock expression of *degP* may lead to the identification of novel stress-responsive signal transduction systems.

Use of stress-responsive pathways to identify extracytoplasmic folding catalysts

Our understanding of protein folding processes in the extracytoplasmic compartment has long lagged behind our understanding of cytoplasmic folding processes and agents. The energy poor nature of the periplasmic space makes it unlikely that extracytoplasmic counterparts of the cytoplasmic chaperones exist (Wülfing and Plückthun, 1994), and folding pathways unique to the extracytoplasmic compartment, such as porin assembly, are

likely to require unusual folding agents (Pugsley, 1993). Prior to the identification of the σ^E and Cpx pathways, the periplasmic disulfide bond isomerases (Dsbs) (Bardwell et al., 1991; Missiakas et al., 1994) were the only proteins known to assist in general protein folding in the periplasm. By isolating genes whose expression levels alter σ^E activity and/or are dependent on the σ^E or Cpx pathways for their expression, several new periplasmic folding agents have been identified and our subsequent understanding of periplasmic folding processes has blossomed.

The periplasmic peptidyl prolyl isomerases (PPIases) SurA and FkpA were implicated in extracytoplasmic folding by two independent genetic approaches. Overexpression of either of these proteins was found to decrease σ^E activity in cells constitutively activated for this response (Missiakas et al., 1996), and Tn*10* insertions in the genes encoding SurA or FkpA lead to an induction of σ^E activity (Missiakas et al., 1996; Rouvière and Gross, 1996). Further, *fkpA* expression is under the control of σ^E (Danese and Silhavy, 1997). A third PPIase, PpiD, was recently identified based on both its ability to suppress *surA* mutants in multicopy and the ability of the Cpx pathway to activate its expression (Dartigalongue and Raina, 1998). The putative OMP chaperone Skp/OmpH was originally identified based on its ability to selectively bind to OMPs (Chen and Henning, 1996), and the findings that σ^E activity is induced in cells lacking Skp (Missiakas et al., 1996) and that overexpression of Skp improves the efficiency of both phage display and the periplasmic expression of recombinant proteins (Bothmann and Pluckthun, 1998) provide functional evidence that Skp is involved in periplasmic folding. Similar studies aimed at identifying genes whose inactivation induces the Cpx pathway or whose overexpression can decrease Cpx activity in cells constitutively activated for this system have not been carried out and may lead to the identification of folding catalysts involved in pathways uniquely monitored by the Cpx system.

Of the putative periplasmic folding agents described above, only the role of SurA in periplasmic folding has been studied in detail (Lazar and Kolter, 1996; Rouvière and

Gross, 1996). Analysis of outer membrane protein maturation in cells lacking *surA* revealed a new intermediate in the porin assembly pathway, the folded monomer, and showed that SurA plays a rate limiting role in the formation of this intermediate (Rouvière and Gross, 1996). Because σ^E activity is high in cells lacking SurA, these studies also suggest that the signal being sensed by the σ^E pathway consists of porin intermediates arising prior to the formation of the folded monomer. Detailed analysis of the roles of FkpA, PpiD, and Skp in OMP maturation or other folding processes have not been carried out and will help to increase our understanding of both periplasmic protein folding and the nature of the signals that induce the extracytoplasmic stress response.

The role of proteolysis in the extracytoplasmic stress response

In addition to chaperones and folding catalysts, the cell relies heavily on proteases to maintain optimal protein folding conditions and cellular integrity. Several proteases contribute to the turnover of abnormal cytoplasmic proteins arising both as a result of normal cellular processes and stress conditions (Gottesman and Maurizi, 1992; Gottesman, 1996; Miller, 1996), and not surprisingly, the results presented in Chapter 5 support the idea that multiple proteases also play a role in ridding the cell of abnormal extracytoplasmic proteins. Deletion of a single protease (DegP, Prc/Tsp, OmpT, DegQ/HhoA) does not induce the stress response, indicating that these proteases are likely to have overlapping substrate specificities, and only upon deletion of several of them will these substrates accumulate. In fact, the substrate specificity of DegP and DegQ/HhoA, at least *in vitro*, are almost identical (Kolmar et al., 1996) and overexpression of DegQ/HhoA suppresses the temperature sensitive phenotype of cells lacking either DegP or Prc/Tsp (Bass et al., 1996; Waller and Sauer, 1996). These findings do not suggest that these proteases are equivalent under all growth conditions. For example, the extreme temperature sensitivity of cells lacking DegP underscore the importance of this protease in ridding the cell of abnormal protein generated at high temperature (Lipinska et al., 1989; Strauch et al., 1989).

Results presented in Chapter 5 also support both a housekeeping and a regulatory role for the second essential inner membrane protease, DegS/HhoB. Deletion of DegS/HhoB alone leads to a decrease in σ^E activity, without effecting the Cpx pathway, suggesting a regulatory role for this protease in signal transduction to σ^E (see above for details). Deletion of DegQ/HhoA leads to a slight induction of the Cpx pathway, and removal of DegS/HhoB from these cells leads both to further induction of the Cpx activity and induction of σ^E. Although these results should be interpreted with caution as the effects of possible suppressor mutations in the double mutant strains have not been assessed, they suggest that DegQ/HhoA and DegS/HhoB might normally be involved in the turnover of an overlapping set of abnormal substrate molecules whose accumulation in the double mutant leads to the activation of both signal transduction pathways. Whether their effects on the extracytoplasmic stress response prove to be direct or indirect, the data in Chapter 5 suggests that these proteases play unique roles in regulating protein turnover in the extracytoplasm. Further characterization of their cellular roles and substrates will undoubtedly shed new light on both periplasmic folding processes and the extracytoplasmic stress response.

Table 6-1. Selected ECF sigma factors

Organism	Sigma factor	Cognate regulator[a]	Operon structure[a]	Function	Reference(s)
Alcaligenes entrophus	CnrH[b]	ORF1	*carH orf1*	Co/Ni export	(Liesegang et al., 1993)
Azotobacter vinlandeii	AlgU	MucA	*algU mucA mucB mucC mucD*	Alginate synthesis, encystment	(Martinez-Salazar et al., 1996; Moreno et al., 1998)
Bacillus subtilis[c]	SigX	RsiX	*sigX rsiX*	Iron metabolism	(Brutsche and Braun, 1997; Huang et al., 1997)
Erwinia amylovora	HrpL			Hypersensitive response, virulence	(Wei and Beer, 1995)
Escherichia coli	σE	RseA	*rpoE rseA rseB rseC*	Stress response	(Raina et al., 1995; Rouvière et al., 1995; De Las Peñas et al., 1997; Missiakas et al., 1997)
	FecI	FecR	*fecI fecA*	Iron-citrate uptake	(Enz et al., 1995)
Mycobacterium tuberculosis[d]	SigE			Stress response	(Wu et al., 1997)
Myxococcus xanthus[e]	CarQ	CarR	*carQ carR carS*	Carotenoid production	(McGowan et al., 1993; Ruiz-Vazquez et al., 1993)
Photobacterium SS9	RpoE	Orf2	*rpoE orf2 orf3 orf4*	Cold, high pressure adaptation	(Chi and Bartlett, 1995)
Pseudomonas aeruginosa	AlgU	MucA	*algU mucA mucB mucC mucD*	Alginate production, stress response	(Martin et al., 1993; DeVries and Ohman, 1994; Schurr et al., 1994)
	PvdS			Pyoveridine biosynthesis, exotoxin A synthesis	(Cunliffe et al., 1995; Ochsner et al., 1996)
	FiuI			Iron uptake	(Ochsner and Vasil, 1996)
Pseudomonas fluorescens	PbrA			Iron-stress response	(Sexton et al., 1995; Sexton et al., 1996)
Pseudomonas putida	PupI	PupR	*pupI pupR*	Pseudobactin transport	(Koster et al., 1994)
	PfrI			Iron regulation	(Venturi et al., 1995)

Table 6-1, cont. Selected ECF sigma factors

Organism	Sigma factor	Cognate regulator[a]	Operon structure[a]	Function	Reference(s)
Pseudomonas syringae	HrpL			Hypersensitive response, virulence	(Xiao et al., 1994; Xiao and Hutcheson, 1994)
Rhodobacter sphaeroides	RpoE	ChrR		Cytochrome expression	Newman and Donohue, personal communication
Streptomyces antibioticus	SigE			Antibiotic production	(Jones et al., 1997)
Streptomyces coelicolor	SigE			Agarase expression	(Lonetto et al., 1994)
Synechococcus sp. strain PCC 7002[f]	SigG			Cell division	(Gruber, 1998)
Xanthomonas campestris	RfaY			Lipopolysaccharide biosynthesis, pathogenesis	(Dow et al., 1995)

[a]Cognate regulator and operon structure are stated when known.

[b]Plasmid-encoded

[c]Six other ECF sigmas of unkown function have been identified in the *B. subtilis* genome and are encoded by *sigV, sigW, sigY, sigZ, yhdM, and ylaC* (Sorokin et al., 1997; Huang et al., 1998; Huang and Helmann, 1998).

[d]At least seven other ECF sigmas are encoded in the *M. tuberculosis* genome and several of these are conserved in other mycobacterial species (Cole et al., 1998).

[e]A second ECF sigma of unknown function, RpoE1, has been identified in *M. xanthus* (Ward and Zusman, personal communication).

[f]Two additional ECF sigmas of unknown function, SigG and SigH, have been identified in *Synechococcus* sp. strain PCC 7002 (Gruber, 1998)

Figure 6-1. Model of signal transduction to σ^E. (a) In the presence of low levels of signal, σ^E is sequestered to the membrane by a complex consisting of RseA and B, resulting in low levels of σ^E activity. (b) Exposure to thermal stress or other conditions leading to protein denaturation in the extracytoplasmic compartment leads to the generation of an unknown signal molecule. RseB is titrated away from RseA by interaction with this signal molecule, leaving RseA susceptible to proteolysis by the inner membrane protease DegS/HhoB. (c) At high levels of signal, RseA becomes even more susceptible to degradation by DegS/HhoB either due to direct interaction with the signal molecule or activation of DegS/HhoB by the signal. See text for further details.

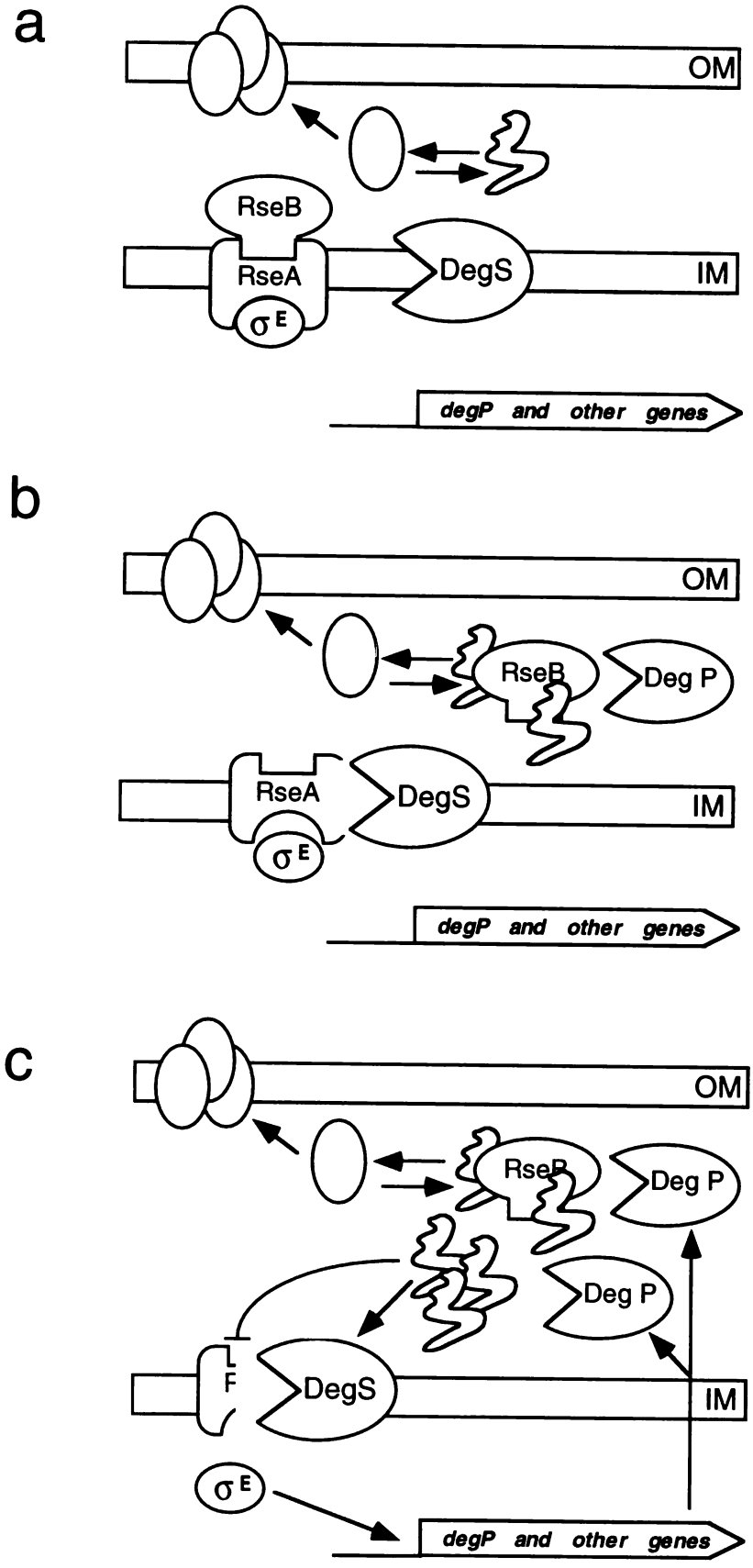
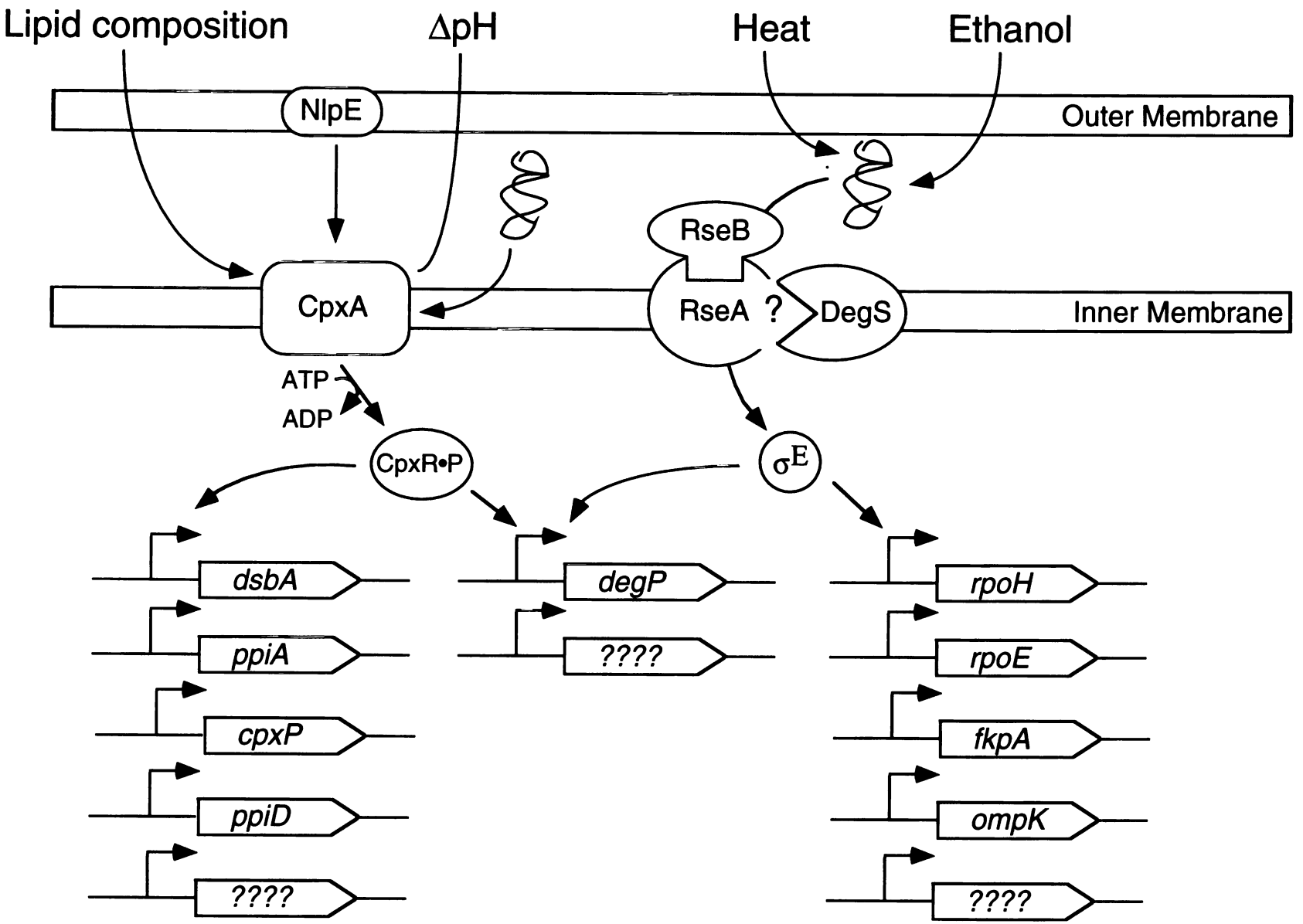

a
OM
RseB
RseA
DegS
IM
σ E
degP and other genes
b
OM
RseB
Deg P
RseA
DegS
IM
σ E
degP and other genes
c
OM
RseB
Deg P
Deg P
L
F
DegS
IM
σ E
degP and other genes

Figure 6-2. Regulation of the extracytoplasmic stress response. The Cpx pathway controls the expression of several periplasmic folding catalysts (DsbA, PpiA, PpiD, DegP, CpxP) in response to basic pH, the accumulation of misfolded pilin subunits, alterations in the lipid content of the outer membrane, and overproduction of the lipoprotein NlpE. The alternate sigma factor σ^E controls its own expression as well as expression of σ^{32}, DegP, FkpA, and OmpK in response to conditions that lead to the accumulation of OMP intermediates in the periplasmic space. See text for further details.


Lipid composition
ΔpH
Heat
Ethanol
NlpE
Outer Membrane
RseB
CpxA
RseA
?
DegS
Inner Membrane
ATP
ADP
CpxR•P
σE
dsbA
ppiA
cpxP
ppiD
????
degP
????
rpoH
rpoE
fkpA
ompK
????

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