Title
Control of the [sigma][superscript E]-dependent extracytoplasmic stress response by regulated proteolysis

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Control of the σ^E-dependent extracytoplasmic stress response by regulated proteolysis

by

Benjamin Michael Alba

DISSERTATION

Submitted in partial satisfaction of the requirements for the degree of

DOCTOR OF PHILOSOPHY

in

Biochemistry and Molecular Biology

in the

GRADUATE DIVISION

of the

UNIVERSITY OF CALIFORNIA, SAN FRANCISCO

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This thesis is dedicated to my parents,

Ted and Roberta

Acknowledgements

The work in this thesis resulted from hard work and perseverance on my part, fruitful collaborations and the unwavering support and guidance from members of the campus community, the Gross Lab, my friends and family.

I thank the members of my thesis committee, Peter Walter and Charly Craik, for taking a genuine interest in my work and for encouragement and guidance along the way.

I would like to thank my most influential mentors for invaluable instruction, technical assistance and/or intellectual input into my work. I thank Carol for providing a laboratory environment in which I could thrive and expand my horizons. Her emphasis upon fostering the development or refinement of skills required for success in science (including oral presentations and writing) has been instrumental in my development as a scientist. I also credit Carol for attracting the laboratory members with whom I have had the pleasure to interact. Most have had not only an exceptional talent for doing science, but they also have had warm and supporting personalities that made the Gross Lab a great place in which to work.

There are many former and current lab members who deserve special recognition. I first thank Lynn Connolly and Alejandro De Las Peñas (Cäno), two of the most effective and unselfish mentors I have ever encountered, for teaching me the fundamentals of molecular biology and bacterial genetics while I worked in the Gross Lab during 1995. They certainly helped me prepare for the transition from college to graduate school-level research. I next thank Christophe Herman for his creative and insightful suggestions throughout my graduate career. His vast knowledge of *E. coli* and

λ phage biology has been indispensable, and his humor constantly enlivened the lab. I also owe a special thanks to Chi Zen Lu, one of the few people I have encountered who exhibits the qualities of a model human being. She truly is a special individual. I thank Lu for her unhesitant experimental assistance and instruction, particularly in protein purification. Additionally, I thank Sarah Ades for helping me develop into a rigorous and critical experimentalist, and I thank Hong Ji Zhong for his cheerful, skillful technical assistance.

The individuals noted above certainly promoted my success in graduate school, but I also owe many thanks to my family. Graduate school can be frustrating and discouraging, such as when one puts in a large amount of work that yields no publishable (or even useful) results or when one considers the terribly slow pace at which research sometimes progresses. I am thankful for my wife Grace May Alba, who never failed to relieve my graduate school-induced irritation and who helped me balance the hard work of graduate school with the pleasures of life. I also thank Grace for her patience throughout my years as a graduate student. I thank Grace's family, the Quetingco's, for the countless diversions such as family activities and parties that helped me re-charge after a week of work.

Finally, I thank my parents, Ted and Roberta Alba, to whom I have dedicated this thesis, for the many years of providing me with the best schooling and home environment in which to learn and grow. Their enduring support, advice and encouragement have been and will continue to be much appreciated.

Statement Regarding Previously Published Material with Multiple Authors

Chapter Two contains the data that I contributed to (Ades, S.E., Connolly, L.E., Alba, B.M. and Gross, C.A. (1999) The *Escherichia coli* σ^E-dependent extracytoplasmic stress response is controlled by the regulated proteolysis of an anti-σ factor. Genes Dev 13:2449-2461), and is reprinted here with permission from Cold Spring Harbor Laboratory Press (page x), who retains all copyright privileges. I conceived of and carried out the experiments in Table 2-1 and Figure 2-1. Chapter Two also contains previously published material from (Alba, B.M., Zhong, H.J., Pelayo, J.C. and Gross, C.A. (2001) *degS* (*hhoB*) is an essential *Escherichia coli* gene whose indispensable function is to provide σ^E activity. Mol Microbiol 40(6): 1323-1333), which is reprinted here with permission from Blackwell Science Ltd. (see page viii), who retains all copyright privileges. I conceived of and carried out nearly all experiments in this publication. Under my guidance, H.J. Zhong assisted with the repetitions of experiments required in Table One, and J.C. Pelayo (a 1999 Summer Research Training Program Student) constructed pBA106 and several strains. I wrote the text, which was edited by Carol A. Gross.

Chapter Three contains work previously published in (Alba, B.M., Leeds, J.A., Onufryk, C., Lu, C.Z., and Gross, C.A. (2002) DegS and YaeL participate sequentially in the cleavage of RseA to activate the σ^E-dependent extracytoplasmic stress. Genes Dev 16:2156-2168), which is reprinted here with permission from Cold Spring Harbor Laboratory Press (see page x) who retains all copyright privileges. This is a co-first

author paper with J.A. Leeds, who provided strains and plasmids (designated JAH). I conceived of all experiments and carried out all experiments except Figure 3-5, which was performed by C.Z. Lu under my direction. C. Onufryk collected additional relevant data that did not appear in the paper. I wrote the entire text, which was edited by Carol A. Gross and J.A. Leeds.

Chapter Four presents a manuscript that was co-submitted to Cell with a manuscript from our collaborator, Robert T. Sauer (Massachusetts Institute of Technology). This manuscript will not be published separately, since the reviewers asked that the two manuscripts be combined into one (Walsh, N. P., Alba, B. M., Bose, B., Gross, C. A., and Sauer R. T. (2003) OMP peptide signals initiate the envelope-stress response by activating DegS protease through relief of inhibitory interactions mediated by its PDZ domain. Cell). I conceived of and performed all of the experiments in Chapter 4 and wrote the entire text. Our collaborators (Nathan Walsh, Baundauna Bose and Robert Sauer) provided information about the relative binding affinities of peptides to the PDZ domain, which was instrumental in the experimental design. The text was edited by Carol A. Gross.

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Control of the σ^E-dependent extracytoplasmic stress response by regulated proteolysis

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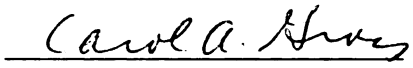

Carol A. Gross, Ph.D.
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Abstract

The *Escherichia coli* σ^E stress-response pathway controls the expression of genes encoding periplasmic folding catalysts, proteases, biosynthesis enzymes for lipid A (a component of lipopolysaccharide or LPS) and a number of other genes, most of which encode proteins that are known or predicted to either function in the envelope or affect envelope functions. RseA, the major negative regulator of σ^E activity, is a membrane-spanning protein whose cytoplasmic face functions as an antisigma factor. When *E. coli* is subjected to heat or other stresses that generate unfolded envelope proteins, especially unfolded outer membrane porins (OMPs), RseA is degraded and σ^E activity is induced. In this thesis, I have dissected the proteolytic pathway that degrades RseA and the signals

that induce this pathway. I present evidence that RseA is degraded by a proteolytic cascade initiated by the inner membrane protease DegS, a member of the HtrA/DegP protease family, and continued by YaeL, an ortholog of S2 protease, which carries out regulated intramembrane proteolysis in mammalian cells. I also show that DegS and YaeL are essential for *E. coli* viability because of their roles in activating σ^E, which is itself essential for *E. coli* viability. Finally, I have dissected one mechanism for inducing σ^E activity. I present evidence that the PDZ domain of DegS inhibits DegS protease activity. Additionally, I show that PDZ domain-binding peptides homologous to the C-termini of OMPs induce σ^E in a PDZ domain-dependent manner. The simplest explanation of these results is that such peptides, and by extension overexpressed and unfolded OmpC, promote DegS-mediated cleavage of RseA and subsequent σ^E activation by antagonizing the autoinhibitory PDZ domain of DegS. These findings significantly advance our understanding of how σ^E-inducing signals are detected and how they are transduced within the σ^E pathway. Moreover, they constitute the first evidence that binding of a ligand to the PDZ domain of a protease promotes degradation of a specific substrate of that protease.

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Chapter One

General Introduction

Summary

The *Escherichia coli* σ^E stress-response pathway controls the expression of genes encoding periplasmic folding catalysts, proteases, biosynthesis enzymes for lipid A (a component of lipopolysaccharide or LPS) and a number of other genes, most of which encode proteins that are known or predicted to function in the envelope. When *E. coli* is subjected to heat or other stresses that generate unfolded envelope proteins, σ^E activity is induced. Four key players in this signal transduction pathway have been identified: RseA, the inner membrane σ^E anti-sigma factor; RseB; and two proteases, DegS and YaeL. The major point of σ^E regulation is the interaction between σ^E and RseA, which is controlled by at least two strategies. Firstly, when envelope stress is present, RseA is destroyed by a proteolytic cascade initiated by DegS. Additionally, RseB, which may be a sensor of unfolded proteins, promotes RseA-dependent inhibition of σ^E activity. In the presence of one σ^E-specific stress, DegS is directly activated to cleave RseA by binding to the stress-inducing signal. Other levels of control upon σ^E activity will undoubtedly become evident upon further study of this stress response pathway.

Introduction

When heat or other stresses damage cellular constituents, cells induce the expression of genes whose products will counteract the damage. Numerous studies of *E. coli* under physiologically stressful conditions have elucidated how cellular damage is sensed and how transcription factors are activated to direct stress responses. As *E. coli* is a Gram-negative bacterium with two compartments, the envelope (including the inner membrane, murein layer, periplasm and outer membrane) and cytoplasm, there are

separate cellular stress response systems serving each compartment. The well-studied σ^{32}-dependent cytoplasmic stress response is induced by heat-shock and stresses that cause protein unfolding in the cytoplasm (Yura and Nakahigashi, 1999). Regulation of the cell envelope stress response is conceptually different from that of the cytoplasmic stress response, since the transcription factors that direct the envelope response reside in the cytoplasm while the stress signals are generated in the envelope. Thus, intercompartmental signal transduction pathways must be employed to activate the relevant transcription factors. There are two well-studied extracytoplasmic stress response pathways, one directed by the ECF (extracytoplasmic function) σ factor σ^E and the other by the two-component CpxAR system (reviewed in Raivio and Silhavy, 2001). This chapter will review recent advances in the understanding of the σ^E signaling system. These developments provide significant insights into the molecular strategies used by cells to convey information across biological membranes.

Roles of *E. coli* σ^E *in vivo*

σ^E activity was found to be required for high temperature expression of *rpoH* (encoding σ^{32}, or σ^H) as well as *degP*, encoding a periplasmic protease required for viability at high temperatures (reviewed in Raivio and Silhavy 2001). This suggested that *E. coli* had at least two heat-inducible regulons that protected the cell from heat-induced damage. The first evidence that σ^E responded specifically to extracytoplasmic stress came from a genetic selection for inducers of σ^E activity (Mecsas et al., 1993). Overexpressed outer membrane porins (OMPs), such as OmpC, strongly induced σ^E activity, and export of the OMPs to the periplasm was required for σ^E induction (Mecsas et al., 1993).

Moreover, strains bearing defective *ompR* alleles, which decreased the levels of porins in the outer membrane, exhibited significantly lower σ^E activity (Mecsas et al., 1993). Thus, σ^E activity is particularly attuned to the levels of OMPs in the envelope, which suggests that σ^E may play a role in OMP biogenesis, perhaps through providing the appropriate levels of folding catalysts for the task.

Other evidence that σ^E activity was modulated by extracytoplasmic stress came from the isolation of mutants lacking functional periplasmic folding catalysts or disulfide bond oxidoreductases, which exhibited higher σ^E activity (Raina et al., 1995; Rouvière and Gross, 1996; Missiakas et al., 1996). These mutants are expected to have elevated levels of unfolded envelope proteins. Strains with a mutation in *rfaD/htrM*, which produce abnormal LPS, had elevated σ^E activity as well (Missiakas et al., 1996). Additionally, strains expressing other periplasmic proteins expected to be unfolded *in vivo* exhibited higher σ^E activity (Betton et al., 1996; Jones et al., 1997).

σ^E is required for viability not only at heat-shock temperatures but also at lower temperatures (De Las Peñas et al., 1997b). A frequently arising suppressor(s) of Δ*rpoE* (the gene encoding σ^E) lethality restores viability only at lower temperatures (De Las Peñas et al., 1997b). The physiological basis for the essentiality of σ^E is unknown, but identifying essential members of its regulon may provide insights. Two studies that characterized the σ^E regulon indicated that σ^E controls the expression of genes encoding periplasmic folding catalysts, biosynthesis genes for lipid A and membrane-derived oligosaccharides, putative lipoproteins, and other proteins with known or predicted periplasmic functions (Dartigalongue et al., 2001b; Rhodius et al. in prep.). The composition of the σ^E regulon suggests that a major σ^E function is to maintain the

integrity of the envelope and that σ^E is essential because the expression of one or more of its regulon members is critical for maintaining envelope integrity.

Regulators of σ^E activity

RseA, RseB and RseC

RseA, RseB and RseC are encoded within the σ^E operon and involved in signal transduction through the inner membrane to σ^E (De Las Peñas et al., 1997a; Missiakas et al., 1997). RseA and RseB are two negative regulators of σ^E activity, while RseC may play a minor positive role in regulating σ^E (De Las Peñas et al., 1997a; Missiakas et al., 1997). RseA is a σ^E-specific anti-sigma factor with a periplasmic C-terminal domain and an N-terminal anti-sigma factor cytoplasmic domain (RseA-cyto) (De Las Peñas et al., 1997a; Missiakas et al., 1997). The crystal structure of RseA-cyto complexed with σ^E indicates that RseA blocks the major binding determinants on σ^E for RNA polymerase (Campbell et al., 2003). The structure also explains previously isolated RseA and σ^E mutants that resulted in, respectively, higher σ^E activity *in vivo* or decreased binding to the RseA-cyto *in vitro* (Missiakas et al., 1997; Tam et al., 2002). Strains lacking *rseA* exhibit a 25-fold increase in basal σ^E activity, which is not further inducible by OmpC overexpression (a specific inducer of σ^E activity) (De Las Peñas et al., 1997a). This indicates that RseA is the major negative regulator of σ^E.

RseB is a soluble periplasmic protein that binds to the periplasmic domain of RseA (De Las Peñas et al., 1997a; Missiakas et al., 1997). Strains lacking *rseB* exhibit a small, 2-fold increase in basal σ^E activity. RseB accumulates in periplasmic inclusion bodies that form when a mutant MalE protein is overproduced (Collinet et al., 2000). Additionally, the interaction between RseA and σ^E is strengthened about 2-fold when

RseB binds to RseA, at least under the high detergent concentrations used (Collinet et al., 2000). There also is an apparent decrease in the amount of σ^E that co-purifies with RseA when RseB is absent (Collinet et al., 2000). Taken together, these data suggest that RseB may act as a sensor of unfolded periplasmic proteins. When stress conditions prevail, RseB is titrated away from RseA by unfolded proteins, resulting in a reduced affinity of RseA for σ^E and, hence, more free σ^E to direct transcription (Fig. 1-1 A, *Left*) (Collinet et al., 2000).

RseB also influences the stability of RseA. A strain lacking *rseB* showed a 2-fold decrease in RseA half-life, which correlated with the approximately 2-fold increase in σ^E activity (Ades et al., 1999). The binding of RseB to RseA may occlude the cleavage site, or, alternatively, RseB may directly regulate DegS activity (Fig. 1-1 A, *Right*).

Regulated proteolysis of RseA

A number of studies have demonstrated that RseA activity is governed by regulated proteolysis. When *E. coli* is subjected to heat-shock temperatures (43°C) or when the outer membrane porin OmpC is overproduced, RseA half-life decreases markedly while σ^E activity increases (Ades et al., 1999; Collinet et al., 2000). RseA is somewhat unstable even in the absence of stress, most likely because of a steady-state, low-level of unfolded proteins as a consequence of normal growth (Ades et al., 1999).

The half-life of RseA is inversely correlated with σ^E activity during the initiation (shift to 43°C), adaptation (prolonged growth at 43°C) and shut-off (shift from 43°C to 30°C) phases of the response, although there are complexities that suggest the existence of other controls upon σ^E activity (Ades et al., 2003). Experiments of Ades and

colleagues demonstrated that the effect of altering the stability of RseA is more complicated than simply changing the amount of free σ^E in the cell by altering the ratio of σ^E to RseA. This result is particularly apparent during the shut-off phase of the stress response. Within five minutes upon entering the shut-off phase, the half-life of RseA increased to a level approximately 10-fold higher than in cells grown at 43°C, and σ^E activity decreased 8-15-fold compared to the stressed cells. However, the relative levels of RseA and σ^E did not change, which suggests that changes in the RseA:σ^E ratio are not sufficient to explain the regulation of σ^E activity during the shut-off phase (Ades et al., 2003). What could account for these observations? Other proteins could modulate the interaction of RseA with σ^E, but a kinetics-based model could also account for the behavior of σ^E activity (Ades et al., 2003). For example, if the dissociation rate of σ^E from RseA were slower than the degradation rate of RseA, then σ^E activity would be determined by the degradation rate of RseA (Ades et al., 2003).

DegS and YaeL sequentially cleave RseA

A growing body of evidence indicates that two inner membrane proteases, DegS and YaeL, participate sequentially in the proteolysis of RseA to activate σ^E. DegS, a member of the HtrA/DegP family of proteases (Clausen et al., 2002), is required for σ^E induction and the concomitant RseA degradation observed when OmpC is overexpressed (Ades et al., 1999). These data suggest that OmpC overexpression induces RseA degradation in a DegS-dependent manner. Most recently, it has been shown *in vitro* that a purified, soluble form of DegS lacking its transmembrane domain directly cleaves the periplasmic domain of RseA (Walsh et al., 2003). Moreover, the observation that *degS* is

essential for cellular viability because it is required to provide σ^E activity further substantiates the importance of DegS-dependent degradation of RseA (Alba et al., 2001).

In addition to DegS, YaeL plays a positive role in regulating the σ^E pathway, as it is required for RseA degradation and σ^E activation in response to OmpC overexpression (Alba et al., 2002; Kanehara et al., 2002). YaeL is an inner membrane Zn^{2+}-metalloprotease that is essential for viability (Dartigalongue et al., 2001b; Kanehara et al., 2001). In the absence of *yaeL*, an RseA degradation intermediate accumulates (Alba et al., 2002; Kanehara et al., 2002). This intermediate contains the cytoplasmic anti-sigma factor domain, the transmembrane domain and a portion of the periplasmic domain. Additionally, the generation of this fragment is dependent upon the presence of *degS* (Alba et al., 2002; Kanehara et al., 2002). These data suggest that RseA is first cleaved within its periplasmic domain by DegS, which generates the intermediate noted above. YaeL then cleaves this intermediate, which ultimately leads to the release of σ^E. The physiological importance of YaeL in activating σ^E is further substantiated by the finding that the lethality of Δ*yaeL* is rescued by a genetic background overexpressing *rpoE* or by mutant backgrounds containing Δ*rseA* (Alba et al., 2002; Kanehara et al., 2002) or either of the suppressor(s) of Δ*rpoE* or Δ*degS* lethalities (Alba et al., 2002).

In contrast to these findings, another report concluded that YaeL was a negative regulator of σ^E activity (Dartigalongue et al., 2001a). Multicopy *yaeL* decreased σ^E activity and steady-state level *in vivo*, while purified YaeL degraded σ^E *in vitro* (Dartigalongue et al., 2001a). However, the *in vivo* results were not reproducible in other laboratories (Alba et al., 2002; Kanehara et al., 2002). While the basis of this discrepancy is not known, it could be related to the use by Dartigalongue and Raina (2001a) of

arabinose-inducible constructs (to overexpress *yaeL*) in an Ara$^-$ strain, which grows poorly in the presence of arabinose. Alternatively, the *yaeL*-expressing plasmid used in the first report may have contained a portion of the downstream open reading frame that could be responsible for their observations.

YaeL, an orthologue of mammalian Site-2 protease

YaeL is an orthologue of the mammalian Site-2 protease (S2P), which participates in regulated intramembrane proteolysis events that activate two integral membrane transcription factors, ATF6 and SREBP. ATF6 is an important regulator of gene expression during the unfolded protein response in the endoplasmic reticulum, while SREBP controls the expression of cholesterol biosynthesis genes (Brown and Goldstein, 1999). These transcription factors are cleaved by two integral membrane proteases, firstly within their lumenal domains and then by S2P within their transmembrane domains (Brown et al., 2000; Ye et al., 2000b). This releases a soluble transcription factor domain that can enter the nucleus. Thus, remarkably, the use of two integral membrane proteases to activate integral membrane transcription factors has been conserved from *E. coli* to humans.

By analogy to the Site-2 protease cleavage site in SREBP, YaeL may cleave RseA at a cysteine residue in itstransmembrane domain, leaving the cytoplasmic anti-sigma factor domain intact. How, then, does σ^E ultimately get released? If YaeL cleaves RseA, then the resulting RseA fragment would have a non-polar tail, which would likely target the intermediate to cytoplasmic proteases that recognize these tails (Alba et al., 2002).

Mechanism of signal transduction by DegS

A recent study that focused on the C-terminal PDZ domain of DegS found that this domain is important for signal-sensing and signal transduction within the σ^E pathway (Walsh et al., 2003). A model was proposed in which σ^E is activated when OmpC-like peptides bind to the PDZ domain and stimulate DegS-dependent cleavage of RseA. Evidence supporting this model includes the following. Strains carrying a variant of DegS lacking its PDZ domain (DegSΔPDZ) exhibit higher basal σ^E activity *in vivo*, which suggests that the PDZ domain plays a negative regulatory role. Also, the PDZ domain of DegS binds selectively to peptides that terminate in a TyrXPhe (YXF) tripeptide, where X is any amino acid (aa) (Walsh et al., 2003). This tripeptide is conserved within most *E. coli* OMPs, the specific modulators of σ^E activity.

The overexpression of a periplasmic, 50 aa-long C-terminal peptide of OmpC, which ends in TyrGlnPhe (YQF), induces σ^E *in vivo* (Walsh et al., 2003). This suggests that the DegS PDZ domain directly senses the OmpC-related signal, at least in part, through binding to the C-terminus of OmpC. Three additional lines of evidence substantiate this idea: a) peptides terminating in tripeptides that are not expected to bind to the PDZ domain do not induce σ^E *in vivo*; b) a peptide that binds more tightly to the PDZ domain induces σ^E more strongly; c) σ^E is generally not induced by the overexpression of inducing peptides in strains carrying only DegSΔPDZ (Walsh et al., 2003).

Taken together, these data suggest that the C-terminus of OmpC binds to the PDZ domain and relieves its autoinhibitory effect, thereby allowing cleavage of RseA (Walsh et al., 2003) (Fig. 1-1 B, *Left*). This model is supported by *in vitro* data showing that the

cleavage of RseA periplasmic domain by soluble DegS is stimulated by peptides homologous to OMP C-termini (Walsh et al., 2003). From a biological perspective, the molecular coupling of stress-sensing with DegS activation is one of the most rapid ways to transduce a stress signal to σ^E. This mechanism of protease activation has broad implications for members of the HtrA/DegP family of proteases, which are found in organisms from bacteria to humans. In the case of human HtrA2 (Li et al., 2002), a regulator of apoptosis, it was hypothesized that ligand binding to its C-terminal PDZ domain relieves PDZ-mediated autoinhibition as well. The recent revelations about DegS confirm that this mode of regulation can be employed.

Concluding remarks

Multiple inputs into the σ^E pathway

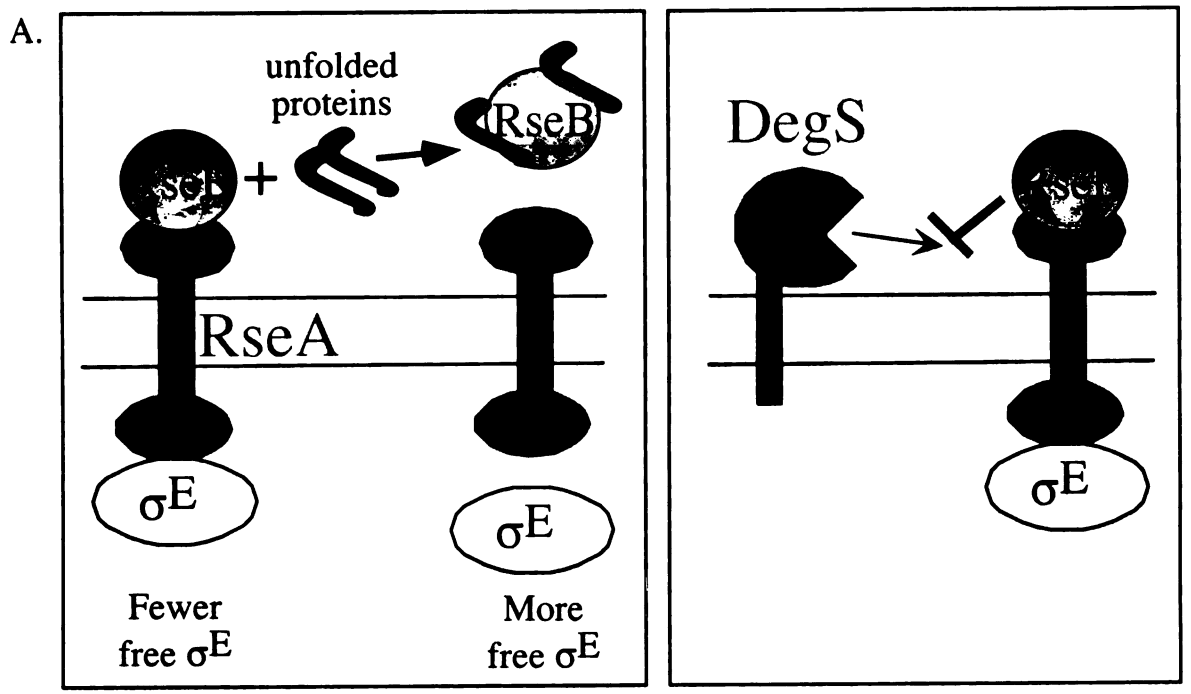
The σ^E pathway apparently has multiple modes of regulation upon the interaction between RseA and σ^E, the major means of regulating σ^E activity. RseB may regulate the interaction between RseA and σ^E both by altering the affinity of RseA for σ^E and by controlling the degradation of RseA. Additionally, activated DegS initiates the proteolytic cascade that ultimately frees σ^E from RseA. Why might σ^E be regulated in multiple ways? RseB and DegS each may preferentially sense a subset of unfolded periplasmic proteins, or, more generally, particular types of envelope damage, thereby allowing multiple 'inputs' into the signal transduction pathway that controls σ^E activity. As RseB may regulate the interaction between RseA and σ^E via two mechanisms, RseB could integrate its sensing of unfolded proteins with inputs from the DegS-dependent degradation pathway to allow a graded response to differing types and/or levels of stress.

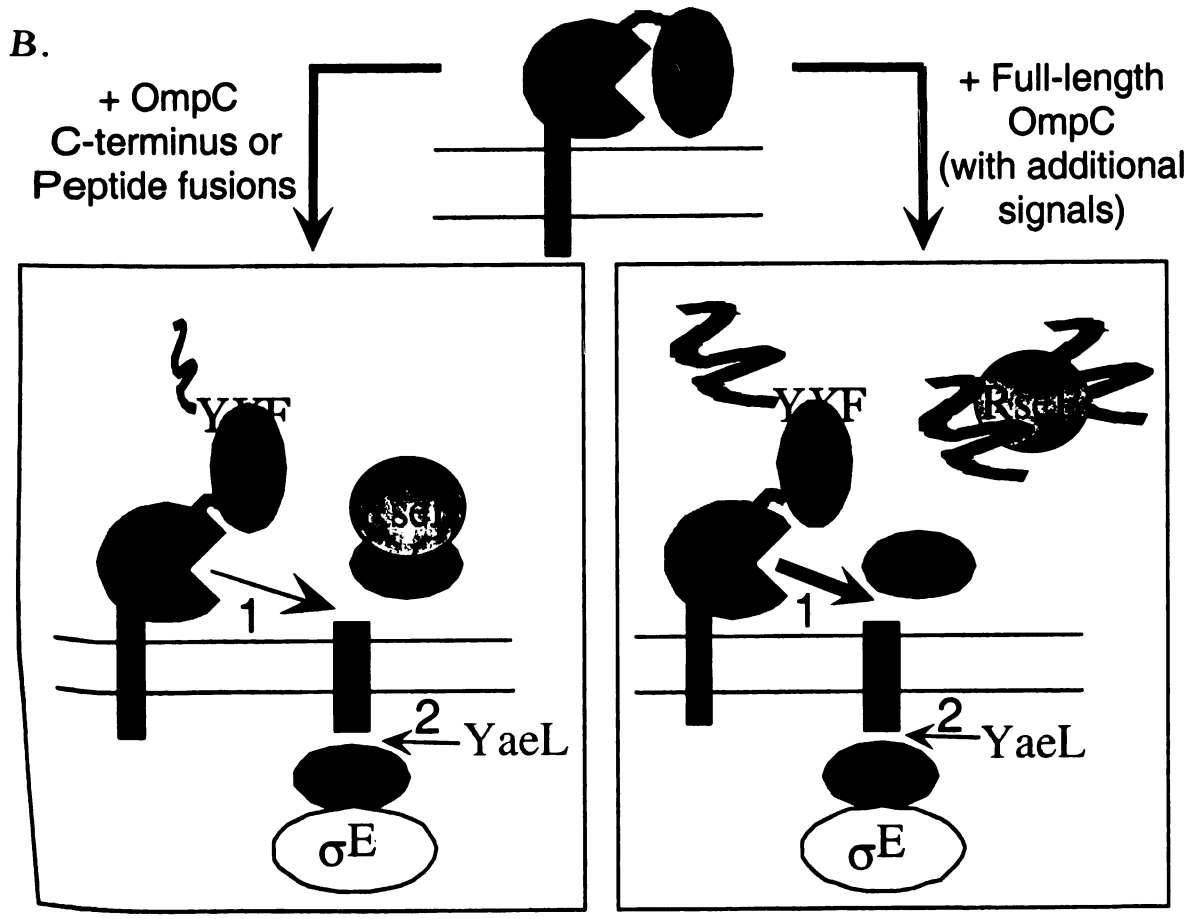
A particularly harsh stress (such as OmpC overexpression) could cause types or levels of damage that would both activate DegS protease activity and titrate RseB away from RseA, thereby maximizing RseA degradation and release of σ^E (Fig. 1-1 B, *Right*). Indeed, overexpression of full-length OmpC still induces σ^E in a DegSΔPDZ background, which indicates that OmpC produces other σ^E-activating signals in addition to the OmpC C-termini (B. Alba and C. A. Gross, unpubl. data; see Chapter 4). These additional signals may be sensed by other components of the pathway, perhaps via an interaction with folding catalysts that regulate σ^E activity. OmpC could also titrate away RseB from RseA, allowing a decrease in the interaction between RseA and σ^E and/or increased RseA cleavage because it becomes more accessible to DegS.

Intriguingly, the σ^E pathway may utilize OMPs as an indirect monitor of the health of the envelope (Walsh et al., 2003). OMP biogenesis is a complex process that requires folding catalysts and LPS for efficient trimerization and insertion into the outer membrane (Danese and Silhavy, 1998). If a stress generates unfolded proteins that increase folding catalyst occupancy or damages LPS integrity, for example, intermediates of OMP biogenesis may accumulate and activate DegS, which then would result in the induction of σ^E-controlled folding catalyst and lipid A biosynthesis genes, whose products will attempt to repair cellular damage. Alternatively, other stresses such as heat-shock, defective LPS, or other unfolded periplasmic proteins may be sensed by the σ^E pathway via different mechanisms. For example, heat-shock may induce or activate other proteases that can cleave RseA or directly weaken the RseA/σ^E interaction between RseA and σ^E. Furthermore, other signaling pathways that sense additional stresses may modulate σ^E-dependent gene expression, either directly through post-translational

modifications of σ^E or coordinately with other transcription factors that act in concert with σ^E.

Figure 1-1. Models showing the multiple controls upon σ^E activity. (A) RseB controls the affinity of RseA for σ^E and the stability of RseA. (*Left*) In the absence of unfolded proteins, RseB is predominantly bound to RseA, resulting in a complex with a higher affinity for σ^E, and, consequently, less free σ^E. Upon binding to unfolded proteins, RseB is titrated away from RseA, resulting in a complex with a lower affinity for σ^E, and, thus, more free σ^E (Collinet et al., 2000). (*Right*) RseB stabilizes RseA, perhaps by occluding the DegS cleavage site in RseA (Ades et al., 1999). (B) Other controls upon RseA degradation (Walsh et al., 2003). (*Left*) YXF C-termini of OMPs that bind to the PDZ domain relieve PDZ-mediated inhibition and activate DegS protease activity, establishing one level of RseA degradation. (*Right*) When full-length OMPs are overproduced, additional signals besides the YXF C-termini feed into the σ^E pathway, perhaps by titrating RseB away from RseA. This leads to an even higher level of RseA degradation because it allows greater accessibility of RseA to DegS. In the ensuing proteolytic cascade, RseA is cleaved in its periplasmic domain by DegS (1) and then near or in its transmembrane domain by YaeL (2). Other proteases may cleave the YaeL-generated RseA degradation intermediate to release σ^E (not shown; Alba et al., 2002; Kanehara et al., 2002).


A.
unfolded
proteins
RseB
+
RseA
σE
σE
Fewer
free σE
More
free σE
DegS
σE


B.
+ OmpC
C-terminus or
Peptide fusions
+ Full-length
OmpC
(with additional
signals)
YYF
1
2
YaeL
σE
YYF
1
2
YaeL
σE

Chapter Two

DegS controls the activity of σ^E by regulating the proteolysis of RseA

Summary

The major negative regulator of σ^E activity, the anti-sigma factor RseA, is rapidly degraded when *Escherichia coli* is subjected to heat-shock temperatures or when the outer membrane porin OmpC is overexpressed (Ades et al., 1999). Here, we show that DegS (HhoB), a putative serine protease related to DegP/HtrA, regulates the basal and induced activity of the essential *Escherichia coli* σ factor σ^E by controlling the proteolysis of RseA. We demonstrate that *degS* is an essential *E. coli* gene, and we show that the essential function of DegS is to provide the cell with σ^E activity. We also show that the putative active site of DegS is periplasmic and that DegS requires its N-terminal transmembrane domain for its σ^E-related function.

Introduction

Escherichia coli has a number of well-characterized cytoplasmic proteases that perform regulatory functions in addition to their housekeeping role of degrading damaged or non-native proteins in the cell. The heat shock-inducible proteases ClpP, Lon, HflB and ClpYQ (HslUV) all modulate the activities of regulatory proteins (Kanemori et al., 1997; Kanemori et al., 1999; Wu et al., 1999). For example, ClpXP participates in the turnover of the stationary phase transcription factor σ^S (Pratt and Silhavy, 1996; Schweder et al., 1996) and Lon controls the activity of both the cell division inhibitor SulA (Mizusawa and Gottesman, 1983) and a regulator of capsular polysaccharide production, RcsA (Torres-Cabassa and Gottesman 1987; Stout et al., 1991). HflB, an essential inner membrane protease with a cytoplasmic active site, degrades the heat shock σ factor σ^{32} (Herman et al., 1995) and the λ phage regulatory protein *c*II (Hoyt et al., 1982; Banuett et al., 1986; Shotland et al., 1997) in addition to performing the general housekeeping task of degrading inner membrane proteins that are not complexed with their normal protein partners (Kihara et al., 1995).

In contrast to the many cytoplasmic proteases whose functions have been well characterized, the dual roles of periplasmic proteases have not been thoroughly explored. To date, only apparent housekeeping roles have been described for DegP and Tsp (Prc). DegP, required for viability at high temperatures and protection from oxidizing conditions, is likely the major protease responsible for degrading damaged or non-native proteins in the envelope (Strauch and Beckwith, 1988; Strauch et al., 1989; Lipinska et al., 1989; Lipinska et al., 1990; Kolmar et al., 1996; Kim et al., 1999; Skorko-Glonek et al., 1999). Tsp recognizes and degrades proteins that have been tagged by SsrA tails,

which are added to incompletely translated proteins (Silber et al., 1992; Keiler et al., 1995; Keiler et al., 1996; Keiler and Sauer, 1996). Another periplasmic protease and DegP paralogue, DegQ/HhoA, has been shown to rescue the conditional lethalities of both *prc* and *degP* deletions, but its *in vivo* functions are unknown (Bass et al., 1996; Waller and Sauer, 1996).

There is evidence that the putative periplasmic serine protease DegS, another DegP paralogue, may possess a regulatory role. DegS is required for normal σ^E activity *in vivo*, as a *degS* deletion results in a five-fold reduction in basal σ^E activity (Ades et al., 1999). The σ^E signal transduction pathway includes the inner membrane σ^E-specific anti-sigma factor RseA, which is rapidly degraded when an inducing stress such as heat shock or the overproduction of an outer membrane porin is present (De Las Peñas et al., 1997a; Missiakas et al., 1997; Ades et al., 1999). The putative active site serine of DegS is required for basal σ^E activity, as mutating it to alanine abolishes the cellular ability to degrade RseA (Ades et al., 1999). This strongly suggests that DegS functions as a protease *in vivo* and that it participates in the degradation of RseA.

degS was initially identified only by its homology to DegP (Bass et al., 1996; Waller and Sauer, 1996). *degS* expression is apparently controlled by σ^{70} and is not heat-inducible (Waller and Sauer, 1996). Waller et al. (1996) noted that their *E. coli degS* deletion strain exhibited a small colony phenotype and that faster growing mutants frequently arose, while Bass et al. (1996) reported that their *E. coli degS* deletion strains were phenotypically variable and that mutants also readily arose. These observations suggest that *degS* plays an important, and possibly essential, role in *E. coli* physiology.

Because DegS is involved in σ^E regulation and because it is critical for normal cellular growth, we sought to understand its physiological role more completely. We show that DegS is required for the induction of σ^E activity and RseA degradation when OmpC is overexpressed. We also used two genetic approaches to demonstrate that *degS* is an essential gene in *E. coli* and showed that the essential function of DegS is to provide the cell with active σ^E, which further substantiates the importance of DegS-dependent RseA degradation. We also show that the protease domain of DegS is periplasmic and that its membrane localization is required for its *in vivo* σ^E-related function.

Results

DegS is required for activation of σ^E when OmpC is overexpressed

As *degS* is required for basal σ^E activity (Ades et al., 1999), we wanted to determine if *degS* is also required for induced σ^E activity. To test this possibility, we overexpressed OmpC, a specific σ^E inducer (Mecsas et al., 1993), and assayed σ^E activity in *degS*$^+$ and Δ*degS* backgrounds. As otherwise wild-type strains deleted for *degS* grow very poorly and accumulate faster-growing suppressor mutations, we transferred Δ*degS* to the "suppressor of Δ*rpoE*" (*sup*$^+$) background (De Las Peñas et al. 1997b), which ameliorated some of the growth defects caused by the Δ*degS* allele. We found that overexpression of OmpC in *sup*$^+$ strains induced σ^E activity, indicating that the σ^E pathway was functional in the *sup*$^+$ background (Table 2-1). In contrast, OmpC overexpression in isogenic *sup*$^+$ Δ*degS* strains failed to induce σ^E activity (Table 2-1). Plasmid-encoded wild-type *degS*, but not *degS* carrying the S201A active site mutation, restored σ^E inducibility to *sup*$^+$ Δ*degS* strains (Table 2-1). Cell fractionation experiments showed that overexpressed OmpC was localized to the outer membrane in both the *degS*$^+$ and Δ*degS* backgrounds, indicating that the lack of σ^E induction in the Δ*degS* background was not due to the failure of OmpC to be overexpressed or localized to the envelope (data not shown). Also, Western blotting showed that wild-type DegS and DegS S210A were expressed at approximately equivalent levels (data not shown). Thus, these data demonstrate that DegS and its putative protease activity is required for σ^E induction in response to OmpC overexpression.

DegS is required RseA degradation

As DegS, including its active site, was required for σ^E induction in response to OmpC overexpression, we considered the possibility that DegS controlled the degradation of RseA. We again used the *sup*$^+$ background, which allowed Δ*degS* cells to grow well in M9 pulse-chase minimal media. We measured the half-life of RseA in *degS*$^+$ and Δ*degS* backgrounds with or without OmpC overexpression. In the absence of stress, RseA was much more stable in the Δ*degS* background than in the wild-type background (Fig. 2-1). When OmpC was overexpressed, we found that RseA was rapidly degraded in the *degS*$^+$ background, but not in the Δ*degS* background (Fig. 2-1). Thus, these data show that DegS is required for both the basal and induced degradation of RseA.

DegS is an essential gene in *E. coli*

The variable characteristics of *degS* deletion strains suggested that *degS* might be an essential gene and that suppressor mutation(s) allowing the cell to bypass the requirement for *degS* arise frequently. We used two genetic tests to determine whether *degS* is an essential gene in *E. coli*: DegS depletion and cotransduction of Δ*degS* with a linked marker. In the depletion strains, the chromosomal wild type *degS* promoter was replaced with the IPTG-inducible promoter $P_{LlacO-1}$ (Lutz and Bujard, 1997) so that expression of DegS could be shut off in the absence of IPTG. We examined the essentiality of *degS* in three common laboratory strain backgrounds, MC1061, MC4100, and MG1655.

We first examined the essentiality of DegS in MC1061 $P_{LlacO-1}$ *degS* and MC4100

$P_{LlacO-1}$ *degS* by comparing the efficiency of plating (EOP) when DegS is absent (no IPTG) to that when DegS is present (IPTG present) (Table 2-2). These experiments were performed on both rich (LB) medium and on M9 minimal medium supplemented with glucose and amino acids (referred to as M9 complete minimal media hereafter) for MC1061 $P_{LlacO-1}$ *degS* but only on rich medium for MC4100 $P_{LlacO-1}$ *degS*. When DegS expression was shut off, few cells were able to grow at any temperature on rich medium, showing EOPs between 10^{-5} and 10^{-3}. MC1061 $P_{LlacO-1}$ *degS* cells growing on M9 complete minimal at 30°C and 37°C also failed to grow when DegS was depleted (Table 2-2). Surprisingly, these cells exhibited an EOP of 0.6 at 43°C, forming small colonies with a jagged morphology (Table 2-2). However, when these colonies were restreaked onto M9 complete minimal media plates incubated at 30°C or 43°C, we estimated that less than 1% of the cells in these colonies were viable. Colony formation may have been due to a physiological alteration that allowed growth for a limited number of doublings before cell death. These results suggest that DegS is required for cell growth at all temperatures tested.

As an additional test for essentiality, we examined the cotransduction of Δ*degS* with the closely linked marker *argR*::Tn*5* (Kan^R) into wild type MC1061 carrying either an empty vector or *degS* on a plasmid. Kanamycin-resistant transductants were selected at 30°C on LB/kanamycin for 24 hours and the presence of the Δ*degS* allele was scored by colony PCR. If *degS* were an essential gene, then cotransduction of the linked loci would occur at the expected frequency (~90%) only when extra *degS* is provided by a plasmid. Indeed, this proved to be the case (Table 2-3). A control transduction indicated that each recipient was equally transducible by P1 phage (data not shown). However,

when transduction plates were incubated for up to 72 hours prior to scoring, Δ*degS* colonies were observed, sometimes reaching a frequency of up to 30%. This frequency is significantly lower than the expected, and evidence presented later indicates that these small late-arising colonies are able to grow because they contain a suppressor mutation. Taken together, the results of the two genetic tests indicate that *degS* is an essential gene in MC1061 and MC4100.

We employed the same two genetic tests to ask whether *degS* is essential in MG1655 $P_{LlacO-1}$ *degS*, a third common laboratory strain. As was the case for the other two strains, MG1655 $P_{LlacO-1}$ *degS* was unable to grow at 42°C when DegS was depleted (Table 2-2). However, when DegS was depleted at 30°C and 37°C, the EOPs were approximately 1, although the colonies were one-third to one-half the size of colonies formed when DegS was present (Table 2-2). One possible explanation is that the MG1655 requirement for DegS is lower than that of MC1061 and MC4100, so that the residual expression from repressed $P_{LlacO-1}$ is sufficient for survival at lower temperatures. If so, we would expect that cells completely lacking DegS (because the *degS* gene had been removed) would be unable to grow. Consistent with this idea, we were unable to cotransduce Δ*degS* with the linked marker *argR::*Tn*5* into MG1655, although a control P1 transduction indicated that the MG1655 strain could be transduced by P1 phage (data not shown). Thus, DegS is also essential in MG1655. As three commonly used strains of *E . coli* all required some DegS for growth, we conclude that *degS* is an essential gene.

We also examined the *in vivo* effect of DegS depletion. We grew the MC1061 DegS depletion strain to mid-exponential phase in M9 complete minimal media plus IPTG, harvested the cells, washed away the IPTG, and then resuspended the cells in M9

complete minimal media with or without IPTG. Samples were taken during the course of depletion and analyzed by Western blotting. After approximately 45 minutes of depletion, the amount of DegS had dropped significantly (Fig. 2-2A), whereas in the presence of IPTG, DegS remained constant (Fig. 2-2B), indicating that this strain significantly shut off *degS* expression under depletion conditions. As the DegS-depleted culture reached stationary phase, significant cell lysis was observed, a clear indication of the lethality of DegS depletion.

DegS is essential because it is required to provide active σ^E

As noted above, the *sup*$^+$ background ameliorates the growth defects of the Δ*degS* allele, although *sup*$^+$ Δ*degS* strains still exhibit the small colony phenotype (Waller and Sauer et al., 1996; data not shown). This suggests that the functions of DegS and σ^E are vitally linked *in vivo*. As σ^E is an essential sigma factor in *E. coli* (De Las Peñas et al., 1997b), it is possible that the indispensable function of DegS is to provide the cell with active σ^E by degrading RseA. If so, *degS* should not be essential in a strain background with high σ^E activity. To address this possibility, DegS was depleted in a strain lacking RseA (Δ*rseA* background) in which the absence of the RseA anti-sigma factor results in high constitutive σ^E activity (De Las Peñas et al., 1997a; Missiakas et al., 1997). At all temperatures tested (30°C, 37°C and 43°C), the EOP was approximately 1, indicating that DegS is not essential in this background (Table 2-2). As a control for DegS depletion, Western blotting indicated that DegS was below the level of detection in the Δ*rseA* background grown in the absence of IPTG (data not shown).

A caveat to the conclusion that regulating σ^E activity is the only essential function

of DegS is that members of the σ^E regulon are overexpressed in the Δ*rseA* strain. Overexpression of one or more regulon members could suppress the lethality caused by loss of DegS. Therefore, we also determined whether *degS* is essential in a background in which there is a significantly reduced requirement for σ^E activity. OmpR controls the production of the OmpC and OmpF porins, whose biogenesis is thought to be monitored by the σ^E pathway (Hall and Silhavy, 1981; Mecsas et al., 1993). In the absence of *ompR*, *ompC* and *ompF* are virtually unexpressed and basal σ^E activity is significantly lower than that in a wild type strain, suggesting that a Δ*ompR* strain has a reduced requirement for σ^E (Mecsas et al., 1993). Indeed, when DegS was depleted in this strain background, there was no reduction in the ability to form colonies at either 30°C or 37°C (Table 2-2), even though Western blotting indicated that DegS was below the level of detection (data not shown). However, at 43°C, when the heat shock functions of the σ^E pathway are indispensable, the EOP is 10^{-4} (Table 2-2). Thus, in Δ*ompR*, DegS can be depleted when there is a reduced need for σ^E (30°C and 37°C), but not at heat shock temperatures when σ^E activity must be induced to protect the cell from heat stress. Taken together, these results suggest that the essential function of DegS is to provide the cell with active σ^E.

We searched for other *degS* phenotypes using the Δ*rseA* DegS depletion strain (CAG43196) grown in the absence of IPTG at 30°C. As the σ^E-related *degS* phenotypes are eliminated by removing RseA, this background allows us to assess whether there are σ^E-independent *degS* phenotypes. We screened for an increased sensitivity of the strain in the absence versus the presence of IPTG to heavy metals [Cu(II), Ni(II), Cr(II), Co(II),

Fe(II), Cd(II)], antibiotics (ampicillin and rifampicin), and other chemicals (high NaCl, EDTA, SDS, sodium deoxycholate, DTT, H_2O_2, HCl, KOH, and NH_4Cl), but we did not uncover any other *degS* phenotypes (data not shown).

Suppressors of Δ*degS* also suppress Δ*rpoE*

We had previously found that *rpoE* is an essential gene but that frequently arising suppressors allow Δ*rpoE* strains to grow at 30°C and 37°C but not at 42°C (De Las Peñas et al., 1997b). Here we have presented evidence that DegS is essential and that its essential function is to provide σ^E activity. We therefore considered the possibility that the Δ*degS* strains that arise in MC1061 upon prolonged incubation contain the same or similar suppressors to those that allow cells to grow without *rpoE*. If this were the case, then *rpoE* should not be essential in the Δ*degS* background. We tested this idea by using the linked marker cotransduction test that we previously employed to show that *rpoE* was essential (De Las Peñas et al., 1997b). As expected, in wild type MC1061, *rpoE*::ΩCm could not be cotransduced with the approximately 100% linked *nadB3140*::Tn*10* marker (Table 2-4). In contrast, in our standard MC1061 Δ*degS* background (CAG33315), *rpoE*::ΩCm and *nadB3140*::Tn*10* were cotransduced at a frequency of 96%, exhibiting behavior indistinguishable from a strain bearing a suppressor of *rpoE*::ΩCm lethality (Table 2-4) and indicating that this Δ*degS* isolate harbors a suppressor of *rpoE*::ΩCm lethality.

We then asked whether it is generally true that the colonies arising 72 hours following cotransduction of Δ*degS* with the linked marker *argR*::Tn*5* harbor suppressors that bypass the cellular requirement for *rpoE*. We performed the same linked marker

cotransduction test described above on 4 of the late-arising Δ*degS* colonies, except that we screened for the presence of *rpoE*::ΩCm with PCR. These Δ*degS* colonies were poorly transducible in both the control and experimental transductions. However, we tested 9 transductants and found that in every case, the *nadB3140*::Tn*10* transductants also contained *rpoE*::ΩCm. As the two markers were cotransduced into the late-arising Δ*degS* colonies at a 100% frequency, we conclude that they had a suppressor that bypassed the cellular requirement for σ^E.

The putative active site of DegS is in the periplasm

Given that the primary regulatory role of DegS is to modulate the expression of the σ^E regulon, we assessed which features of the protein are important *in vivo*. The location of the putative active site of DegS has implications for the nature of its participation in the degradation of the antisigma factor RseA, an inner membrane protein with a single transmembrane domain separating the periplasmic and cytoplasmic domains (De Las Peñas et al., 1997a; Missiakas et al., 1997). Topology prediction programs suggest that DegS has an N-terminal inner membrane anchor, while the rest of the protein, including the putative active site and a C-terminal PDZ-like domain, is predicted to be in the periplasm (Jones et al., 1994; Waller and Sauer, 1996). Consistent with its predicted location as an inner membrane protein, we (unpublished results) and Waller et al. (1996) found that DegS cofractionates with *E. coli* membranes and that, as expected for an inner membrane protein, the N-terminal signal sequence was not removed (Waller and Sauer, 1996).

We confirmed the topology of DegS through the use of DegS-PhoA (alkaline phosphatase) fusions. As PhoA is highly active when localized in the periplasm, the activity of the fusion is indicative of the cellular location of that portion of the protein containing PhoA (Manoil and Beckwith, 1986). *phoA* lacking its signal sequence was inserted either immediately distal to the predicted N-terminal membrane anchor or in the PDZ-like domain at the C-terminus of *degS* (Fig. 2-3A). Both fusions yielded high PhoA activities relative to the negative control, PhoA lacking its signal sequence, indicating that these regions of DegS are on the periplasmic side of the membrane (Fig. 2-3B). Western blotting confirmed that PhoA lacking its signal sequence was produced at a level nearly equivalent to that of each fusion (data not shown). As there are no additional predicted transmembrane sequences between the sites of the two fusions, we conclude that the entire protein distal to the membrane anchor is on the periplasmic side of the membrane.

DegS requires its membrane anchor for σ^E-related activity

We assessed the importance of the membrane localization of DegS in regulating the σ^E pathway by comparing the ability of DegS with and without its N-terminal transmembrane domain to restore wild type basal σ^E activity to the MC1061 Δ*degS* strain (CAG33315). The basal level of σ^E activity in this strain is significantly lower than that of MC1061 *degS*$^+$, and this low activity is fully complemented by plasmid-encoded wild type DegS (Ades et al., 1999). We fused *degS* lacking its N-terminal transmembrane domain (ΔTM-DegS) to the heterologous signal sequence from the periplasmic protein PelB. Although plasmid-encoded ΔTM-DegS was exported to the envelope and produced

at a level at least ten times that of wild type DegS (data not shown), it failed to fully restore the basal σ^E activity of the Δ*degS* strain to the wild type level (Fig. 2-4). This indicates that DegS requires its transmembrane domain for wild type regulation of the σ^E pathway. The partial complementation exhibited by ΔTM-DegS may be a consequence of its overproduction.

Discussion

Mechanism of RseA degradation

We have demonstrated that DegS is required for σ^E induction and RseA degradation in response to OmpC overexpression. As the active face of DegS is located in the periplasm, DegS likely controls RseA stability via a functional interaction with the periplasmic domain of RseA. Since the active site serine of DegS is required for σ^E induction and RseA degradation, DegS most likely cleaves RseA directly, perhaps within its periplasmic domain. If DegS cuts RseA within its periplasmic domain, what is the fate of the cytoplasmic, anti-sigma factor domain that is sufficient to inhibit σ^E activity? DegS-mediated cleavage of RseA may inactivate the cytoplasmic domain, perhaps by causing a conformational change that releases σ^E. Alternatively, DegS could simply degrade the entire protein, or DegS may work in conjunction with another protease that cleaves/degrades the cytoplasmic domain. In this case, DegS may generate an RseA fragment that is subsequently recognized by the second protease, or the activity of the second protease may be coordinated with that of DegS.

degS essentiality

We have shown that *degS* is an essential gene in *E. coli*. If *degS* is confirmed to be a serine protease *in vitro*, then DegS would be the second known essential *E. coli* protease after HflB (Akiyama et al., 1994). We have also shown that the essential role of DegS is to provide the cell with σ^E activity, which is itself essential for viability (De Las Peñas et al., 1997b). Since DegS controls the stability of RseA, which inhibits σ^E-

dependent transcription, the absence of DegS results in much lower σ^E activity (Ades et al., 1999). We suggest that DegS-depleted strains die because of the loss of at least one essential activity of a σ^E regulon member. As σ^E regulon members are involved in extracytoplasmic functions, we expect that the ultimate cause of lethality is a derangement of the structural integrity of the outer membrane or periplasmic cellular functions(s).

Cells apparently require less DegS when σ^E activity is low. This is clearly demonstrated by comparing the requirements of wild type MC1061 and MC1061 Δ*ompR* for DegS. The basal activity (activity under non-stress conditions) of σ^E in MC1061 Δ*ompR* is 5-fold lower than that in wild type MC1061. The σ^E pathway is thought to monitor and promote outer membrane porin biogenesis; thus, the very low levels of OmpC and OmpF in the Δ*ompR* strain must lead to a reduced need for σ^E (Mecsas et al., 1993). Here we have shown that the reduced need for active σ^E correlates with a reduced need for DegS. Upon DegS depletion at 30°C and 37°C, MC1061 Δ*ompR* survives, while wild type MC1061 does not. However, at the heat shock temperature of 43°C, *degS* becomes essential even in the Δ*ompR* background because the stress-combative functions of the σ^E pathway must be induced. A graded need for DegS is also likely to explain why, under normal growth conditions at 30°C and 37°C, the MG1655 DegS depletion strain survives, presumably from residual *degS* expression even in the repressed state.

A key genotype difference between MG1655 and both MC1061 and MC4100 could account for the differential requirement for DegS. MG1655 is *relA*$^+$ *spoT*$^+$, while

MC1061 and MC4100 are *relA1* and/or *spoT1* (Casadaban, 1976; Casadaban and Cohen, 1980; Jensen, 1993), which renders them defective in mounting the stringent response (reviewed in Cashel *et al.* 1996). *relA1* and/or *spoT1* strains have difficulty adjusting to nutrient limitation and other stresses. Consequently, MC4100 and MC1061 could have a greater requirement for the σ^E stress-responsive pathway. Taken together, our results are consistent with the idea that there is a graded requirement for DegS which is directly related to the need for σ^E activity. This correlation supports our conclusion that the essential role of DegS is to activate the σ^E pathway.

Cells can circumvent the need for DegS with suppressor mutations. These suppressors are functionally equivalent to the suppressor(s) of Δ*rpoE* lethality that we have previously identified (De Las Peñas et al., 1997b). Our results indicate that Δ*degS* suppressors arise at a variable frequency. In the linked marker cotransduction experiments to introduce the Δ*degS* allele, MC1061 transductants carrying suppressor mutations arose frequently, but MG1655 transductants with suppressor mutations were not observed. Knowing the mechanism of suppression might facilitate understanding this variability. These suppressors may bypass the requirement for σ^E activity by facilitating the σ^E-independent expression of essential σ^E regulon members and/or by lowering the physiological requirement for σ^E.

degS-deleted or DegS-depleted cells accumulate suppressors at higher frequencies than other gene deletions. For example, suppressors of the temperature sensitivity of Δ*rpoH* (σ^{32}) arise at frequencies between 10^{-9} and 10^{-7} and involve the overexpression of two chaperones, *groEL* and *dnaK* (Kusukawa and Yura, 1988). In contrast, suppressors of Δ*rpoA* (α subunit of RNA polymerase) do not exist because no mutation can bypass

the requirement for this fundamental component of the transcription apparatus. These examples suggest that essential genes have differing levels of importance *in vivo*. While RpoA can never be replaced by another mutation, the requirement for σ^{32} can be bypassed by a change in the regulation of two of its downstream regulon members such that they are overexpressed by other σ factors. In the case of Δ*degS*, it is possible that other pathways that are normally minor contributors to the expression of essential σ^E regulon members may undergo mutations that facilitate survival through σ^E-independent mechanisms.

DegS topology and localization

We have shown that DegS requires its membrane anchor for its wild type σ^E-related activity and that DegS has a periplasmic active site and PDZ-like domain. The partial complementation of Δ*degS* by ΔTM-DegS suggests that when produced at a high level, ΔTM-DegS can alter the stability of RseA. We have not yet determined if this partial complementation is due to soluble ΔTM-DegS or to ΔTM-DegS that may be membrane-associated independently of the N-terminal portion of DegS.

As an inner membrane protein, DegS is in an ideal location for its participation in a signal transduction pathway that senses periplasmic stress and transduces it across the inner membrane to the cytoplasm. Presumably, DegS must be in close spatial proximity to RseA to regulate its stability. If DegS directly cleaves RseA, then the active site of DegS would have to be in the appropriate position to reach the RseA cleavage site. The colocalization of RseA and DegS in the inner membrane may poise DegS for precise and immediate cleavage of RseA in response to a periplasmic signal. Their colocalization

may also raise their local concentrations and facilitate interaction. Drawing a parallel between HflB and DegS, the membrane anchor of DegS could also facilitate its oligomerization into an active ring structure (Akiyama et al., 1995; Akiyama et al., 1998; Makino et al., 1999).

Certain DegP orthologues in other bacteria and eukaryotes may also be membrane-localized. hHtrA2, one of three known human HtrA-like proteins, and YkdA of *B. subtilis* are predicted to have membrane-spanning domains (Gray et al., 2000; Noone et al., 2000). $HtrA_{LI}$ from *Lactococcus lactis* is predicted to be a surface protease and has recently been shown to be required for the processing or maturation of two exported *L. lactis* proteins (Poquet et al., 2000). It will be interesting to determine if a subset of DegP family members, including DegS and $HtrA_{LI}$, has become specialized membrane-localized proteins that process, degrade or regulate only other membrane or secreted proteins.

Experimental Procedures

Materials

Luria-Bertani (LB) and M9 minimal media were prepared as described (Sambrook et al., 1989). Unless noted otherwise, M9 minimal media were supplemented with 0.2% glucose, 1 mM $MgSO_4$, vitamins and all amino acids (40 μg/ml) (referred to in text as M9 complete minimal media). When required, the media were supplemented with 30 μg/ml kanamycin (Kan), 20 μg/ml chloramphenicol (Cm), 10 μg/ml tetracycline (Tet), 100 μg/ml ampicillin (Ap), and/or 20 μg/ml spectinomycin (Spec), unless noted otherwise. The PhoA substrate Sigma-104, iodoacetamide and $CuCl_2$, $NiCl_2$, $CrCl_2$, $CoCl_2$, $FeCl_2$, and $CdCl_2$ were purchased from Sigma-Aldrich.

Strains

Bacterial strains used in this study are listed in Table 2-5. To prepare the appropriate strain for the Δ*degS argR*::Tn*5* cotransduction experiments, our Δ*degS* strain (Ades et al., 1999) was transduced to *argR*::Tn*5* with lysate grown on DS954 (*argR*::Tn*5*). Kanamycin resistant transductants were screened for linkage to Δ*degS* by colony PCR.

Strain CAG43173 was constructed as follows. pBA106 was digested with *Eco*RI/*Pst*I, yielding an approximately 3.8 kb fragment, which carries Ω spectinomycin-$P_{LlacO-1}$ *degS* flanked by approximately 850 base pairs of *degQ* and *degS* sequence to facilitate homologous recombination. The 3.8 kb fragment was electroporated into the hyper-recombinogenic strain KM9 (Murphy, 1998), and recombinants were selected on

LB/ Spec/ 1 mM IPTG. $Spec^R/Amp^S$ transformants were subjected to colony PCR to confirm the presence of $P_{LlacO-1}$ *degS*.

CAG43187, CAG43189, CAG43190 and CAG43248 were constructed by transducing CAG48026, CAG48027, CAG48028 and CAG43244, respectively, with a P1 lysate grown on CAG43173. $Spec^R$ transductants were screened for the presence of the chromosomal $P_{LlacO-1}$ *degS* by colony PCR. To construct CAG43196, CAG43187 was transduced to Δ*rseA nadB*::Tn*10* with lysate grown on CAG22357. The cotransduction of the Δ*rseA* allele was confirmed by colony PCR. To construct CAG43219, CAG43187 was transduced to *ompR*::Tn*10* with lysate grown on SG460. As with the *ompR*::Mu allele (Mecsas et al., 1993), *ompR*::Tn*10* exhibits a five-fold decrease in basal σ^E activity (data not shown). The above transductants were selected on LB/ Tet/ Kan/ 1 mM IPTG at 30°C.

Plasmids

pBA106, carrying $P_{LlacO-1}$ *degS*, was constructed in 4 steps. All PCR steps employed *Pfu* polymerase (Stratagene) and purified MC1061 genomic DNA as a template, unless noted otherwise. Firstly, Ω spectinomycin was amplified with OC31 (5'-CCGTCTAGAGCTTTATGCTTGTAAACCGTTTTGTG-3') and cloned into *Xba*I of pUC18. Secondly, $P_{LlacO-1}$ was generated by PCR using OC57 (5' GCCCTGCAGCCATGGAGATCTGGTCAGTGCGTCCTGCTGATGTGCTCAGTATC TTGTTATCCGCTCACAAT-3') and OC58 (5'CCGTCTAGAATAAATGTA-GCGGATAACATTGACATTGTGAGCGGATAACAAGATACTG-3') (Lutz and Bujard, 1997). The $P_{LlacO-1}$ was cloned into *Xba*I/*Pst*I of the first intermediate above.

Thirdly, DegS1 (5'-GGGCCATGGGCTGCTGCCGTTCCC-3') and DegS2 (5'-GGGCTGCAGCCGC-CGATACCAATG-3') were used to amplify a portion of the *degS* locus, including the *degS* ribosome binding site and approximately 840 bp downstream of *degS* promoter. This product was cloned into the *NcoI*/*Pst*I sites of the second intermediate above. Fourthly, DegQ1 (5'-GGGGAATTCCCTTGGGCAAACCGCC-3') and DegQ3 (5'-GGGGGTACCGTAGCCTGATGCCCGG-3') amplified a portion of the *degQ* locus, including the sequence directly upstream of the *degS* promoter and extending approximately 880 bp upstream of the *degS* promoter. This product was cloned into *Eco*RI/*Kpn*I of intermediate 3 above, resulting in pBA106. All products were confirmed by DNA sequencing.

The *degS-phoA* fusions were constructed as follows. To make pBA108, *phoA* lacking its signal sequence was amplified using primers PhoA1 (5'-CCCGGATCCGCTCAGGGCGATATTACTGCACCC-3' and PhoA2 (5'-CCCGGATCCTTTCAGCCCCAGAGCGGCTTTCAT-3') and cloned into the *Bam*HI site of pLC259. To make pBA115, primers similar to PhoA1 and 2, but with a *Pst*I site, were used, and the product was cloned into the *Pst*I site of pLC259. To make pBA111, PhoA9 (5'-GCTCTAGAATGGCTCAGGGCGATATTACT-3') and PhoA10 (5'-GCGGTACCTTATTTCAGACCCAGAGCGGC-3') were used and the product was cloned into *Xba*I and *Kpn*I of pSU21. pBA123, confirmed by DNA sequencing, was made in two steps. The *pelB* leader sequence was amplified from pET25b(+) (Novagen) with PelB1 (5'-CCAAGCTTTTTAAGAAGGAGATATAC-3') and PelB2 (5'-CCGGATCCAATTCTAGACGAATTAATTCCGAT-3') and cloned into *Hind*III and *Bam*HI of pSU21, generating intermediate A. Next, (ΔTM)*degS* was amplified with

DegS105 (5'-CCTCTAGACTGCGCAGCCTTAACCCGCTT-3') and DegS75 (5'-GGGGTACCGAGCGCACGACTTAATTGGTTG-3') and cloned into *Xba*I and *Kpn*I of intermediate A. The (ΔTM)*degS* truncation site was chosen based on the MEMSAT topology prediction program which predicts where the N-terminal membrane-spanning helix ends (Jones et al., 1994).

Cotransductions

We first linked Δ*degS* from CAG33315 to *argR*::Tn*5* (from DS954) by standard P1 transduction (Miller 1972), generating CAG43081-3. We used our Δ*degS* isolate CAG33315 (Ades et al., 1999), derived from the Sauer laboratory's deletion strain (Waller and Sauer, 1996), since we have found that it is the most stable isolate when grown in liquid LB or on LB plates. CAG33330 and CAG33333 were infected with P1 lysate grown on CAG43081-3 by a standard P1 transduction protocol. Kan^R transductants were selected at 30°C on LB/ Cm/ Kan. After approximately 24 hours or longer, Kan^R transductants were streak-purified and then tested for canavanine resistance on M9 complete glucose minimal medium (lacking L-arginine) plus 100 μg/ml L-canavanine to confirm that they were *argR*$^-$ (Tian and Maas, 1994). Next, they were PCR-screened for the cotransduction of the Δ*degS* allele. The frequency of cotransduction of Δ*degS* with *argR*::Tn*5* was calculated by dividing the number of Δ*degS* *argR*::Tn*5* transductants by the total number of *argR*::Tn*5* transductants. This was repeated twice and yielded similar results.

The Δ*rpoE*:ΩCm *nadB3140*::Tn*10* cotransduction was done as described previously (De Las Peñas et al., 1997b), except that transductants were selected at 30°C on LB/Tet for 24 hours and PCR-screened for the presence of Δ*rpoE*:ΩCm.

Genetics with DegS depletion strains

To determine if *degS* is essential using the *degS* depletion strains, efficiencies of plating (EOP) with and without IPTG were performed as follows. 1 ml of each overnight culture, grown at 30°C in LB/ Kan/ 1 mM IPTG, was pelleted in a microcentrifuge and washed 3 times in 1 ml LB to remove IPTG from the suspension. The washed cultures were ten-fold serially diluted to 10^{-7}. 100 µl of the 10^{-7} - 10^{-5} dilutions was plated on LB/Kan and LB/Kan/1 mM IPTG and incubated at 30°C, 37°C, and 43°C for approximately 24 hours or longer. Alternatively, 10 µl of dilutions 10^{-1} - 10^{-7} were spotted onto the above plates. The EOPs were calculated by dividing the number of colony forming units (cfus) on LB/ Kan by the number of cfus on LB/ Kan/ 1 mM IPTG. For the EOP of 43187 on M9 complete minimal plates, all of the above steps were performed with M9 complete minimal media. EOPs were repeated at least three times, except for CAG43189 which was repeated two times. Data are the average EOPs.

Other *degS* phenotypes were sought by looking for sensitivities to 1M each of $CuCl_2$, $NiCl_2$, $CrCl_2$, $CoCl_2$, $FeCl_2$, $CdCl_2$, and 4M NaCl, 0.5 M EDTA 20 % SDS, 1M DTT, ampicillin (100 mg/ml), rifampicin (100 mg/ml), H_2O_2 (30%), 1M KOH, 1 M HCl, 10% sodium deoxycholate and 1M NH_4Cl (1M). Solutions were spotted onto Whatman paper discs, which were allowed to dry before being placed onto lawns of CAG43196 on

LB/ Kan +/- 1 mM IPTG. Zones of clearing on +/- 1 mM IPTG were compared; no differences were observed.

DegS depletion in vivo

CAG43248 was grown in M9 complete minimal media with Kan and 1 mM IPTG at 37°C to an OD_{450} of approximately 0.35 - 0.5. The culture was poured onto a 0.45 μm Millipore filter (Millipore Corp.) in a Nalgene (Nalge Nunc International) filtering system and washed with 10-15 ml of 37°C M9 complete minimal media (without IPTG) to remove IPTG from the cells. The cells were resuspended in M9 complete minimal media and diluted to OD_{450} ~ 0.04 in 37°C M9 complete minimal plus Kan +/- 1 mM IPTG. At time points after subculturing, aliquots were sampled for anti-DegS Western blots and processed as below. The depletion was repeated 3 times with similar results.

Western blotting of DegS

During the course of the DegS depletion, 900 μl samples were mixed with 100 μl of ice-cold 50% TCA and stored on ice. TCA precipitates were pelleted by centrifugation at 17,500 x g for 25 minutes at 4°C 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/μl. 10^8 cells from each sample were run on 12 % Tris-glycine SDS-polyacrylamide gels and the proteins were transferred to nitrocellulose. The blots were blocked overnight in Tris-buffered saline with Tween-20 (10 mM Tris pH 8.0, 150 mM NaCl, 0.2% Tween-20 [TBST]) plus 10% nonfat dry milk. Next, the blots were incubated for one hour in TBST with a 1:2000 dilution of the primary DegS-PDZ domain

antisera (raised against the purified 6HIS-tagged PDZ-like domain of DegS from CAG33350) and washed three times for ten minutes each with TBST. They were subsequently incubated with the anti-rabbit horseradish peroxidase-conjugated secondary antibody (Amersham Life Sciences), diluted 1:10000 in 1X TBST plus 10% nonfat dry milk, for 1 hour at room temperature and washed as before. The blots were developed with an ECL (enhanced chemiluminescence) kit (Amersham Pharmacia Biotech).

Alkaline phosphatase assays

Assays were performed and activities were calculated as previously described (Gutierrez et al., 1987). We used strain CC118 (Manoil and Beckwith, 1986) as the background for these assays. Freshly prepared iodoacetamide was added (final concentration 1 mM) to the harvested cells and reactions to diminish the activation of cytoplasmic PhoA (Derman and Beckwith, 1995). Assays were repeated 2 times. The means and standard deviations are shown.

β-galactosidase assays

Cultures were grown in LB/Cm at 30°C without IPTG. Assays were performed as previously described (Mecsas et al., 1993; Ades et al., 1999). We repeated the assays on cultures grown in the presence of 1 mM IPTG and observed the same activities (data not shown).

For Table 2-1, fold effects were determined by comparing the slopes (determined by linear regression analysis) of the initial linear portion (in early log phase) of the plots of β-galactosidase activity/ 0.5 ml cells versus OD_{600}. This was necessary because σ^E

activity increases as cells enter mid-log growth phase (J. Mecsas and C. A. Gross, unpubl. observations).

Determination of the half-life of RseA by pulse-chase-immunopreciptation

All strains to be assayed were grown at 30 °C in M9 minimal media lacking methionine and cysteine except Δ*degS* strains which were grown in M9 pH 7.5 (titrated with Tris-buffer) lacking methionine and cysteine to an OD_{450} of 0.15-0.3. Cells were pulse-labeled for 1 minute with EasyTag Expre^{35}S ^{35}S protein labeling mix (NEN Life Sciences Products). An 800 μl sample was removed (t = 0) and a cold chase of methionine and cysteine was added to give a final concentration of 0.1%. At given times after the chase, 900 μl samples were removed. To initiate the extracytoplasmic stress response at the appropriate time either before or after addition of the protein labeling mix, the temperature was shifted to 43 °C or *ompC* expression was induced by the addition of IPTG to 2mM. All samples were added to 100 μl of ice-cold 50% TCA and incubated on ice for > 15 minutes. Samples were centrifuged to pellet the precipitated proteins and pellets were resuspended by vortexing and boiling for 5 minutes in 50 μl of 50 mM Tris-HCl pH 7.5, 2% SDS, 1 mM PMSF, and 10 mM EDTA. 750 μl of RIPA (50 mM Tris pH 7.5, 150 mM NaCl, 1% NaDeoxycholate, 1% Triton X-100, 0.1% SDS) was then added to the samples and a 5 μl aliquot of each was counted in a scintillation counter. Equal numbers of cpm were immunoprecipitated using 2 μl of a polyclonal anti-RseA periplasmic domain antibody and 25 μl of a 1:1 suspension of Protein A-conjugated sepharose beads in RIPA (Sigma). The volumes of the samples were adjusted with RIPA to 500μl. As an internal control, an aliquot of a pulse-labeled strain (CAG33149) induced for the overexpression of the RseA periplasmic domain was added to each sample prior to immunoprecipitation. The samples were rocked at 4° C for 1-3 hours, and then the beads and associated immune complexes were harvested by centrifugation

and washed 3 times with 900 μl of RIPA buffer. Immunoprecipitated proteins were eluted from the beads by the addition of 30 μl of Laemmli sample buffer, boiled for 5 minutes, then loaded onto 15% SDS Tris-glycine gels. Immunoprecipitated proteins were visualized with the Molecular Dynamics Storm 560 Phosphorimager scanning system. The intensity of the band corresponding to full length RseA protein was normalized to the intensity of the periplasmic domain RseA standard in each lane after background correction using the program ImageQuant 1.2. Half-lives were determined by fitting to the exponential decay equation. Experiments were conducted a minimum of two times each.

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Table 2-1. DegS is required for signal transduction to σ^E

Strain[a]	Relevant Genotype			Induction of OmpC	σ^E activity	Fold Induction
CAG41073	*sup*$^+$ *degS*	p*ompC*		-	8	
				+	306	38
CAG43150	*sup*$^+$ Δ*degS*	p*ompC*		-	2	
				+	3	1.5
CAG43133	*sup*$^+$ Δ*degS*	p*ompC*	p*degS*	-	6	
				+	64	11
CAG43134	*sup*$^+$ Δ*degS*	p*ompC*	p*degS S201A*	-	2	
				+	1	0.5
CAG41323	*sup*$^+$ Δ*degS*	p*ompC*	vector	-	3	
				+	1	0.3
CAG43126	*sup*$^+$ *degS*	p*ompC*	vector	-	35	
				+	202	6[b]

σ^E activity was measured by monitoring β-galactosidase activity from a single copy σ^E-dependent *lacZ* reporter gene in Φλ[*rpoHP3-lacZ*] as described in Methods. OmpC expression was induced by the addition of IPTG as indicated, and fold induction was determined by calculating the ratio of β-galactosidase activity in the presence or absence of IPTG.

[a] All strains carry the suppressor of Δ*rpoE* and the plasmid p*ompC* (pEMC1).

[b] The basal level of β-galactosidase activity is increased in this strain. This effect is dependent on the presence of p*ompC*, the vector plasmid pSU21, and a chromosomal copy of *degS*. As a result, the fold induction is lower in this strain than in strains lacking the vector plasmid pSU21 (see the first row of this table). Interaction between the two plasmids in the suppressor strain may also account for the lack of complete induction in the p*degS* strain.

Table 2-2. Efficiency of plating (EOP) of DegS depletion strains on LB/ kanamycin or M9 complete minimal/ kanamycin in the absence versus the presence of 1 mM IPTG at 30°C, 37°C, and 43°C. EOPs were determined as described in Experimental Procedures.

Strain background (all contain $P_{LlacO-1}$ *degS*)		EOP [(# cfus[1] –IPTG)/(# cfus +IPTG)] 30°C	37°C	43°C
MC1061 (43187)	LB	2.7×10^{-3}	5.8×10^{-3}	1.8×10^{-3}
	M9	1.4×10^{-3}	1.6×10^{-2}	0.60
MC4100 (43189)	LB	5.5×10^{-4}	1.7×10^{-4}	8.6×10^{-5}
MG1655 (43190)	LB	0.80	1.0	3.8×10^{-3}
MC1061 Δ*rseA* (43196)	LB	0.93	1.2	0.81
MC1061 Δ*ompR* (43219)	LB	1.0	0.85	8.2×10^{-4}

[1] colony forming units

Table 2-3. Cotransduction of Δ*degS* with *argR*::Tn*5* into MC1061 indicates that *degS* is essential (See Experimental Procedures). A control transduction showed that each strain is equally transducible (data not shown).

Recipient	# *argR*$^+$ colonies	# Δ*degS* colonies	%Linkage
MC1061 + vector (33330)	7	0	0
MC1061 + p*degS* (33333)	35	32	91

Table 2-4. Cotransduction of *rpoE*::Cm with *nadB3140*::Tn*10* into MC1061 Δ*degS* indicates that MC1061 Δ*degS* harbors a suppressor(s) of the lethality of *rpoE*::ΩCm.

	Colonies obtained with donor P1(*nadB3140*::Tn*10 rpoE*::ΩCm)		
Recipient	Tet^R	Cm^R	% Linkage
Δ*degS* (CAG33315)	50	48	96
Controls			
MC1061 wild type (CAG16037)	4	1	25
rpoE$^+$ with suppressor of *rpoE*::Cm (CAG41001)	50	50	100

Recipients were infected with the donor P1 phage noted below in which *nadB3140*::Tn*10* and *rpoE*::ΩCm are 90 - 100% linked (De Las Peñas et al., 1997b). 207, 421, and 4 tetracycline resistant (Tet^R) CAG33315, CAG41001, and CAG16037 transductants, respectively, were obtained by selection at 30°C on LB/ tetracycline for approximately 24 hours. Only 50 CAG33315 and CAG16037 transductants were further tested and screened for chloramphenicol resistance (Cm^R). A control transduction showed that each strain was equally transducible (data not shown).

Table 2-5. Strains and plasmids used in this work.

Strain/plasmid	Relevant genotype	Source/reference
Strain		
CC118	*araD*139 Δ(*ara-leu*)7697 Δ*lacX*74 *phoA*D20 *galE galK thi rpsE rpoB argE*(Am)	(Manoil and Beckwith, 1986)
DS954	AB1157 *recF lacI*^{q} *lacZ*ΔM15 *sup*^{o} *argR*::Tn*5*(A3), Kan^R	Prof. David Sherratt
MC1061	*araD* Δ(*ara-leu*)7697 Δ(*codB-lacI*) *galK*16 *galE*15 *mcrA*0 *relA*1 *rpsL*150 *spoT*1 *mcrB*9999 *hsdR*2	(Casadaban and Cohen, 1980; *E. coli* Genetic Stock Center)
MC4100	*araD*111 Δ(*argF-lac*)169 *flhD*5301 *fruA*25 *relA*1 *rpsL*150 *rbsR*22 *deoC*1	(Casadaban, 1976; *E. coli* Genetic Stock Center)
MG1655	*rph*-1	(Guyer et al., 1981; Jensen et al., 1993; *E. coli* Genetic Stock Center)
KM9	AB1157 Δ(*recC ptr recB recD*):: P_{lac}-*bet exo kan recJ*	(Murphy, 1998)
SG460	*ompR*::Tn*10*, Tet^R	Prof. Tom Silhavy laboratory
CAG16037	MC1061 [Φλ*rpoH* P3::*lacZ*]	(Mecsas et al., 1993)
CAG33315	MC1061 Δ*degS* [Φλ*rpoH* P3::*lacZ*]	(Ades et al., 1999)
CAG22357	16037 Δ*rseA nadB*::Tn*10*, Tet^R	This work
CAG33149	BL21(DE3) pLC234, Kan^R	(Ades et al., 1999)
CAG33324	MC1061 Δ*degS* pSU21	This work
CAG33325	MC1061 Δ*degS* pLC259	This work
CAG33330	16037 pSU21, Cm^R	This work
CAG33333	16037 pLC259, Cm^R	This work
CAG33350	BL21 λDE3 pLC263, Kan^R	This work
CAG41001	MC1061 *rpoE*$^+$ with suppressor of *rpoE*::ΩCm	This work
CAG41073	16037 *sup*$^+$ pPLT13 pEMC1, Kan^R Ap^R	(Ades et al., 1999)
CAG43126	41073 pSU21, Ap^R Kan^R Tri^R Cm^R	(Ades et al., 1999)
CAG43132	43150 pSU21, Ap^R Kan^R Tri^R Cm^R	(Ades et al., 1999)
CAG43133	43150 pLC259, Ap^R Kan^R Tri^R Cm^R	(Ades et al., 1999)
CAG43134	43150 pLC261, Ap^R Kan^R Tri^R Cm^R	(Ades et al., 1999)
CAG43150	41073 Δ*degS argR*::trimethoprim, Ap^R Kan^R Tri^R	(Ades et al., 1999)
CAG43081-3	33315 *argR*::Tn*5*, Kan^R	This work
CAG43173	KM9 $P_{LlacO-1}$ *degS*	This work
CAG43187	48026 $P_{LlacO-1}$ *degS*, Kan^R Spec^R	This work

CAG43189	48027 $P_{LlacO-1}$ *degS*, Kan^R $Spec^R$	This work
CAG43190	48028 $P_{LlacO-1}$ *degS*, Kan^R $Spec^R$	This work
CAG43196	43187 Δ*rseA nadB*::Tn*10*, Kan^R Tet^R $Spec^R$	This work
CAG43210	CC118 pBA108, Cm^R	This work
CAG43213	CC118 pBA111, Cm^R	This work
CAG43219	43187 *ompR*::Tn*10*, Kan^R Tet^R $Spec^R$	This work
CAG43224	MC1061 [Φλ*rpoH* P3::*lacZ* Δ(*lacY*)::*cat*], Cm^R	This work
CAG43244	43224 pJM100, Kan^R Cm^R	This work
CAG43248	43244 $P_{LlacO-1}$ *degS*, Kan^R $Spec^R$	This work
CAG43228	CC118 pBA115, Cm^R	This work
CAG43276	CC118 pSU21, Cm^R	This work
CAG43302	33315 pBA123, Cm^R	This work
CAG48026	MC1061 pJM100, Kan^R	This work
CAG48027	MC4100 pJM100, Kan^R	This work
CAG48028	MG1655 pJM100, Kan^R	This work
Plasmid		
pSU21	Cloning vector, p15a ori, *lac* promoter, Cm^R	(Bartolomé et al., 1991)
pEMC1	*ompC* in pKK223-3, Ap^R	(Catron and Schnaitman, 1997)
pET25b(+)	Protein expression vector, Ap^R	Novagen
pET28b	Protein expression vector, Kan^R	Novagen
pJM100	pACYC177 derivative with $lacI^q$, Kan^R	(McCarty and Walker, 1994)
pPLT13	Mini-F carrying $lacI^q$, Km^R	(Tavormina et al. 1996)
pLC234	*rseA* periplasmic domain with N-terminal 6-histidine tag	(De Las Peñas et al. 1997a)
pLC259	*degS* and *degS* promoter in pSU21, Cm^R	(Ades et al., 1999)
pLC261	*degS S201A* and *degS* promoter in pSU21, Cm^R	(Ades et al., 1999)
pLC263	PDZ-like domain of *degS* in pET28b, Kan^R	This work
pBA106	$P_{LlacO-1}$ *degS* in pUC18, Ap^R $Spec^R$	This work
pBA108	signal sequence-less *phoA* in *Bam*HI of pLC259, Cm^R	This work
pBA111	signal sequence-less *phoA* in pSU21, Cm^R	This work
pBA115	signal sequence-less *phoA* in *Pst*I of pLC259, Cm^R	This work
pBA123	(ΔTM)*degS* in pSU21, Cm^R	This work

Figure 2-1. RseA stability in cells lacking DegS. *sup*$^+$ *degS* (CAG41073, filled squares) or *sup*$^+$ *ΔdegS* (CAG43150, open squares) cells containing p*ompC* were grown to early log phase at which point IPTG was added to induce overexpression of *ompC*. The rate of RseA degradation after induction of the extracytoplasmic stress response was determined by pulse-chase immunoprecipiation analysis as described in Methods. A representative data set is shown.

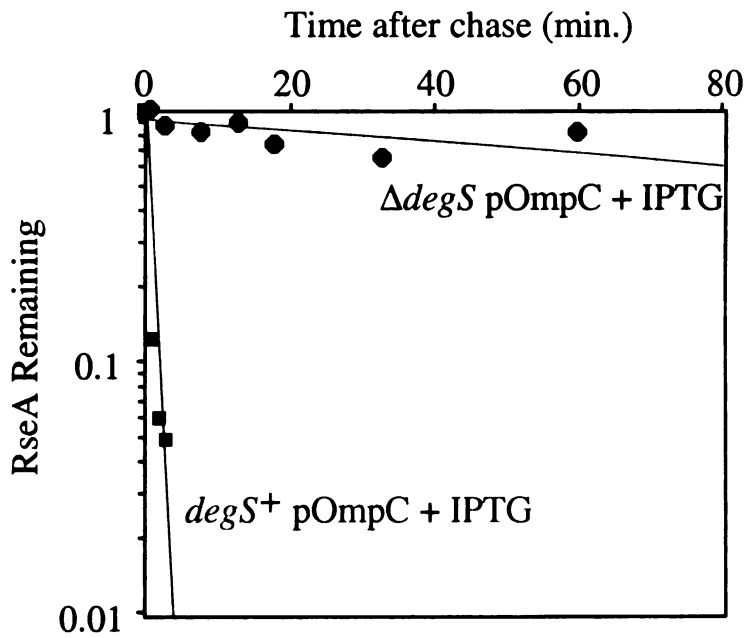
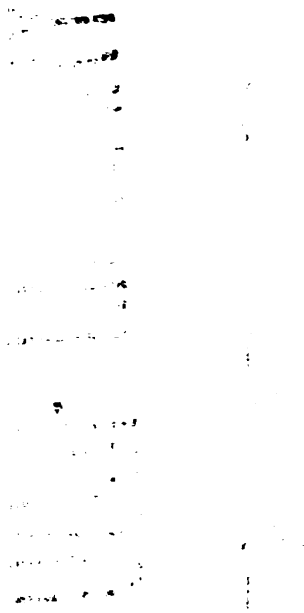
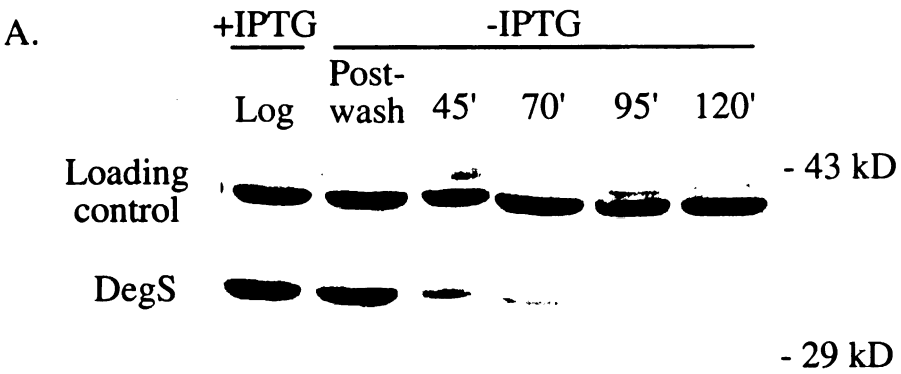

Time after chase (min.)
0
20
40
60
80
1
0.1
0.01
RseA Remaining
ΔdegS pOmpC + IPTG
degS+ pOmpC + IPTG

Figure 2-2. DegS is depleted *in vivo*. (A and B) Western blots of TCA-precipitated samples from the MC1061 DegS depletion strain (CAG43248). CAG43248 was grown in M9 complete minimal media plus 1 mM IPTG to mid-logarithmic growth phase before being washed free of IPTG, as detailed in Experimental Procedures. The washed cells were resuspended in the same media with (B) or without (A) 1 mM IPTG and the culture was sampled periodically for Western blotting. *Log* is the DegS level prior to washing and *Post-wash* is its level after removal of IPTG but prior to subculturing. A non-specific band serves as a loading control.




A.
+IPTG
-IPTG
Log
Post-wash
45'
70'
95'
120'
Loading control
DegS
- 43 kD
- 29 kD

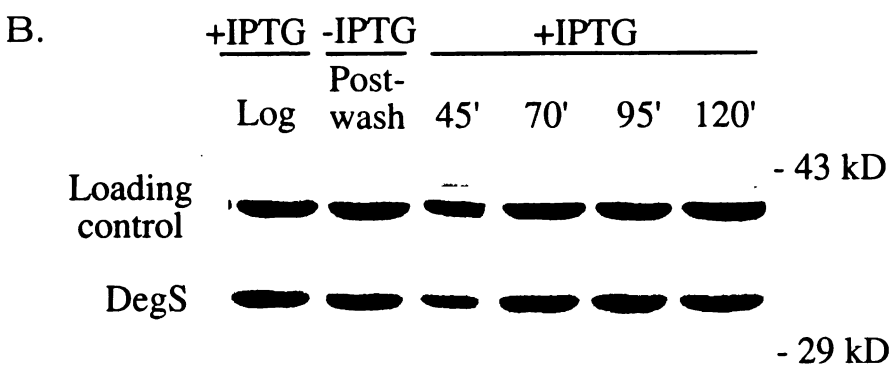
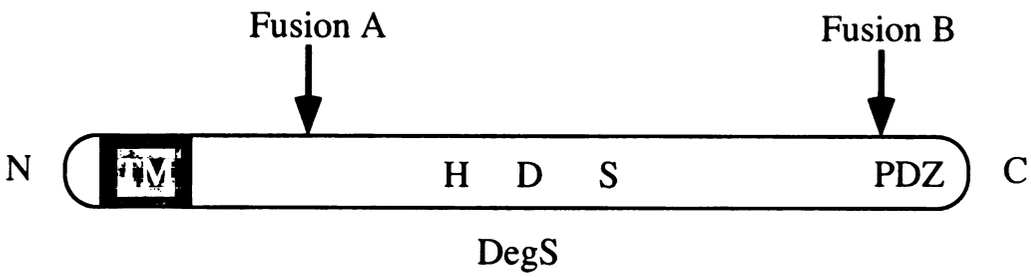

B.
+IPTG
-IPTG
+IPTG
Log
Post-wash
45'
70'
95'
120'
Loading control
DegS
- 43 kD
- 29 kD

Figure 2-3. DegS-PhoA fusions indicate that DegS has a periplasmic active site and PDZ-like domain. (A) Schematic diagram showing where mature *phoA* has been fused to *degS*. In fusions A and B, mature *phoA* is fused to serine 81 (CAG43210) and glutamine 345 (CAG43228), respectively, of DegS. TM indicates the transmembrane domain while the H, D, and S are the residues of the putative serine protease catalytic triad. PDZ signifies the PDZ-like domain. (B) Alkaline phosphatase activities of fusions A and B and *phoA* lacking its signal sequence (CAG43213). Activities were determined according to Gutierrez et al. (1987) and Derman and Beckwith (1995).

A.


Fusion A
Fusion B
N
TM
H
D
S
PDZ
C
DegS

B.

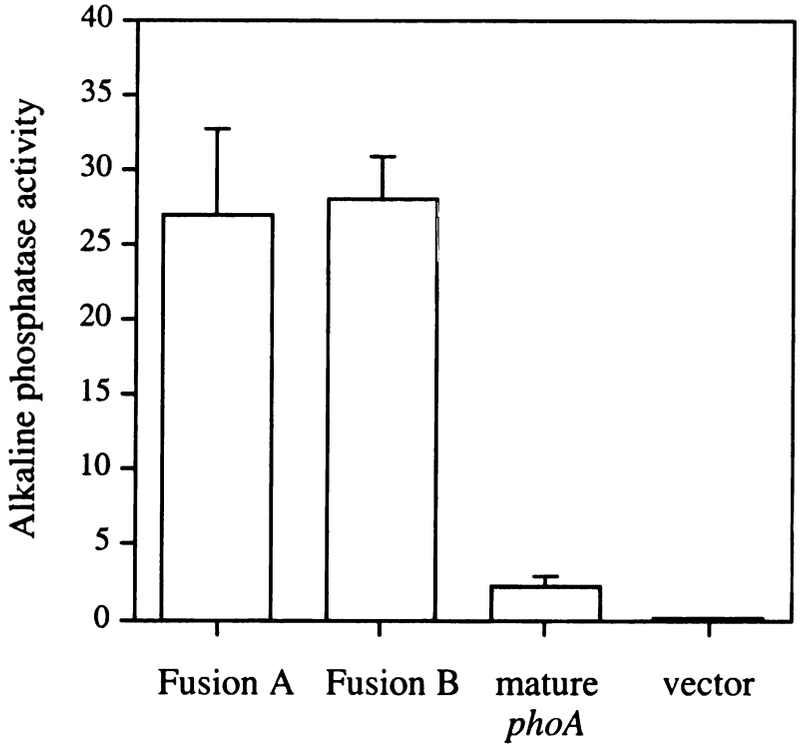
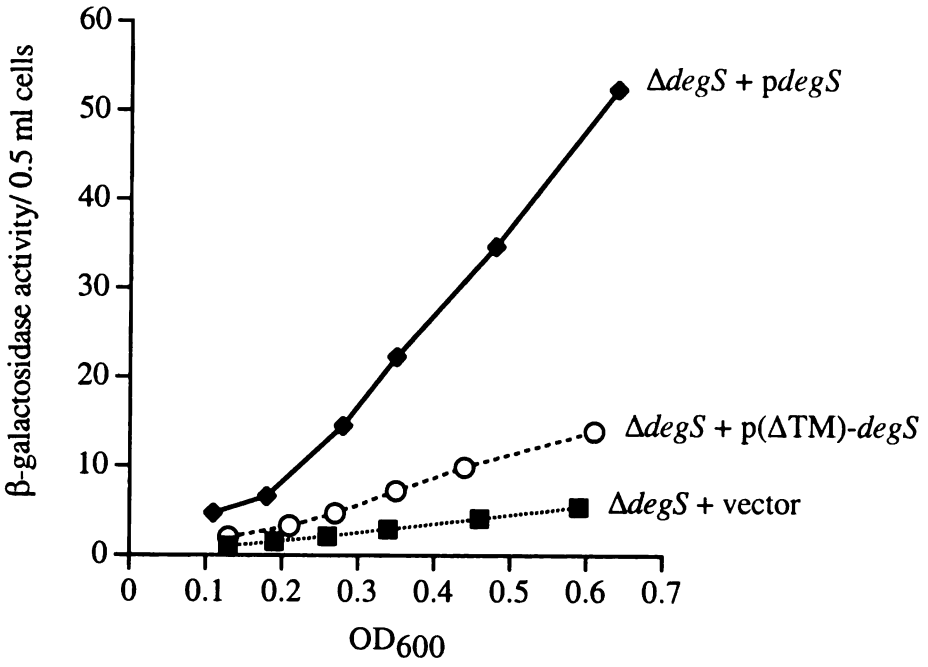

Alkaline phosphatase activity
40
35
30
25
20
15
10
5
0
Fusion A
Fusion B
mature
phoA
vector

Figure 2-4. Basal σ^Eactivity in Δ*degS* is not restored to wild type level by Δ(TM)-*degS*. σ^E activity was assayed by monitoring β-galactosidase production from a single copy Φλ[*rpoH*P3-*lacZ*] fusion in Δ*degS* + p*degS* (CAG33325), Δ*degS* + pΔ(TM)-*degS* (CAG43302), and Δ*degS* + vector (CAG33324). β-galactosidase activities were determined as described in Experimental Procedures.


β-galactosidase activity/ 0.5 ml cells
60
50
40
30
20
10
0
0
0.1
0.2
0.3
0.4
0.5
0.6
0.7
OD600
ΔdegS + pdegS
ΔdegS + p(ΔTM)-degS
ΔdegS + vector

Chapter Three

DegS and YaeL participate sequentially in the cleavage of RseA to activate the σ^E-dependent extracytoplasmic stress response

Summary

All cells have stress response pathways that maintain homeostasis in each cellular compartment. In the Gram-negative bacterium *Escherichia coli*, the σ^E pathway responds to protein misfolding in the envelope. The stress signal is transduced across the inner membrane to the cytoplasm via the inner membrane protein RseA, the anti-sigma factor that inhibits the transcriptional activity of σ^E. Stress-induced activation of the pathway requires the regulated proteolysis of RseA. In this report we show that RseA is degraded by sequential proteolytic events controlled by the inner membrane-anchored protease DegS and the membrane-embedded metalloprotease YaeL, an orthologue of mammalian Site-2 protease (S2P). This is consistent with the mechanism of activation of the mammalian unfolded protein response ATF6 transcription factor by Site-1 protease and S2P. Thus, mammalian and bacterial cells employ a conserved proteolytic mechanism to activate membrane-associated transcription factors that initiate intercompartmental cellular stress responses.

Introduction

Stress response pathways that sense protein misfolding and other cellular damage in one compartment of the cell and transduce this signal to another cellular compartment are essential for balanced cell growth. Eukaryotic cells sense protein misfolding in the endoplasmic reticulum (ER) and transduce this signal to the nucleus of the cell to generate an appropriate response. This process has been called the unfolded protein response (UPR) (Patil and Walter, 2001). Likewise, Gram-negative bacteria sense unfolded proteins in the envelope compartment of the cell and transduce this signal to the cytoplasmic compartment to generate a response. This process has been termed the extracytoplasmic stress response. In this report, we show that the σ^E-dependent *E. coli* extracytoplasmic stress response has a proteolytic regulatory element that is similar to that of the ATF6-dependent response of mammalian cells during the UPR (Ye et al., 2000b) and the sterol regulatory element-binding protein (SREBP) dependent response to sterol deprivation (Brown et al., 2000).

In mammals, the UPR is initiated in part by the processing of ATF6, an integral membrane protein with an N-terminal, cytoplasmic basic leucine zipper (bZIP) transcription factor domain (Haze et al., 1999). The C-terminal domain of ATF6 projects into the ER and is positioned to sense stress in that cellular compartment (Haze et al., 1999). During ER stress, the bZIP domain is released by sequential proteolytic events controlled by the Site-1 (S1P) and Site-2 (S2P) proteases, which also process SREBPs in response to sterol deprivation (Rawson et al., 1997; Sakai et al., 1998; Ye et al., 2000b). First, the membrane-anchored serine protease S1P cleaves ATF6 in its lumenal domain

(Ye et al., 2000b). For this cleavage event to occur, ATF6 must transit to a post-ER compartment in or near the Golgi complex where S1P and S2P are active (DeBose-Boyd et al., 1999; Ye et al., 2000b; Chen et al., 2002). Following S1P cleavage, the remaining integral membrane fragment of ATF6 becomes a substrate for regulated intramembrane proteolysis (Rip) by S2P, an integral membrane metalloprotease that cleaves ATF6 within its membrane-spanning region (Ye et al., 2000b). The released N-terminal domain travels to the nucleus where it activates transcription of chaperone-encoding genes and other key regulators of the response (Haze et al., 1999).

The extracytoplasmic stress response in *E. coli* is induced by excessive amounts of unfolded proteins in the envelope of the cell, particularly unfolded outer membrane porins, which are an abundant component of the outer membrane of Gram-negative bacteria (Mecsas et al., 1993; Betton et al., 1996; Missiakas et al., 1996; Rouvière and Gross, 1996; Jones et al., 1997). This response is initiated by activating the transcription factor σ^E, an alternative σ factor that is required not only for the stress response but is also essential for *E. coli* viability under normal conditions (De Las Peñas et al., 1997b). σ^E directs the expression of genes encoding envelope-localized chaperones, protein folding catalysts and proteases, as well as genes involved in lipid and lipopolysaccharide metabolism and cell wall biogenesis (Dartigalongue et al., 2001b).

Under non-stress conditions, the activity of σ^E is negatively regulated by two proteins, RseA and RseB, which are encoded along with *rpoE* (σ^E gene) in a single operon. RseA, an inner membrane protein with one transmembrane domain, a cytoplasmic and a periplasmic domain, is the major negative regulator of σ^E (De Las Peñas et al., 1997a; Missiakas et al., 1997). The N-terminal cytoplasmic domain of RseA

is an anti-sigma factor that binds to cytoplasmic σ^E and is sufficient to inhibit σ^E *in vivo* and *in vitro* (De Las Peñas et al., 1997a; Missiakas et al., 1997). The C-terminal domain of RseA projects into the periplasm and is positioned to sense stress in the envelope compartment (De Las Peñas et al., 1997a; Missiakas et al., 1997). This periplasmic domain of RseA interacts with RseB, an auxiliary negative regulator that may act as a sensor of unfolded proteins (De Las Peñas et al., 1997a; Missiakas et al., 1997; Collinet et al., 2000). When *E. coli* is subjected to heat shock, or when the outer membrane porin OmpC is overproduced, RseA is rapidly degraded (Ades et al., 1999). This frees σ^E to associate with RNA polymerase and direct the transcription of its regulon.

As the proteolysis of RseA is the central point of regulation in the σ^E pathway, we have been identifying proteins required for RseA degradation (Ades et al., 1999). We found that DegS, an inner membrane protease that is a member of the large DegP/HtrA family of serine proteases (Waller and Sauer, 1996; Pallen and Wren, 1997), is required for RseA degradation (Ades et al., 1999). Like σ^E, DegS is required for viability (Alba et al., 2001). The essential function of DegS is to provide σ^E activity through the degradation of RseA, as *degS* null mutants are viable both in suppressor strains that no longer require σ^E activity for cell growth at low temperature or in strains lacking the negative regulator RseA (De Las Peñas et al., 1997a; Alba et al., 2001). In suppressor strains carrying a deletion of *degS* or a mutation in the DegS active site serine, RseA is not degraded and σ^E activity is not increased during inducing conditions (Ades et al., 1999). Thus, in the absence of DegS, σ^E is almost fully inhibited by RseA (Ades et al., 1999; Alba et al., 2001). As its proteolytic domain is periplasmically localized, DegS is likely to initiate degradation in the periplasmic domain of RseA (Alba et al., 2001).

However, since the cytoplasmic domain of RseA alone is sufficient to inhibit σ^E (De Las Peñas et al., 1997a; Missiakas et al., 1997), it must also be degraded to release σ^E (Ades et al., 1999). Either DegS or other proteases working in coordination with DegS must perform this function (Alba et al., 2001). We took a candidate approach to look for other *E. coli* proteases that participate in RseA degradation.

We examined the involvement of YaeL, which is an inner membrane protein and an S2P orthologue, in RseA degradation (Lewis and Thomas, 1999; Rudner et al., 1999; Kanehara et al., 2001). YaeL possesses the conserved signature amino acids of proteases that carry out Rip (Lewis and Thomas, 1999; Rudner et al., 1999; Brown et al., 2000; Kanehara et al., 2001). YaeL is essential for cell growth (Dartigalongue et al., 2001b; Kanehara et al., 2001) and is a member of the σ^E regulon (Dartigalongue et al., 2001b). In this report we provide evidence to support a role for YaeL, along with DegS, in the sequential cleavages of RseA. Thus, activation of the *E. coli* extracytoplasmic stress response, like activation of ATF6 in the mammalian UPR, requires cleavage first by a membrane-anchored serine protease and subsequently by a membrane-embedded metalloprotease, to release the active transcription factor. YaeL joins a growing list of bacterial S2P orthologues that play important regulatory roles. These proteases include SpoIVFB, which is required to process the *Bacillus subtilis* sporulation factor σ^K to its active form (Rudner et al., 1999; Yu and Kroos, 2000), and Eep, which is required to produce an 8 amino-acid phermone in *Enterococcus faecalis* (Dunny and Leonard, 1997; An et al., 1999; Brown et al., 2000).

Results

σ^E activity decreases during depletion of YaeL

We tested whether YaeL plays a regulatory role in the extracytoplasmic stress response pathway by depleting YaeL *in vivo* and assaying σ^E activity with a reporter construct. This reporter contains a minimal σ^E promoter driving expression of *lacZ* and has been extensively utilized to monitor σ^E activity (Mecsas et al., 1993; Ades et al., 1999). The construct is carried by a λ phage located at the λ attachment site in the chromosome. We grew the YaeL depletion strain (CAG43509), which has a chromosomal *yaeL*::*kanR* and a plasmid carrying wild type (wt) *yaeL*, to mid-exponential growth phase in rich medium supplemented with arabinose. The addition of arabinose is required to induce the complementing copy of *yaeL* that is under the control of the P_{ara} promoter. Following growth, cells were collected, washed, and resuspended in rich medium containing arabinose or the P_{ara} repressor glucose. We maintained the cultures in mid-exponential growth phase by diluting the cultures with pre-warmed media. Growth curves for each successive subculture are shown in Figure 3-1A. We observed that cells grown in the presence of glucose eventually cease growing and lyse, as previously described (Kanehara et al., 2001). The differential rate of β-galactosidase synthesis (the measure of σ^E activity) for each successive subculture in glucose, shown in Figure 3-1B, indicates that σ^E activity began to decrease starting in the third subculture. Notably, σ^E activity (Fig. 3-1B) decreased prior to the decrease in growth rate, which was not apparent until the fourth subculture (Fig. 3-1A). In contrast, σ^E activity in the arabinose-containing culture did not change (Fig. 3-1A; data not shown). We confirmed

that the YaeL protein level decreased during the course of depletion (Fig. 3-1C) and remained constant under inducing conditions (data not shown). We note that in longer exposures of the Western blot (data not shown), YaeL remains detectable until the end of glucose subculture #2, which is consistent with the decrease in σ^E activity during subculture #3 (Fig. 3-1B). The data in Figure 3-1 were obtained in strain MC1061; similar data were obtained in strain MG1655 (data not shown). Together, these data suggest that YaeL is an activator of the σ^E pathway.

The essential function of YaeL is to maintain σ^E activity

σ^E activity is essential for *E. coli* viability (De Las Peñas et al., 1997b). As σ^E activity decreases upon YaeL depletion, we considered the possibility that the essential function of YaeL is to maintain σ^E activity. If this were true, then *yaeL* would not be essential in suppressor strains that no longer require σ^E activity for cell growth at low temperature (hereafter called *sup*$^+$ strains and described in De Las Peñas et al., 1997b). Consistent with this idea, *yaeL*::*kanR* could be moved into the *sup*$^+$ background but not into the isogenic wt strain using P1 phage-mediated transduction (Table 3-1). Likewise, we could transduce *yaeL*::*kanR* into Δ*degS*, which also harbors a suppressor that bypasses the need for σ^E activity in *E. coli* (Alba et al., 2001). As YaeL is likely to function as a protease, it might increase σ^E activity by participating in the degradation of RseA. If so, *yaeL* should not be essential in strains lacking *rseA*, as such strains have high, constitutive σ^E activity. This hypothesis is supported by results presented in Table 3-1. *yaeL*::*kanR* also could be transduced into a wt *E. coli* background that bypassed the need for RseA degradation because σ^E was overexpressed (Table 3-1).

We reconstructed the YaeL depletion system in the Δ*rseA* strain, the *sup*$^+$ strain and Δ*degS* strain (with its associated suppressor of σ^E essentiality) and then determined their efficiencies of plating (EOP) on LB in the absence of YaeL (LB/-arabinose) versus the presence of YaeL (LB/+arabinose). This allowed us to quantify the extent to which such genetic backgrounds dispensed with the need for YaeL (Table 3-2). The Δ*rseA* and Δ*degS* strains efficiently bypassed the need for YaeL at all temperatures tested, exhibiting EOP around 1. The *sup*$^+$ strain efficiently bypassed the need for YaeL at 30°C and 37°C, but not at 42°C. This was expected as *sup*$^+$ does not efficiently bypass the need for σ^E at 42°C (De Las Peñas et al., 1997b). In sharp contrast, the EOP following YaeL depletion in the wt MC1061 background was 10^{-4}-10^{-3} at each temperature tested (Table 3-2). These data indicate that the essential function of YaeL is efficiently bypassed either by removing the need for σ^E activity or by removing the requirement for RseA proteolysis to generate σ^E activity. Our results support the model that the essential function of YaeL is to provide the cell with σ^E activity through the proteolysis of RseA.

We previously showed that *sup*$^+$ strains having either a Δ*degS* allele or expressing DegS201A (which alters the protease active site serine to alanine) exhibited reduced basal σ^E activity and were unable to induce σ^E activity in response to the overexpression of OmpC (Ades et al., 1999). If YaeL also functions in RseA degradation, then *E. coli* carrying the null *yaeL* allele or expressing YaeL E23A (which alters the protease active site glutamic acid to alanine) should exhibit the same phenotypes. *sup*$^+$ strains harboring the *yaeL::kanR* allele (data not shown) or expressing YaeL E23A in an otherwise null *yaeL* background exhibit lower basal σ^E activity than one expressing wt YaeL (Fig. 3-2). This reduction in basal activity is similar to that exhibited by strains with a Δ*degS* allele

or DegS201A. Additionally, the uncomplemented *yaeL::kanR* strain (data not shown) and the *yaeL::kanR* strain expressing YaeL E23A were unable to induce σ^E activity in response to the overexpression of OmpC (Fig. 3-2). A Western blot confirmed that the steady-state level of YaeL E23A was at least as high as that of wt YaeL (data not shown). These results support the hypothesis that the proteolytic activity of YaeL is required for both basal and induced σ^E activity.

In addition to the nonconserved E23A substitution, we tested a conserved YaeL active site substitution (E23D) for its effect on YaeL activity. For two YaeL orthologues, mammalian S2P and *B. subtilis* SpoIVFB, changing the active site glutamic acid residue to aspartic acid (E→D) does not abolish activity, although in other metalloproteases such a substitution is not tolerated (Rawson et al., 1997; Rudner et al., 1999). We found that the *yaeL::kanR* mutant expressing YaeL E23D was able to induce the σ^E pathway when OmpC was overproduced (Fig. 3-2). By comparison, the S2P E172D mutant exhibited an activity only slightly lower than wt S2P (Rawson et al., 1997). Thus, the YaeL-like metalloproteases tested to date can tolerate the E→D substitution, most likely because the carboxylic acid group of aspartic acid can functionally substitute for that of glutamic acid (Rawson et al., 1997; Rudner et al., 1999).

YaeL functions downstream of DegS in the RseA proteolytic pathway

Because DegS has an active site in the periplasm and is required for any proteolysis of RseA (Ades et al., 1999; Alba et al., 2001), we imagined that DegS-dependent processing would remove the periplasmic portion of RseA, allowing subsequent cleavage by YaeL. If so, strains lacking YaeL should accumulate an RseA

fragment which contains the transmembrane and cytoplasmic domains. The following series of experiments supports this idea.

We used Western blotting to ask whether a fragment containing the cytoplasmic domain of RseA accumulates in strains lacking active YaeL. We analyzed the strains without and with OmpC overexpression, the σ^E-inducing signal. Strains expressing YaeL E23A (Fig. 3-3A, lanes 3 and 4) but not wt YaeL (Fig. 3-3A, lanes 1 and 2) exhibited high levels of an RseA fragment that was reactive to antisera against the cytoplasmic domain of RseA (Fig. 3-3A, lanes 3 and 4), but not the periplasmic domain of RseA (data not shown). With OmpC overexpression in the YaeL E23A background, the fragment accumulated to an even higher level, while the level of full-length RseA dropped to a low level (Fig. 3-3A, lane 4). The fragment still retains its anti-sigma factor activity since σ^E activity is not induced by OmpC overexpression (Fig. 3-2). The level of RseA after OmpC overexpression in wt cells is as expected from previous studies (Ades et al., 1999). The low level of full-length RseA in YaeL E23A (lane 4) is likely a consequence of its conversion to the RseA fragment and the reduced expression of *rseA* from the σ^E operon. This same fragment was produced in reduced amounts in strains expressing the YaeL E23D variant (with or without OmpC overexpression), which suggests that the E23D substitution, although conservative, slightly impairs the proteolytic activity of YaeL (Fig. 3-3A, lanes 5 and 6). As expected, DegS-dependent cleavage of RseA is a prerequisite for cleavage by YaeL, as strains lacking both *degS* and *yaeL* exhibited only full-length RseA with or without OmpC overexpression (Fig. 3-3B), and σ^E activity did not increase after OmpC overexpression (data not shown). These results are consistent with the model that RseA is processed sequentially, first by DegS and then by YaeL.

The model describing sequential processing would be strengthened if we could demonstrate a precursor-product relationship between full-length RseA and the RseA fragment present in cells lacking YaeL activity. When the inducing signal caused by OmpC overexpression was provided to cells carrying YaeL E23A and active DegS, full-length RseA is virtually absent after 60'and the RseA fragment is pronounced (Fig. 3-3A, lane 4). To quantify this conversion, we performed quantitative Western blotting at various timepoints following induction. These data demonstrate that as full-length RseA disappeared, the RseA fragment accumulated (Fig. 3-4A), with nearly all of the cytoplasmic domain from full-length RseA ending up in the fragment (Fig. 3-4B). Continued low-level synthesis of full-length RseA during the course of this experiment may account for the fact that slightly more RseA ends up in the fragment than disappears from the amount of full-length RseA present at any particular time. A precursor-product relationship is also indicated by the fact that upon depletion of YaeL, full-length RseA disappeared and the RseA fragment appeared (Fig. 3-1C). Additionally, Kanehara et al. (2002) have used a pulse-labeling protocol to demonstrate a precursor-product relationship. Taken together, these data strongly support the idea that, following induction, DegS processing creates a smaller fragment of RseA, which is subsequently processed by YaeL.

Assuming that the observed RseA fragment does not have an aberrant mobility, it appears to be slightly larger (>15 kD) than the expected size of the cytoplasmic domain alone (12 kD), suggesting that the fragment contains a portion of the transmembrane domain as well as the cytoplasmic domain. We used cellular fractionation and Western blotting to determine the location of the RseA fragment (Fig. 3-5). We found that the

RseA fragment was present in the inner membrane fraction and absent from the cytoplasmic, periplasmic and outer membrane fractions. Control experiments indicated that, as expected, the known inner membrane protease, FtsH/HflB was present only in the inner membrane fraction and that the cytoplasmic protein HtpG and the periplasmic protein MalE were absent from this fraction. We note that this fragment retained its anti-sigma factor activity (Fig. 3-2), consistent with previous studies indicating that the cytoplasmic domain of RseA is sufficient for its anti-sigma activity (De Las Peñas et al., 1997a; Missiakas et al., 1997).

DegS and YaeL are not limiting for σ^E activity

Activation of σ^E is controlled by a proteolytic cascade. We asked whether the proteases carrying out this function are present in limiting amounts in the cell. We overexpressed DegS and YaeL separately or in combination and saw no increase in the steady-state activity of σ^E in the cell (Fig. 3-6). Western blots confirmed that the accumulation of YaeL and DegS increased upon overexpression (data not shown). These data indicate that simply increasing the amount of these proteases in the absence of stress is not sufficient to induce σ^E. Instead, an inducing signal that either alters the activity of the proteases or renders RseA accessible to DegS action is required for activation.

Discussion

The extracytoplasmic stress response in *E. coli* is controlled by the regulated proteolysis of RseA (Ades et al., 1999), a membrane-spanning anti-sigma factor that sequesters σ^E, the transcription factor responsible for up-regulating expression of the genes involved in this stress response activity (De Las Peñas et al., 1997a; Missiakas et al., 1997). Upon RseA degradation, σ^E is free to associate with RNA polymerase and initiate transcription. The principal finding of this work is that RseA is degraded by sequential proteolytic events controlled by the integral membrane proteases DegS and YaeL, an orthologue of S2P. The mechanism for activating σ^E is remarkably similar to that for activating the mammalian transcription factors ATF6 and SREBP, which control the unfolded protein response and cholesterol homeostasis, respectively.

RseA is cleaved sequentially by DegS and YaeL

We have presented evidence that YaeL participates in the degradation of RseA. We first showed that the essential function of YaeL, like that previously demonstrated for DegS (Alba et al., 2001), is to provide σ^E activity by participating in the degradation of RseA. We demonstrated this by showing that YaeL is not essential when: (1) a suppressor (*sup*$^+$) bypasses the need for σ^E activity or (2) the necessity for RseA degradation is bypassed by genetically eliminating RseA from cells. We then showed that DegS functions upstream of YaeL in the degradation pathway. We demonstrated this by following the fate of RseA in *sup*$^+$ cells (in which neither YaeL nor DegS is essential) after a σ^E-inducing signal was provided. In *sup*$^+$ cells expressing DegS and YaeL, degradation of RseA accelerates and proceeds without the accumulation of intermediates

upon induction (Ades et al., 1999). DegS is required for initial processing of RseA since *sup*$^+$ cells lacking DegS do not degrade RseA upon induction (Ades et al., 1999). YaeL is required for a subsequent processing event since *sup*$^+$ cells lacking YaeL rapidly degrade the periplasmic domain upon induction but accumulate the RseA fragment that contains the transmembrane portion and cytoplasmic domain. These data indicate that YaeL processes the RseA fragment generated by DegS action, thereby functioning downstream of DegS in the RseA degradation pathway.

In direct contrast to our finding that YaeL is necessary to activate σ^E, a recent report found that overexpressing YaeL decreased the activity and amount of σ^E. The authors concluded that YaeL negatively regulates σ^E (Dartigalongue et al., 2001a). Upon YaeL overexpression, we observed neither a decrease in the activity nor amount of σ^E in two different *E. coli* strain backgrounds, MC1061 and MG1655 (Figure 3-6 and data not shown). We also did not observe the decreased σ^{32} activity upon YaeL overexpression as reported by Dartigalongue et al. (2001a) (data not shown). In that report, overexpression of YaeL by arabinose in an *araD*$^-$ background (in which arabinose is toxic; Englesberg et al. 1962) and using YaeL overexpression constructs containing a portion of the downstream gene may account for some of these discrepancies. We note that Kanehara et al. (2002) have independently reached conclusions similar to ours concerning the role of YaeL in regulating σ^E activity.

Pathways for maintaining homeostasis in the *E. coli* envelope and mammalian ER have a similar means of activation

The envelope of Gram-negative bacteria and the ER of eukaryotes have functional similarities. Both cellular compartments are oxidizing and facilitate disulfide bond formation in proteins. Both compartments are sites of protein folding and assembly. In *E. coli*, outer membrane proteins and periplasmic proteins fold in the envelope. In eukaryotic cells, all proteins destined for secretion, the ER lumen or the Golgi are folded in the ER. The compartments are also sites of assembly of membrane components: assembly of the bacterial outer membrane takes place in the envelope, and assembly of lipid bilayers occurs in the ER. Integral membrane transcription factors respond to changing physiological states of the ER and envelope. SREBPs maintain ER lipid homeostasis by responding either to the levels of sterols in mammals (Brown and Goldstein, 1999) or phosphatidylethanolamine in *Drosophila* (Dobrosotskaya et al., 2002). ATF6 responds to changes in ER protein homeostasis by initiating the UPR upon increases in the level of unfolded proteins (Haze et al., 1999). In *E. coli,* the transcription factor σ^E, which is indirectly tethered to the membrane via RseA, responds to fluctuations in homeostasis in the envelope. Interestingly, genes whose expressions are controlled by σ^E overlap the classes of genes whose expressions are controlled by both SREBP (e.g. genes encoding lipid-associated functions) (Brown and Goldstein, 1999) and ATF6 (e.g. genes encoding chaperones and protein folding catalysts) (Yoshida et al., 1998). σ^E regulates genes important for porin biogenesis and for the production of the lipid A component of the outer membrane (Dartigalongue et al., 2001b). Thus, the σ^E pathway

regulates the expression of classes of genes that are controlled by at least two separate pathways in higher eukaryotes.

During the ER and envelope responses, proteolysis releases membrane-bound transcription factors so that they can carry out transcription. Proteolysis directly releases the transcription factor domains of SREBP and ATF6 from the membrane so that they can travel to the nucleus and activate transcription (Wang et al., 1994; Sakai et al., 1996; Haze et al., 1999; Ye et al., 2000b). Proteolysis indirectly releases σ^E by degrading the anti-sigma factor RseA so that σ^E can initiate transcription (Ades et al., 1999). The proteolytic process that releases the three membrane-bound transcription factors is remarkably similar. In each case, the initial processing event is carried out by an integral membrane serine protease. S1P cleaves ATF6 and SREBP in their lumenal domains after transit from the ER to the Golgi (Ye et al., 2000b; Chen et al., 2002), and DegS cleaves RseA in its periplasmic domain. In each case, the second processing event is dependent upon the first and is carried out by a membrane-embedded metalloprotease. Thus, without cleavage at the first site, there is very little cleavage of ATF6 or SREBP by S2P. Likewise, DegS cleavage of RseA is a prerequisite for YaeL cleavage. Finally, in each case, it is the initial processing event that appears to be regulated. For SREBP, an associated regulatory protein (SCAP), which is responsive to sterol levels, causes transit of SREBPs to the Golgi so that they can be cleaved by S1P (DeBose-Boyd et al., 1999; Nohturfft et al., 1999). ATF6 is hypothesized to be escorted to the Golgi in a similar, but SCAP-independent, fashion (Ye et al., 2000b; Chen et al., 2002). For RseA, we demonstrate here that the inducing signal promotes the first DegS-dependent cleavage event, even when YaeL is absent.

YaeL and DegS are part of the RseA degradation pathway whose complete mechanism is undetermined

Two important issues remain to be addressed before we fully understand the regulated degradation of RseA, which is the major mechanism for regulating the activity of σ^E. First, what are the molecular details of the DegS and YaeL contributions to the RseA degradation pathway? Second, how do these two proteases cooperate to degrade RseA?

We do not know where DegS cleaves within RseA. Our data indicate that the initial RseA degradation product resulting from DegS activity is a membrane-localized fragment consisting primarily of the cytoplasmic domain. A VSLG sequence about 30 amino acids downstream of the membrane-spanning region is similar to the VLS sequence at which S1P cuts SREBP-2 (Duncan et al., 1997). A DegS cut at this position would yield a fragment of about 16.7 kD, which is consistent with the apparent size of the released RseA fragment we observed. Existing data do not address whether DegS fully degrades the periplasmic domain or allows another periplasmic protease to complete the degradation.

We do not know whether YaeL degrades the cytoplasmic fragment of RseA or simply clips it. YaeL has the protease active site motifs and hydropathy plot characteristic of S2P. Moreover, our mutational studies indicating that altering the active site glutamic acid to alanine eliminates protease activity whereas altering it to aspartic acid slightly reduces activity are consistent with similar studies in other S2P orthologues. By analogy to S2P (Duncan et al., 1998) and SpoIVFB (Rudner et al., 1999), we favor the idea that

YaeL clips RseA either within or close to the membrane-spanning segment, leaving the cytoplasmic domain associated with σ^E. In this case, how might the remainder of RseA be degraded so that σ^E is released? We note that S2P cuts at a hydrophobic residue followed by a cysteine, although there is not a stringent preference for the cysteine (Duncan et al., 1998). The RseA transmembrane segment contains a cysteine. If YaeL cuts immediately before the cysteine, it would generate an RseA fragment with a non-polar tail of GVAA. Nonpolar tails are substrates for the ClpAP and ClpXP proteases, which bind, unfold and then degrade such substrates (Gottesman et al., 1998). Thus, ClpAP or ClpAP could degrade such a fragment. Since σ^E lacks a non-polar tail, it would not be a substrate of these proteases and would be released during the process of degrading the RseA fragment.

Most importantly, we do not know the functional relationship between DegS and YaeL. The data presented here indicate that whereas DegS can act in the absence of YaeL, the converse is not true. In the absence of YaeL, DegS can both receive an inducing signal and generate a membrane-localized RseA fragment. However, there is no evidence of YaeL function in the absence of DegS, as we do not observe activation of the σ^E pathway in response to the OmpC inducing signal. We imagine three possible mechanisms by which DegS can influence YaeL activity. First, DegS cleavage of the periplasmic domain of RseA may alter the membrane-spanning region so that it is accessible to YaeL function, as has been suggested for S1P. In that case, S1P cleavage close to the membrane is thought to alter the conformation of an α-helical membrane-spanning segment of SREBP such that the S2P cleavage site is either more accessible or more easily denatured (Ye et al., 2000a). Second, DegS might generate a signal that is

necessary to activate YaeL. A precedent for this idea comes from investigations of the pathway producing the activated σ^K transcription factor required for *B. subtilis* sporulation. Here, the upstream serine peptidase SpoIVB promotes SpoIVFB-dependent processing of the inactive pro-σ^K to active σ^K by an as yet unknown mechanism (Cutting et al., 1991; Lu et al., 1995; Rudner et al., 1999; Wakeley et al., 2000; Hoa et al., 2002). Finally, YaeL might require an interaction with DegS in order to be activated to cleave RseA. Interestingly, both DegS and YaeL have periplasmically localized PDZ domains. In eukaryotes, PDZ domains mediate the building of protein complexes, especially those involved in signal transduction (Harris and Lim, 2001). In bacteria, PDZ domains in proteases can mediate substrate recognition (Beebe et al., 2000; Krojer et al., 2002). Interactions between the PDZ domains of DegS and YaeL may alter the activities or substrate recognition properties of YaeL. Mutationally altering or replacing the PDZ domain of YaeL with heterologous sequences inactivates the protein (Dartigalongue et al., 2001a; Kanehara et al., 2001), providing support for the idea that the PDZ domain might play an important role in RseA degradation.

We are continuing to investigate the features of DegS and YaeL that are required for signal transduction in the σ^E pathway. Given the mechanistic similarities to the mammalian systems, further study of DegS and YaeL may suggest how two proteases work together to activate membrane-localized transcription factors.

Experimental Procedures

Media and antibiotics

Luria-Bertani (LB) was prepared as described (Sambrook et al., 1989). When required, the media were supplemented with the following: 30 μg/ml kanamycin (Kan); 20 μg/ml chloramphenicol (Cm) (for pBAD33-containing strains) or 10 μg/ml Cm (for pBAD45-containing strains); 100 μg/ml ampicillin (Ap) or 50 μg/ml ampicillin (Ap) for CAG43514-based strains. A final percentage of 0.2 % L-(+)-arabinose was used to induce the expression of *yaeL* and *ompC* from the arabinose-inducible promoter P_{ara}.

Strains

Strains used in this study are listed in Table 3-3. To make the *yaeL::kanR* strain, JAH175 was transformed with pJAH118 to create JAH152. These clones were then made competent for transformation in the presence of arabinose to induce the lambda phage *red/gam* genes and the plasmid copy of *yaeL*. JAH152 was transformed (in the presence of arabinose to induce the complementing *yaeL* gene on pJAH118) with 50 ng of linear PCR product containing a *kanR* cassette flanked by regions of homology to the 3' end of the *cdsA* gene, found upstream of *yaeL*, and homology to the 5' end of *yzzN*, found immediately downstream of *yaeL*. The *kanR* cassette was amplified from FED326 using primers: *yzz/kan* 3'

ggcgctgctaaacagcagcgacgctatgagcaacttttcatcgccatcgttattatgcgttcttcctaactaactcTCAtctgaT**TAGAAAAACTCATC** containing 15 nt of homology to *kanR* (bold) plus a stop codon and 77 nt homology to *yzzN* (underlined); and *cds/kan* 5'

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ggcgctgctaaacagcagcgacgctatgagcaacttttcatcgccatcgttattatgcgttcttcctaactaactcTCAtctga**T**

TAGAAAAACTCATC containing 15 nt of homology to *kanR* (bold) plus a stop codon and 77 nt homology to *yzzN* (underlined); and *cds/kan* 5'

AttgatagcctgacggctgcggtaccggtctttgcttgcttgttgttactggtattcaggacgcttTAAcggaaggtaat**GGG AAAGCCACGTTGTGTC** containing 19 nt of homology to *kanR* (bold) and 66 nt of homology to *cdsA* (underlined) plus a ribosome binding site and linker. JAH152 transformants that grew on kanamycin were restreaked in the presence of 0.2% arabinose or 0.2% glucose, and only those grown in the presence of arabinose would restreak, indicating that expression of the complementing copy of *yaeL* was essential for growth of the newly constructed *yaeL::kanR* deletion strain. *yaeL::kanR* was moved into various backgrounds by standard P1 transduction (Miller 1972). When moved into CAG41001 (*sup*$^+$), the transductants were much smaller and somewhat more heterogeneous than those in the Δ*rseA* and Δ*degS* backgrounds, although in each case, the transductants were visible after an overnight's growth at 30°C.

CAG43540, 43541 and 43553 were made by transforming CAG43514 with pJAH322, pJAH340 and pJAH325, respectively, and selecting on Ap/Cm. In general, all three strains grew very slowly on selective solid media. CAG43541 and empty vector-containing strains were notably smaller than those of 43540 and 43553. We noticed some heterogeneity in the colony sizes of transformants, although the heterogeneity was more pronounced among the pJAH340 and empty vector transformants. Therefore, to ensure that our results were not isolate-specific, we tested multiple independent isolates from each transformation in σ^E activity assays and for the generation of the RseA fragment. Each strain's respective group of independent isolates exhibited similar phenotypes.

Plasmids

To make pJAH118 and pJAH184, the *yaeL* gene was amplified from *E. coli* MG1655 with the primers YaeL (L) 5'-GGAATTCATGCTGA-GTTTTCTCTGGGATTTGGC-3' and YaeL (R) 5'-GGAATTCTCATAA-CCGAGAGAAATCATTG-3'. The product was cloned into *Eco*RI of each plasmid. To make pBA114, the *ompC*-containing *Hin*DIII fragment from pEMC1 (Catron and Schnaitman 1987) was cloned into *Hin*DIII of pBAD33. A two-step PCR procedure was used to make pJAH325 (*yaeL E23D*) and pJAH340 (*yaeL E23A*). In the first step, two individual PCRs created an overlapping region of homology that contained the desired mutation. The first round of PCR used pJAH184 as a template. For pJAH325: the two individual PCRs were: (1) *araC down* 5' primer 5'-GACTAGGCCTGATATAGGCGCCAGCAACCG-3'and 3' primer 5'-CAGAAATGACCAAAATCATGCACG-3'; (2) 5'-CGTGCATGATTTTGGTCATTTCTG-3'and YaeL (R). These two products were gel-purified and, in Step 2, mixed with the outside primers YaeL (L) and YaeL (R), which were then extended across the annealed products from the first round to generate the complete gene. For pJAH340: the 1st step used (1) *araC down* and 5'-CAGAAATGACCAAATGCATGCACG-3'; (2) 5'-CGTGCATGCATTTGGTCATTTCTG-3' and YaeL (R). The 2nd step was like that for pJAH325. The final products were cloned into *Eco*RI of pDSW206 (YaeL E23D) or pDSW204 (YaeL E23A).

Western blotting

During the course of YaeL depletion (Fig. 3-1) or during the course of OmpC overexpression (Fig. 3-4), 900 µl samples were removed and mixed with 100 µl of ice-cold 50% trichloroacetic acid (TCA) and stored on ice. Samples in Fig. 3-3 were harvested at 1 hr. following the addition of arabinose to overexpress OmpC. TCA precipitates were processed as described in Alba et al. (2001), run on 15% polyacrylamide gels and transferred to nitrocellulose. An equal number of cells was loaded for each sample. Western blotting conditions were described previously (Alba et al., 2001). The following dilutions of primary antisera (all rabbit) were used: anti-YaeL (1:5000); anti-RseA cytoplasmic domain (1:5000); anti-HtpG (1:10000); and anti-FtsH/MalE mixture (1:10000). The secondary antibody (used at 1:10000) was an anti-rabbit horseradish peroxidase-conjugated antibody from Amersham Life Sciences. Western blots in Figure 3-4 were developed with the SuperSignal West Dura Extended Duration Substrate from Pierce. We used the *Epi Chemi II Darkroom* (UVP Laboratory Products) to capture the light emitted from the blots and quantitated the bands using the associated software (Labworks). All other Western blots were developed with an enhanced chemiluminescence kit (Amersham Pharmacia Biotech) and exposed to film.

The YaeL antibody was raised in a rabbit (Covance, Inc.) against a nickel column-purified N-terminally 6His-tagged YaeL periplasmic domain. The coding sequence of the domain, which includes nucleotides 384-1116 (amino acid residues 127-372), was PCR-amplified and cloned into *Bam*HI and *Hin*DIII of pQE30 (Qiagen).

Genetics addressing yaeL *essentiality*

To determine if *yaeL* is essential in various backgrounds by using the *yaeL* depletion strains, efficiencies of plating (EOP) on LB with and without arabinose were performed as follows. 1 ml of each overnight culture, grown at 30°C in LB/ Cm/arabinose, was pelleted in a microcentrifuge and washed 3 times in 1 ml LB to remove arabinose from the suspension. The washed cultures were ten-fold serially diluted to 10^{-7}. 10 µl of dilutions 10^{-1}-10^{-7} were spotted onto LB/ Cm +/- arabinose plates and incubated at 30°C, 37°C and 43°C. EOP were calculated by dividing the number of colony forming units (cfu) in the absence of arabinose by the number of cfu in the presence of arabinose. Cfu were counted after approximately 20–22 hrs. of incubation and did not change upon prolonged incubation. EOP were repeated at least three times, except for CAG43560, which was done twice. Data presented are the average EOP.

P1 transductions using JAH301 as a donor were performed by a standard procedure (Miller 1972). Transductants were selected on LB/Kan at 30°C. Plates were scored after 20-22 hours of growth. CAG41001 *yaeL::kanR* transductants were much smaller and somewhat more heterogeneous in size than the others.

YaeL depletion in vivo

CAG43509 was grown at 30°C in LB/ Cm/arabinose to an OD_{600} of approximately 0.25 - 0.3. The culture was poured onto a 0.45 µm Millipore filter (Millipore Corp.) in a Nalgene (Nalge Nunc International) filtering system and washed with 10-15 ml of 30°C LB to remove the arabinose. The cells were resuspended in 30°C LB/ Cm containing arabinose or 0.2% glucose to an OD_{600} ~ 0.04. At time points after

subculturing, aliquots were sampled for Western blots and β-galactosidase assays. The culture was maintained in exponential growth phase by periodically diluting the culture (to OD_{600} ~ 0.04) as follows. An appropriate volume of culture was removed, leaving the subsequent subculture "starter" behind and shaking; the removed volume was quickly replaced with fresh, pre-warmed media. We have observed that the alternative dilution method, removing the starter culture by pipet and transferring it to a new flask, can cause a large decrease in σ^E activity (unpublished observations).

β-galactosidase assays

Overnight cultures were diluted to an OD_{600} ~ 0.04 and grown at 30°C in LB with the appropriate antibiotics. In experiments involving the overexpression of OmpC (Fig. 3-2), cultures were grown to OD_{600} ~ 0.15 and sampled for initial σ^E activity. Arabinose was then added to induce the overexpression of OmpC. Additional samples were collected at subsequent time points. In Fig. 3-6, cultures were grown to OD_{600} ~ 0.15, sampled for initial σ^E activity and induced with arabinose to overexpress YaeL; additional samples were collected at subsequent time points. Graphs plot β-galactosidase activity/sample versus sample OD_{600}, the slope of which is the differential rate of β-galactosidase synthesis and a measure of σ^E activity. Assays were performed as previously described (Miller 1972; Mecsas et al., 1993; Ades et al., 1999).

Cellular fractionation

A 10 ml (LB/Cm/30°C) culture of CAG43514 was grown to OD_{600} ~ 0.25 and induced with arabinose to overexpress OmpC. After 1 hr., cells were harvested and

fractionated as described in Mecsas et al. (1993). Samples were run on 15% polyacrylamide gels and analyzed by Western blotting.

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Table 3-1. P1 transduction of *yaeL::kanR* into *E. coli* strains grown on LB at 30°C.

Recipient	Strain	# Kan^R colonies obtained following transduction with P1 grown on donor strain *yaeL::kanR*[a]
Wild type	CAG16037	0
Δ*rseA*	CAG22968	~100 - 150
sup$^+$ of Δ*rpoE*	CAG41001	~100
Δ*degS*[b]	CAG33315	~100 - 150
p*rpoE*	CAG25187	
0 mM IPTG		0
0.1 mM IPTG		~100

[a] Strain JAH301 was the donor.

[b] Contains a suppressor of Δ*degS* lethality

P1 transductions were done twice and performed as described in Materials and Methods.

Table 3-2. Efficiencies of plating (EOP) of YaeL depletion strains in the absence (complementing *yaeL* repressed) versus the presence of arabinose (complementing *yaeL* induced) at 30°C, 37°C and 43°C.

		Efficiencies of Plating (# cfu[a] - arabinose/ #cfu + arabinose)		
Background	Strain	30°C	37°C	43°C
Wild type	CAG4350	1.2×10^{-4}	1.9×10^{-3}	5.7×10^{-4}
Δ*rseA*	CAG4355	1.7	1.2	0.6
sup$^+$ of Δ*rpoE*	CAG4354	0.8	0.4	8.6×10^{-4}
Δ*degS*[b]	CAG4356	0.9	0.6	0.9

[a] Colony-forming units

[b] Contains a suppressor of Δ*degS* lethality

EOP were performed as described in Materials and Methods. Average EOP are shown.

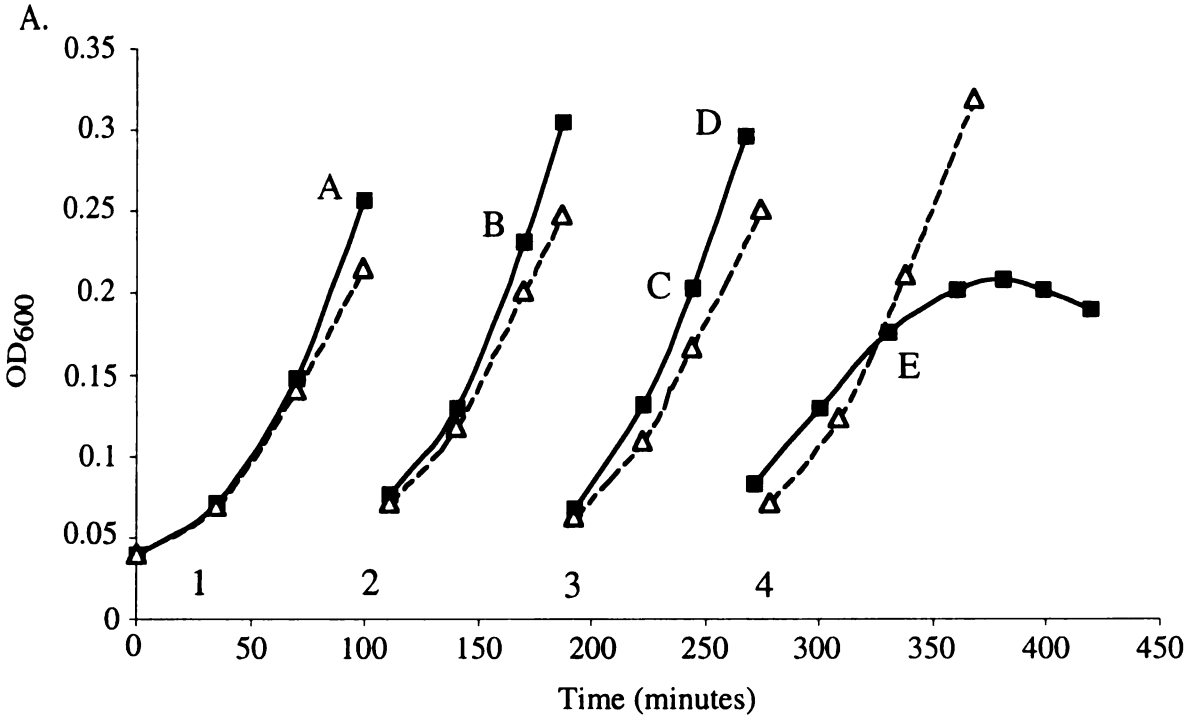
Table 3-3. Strains and plasmids used in this study.

Strain / plasmid	Relevant genotype	Source / reference
Strain		
MC1061	*araD* Δ(*ara-leu*)7697 Δ(*codB-lacI*) *galK*16 *galE*15 *mcrA*0 *relA*1 *rpsL*150 *spoT*1 *mcrB*9999 *hsdR*2	(Casadaban and Cohen, 1980; *E. coli* Genetic Stock Center)
CAG16037	MC1061 [Φλ*rpoH* P3::*lacZ*]	(Mecsas et al., 1993)
CAG22216	16037 *rpoE*::ΩCm, Cm^R	(Rouvière et al., 1995)
CAG22968	16037 Δ*rseA nadB*51::Tn*10*, Tet^R	this work
CAG25187	16037 pLC245, Ap^R	this work
CAG33315	MC1061 Δ*degS* [Φλ*rpoH* P3::*lacZ*]	(Ades et al., 1999)
CAG33330	16037 pSU21, Cm^R	(Alba et al., 2001)
CAG33333	16037 pLC259, Cm^R	(Alba et al., 2001)
CAG41001	MC1061 $rpoE^+$ with suppressor of *rpoE*::ΩCm	(Alba et al., 2001)
CAG43471	41001 *yaeL*::*kanR*, Kan^R	this work
CAG43509	16037 *yaeL*::*kanR* pJAH184, Cm^R Kan^R	this work
CAG43512	33333 pJAH118, Ap^R Cm^R	this work
CAG43514	43471 pBA114, Kan^R Cm^R	this work
CAG43540	43514 pJAH322, Cm^R Ap^R Kan^R	this work
CAG43541	43514 pJAH340 Cm^R Ap^R Kan^R	this work
CAG43549	41001 *yaeL*::*kanR* pJAH184, Cm^R Kan^R	this work
CAG43550	22968 *yaeL*::*kanR* pJAH184, Cm^R Kan^R Tet^R	this work
CAG43551	33315 *yaeL*::*kanR* pBA114, Cm^R Kan^R	this work
CAG43552	33315 *yaeL*::*kanR* pBAD33, Cm^R Kan^R	this work
CAG43553	43514 pJAH325, Cm^R Ap^R	this work
CAG43560	33315 *yaeL*::*kanR* pJAH184, Cm^R Kan^R	this work
CAG43576	33330 pJAH118, Ap^R Cm^R	this work
KS272	F^- Δ*lacX74 galE galK thi rpsL* Δ*phoA* (*Pvu*II)	(Strauch and Beckwith, 1988)
JAH184	KS272 pJAH184, Cm^R	this work
JAH301	JAH184 *yaeL*::*kanR*, Kan^R Cm^R	this work
JAH175	KS272 pKOBEG, Cm^R	this work

FED326	MC1000 *dsbD trxA* Δ(λ *attL-lon*)::pFK79 (pBAD18-kan Ω *trxA*$^+$)	F. Katzen, Beckwith lab (unpublished)
Plasmid		
pKOBEG	*gam bet exo* under P_{ara} control, Cm^R	(Chaveroche et al., 2000)
pJAH118	*yaeL* in pBAD24, Ap^R	this work
pJAH184	*yaeL* in pBAD45, Cm^R	this work
pJAH322	*yaeL* in pDSW206, Ap^R	this work
pJAH325	*yaeL E23D* in pDSW206, Ap^R	this work
pJAH340	*yaeL E23A* in pDSW204, Ap^R	this work
pLC245	*rpoE* in p*Trc99*A, Ap^R	this work
pBA114[a]	*ompC* in pBAD33, Cm^R	this work
pBAD24	Vector, pBR322 ori, P_{ara}, Ap^R	(Guzman et al., 1995)
pBAD45	Vector, p15A ori, P_{ara} Cm^R	Beckwith lab
pDSW204	Derivative of p*Trc99*A, Ap^R	(Weiss et al., 1999)
pDSW206	Derivative of p*Trc99*A, Ap^R	(Weiss et al., 1999)
pSU21	Cloning vector, p15a ori, *lac* promoter, Cm^R	(Bartolomé et al., 1991)
p*Trc99* A	Vector, pBR322 ori, Ap^R	Amersham Pharmacia Biotech

[a] Referred to as p*ompC* in text.

Figure 3-1. *In vivo* depletion of YaeL. (A) Growth of YaeL depletion strain (CAG43509) in *yaeL*-inducing (LB/0.2% arabinose; closed squares) or repressing (LB/0.2% glucose; open triangles) media at 30°C. The depletion strain carries *yaeL::kanR* on its chromosome and a complementing plasmid encoding *yaeL* expressed from an arabinose-inducible promoter P_{ara}. The depletion was performed as described in Materials and Methods. To maintain the cultures in exponential phase, they were subcultured by dilution into the same pre-warmed media. Numbers 1-4 designate the subcultures, and letters A-E identify the time points at which samples for Western blotting were removed. (B) σ^E activity during YaeL depletion. Samples from the LB/glucose and LB/arabinose subcultures in (A) were assayed for σ^E activity by monitoring β-galactosidase activity produced from a single-copy [Φλ*rpoH* P3::*lacZ*] fusion. For simplicity, only σ^E activity from the first arabinose subculture is shown since all subsequent arabinose subcultures had equivalent activities. Assays were performed as described in Materials and Methods. (C) *In vivo* steady-state levels of YaeL, RseA, and RseA fragment during depletion. At the time points identified in (A), samples were removed from the subcultures and analyzed by Western blotting using anti-YaeL and anti-RseA cytoplasmic domain antisera (see Materials and Methods). Asterisk indicates a non-specific background band which controls for loading error.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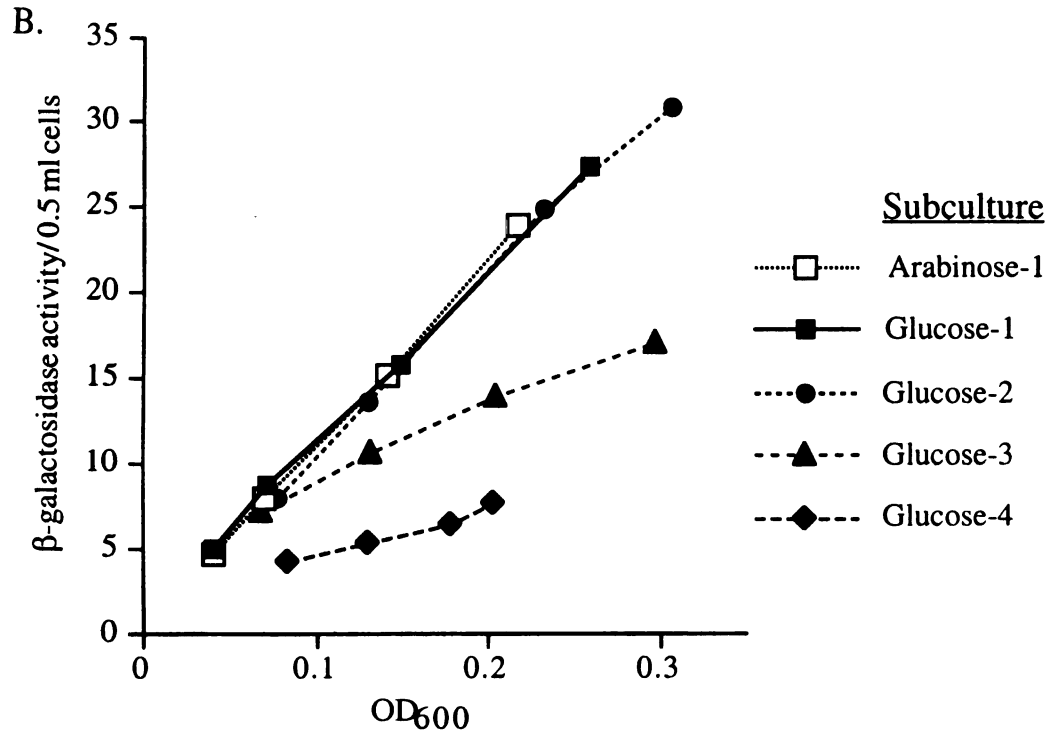
Time (minutes)


B.
35
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β-galactosidase activity/0.5 ml cells
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0.1
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OD600
Subculture
Arabinose-1
Glucose-1
Glucose-2
Glucose-3
Glucose-4

C.

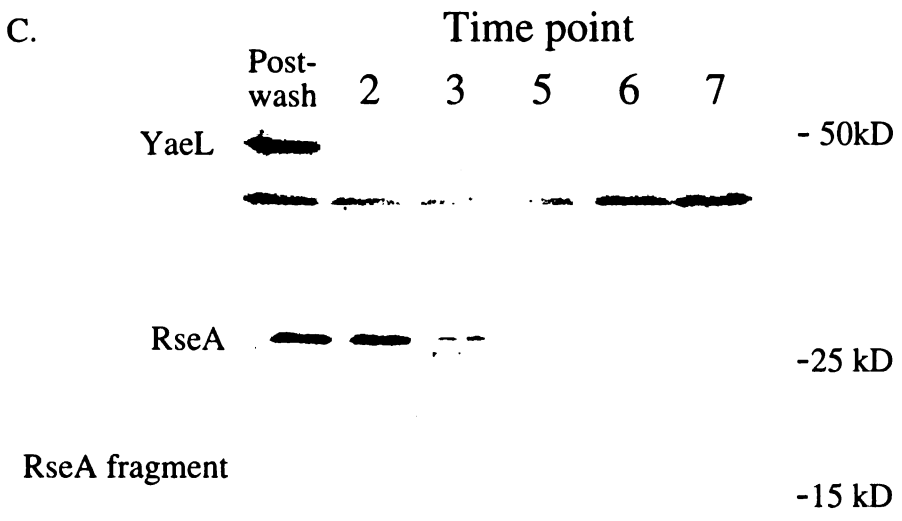

Time point
Post-wash
2
3
5
6
7
YaeL
- 50kD
RseA
-25 kD
RseA fragment
-15 kD

Figure 3-2. Wild type YaeL and YaeL E23D, but not the active site mutant YaeL E23A, restore σ^E activity to a *yaeL::kanR* strain. *sup*$^+$ *yaeL::kanR* strains carrying p*ompC* and pJAH322 (*yaeL*;■/□, CAG43540) or pJAH340 (*yaeL E23A* ; ●/○, CAG43541) or pJAH325 (*yaeL E23D*; ▲/Δ, CAG43553) were assayed for basal and induced σ^E activity by monitoring β-galactosidase activity produced from a single-copy [Φλ*rpoH* P3::*lacZ*] fusion, as described in Materials and Methods. The σ^E inducing signal was provided by overexpressing OmpC with 0.2% arabinose, which was added immediately following the first assay time point (OD_{600}~0.15). Solid symbols contained arabinose (and overexpressed OmpC) while open symbols did not. The inset shows the very low β-galactosidase activity of the *yaeL E23A* strain.

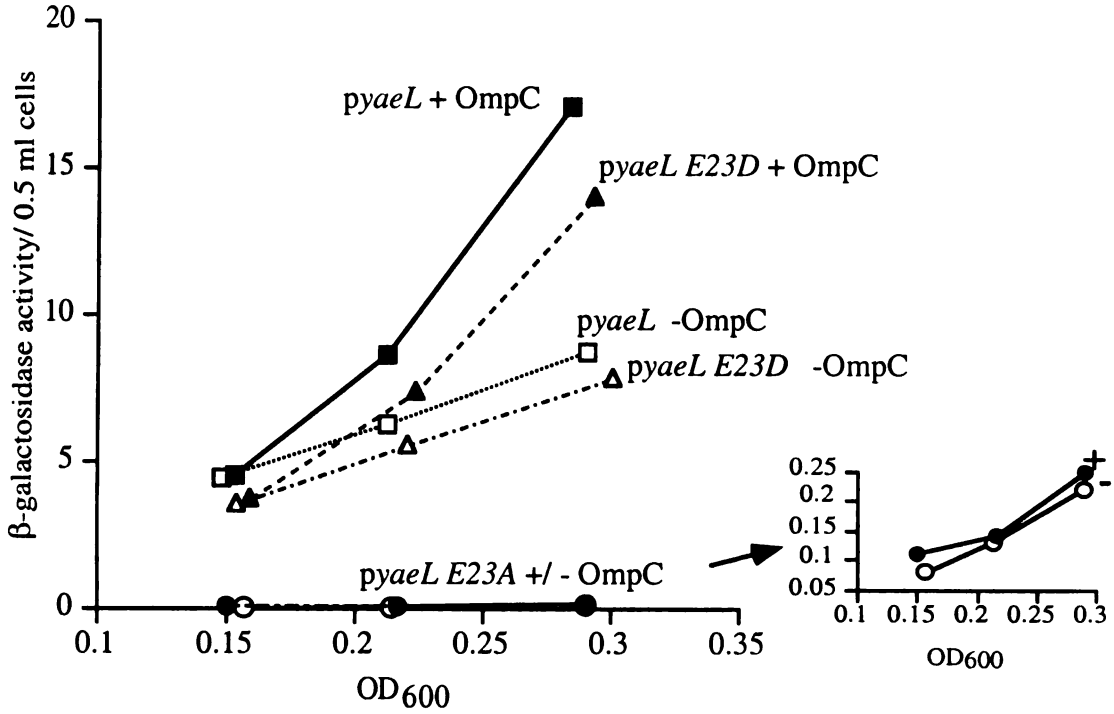
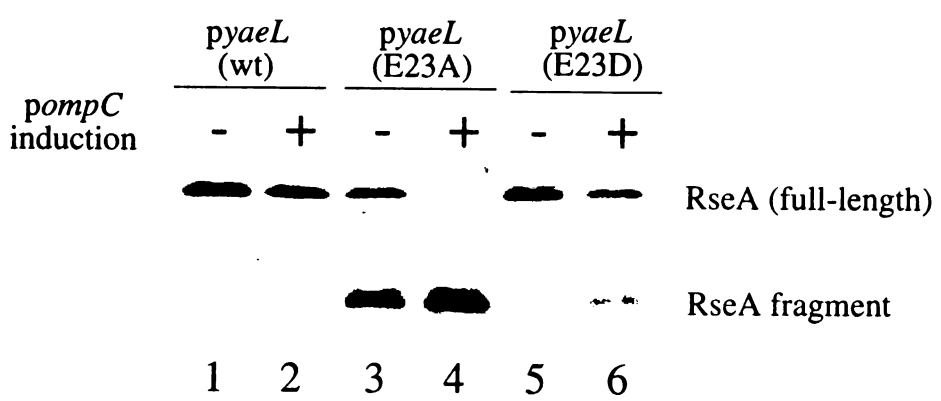

β-galactosidase activity/ 0.5 ml cells
20
15
10
5
0
0.1
0.15
0.2
0.25
0.3
0.35
OD600
pyaeL + OmpC
pyaeL E23D + OmpC
pyaeL -OmpC
pyaeL E23D -OmpC
pyaeL E23A +/ - OmpC
0.25
0.2
0.15
0.1
0.05
0.1 0.15 0.2 0.25 0.3
OD600
+
-

Figure 3-3. YaeL is involved in degradation of RseA. (A) Western blotting (with anti-RseA cytoplasmic domain) of samples harvested from *sup*$^+$ *yaeL*::*kanR* strains carrying p*ompC* and pJAH322 (*yaeL*; lanes 1 and 2) or pJAH340 (*yaeL E23A*; lanes 3 and 4) or pJAH325 (*yaeL E23D*; lanes 5,6) grown with or without overexpression of OmpC for 60 minutes. These samples are from the strains assayed for σ^E activity in Fig. 3-2. (B) Western blotting (with anti-RseA cytoplasmic domain) of samples harvested from Δ*degS* *yaeL*::*kanR* strains with p*ompC* (lanes 1 and 2; CAG43551) or empty vector (lanes 3 and 4; CAG43552). Lane 5 shows a reference sample containing both full-length RseA and the RseA fragment. In both 3A and 3B, arabinose was added to induce the overexpression of OmpC (see Materials and Methods). Cell fractionation experiments confirmed that overexpressed OmpC was present in the outer membrane fraction of the Δ*degS* *yaeL*::*kanR* strain carrying p*ompC* (data not shown).

A.


pyaeL (wt)
pyaeL (E23A)
pyaeL (E23D)
pompC induction
- + - + - +
RseA (full-length)
RseA fragment
1 2 3 4 5 6

B.

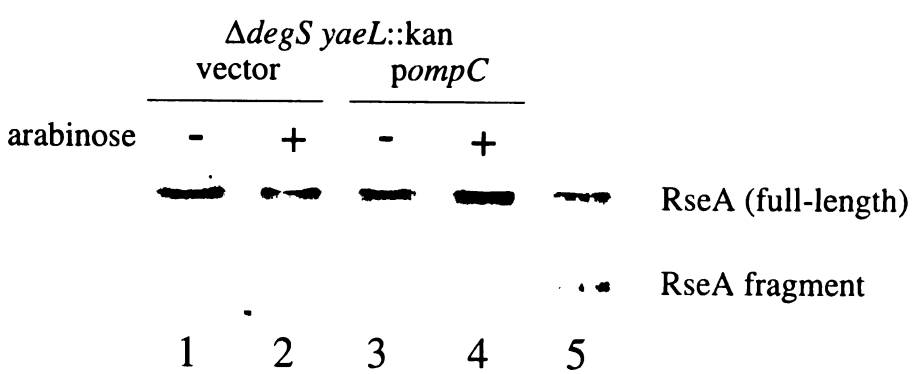
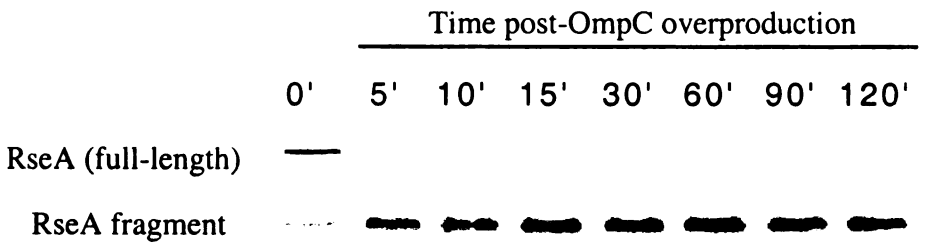

ΔdegS yaeL::kan
vector
pompC
arabinose
- + - +
RseA (full-length)
RseA fragment
1 2 3 4 5

Figure 3-4. Full-length RseA decreases and the RseA fragment increases following OmpC overexpression. (A) Western blotting (with anti-RseA cytoplasmic domain) of samples harvested from *sup*$^{+}$ *yaeL*::*kanR* p*ompC* pJAH340 (*yaeL E23A* ; CAG43541) at various time points after OmpC overexpression is induced. (B) Quantitation of full-length RseA and RseA fragment levels. Blots were quantified as described in Materials and Methods. The intensity of each band was normalized to that of a background band (not shown) to control for loading errors.

A.


Time post-OmpC overproduction
0'
5'
10'
15'
30'
60'
90'
120'
RseA (full-length)
RseA fragment

B.

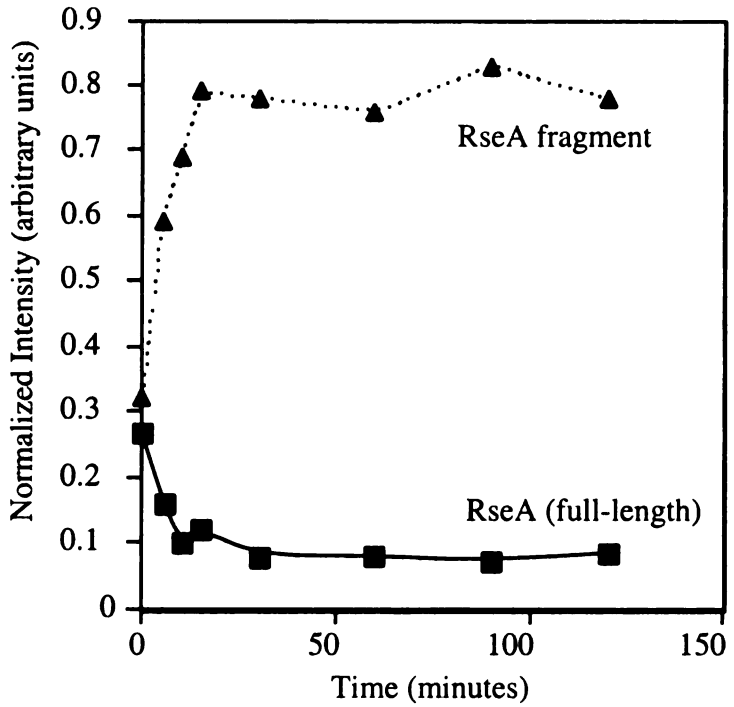

Normalized Intensity (arbitrary units)
0.9
0.8
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0
0
50
100
150
Time (minutes)
RseA fragment
RseA (full-length)

Figure 3-5. RseA fragment is localized to the inner membrane. Strain *sup*$^{+}$ *yaeL*::*kanR* p*ompC* (CAG43514) was grown to mid-log phase and p*ompC* was induced with arabinose for 1 hr. prior to fractionation, as detailed in Materials and Methods. The cells were fractionated into four components: periplasm, cytoplasm, inner and outer membrane. Western blots were probed with anti-HtpG (cytoplasmic protein), anti-FtsH (inner membrane protein), anti-MalE (periplasmic protein), and anti-RseA cytoplasmic domain antisera.

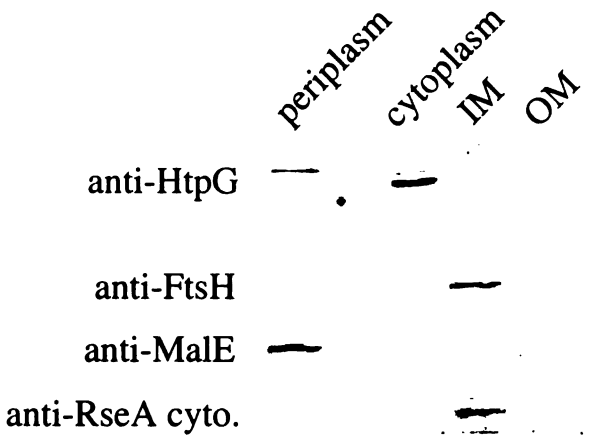
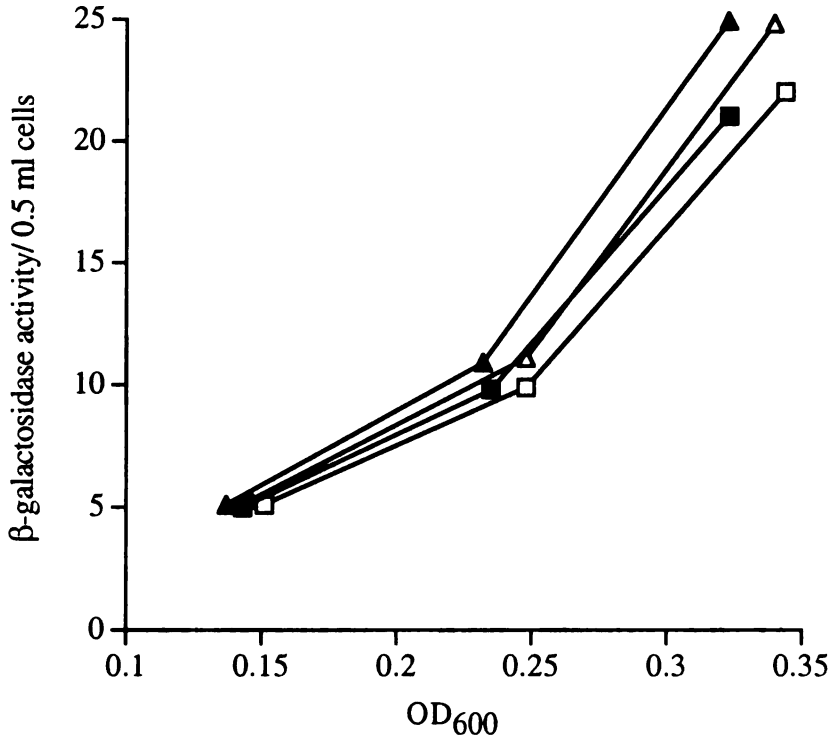

periplasm
cytoplasm
IM
OM
anti-HtpG
anti-FtsH
anti-MalE
anti-RseA cyto.

Figure 3-6. Overexpression of DegS or YaeL separately or together does not affect σ^E activity. Wild type strains with a *yaeL*-expressing plasmid (▲/Δ; CAG43576) or *degS*-expressing plasmid plus a *yaeL*-expressing plasmid (■/□; CAG43512) were grown to early log phase. At an OD_{600}~0.15, arabinose was added to overexpress YaeL, and σ^E activity was by monitored β-galactosidase assays (See Materials and Methods). DegS was constitutively overexpressed in CAG43512. Solid symbols contained arabinose (and overexpressed YaeL) while open symbols did not. Strains containing the empty vectors exhibited nearly identical activities and are not shown for simplicity.


β-galactosidase activity/ 0.5 ml cells
25
20
15
10
5
0
0.1
0.15
0.2
0.25
0.3
0.35
OD600

Chapter Four

PDZ domain-mediated inhibition of DegS protease activity is relieved upon binding OMP C-termini, thereby activating the σ^E-dependent extracytoplasmic stress response

Summary

The envelope stress response in *Escherichia coli* is controlled in part by a signal transduction pathway that activates the σ^E transcription factor. σ^E is normally sequestered by its membrane-spanning anti-sigma factor RseA. During envelope stress, a proteolytic cascade initiated by the HtrA/DegP family member DegS, and propagated by YaeL, an ortholog of Site-2 protease (S2P), destroys RseA. Our *in vivo* evidence indicates that the PDZ domain of DegS normally inhibits its protease activity and that binding of the extreme C-terminal amino acids of outer membrane porins (OMPs) to the PDZ domain constitutes an inducing signal. We suggest that binding of OMP C-termini antagonizes the negative regulatory role of the PDZ domain, thereby activating DegS and this proteolytic cascade. A similar mechanism may be commonly employed by HtrA/DegP proteases to activate stress responses.

Introduction

Signal transduction across cellular membranes employs membrane-spanning proteins that facilitate communication between the cell surface and cytoplasmic factors or between intracellular compartments. One class of transmembrane signal transduction systems utilizes integral membrane proteases that receive signals on one side of a membrane and subsequently cleave integral membrane proteins, releasing soluble effector domains on the other side. In the Gram-negative bacterium *Escherichia coli*, the σ^E extracytoplasmic stress response pathway is an intercompartmental signaling pathway that employs a proteolytic cascade as a part of its signal transduction mechanism.

Misfolding of envelope proteins activates the σ^E transcription factor whose regulon encodes proteases and chaperones that combat cellular stress, as well as biosynthesis enzymes for lipid A (a component of lipopolysaccharide, or LPS) (Mecsas et al., 1993; Betton et al., 1996; Jones et al., 1997; Missiakas et al., 1997; Dartigalongue et al., 2001b; V. Rhodius et al., in prep.). Specific activators of σ^E include overexpressed outer membrane porins (OMPs) such as OmpC, OmpF and OmpX (Mecsas et al., 1993). Besides its role in directing the extracytoplasmic stress response, σ^E is essential for viability under normal laboratory conditions (De Las Peñas et al., 1997b). σ^E is negatively regulated by the inner membrane anti-sigma factor RseA, which has a cytoplasmic domain that binds to σ^E and blocks its transcriptional (De Las Peñas et al., 1997a; Missiakas et al., 1997). The periplasmic domain of RseA binds to RseB, a soluble periplasmic protein, which is another negative regulator of σ^E transcriptional (De Las Peñas et al., 1997a; Missiakas et al., 1997). RseB stabilizes RseA (Ades et al., 1999) and may strengthen the association of RseA with σ^E (Collinet et al., 2000). When OmpC is overexpressed, RseA is rapidly degraded and σ^E activity increases (Ades et al., 1999).

RseA degradation depends upon sequential cutting by at least two inner membrane proteases. RseA is cleaved first in its periplasmic domain by DegS, a member of the DegP/HtrA family of serine proteases (Alba et al., 2002; Clausen et al., 2002; Kanehara et al., 2002). DegS has an N-terminal membrane anchor, a periplasmic active site and a C-terminal periplasmic PDZ domain (Bass et al., 1996; Waller and Sauer, 1996; Alba et al., 2001). YaeL, an integral membrane Zn^{2+}-metalloprotease homologous to eukaryotic Site-2 protease (S2P), then cleaves either within the transmembrane domain of RseA or in the cytoplasmic domain adjacent to the membrane (Kanehara et al., 2001; Alba et al., 2002; Kanehara et al., 2002). Interestingly, both DegS and YaeL are essential proteins (Alba et al., 2002; Kanehara et al., 2002) whose indispensable function is to activate σ^E through RseA cleavage (Alba et al., 2002).

As DegS performs the initial cleavage of RseA, its activity is likely regulated in a signal-dependent fashion. Thus, dissecting DegS features that are required for wild-type (wt) σ^E activity should provide further insights into the mechanism of signal transduction within the σ^E pathway. In earlier work, we found that cells require DegS, including its active site serine and membrane anchor, for wt σ^E activity (Ades et al., 1999; Alba et al., 2001). The remaining feature, a C-terminal PDZ domain, is interesting because PDZ domains in eukaryotes often facilitate the organization of proteins in signal transduction pathways (Harris and Lim, 2001), and, in bacteria, the PDZ domain of the Tsp protease mediates substrate binding (Beebe et al., 2000). In this work, we present *in vivo* evidence that the PDZ domain of DegS plays an inhibitory role within the pathway, which is relieved by interaction of this domain with peptide sequences at the C-termini of OMPs. The *in vitro* studies presented in Walsh et al. (2003) support this model.

Results

Cells expressing DegS lacking its PDZ domain exhibit higher basal σ^E activity

To probe the role of the DegS PDZ domain, we expressed a plasmid-borne DegSΔPDZ variant and assayed a chromosomal *lacZ* reporter under σ^E control (Fig. 4-1B). Δ*degS* strains expressing plasmid-borne *degSΔPDZ* exhibited a basal σ^E activity that was approximately 1.5 to 2.0-fold higher than that of a wt strain carrying only the plasmid vector, as determined by expression of a σ^E-specific *lacZ* transcriptional reporter located on the chromosome (Fig. 4-1A). Plasmid-borne *degSΔPDZ* in the wt background similarly resulted in increased basal σ^E activity (Fig. 4-1A), which indicates that *degSΔPDZ* is dominant over wt *degS* and suggests that *degSΔPDZ* is a gain-of-function allele. Further overexpression of *degSΔPDZ* (caused by inducing its P_{trc} promoter with IPTG) did not result in an additional increase in basal σ^E activity (data not shown). As expected from previous results, plasmid-borne wt *degS* restored normal basal σ^E activity to a Δ*degS* strain, but did not alter the basal σ^E activity of a wt strain, whether or not expression was induced with IPTG (Fig. 4-1A and data not shown). Western blotting with antibodies against the C-terminal hexahistidine tags of DegS and DegSΔPDZ indicated that the *in vivo* steady-state level of DegSΔPDZ was significantly lower than that of DegS (Fig. 4-1B). Thus, DegSΔPDZ increased basal σ^E activity despite being present at a lower steady-state level than DegS. Taken together, these data suggest that the PDZ domain of DegS plays a negative regulatory role in the σ^E signal transduction pathway, possibly by inhibiting the protease activity of DegS itself.

Peptides homologous to the C-terminus of OmpC induce σ^E

Overexpressed OMPs are a major inducer of the σ^E pathway, suggesting that an aberrant fate of OMPs is an inducing signal (Mecsas et al., 1993). The finding that the

PDZ domain of DegS bound selectively to small peptides that are homologous to the C-termini of OMPs (Walsh et al., submitted) coupled with the finding that the PDZ domain regulates DegS activity (Fig. 4-1) suggested that the σ^E pathway may sense OMP overproduction via a direct interaction between DegS and OMP C-termini. To address this possibility, we fused the C-terminal 50 amino acids (aa) of OmpC to an N-terminal *pelB* leader sequence, which will export the OmpC peptide to the periplasm (Fig. 4-2A). The PelB leader sequence should be removed by leader sequence peptidases, leaving the OmpC peptide in the periplasm. We tested whether overexpression of the *pelB-ompC* fusion constituted a σ^E-inducing signal by measuring the accumulation of β–galactosidase from a chromosomal σ^E-dependent *lacZ* transcriptional reporter as a function of cell growth after transcriptional induction of the *pelB-ompC* fusion. This "differential rate of LacZ synthesis" measures how σ^E activity changes in response to a signal. Overexpression of the C-terminal 50 aa of wt OmpC (ending in YQF) induced σ^E about 2-fold relative to the empty vector control, and induction was dependent upon a functional DegS (Fig. 4-2B).

Three lines of evidence suggested that the extreme C-terminal amino acids of the 50 aa OmpC peptide were critical for induction. First, overexpression of a mutant peptide in which the wt C-terminal YQF sequence was changed to YYF, which bound about 30-fold more tightly to the DegS PDZ domain *in vitro* (Walsh et al., 2003), increased induction *in vivo* to a level about 6-fold higher than that of the empty vector (Fig. 4-2B). Second, C-termini expected to bind more poorly to the PDZ domain eliminated induction. When the YQF moiety was deleted (ΔYQF) or the last phenylalanine was substituted with aspartate (YQD), overexpression of these mutant peptides failed to induce σ^E (Fig. 4-2B). Finally, we tested whether a YYF sequence internal to the wt peptide (YYF_{int}) was important for induction. Overexpression of a peptide containing a YYF_{int}→AAA mutation induced σ^E approximately as well as the YQF peptide (data not shown).

A caveat of the peptide overexpression experiments was our inability to directly measure the cellular levels of the overexpressed peptides to rule out the possibility that differences in inducibility resulted from differences in their levels of accumulation. To partially address this concern, we overexpressed peptides in Δ*tsp*, Δ*degP* and Δ*tsp* Δ*degP* backgrounds, which lack well-characterized periplasmic proteases (Lipinska et al., 1989; Strauch et al., 1989; Hara et al., 1991), and found that the overexpressed peptides behaved as they did in the wt background (data not shown).

OMP peptides fused to cytochrome-b_{562} induce σ^E

To quantify accumulation of the inducing OMP peptides, we fused them to the C-terminus of cytochrome-b_{562} (Fig. 4-3A), with expression controlled by an arabinose-inducible promoter. Cytochrome-b_{562} is a soluble, periplasmic protein whose *in vivo* level can be monitored either by polyacrylamide gel electrophoresis or by a spectrophotometric assay for cytochrome-b_{562} absorbance (Brunet et al., 1993; Keiler and Sauer, 1996). Overexpression of cytochrome-b_{562} itself did not induce σ^E (Fig. 4-3B). Overexpression of the cytochrome-peptide fusions ending in YQF or YQM (which bound about 2-fold more weakly than YQF to the PDZ domain *in vitro*; Walsh et al., 2003) or with the YYF_{int} →AAA substitution, induced σ^E approximately 4 to 5-fold relative to vector alone (Fig. 4-3B). This was about twice the induction level observed with these peptides alone and was approximately equivalent to the σ^E induction caused by overproduction of full-length OmpC (Fig. 4-3B). The YYF fusion induced σ^E even more strongly (14-fold relative to empty vector) (Fig. 4-3B), consistent with the approximately 3-fold higher induction exhibited by the YYF peptide as compared to the YQF peptide (Fig. 4-2B). We note that addition of arabinose transiently decreases σ^E activity, which may explain the apparent lag in induction by the fusions or OmpC (Fig. 4-3B and data not shown). Overexpression of cytochrome-peptide fusions ending with ΔYQF or YQD did not

induce σ^E (Fig. 4-3B). Thus, these results qualitatively reproduce the peptide-alone experiments, although at twice the induction levels.

We prepared periplasmic extracts from each strain assayed in Fig. 4-3B and examined accumulation of the cytochrome-peptide fusion proteins on a polyacrylamide gel. Each fusion protein accumulated to a nearly equivalent level *in vivo*, as determined by densitometry of the fusion proteins and normalization to a non-specific protein to account for loading error (Fig. 4-3C). Additionally, spectrophotometric assays for cytochrome-b_{562} levels in these extracts indicated that strains carrying each of the fusion proteins had nearly equivalent levels (data not shown). Taken together, these data support the idea that the identity of the extreme C-terminal residues, rather than the level of accumulation of the fusion protein, determines σ^E–induction activity.

The DegS PDZ domain is required for normal σ^E induction by the cytochrome-peptide fusions

The simplest model consistent with the data presented so far is that binding of the C-terminal amino acids of the OmpC peptide to the DegS PDZ domain relieves PDZ-mediated inhibition of DegS protease activity, thereby promoting degradation of RseA and induction of σ^E activity. The strong prediction of this model is that the cytochrome-peptide fusion proteins should be unable to normally induce σ^E activity when strains carry only DegSΔPDZ. This proved to be the case. Overexpression of the cytochrome-YQF and YYF fusions generally failed to induce σ^E in a Δ*degS* strain expressing plasmid-borne *degSΔPDZ* (Fig. 4-4A), but did so when such strains expressed wt *degS* (Fig. 4-4B). Failure of the fusions to induce σ^E activity in the DegSΔPDZ strains was not overcome by overexpressing DegSΔPDZ (data not shown), which suggested that the inability to induce σ^E activity did not result from limiting amounts of DegSΔPDZ.

Interestingly, a minor phenotypic class among independent isolates of strains bearing DegSΔPDZ and overexpressing the YQF or YYF fusions exhibited atypical induction of σ^E activity. Whereas the major class (10/14 isolates) (Fig. 4-4A and data not shown) exhibited absolutely no induction of σ^E by the fusions, the minor class (4/14 isolates) exhibited an aberrant induction, which was approximately 2 to 3-fold lower (for YQF overexpression) and delayed in comparison to induction in the DegS background (Fig. 4-4C and data not shown). DNA sequencing of DegSΔPDZ-expressing plasmids re-isolated from the minor class revealed no coding sequence changes, and retransformation of these plasmids into the parental background yielded isolates in both classes (data not shown). Taken together, these data suggest that normal induction of σ^E activity by the fusions requires the PDZ domain of DegS. However, these fusion proteins can occasionally utilize a different pathway, independent of the PDZ domain, to slightly induce σ^E activity. We suggest that the slight induction in the minor class occurs through a different mechanism that is dependent upon an epigenetic or genetic alteration in these isolates.

Overexpression of OmpC induces σ^E in strains expressing DegSΔPDZ

The experiments described above demonstrated that the PDZ domain of DegS was required for the normal induction of σ^E caused by overexpression of the C-terminal 50 aa of OmpC. However, it also seemed possible that full-length OmpC contained additional inducing signals that are sensed independently of the DegS PDZ domain. We tested this possibility by overexpressing OmpC in backgrounds expressing either plasmid-borne wt DegS or DegSΔPDZ. Surprisingly, OmpC overexpression induced σ^E approximately equivalently in both strain backgrounds (Fig. 4-5). This suggests that full-length OmpC contains one or more inducing signals that are sensed by the σ^E pathway independently of the DegS PDZ domain. Transduction of these other signals still requires

some feature of DegS, as σ^E activity is not induced by OmpC overexpression in Δ*degS* strains (Ades et al., 1999).

Discussion

Membrane-spanning proteins are ubiquitously employed in the signal transduction cascades that coordinate processes between cellular compartments. However, the signals initiating these cascades are rarely understood in molecular detail. The principal contribution of this work is to define the initial step of the DegS-dependent signal transduction pathway that activates the σ^E transcription factor in response to an envelope stress in *E. coli.* Under normal conditions, σ^E is sequestered by RseA, a membrane-spanning σ^E-specific anti-sigma factor; during stress, a proteolytic cascade initiated by the DegS protease destroys RseA. Here we present *in vivo* evidence that binding of the extreme C-terminal amino acids of OMPs to the PDZ domain of DegS constitutes an inducing signal and that the PDZ domain of DegS normally inhibits the activity of this protease. Taken together, these observations suggest that OmpC binding to the PDZ domain antagonizes its negative regulatory role thereby activating the proteolytic cascade that releases σ^E to promote transcription. The *in vitro* studies presented in Walsh et al. (2003) support this model.

The OMP inducing signal

The very first physiological study of the envelope stress response established that the state of OMPs was critical to the response: (1) an unbiased genetic selection predominantly identified overexpression of OMPs as inducers of this response, and (2) a deletion of *ompR*, which decreases OmpC and OmpF expression, resulted in a 4-fold decrease in the basal rate of σ^E expression (Mecsas et al., 1993). This suggested that an OMP-associated signal was the major inducer of σ^E during normal cell growth. The studies presented here and in Walsh et al. (2003) indicate that at least one OMP signal

resides in the extreme C-terminus of OMPs and works by directly interacting with the PDZ domain of the DegS protease.

The extreme C-termini of OMPs are implicated as inducing signals by the observation that overexpression of the last 50 aa of OmpC is sufficient for induction. The C-terminal three amino acids are most important because deleting the C-terminal YQF or substituting the terminal F with D abolished induction without significantly altering the expression levels of these fusion proteins. Note that it is unlikely that we can assess the role of the very C-terminal amino acids in their native context of the full-length OMP. As OMP C-termini are important for the proper assembly of native porins (Struyvé et al., 1991; Misra, 1993; de Cock et al., 1997; Misra et al., 2000), σ^E induction by such mutant OMPs could be due to any one of a number of signals resulting from the general perturbation of OMP biogenesis.

We present two lines of evidence that induction by the C-terminal amino acids of OMPs is a direct result of their binding to the PDZ domain of DegS *in vivo*. First, induction properties are consistent with two characteristics of peptide binding to this PDZ domain established in Walsh et al. (2003): a) the peptide that binds most tightly to the PDZ domain *in vitro* (YYF) is also the best inducer *in vivo*, and b) as expected from the fact that the C-terminal α-carboxylate is an essential binding determinant, an internal YYF motif does not contribute to induction by the C-terminal OmpC fragment. Second, removing the PDZ domain from DegS eliminates the ability of the pathway to respond to the inducing signal encoded in the C-terminal amino acids of the OmpC fragment. Monitoring OMP C-termini is a sensible way for the cell to keep track of OMP folding. Based upon homology to OmpF, for which a structure at atomic resolution has been

solved, the C-termini of native OMPs likely are buried within the trimer interface (Cowan et al., 1992). Thus, an increase in the abundance of free C-termini could indicate a build-up of non-native species in the folding pathway, as well as of unfolded off-pathway species. These exposed OMP C-termini could then interact with the DegS PDZ domain and induce RseA degradation.

Cells containing DegSΔPDZ can respond to other inducing signals generated by OmpC overexpression, indicating that lack of the PDZ domain renders DegS blind only to the particular signal encoded in the C-terminal OmpC fragment, rather than generally incompetent in responding to signals. The transduction of these additional signals is still dependent upon some aspect of DegS, as σ^E activity in cells lacking *degS* is not induced by OmpC overexpression (Ades et al., 1999). These additional OmpC-encoded signals may be sensed indirectly by the σ^E pathway, perhaps by folding catalysts, or they might be sensed directly by interacting with other components of the proteolytic cascade, including the protease domain of DegS. Alternatively, such signals could increase RseA susceptibility to DegS-dependent cleavage either by binding directly to RseA or by binding to RseB and titrating it away from RseA. The RseB titration model has been proposed as a mechanism for induction by other classes of misfolded envelope proteins (Collinet et al., 2000). The idea of multiple OmpC-related inputs into the σ^E pathway is attractive because it accommodates the multiple stages of OmpC maturation and suggests that the entire OMP biogenesis pathway might be monitored by the σ^E pathway.

The demonstration that the DegS PDZ domain is a key sensor of OMP C-termini explains why the cellular level of OMPs modulates σ^E activity. We speculate that this same circuit is used as a more general monitor of extracytoplasmic stress. Why might the

cell use a restricted set of proteins — OMPs and other proteins with YXF C-termini — to sense compartmental stress? We suggest that the nature of the OMP maturation process makes it a sensitive indicator of the health of the envelope. Maturation of OMPs not only requires LPS (Ried et al., 1990; Sen and Nikaido, 1991; Laird et al., 1994; Kloser et al., 1998), but is also believed to utilize many if not all of the chaperones and folding catalysts (e.g. FkpA, SurA and Skp) encoded in the σ^E regulon (Missiakas et al., 1996; Rouvière and Gross, 1996; Schafer et al., 1999; Behrens et al., 2001; Harms et al., 2001). Thus, any envelope stresses altering the availability of either LPS or periplasmic chaperones should perturb normal OMP maturation. Because OMPs are so abundant, and most have YXF C-termini (Walsh et al., 2003), even a small perturbation in their rate of maturation should lead to the accumulation of sufficient OMP biogenesis intermediates to induce σ^E activity. These considerations suggest that the folding state of OMPs could serve as a sensitive indicator of two parameters: a) whether the general level of unfolded proteins has exceeded chaperone capacity of the extracytoplasmic compartment, and b) the integrity or level of the LPS component of the outer membrane (Fig. 4-6A). Although this strategy achieves the same end result, it differs from those described so far, in which stress activates a response when a general increase in unfolded proteins titrates chaperones away from negative regulatory roles in the stress response (Straus et al., 1989; Straus et al., 1990; Mogk et al., 1997; Tomoyasu et al., 1998; Bertolotti et al., 2000).

Role of the DegS protease in regulating the envelope stress response

DegS is a member of the large DegP/HtrA family of serine proteases, which are distinguished by having a trypsin-like protease domain coupled to one or more C-terminal PDZ domains (Clausen et al., 2002). This protease family is widely distributed in prokaryotes and eukaryotes. Interestingly, in most cases studied, these proteases are involved in stress responses. Bacterial DegP orthologues are implicated in response to heat stress, osmotic shock and pH alterations; human orthologues are involved in heat shock, osteoarthritis and apoptosis (Clausen et al., 2002). The fact that DegS activity is a major determinant of the envelope stress response fits with the general function of this protein family.

Our finding that removing the DegS PDZ domain increased DegS activity *in vivo* suggested that DegS protease activity is negatively regulated by the PDZ domain. That σ^E induction by the overexpression of fusion proteins with OMP C-terminal peptides requires the PDZ domain suggests that OMP C-termini are sensed by the PDZ domain. The simplest model consistent with these observations is that binding of OMP C-termini to the DegS PDZ domain relieves the PDZ domain-mediated inhibition of DegS protease activity (Fig. 4-6B). This model is supported by the *in vitro* data in Walsh et al. (2003).

Structural and biochemical studies of *E. coli* DegP and human HtrA2 provide a context for our results (Krojer et al., 2002; Li et al., 2002). The basic functional unit of these proteases is a trimer, as is also the case for DegS (Walsh et al., 2003). A key feature of these structures is the position of the PDZ domains relative to the protease active sites. In DegP, they are critical for proteolysis, presumably because they present substrates, but they also exist in a closed form that limits access to the active site (Spiess et al., 1999; Krojer et al., 2002). In HtrA2, the PDZ domain is positioned to occlude the active site;

consistent with this idea, the activity of HtrA2 increases upon deletion of this domain (Li et al., 2002). By analogy to these results, we suggest that the PDZ domains of the DegS trimer are mobile and can exist in a conformation that either blocks active site access (closed) or one that permits access (open). Binding of the OMP C-termini to the PDZ domain would stabilize the open configuration and allow proteolysis of RseA. Interestingly, Li et al. (2002) hypothesized that HtrA2 might be activated by ligand binding to the PDZ domain. Our finding that DegS utilizes this mechanism validates the feasibility of this proposal.

Although the DegS PDZ domain may be solely a negative regulatory element, it may have a positive function as well. Deletion of the domain results in only a 1.5 to 2.0-fold increase in σ^E activity, which is not further increased by the overexpression of DegSΔPDZ. As this extent of induction is far less than maximal, the PDZ domain may play some role in presenting substrate or in repositioning the active site for maximal activity. Alternatively, the higher activity of DegSΔPDZ may be counteracted by RseB or other controls upon RseA accessibility to DegS cleavage.

DegS is an integral component of the σ^E envelope stress response: it is a direct sensor of envelope stress and a direct regulator of RseA degradation. The level of DegS itself does not appear to be stress-responsive, as *degS* is neither under σ^E control nor heat-inducible (Waller et al., 1996; V. Rhodius et al., in prep.). Instead, the activity of DegS is modulated by stress. In this work and in Walsh et al. (2003), we have shown that one way to activate DegS is through binding peptide sequences found in OMP C-termini. If DegS were present in excess of OMP-related stress signals, then activation of pre-existing DegS would allow sensitive detection of varying amounts of OMP signals.

Additionally, regulation of the protease domain by the sensor (PDZ) domain allows rapid coupling of stress-sensing and subsequent σ^E induction.

The σ^E stress response is graded, as the half-life of RseA ranges from as little as one or two minutes to as much as one hour, depending upon growth conditions (Ades et al., 2003). Our demonstration that the amount of σ^E induction by peptides varies in accord with their affinities for the PDZ domain (Walsh et al., 2003) suggests that the PDZ domain contributes to the capacity of this system to carry out a graded response to stress. Relief from PDZ-domain mediated autoinhibition of DegS protease activity should be responsive to the prevalence of free OMP C-termini, thus contributing to the variable output of the system. The strategies we have elaborated here for DegS may be generally used by other HtrA/DegP family members to achieve both sensitivity and rapid signal transduction in responses as diverse as thermotolerance and apoptosis.

Materials and Methods

Media and antibiotics

Luria-Bertani (LB) broth was prepared as described (Sambrook et al., 1989). When required, media were supplemented with: 20 μg/ml chloramphenicol (Cm) or 100 μg/ml ampicillin (Ap). A final percentage of 0.2% L-(+)-arabinose was used to induce the overexpression of *ompC* and the cytochrome-peptide fusions from the arabinose-inducible promoter P_{ara}. A final concentration of 1 mM isopropyl-β-D-thiogalactoside (IPTG) was used to induce the expression of the *pelB-peptide* fusions from the P_{trc} promoter.

Strains and plasmids

The strains and plasmids used are listed in Table 1. All plasmids were confirmed by DNA sequencing. The Quikchange (Qiagen) protocol was used to make pBA169, a p*Trc99*A derivative that lacks *Nco*I. pBA152 was made as follows. The *pelB* leader sequence was PCR-amplified from pET25b(+) (Novagen) with primers *pelB3* 5' CCGAGCTCTTTAAGAAGGAGATATAC 3' and *pelB4* 5' CCGGATCCCGAATTAATTCCGAT 3' and cloned into *Sac*I/*Bam*HI of pSU21 (Bartolomé et al. 1991). Next, a C-terminual portion of OmpC (from Y318 to F367) was amplified with primers *ompC2* 5' CAGGATCCTATGTTGATGTTGGT 3' and *ompC5* 5' CCTCTAGATCATTAGAACTGGTAAACCAG 3' and cloned into *Bam*HI/*Xba*I of this plasmid. This generated a fusion between the *pelB* leader and the *ompC* peptide coding sequences (the intervening *Bam*HI site introduces a Gly-Ser linker). Finally, the

*Eco*RI/*Hin*dIII fragment containing the fusion sequence was cloned into p*Trc99*A. pBA161, pBA166 and pBA168 were generated with the Quikchange protocol (Qiagen) using pBA152 as the template.

pBA174 was made as follows. The cytochrome b_{562} gene was amplified from pRW-1 (Brunet et al., 1993; Keiler and Sauer, 1996) with *cyt1* 5' CCGAATTCATGCGTAAAAGCCTG 3' and *cyt2* 5' CTGGATCCACGATACTTCTGGTG 3' and cloned into *Eco*RI/*Bam*HI of a pBA152 derivative lacking *Nco*I. The resulting plasmid was then used as a PCR template in PCR with primers *cyt5* 5' CCGAGCTCAGGAGGAATTCATGCGTAAAAGCCTGTTA 3' and *Trc2* 5' CCGGTACCGCCAAAACAGCCAAGCTT 3', which amplified the cytochrome-peptide fusion coding sequence. This product was cloned into *SacI*/*Kpn*I of pBAD33. pBA180, pBA181, pBA182, pBA184, and pBA190 were generated with the Quikchange protocol (Qiagen) using pBA174 as the template.

To make pBA175, wt cytochrome-b_{562} was first amplified from pRW-1 with primers *cyt1* and *cyt3* 5' GCTCTAGATTAACGATACTTCTG 3' and cloned into *Eco*RI/*Xba*I of pBA169. This plasmid was then used as a PCR template in PCR with *cyt5* and *Trc2*, and the product was cloned into *SacI*/*Kpn*I of pBAD33.

To make pBA191, the *Eco*RI/*Hin*dIII fragment from pLC260 was cloned into the same sites of pBA169. pLC260, which encodes a C-terminally 6His-tagged DegS, was made by cloning the PCR product obtained with primers *DegS70* 5' GGAATTCAAGGAGAGACGCCTCCATCATGTTTGTG 3' and *DegS71* 5' CCCAAGCTTTTAGTGGTGGTGGTGGTGGTGATTGGTTGCCGGATATTCCTGAA TGGTG 3' into EcoRI/HindIII of p*Trc99*A.

To make pBA192, primers *DegS118* (similar to *DegS70* but with SacI) and *DegS121* 5' CCAAGCTTTTAGTGGTGGTGGTGGTGGTGACCATCGCGGATCAG 3' were used to amplify DegSΔPDZ (truncated at G252) from MC1061 genomic DNA. The product was cloned into pBAD33, generating pBA179. Then, the *Sac*I/*Hin*dIII fragment of this plasmid was cloned into pBA169.

Western blotting

900 µl samples were mixed with 100 µl of cold 50% trichloroacetic acid and stored on ice for an hour or placed at –20°C overnight before being processed as described (Alba et al., 2001). Equal numbers of cells from each sample were run on 12% polyacrylamide gels. Blots were blocked overnight at 4°C in 3% bovine serum albumin. For the primary incubation, a 1/3000 dilution (in Tris-buffered saline with 0.1% Tween-20 [TBST]) of anti-pentaHis (Qiagen) was used. For the secondary incubation, a 1/10000 of horseradish peroxidase-conjugated anti-mouse (in TBST with 10% non-fat dry milk) was used. Blots were developed with the SuperSignal West Dura Extended Duration Substrate from Pierce.

Periplasmic extracts and cytochrome-b_{562} assays

Periplasmic extracts were obtained as described (Keiler and Sauer, 1996). Cells were grown in LB at 30°C with 5 µg/ml $FeCl_2$. Equal numbers of cell equivalents were loaded on 15% polyacrylamide gels (19:1 acrylamide) and were stained with Coomassie Blue. Bands were quantitated by densitometry (Alpha Innotech Corp.).

Spectrophotometric assays for cytochrome-b_{562} absorbance at 426 nm were performed as described (Keiler and Sauer, 1996).

β-galactosidase assays

For all experiments, overnight cultures were diluted to an OD_{600} ~ 0.03 and grown at 30°C in LB with the appropriate antibiotics. In Fig. 4-1, cultures were grown to OD_{600} ~ 0.2-0.25, and β-galactosidase activities were measured. The data, in Miller units, show the means and standard deviations of activities from 3–4 independent experiments (Miller 1972). We also grew these strains in the presence of 1 mM IPTG to overexpress *degS* and *degSΔPDZ* (and overexpression was confirmed by Western blotting) and observed no significant change in activities (data not shown). In experiments involving the overexpression of *ompC* or the fusions, cultures were grown to OD_{600} ~ 0.12 and sampled for initial σ^E activities. Arabinose was then added to induce their overexpression. Additional samples were collected at subsequent time points. In Fig. 4-2B, cultures were grown to OD_{600} ~ 0.12, sampled for initial σ^E activity and induced with IPTG to overexpress the peptides; additional samples were collected at subsequent time points. Assays were performed as previously described (Miller 1972; Mecsas et al., 1993; Ades et al., 1999). All experiments were repeated at least 3 times, with nearly identical results; data from representative experiments are shown.

We note that the addition of IPTG or arabinose to cultures slightly decreases σ^E activity, as seen in the empty vector control (Fig. 4-2B and data not shown). Thus, to better quantitate σ^E activity induction ratios of the peptides or fusions relative to the empty vectors, we compared the slopes of the lines fit to data points two through four for each strain.

Acknowledgements

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

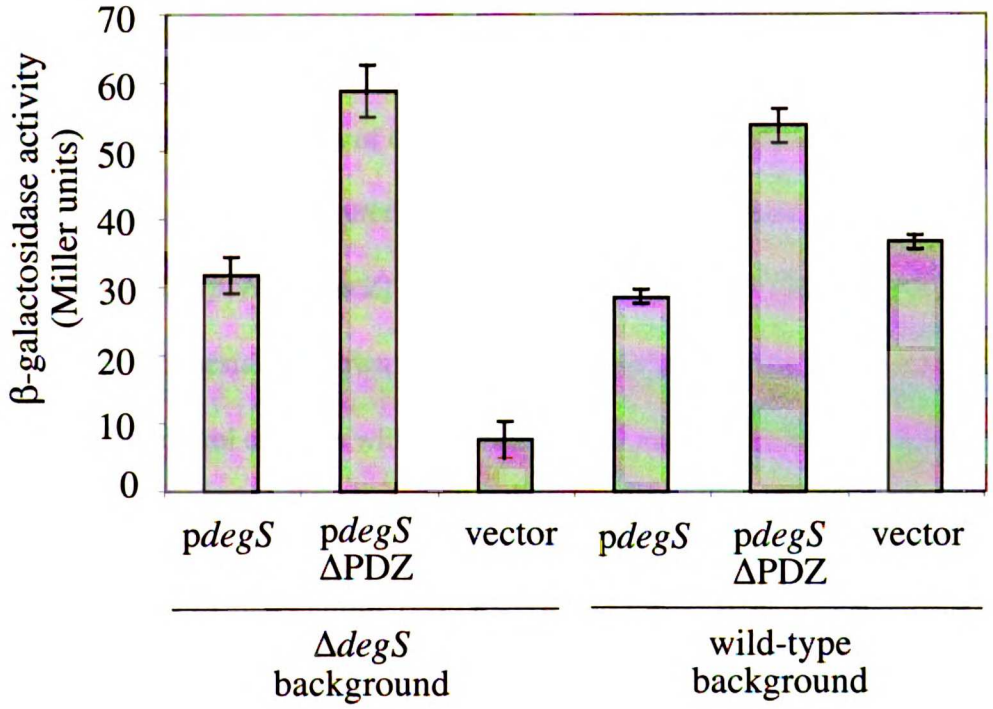
Strain / plasmid	Relevant genotype	Source / reference
Strain		
MC1061	*araD* Δ(*ara-leu*)7697 Δ(*codB-lacI*) *galK*16 *galE*15 *mcrA*0 *relA*1 *rpsL*150 *spoT*1 *mcrB*9999 *hsdR*2	(Casadaban and Cohen, 1979); *E. coli* Genetic Stock Center
CAG16037	MC1061 [Φλ*rpoH* P3::*lacZ*]	(Mecsas et al., 1993)
CAG22376	16037 pTrc99a, Ap^R	this work
CAG33315	MC1061 Δ*degS* [Φλ*rpoH* P3::*lacZ*]	(Ades et al., 1999)
CAG43216	16037 pBA114, Cm^R	this work
CAG43335	16037 pBAD33, Cm^R	this work
CAG43379	16037 pBA152, Ap^R	this work
CAG43397	16037 pBA161b, Ap^R	this work
CAG43398	33315 pBA152, Ap^R	this work
CAG43450	16037 pBA166, Ap^R	this work
CAG43452	16037 pBA168, Ap^R	this work
CAG43472	16037 pBA174, Cm^R	this work
CAG43473	16037 pBA175, Cm^R	this work
CAG43581	16037 pBA180, Cm^R	this work
CAG43582	16037 pBA181, Cm^R	this work
CAG43583	16037 pBA182, Cm^R	this work
CAG43584	16037 pBA184, Cm^R	this work
CAG43585	16037 pBA190, Cm^R	this work
CAG43586	16037 pBA191, Ap^R	this work
CAG43587	16037 pBA192, Ap^R	this work
CAG43588	33315 pBA191, Ap^R	this work
CAG43589	33315 pBA192, Ap^R	this work
CAG43591b	43588 pBA114, Ap^R Cm^R	this work
CAG43592b	43588 pBA174, Ap^R Cm^R	this work
CAG43594a	43588 pBA182, Ap^R Cm^R	this work
CAG43597b	43589 pBA114, Ap^R Cm^R	this work
CAG43602a	43589 pBA174, Ap^R Cm^R	this work
CAG43603a	43589 pBA182, Ap^R Cm^R	this work
CAG43604	16037 pBA169, Ap^R	this work

CAG43605	33315 pBA169, Ap^R	this work
CAG43611	33315 pBA174, Cm^R	this work
CAG43613a	43611 pBA191, Ap^R Cm^R	this work
CAG43614a	43611 pBA192, Ap^R Cm^R	this work
Plasmids		
pBA114	*ompC* in pBAD33, Cm^R	(Alba et al., 2002)
pBA169	p*Trc99*A Δ*Nco*I, Ap^R	this work
pBA152	YQF peptide in p*Trc99*A, Ap^R	this work
pBA161b	ΔYQF peptide in p*Trc99*A, Ap^R	this work
pBA166	YYF peptide in p*Trc99*A, Ap^R	this work
pBA168	YQD peptide in p*Trc99*A, Ap^R	this work
pBA174	Cyt[1]-YQF peptide in pBAD33, Cm^R	this work
pBA175	wt cytochrome in pBAD33, Cm^R	this work
pBA180	Cyt-YYF_{int}→AAA peptide in pBAD33, Cm^R	this work
pBA181	Cyt-ΔYQF peptide in pBAD33, Cm^R	this work
pBA182	Cyt-YYF peptide in pBAD33, Cm^R	this work
pBA184	Cyt-YQD peptide in pBAD33, Cm^R	this work
pBA190	Cyt-YQM peptide in pBAD33, Cm^R	this work
pBA191	DegS-6His in pBA169, Ap^R	this work
pBA192	DegSΔPDZ-6His in pBA169, Ap^R	this work
pBAD33	Vector, pACYC ori, P_{ara}, Cm^R	(Guzman et al., 1995)
p*Trc99*A	Vector, pBR322 ori, Ap^R	Amersham Pharmacia Biotech
pRW-1	cytochrome-b_{562} in a pEMBL-18-derived plasmid, Ap^R	(Brunet et al., 1993; Keiler and Sauer, 1996)

[1] cytochrome-b_{562}

Figure 4-1. Cultures expressing DegSΔPDZ have higher basal σ^E activity than those expressing wild-type (wt) DegS. (A) Strains carrying p*degS*, p*degSΔPDZ*, or empty vector (Δ*degS* background: 43588, 43589, 43605, respectively; wt background: CAG43586, 43587, 43604, respectively) were grown to early exponential growth phase in LB at 30°C, and σ^E activity was measured by monitoring β-galactosidase activity produced from a single-copy [Φλ*rpoH* P3::*lacZ*] σ^E-dependent reporter gene. The average activities in Miller units (with standard deviations) are shown for each strain. (B) Anti-histidine tag Western blot (described in Materials and Methods) showing steady-state *in vivo* levels of DegS and DegSΔPDZ in the strains listed in (A). The asterisks indicate non-specific background bands.

A.


β-galactosidase activity (Miller units)
70
60
50
40
30
20
10
0
pdegS
pdegS ΔPDZ
vector
pdegS
pdegS ΔPDZ
vector
ΔdegS background
wild-type background

B.

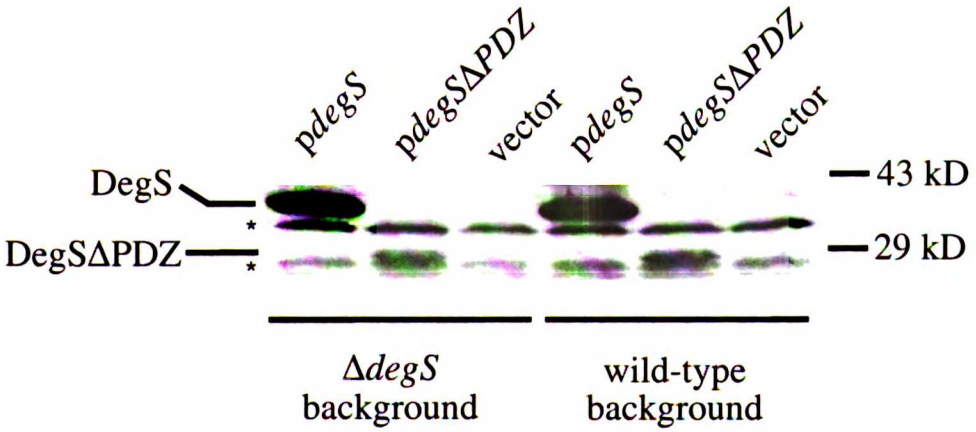

pdegS
pdegSΔPDZ
vector
pdegS
pdegSΔPDZ
vector
DegS
DegSΔPDZ
43 kD
29 kD
ΔdegS background
wild-type background

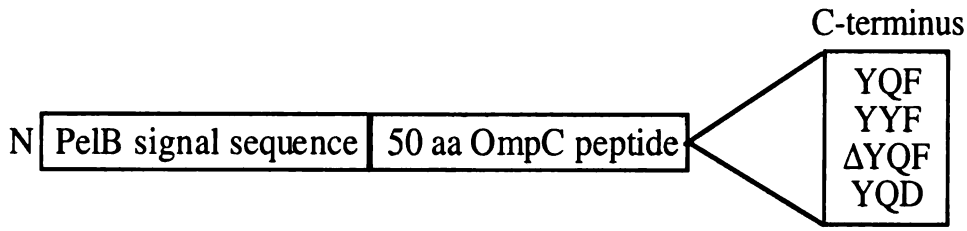
Figure 4-2. Overexpression of OmpC-like C-terminal peptides in the periplasm induces σ^E. (A) Schematic diagram of the OmpC peptides fused to the PelB signal sequence, which directs their export to the periplasm. YQF is the wt C-terminal tripeptide of OmpC. (B) Strains were grown to early exponential growth phase in LB at 30°C, and IPTG was added to induce the overexpression of the PelB-peptide fusions, as detailed in Materials and Methods. σ^E activity was measured by monitoring β-galactosidase activity produced from a single-copy [Φλ*rpoH* P3::*lacZ*] σ^E-dependent reporter gene. The differential rates of β-galactosidase synthesis are shown for a representative experiment. For simplicity, the activities of the control cultures without IPTG (which had activities equivalent to that of the vector-bearing strain) are not shown. The wt background carried empty vector (CAG22376, closed squares) or plasmids expressing peptides ending in YQF (CAG43379, closed diamonds), ΔYQF (CAG43397, open triangles), YYF (CAG43450, closed circles), or YQD (CAG43452, open diamonds). The Δ*degS* background carried a plasmid expressing the YQF peptide (CAG43398, open squares).

A.

N

B.

A.


C-terminus
N
PelB signal sequence
50 aa OmpC peptide
YQF
YYF
ΔYQF
YQD

B.

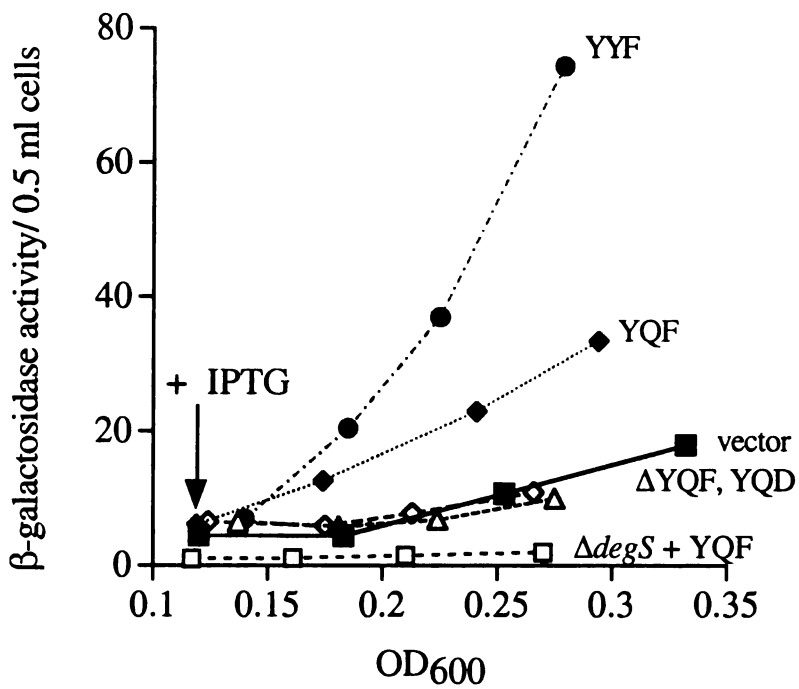
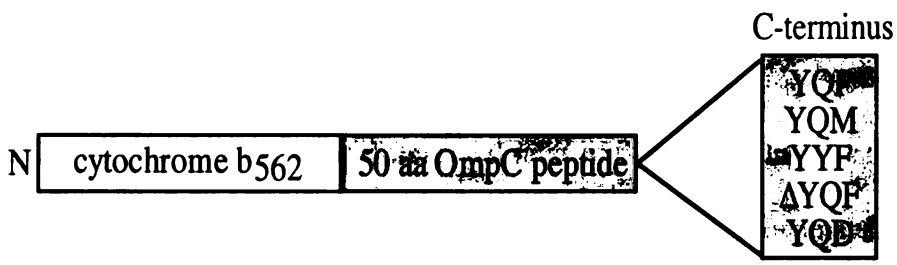
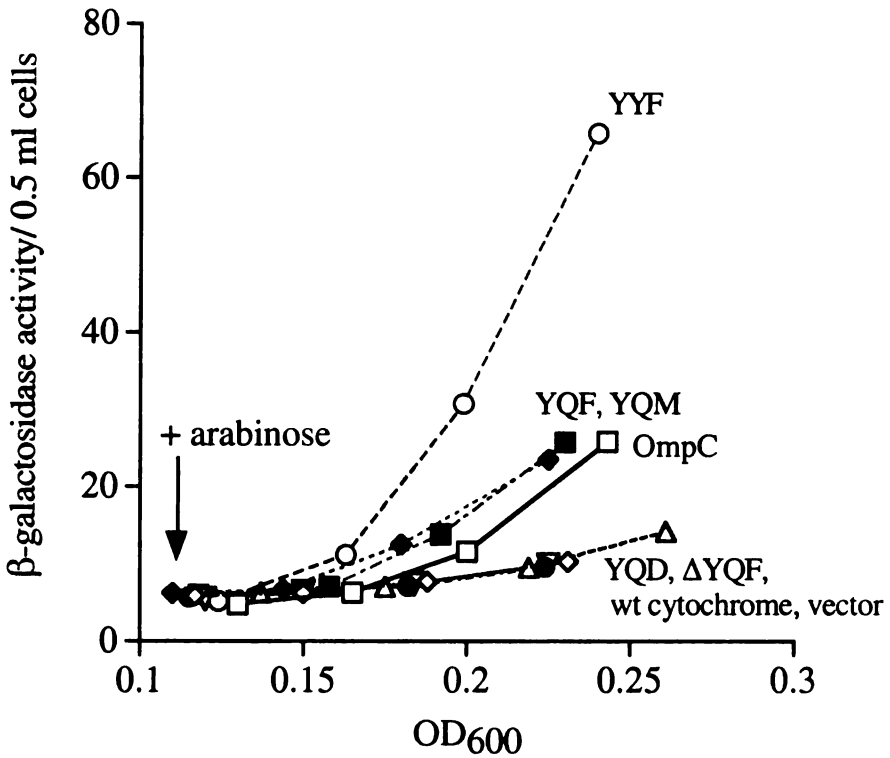

β-galactosidase activity/ 0.5 ml cells
80
60
40
20
0
YYF
YQF
+ IPTG
vector
ΔYQF, YQD
ΔdegS + YQF
0.1
0.15
0.2
0.25
0.3
0.35
OD600

Figure 4-3. Overexpression of cytochrome-b_{562}-OmpC peptide fusions induces σ^E. (A) Schematic diagram of the cytochrome b_{562} fused to OmpC peptides. YQF is the wt C-terminal tripeptide of OmpC. YYF_{int}→AAA (not shown) is located very near the N-terminus of the peptide. (B) Strains were grown to early exponential growth phase in LB at 30°C, and arabinose was added to induce the overexpression of the cytochrome-peptide fusions, as detailed in Materials and Methods. σ^E activity was monitored as in Fig. 4-2. The differential rates of β-galactosidase synthesis are shown for a representative experiment. For simplicity, the activities of the control cultures without arabinose (which had activities equivalent to that of the vector-bearing strain) are not shown. The wt background carried empty vector (CAG43335, open diamonds) or plasmids expressing fusions terminating in YQF (CAG43472, closed diamonds), YYF_{int}→AAA (CAG43581, closed triangles), ΔYQF (CAG43582, closed circles), YYF (CAG43583, open circles), YQD (CAG43584, inverted open triangles), YQM (CAG43585, closed squares), wt cytochrome-b_{562} (CAG43473, open triangles), or full-length OmpC (CAG43216, open squares). (C) Steady-state levels of each fusion and wt cytochrome-b_{562}. Coomassie-stained gel of periplasmic extracts from the strains in (A). Extracts were prepared as described in Materials and Methods. Protein bands were quantitated by densitometry and normalized to a non-specific band (not shown) to account for loading error. The normalized intensity of each band is shown below each lane. A representative gel is shown.

A.


C-terminus
N
cytochrome b562
50 aa OmpC peptide
YQF
YQM
YYF
ΔYQF
YQD

B.


β-galactosidase activity/ 0.5 ml cells
80
60
40
20
0
0.1
0.15
0.2
0.25
0.3
OD600
YYF
YQF, YQM
OmpC
+ arabinose
YQD, ΔYQF,
wt cytochrome, vector

C.

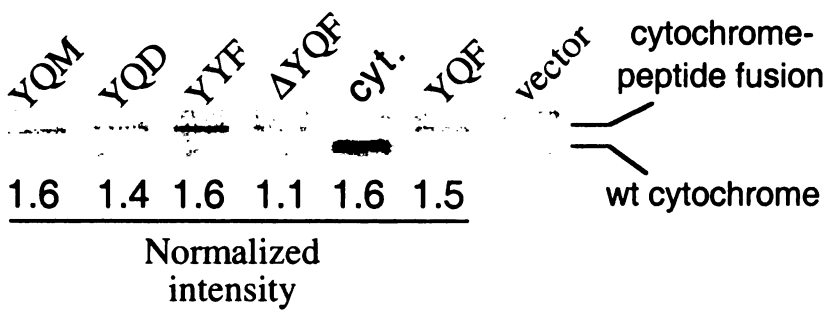
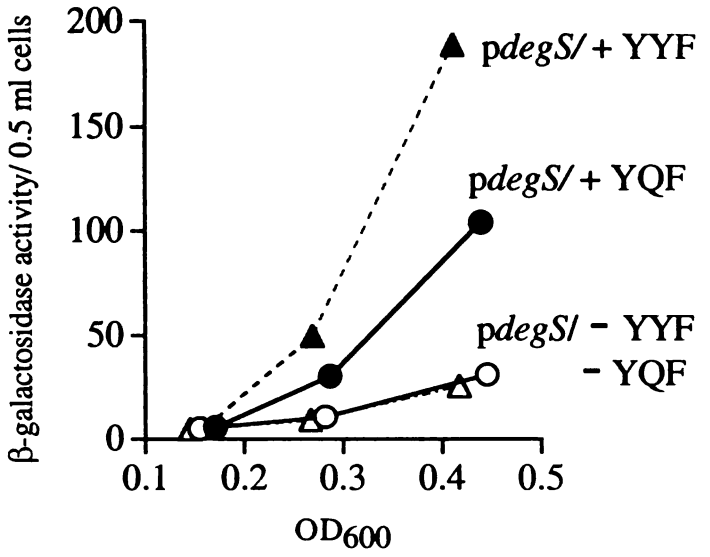
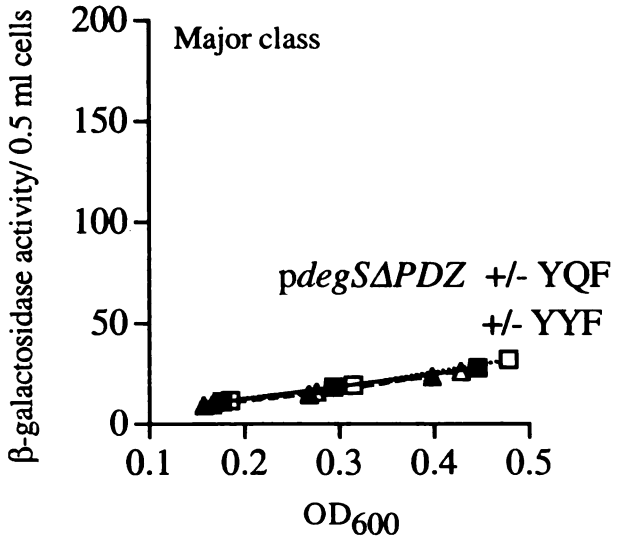

YQM
YQD
YYF
ΔYQF
cyt.
YQF
vector
cytochrome-
peptide fusion
wt cytochrome
1.6
1.4
1.6
1.1
1.6
1.5
Normalized
intensity

Figure 4-4. Normal activation of σ^E by overexpressed fusions requires the PDZ domain of DegS. All strains were grown to early exponential growth phase in LB at 30°C, and arabinose was added to induce the overexpression of the cytochrome-peptide fusions. σ^E activity was monitored as in Fig. 4-2. The differential rates of β-galactosidase synthesis for representative experiments are shown. (A and C) Strains with a chromosomal Δ*degS* allele and carrying p*degSΔPDZ* were transformed with plasmids overexpressing the YQF and/or YYF fusions. An example of the behavior of the major class (A) of Δ*degS* transformants bearing p*degSΔPDZ* and a plasmid overexpressing the YYF fusion (CAG43603a, triangles) or YQF fusion (CAG43602a, squares). An example of the behavior of the minor class (C) of transformants carrying a plasmid overexpressing the YQF fusion (CAG43614a, circles). For comparison, a Δ*degS* strain bearing p*degS* and overexpressing the YQF fusion is shown (CAG43613a, squares). (B) Strains with a chromosomal Δ*degS* allele and carrying p*degS* were transformed with a plasmid overexpressing the YYF fusion (CAG43594, triangles) or YQF fusion (CAG43592b, circles). Solid symbols contained arabinose (and the overexpressed fusion) while open symbols did not.

A.


β-galactosidase activity/ 0.5 ml cells
200
150
100
50
0
0.1
0.2
0.3
0.4
0.5
OD600
pdegS/ + YYF
pdegS/ + YQF
pdegS/ - YYF
- YQF

B.


β-galactosidase activity/ 0.5 ml cells
200
150
100
50
0
0.1
0.2
0.3
0.4
0.5
OD600
Major class
pdegSΔPDZ +/- YQF
+/- YYF

C.

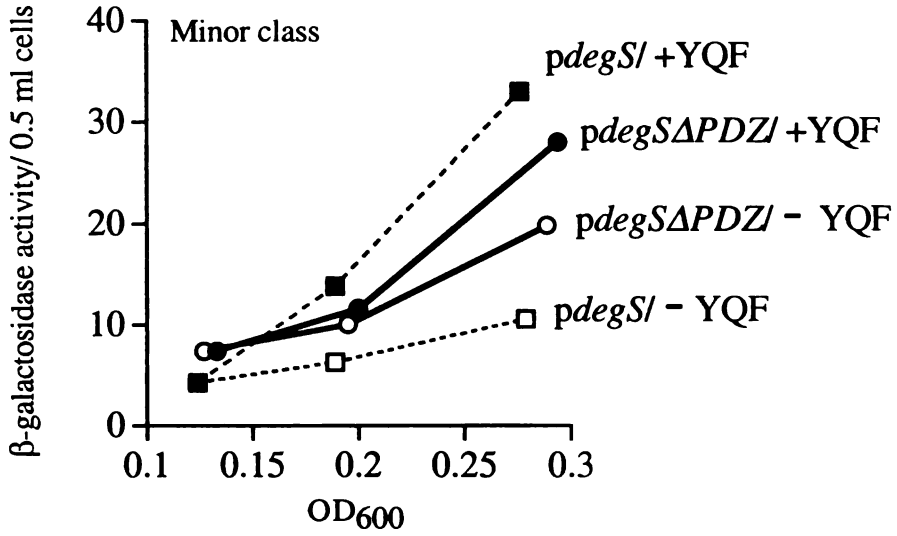

β-galactosidase activity/ 0.5 ml cells
40
30
20
10
0
0.1
0.15
0.2
0.25
0.3
OD600
Minor class
pdegS/ +YQF
pdegSΔPDZ/ +YQF
pdegSΔPDZ/ - YQF
pdegS/ - YQF

Figure 4-5. Full-length OmpC activates σ^E in the DegSΔPDZ background. Δ*degS* strains bearing p*ompC* and carrying p*degS* (CAG43591b, triangles) or p*degSΔPDZ* (CAG43597b, circles) were grown to early exponential growth phase in LB at 30°C, and arabinose was added to induce the overexpression of OmpC. σ^E activity was monitored as in Fig. 4-2. The differential rates of β-galactosidase synthesis are shown for a representative experiment. Solid symbols contained arabinose (and overexpressed OmpC) while open symbols did not.

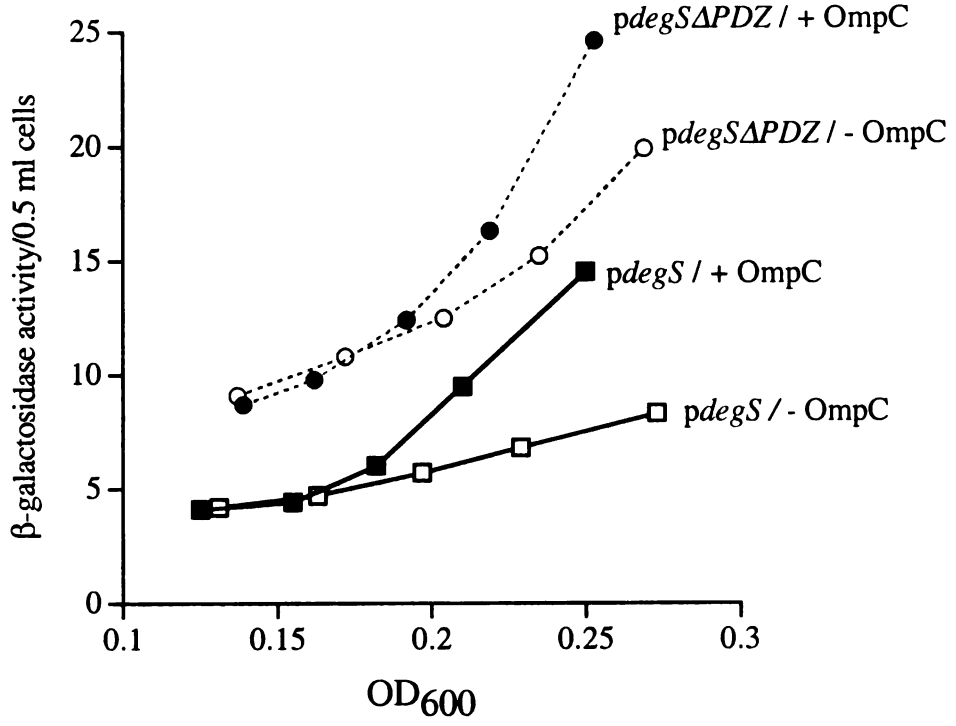
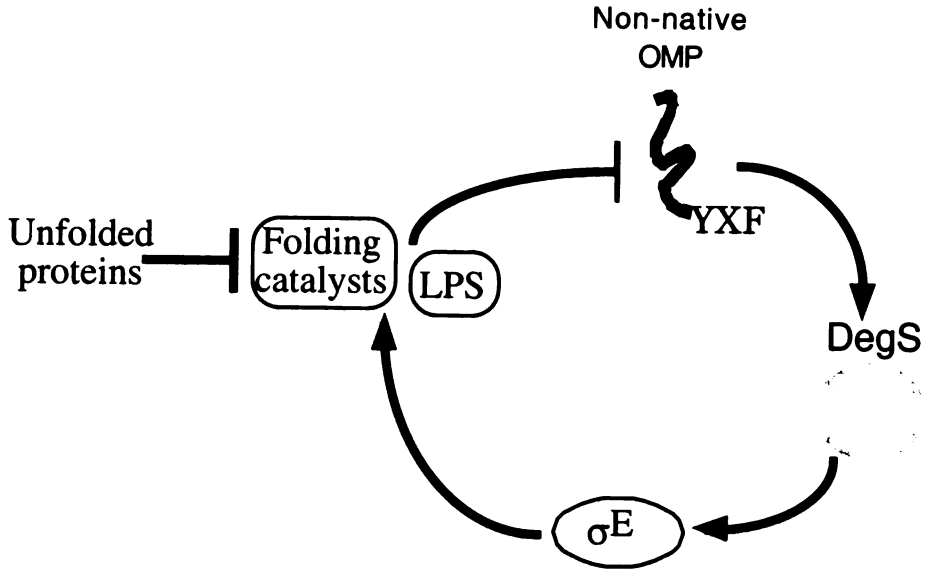
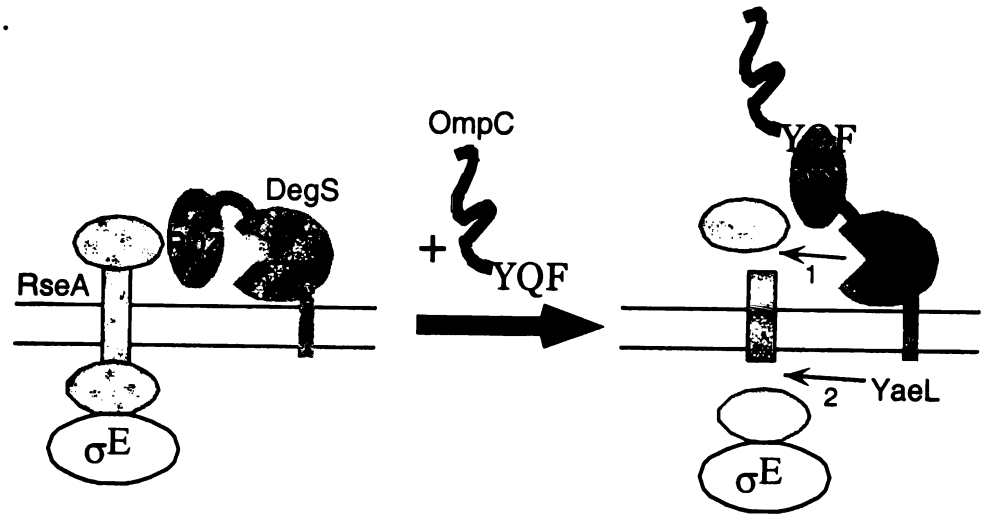

pdegSΔPDZ / + OmpC
pdegSΔPDZ / - OmpC
pdegS / + OmpC
pdegS / - OmpC
β-galactosidase activity/0.5 ml cells
25
20
15
10
5
0
0.1
0.15
0.2
0.25
0.3
OD600

Figure 4-6. Models. (A) The σ^E pathway may use OMPs with YXF C-termini as indirect sensors of unfolded extracytoplasmic proteins and/or LPS integrity. Since folding catalysts and LPS contribute to proper OMP biogenesis, stresses that increase the occupancy of folding catalysts and/or alter the integrity or flux of LPS would increase the levels of non-native OMPs. These OMPs would then induce DegS-catalyzed cleavage of RseA and induction of σ^E activity, which, in turn, would increase the expression of genes encoding folding catalysts and those involved in lipopolysaccharide (LPS) biosynthesis. (B) The DegS PDZ domain as a negative regulator of DegS protease activity. The protease activity of DegS is inhibited by the PDZ domain, perhaps through active site occlusion. When a non-native OMP with a YXF terminus binds to the PDZ domain, a rearrangement of the PDZ domain occurs such that DegS is activated. In the ensuing proteolytic cascade, RseA is cleaved in its periplasmic domain by DegS (1) and then near or in its transmembrane domain by YaeL (2). Other proteases may cleave the YaeL-generated RseA degradation intermediate to release σ^E (not shown; Alba et al., 2002; Kanehara et al., 2002).

A.


Non-native
OMP
YXF
Unfolded
proteins
Folding
catalysts
LPS
DegS
σE

B.


DegS
RseA
σE
OmpC
+
YQF
YQF
1
2
YaeL
σE

Chapter Five

Conclusion and Future Directions

Conclusion and Future Directions

The σ^E-dependent extracytoplasmic stress response uses the regulated proteolysis of RseA to transduce stress signals to σ^E (Ades et al., 1999). In this thesis, I have shown that two inner-membrane proteases, DegS and YaeL, are involved in the degradation of RseA and concomitant σ^E activation in response to OmpC overexpression (Ch. 2 and 3) (Alba et al., 2002). I have shown that both *degS* and *yaeL* are essential for *E. coli* viability because of their roles in providing the cell with σ^E activity, which is itself essential for viability (Ch. 2 and 3) (De Las Peñas et al., 1997b; Alba et al., 2001; Alba et al., 2002). I have also presented evidence suggesting that the PDZ domain of DegS plays a role in sensing the C-termini of overexpressed OmpC porins (Ch. 4) (Walsh et al., 2003). The current working model for the regulated proteolysis of RseA in response to OmpC overexpression is the following: the binding of OmpC termini to the DegS PDZ domain relieves PDZ-mediated inhibition of DegS protease activity, allowing DegS to cleave RseA within its periplasmic domain (Walsh et al., 2003). Subsequently, YaeL cleaves RseA within or near its transmembrane domain. This ultimately leads to the release of σ^E from the cytoplasmic anti-sigma factor domain of RseA, presumably because the YaeL-generated RseA fragment has a C-terminal tail that tags the cytoplasmic domain for degradation by cytoplasmic proteases (Alba et al., 2002). Consistent with this idea, RseA has been found associated with inactive ClpXP protease *in vivo* (Flynn et al., 2003).

Comparison of the σ^E pathway with other stress-response pathways

Cells use multiple strategies to sense stress and alter gene expression so that they can combat stress-induced damage. One of these strategies seems to be conserved from prokaryotes to eukaryotes: the activities of heat-shock transcription factors are negatively regulated by the chaperones that repair misfolded proteins. In a widely accepted model for such regulation, unfolded proteins activate heat-shock transcription factors by titrating the inhibitory chaperones away from them (Morimoto, 1998; Yura and Nakahigashi, 1999). In *E. coli*, DnaK, DnaJ and GroEL (E. Guisbert, C. Herman and C. A. Gross, in prep.) repress σ^{32} activity in the absence of stress, most likely by preventing σ^{32} from binding to core RNA polymerase (RNAP) and/or by targeting σ^{32} for degradation (Straus et al., 1989; Straus et al., 1990; Tomoyasu et al., 1998). When various unfolded proteins accumulate, they titrate DnaK/J and GroEL away from σ^{32}, thereby allowing σ^{32} to associate with RNAP. When the level of unfolded proteins decreases, the chaperones resume their inhibition of σ^{32} activity, thereby turning off the response. Since σ^{32} controls the expression of *dnaK*, *dnaJ* and *groEL*, the system includes a negative feedback loop that helps to tightly control σ^{32} activity.

In eukaryotes, there are additional examples of chaperones functioning as negative regulators of stress response transcription factors. In certain systems employing HSF (heat shock transcription factor), there is evidence that chaperones are bound to inactive forms of HSF and while the HSF-dependent response is waning (Morimoto, 1998). Additionally, overexpression of Hsp70 in some systems can block heat shock-induced gene expression (Morimoto, 1998). In the mammalian endoplasmic reticulum unfolded protein response (UPR), the lumenal chaperone BiP is thought to inhibit the

activity of the Ire1 kinase/ endonuclease, which is required for the expression of the UPR transcription factor XBP1 (Bertolotti et al., 2000; Patil and Walter, 2001; Yoshida et al., 2001; Lee et al., 2002).

There are known parallels (and potentially others) between these stress response regulatory systems and that of the σ^E pathway. σ^E activity is controlled in part by a negative feedback loop that includes RseA and RseB, both of which are encoded by the σ^E operon. Thus, as in the case of the σ^{32} system, σ^E activity is tightly controlled in part because σ^E directs the expression of its negative regulators. Intriguingly, RseB has been found associated with an overexpressed mutant protein *in vivo* (Collinet et al., 2000). One possible explanation for this observation is that RseB might have chaperone activity. If so, its role in σ^E regulation may indeed be analogous to that of the cytoplasmic chaperones in the σ^{32} system. It is also possible that known periplasmic chaperones and folding catalysts (e.g. Skp and SurA) negatively regulate σ^E activity, perhaps by stabilizing RseA.

While these parallels may exist, there are interesting differences between the σ^E pathway and cytoplasmic stress response pathways, such as the σ^{32} pathway. Since the signals that activate σ^E are extracytoplasmic and σ^E is cytoplasmic, stress signals must be transduced to σ^E across the inner membrane. To efficiently and rapidly transduce such signals to σ^E, it is reasonable to expect that σ^E would communicate directly with an inner membrane signal-transducer. From this perspective, the use of an inner membrane protein like RseA is warranted. The cytoplasmic domain of RseA binds to σ^E and inhibits its activity, while the periplasmic domain of RseA interacts with periplasmic "sensors"

(DegS, RseB, etc.) of unfolded proteins or other stress signals. Instead of directly modulating σ^E activity, the sensors inactivate RseA, which, in turn, activates σ^E.

The σ^E pathway and those noted above might also differ with regards to the classes of unfolded proteins that are monitored by the pathways. As OMPs are the major modulators of σ^E activity, and since OMP maturation is dependent upon periplasmic folding catalysts and normal lipopolysaccharides, the σ^E pathway may primarily monitor the folding state of porins to *indirectly* monitor the general health of the envelope (Ch. 4; Walsh et al., 2003).

Future Directions

Our understanding of σ^E regulation could be advanced even further by exploring ideas generated during the course of my work. One focus could be the mechanism by which full-length OmpC induces RseA degradation and activates σ^E. Our model for the activation of σ^E by the overexpression of OmpC (Ch. 4) involves the removal of two levels of negative control upon RseA degradation: a) PDZ-mediated inhibition of DegS protease activity; b) RseB-mediated occlusion of the site at which DegS cleaves RseA. A key prediction of this model is that σ^E activity in a strain lacking *rseB* and carrying DegSΔPDZ would be high and not further inducible by OmpC overexpression. As shown in Appendix B, I obtained preliminary results suggesting that the Δ*rseB degSΔPDZ* double mutant indeed has high σ^E activity, which is greater than that of either of the mutants alone. Additional experiments will be required to determine if σ^E activity in this genetic background is further inducible by OmpC overexpression. If σ^E activity is not further inducible, it will be important to test whether RseB associates with unfolded

OmpC and if OmpC overexpression reduces the amount of RseB that associates with RseA *in vivo*. Certainly, an *in vitro* reconstituted degradation system such as that used in Walsh et al. (2003) could be used to test this model.

Another focus could be the identification of OmpC species that are sensed by the σ^E pathway. We showed *in vivo* that the C-terminus of OmpC is an important component of the signal that activates σ^E, presumably through DegS-mediated cleavage of RseA (Ch. 4). As the C-termini of trimeric OmpC are likely buried within the trimer interface (based upon homology to OmpF; Cowan et al., 1992), it is likely that non-native folding intermediates bind to the PDZ domain of DegS and trigger cleavage of RseA. These species could be monomers, dimers, or metastable trimers, all of which have been observed *in vivo* (Pugsley, 1993; Danese and Silhavy, 1998). As above, an *in vitro* degradation system could be used to determine which species are activators of DegS, assuming that purified, homogeneous OmpF/C species could be obtained. Using an *in vivo* approach, it may be possible to use certain OmpF or OmpC mutants from the lab of Rajeev Misra to identify the types of OmpF/C species that most strongly induce σ^E activity (Misra, 1993; Xiong et al., 1996; Misra et al., 2000). For example, there are OmpF mutants that affect the kinetics of OmpF trimerization and induce *degP* expression (likely due to σ^E-dependent expression) (Misra et al., 2000). However, these OmpF mutants (Misra et al., 2000) have amino acid substitutions at the C-terminal residue, the highly conserved phenylalanine that is a critical part of the σ^E-inducing signal encoded within the OmpC C-terminus (Walsh et al., 2003). Thus, to differentiate between σ^E-inducing signals at the C-termini from those arising because of trimerization defects, it

will be important to determine whether such OmpF mutant C-termini induce σ^E activity in the context of cytochrome-porin fusions (Walsh et al., 2003) and/or full-length OmpF.

A third focal point could be the dissection of the mechanism by which DegS and YaeL cooperate to cleave RseA. YaeL-dependent cleavage of RseA requires prior cleavage by DegS within the periplasmic domain of RseA, although it is not known why this is the case (Alba et al., 2002; Kanehara et al., 2002). Some possibilities are discussed here. The DegS-generated intermediate could be a YaeL-specific substrate. The "new" C-terminus of RseA generated by DegS action could be recognized by the periplasmic PDZ domain of YaeL (Walsh et al., 2003), thus facilitating the YaeL-dependent cleavage step. Alternatively, the DegS-generated intermediate could have an altered conformation that facilitates YaeL cleavage. Studies of Site-2 protease (S2P) and signal peptide peptidase (SPP) in mammalian cells, both of which cleave near or within transmembrane domains of their substrates (Ye et al., 2000b; Lemberg and Martoglio, 2002; Wolfe and Selkoe, 2002), provide clues about how YaeL might cleave RseA. S2P and SPP, like YaeL, require prior cleavage of their substrates by other proteases before they can act. The scissile bonds of S2P and SPP substrates (SREBPs and signal peptides, respectively) are located in transmembrane domains, which are predicted to be α-helical and, consequently, poorly accessible to proteases (Paetzel et al., 1998; Brown et al., 2000; Ye et al., 2000; Lemberg and Martoglio, 2002). It was proposed that the first cleavage events somehow lead to the partial unwinding of the α-helix, thereby exposing the scissile bond. Thus, by analogy to these models, DegS-mediated cleavage of RseA may cause an unwinding of the transmembrane helix, allowing YaeL to subsequently cleave RseA.

While YaeL action may be facilitated by a conformational change in the RseA substrate, it is possible that DegS activates YaeL to cleave RseA. Once DegS is activated by the OmpC signal, DegS (or the DegS/OmpC complex) may then be capable of activating YaeL. An interaction between the PDZ domain of DegS and the periplasmic PDZ domain of YaeL may contribute to the coordination of both proteases. If this were true, then one might be able to observe an interaction (*in vivo* and *in vitro*) between DegS and YaeL, and/or an interaction between the purified PDZ domains. Additionally, the DegSΔPDZ mutant may affect YaeL activity *in vivo*. The absence of the DegS PDZ domain may uncouple YaeL activity from that of DegS, leading to YaeL-mediated cleavage of RseA without prior cleavage by DegS. β-galactosidase assays for σ^E activity and anti-RseA Western blots could reveal such effects.

How widespread is this pathway?

A survey of sequenced bacterial genomes revealed that other members of the gamma branch (γ) and one from the beta (β) subdivision of proteobacteria have orthologues of *rpoE* (σ^E), *rseA*, *rseB*, *degS* and *yaeL* (Table 5-1). In most of these bacteria, *rpoE*, *rseA*, *rseB* and *rseC* appear to be arranged in operons, except for *Haemophilus influenzae* (Table 5-1). It is difficult to predict whether the *degS* orthologues have functions similar to those of *E. coli degS* because many bacteria have multiple members of the HtrA/DegP family. Some of these orthologues may function like HtrA/DegP, which is required for degrading misfolded proteins, while others may participate in the regulation of RseA stability. The majority of *degS* orthologues with the highest homology scores apparently encode proteins with hydrophobic N-termini

suggestive of inner membrane anchors, which is consistent with them being functionally similar to *degS*. Moreover, most RseA orthologues contain the aa residues Val-Ser or Val-Leu (in *P. aeruginosa* MucA) approximately 30 aa from the transmembrane domain, an observation consistent with the cleavage of *E. coli* RseA by DegS after such a Val residue (Walsh et al., 2003).

The regulation of *P. aeruginosa* AlgU (σ^E orthologue), which controls mucoidy, has also been studied extensively (Hughes and Mathee, 1998; see also L. E. Connolly, Ph.D. thesis, 1998, UCSF). Mutant *mucA* alleles found in natural isolates cause high AlgU activity and mucoidy *in vivo*. Many of these alleles encode unstable proteins that have truncated periplasmic domains (Boucher et al., 1997). Δ*mucB* (*rseB* orthologue) alleles elevate AlgU activity (Schurr et al., 1996), and MucA may be more unstable in the absence of MucB (Rowen and Deretic, 2000). Although there are other models explaining the control of AlgU activity (Mathee et al., 1997; Rowen and Deretic, 2000), these observations suggest that MucA stability might be an important determinant of AlgU activity. However, deleting either *algW* or *mucD* (two *degS/htrA* orthologues) induces mucoidy (Boucher et al., 1996), rather than suppresses it, which is inconsistent with either of these orthologues being a positive regulator of AlgU, akin to *E. coli* DegS. Thus, it is unclear if MucA proteolysis plays a role in regulating AlgU activity.

While other orthologues may function like those in *E. coli*, the signals that are sensed by other σ^E pathways may not be the same as those in *E. coli*. Each bacterium may have adapted this signal transduction mechanism to sense and respond to different physiological signals. To accomplish this, the ligand specificity of the DegS PDZ domain could be altered, and/or the relative contributions of RseB and DegS orthologues to σ^E

regulation may be different. If these bacteria indeed employ such a signal transduction mechanism, then determining the inducing signals will yield exciting insights into the versatility of such a signaling system and the range of biological roles played by σ^E orthologues.

Table 5-1. *rpoE, rseA, rseB, degS* and *yaeL* orthologues[1] in other bacteria.

Bacterium (proteobacteria subdivision)	*rpoE*	*rseA*	*rseB*	*rseC*	*degS*	*yaeL*
E. coli, Haemophilus influenzae[2], *Salmonella enterica, S. typhimurium, Shewanella oneidensis, Shigella flexneri, Pasturella multicida, Pseudomonas aeruginosa*[3], *Vibrio cholerae, Yersinia pestis(all γ)*	+	+	+	+	+	+
Xanthomonas campestris (γ)	+	+	-	-	+	+
Ralstonia solanacereum (β)	+	+	+	-	+	+

[1] Deposited in NCBI and/or TIGR CMR databases
[2] Contains two *rseC* orthologues, neither of which is in *rpoE* operon
[3] *rpoE, rseA, rseB, rseC* are known as *algU, mucA, mucB, mucC*; highest scoring *degS* orthologue is *algW*.

Table 5-1. *rpoE, rseA, rseB, degS* and *yaeL* orthologues[1] in other bacteria.

Bacterium (proteobacteria subdivision)	*rpoE*	*rseA*	*rseB*	*rseC*	*degS*	*yaeL*
E. coli, *Haemophilus influenzae*[2], *Salmonella enterica*, *S. typhimurium*, *Shewanella oneidensis*, *Shigella flexneri*, *Pasturella multicida*, *Pseudomonas aeruginosa*[3], *Vibrio cholerae*, *Yersinia pestis*(all γ)	+	+	+	+	+	+
Xanthomonas campestris (γ)	+	+	-	-	+	+
Ralstonia solanacereum (β)	+	+	+	-	+	+

[1] Deposited in NCBI and/or TIGR CMR databases
[2] Contains two *rseC* orthologues, neither of which is in *rpoE* operon
[3] *rpoE, rseA, rseB, rseC* are known as *algU, mucA, mucB, mucC*; highest scoring *degS* orthologue is *algW*.

Appendix A

σ^E activity during depletion of DegS

Chapter 2 describes how I used a DegS depletion strain to show that DegS is essential for *E. coli* viability (Table 2-2). In that experiment, Western blotting confirmed that DegS was depleted when *degS* expression was shut-off (Fig. 2-2). This appendix reports experiments that monitor σ^E activity and growth rate following shut-off of *degS* expression. All procedures were as described in Chapter 2.

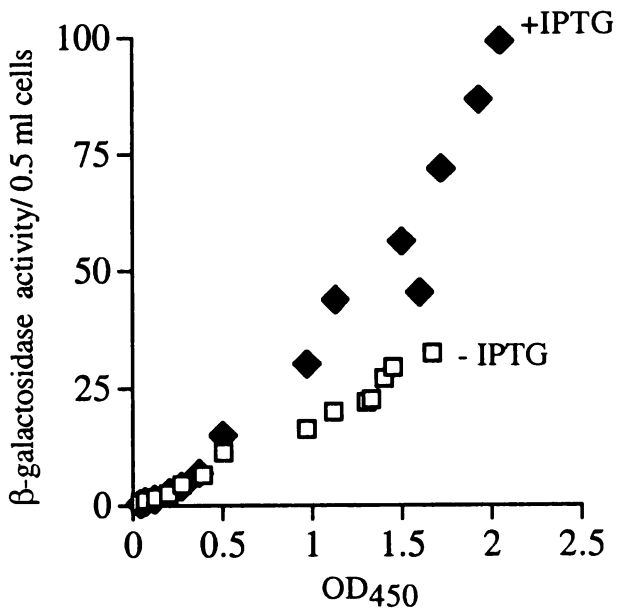
I grew the DegS shut-off strain (CAG43248) to early log phase (OD_{450} ~ 0.25) in M9 minimal medium at 30°C under inducing conditions (+IPTG) filtered the culture, and resuspended the washed cells in pre-warmed media without IPTG (see 12/12/01; Vol. 3, p. 42). These cells were used to inoculate four flasks, two of which contained IPTG, to OD_{450} ~ 0.04. Two flasks (+/-IPTG) were allowed to grow into stationary phase (undiluted), while the other two flasks (+/-IPTG) were continually diluted (see Alba et al., 2002) to maintain the culture in log phase (OD_{450} ~ 0.04 – 0.3). This allowed us to monitor σ^E activity during DegS depletion in log phase and as cultures approached and entered stationary phase.

We found that σ^E activity decreased as DegS was depleted both in cultures maintained in log phase and in undiluted cultures that entered stationary phase (no IPTG; Fig. A-1, A and B). In contrast, wild-type σ^E activities were observed in cultures with DegS present (with IPTG; Fig. A-1, A and B). This is consistent with our earlier finding that DegS is required for RseA degradation and normal σ^E activity. The growth curves of the diluted cultures indicated that growth rates remained unchanged in the presence of IPTG but eventually slowed during the second dilution in the absence of IPTG (Fig. A-1,

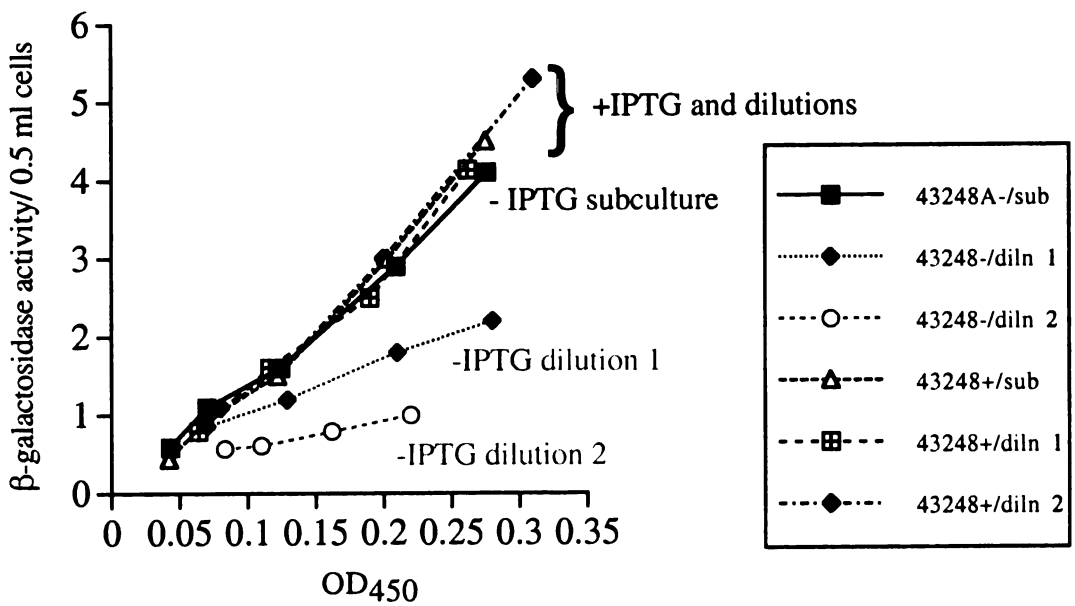
C). This drop in growth rate corresponded with the lowest level of σ^E activity measured in this experiment (Fig. A-1, B). We also observed cell lysis in the log phase culture early in the third dilution as well as in the undiluted (OD_{450} ~ 1.3) DegS-depleted cultures. The lysis was not evident as a dramatic drop in OD_{450}, but, rather, as clumps of stringy cell debris in the flasks. Taken together, these data suggest that DegS, and by extension RseA proteolysis, is required to provide σ^E activity during both log and stationary phases and that DegS depletion in M9 minimal media results in cell death.

σ^E activity decreases as DegS is depleted in undiluted (A) and diluted (B) cultures

A.

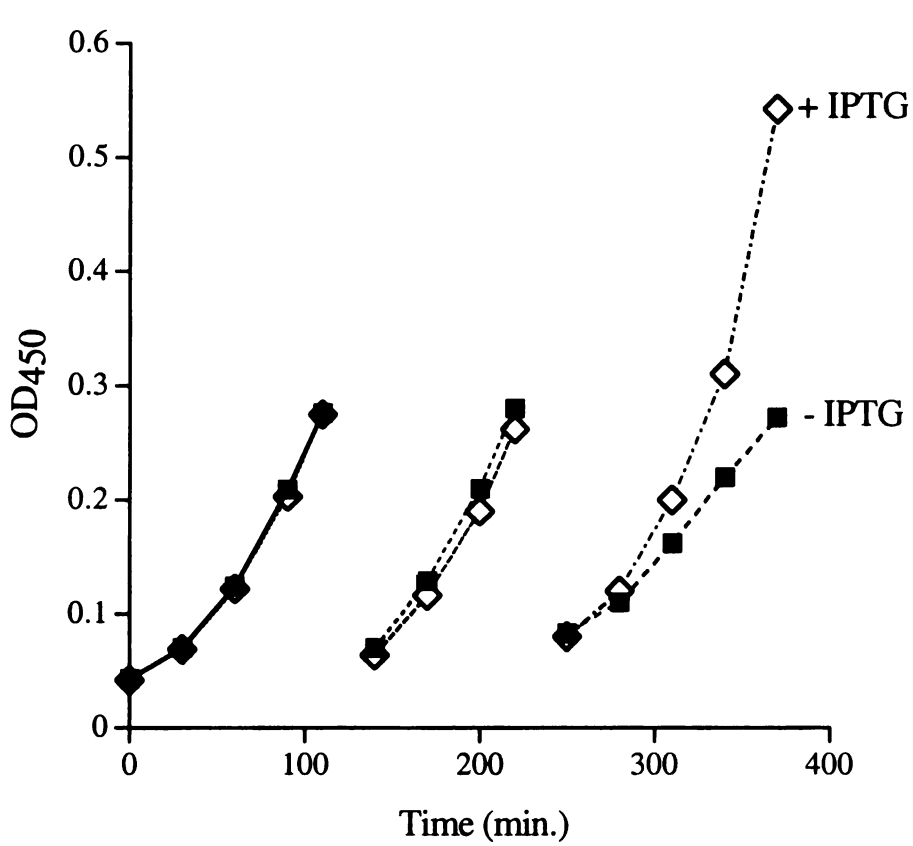


B.



Growth curves of cultures during DegS depletion (-IPTG) and DegS expression (+IPTG).

C.



Appendix B

Combination of *degSΔPDZ* and Δ*rseB* alleles strongly elevates σ^E activity

In Chapter Four we proposed that OmpC overexpression activates σ^E by: a) removing PDZ-mediated inhibition of DegS protease activity; and b) titrating RseB away from RseA, which uncovers the periplasmic site in RseA at which DegS cleaves (Walsh et al., 2003). A strong prediction of this model is that combining the Δ*rseB* and *degSΔPDZ* mutations would fully activate the σ^E pathway.

As a first attempt to test this model, I constructed strains that carry both the Δ*rseB* and *degSΔPDZ* mutations in an otherwise wild-type background (i.e. not Δ*degS*). Note that in these experiments (5/2/01;Vol. 3, p. 4), I used my original p*degSΔPDZ* (pBA126), which seemed to produce a less stable DegSΔPDZ protein than that produced by pBA192, which was used in Walsh et al. (2003). Without overexpression of p*degSΔPDZ* in the Δ*rseB* background (-IPTG, open triangles), σ^E activity was elevated 3-fold relative to wild-type (closed squares) (Fig. B-1). When *degSΔPDZ* was overproduced in the Δ*rseB* background (+IPTG, closed triangles), σ^E activity was elevated 5-fold relative to wild-type activity (Fig. B-1). By comparison, σ^E activity in Δ*rseB* alone (open squares) and p*degSΔPDZ* alone was lower (Fig. B-1; see Fig. B-2, open circles, for p*degSΔPDZ*). The 5-fold elevation of σ^E activity in the Δ*rseB* background with p*degSΔPDZ* overexpression is in the range of the activity generated when full-length OmpC is overexpressed (data not shown). These data suggest that removal of the PDZ domain and of RseB may indeed be sufficient to fully activate σ^E activity. However, one must test

whether OmpC overexpression in the double mutant background can further induce σ^E activity (such experiments need to be performed in the Δ*degS* background as well). Also, it would be ideal if one could show that RseB associates with overexpressed OmpC *in vivo* and *in vitro*.

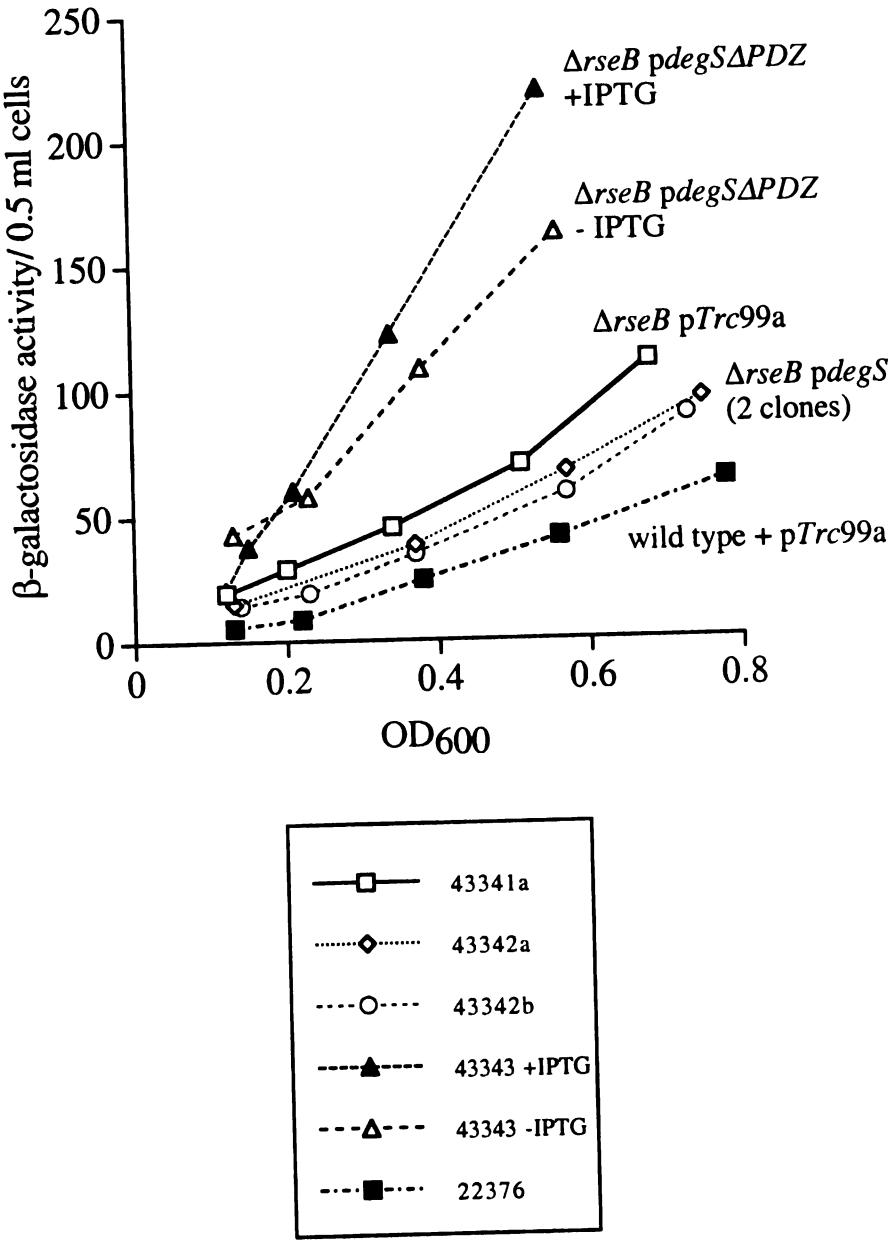
Overexpression of *degSΔPDZ S201A* elevates basal σ^E activity

We previously showed that full-length DegS lacking its active site serine (DegS S201A) could not restore basal or induced σ^E activity to Δ*degS* strains (Ades et al., 1999). This is consistent with DegS playing a proteolytic role in regulating σ^E activity. Here, I tested whether the DegS active site mutant in the context of DegSΔPDZ could complement Δ*degS* (7/19/01; Vol. 3, p. 23). I transformed Δ*degS* with pBA126 (p*degSΔPDZ*), pBA142 (p*degSΔPDZ S201A*), and pLC260 (wild-type p*degS*). I assayed σ^E activity with (+ IPTG) or without (-IPTG) overexpression of *degS* or p*degSΔPDZ*. Unexpectedly, overexpressed *degSΔPDZ S201A* (closed triangles) elevated basal σ^E activity to a level equivalent to that generated by overexpressed *degSΔPDZ* (closed circles) (Fig. B-2). In the absence of IPTG, *degSΔPDZ* partially complemented Δ*degS* as usual (open circles, Fig. B-2; also see 6/20/01; Vol. 3, p. 16). Non-induced *degSΔPDZ S201A* also partially complemented Δ*degS*, but to a lesser extent (open triangles, Fig. B-2). Western blots showed that both *degSΔPDZ S201A* and *degSΔPDZ* accumulated to low cellular levels in the absence of IPTG (data not shown). *degSΔPDZ S201A* accumulated to a level even lower than that of *degSΔPDZ*, suggesting that the former is more unstable than the latter. In the presence of IPTG, both proteins were overproduced and accumulated to nearly equivalent levels (data not shown). Taken together, these data suggest that *degSΔPDZ S201A* can increase σ^E activity in a proteolysis-independent manner.

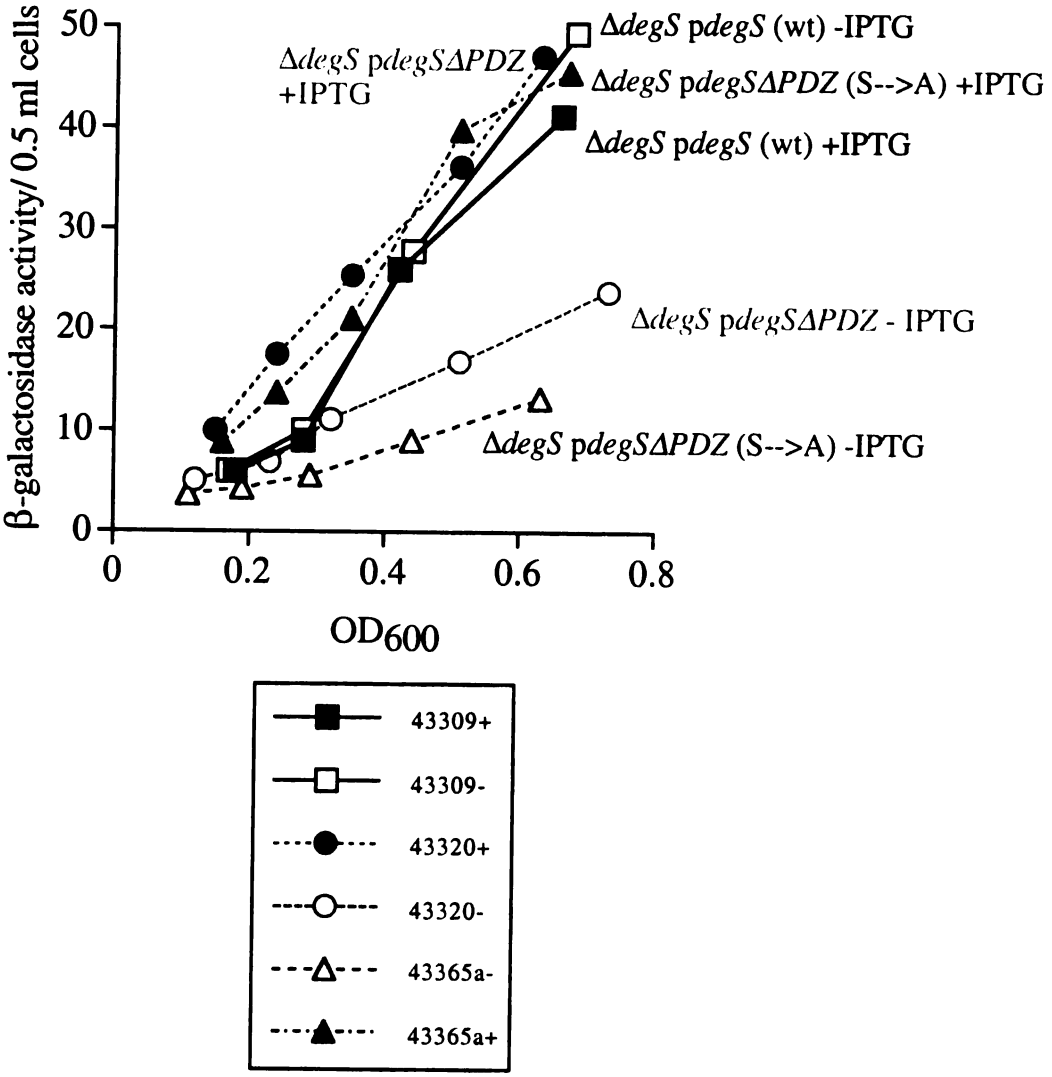
I next tested whether *degSΔPDZ S201A* could restore σ^E-inducibility to a Δ*degS* strain. I overexpressed *ompC* in the *degSΔPDZ S201A* and *degSΔPDZ* backgrounds and assayed σ^E activity (7/20/01; Vol. 3, p. 23). For simplicity, only the cultures overexpressing OmpC are shown in Fig. B-3. σ^E-inducibility by OmpC was restored by *degSΔPDZ* (circles), but not by *degSΔPDZ S201A* whether or not *degSΔPDZ S201A* was itself overexpressed by IPTG (triangles) (Fig. B-3). This is consistent with the protease activity of DegS being required for OmpC-mediated induction of σ^E activity (Ades et al., 1999).

Why does *degSΔPDZ S201A* affect basal but not induced σ^E activity? Since RseA, RseB, σ^E and presumably DegS are closely associated *in vivo*, it is possible that DegSΔPDZ S201A destabilizes the RseA/σ^E and/or RseA/RseB complexes, leading to increased free σ^E. Alternatively, DegSΔPDZ S201A could affect σ^E activity independently of RseA, or it could affect YaeL activity. However, the failure of DegSΔPDZ S201A to allow induction of σ^E by OmpC suggests that DegS-dependent proteolysis of RseA is still required for σ^E induction in response to OmpC overexpression. Pulse-chase analyses of RseA in the presence of DegSΔPDZ S201A will help determine the mechanism by which this DegS mutant affects σ^E activity.

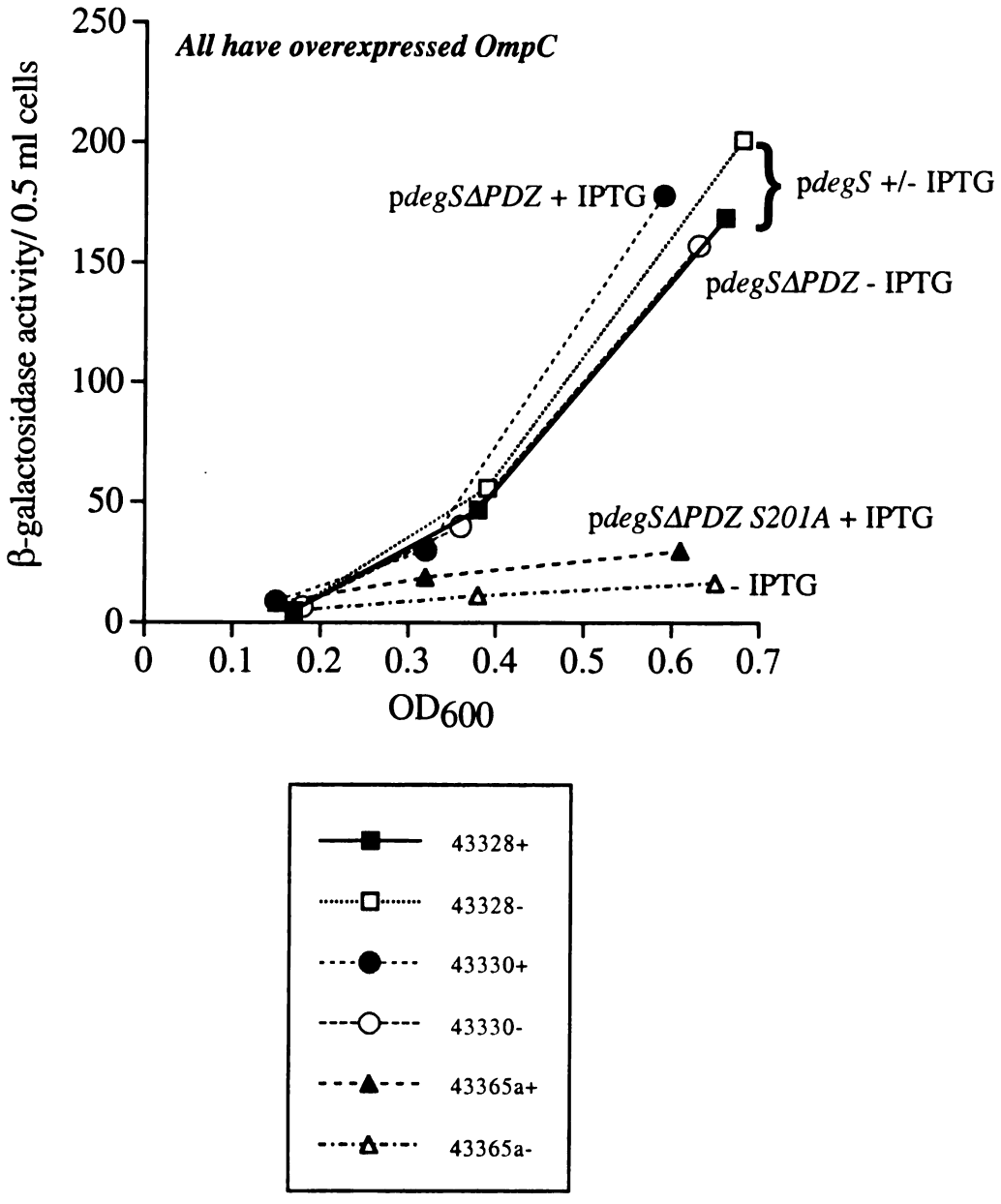
Combination of *degSΔPDZ* and Δ*rseB* alleles strongly elevates σ^E activity



Overexpression of *degSΔPDZ S201A*
elevates σ^E activity



DegSΔPDZ S201A does not restore σ^E inducibility to Δ*degS* a strain.



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