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Pushing the Envelope:

Extracytoplasmic Stress Responses

in

Escherichia coli

Paul Nicola Danese

A DISSERTATION
PRESENTED TO THE FACULTY
OF PRINCETON UNIVERSITY
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Abstract

The results of this dissertation can be classified into two general categories that address the following question: How does the gram-negative bacterium, *Escherichia coli* respond to protein-mediated stresses that originate from its extracytoplasmic subcellular compartments?

First, I have shown that *E. coli* activates the Cpx two-component signal transduction system in response to certain extracytoplasmic protein-mediated stresses. The Cpx pathway functions in parallel with a second signal transduction system that modulates the activity of the heat-shock σ factor, σ^E, in response to extracytoplasmic stresses. The stimuli that activate these two signal transduction pathways can be grouped into three classes: 1) those that stimulate only the Cpx pathway [*e.g.*, overproduction of the envelope lipoprotein, NlpE], 2) those that stimulate only the σ^E modulatory system [*e.g.*, overproduction of outer-membrane porin proteins], and 3) those that stimulate both signal transduction systems [*e.g.*, overproduction of the P-pilus subunit, PapG]. Taken together, these results indicate that *E. coli* possesses at least two signal transduction systems that monitor the state of its exported proteins.

Second, the results presented in this dissertation indicate that there are at least three types of genes whose transcription can be stimulated in response to extracytoplasmic stresses: 1) genes whose transcription is activated solely by the Cpx signal transduction system (*cpxP*, *dsbA*), 2) genes whose transcription is activated solely by σ^E (*fkpA*), and 3) genes whose transcription is jointly regulated by both σ^E and Cpx (*degP*). Most of these genes specify extracytoplasmic protein folding and protein turnover agents, implying that *E. coli* responds to extracytoplasmic protein-mediated stresses by increasing the synthesis of proteins that can either refold or destroy damaged extracytoplasmic proteins.

"I believe an intelligent being governs over everything,

except certain parts of New Jersey."

-Woody Allen

Quando sei l'incudine, statti,

e

quando sei martello, batti.

First, I thank Tom (my advisor, not my boss) Silhavy. I am one of the lucky few that have been privy to your imagination, your knowledge, and (as far as I'm concerned) your bacterium. To paraphrase a slogan from the 1960's, you have shown me that 'Lac is beautiful.' However, in my opinion, I am most lucky because I can tell that you have a good heart - your door is always open, you always have something good to say (in your own laconic way), and I can tell that you always have everyone's best interest in mind. It is this attitude that I try to emulate. If I had to do it all over again, I would pick the Silhavy lab with even more certainty, and this is the strongest endorsement that I can provide.

Second, I would like to thank my family. I thank Leslie for her love and support over the past six years (and in the future), for putting up with me when I was in a foul mood (99% of the time) and when I was in an okay mood (the other 1%). I also thank my parents (Mom & Pop), my brothers (Ant & Dave) and the rest of my family for their love and support over the past 28 years. I couldn't have done it without you guys.

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book. **Andy Kirby**: Thanks for calling me at work at 11:30 PM on weeknights, thus preventing my departure from lab. Thanks for pretending to stab I. G. in the head while I was talking to her. Thanks for being an all-around disruptive force in lab (sarcasm). **Gerry Waters and Mike Hecht**: Thanks to both of you for your positive and cheery attitudes, for your help throughout the past 6 years, for serving on my generals committee, and for your reference letters. You have both been an enormous help. **Ann Flower**: Thanks for letting me install your fence (whooooooboy that was fun!), thanks for your help getting started in the lab (without your help I'd still be thinking that P1 was a pipetman that dispensed 1 µl of liquid. I now realize that it's a pipetman that dispenses 1 liter of liquid). **Katherine Gibson**: Thanks for joining the lab and for your superb musical taste. **Tracy Raivio & Jill Riess**: Thanks for taking over the *cpx* project with unbridled enthusiasm. **John Denis**: Thanks for attending all the parties, and for rolling your eyes when the women started talking about great shopping deals. **Crane & Co.**: Thank you for your generous donation of 10,000 sheets of primo Thesis Paper - you guys are the best! **Laura Davis**: Thanks for finding NlpE. **Jon Beckwith**: Thanks for your help on the GroE paper. **Vanessa Register**: Thanks for making all that media. Your perfectionistic attitude is a plus. **Liya Shi**: Thanks for being a superb lab manager, for not screaming at me when I left the -20°C freezer-door open, and thanks for your help purifying my plasmid DNA. **Susan Direnzo**: Thanks for the worldly advice, and for your help with my manuscripts (you give Tom just enough power to fool him into thinking he runs the place). **Tom Mason and Susan Leonhardt**: Thanks for providing me with a solid foundation in the ways of microbial genetics and molecular biology. **Bonnie Bassler and Jim Broach**: Thanks for accepting the thankless task of reading 250 pages of my writing. Your comments were both important and constructive. **Chris Murphy**: Thanks for your help and faith with the GroE project. **Sue Biggins**: Thanks for allowing me to become the 3rd amigo. **Sam Halloway**: Thanks for your help over the past 6 years, for finding my wallet, and for not blowing a head-gasket every time I dropped spare change on the floor near my desk. **Dan Popkin**: Thanks for being goofier than I could ever be.

This is what some reviewers had to say after perusing Paul's dissertation!

"Reads like the Japanese portion of a VCR assembly manual."
-Gerry Waters, Princeton University

"It was full of pages."
Andy Kirby, Cambridge, Mass.

Table of Contents

One. Introduction

All cells sequester biological activities in distinct subcellular compartments that are typically bounded by lipid bilayers. Through this type of compartmentalization, cells enhance the functions of many activities by concentrating them, and they avoid such problems as the intermingling of incompatible reactions.

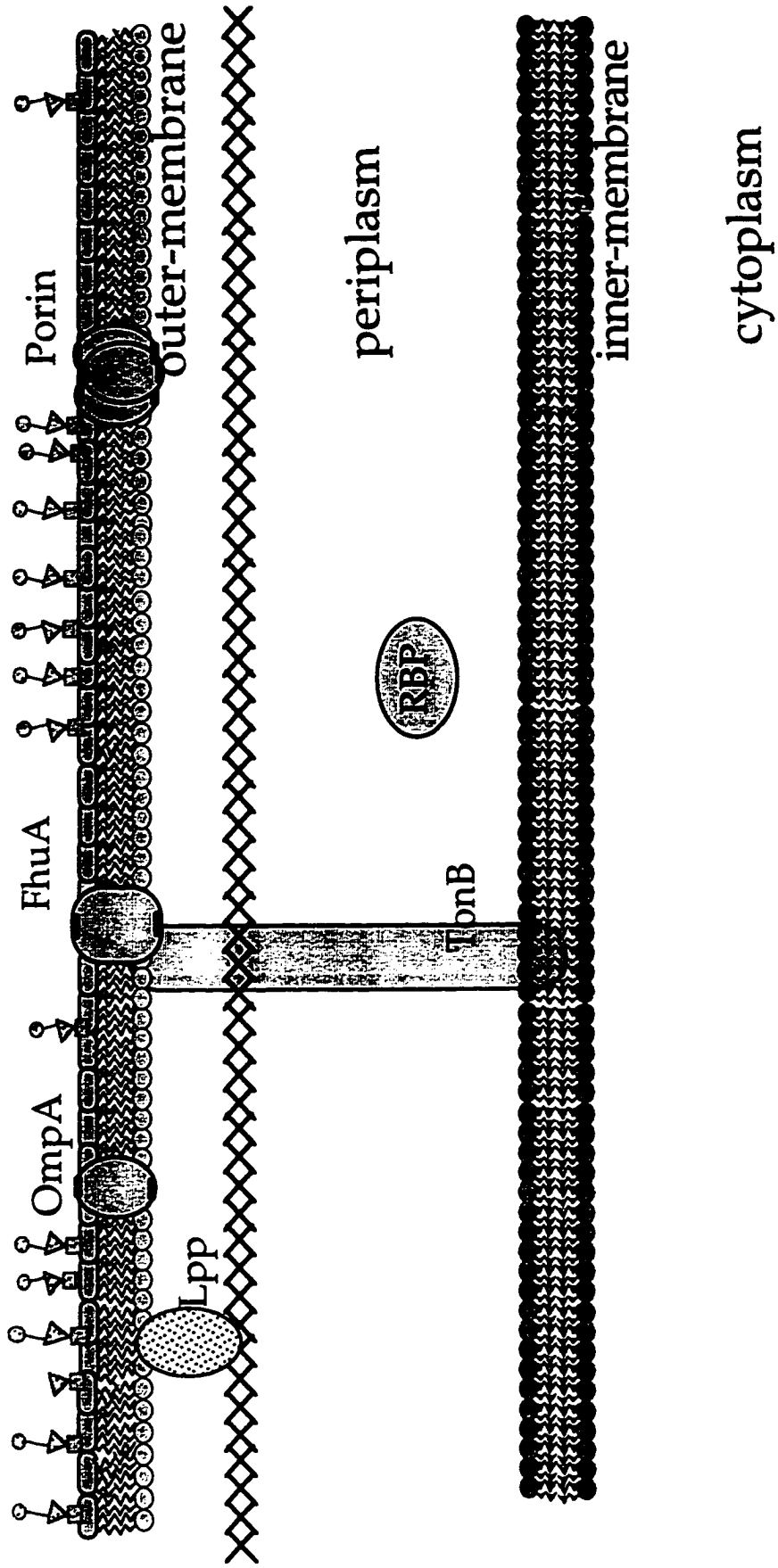
If we take into account the notion that lipid bilayers can also be considered as compartments in and of themselves, then the bacterium *Escherichia coli* can be divided into four regions: cytoplasm, inner membrane, periplasm and outer membrane (Figure 1.1). These final three extracytoplasmic compartments can also be viewed as one large structure that envelops and separates the cytoplasm from the external environment. Hence, the inner membrane, periplasm and outer membrane are collectively known as the bacterial envelope [Bayer 1974].

While compartmentalization is necessary for cellular viability, it also poses a challenge for the cell. For example, although *E. coli* possesses the four compartments described above, all of its proteins are synthesized in the cytoplasm. Hence, proteins required to function in the inner membrane, periplasm or outer membrane must be actively exported to these specific destinations.

To be transported to *E. coli*'s periplasm or outer membrane, proteins must initially be threaded through the inner membrane in a relatively unfolded state [Murphy and Beckwith 1996]. Consequently, as these proteins emerge from the inner membrane, they must refold as they journey on to their final destination. Thus, protein targeting and protein folding in the periplasm and outer membrane are interwoven processes. For example, envelope proteins that cannot fold properly are often improperly targeted [*e.g.*, see Bosch *et al.* 1986]. Conversely, proteins that are improperly targeted will often remain in an unfolded state [*e.g.*, see Hengge and Boos 1985].

Although *E. coli* is arguably the most thoroughly characterized living organism, the processes of envelope-protein targeting and assembly are still poorly understood. The general goal of this thesis has been to analyze how *E. coli* responds when the targeting and assembly of

Figure 1.1. The four major subcellular compartments of *E. coli*. The cytoplasm, inner membrane, periplasm and outer membrane are diagrammed. The bacterial envelope is the collection of the inner membrane, periplasm and outer membrane. Various envelope proteins are diagrammed in their appropriate compartments. The peptidoglycan layer is shown as a hatched structure associated with Braun's lipoprotein. Abbreviation: RBP (Ribose binding protein). All other terms are given in the text.



periplasmic and outer membrane proteins is perturbed. Does *E. coli* monitor these perturbations? If so, how does *E. coli* respond?

The indirect goals of addressing these questions are two-fold. First, if *E. coli* does monitor such events, it provides an opportunity to study subcellular communication. In other words, how does the cytoplasm "know" that proteins have been perturbed on the other side of the inner membrane? Second, by perturbing the targeting and/or folding of envelope proteins, one can potentially identify factors involved in assisting these processes. The identification of such factors is the first major step in understanding the molecular assembly of the cell.

In this chapter, I provide a brief description of the major periplasmic and outer membrane proteins found in *E. coli*. I then describe our current knowledge of the targeting and assembly of these proteins. Finally, I discuss the various signal transduction systems that monitor the physiology of periplasmic and outer membrane proteins in *E. coli*.

PERIPLASMIC PROTEINS

The periplasm is a diverse molecular community, whose residents play a variety of roles important for the viability of *E. coli*. For example, the peptidoglycan layer, which molds the structural framework of the cell, is found in the periplasm (Figure 1.1). In addition, many periplasmic proteins are involved in promoting the biogenesis of extracellular capsule polysaccharides, the outer membrane and the periplasm itself [Oliver 1996].

In one sense, the periplasm is a buffer zone between the bacterial cytoplasm and the external environment. Indeed, since the outer membrane is studded with diffusion channels, the periplasmic fluid is actually continuous with the external milieu. Hence, it is not surprising that the periplasm is dominated by proteins whose function is to coordinate the movement of various compounds across the inner membrane.

A comprehensive description of all periplasmic proteins would be unwieldy, and thus, I will limit my descriptions to the most prevalent periplasmic proteins and those that bear directly on this study.

Periplasmic Binding Proteins

The largest class of periplasmic proteins is the periplasmic binding protein family. As their name suggests, these proteins bind specific solutes and facilitate their transport across the inner membrane (Figure 1.1). There are more than 30 types of periplasmic binding protein in *E. coli* alone, and their ligands range from sugars such as ribose (RBP) to metals like nickel (NikA) [Oliver 1996].

The structures of several periplasmic binding proteins have been determined by crystallographic methods, and these studies indicate a common overall structure for this class of proteins. Specifically, most periplasmic binding proteins possess two globular domains that are attached by a flexible hinge. Ligand binding occurs at the hinge, whereupon the globular domains snap together and fasten the ligand in place [reviewed in Pugsley 1993]. The protein/ligand complex is then able to interact with its cognate transporter in the inner membrane, thereby facilitating movement of the ligand into the cytoplasm.

Catabolic Periplasmic Enzymes

A second major class of periplasmic proteins functions to break down various polymers and smaller substrates found in the periplasm. The best-characterized member of this group is alkaline phosphatase, a homodimeric phosphomonoesterase that releases inorganic phosphate (P_i) from a variety of compounds [Oliver 1996].

The folding and assembly of alkaline phosphatase has been extensively characterized, and it is one of the best-studied periplasmic enzymes in this regard. Each alkaline phosphatase subunit contains a unique phosphomonoesterase reaction center and each assumes the rather common α/β barrel fold found in many soluble proteins [Sowadski *et al.* 1985].

Each subunit also contains an intramolecular disulfide bond required for alkaline phosphatase activity. Interestingly, these disulfide bonds impart a rather useful property upon alkaline phosphatase. Specifically, alkaline phosphatase can only assume a functional state in the relatively oxidizing environment of the periplasm. If alkaline phosphatase is retained in the bacterial cytoplasm, it remains unfolded and bereft of function [Derman and

Beckwith 1991]. This property has been cleverly exploited to map the two-dimensional topology of a variety of bacterial inner-membrane proteins [Traxler *et al.* 1993]. By randomly fusing the mature portion of alkaline phosphatase to a target inner-membrane protein, one can determine which regions of this target protein are found in the periplasm. This is done simply by determining which hybrid molecules display relatively high alkaline phosphatase specific activity.

OUTER MEMBRANE PROTEINS

Although the periplasmic fluid is continuous with the external milieu, it is also a compartment distinct from this surrounding medium. The boundary that maintains this distinction is the outer membrane. Indeed, the outer membrane is the first major barrier that separates *E. coli* from its environment.

Although E. *coli* utilizes the outer membrane as a barrier, the cell must still extract nutrients from its environment, it must still dispose of its waste and it must still communicate with its neighbors. Accordingly, the bacterium must also allow the selective transport of its waste, nutrients and signalling compounds across the outer membrane. Given this role, it is no surprise that the protein composition of the outer membrane is dominated by channels that bridge the external environment with the periplasm.

Below, I briefly describe the form and function of *E. coli*'s most prevalent outer membrane proteins.

Porins

Porins are homotrimeric proteins that form channels for the passive diffusion of solutes between the external environment and the periplasm (Figure 1.1) [Nikaido 1996]. There are two general classes of porin: 1) those like OmpF and OmpC that form relatively non-specific channels with a size limit of approximately 600 Da [Rosenbusch 1990], and 2) those like LamB and PhoE that are more selective with regard to the compounds that can diffuse through them; LamB forms channels for the uptake of maltose and its oligomers, while PhoE is an anion-specific channel [Nikaido 1996]. Despite their divergent functions, the porins are remarkably

similar in their tertiary structure: OmpF, LamB and PhoE subunits all assume a ß-barrel fold (see below) [Cowan *et al.* 1992, Schirmer *et al.* 1995].

High-Affinity Receptors

Certain externally-available compounds such as vitamin B_{12} are too large to be transported through the porins. In these cases, high-affinity outer membrane binding proteins are used for substrate uptake (Figure 1.1). For example, BtuB, FhuA and FadL facilitate the import of B_{12}, ferrichrome and long-chain fatty acids, respectively [Nikaido 1996]. These receptors are dissimilar to the porins in at least two major qualities. First, they bind their ligands tightly, with K_D values in the nanomolar range [*e.g.*, see Bradbeer 1986]. Second, these receptors typically require an accessory protein, TonB, to facilitate entry of their ligand into the cell (Figure 1.1) [Postle 1993].

Braun's Lipoprotein

Braun's Lipoprotein, or Lpp, is one of the most abundant proteins found in *E. coli*. It is attached to the outer membrane primarily through fatty acyl and diglyceride moieties that are bound to Lpp's amino-terminal cysteine residue. The proteinaceous portion of Lpp is found in the periplasm, where approximately one-third of Lpp molecules are covalently attached to the peptidoglycan layer (Figure 1.1). Hence, Lpp fastens the outer membrane to the cell wall, thereby increasing the structural integrity of this membrane [Nikaido 1996].

OmpA

OmpA is an abundant outer membrane protein that also functions as a weak, non-specific solute diffusion channel [Sugawara and Nikaido 1992]. Although the OmpA polypeptide is similar in size to the porin subunits, OmpA appears to function as a monomer (Figure 1.1) [Nikaido 1996]. OmpA is also important for maintaining the structural integrity of the outer membrane, as strains lacking both OmpA and Lpp tend to lose portions of their outer membrane to the surrounding medium [Sonntag *et al.* 1978]. Interestingly, spectroscopic studies indicate that OmpA contains a high degree of ß secondary structure, much like the porins

[Vogel and Jahnig 1986]. This suggests that a defining feature of integral outer membrane proteins may be a richness in ß structure.

Higher Resolution Structures of the Porins

As mentioned, initial spectroscopic studies indicated that the porins are rich in ß secondary structure [Rosenbusch 1974, Kleffel *et al.* 1985, Vogel and Jahnig 1986]. In conjunction with these studies, sequence analysis has shown that the porins are remarkably hydrophilic for membrane proteins [Chen *et al.* 1979, 1980, Movva *et al.* 1980, Clement and Hofnung 1981, Overbeeke *et al.* 1983, Mizuno *et al.* 1983]. The high degree of ß structure and the hydrophilicity of the porins contrasts sharply with the classical hydrophobic and alpha-helical features of inner-membrane proteins [Kadner 1996].

How then, do the hydrophilic porins span the outer membrane? Fortunately, the porins are a rare example of membrane proteins whose structures have been determined by X-ray crystallography. The first such examination was described by Weiss and coworkers [1991a, b], who solved the structure of porin from *R. capsulatus*. Like its *E. coli* counterparts, the *R. capsulatus* porin is a homotrimeric outer membrane protein, whose subunits each form a 16-stranded anti-parallel ß-barrel. Interestingly, this general structure has now been observed by crystallographic methods in three other porins, OmpF, PhoE and LamB [Cowan *et al.* 1992, Schirmer *et al.* 1995]. LamB is slightly unusual in that each subunit forms an 18-stranded ß-barrel instead of the typical 16-stranded form seen for the others [Schirmer *et al.* 1995].

Aside from the overall fold, the porins also display other common qualities. The cell-surface-exposed loops that connect individual ß strands tend to be long. In some cases, either the cell-surface-exposed loop or a membrane-embedded strand will partially occlude the subunit pore, restricting access to the channel [Cowan and Schirmer 1994]. Aromatic residues form a ring around the porin subunits at the interface between the lipid bilayer and the hydrophilic environment. This aromatic girdle presumably impedes movement of the porin subunit out of the plane of the membrane [Cowan and Schirmer 1994].

The crystal structures also explain how such hydrophilic proteins can be stably associated with the membrane. Each ß strand of a porin subunit tends to be an array of alternating hydrophobic and hydrophilic residues. The hydrophilic residues primarily form inter-strand hydrogen bonds while their hydrophobic neighbors insert into the non-polar milieu of the bilayer [Cowan *et al.* 1992].

Although the crystal structures explain how the hydrophilic porin trimers can remain stably inserted in the outer membrane, they fail to address a more dynamic phenomenon of porin assembly. Specifically, how and where do the ß-barrels form? For entropic reasons, the single ß strands of porin subunits cannot be inserted into the outer membrane unless the hydrophilic portions of each strand are shielded from the bilayer. If the individual strands are not shielded, it may be necessary to form the overall ß-barrel fold of the porin subunit prior to insertion into the outer membrane. If this latter scenario is correct, the folded structure would insert into the membrane *in toto*.

From the description above, it is clear that many fundamental aspects of porin assembly are still poorly understood. Indeed, the assembly of both periplasmic and outer membrane proteins in general, is only understood at a rudimentary level. However, before I review our current understanding of periplasmic- and outer membrane-protein assembly, it is necessary to describe the possible routes by which these proteins travel to their specific destinations.

Periplasmic and Outer Membrane-Protein Targeting

As mentioned earlier in this chapter, periplasmic and outer membrane proteins are initially synthesized in the bacterial cytoplasm, and they are then actively exported to their proper destinations. The process by which these proteins are targeted to and through the Sec machinery in the inner membrane is fairly well-understood [For a review, see Murphy and Beckwith 1996]. In contrast, the movement of periplasmic and outer membrane proteins *after* their association with the Sec machinery is enigmatic at best.

Traveling to the periplasm

For example, how are periplasmic proteins released from the inner-membrane and how are they ultimately moved to the periplasm? There is some evidence suggesting that the inner-membrane protein, SecD, is responsible for releasing periplasmic proteins from the inner membrane. Anti-SecD antibodies inhibit the release of mature maltose-binding protein (a periplasmic binding protein) from the inner membrane of *E. coli* spheroplasts, suggesting that SecD is involved in releasing, and perhaps folding exported proteins as they emerge from the Sec machinery [Matsuyama *et al.* 1993]. However, other biochemical functions have also been ascribed to SecD. For example, Arkowitz and Wickner [1994] suggest that SecD couples the proton-motive force to protein translocation across the inner membrane. Additionally, a study by Economou and coworkers [1995] suggests that SecD regulates the insertion and de-insertion of the SecA protein into and out of the inner membrane.

From this information, it is safe to say that the function of SecD is still a matter of debate. Encouragingly, however, *secD* null strains are viable [Pogliano and Beckwith 1994], and thus, it should be relatively straightforward to test some of the models for SecD function. For example, do *secD* null strains display defects in the release of mature folded periplasmic proteins from the inner membrane? The answer to this question would ultimately support or refute the release/folding function attributed to SecD.

In general, the mechanism by which periplasmic proteins move from the inner membrane to the periplasm is not clear. Are there *cis*-acting determinants found within a periplasmic polypeptide (aside from a signal sequence) that direct it to the periplasm? The experimental data addressing this question are scant at best.

Despite the relative dearth of data, the targeting of periplasmic proteins is now commonly regarded as a default process [*e.g.*, see Oliver 1996]. This view is based largely on two general observations. First, in contrast to the study by Summers and Knowles, other cytoplasmic proteins *can* be targeted to the periplasm with the simple addition of a cleavable signal sequence [*e.g.*, see Karim *et al.* 1993]. Thus, the ability of a cytoplasmic protein to be

routed to the periplasm depends on the protein in question. In addition, various outer membrane proteins will mislocalize to the periplasm when portions of their mature sequences are deleted [Bosch *et al.* 1986, Klose *et al.* 1988].

Taken together, these analyses provide three major implications. First, the periplasm *can* be the default address for cytoplasmic proteins, provided that they are fitted with an appropriate signal sequence. Second, in contrast to periplasmic proteins, outer membrane proteins require *cis*-acting determinants (aside from a signal sequence) for their proper targeting. Third, the periplasm is clearly the default address for proteins that are normally exported across the inner membrane (*e.g.*, mutant outer membrane proteins).

Since deletions within the mature sequences of various outer membrane proteins cause them to mislocalize to the periplasm, does this mean that outer membrane proteins normally travel through the periplasm to reach their final destination? Surprisingly, this question has been, and is still a matter of intense debate.

Traveling to the Outer Membrane

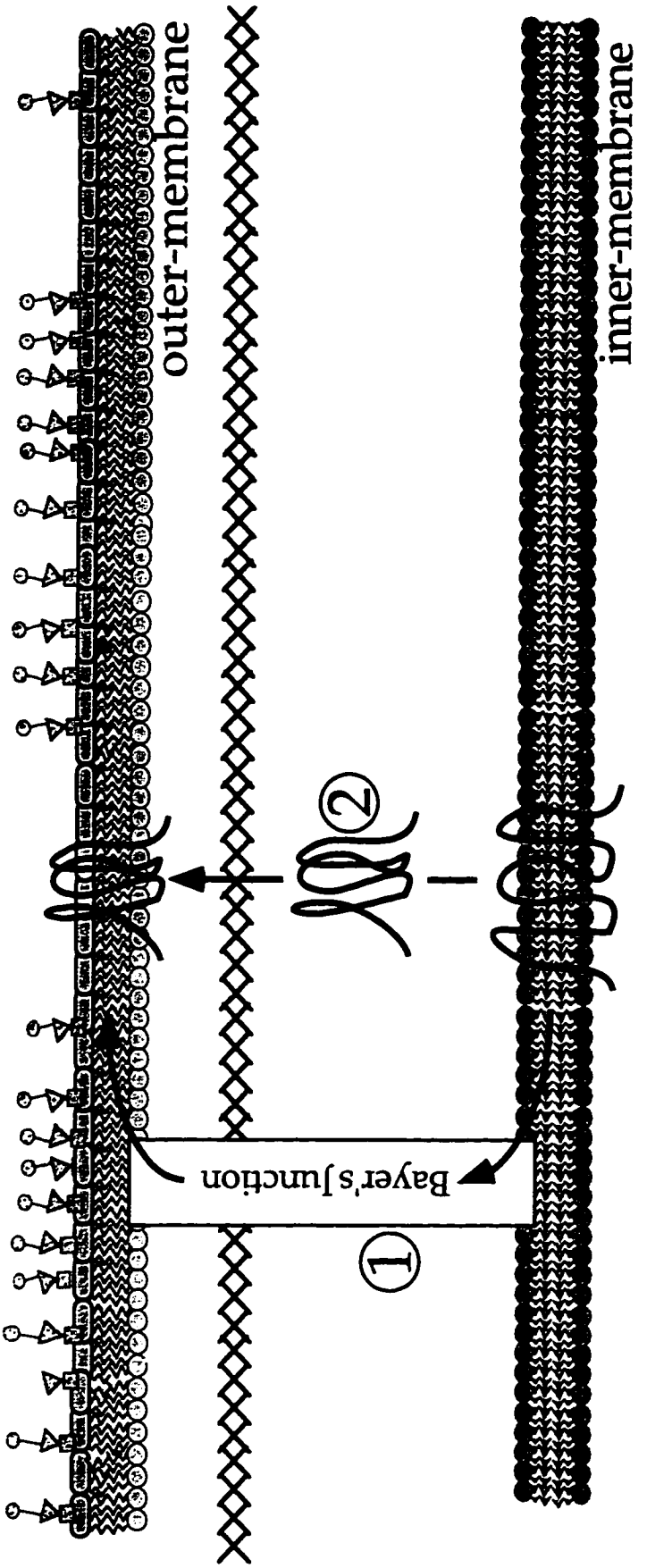
Bayer's junctions versus Periplasmic Intermediates

Two general paths have been proposed for the translocation of outer membrane proteins from the inner to the outer membrane. The Bayer's Junction model postulates that outer membrane proteins are ferried to the outer membrane *via* zones of adhesion between the inner and outer membranes [Bayer 1968, 1979]. Alternatively, the Periplasmic Intermediate model suggests that outer membrane proteins are ejected from the inner membrane, transit through the periplasm, navigate the sieve-like structure of the peptidoglycan layer [Decad and Nikaido 1976] and are subsequently inserted into the outer membrane *via* an unknown process (Figure 1.2) [*e.g.*, see Nikaido 1996].

Zones of adhesion (Bayer's junctions)

Bayer and others [Bayer 1968, Crowlesmith *et al.* 1978, Kroncke *et al.* 1990] have observed zones of adhesion between the inner and outer membranes in electron micrographs of plasmolyzed *E. coli* cells. These Bayer's junctions appear to bridge the two membranes at more

Figure 1.2. The Bayer's Junction (1) and Periplasmic Intermediate (2) models for outer-membrane-protein targeting. A Bayer's Junction is depicted as a rectangle bridging the inner and outer membranes.



than 50 sites per cell [Bayer 1994]. However, the *in vivo* existence of these sites has been disputed [Kellenberger 1990, Hobot *et al.* 1984]. For example, Kellengerger notes that Bayer's junctions are only observed when certain cryo-fixation techniques are used for sample preparation. Bayer [1991] has attempted to rebut these deconstructionistic attacks by noting that when relatively rapid cryo-fixation techniques are employed, zones of adhesion *are* observed. The interpretation of this result is that rapid fixation techniques preserve the *in vivo* state of the cell with higher fidelity. Despite this rebuttal, the debate still rages.

An important issue may be lost in the midst of this quarrel: even if Bayer's junctions exist, is there any evidence suggesting that they are involved in shuttling outer membrane proteins to the outer membrane?

It seems relatively straightforward to distinguish between the Bayer's Junction and Periplasmic Intermediate models. One could simply identify intermediates in the process of outer membrane-protein targeting and determine if these intermediates fractionate with the membrane or with the periplasm. Indeed, several studies have been performed to distinguish between these two models. Although most studies suggest that at least part of the journey to the outer membrane proceeds through the periplasm, many of these studies are equivocal in their discrimination between the two models. Thus, while it seems likely that the Periplasmic Intermediate model will emerge victorious in some form, the Bayer's junction model has not been completely ruled out.

Membrane-anchoring studies

MacIntyre and coworkers [1988] have shown that insertion of a hydrophobic domain into OmpA anchors this outer membrane protein to the inner membrane. Nikaido [1996] argues that if Bayer's junctions are used for outer membrane-protein targeting, then a membrane-anchored OmpA protein *should* still be targeted to the outer membrane. Since the anchored OmpA protein is not targeted to the outer membrane, OmpA probably migrates *via* a periplasmic route. However, this reasoning assumes that the information directing OmpA to

the outer membrane will override the inner-membrane-anchoring information provided by the hydrophobic insertion. There is no evidence to support (or refute) this assumption.

Moreover, similar studies have yielded different results. For example, Carlson and Silhavy [1993] constructed LamBA23D, a mutant form of the maltoporin, LamB. LamBA23D contains a mutation which impairs its signal-sequence cleavage, and thus, the signal sequence of LamBA23D acts largely as an inner-membrane-anchoring domain. In this case, however, a significant fraction of the LamBA23D precursor cofractionates with the outer membrane. Additionally, Shinkai and coworkers [1989] inserted a hydrophobic membrane-spanning domain into the mature sequence of OmpC, and noted that this hydrophobic domain did not impede export of OmpC across the inner membrane and into the outer membrane.

Taken together, these studies are difficult to interpret. Does each membrane-spanning domain completely impede movement of each protein out of the inner membrane? If not, then these outer membrane proteins could still proceed through the periplasm. In addition, Carlson and Silhavy [1993] note that the fractionation technique used to determine the subcellular localization of LamBA23D may be misleading. For example, imagine that the signal sequence of LamBA23D causes the molecule to remain tethered to the inner membrane while the mature portion of the protein reaches through the periplasm and into the outer membrane. Under these circumstances, fractionation of LamBA23D could generate proteins that are associated with both the inner and outer membranes [Carlson and Silhavy 1993].

In general, the strength of the membrane-anchoring domain will influence the ability of a particular outer membrane protein to be properly targeted. Since it is difficult to gauge the strength of the membrane-anchoring domains used in the studies described above, these studies do not conclusively distinguish between the Bayer's Junction and Periplasmic Intermediate models.

Deletion analysis

Deletion analyses have also been utilized in an attempt to distinguish between the Bayer's Junction and Periplasmic Intermediate models. For example, deletions within the

mature sequences of OmpA, PhoE and FhuA cause these proteins to localize to the periplasm [Freudl *et al.* 1985, Bosch *et al.* 1986, Coulton *et al.* 1988, Klose *et al.* 1988, Agterberg *et al.* 1989]. It seems reasonable to assume that these proteins lack the information to move from the periplasm to the outer membrane - *i.e.*, they are "dead-end" intermediates. However, since these "dead-end" species cannot be chased into the outer membrane, their classification as intermediates can always be disputed. More evidence is needed.

Other studies

Freudl *et al.* [1986] demonstrated that when OmpA is overproduced, a fraction of the overproduced species is found in the periplasm, again suggesting that this periplasmic form is an intermediate on the road to the outer membrane. Jackson *et al.* [1986] used differential detergent solubility to demonstrate that FhuA proceeds through a membrane-free compartment before it is stably inserted into the outer membrane. This membrane-free compartment is most likely the periplasm.

Halegoua and Inouye [1979] inhibited the incorporation of OmpA into the outer membrane through the addition of phenethyl alcohol to growing cultures. OmpA synthesized under these conditions co-fractionated with periplasmic proteins, again suggesting a periplasmic path to the outer membrane.

Sen and Nikaido [1990] have shown that *E. coli* spheroplasts secrete a water-soluble OmpF monomer into the surrounding medium. This monomer can trimerize and can incorporate into membrane vesicles in the presence of low levels of detergent. This study also implies that OmpF monomer proceeds through the periplasm before its insertion into the outer membrane. Bayer [1994], however, suggests that the detergent required to insert OmpF into membrane vesicles may mimic the function of junctions that bear his name.

Finally (and most compellingly), Fourel *et al.* [1992] followed the maturation of OmpF by examining the formation of various OmpF epitopes with a repertoire of monoclonal antibodies. This study noted that a metastable species of wild-type OmpF trimer is found in

periplasmic fractions, suggesting that at least part of the journey to the outer membrane proceeds through the periplasm.

In sum, most of the available data support the notion that outer membrane proteins proceed through the periplasm prior to insertion into the outer membrane. However, it should be noted that the Bayer's Junction and Periplasmic Intermediate models are not mutually exclusive. The path to the outer membrane could proceed partly through the periplasm and partly through Bayer's Junctions. Also, a subset of outer membrane proteins could traverse the periplasm, while others could use Bayer's junctions.

How can we distinguish amongst these possibilities? First, a wide range of outer membrane proteins needs to be examined. Do OmpA, BtuB and OmpF all proceed through the periplasm? Second, we need to identify all intermediates in the process of outer membrane-protein targeting. Are all post-signal-sequence-cleavage intermediates found in the periplasm, or are some still membrane bound? These types of analyses should ultimately clarify the outer membrane-protein-targeting process.

The Search for Outer membrane-Protein-Targeting Signals

Regardless of the route that outer membrane proteins take from the inner to the outer membrane, the mature sequences of outer membrane proteins must possess information that targets them to the outer membrane.

Where does this information reside? Two general approaches have been employed to address this question. The first class of approaches fused various portions of outer membrane proteins to reporter proteins in an attempt to identify sequences that could target a reporter to the outer membrane. The second class of approaches utilized deletion analysis to identify domains that were required for the proper localization of outer membrane proteins.

Protein fusion studies

Benson *et al.* [1984] fused ß-galactosidase (encoded by *lacZ*) to the carboxy-terminus of various LamB fragments. Using sucrose-density centrifugation to fractionate inner and outer membranes, the authors noted that the first 49 amino acids of the mature LamB sequence were sufficient to target the ß-galactosidase moiety to the outer membrane. However, subsequent

immuno-electron microscopy studies [Tommassen *et al.* 1985, Tommassen and de Kroon 1987, Voorhout *et al.* 1988] suggested that at least some of the LamB-LacZ fusion proteins analyzed in the study by Benson *et al.* was associated in membrane-like structures *within* the cytoplasm. Hence, the results reported by Benson *et al.* [1984] may have reflected a situation in which the LamB-LacZ fusion protein formed cytoplasmic aggregates that cofractionated with the outer membrane. Alternatively, it is possible that the fractionation studies of Benson *et al.* [1984] more accurately reflected the location of LamB-LacZ, and that the microscopy studies highlighted the location of a minor portion of this fusion protein. This dispute over the localization of LamB-LacZ has never been resolved, and thus, it is still unclear as to whether the first 49 amino acids of the mature sequence of LamB are utilized for targeting to the outer membrane.

Coulton *et al.* [1988] performed a similar study to that described above. In this case, the periplasmic protein alkaline phosphatase (AP) was fused to the carboxy-terminus of various FhuA fragments. The authors noted that a FhuA-AP fusion containing the first 88 amino acid residues of FhuA was periplasmically localized. In contrast, a FhuA-AP fusion containing an additional 100 residues from the FhuA protein caused the fusion to localize to the outer membrane. Coulton *et al.* suggest that there may be an outer membrane-targeting sequence within this 100 residue region. However, this region has not been examined to further delineate such a putative targeting sequence. Moreover, it is distinctly possible that these 100 additional residues cause the FhuA-AP fusion to aggregate and non-specifically associate with the outer membrane.

Deletion studies

Various deletion analyses have been performed on outer membrane proteins in order to identify regions important for their proper localization. For example, Bosch and coworkers have performed extensive deletion analysis throughout the PhoE mature sequence, only to conclude that deletions extending into the membrane-spanning domains of PhoE interfere with outer membrane localization [Bosch *et al.* 1986, 1988, 1989, Agterberg *et al.* 1989].

Several similar studies have also been employed with OmpA. Klose *et al.* [1988a, b] used immuno-electron microscopic techniques to show that the region between residues 154 and 180 of the OmpA sequence is needed for outer membrane association. This region corresponds to the last putative membrane-spanning domain of OmpA. Hence, it seems likely that such a carboxy-terminal deletion severely alters the overall structure, and by extension, the localization of OmpA.

Similar results have also been observed when amino-terminal deletions are generated in OmpA. Small deletions which presumably do not perturb the overall structure of OmpA do not interfere with its outer membrane localization. However, larger amino-terminal deletions, which presumably extend into OmpA's membrane-spanning regions, cause the protein to localize to the periplasm [Freudl *et al.* 1985].

There are inherent caveats associated with these deletion analyses. Even if a specific outer membrane-targeting sequence exists, this sequence would be molded into the overall structure of the mature protein. Thus, it is difficult to determine if deletions prevent outer membrane targeting because they remove a targeting signal or because they destabilize the outer membrane protein. Accordingly, it is necessary to demonstrate that a particular sequence is sufficient to target a non outer membrane protein to the outer membrane. However, even a positive result in this scenario must be cautiously interpreted. Does a given sequence target a protein to the outer membrane, or does it merely cause the protein to non-specifically aggregate with the outer membrane?

Taken together, these studies imply that it is the overall structure of outer membrane proteins that targets them to the outer membrane. For example, this targeting information may reside in the predilection of outer membrane proteins to assume ß secondary structures. In addition, we may ultimately find that factors required for targeting outer membrane proteins to their proper destination will recognize these higher-order structures.

Porin Assembly Intermediates

Interwoven with the targeting of outer membrane proteins is their assembly into the outer membrane. With respect to their assembly, the porins have been the most extensively characterized outer membrane proteins. Presently, four general assembly intermediates have been described for one or more of the porins: 1) mature (signal-sequence processed) unfolded monomer, 2) folded monomer, 3) dimer, and 4) metastable trimer. The metastable trimer is ultimately transformed into a stable native trimer. These four putative general intermediates in porin trimerization are diagrammed in Figure 1.3.

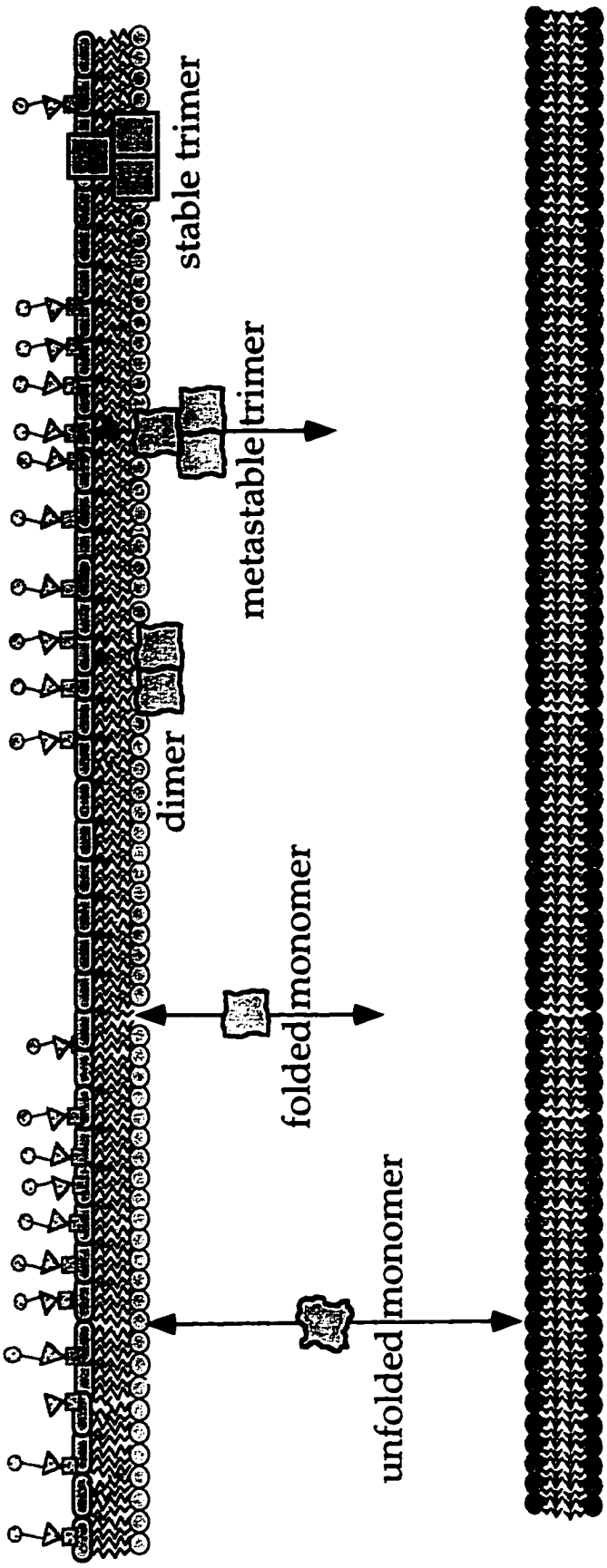
Unfolded monomer

Outer membrane proteins must remain in a relatively unfolded state when translocating through the Sec machinery in the inner membrane [Murphy and Beckwith 1996]. Thus, as porins emerge from the inner membrane they are transiently found in an unfolded state. Such unfolded porin monomers have been observed with OmpF, [Pagès and Bolla 1988, Fourel *et al.* 1992], OmpC [Pagès and Bolla 1988], and LamB [Vos-Schepterkeuter and Witholt 1984, Misra *et al.* 1991, Stader and Justice 1994].

Clearly, this unfolded intermediate must be found in the inner membrane at some point during the targeting process (*e.g.*, see Stader and Justice [1994]). However, the study by Misra *et al.* [1991] suggests that the porin monomer is also found at the outer membrane. Misra and coworkers used a temperature-sensitive LamB protein that reversibly accumulated LamB monomer at the restrictive temperature. Performing a rather thorough fractionation analysis, the authors noted that the LamB monomer species associated with the outer membrane. Thus, the LamB monomer contains all the information necessary to localize to the outer membrane.

In contrast, Fourel *et al.* [1992] observe the unfolded OmpF monomer in periplasmic fractions. However, the results of Fourel *et al.* [1992] and Misra *et al.* [1991], are not irreconcilable. If OmpF and LamB proceed through the same assembly pathway, the rapidity of the wild-type trimerization process may mask the ability of monomeric porin to associate with the outer membrane. The temperature-sensitive LamB protein may have simply revealed this capability.

Figure 1.3. All known porin assembly intermediates are shown (unfolded monomer, folded monomer, dimer and metastable trimer). The arrows associated with each intermediate delineate the compartments in which each particular intermediate has been observed. For example, folded porin monomers have been observed within the periplasm and the outer membrane.



Folded monomer

Several studies have described porin monomers that display structural features that are normally found within the subunits of native trimers. For example, Sen and Nikaido [1991b] have observed that *in vitro* synthesized OmpF assumes a relatively folded conformation that is recognized by antibodies that bind to native epitopes of the OmpF trimer. Fourel *et al.* [1992] observed a similar OmpF folded monomer *in vivo*, noting that this species was found in the periplasm. Such folded monomer species have also been observed with PhoE [de Cock *et al.* 1990] and LamB [P. Rouvière and C.A. Gross, personal communication], suggesting that this species may be a common intermediate in porin assembly.

Dimeric porin

Little is known about the transition from porin monomer to the trimeric state. However, two studies [Reid *et al.* 1988, Rocque and McGroarty 1989] describe OmpF and OmpC dimers that are weakly associated with the outer membrane. Although similar dimeric oligomers have not been described for other porins, it is distinctly possible that these dimers are intermediates in the porin assembly process.

Metastable trimer

Prior to assuming a stable native structure, the porin trimers exist in a metastable state [*e.g.*, see Vos-Scheperkeuter and Witholt 1984, Reid *et al.* 1988, Fourel *et al.* 1992]. The metastable trimers display many conformational epitopes found in the native structure. However, these metastable intermediates dissociate in SDS at temperatures above 50°C, whereas native porins are stable in SDS at temperatures up to 70°C [Vos-Scheperkeuter and Witholt 1984, Reid *et al.* 1988, Fourel *et al.* 1992].

FACILITATORS OF PERIPLASMIC AND OUTER MEMBRANE PROTEIN BIOGENESIS AND TURNOVER

Protein-folding agents

As mentioned above, periplasmic and outer membrane proteins are translocated across the inner-membrane *via* the Sec machinery [Murphy and Beckwith 1996]. As these proteins emerge from the inner membrane, they are greeted by an environment that is vastly different from the cytoplasm. For example, disulfide-bond formation between cysteine side chains in

polypeptides is favored in the envelope because of its oxidizing potential [Derman and Beckwith 1991]. Also, estimates of polypeptide diffusion in the periplasm suggest that this compartment is a gel-like medium with macromolecular diffusion rates that are 100-fold lower than that observed for cytoplasmic proteins [Brass *et al.* 1986]. Moreover, the periplasm and outer membrane are in direct contact with the surrounding medium. Consequently, the residents of these compartments must bear the brunt of all environmental fluctuations. Given such conditions, it seems reasonable to assume that the folding, targeting and assembly of periplasmic and outer membrane proteins must be carefully regulated.

Consistent with this notion, several factors found in the periplasm and outer membrane have been implicated (or suggested to be involved) in periplasmic- and outer membrane-protein targeting and assembly. Below, I discuss the evidence (or lack thereof) for the involvement of these factors in the biogenesis of periplasmic and outer membrane proteins.

AsmA

Rajeev Misra's laboratory has attempted to identify factors involved in porin assembly. In one clever study, Misra [1993] selected for mutations that could suppress the inability of a thermosensitive $OmpF^{Dex}$ protein to assemble in the outer membrane (the $OmpF^{Dex}$ protein alters the permeability of the OmpF porin, allowing growth on maltodextrins even in the absence of the maltoporin, LamB). The *asmA* locus (*a*ssembly *s*uppressor *m*utation) was identified in this way [Misra 1993, Misra and Miao 1995]. Misra's laboratory has subsequently shown that the *asmA* suppressor mutation is capable of suppressing a variety of porin assembly defects, including those caused by mutations affecting the biosynthesis of the major outer membrane lipopolysaccharide, LPS [Laird *et al.* 1994, Xiong *et al.* 1996]. Surprisingly, the *asmA* suppressor mutations are null alleles, suggesting that the wild-type AsmA protein may perform a proofreading function to prevent mutant porins from assembling in the outer membrane [Misra and Miao 1995]. AsmA appears to be an exported protein, which is consistent with its proposed role in porin biogenesis, but its precise function in this process is presently a matter of speculation.

Enzymes involved in disulfide-bond formation

As mentioned in the beginning of this section, disulfide-bond formation between cysteine residues is favored in the relatively oxidizing environments of the periplasm and outer membrane [Derman and Beckwith 1991]. At first glance, it would seem that such disulfide-bond formation would occur spontaneously because of the oxidizing potential of molecular oxygen found in the envelope. However, this is not so. *E. coli* synthesizes several enzymes that catalyze the efficient formation of disulfide bonds in envelope proteins [Grauschopf *et al.* 1995].

DsbA

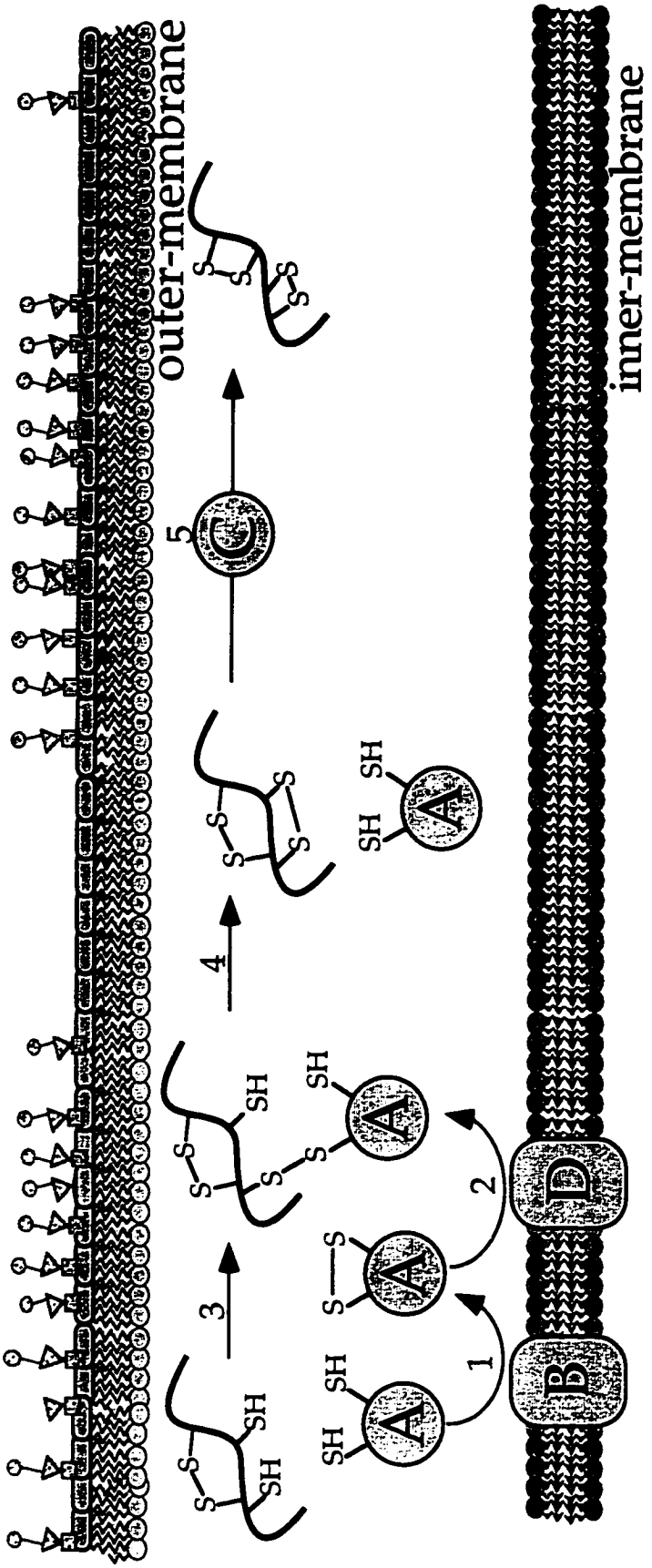
Chief among these is DsbA, a periplasmic protein that catalyzes disulfide-bond formation in various envelope proteins [Bardwell *et al.* 1991, Kamitani *et al.* 1992]. *dsbA* null strains are severely defective for disulfide-bond formation in proteins such as alkaline phosphatase, ß-lactamase and OmpA [Bardwell *et al.* 1991, Kamitani *et al.* 1992] . As a consequence, the folding and stability of these proteins is also impaired in *dsbA* mutants [Bardwell *et al.* 1991].

Purified DsbA rapidly oxidizes cysteine residues of several proteins *in vitro*, including alkaline phosphatase, RNase A and Bovine Pancreatic Trypsin Inhibitor, implying that DsbA is the direct catalyst of disulfide-bond formation *in vivo* [Akiyama and Ito 1993, Frech and Schmid 1995, Zapun and Creighton 1994].

The active site of DsbA consists of two cysteine residues (C30 and C33) that can form a disulfide-bonded pair. This disulfide bond is unstable and C30 will preferentially form a disulfide bond with cysteine sulfhydryl groups of other polypeptides. However, even this intermolecular disulfide bond is relatively unstable, and as a consequence this bond is rapidly transferred to the target polypeptide, thereby catalyzing disulfide-bond formation in the target protein (Figure 1.4) [Bardwell 1994].

The instability of the C30-C33 and C30-Target disulfide bonds is at least partly due to the folding characteristics of DsbA. Mutational analysis indicates that the sulfhydryl groups of the reduced C30 and C33 residues stabilize the folded state of DsbA [Zapun *et al.* 1994]. This

Figure 1.4. A model for disulfide-bond formation in the bacterial envelope. 1) The oxidation of reduced DsbA by DsbB. 2) The reduction of the C30-C33 disulfide bond in DsbA by DsbD. 3) The creation of a disulfide bond between C30 of DsbA and a cysteine sulfhydryl of a target periplasmic protein. 4) Creation of a disulfide bond between the two cysteine sulfhydryl groups of the target protein. 5) Disulfide-bond isomerization catalyzed by DsbC. Abbreviations: DsbA, DsbB, DsbC and DsbD are denoted as A, B, C and D, respectively. SH and S represent reduced and oxidized cysteine residues, respectively.



increased stability could account for the rapid transferal of the disulfide bond from oxidized DsbA to target polypeptides.

DsbB

Since the oxidized form of DsbA is relatively unstable, there must be an external factor which drives its oxidation. This external factor is the inner-membrane protein DsbB (Figure 1.4). *dsbB* null strains display similar defects in disulfide-bond formation for envelope proteins, and they also accumulate the reduced form of DsbA [Bardwell *et al.* 1993, Dailey and Berg 1993, Missiakas *et al.* 1993]. Although there is no direct biochemical evidence supporting this notion, it seems likely that DsbB utilizes a membrane energy source (perhaps the electron transport chain) to drive the oxidation of DsbA [Bardwell 1994].

DsbC

Additional genes involved in disulfide-bond formation are still being identified. For example, Missiakas *et al.* [1994] identified DsbC, another periplasmic disulfide bond oxidoreductase. *In vitro*, DsbC and DsbA act synergistically to promote protein folding [Zapun *et al.* 1995], and it is presently believed that DsbC is a protein disulfide isomerase, shuffling the disulfide bonds generated by DsbA until the appropriate combination is created (Figure 1.4) [Bardwell 1994, Zapun *et al.* 1995].

DsbD

The fourth, and most recently identified, protein involved in disulfide-bond formation is DsbD, an inner-membrane disulfide bond oxidoreductase [Missiakas *et al.* 1995]. Interestingly, the *dsbD* null causes DsbA to accumulate in its oxidized form [Missiakas *et al.* 1995], suggesting that DsbD may facilitate disulfide-bond formation by reducing the C30-C33 disulfide bond found in oxidized DsbA (Figure 1.4). Surprisingly, DsbD is essential for the growth of *E. coli* at high-temperatures [Missiakas *et al.* 1995]. However, we do not understand why DsbD is required at high temperatures while the other Dsb proteins are not. Clearly, suppressor analysis of the *dsbD* null would be informative in this regard.

EcpD

EcpD is a chromosomally-encoded *E. coli* PapD homolog [Raina *et al.* 1993]. The function of EcpD is not known, but its homolog, PapD is involved in assembling pilus structures on the surface of the outer membrane of some pathogenic *E. coli* strains [*e.g.*, see Kuehn *et al.* 1993]. Thus, by homology it has been speculated that EcpD may promote the assembly of outer membrane proteins in non-pathogenic strains of *E. coli* [Raina *et al.* 1993]. However, this possibility has not yet been investigated.

Lipopolysaccharide

Lipopolysaccharide (LPS) is the dominant lipid species found in the outer-leaflet of *E. coli*'s outer membrane [Nikaido and Vaara 1985]. Given its subcellular location, it is not surprising that LPS is found tightly associated with porin trimers. Indeed, Rocque *et al.* [1987] suggested that LPS would be intimately involved in assembling and maintaining porin structure. This notion has ultimately been corroborated both by *in vitro* and *in vivo* studies.

For example, functional LPS is required for the efficient trimerization of porins in membrane vesicles *in vitro* [Schindler and Rosenbusch 1981, Sen and Nikaido 1991a]. Furthermore, LPS mutants that are unable to complete the synthesis of their polysaccharide moieties also display inefficient porin trimerization [Ried *et al.* 1990, Laird *et al.* 1994]. These data indicate that functional LPS is required for the stable assembly of the porins in the outer membrane.

In addition to this function, there is also some evidence that LPS *biosynthesis* is linked to porin assembly. Bolla and coworkers [1988] showed that cerulenin, an antibiotic that indirectly blocks LPS synthesis, concomitantly caused the accumulation of OmpF and OmpC monomers in *E. coli*. This information suggests that the porin monomers may require association with newly synthesized LPS prior to their trimerization.

p20

Matsuyama *et al.* [1995] have provided an interesting story describing the biogenesis of Braun's lipoprotein (Lpp). These authors noted that *E. coli* spheroplasts (lacking portions of their outer membrane and devoid of periplasmic contents) retained their newly synthesized

Lpp molecules at the surface of the inner membrane. Matsuyama and coworkers then purified a periplasmic protein, p20, that solubilized the inner-membrane-bound Lpp molecules. p20 appears to function as a periplasmic usher and assembly factor, shuttling Lpp from the inner membrane and properly assembling it into outer membrane vesicles *in vitro*. We now await genetic analysis to corroborate this biochemical study.

Peptidyl-prolyl *cis/trans* isomerases

It is well-established that the peptide bond displays a partial double-bond character (Figure 1.5) that forces its neighboring carbonyl and substituted-amino groups into a planar structure [Pauling 1960]. This partial double-bond character consequently restricts the movement of the carbonyl oxygen and the substituted-amino hydrogen, allowing them to assume only a *cis* or *trans* conformation with respect to each other. For most dipeptide units, these oxygen and hydrogen atoms are almost always found in the *trans* conformation [Fersht 1985]. However, peptide bonds formed from X-proline dipeptide units (X signifies any residue) are the exception to this rule. The pyrrolidine ring of the prolyl residue assumes either the *cis* or *trans* conformation with respect to the oxygen of the neighboring carbonyl group, depending on the specific polypeptide in question (Figure 1.5) [Levitt 1981].

The *cis/trans* isomerization reaction for prolyl peptides is slow, and this interconversion is known to impede certain protein folding reactions [Brandts *et al.* 1975, Jullien and Baldwin 1981]. Given this information, it is not surprising that peptidyl-prolyl *cis/trans* isomerases (PPIases) exist in all organisms examined thus far [Galat 1993]. These PPIases have been shown to accelerate certain protein folding reactions *in vitro* [*e.g.*, see Lang *et al.* 1987, Bang and Fischer 1991, Veeraraghavan and Nall 1994], and it is assumed that they perform such functions *in vivo* as well [Nilsson and Anderson 1991].

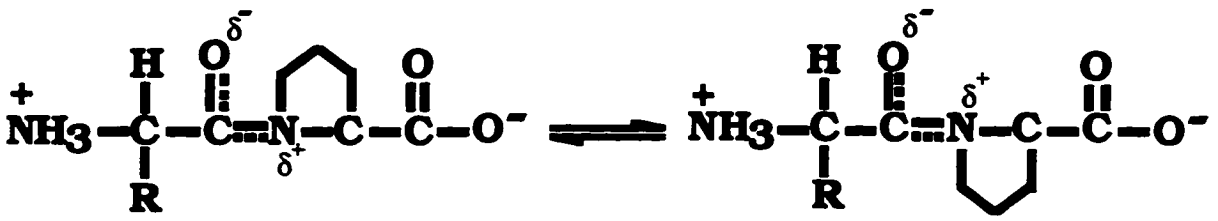


Figure 1.5 *cis/trans* isomerization of a prolyl dipeptide. Dashed lines (---) signify the partial double-bond characteristics of the carbonyl group and the peptide bond. δ^+ and δ^- represent the partial positive and negative charges associated with the oxygen and nitrogen atoms within the dipeptide.

The PPIases were originally identified in higher eukaryotes as targets of the immunosuppressive drugs, cyclosporin and FK506 [Fischer *et al.* 1984]. Accordingly, PPIases were originally grouped into two classes based on sequence homology: cyclophilins and FK506-binding proteins (FKBPs) [Heitman *et al.* 1992]. Subsequent biochemical analysis has identified a third family of PPIases, the Parvulin class. This family is based on the 10-kilodalton *E. coli* PPIase, Parvulin, which bears no sequence relatedness to the cyclophilins or FKBPs [Rudd *et al.* 1995].

SurA

The most intriguing PPIase yet identified in *E. coli's* periplasm is SurA, an abundant Parvulin-like protein that is required for survival of *E. coli* in stationary phase [Tormo *et al.* 1990, Rudd *et al.* 1995]. Oddly, SurA does not appear to serve a specific stationary phase function. Instead, it appears that the inability of *surA* mutants to survive stationary phase reflects their hypersensitivity to alkaline pH. As *E. coli* strains quiesce in stationary phase after growth in Luria broth, they increase the pH of the surrounding medium. Consequently, *surA* mutants inadvertently commit the bacterial version of suicide in stationary phase [Lazar and Kolter 1995].

The true function of SurA appears to be assisting in the assembly of outer membrane proteins [Lazar and Kolter 1995, P. Rouvière and C.A. Gross, personal communication]. *surA*

null strains display a wide range of outer membrane-permeability defects, including hypersensitivity to detergents and hydrophobic dyes. More compelling, though, is the inefficient trimerization of LamB and OmpF in *surA* null strains. These *surA* null strains accumulate an unfolded LamB monomer species, suggesting that SurA specifically catalyzes the conversion of unfolded LamB monomer to a more compact state [P. Rouvière and C.A. Gross, personal communication]. I note, however, that this model has not (yet) been corroborated by direct biochemical analysis.

It is not immediately obvious why the *surA*-associated defect in outer membrane-protein assembly would confer a hypersensitivity to alkaline pH. However, I offer one speculative explanation. Alkaline conditions may simply interfere with the low level of outer membrane proteins that *are* properly assembled in the absence of SurA, thus decreasing the amount of functional outer membrane proteins to a lethal state.

Imp

The *surA* gene is found in a complex operon near the one minute region of the *E. coli* chromosome [Tormo *et al.* 1990]. *imp*, the gene immediately upstream of *surA* deserves some mention here. *imp* specifies a large outer membrane protein, and certain *imp* mutant strains display *i*ncreased outer membrane *p*ermeability [Sampson *et al.* 1989]. All attempts to create null mutations in *imp* have been met with steely resistance from *E. coli*, suggesting that this gene is essential for viability [S.A. Benson, personal communication]. Since Imp is 1) an outer membrane protein, 2) probably essential for viability, and 3) cotranscribed with *surA*, I suggest that Imp may be involved in outer membrane-protein assembly as well. However, this guilt-by-association reasoning needs the support of experimental evidence. The simplest way to test this model is to engineer a strain that can be conditionally depleted of Imp. One may then ask if depletion of Imp interferes with the assembly of any outer membrane proteins.

Other peptidyl-prolyl isomerases

Aside from SurA, there are at least two other PPIases found in *E. coli's* periplasm: RotA and FkpA. RotA was initially purified as a periplasmic cyclophilin homolog [Liu and Walsh 1990]. *rotA* null strains have been examined for defects in outer membrane-protein

targeting and assembly [Kleerebezem *et al.* 1995], with the conclusion that *rotA* mutants do not display defects in this process.

FkpA is a FKBP member [Missiakas *et al.* 1996] and is most homologous to the Mip family of FKBPs found in *L. pneumophila* [Horne and Young 1995]. Mip is an outer membrane protein required for survival of *L. pneumophila* in macrophages (*m*acrophage *i*nfectivity *p*otentiator) [Fischer *et al.* 1992]. Null mutations in *fkpA* confer no known growth defects. However, Missiakas *et al.* [1996] have recently reported that overproduction of FkpA can alleviate certain defects in the assembly of outer membrane proteins.

Perusing the *E. coli* sequence database, I have been struck by the large number of putative PPIases found throughout the genome (at least seven putative PPIases have been sequenced in *E. coli* alone). Three PPIases reside in the bacterial envelope (SurA, RotA and FkpA), and sequencing efforts suggest that others will ultimately be found in this subcellular location as well. Why are there so many PPIases in *E. coli*?

Perhaps peptidyl-prolyl *cis/trans* isomerization is such an important aspect of protein folding that it must be regulated by a battalion of catalysts. Alternatively, each PPIase may be required for folding a specific subset of proteins within the cell. If so, it is possible that the true substrates of RotA, for example, have been overlooked. A third possibility posits that the isomerase activity found in the PPIases is misleading. Each PPIase member may not necessarily be involved in protein folding. Rather, some members may use their peptidyl-prolyl isomerization domains simply to bind polypeptide substrates and then elicit some other (non-folding) alteration on their target.

Skp

Using affinity chromatography, Chen and Henning [1996] purified a periplasmic protein, Skp, that selectively bound to denatured OmpF protein. On the assumption that Skp might serve as a chaperone for outer membrane-protein assembly, the authors created a *skp* null strain. Chen and Henning [1996] claim that *skp* null strains contain lower concentrations of outer membrane proteins such as OmpF and OmpC. However, the reduction in outer membrane-

protein levels is weak and may be attributable to the lack of an internal loading standard for proper comparison of outer membrane-protein levels between strains [see Figure 6 of Chen and Henning 1996]. Further analysis of the *skp* null mutant is needed. If the *skp* null strain is truly defective in outer membrane-protein targeting and/or assembly, we must identify the precise step(s) at which the defects arise. Also, a biochemical function for Skp has not been described. Does Skp merely usher outer membrane proteins through the periplasm or does it also assist in their folding?

We now know of some factors that facilitate the targeting, folding and assembly of periplasmic and outer membrane proteins. For example, LPS and SurA are required for efficient porin trimerization, and DsbA is needed for the folding of various envelope proteins. However, it seems likely that other factors involved in the targeting, folding and assembly of periplasmic and outer membrane proteins are still at large. There are three main reasons for suggesting this. First, many steps have been identified in the process of porin trimerization. If SurA catalyzes the formation of one of these steps (unfolded monomer to folded monomer), it seems reasonable to assume that other factors are required to catalyze the remaining reactions. Second, The Dsb proteins catalyze one specific aspect of protein folding - namely, disulfide-bond formation. Clearly, there are other aspects to protein folding that might also require catalysis. Third, none of the proteins described above is essential for the viability of *E. coli*. Does this mean that periplasmic and outer membrane proteins are unnecessary for the survival of *E. coli*? It seems more reasonable to assume that the processes of periplasmic and outer membrane protein targeting, folding and assembly are catalyzed by a redundant array of proteins. Indeed, this view would help to explain the large number of PPIases found in the bacterial envelope.

Periplasmic and outer membrane proteases

Just as molecular chaperones are needed to breathe form and function into newly synthesized proteins, proteases are marshaled to action when proteins are no longer needed by the cell. Proteases are also utilized when proteins have been irreparably damaged in the line

of duty. The bacterial envelope, which houses at least eight proteases, is no stranger to the phenomenon of proteolysis.

The Undertaker of the Periplasm - DegP

From the standpoint of viability, *degP* encodes the most important periplasmic protease yet identified in *E. coli*. DegP (a.k.a. HtrA) is a periplasmic serine endoprotease that is required for the viability of *E. coli* at temperatures above 40°C (*h*igh *t*emperature *r*equirement) [Strauch and Beckwith 1988, Lipinska *et al.* 1988, 1989, 1990, Strauch *et al.* 1989].

Why is DegP essential for high-temperature growth of *E. coli*? There are several studies that imply that DegP's primary function is to degrade abnormal or misassembled envelope proteins [Strauch and Beckwith 1988, Bakker *et al.* 1991, Misra *et al.* 1991, Reed and Cronan 1991, Zdanovsky *et al.* 1992, Meerman and Georgiou 1994, Lazar and Kolter 1995]. One can imagine that at high temperatures, protein folding is somewhat compromised. Under such circumstances, proteases such as DegP may be required to destroy misfolded proteins before they can damage their surroundings.

Consistent with this hypothesis, Strauch and coworkers [1988, 1989] noted that the growth defect of *degP* null strains was alleviated by an *lpp* (specifies Braun's lipoprotein) null mutation. *lpp*-minus strains release their periplasmic contents into the surrounding medium because of a weakened outer membrane. It is believed that with fewer resident periplasmic proteins, DegP is rendered non-essential, perhaps because there are fewer proteins that can damage the periplasm at high temperatures [Strauch *et al.* 1989].

SohA

A second genetic study sought extragenic mutations that could suppress the temperature-sensitive growth phenotype of a *degP* null strain [Baird and Georgopoulos 1990]. In this way, SohA was identified (*s*uppressor *o*f *h*trA). SohA appears to be a cytoplasmic protein with no homology to any protein presently in the sequence databases [Kiino *et al.* 1990]. How then, do *sohA* suppressor mutations function? Snyder and Silhavy [1992] demonstrated that the *sohA* suppressor mutations (a.k.a. *prlF*) hyperactivate the *cytoplasmic* protease Lon. It is through this hyperactivation that suppression of the *degP*-associated growth defect is

mediated. Presently, we do not understand how activation of a cytoplasmic protease can relieve the requirement for a periplasmic protease. However, Snyder and Silhavy [1992] note that *sohA* suppressor strains increase the degradation of certain exported precursor proteins. It is therefore conceivable that the *sohA* mutations suppress the growth defect of *degP*⁻ strains simply by lowering the flux of proteins to the bacterial envelope.

SohB

A third genetic study identified *sohB* as a multicopy suppressor of the growth defect conferred by *degP* null mutations [Baird *et al.* 1991]. Although the SohB protein has not been well-characterized, it is homologous to protease IV, an inner-membrane protein that digests cleaved signal peptides in *E. coli*.

From these suppressor analyses, we are led to the mundane conclusion that DegP is essential for high-temperature growth of *E. coli* because of its proteolytic activity. However, there are some speculative challenges to this "protease-centric" view of DegP function. In our laboratory, Christine Cosma has engineered a DegP protein that lacks a serine residue required for proteolytic activity. Surprisingly, high-level expression of this protein stabilizes the mutant maltoporin, LamBA23D. Also, this protease-deficient form of DegP partially complements the temperature-sensitive phenotype associated with the *degP* null mutation [Christine Cosma, personal communication]. These results have been interpreted to indicate a non-proteolytic, chaperoning function for DegP. In support of this hypothesis, a naturally-occurring DegP homolog in *Rickettsia* lacks all three residues of the putative catalytic triad for serine-mediated proteolysis [Bass *et al.* 1996].

This dual protease/chaperone function has previously been described for other proteases. For example, there is evidence that the ClpA protease regulatory subunit (as well as other Clp homologs) possesses protein folding abilities [Squires and Squires, 1992, Wickner *et al.* 1992, Moczko *et al.* 1995, Schmitt *et al.* 1995]. Such a dual function is an economical approach to protein folding and turnover: if the chaperone cannot fold a protein, it simply

destroys it. Indeed, DNA polymerase possesses both polymerase and exonuclease activities for similar purposes [Deutscher and Kornberg 1969].

However, before I bestow chaperoning powers upon DegP, I must note that the concept of DegP as a molecular chaperone is still in its infancy. More direct analysis is needed. Specifically, DegP must be shown to fold a natural substrate before we can conclude that this protease doubles as a chaperone.

DegP homologs within *E. coli*

Recently, two DegP homologs, DegQ and DegS, were found in *E. coli* by two different approaches: 1) They were identified as minor contaminants of the purified DegP protein, and 2) They were cloned as multicopy suppressors of a growth defect conferred by a strain deficient in another periplasmic protease, Tsp (see below) [Bass *et al.* 1996, Waller and Sauer 1996]. Although null mutations in *degQ* cause no known growth defect, multiple copies of *degQ* suppress the temperature-sensitive phenotype of *degP* null strains. In contrast, *degS* null strains display a slow-growth phenotype, but the *degS* locus is not a multicopy suppressor of a *degP* null [Waller and Sauer 1996].

DegP homologs in other species

DegP homologs have also been found in a variety of other prokaryotic species, including several pathogens [Johnson *et al.* 1991, Clayton *et al.* 1993, Baumler *et al.* 1994, Tatum *et al.* 1994, Boucher *et al.* 1996, Glazebrook *et al.* 1996, Martinez-Salazar *et al.* 1996]. Not surprisingly, many DegP homologs of pathogenic bacteria are required for their virulence, and it seems likely that this necessity is indirect [Johnson *et al.* 1991, Baumler *et al.* 1994, Boucher *et al.* 1996]. Specifically, since *degP* null strains grow poorly at high temperature, the growth of *degP*$^-$ pathogens may simply be compromised within the warm confines of a host.

OmpT and OmpP

OmpT is an outer membrane serine endoprotease that primarily cleaves peptide bonds between paired basic residues [Sugimura and Higashi 1988, Sugimura and Nishihara 1988]. Although the precise physiological function of OmpT is unknown, it appears that this protease is utilized to destroy abnormal envelope proteins. This view is based largely on the fact that

OmpT degrades a variety of fusion proteins found in the envelope [*e.g.*, see Baneyx and Georgiou 1990].

OmpT is also somewhat of a rogue protease. Upon cell lysis and during protein purification procedures, OmpT has been known to proteolytically cleave SecY [Akiyama and Ito 1990], T7 RNA polymerase [Grodberg *et al.* 1988], *E. coli* initiation factor 2 [Lassen *et al.* 1992] and several other proteins [Miller 1996]. Accordingly, *ompT*⁻ strains are often utilized for protein expression and purification procedures.

The steady state level of OmpT is thermoregulated in a qualitatively similar manner to DegP. Specifically, OmpT levels increase with rising temperature [Rupprecht *et al.* 1983]. However, the mechanism of this regulation has not been examined.

OmpP is a recently identified outer membrane serine endoprotease that is highly homologous to OmpT. OmpP is also thermoregulated much like OmpT, suggesting that these two proteins (and perhaps others) perform a redundant proteolytic function in the envelope [Kaufmann *et al.* 1994].

Protease III

Protease III is a non-essential, periplasmic, zinc metalloendoprotease with a conflicting biochemical history. A number of studies indicate that protease III preferentially degrades low molecular weight polypeptides (less than 7 kDa in size) [*e.g.*, see Cheng and Zipser 1979], while one study has suggested that its preferential targets are large molecular weight proteins [Baneyx and Georgiou 1991]. However, this latter study focused on only one substrate, and the effect of protease III on this single substrate was weak. Taken together, these results argue that this latter study is flawed. Thus, although the *in vivo* targets of protease III are presently unknown, they are likely to include low molecular weight polypeptides.

Tsp

Tsp (*t*ail *s*pecific *p*rotease) is a periplasmic protease that was initially purified as an activity that selectively digested polypeptides containing small uncharged residues at their carboxy-termini [Keiler and Sauer 1996]. Keiler *et al.* [1996] recently demonstrated a profound

function for Tsp. When mRNA transcripts are generated without a stop codon, a non-functional polypeptide may be synthesized. When such events occur in *E. coli*, the polypeptides are tagged at their carboxy-termini with a novel oligopeptide that signals for their degradation. The protease responsible for recognizing this oligopeptide in *exported* proteins is Tsp, suggesting that Tsp is important for degrading improperly synthesized envelope proteins.

The descriptions provided above indicate that these proteases serve important and fundamental functions within the bacterial envelope. For example, Tsp destroys abnormally synthesized proteins, while DegP's proteolytic activity is essential at high temperatures. But how essential is the overall process of protein turnover in the envelope? None of the envelope proteases presently identified is completely essential for the viability of *E. coli*. Does this mean that *E. coli* does not need to recycle its envelope proteins? This seems unlikely. Indeed, strains of *E. coli* have been created that harbor multiple null mutations in genes specifying envelope proteases, and these strains are rather sick. Specifically, with each addition of a new protease-null mutation, the growth rate of these strains is reduced [Baneyx and Georgiou 1991]. This implies that the successive removal of each new protease makes the chore of protein turnover that much more ponderous for the remaining envelope proteases. Accordingly, it seems likely that protein turnover is catalyzed by an array of proteases that serve redundant functions.

MONITORING ENVELOPE-PROTEIN PHYSIOLOGY

In the first portion of this chapter, I described various factors involved in the targeting, folding, assembly and turnover of periplasmic and outer membrane proteins. Below, I describe the signal transduction systems that *E. coli* employs to monitor the physiological state of its periplasmic and outer membrane proteins. I will ultimately show that two of these signal transduction systems control the synthesis of some of the aforementioned facilitators of extracytoplasmic protein targeting, folding, assembly and turnover.

σ^E

σ^{32}, the classical heat-shock σ factor, controls the transcription of at least 25 heat-shock-inducible loci in *E. coli* [Gross 1996]. Because of the heat-shock-inducible nature of the σ^{32} regulon, several groups have intensively scrutinized the transcriptional regulation of *rpoH*, the gene encoding σ^{32}. Surprisingly, this analysis of *rpoH* transcription has led to the identification of another σ factor that controls a second heat-shock regulon.

Erickson and coworkers [1987] were the first to identify the promoters that drive *rpoH* transcription, noting that *rpoH* possessed at least three promoters (*P1*, *P2* and *P3*). The $rpoHp_1$ and $rpoHp_2$ promoters are recognized by RNA polymerases containing the standard "housekeeping" σ factor, σ^{70}. The $rpoHp_3$ promoter is more unusual. Erickson and coworkers [1987] noted that at the lethal temperature of 50°C, while the *P1* and *P2* promoters ceased to function, the *P3* promoter continued to drive *rpoH* transcription.

RNA polymerase molecules (E) containing the σ^E subunit ($E\sigma^E$) were subsequently purified as the activity responsible for recognizing the $rpoHp_3$ promoter [Erickson and Gross 1989]. After purifying $E\sigma^E$, Erickson and Gross made a profound observation: the promoter recognized by $E\sigma^E$ was remarkably similar to three other promoters found in *Streptomyces coelicolor* and *S. liquefaciens*. These *Streptomyces* promoters direct the transcription of genes encoding periplasmic hydrolases [Buttner *et al.* 1988, Givskov *et al.* 1988]. This observation is profound because the *degP* locus is also transcribed by $E\sigma^E$ [Lipinska *et al.* 1988, Erickson and Gross 1989]. In other words, this group of promoters directs the synthesis of a collection of periplasmic hydrolases, intimating a connection between $E\sigma^E$ and the extracytoplasmic milieu.

rpoE, the gene encoding σ^E, has since been cloned, and *rpoE* null strains have been created [Hiratsu *et al.* 1995, Raina *et al.* 1995, Rouvière *et al.* 1995]. Since *degP* null strains are unable to grow at temperatures above 40°C [Lipinska *et al.* 1989], it seems reasonable to predict that *rpoE* null strains would also be unable to grow at these temperatures. However, *rpoE* null

strains are even more heat-sensitive; they will not grow above 37°C, suggesting that σ^E controls the synthesis of other gene products required for the high-temperature growth of *E. coli* [Hiratsu *et al.* 1995, Raina *et al.* 1995, Rouvière *et al.* 1995]. Consistent with this view, Rouvière and coworkers [1995] have shown that overproduction of σ^E stimulates the synthesis of at least 11 other proteins.

Since σ^E and σ^{32} both control heat-shock regulons, it seems likely that the global functions of these two σ factors are similar. σ^{32} controls the synthesis of a host of cytoplasmic heat-shock proteins that are primarily involved in protein turnover (*e.g.* Lon) and protein folding (*e.g.* GroEL/ES, DnaK). By analogy, I suggest that σ^E may serve a complementary function to that performed by σ^{32}. Specifically, σ^E may control the synthesis of a collection of *extra*cytoplasmic heat-shock proteins that perform protein folding and proteolysis functions.

But what are the other genes controlled by $E\sigma^E$? Until this study, σ^E was only known to control the synthesis of three proteins: σ^{32}, DegP and σ^E itself [Raina *et al.* 1995, Rouvière *et al.* 1995]. This information alone is hardly a sterling endorsement for the hypothesis that σ^E is primarily involved in the physiology of *E. coli's* extracytoplasmic compartments. However, the contribution of σ^E to σ^{32} synthesis is negligible even under standard heat-shock conditions. It is only under extreme heat-shock (50°C) that σ^E is appreciably responsible for σ^{32} synthesis [Erickson *et al.* 1987]. Keeping this in mind, we may ultimately find that the regulation of σ^{32} synthesis by σ^E is the exception rather than the rule.

Moreover, several lines of evidence point to a role for σ^E in maintaining the physiology of periplasmic and outer membrane proteins. First, the fact that σ^E regulates *degP* transcription supports this notion. Second, *rpoE* null strains are hypersensitive to bile-salt detergents and a variety of hydrophobic dyes, suggesting that they possess outer membrane-permeability defects [Rouvière *et al.* 1995]. Furthermore, sequence analysis of σ^E indicates that

it belongs to a family of prokaryotic σ factors that are each involved in controlling extracytoplasmic functions [Lonetto *et al.* 1994].

In addition, σ^E activity is modulated by the production of outer membrane proteins. Specifically, overproduction of a wide array of outer membrane proteins stimulates σ^E activity, while decreased synthesis of outer membrane proteins concomitantly decreases σ^E activity [Mecsas *et al.* 1993]. Since σ^E controls the synthesis of DegP, one can imagine that *E. coli* would require more DegP to proteolyze any excess outer membrane proteins synthesized by the cell. Conversely, if the cell is synthesizing fewer outer membrane proteins, less DegP is needed for outer membrane protein turnover. Thus, *E. coli* possesses a signal transduction system that can modulate σ^E activity according to outer membrane protein levels (Figure 1.6).

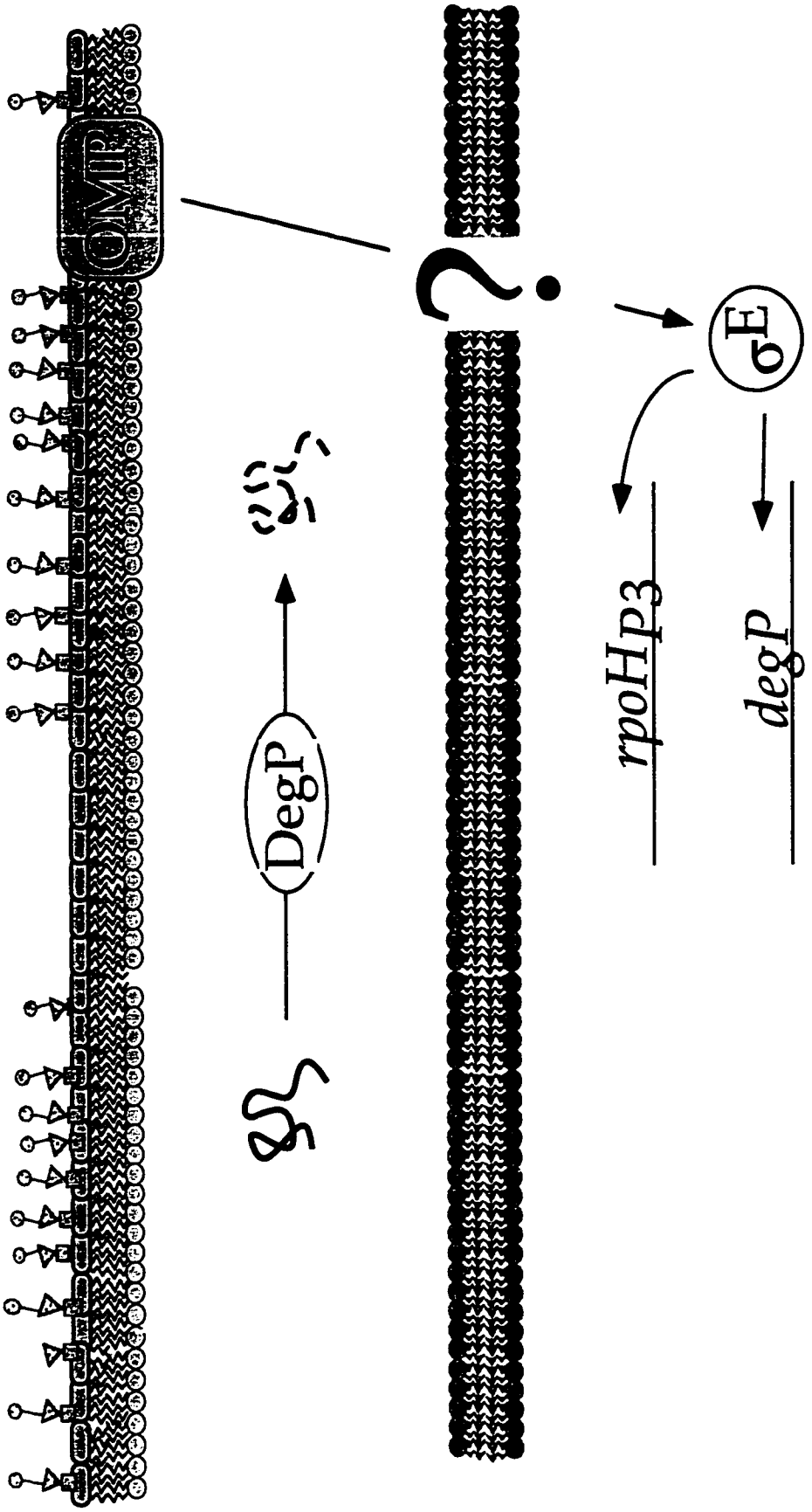
Finally, Chapter 7 of this study demonstrates that σ^E controls the synthesis of a periplasmic peptidyl-prolyl *cis/trans* isomerase, suggesting a role for σ^E in controlling protein folding within the bacterial envelope. Taken together, these results suggest that the primary function of σ^E is to maintain the integrity of the bacterial periplasm and outer membrane. The identification and characterization of the remainder of the σ^E regulon will ultimately verify or refute this hypothesis.

The Cpx Proteins

The *cpxA* locus was initially identified by mutations that impaired the ability of *E. coli* to transfer conjugative (F) plasmids to recipient strains (*c*onjugative *p*lasmid *e*xpression) [McEwen and Silverman 1980a]. These *cpxA* mutant strains fail to accumulate TraJ, a transcription factor required for the synthesis of F-pilus subunits [Silverman *et al.* 1993]. Without F-pili, there is no conduit for the transfer of F-plasmid DNA from donor to recipient cells. Thus, it was initially believed that *cpxA* was a host locus intimately involved in bacterial conjugation [McEwen and Silverman 1980a].

However, soon after these initial discoveries, several other phenotypes were also found associated with these same *cpxA* mutations. Depending upon the particular *cpxA* allele,

Figure 1.6. The σ^E signal transduction system. The figure shows that an unidentified signal transduction system (denoted as a question-mark) modulates σ^E activity in response to the expression levels of outer-membrane proteins. σ^E is used to direct transcription from the *degP* and *rpoHP3* promoters.



any or all of the following phenotypes are conferred: 1) increased resistance to the antibiotic amikacin, 2) inefficient transport of proline, lactose and other compounds across the inner membrane using ion-driven transport systems, 3) temperature-sensitive isoleucine/valine auxotrophy, 4) partial resistance to colicin A, 5) ability to utilize L-serine as a sole carbon source, 6) inability to grow on succinate and other non-fermentable carbon sources, 7) instability of inner- and outer membrane proteins, including OmpF and Lpp, 8) and lowered anaerobic growth rate on glucose [McEwen and Silverman 1980b, 1982, Sutton *et al.* 1982, McEwen *et al.* 1983, Rainwater and Silverman 1990].

These observations certainly obscured the precise function of the CpxA protein. How can *cpxA* mutations affect so many diverse processes? The first hint of an answer to this question came from the sequence of CpxA. CpxA was found to be an inner-membrane protein homologous to the two-component sensor family of histidine kinases [Albin *et al.* 1986, Weber and Silverman 1988]. These two-component sensors are typically situated in the inner membrane where they sense an external parameter (*e.g.*, osmolarity, nutrient concentrations). The sensors then communicate this information to a cognate response regulator in order to elicit an appropriate response to the stimulus [Stock *et al.* 1990]. Thus, by homology, it appeared that CpxA served a sensory and regulatory function.

But how can a regulatory protein affect all of the processes described above? The second hint of an answer came from other genetic studies performed on *cpxA* mutant strains. First, it was noted that the *cpxA* alleles that conferred the array of phenotypes described above, were codominant to a wild-type *cpxA* allele, suggesting that the *cpxA* mutations were gain of function alleles [McEwen and Silverman 1980a, Cosma *et al.* 1995]. In support of this view, a large deletion, devouring *cpxA* and several other adjacent genes, does not affect any of the processes altered by the gain of function *cpxA* alleles [Rainwater and Silverman 1990, Dong *et al.* 1993]. Assuming that the other genes removed in this deletion do not affect the processes in question, we are led to the conclusion that only the gain of function *cpxA* alleles alter the

processes described above (I grant that this is a generous assumption, but Chapter 3 will verify this view).

From these results, it seems likely that the gain of function *cpxA* alleles are altering processes in which they are not normally involved. This could explain how the *cpxA* mutations affect so many diverse processes. Indeed, this is not unprecedented behavior for a two-component sensor. The *E. coli* osmosensor, EnvZ, can be altered by mutation to hyperactivate its cognate response regulator, OmpR. This hyperactivation of OmpR ultimately alters transcription of EnvZ's normal downstream targets, *ompF* and *ompC*, as well as other loci [Slauch *et al.* 1988]. Interestingly, CpxR, the putative cognate response-regulator of CpxA, is most homologous to the OmpR transcription factor [Dong *et al.* 1993].

Although CpxA is clearly an inner-membrane sensor, the initial genetic studies have never provided an example of a stimulus that affected the wild-type CpxA protein (only the hyperactivated *cpxA* alleles have been examined). In Chapters 3, 6 and 7, I provide evidence that wild-type CpxA monitors the levels of certain envelope proteins. In response to these protein levels, CpxA regulates the transcription of *degP* and *dsbA*, two genes whose products are involved in protein turnover and protein folding within the envelope.

The Phage-Shock Response - PspA

PspA is a periplasmic protein whose synthesis is massively induced under various stressful conditions, including: 1) infection by filamentous phages (*p*hage-*s*hock *p*rotein), 2) heat, 3) ethanol treatment, 4) osmotic shock and 5) high-level expression of the mutant maltoporin, LamBA23D [Brissette *et al.* 1990, Carlson and Silhavy 1993]. Despite the information indicating that PspA is stress-inducible, its function remains elusive. Tommassen's group suggests that PspA is involved in maintaining the proton-motive force across the inner membrane [Kleerebezem *et al.* 1996]. However, the Model laboratory notes that *pspA* null strains are hypersensitive to alkaline pH in stationary phase cells [Weiner and Model 1994]. Thus, we must wait for other studies to clarify the function of PspA.

Oddly, transcription of the *pspA* gene is not initiated by one of the more standard stress-responsive σ factors. Instead, its transcription is directed by σ^{54}, which is normally consigned to regulating transcription of genes whose products are involved in nitrogen metabolism [Weiner *et al.* 1991]. It is presently unclear whether σ^{54} directly induces PspA synthesis in response to the extracytoplasmic stresses described above, or whether this σ factor works in concert with another, as yet unidentified, factor.

Extractyoplasmic events that alter periplasmic and outer membrane protein synthesis

There are two main groups of studies indicating that *E. coli* alters the synthesis of its periplasmic and outer membrane proteins in response to the physiological state of these proteins.

The first group of studies was performed by Hengge-Aronis and Boos in the mid-1980's. In the process of examining a carboxy-terminal truncated form of the periplasmic protein, GlpQ, these authors noted that this protein remained associated with the inner-membrane in a protease-sensitive state. Moreover, expression of this protein inhibited the synthesis of other periplasmic proteins at the translational level, indicating that *E. coli* can monitor defects in the targeting and/or assembly of its periplasmic proteins. Interestingly, the synthesis of outer membrane proteins is unaffected by the mutated GlpQ protein [Hengge and Boos 1985, Hengge-Aronis and Boos 1986].

Carl Schnaitman's laboratory has observed a similar phenomenon that occurs with outer membrane proteins. For example, the *ompCtd* mutation, which impairs the assembly of OmpC, decreases the synthesis of other outer membrane proteins at the translational level. This inhibitory phenomenon does not affect periplasmic proteins, and it can also be observed simply by overproducing OmpC [Catron and Schnaitman 1987, Click *et al.* 1988, Click and Schnaitman 1989].

Taken as a whole, these results indicate that *E. coli* has fine-tuned its sensory capabilities to the point where it can distinguish between defects in periplasmic protein targeting and outer membrane protein targeting.

CONCLUSIONS

At this inception of this study, the evidence indicating that the physiology of periplasmic and outer membrane proteins was monitored by cytoplasmic factors was scant. The gene encoding σ^E had not been cloned, and the primary function of this σ factor was difficult to decipher because only two of its regulatory targets (*degP* and *rpoH*) were known. Additionally, the study by Mecsas *et al.* [1993], which indicated that σ^E activity was affected by the synthesis of outer membrane proteins, was not yet published.

The study described in this dissertation initially utilized a *degP-lacZ* operon fusion as a reporter for *degP* transcription. There were three main reasons for analyzing *degP* transcription. First, I thought it was likely that σ^E activity would be attuned to the physiology of envelope proteins. This opinion was based largely on that fact that σ^E was heat-shock inducible, and that it controlled the synthesis of the DegP protease. Since σ^E had not been cloned, I needed an indirect reporter (*degP*) for its activity. Second, If *E. coli* employed signal transduction systems that monitored the state of its envelope proteins, it seemed reasonable to assume that proteases such as DegP would fall under the control of these systems. Accordingly, *degP* transcription provided an opportunity to identify and analyze these putative signal transduction systems. Third, I reasoned that defects in the targeting, folding and assembly of envelope proteins would most likely require increased synthesis of proteases like DegP. Thus, *degP* transcription was also a potential gateway to identifying factors involved the biogenesis of envelope proteins.

In general, my goal was to identify and analyze mutations that altered the transcription of *degP-lacZ* with the hope of addressing the three issues described above. These analyses are described in Chapters Three through Seven.

Chapter Three illustrates that *degP* transcription is not only controlled by σ^E, but it is also regulated by the Cpx two-component signal transduction system. This work was performed in conjunction with Christine Cosma, William B. Snyder and Laura J.B. Davis, all present and

former members of the Silhavy Laboratory. Christine and Bill demonstrated that gain of function *cpxA* mutations could suppress various envelope protein-mediated toxicities. Bill then showed that this suppression was partially dependent on the integrity of the *degP* locus. This provided the impetus for examining the effect of the Cpx system on *degP* transcription.

Chapter Four describes the identification of *cpxP*, a second locus controlled by the Cpx system. *cpxP* encodes a periplasmic protein that, like DegP, can also help to ameliorate envelope protein-mediated toxicities. Surprisingly, *cpxP* transcription is stimulated by alkaline pH and *cpxP* null strains are hypersensitive to growth in basic media, suggesting a role for the CpxP protein in combating stress created by alkaline conditions.

Chapter Five describes a search for mutations that increase *degP-lacZ* transcription. This study was initiated by George R. Oliver, a senior undergraduate in the Silhavy laboratory. The mutations described in this chapter interfere with the synthesis of an outer membrane glycolipid, the Enterobacterial Common Antigen (ECA). These mutations perturb the physiology of the outer membrane, and they stimulate *degP* transcription by activating both σ^E and the Cpx signal transduction system.

Chapter Six stems from initial studies performed by C. Hal Jones, a postdoctoral fellow in Scott Hultgren's laboratory at Washington University (Saint Louis). This chapter demonstrates that the Cpx and σ^E pathways can be simultaneously stimulated by overproduction of the pathogenic pilus subunit, PapG.

Taken together, Chapters Five and Six imply that even though σ^E and Cpx function in parallel to regulate *degP* transcription, these signal transduction systems *can* respond to the same extracytoplasmic perturbations.

Chapter Seven further supports the notion that σ^E and Cpx are primarily involved in controlling protein folding events within the bacterial envelope. Specifically, this chapter shows that: 1) σ^E controls the synthesis of FkpA, a periplasmic peptidyl-prolyl *cis/trans* isomerase, and 2) Cpx controls the synthesis of DsbA, a periplasmic enzyme that catalyzes disulfide-bond formation in various envelope proteins.

Chapter Eight provides a review and discussion of the previous seven chapters.

Two. Materials & Methods

MEDIA, REAGENTS AND ENZYMES

Media were prepared as described by Silhavy *et al.* [1984]. Liquid cultures were grown either in Luria broth or M63 minimal media supplemented with thiamine (50μg/ml) and 0.4% of the indicated carbon source. Unless specifically noted, the final concentration of antibiotics used in the growth media was as follows: ampicillin, 50μg/ml; kanamycin, 50μg/ml; tetracycline, 20μg/ml; spectinomycin, 50μg/ml; chloramphenicol, 20μg/ml. Standard microbiological techniques were used for strain construction and bacterial growth [Silhavy *et al.* 1984]. 5-bromo, 4-chloro, 3-indolyl-D-galactoside (X-Gal) was purchased from Fischer.

STRAINS AND PHAGE

All strains are described in Table 2.1. Strains described in Figure 3.7 contain the *degP-lacZ* fusion in strain MC1061. The use of the MC1061 background for experiments described in Figure 3.7 was necessitated because of the instability of the *rpoE::cam* mutation in the MC4100 background.

λRS88, λRS45, λNK1324 and λp*lac*Mu53 have been described elsewhere [Bremer *et al.* 1985, Simons *et al.* 1987, Kleckner *et al.* 1991]. Lysogenization of λRS88[*degP-lacZ*] was performed as described by Simons *et al.* [1987]. All λRS45 and λRS88 operon fusions were shown to be located in single copy at the *λatt* locus by P1 transduction.

MISCELLANEOUS PLASMID CONSTRUCTION AND PRIMER INFORMATION

All plasmids are described in Table 2.2.

All primer sequences are provided in Table 2.3.

pND12

pND12, which contains the *rpoE* locus subcloned into the pBR322 vector, was constructed as follows: *rpoE* was amplified from the chromosome of MC4100 by PCR using the Rpoe3 and Rpoe5 primers. The amplified DNA was then subcloned into the *Bam*HI and *Sal*I sites of pBR322, creating pND12. pND12 places *rpoE* under its own transcriptional control.

Table 2.1 Strains Described in This Study

Name	*Genotype*[a]	*Reference*
CLC198	MC4100, *degP*::Tn*10*	Christine Cosma
MC1061	F$^-$, *araD139, Δ(ara-leu)7696, galE15, galK16, Δ(lac)X74, rpsL* (Str^R), *hsdR2*, (r$_K^-$ m$_K^+$), *mcrA, mcrB1*	Silhavy *et al.* 1984
MC4100	F$^-$, *araD139, Δ(argF-lac)U169, rpsL150* (Str^R), *relA1, flbB5301, deoC1, ptsF25, rbsR*	Silhavy *et al.* 1984
PND2000	MC4100, λRS88[*degP-lacZ*]	This study
PND242	PND2000, *cpxA::cam*	This study
PND257	PND2000, *ompR*::Tn*10*	This study
PND325	PND2000, *cpxR::Ω*	This study
PND381	MC4100, λRS45[*rpoHp3-lacZ*]	This study
PND393	PND381, *cpxR::Ω*	This study
PND394	PND381, *cpxA::cam*	This study
PND421	PND2000, *cpxA::cam, zej*::Tn*10*, Δ[*pta, ackA, hisQ,hisP*]	This study
PND422	PND2000, *cpxA::cam, zej*::Tn*10*	This study
PND423	PND2000, *ompR101*	This study
PND495	PND2000, *zej*::Tn*10*	This study
PND496	PND2000, *cpxR::Ω, zej*::Tn*10*	This study
PND497	PND2000, *ara*$^+$, *zej*::Tn*10*, Δ[*pta, ackA, hisQ, hisP*]	This study
PND498	PND497, *cpxA::cam*	This study
PND499	PND497, *cpxR::Ω*	This study
PND500	PND381, *ara*$^+$	This study
PND541[b]	MC4100, *ara*$^+$, *nadA*::Tn*10 Δ(gal-att-bio)*	This study
PND788	PND257, *rffT::cam*	This study
PND789	PND257, *rffH::cam*	This study
PND790	PND257, *rffA::cam*	This study
PND818	MC1061, *rpoE::cam*, λRS88[*degP-lacZ*]	This study
PND819	MC1061, λRS88[*degP-lacZ*]	This study
PND900	MC4100, *ara*$^+$	This study
PND2000	MC4100, λRS88[*degP-lacZ*]	This study
SP1	MC4100, *ara*$^+$, λp*lac*Mu53[*cpxP-lacZ*]	This study
SP7	SP1, *cpxA::cam*	This study

SP9	WBS164, *degP*::Tn*10*	This study
SP10	WBS164, λp*lac*Mu53[*cpxP-lacZ*]	This study
SP24	WBS164, *degP*::Tn*10*, λp*lac*Mu53[*cpxP-lacZ*]	This study
SP34	MC4100, *ara*$^+$, *zej*::Tn*10*, λp*lac*Mu53[*cpxP-lacZ*]	This study
SP35	SP34, Δ[*pta, ackA, hisQ, hisP*]	This study
SP36	SP34, *cpxA::cam*	This study
SP37	SP34, *cpxA::cam*, Δ[*pta, ackA, hisQ, hisP*]	This study
SP150	PND257, *cpxR::Ω*	This study
SP171	PND423, *rffT::cam*	This study
SP172	PND423, *rffH::cam*	This study
SP173	PND423, *rffA::cam*	This study
SP179	PND423, *rffT::cam*, *rfe*::Tn*10*	This study
SP182	PND423, *rffT::cam*, *rff*C::Tn*10*	This study
SP185	PND423, *rffH::cam*, *rfe*::Tn*10*	This study
SP188	PND423, *rffH::cam*, *rffA*::Tn*10*	This study
SP190	PND423, *rffA::cam*, *rfe*::Tn*10*	This study
SP193	PND423, *rffA::cam*, *rff*C::Tn*10*	This study
SP231	SP150, *rffT::cam*	This study
SP232	SP150, *rffH::cam*	This study
SP233	SP150, *rffA::cam*	This study
SP245	MC4100, *ompR*::Tn*10*, λRS45[*rpoHp3-lacZ*]	This study
SP282	SP245, *rffT::cam*	This study
SP283	SP245, *rffH::cam*	This study
SP284	SP245, *rffA::cam*	This study
SP298	MC4100, *ompR101*, *zja*::Tn*10*, *lamBΔ60*, λRS88[*degP-lacZ*]	This study
SP299	MC4100, *ompR101*, *zja*::Tn*10*, *lamB*$^+$, λRS88[*degP-lacZ*]	This study
SP300	SP298, *rffT::cam*	This study
SP301	SP299, *rffT::cam*	This study
SP302	SP298, *rffH::cam*	This study
SP303	SP299, *rffH::cam*	This study
SP304	SP298, *rffA::cam*	This study
SP305	SP299, *rffA::cam*	This study
SP332	PND2000, *rffT::cam*	This study
SP333	PND2000, *rffH::cam*	This study
SP334	PND2000, *rffA::cam*	This study

SP558	PND2000, *cpxA::cam*	This study
SP559	PND2000, *cpxR::Ω*	This study
SP569	MC4100, *ara*$^+$, *ilv*::Tn*10*, *ΔuncBC*, λp*lac*Mu53[*cpxP-lacZ*]	This study
SP594	MC4100, λRS88[*cpxP-lacZ*]	This study
SP616	MC4100, λRS45[*rpoH*$_{P3}$-*lacZ*]	This study
SP619	SP594, *cpxA::cam*	This study
SP620	SP594, *cpxR::Ω*	This study
SP627a	MC4100, *ara74::cam*[c]	This study
SP702	MC4100, *ara*$^+$, *zej*::Tn*10*, Δ[*pta*, *ackA*, *hisQ*, *hisP*],λRS88[*cpxP-lacZ*]	This study
SP704	SP702, *cpxA::cam*	This study
SP706	SP702, *cpxR::Ω*	This study
SP766	MC4100, *cpxR::Ω*	This study
SP779	MC4100, λRS88[*degP-lacZ*], *ara74::cam*[c] *zab*::Tn*10*	This study
SP781	SP779, *cpxR::Ω*	This study
SP887	MC4100, λRS88[*fkpA-lacZ*]	This study
SP921	SP887, *surA::kan*	This study
SP940	SP887, *degP*::Tn*10*	This study
SP942	SP921, *degP*::Tn*10*	This study
SP969	MC4100, λRS88[p*orfA-dsbA-lacZ*]	This study
SP971	SP969, *cpxA24*	This study
SP994	SP969, *ara74::cam*[c] *zab::*Tn*10*	This study
SP995	SP994, *cpxA::cam*	This study
SP996	SP994, *cpxR::Ω*	This study
WBS164	MC4100, Φ(*lamB-lacZX90*) Hyb10-1[λp1(209])	Snyder and Silhavy, 1995

[a]Abbreviations: *cam*, chloramphenicol resistance; *kan*, kanamycin resistance; *Ω*, spectinomycin/streptomycin resistance; Tn*10*, tetracycline resistance.

[b]SP541 harbors plasmid pND18 which expresses the *nlpE* gene under the control of the arabinose promoter, *pBAD* (see Table 2.2 for details).

[c]The *ara74::cam* allele confers arabinose-resistance upon MC4100, as well as arabinose auxotrophy.

Table 2.2 Plasmids Used in This Study

Name	*Parent vector*	*Comments*	*Drug resistance*[a]	*Reference*
pBAD18	----------	Used for expressing genes with the arabinose-inducible *pBAD* promoter.	Amp	Guzman *et al.* 1995
pBluescript II	----------	Cloning vector. Control vector for pJKI10.	Amp	Stratagene
pBR322	----------	Cloning vector.	Amp, Tet	Bolivar 1978
pCG242[b]	pSB118	Overexpresses *osmB*.	Amp	Jung *et al.* 1989
pCH215	pBAD18	Synthesizes SecY-AP fusion. AP moiety is fused to cytoplasmic loop 5 of SecY.	Amp	Chris Harris
pFY107[b]	pUC9	Overexpresses *nlpA*.	Amp	Yu *et al.* 1986
pGP1-2	----------	Generates T7 RNA polymerase for expression of *nlpD*.	Kan	Ichikawa *et al.* 1994
pHJ8	pMMB66	Overexpresses *papG* from an IPTG-inducible promoter.	Amp	C. Hal Jones
pJE100	pBR322	Overexpresses *ompX*.	Amp	Mecsas *et al.* 1993
pJKI10[b]	pBluescriptII	Overexpresses *nlpD*.	Amp	Ichikawa *et al.* 1994
pKEN125[b]	pBR322	Overexpresses *lpp*.	Amp	Nakamura *et al.* 1982
pLD404[b]	pBR322	Overexpresses *nlpE*.	Amp	Snyder *et al.* 1995
pLG338	----------	Cloning vector.	Kan	Stoker *et al.* 1982
pMAK705	----------	Temperature-sensitive replicon for integration of DNA into the *E. coli* chromosome.	Cam	Hamilton *et al.* 1989
pMMB66	----------	Control vector for pHJ8.	Amp	C. Hal Jones
pND8	pTSA29	Contains *cpxA* open reading frame and surrounding sequence. Used to create *cpxA* null mutation.	Amp	This study
pND9	pND8	Contains chloramphenicol resistance cassette cloned into *Eco*RI site of *cpxA* open reading frame. Used to create *cpxA* null mutation.	Amp, Cam	This study
pND10	pBR322	Contains *cpxR* and surrounding sequence cloned into *Bam*HI site of pBR322.	Amp	This study
pND12	pBR322	Overexpresses *rpoE*.	Amp	This study
pND16	pMAK705	Contains *cpxR* and upstream sequences cloned into *Bam*HI site of pMAK705. Used to create *cpxR* null mutation.	Cam	This study
pND17	pND16	Contains the Ω fragment inserted within the *Xho*I site located at the 21st codon of the *cpxR* open reading frame. Used to create *cpxR* null mutation.	Cam, Spec	This study

pND18	pBAD18	Expresses *nlpE* under the control of the *pBAD* promoter.	Amp	This study
pND22	pRS415	*cpxP-lacZ* operon fusion vector.	Amp	This study
pND23	pBR322	Contains 5′ end of *cpxP* open reading frame. Used to create CpxP-AP fusion.	Amp	This study
pND24	pND23	Produces CpxP-AP protein fusion.	Amp	This study
pND26	pMAK705	Contains 3′ terminal third of *cpxP* open reading frame. Used to create *cpxP* deletion/insertion allele.	Cam	This study
pND27	pND26	Fuses 5′ terminal third of *cpxP* open reading frame with 3′ terminal third of *cpxP* open reading frame. Used to create *cpxP* deletion/insertion allele.	Cam	This study
pND28	pRS415	*fkpA-lacZ* fusion vector.	Amp	This study
pND30	pND27	Inserts kanamycin resistance cassette into *Bgl*II site of pND27. Used to create *cpxP* deletion/insertion allele.	Cam, Kan	This study
pND31	pRS415	p*orfA-dsbA* operon fusion vector.	Amp	This study
pOW3[b]	pBR322	Overexpresses *traT*.	Amp	Ogata *et al.* 1982
pPHO7	----------	Contains alkaline phosphatase open reading frame deleted for the signal sequence coding region.	Amp	Gutierrez and Devedjian 1989
pPR272	pLG338	Overexpresses *ompF*.	Kan	Misra and Reeves 1987
pRAM1005	----------	Parent vector of pRAM1006.	Amp	Misra and Benson 1988
pRAM1006	pRAM1005	Overexpresses *ompC*.	Amp	Misra and Benson 1988
pRC2[b]	pLG338	Overexpresses *pal*.	Kan	Chen and Henning 1987
pRS415	----------	*lacZ* operon fusion vector.	Amp	Simons *et al.* 1987
pSB118	----------	Control vector for pCG242	Amp	Jung *et al.* 1989
pSINATRA131	pRS415	*degP-lacZ* operon fusion vector.	Amp	This study
pTSA29	----------	Temperature-sensitive replicon for integration of DNA into the *E. coli* chromosome.	Amp	G. Phillips
pUC4K	----------	Cloning vector. Contains kanamycin resistance cassette.	Kan	Pharmacia
pUC9	----------	Control vector for pFY107.	Amp	Pharmacia

[a]Abbreviations: Amp, ampicillin; Cam, chloramphenicol; Kan, kanamycin; Spec, spectinomycin; Tet, tetracycline

[b]The expression levels of the various lipoproteins are at least 10-fold greater than with control plasmids, spanning a range that exceeds (Lpp), is comparable to (NlpA, NlpD), or is less than (TraT, Pal, OsmB) the level of NlpE when it is overproduced [Nakamura *et al.* 1982, Ogata *et al.* 1982, Yu *et al.* 1986, Chen and Henning 1987, Jung *et al.* 1989, Ichikawa *et al.* 1994].

Table 2.3 Primers Used in This Study

Name	*Sequence*[a]	*Comments*	*Restriction sites*
CAM-5′[b]	CTGACGGGGTGGTGCGTAACGGC	Used to amplify DNA flanking the Tn*cam* transposon.	
CAM-5′[b]	CATTTATTCTGCCTCCCAGAGCC	Used to amplify DNA flanking the Tn*cam* transposon.	
Cpx2828	CTGGTAAGCTTTGATGGTTTCG	Used to create pND23 and pND27 (CpxP-AP, *cpxP::kan*).	*Hind*III
Cpx37	TGTCACGGTACCCCCGACGCCTGG	Used to create pND26 (*cpxP::kan*).	*Kpn*I
Cpxa3	CCGGAGTGTAGGCCTGATAAG	Used to create pND8 (*cpxA::cam*).	*Stu*I
Cpxkpn	GTTAACCGGTACCGAGTTTACCCTGC	Used to create pND8 (*cpxA::cam*).	*Kpn*I
Cpxr3	CGTTGGGCGGATCCTTCGCCAGCTCCG	Used to create pND10 (*cpxR* in pBR322).	*Bam*HI
Cpxr5	GATATCCACCAGCGGATCCACCAGCGCG	Used to create pND10 (*cpxR* in pBR322).	*Bam*HI
Dsba3	CAGCGCCAGCGAATTCTTTTTCATG	Used to create pND31 (p*orfA-dsbA-lacZ*).	*Eco*RI
Dsba5	CGTCGTCATCGAATTCACCGATATCG	Used to create pND31 (p*orfA-dsbA-lacZ*).	*Eco*RI
Finko	TGAAGGATCCCGAGAAAGATCTACAACGAATG	Used to create pND26 (*cpxP::kan*).	*Bam*HI, *Bgl*II
Finlac	CTCAAGGCCGAGAATTCGATCAAG	Used to create pND22 (*cpxP-lacZ*).	*Eco*RI
Finpho	CATTAACAGGATCCTGTTCGTGCC	Used to create pND22, pND23 and pND27 (*cpxP-lacZ*, CpxP-AP, *cpxP::kan*).	*Bam*HI
Fkpalac3	CAAAAGTGATTGGTGGATCCAGGGCAAC	Used to create pND28 (*fkpA-lacZ*).	*Bam*HI
Fkpa5	CTTCAATGGTGAATTCCTGAAAAG	Used to create pND28 (*fkpA-lacZ*).	*Eco*RI
Htra5	GCGTGGGATGAATTCCGACGTCTGATGG	Used to create pSINATRA131 (*degP-lacZ*).	*Eco*RI
Htramid	CCATGTTACCCGGGATAGCAAAACCG	Used to create pSINATRA131 (*degP-lacZ*).	*Sma*I
Nlpe3	GATGCGGCGTAAAAGCTTTATCCGGCC	Used to create pND18 (*nlpE* in pBAD18).	*Hind*III
Nlpe5	TCAAGCGTGAAGTCGACGCGCGGCAAAGTG	Used to create pND18 (*nlpE* in pBAD18).	*Sal*I
Rff5880	GGCTGCGTGCCGCAGG	Used to map Tn*cam* insertions in *rff/rfe* gene cluster.	
Rff7216	GCGCCGTGGTATGCGCC	Used to map Tn*cam* insertions in *rff/rfe* gene cluster.	
Rff8605	CGCCTGCTGCGCCTGCC	Used to map Tn*cam* insertions in *rff/rfe* gene cluster.	
Rff9854	CTGGGCGCGGCGCAGGC	Used to map Tn*cam* insertions in *rff/rfe* gene cluster.	
Rff11160	CGGCTTCCCGCTGACC	Used to map Tn*cam* insertions in *rff/rfe* gene cluster.	
Rff12431	GATATGCAGCACGCCC	Used to map Tn*cam* insertions in *rff/rfe* gene cluster.	
Rpoe3	CTATCCAGCGTGTCGACATCCATTAAAGCGG	Used to create pND12 (*rpoE* in pBR322).	*Sal*I
Rpoe5	GCATGACAAACAAAAACGGATCCGTTACGGAAC	Used to create pND12 (*rpoE* in pBR322).	*Bam*HI

[a]All sequences are listed 5′ to 3′. Restriction sites that were intentionally incorporated into the primers are underlined.

[b] Source of CAM-5′ and CAM-3′ is Leslie Pratt.

pND18

pND18 was constructed as follows: *nlpE* was amplified from the chromosome of MC4100 by the polymerase chain reaction (PCR) using the Nlpe5 and Nlpe3 primers. The amplified DNA was then subcloned into the *Sal*I and *Hind*III sites of pBAD18, creating pND18. pND18 places *nlpE* under the transcriptional control of the *araB* promoter [Guzman *et al.* 1995].

Construction of the plasmid producing the CpxP-AP fusion protein

The 5' region of the *cpxP* open reading frame, along with 1031 nucleotides of upstream, noncoding sequence was amplified by PCR using the Cpx2828 and Finpho primers. The amplified DNA was digested with *Hin*dIII and *Bam*HI and was subcloned into the corresponding restriction sites of pBR322, generating pND23. pND23 contains the first 71 codons of the *cpxP* open reading frame. A 2.6 kilobase *Bam*HI fragment containing the alkaline phosphatase open reading frame (without the signal-sequence coding region) was removed from plasmid pPHO7 [Gutierrez and Devedjian 1989] and subcloned into the *Bam*HI site of pND23. The proper insertion and orientation of this *phoA* coding sequence was confirmed by restriction analysis. In this way, pND24 was generated. pND24 fuses the first 71 codons of the *cpxP* open reading frame with the coding sequence for the mature portion of alkaline phosphatase.

CONSTRUCTION OF CHROMOSOMAL NULL MUTATIONS

cpxA::cam

The chromosomal *cpxA* locus was amplified by PCR using the Cpxkpn and Cpxa3 primers. The amplified *cpxA* DNA was subcloned into the *Kpn*I and *Sma*I sites of pTSA29 (Phillips and Probst, manuscript in preparation), creating pND8. The pTSA29 vector is temperature-sensitive for replication. Strains grown in media selecting for this plasmid at the restrictive temperature (42°C) must integrate the plasmid into the host chromosome to maintain viability. This temperature-sensitive replication provides a means for replacing chromosomal DNA with homologous plasmid-encoded DNA [Hamilton *et al.* 1989].

pND8 contains a unique *Eco*RI restriction site within the open reading frame of *cpxA*. This *Eco*RI site lies upstream of the codon for the conserved histidine residue of two-component sensors [Weber and Silverman 1988, Stock *et al.* 1990]. The chloramphenicol antibiotic resistance cassette described by Fellay *et al.* [1987] was inserted within the *cpxA* open reading frame at the unique *Eco*RI site, creating pND9. This insertion/disruption of *cpxA* was then recombined onto the chromosome of MC4100 as described by Hamilton *et al.* [1989].

cpxR::Ω

cpxR was amplified from the MC4100 chromosome using primers Cpxr5 and Cpxr3. The amplified *cpxR* DNA was subcloned into the *Bam*HI site of pMAK705, creating pND16. pND16 contains a unique *Xho*I restriction site 21 codons downstream of the 5' end of the *cpxR* open reading frame. pND16 was digested with *Xho*I, the 3' overhangs were filled-in with Klenow fragment [Sambrook *et al.* 1989], and the resulting fragment was ligated to the spectinomycin-resistance cassette described by Fellay *et al.* [1987]. This subcloning created pND17. This *cpxR* disruption was also recombined onto the chromosome as described [Hamilton *et al.* 1989]. The chromosomal disruptions of *cpxR* and *cpxA* were confirmed by Southern hybridization. The insertion within the *cpxR* open reading frame is polar and strains containing this insertion are *cpxR*$^-$ and *cpxA*$^-$. When a *cpxR* null strain is complemented with plasmid pND10 (containing *cpxR*), the strain behaves as a *cpxA*$^-$ strain in that *degP-lacZ* transcription is dramatically increased in the presence of carbon sources such as D-glucose (See Figure 3.4 and the section in Chapter 3 titled, "Acetyl-phosphate can stimulate *degP* transcription through CpxR").

***cpxP::kan* deletion/insertion**

The plasmid used to create the *cpxP::kan* mutation was generated in three steps. First, the 3' end of the *cpxP* open reading frame was amplified by PCR using the Finko and Cpx37 primers. The amplified DNA was digested with *Bam*HI and *Kpn*I restriction endonucleases and subcloned into the *Bam*HI and *Kpn*I sites of pMAK705 [Hamilton *et al.* 1989], generating pND26. A portion of the 5' end of the *cpxP* open reading frame was then amplified *via* PCR using the Cpx2828 and Finpho primers. The amplified DNA was digested with *Hin*dIII and

*Bam*HI and subcloned into the *Hin*dIII and *Bam*HI sites of pND26, generating pND27. pND27 contains the 5' and 3' ends of the *cpxP* open reading frame, but it lacks an internal region spanning from codon 72 to codon 144. pND27 was then digested with *Bgl*II, removing an additional 12 codonds from the internal portion of the *cpxP* open reading frame. The kanamycin-resistance cassette of pUC4K [Pharmacia] was then isolated as a *Bam*HI fragment and subcloned into the large *Bgl*II fragment of pND27, generating pND30. The kanamycin-resistance cassette of pND30 replaces codons 63 to 147 of the *cpxP* open reading frame. This *cpxP::kan* deletion/insertion was recombined onto the chromosome of MC4100 as described by Hamilton *et al.* [1989]. The proper location of the *cpxP::kan* mutation was confirmed by linkage analysis and PCR.

CONSTRUCTION OF *lacZ* FUSIONS

All *lacZ* fusions derived from pRS415 were recombined onto λRS88 as described by Simons *et al.* [1987], and the resulting recombinant phage were introduced at the λ*att* site of MC4100.

degP-lacZ

The *degP-lacZ* fusion was created by amplifying the chromosomal *degP* locus of MC4100 by PCR using the Htra5 and Htramid3 primers. This amplified DNA was subcloned into the *Eco*RI and *Sma*I sites of pRS415 [Simons *et al.* 1987], creating pSINATRA131. The nucleotide sequence of the *degP* insert of pSINATRA131 was confirmed by dideoxy nucleotide sequencing. The subcloned *degP* insert includes nucleotides from position -122 with respect to the *degP* transcription start site to position +845 with respect to this same site.

fkpA-lacZ

The FkpA5 and Fkpalac3 primers were used to amplify the promoter region and a portion of the *fkpA* open reading frame from the chromosome of MC4100. The amplified DNA was subcloned into the *Eco*RI and *Bam*HI sites of pRS415, generating pND28. This amplified DNA includes nucleotides from position -286 with respect to the *fkpA* translational start site to position +55 with respect to this same site.

λRS88[*cpxP-lacZ*]

The Finlac and Finpho primers were used to amplify the promoter region and a portion of the *cpxP* open reading frame from the chromosome of MC4100. This amplified DNA was digested with *Eco*RI and *Bam*HI and subcloned into the corresponding restriction sites of pRS415, generating pND22. This amplified DNA includes nucleotides from position -410 with respect to the *cpxP* translation start site to position +214 with respect to this same site.

p*orfA-dsbA-lacZ*

The Dsba5 and Dsba3 primers were used to amplify from the promoter region of *orfA* (the gene immediately upstream of *dsbA*) through to the beginning of the *dsbA* coding sequence. This DNA was amplified from the chromosome of MC4100 and subcloned into the *Eco*RI site of pRS415, generating pND31. The proper orientation of the insert was confirmed by restriction analysis. pND31 drives *lacZ* transcription from both promoters that are known to initiate transcription of *dsbA*, p*orfA* and p*dsbA* [Belin and Boquet 1994]. The amplified DNA contains nucleotides from position -422 with respect to the translational start site of *orfA* to position +86 with respect to the start site of translation of ***dsbA***.

GENETIC SCREENS

The screen for Cpx-regulated *lacZ* operon fusions (Chapter Four)

A collection of *lacZ* operon fusions, positioned throughout the genome of strain PND541 (MC4100, *ara*$^+$, *nadA*::Tn*10* Δ[*gal-att-bio*]), was generated *via* infection with λp*lac*Mu53 as described by Bremer *et al.* [1985]. Note that PND541 harbors the pND18 plasmid, which expresses the *nlpE* locus under the control of the arabinose promoter, *pBAD*. Since the expression of *nlpE* is under the control of *pBAD*, the Cpx signal transduction system of PND541 can be conditionally activated simply by growing this strain in the presence of arabinose.

Using λp*lac*Mu53, 12 independent pools of *lacZ* operon fusions were generated throughout the genome of PND541. From these 12 pools, a total of 13,213 colonies were individually streaked on two types of media: 1) Luria broth containing 1.6μg/ml X-Gal and 2) the same medium supplemented with 0.4% L-arabinose. The colonies streaked on these media

were incubated at 30°C for 48 hours. After this incubation period, the colonies were screened for those that displayed a qualitative increase in Lac activity on the medium supplemented with arabinose. 107 colonies satisfying this criterion were subjected to further examination.

Specifically, each of the 107 λ*plac*Mu53-generated *lacZ* operon fusions was transferred to PND900 (MC4100, *ara*$^+$), and these 107 new strains were each transformed with pBR322 (control for pLD404) or pLD404. pLD404 constitutively overproduces the NlpE [Snyder *et al.* 1995] protein and thus, it provides a means to distinguish between those *lacZ* fusions whose transcription is induced by arabinose from those fusions whose transcription is induced by overproduction of NlpE.

Of the 107 strains tested, one possessed a *lacZ* operon fusion whose transcription was induced when transformed with pLD404. This fusion was renamed *cpxP-lacZ* (*f*usion *i*nduced by *N*lp*E*), and the strain harboring this fusion was renamed SP1 (MC4100, *ara*$^+$, λ*plac*Mu53[*cpxP-lacZ*]).

A screen for negative regulators of *degP* transcription (Chapter Five)

We infected nine independent cultures of MC4100 with λNK1324 as described [Kleckner *et al.* 1991]. Cells were plated on Luria agar containing chloramphenicol to select for cells carrying Tn*10cam* insertions. The resulting transductants were grouped into nine independent pools. Each pool contained at least 1200 independent colonies.

P1*vir* lysates were prepared on each pool as described by Kleckner *et al.* [1991]. The P1 lysates were then used to transduce the Tn*10cam* insertions into PND257 (MC4100, *ompR*::Tn*10*, λRS88[*degP-lacZ*]). Transductants were plated on Luria agar containing chloramphenicol and 1.4 μg/ml X-Gal. The parent strain (PND257) is phenotypically lac$^-$ (white) on this medium, providing a simple screen for mutants with increased *degP* transcription. Eight Lac$^+$ (blue) colonies were isolated from each of the nine pools and further analyzed.

The Tn*10cam* insertions within each lac$^+$ mutant were re-introduced into the parent strain (PND257) to determine whether the increase in *lac* activity was due to the specific Tn*10cam* insertion. Those mutant strains whose increased Lac activity resulted from the

Tn*10cam* insertion were further analyzed. These rebuilt strains were used for subsequent analyses. Each insertion mutation was numbered #XY. The X value indicates the pool number (1-9) from which the mutation was isolated, while the Y value indicates the isolate number (1 - 8) from the given pool.

PCR MAPPING OF THE Tn*10cam* INSERTIONS

The precise position of each Tn*10cam* insertion within the *rff/rfe* gene cluster was determined as follows. The six Rff primers (Rff5880, Rff7216, Rff8605, Rff9854, Rff11160 and Rff12431) were each used in combination with the CAM-5' and CAM-3' primers to amplify DNA from the *rff/rfe* gene cluster in each Tn*10cam* mutant strain. The CAM-5' and CAM-3' primers hybridize, respectively, to the 5' and 3' ends of the Tn*cam* transposon of λNK1324. Therefore, amplification of chromosomal DNA from Tn*10cam* mutant strains using these primers allows the precise positioning of each Tncam insertion within the *rff/rfe* gene cluster.

Amplified DNA was examined by electrophoresis in a 1% agarose gel.

MALTOSE-SENSITIVITY DISC ASSAYS

Chapter Four

Maltose-sensitivity disc assays for Chapter Four were performed as follows. Each strain was grown to saturation overnight at 37°C in 5ml of M63 minimal medium supplemented with 0.4% glycerol, Luria broth (final concentration 1%) and ampicillin. 3 ml of molten F-top agar (55°C) was mixed with 100µl of each overnight culture and immediately spread onto M63 minimal agar supplemented with 0.4% glycerol and ampicillin (warmed to 23°C). The top agar was allowed to solidify for 2 minutes. A Schleicher and Schuell analytical paper filter disc (7mm diameter) was then placed in the middle of the M63 glycerol plate. 10µl of 40% maltose (w/v) was placed on the filter disc and the plates were incubated overnight at 37°C. The zone of clearing, which is defined as the diameter of inhibited growth minus the diameter of the filter disc, was measured 18 h after the inception of incubation. Each value shown in Figure 4.7 is the average of four replicate experiments. The error bars represent the standard deviation from each average.

Chapter Five

Maltose-sensitivity disc assays for Chapter Five were performed as follows. Each strain was grown to saturation overnight at 37°C in 5ml of Luria broth. 3 ml of molten Luria Top agar (55°C) was mixed with 100µl of an overnight culture and immediately spread onto Luria agar (warmed to 23°C). The top agar was allowed to solidify for 2 minutes. A Schleicher and Schuell analytical paper filter disc (7mm diameter) was then placed in the middle of the Luria agar plate. 10µl of 20% maltose (w/v) was placed on the filter disc and the plates were incubated overnight at 37°C. The zone of clearing, which is defined as the diameter of inhibited growth minus the diameter of the filter disc, was measured 18 h after the inception of incubation. Each value shown in Figure 5.7 is the average of four replicate experiments. The error bars represent the standard deviation from each average.

ENZYMATIC ASSAYS

Alkaline phosphatase assays

Alkaline phosphatase assays were performed as described by Snyder and Silhavy [1995]. All assays were performed in the presence of 5mM iodoacetamide.

ß-galactosidase assays

Cells were grown overnight in Luria broth or M63 minimal media supplemented with 0.4% of a given carbon source (see figure legends for details). Cells were then subcultured (1:40) into 2 ml of the same media and grown to midlog phase. ß-galactosidase activities were determined utilizing a microtiter plate assay [Slauch and Silhavy, 1991]. ß-galactosidase activities are expressed as (units/A_{600}) x 10^3, where units = µmol of product formed per minute. A minimum of four independent assays were performed on each strain, and the results were averaged to obtain the indicated activities. Error bars indicate the standard deviation. The absence of error bars indicates that the standard deviation fell below the resolution limit of the graphing program.

Measuring *cpxP-lacZ* transcription as a function of pH

The buffering of Luria broth with 100 mM sodium phosphate necessarily changes sodium ion concentration as well as the pH of the medium. Thus, it was important to determine if alterations in sodium ion concentration affected transcription of *cpxP-lacZ*. Alterations in sodium ion concentration do not alter transcription from either *cpxP-lacZ* operon fusion (data not shown). Thus, the observed changes in *cpxP-lacZ* transcription shown in Figure 4.8 are due to changes in pH.

I also note that in the process of generating data for Figure 4.8, bacterial growth in the buffered Luria broth did not alter extracellular pH by more than 0.1 pH units.

Finally, the pH of the ß-galactosidase assay buffer (Z buffer) was not affected by the residual buffered Luria broth associated with the cell pellets. All ß-galactosidase assays were performed at the same pH (7.1).

Measuring *cpxP-lacZ* transcription in the presence of CCCP

Two aspects of the experiment whose results are depicted in Figure 4.9 must be noted. First, essentially the same results were observed using CCCP concentrations of 25µM, 50µM and 100µM. Second, in all cases (including the experiment depicted in Figure 4.9), the $O.D._{600}$ measurement of cell density continued to rise as a function of time. Thus, the SP569 cultures were not killed during the experiment.

ß-galactosidase assays for Chapter Six

The ß-galactosidase assays performed to generate the data in Chapter Six were performed essentially as described above, but with the following modifications. Strains were subcultured 1:50 from saturated overnight cultures. These strains were grown at 37°C for 60 minutes, at which point IPTG was added to a final concentration of 0.5mM to induce synthesis of the P-pilus Pap proteins. All strains were reincubated for 90 minutes at 37°C before they were harvested for the ß-galactosidase assays.

PROTEIN ANALYSIS

Preparation of whole-cell, periplasmic and spheroplast protein extracts

All procedures were performed on ice and all solutions were chilled on ice unless noted otherwise.

Whole cell extracts were prepared by pelleting 1 ml of cells, resuspending the pellet in loading buffer [Silhavy *et al.* 1984], and boiling for 10 minutes.

Periplasmic and spheroplast protein extracts were prepared as follows. Strains were grown in the appropriate media until they reached an O.D.$_{600}$ of approximately 1.0. At this juncture, 1 ml of each culture was harvested and resuspended in 250 µl of 0.2 M Tris-HCl (pH 8.0). 250 µl of 0.2 M Tris-HCl, 1 M Sucrose (pH 8.0) was then added to each suspension. 2.5 µl of 0.1 M EDTA was subsequently added along with 7.5 µl of lysozyme (4 mg/ml). 500 µl of cold distilled water was then added and the cell suspension was incubated on ice for two minutes. 20 µl of 1M $MgCl_2$ was added to the cell suspension and each mixture was incubated for 30 additional minutes on ice. After this incubation period, each suspension was harvested at 14,000 rpm in a microcentrifuge to pellet spheroplasts. The remaining supernatant contained the bulk of periplasmic proteins. The periplasmic proteins were precipitated from these supernatants with trichloroacetic acid.

Immunoblot Analysis

Protein samples were subjected to SDS polyacrylamide gel electrophoresis and transferred to nitrocellulose membranes. The membranes were blocked in MTS buffer (0.9% NaCl, 0.01M Tris-HCl [pH7.5], 2.5% powdered milk) at 4°C for approximately 12 hours, and then incubated with the primary antibody (either anti-OmpR, anti-alkaline phosphatase or anti-MalE, diluted 1:5000 in MTS) at 4°C overnight. Membranes were washed with wash buffer (0.2% Tween-20, 0.9% NaCl, 0.01M Tris-HCl [pH7.5]) for two hours with at least four changes of buffer, and then incubated as before with horseradish peroxidase-linked secondary antibody (anti-rabbit diluted 1:10000 in MTS). Membranes were washed as before, and

antibody detection was performed as described by the supplier (ECL detection reagents, Amersham).

Amino acid sequence analysis of FkpA and DsbA

Periplasmic protein extracts depicted in Figure 7.1 were subjected to SDS polyacrylamide gel electrophoresis and transferred to Immobilon-P transfer membrane. The membrane was stained with Coomassie Brilliant Blue and the bands marked with arrowheads in Figure 7.1 were excised from the Immobilon-P membrane. The identity of the first 11 residues from each band was determined by Edman Degradation by the Princeton University Synthesis/Sequencing Facility.

PREPARATION OF *E. COLI* RNA, S1 NUCLEASE PROTECTION ASSAYS, AND DNA SEQUENCING

RNA was prepared from strains grown at 30°C in Luria broth as described by Barry *et al.* [1980]. A 340 nucleotide-long fragment, spanning from position 1 to position 340 of the published *degP* sequence [Lipinska *et al.* 1988] was used to create a radioactive probe for S1 experiments in Chapter Three. A 342 nucleotide-long fragment, spanning from position 833 to position 1175 of the published *slyD-fkpA* sequence [Horne and Young 1995] was used to create a radioactive probe for S1 experiments depicted in Chapter Seven. A 425 nucleotide-long fragment, spanning from position 931 to position 1356 of the published *orfA-dsbA* sequence [Belin and Boquet 1994] was used to create a radioactive probe for S1 experiments depicted in Chapter Seven. This 425 nucleotide-long fragment was used to quantify the amount of transcription from the p*orfA* promoter described by Belin and Boquet [1994]. A 270 nucleotide-long fragment spanning the *pspc* promoter was used to create a radioactive probe for S1 experiments. The pspc transcript serves as an internal loading control for quantifying changes in the amount of transcription from the *fkpA* and p*orfA* promoters.

Each probe was phosphorylated with either γ^{32}P-ATP or γ^{33}P-ATP in the forward reaction as described [Sambrook *et al.* 1989]. 60 µg of total RNA was used in each S1 assay and the assays were performed as described in Sambrook *et al.* [1989]. The DNA sequence of *degP*, *fkpA* and *orfA-dsbA* was determined as previously described [Russo *et al.* 1993]. The *degP*,

fkpA and *orfA-dsbA* sequencing reactions and S1 nuclease samples were electrophoresed on 6% polyacrylamide sequencing gels and analyzed using the PhosphorImager ImageQuant™ (Molecular Dynamics) analysis program.

Three. The Cpx Two-Component Signal Transduction Pathway Regulates *degP* Transcription

INTRODUCTION

At the inception of this study, the gene encoding σ^E (*rpoE*) had not been cloned, and the link between σ^E activity and extracytoplasmic events had not been established. Accordingly, I chose to analyze the transcription of a *degP-lacZ* operon fusion as an indirect reporter for σ^E activity. I reasoned that mutations that altered *degP-lacZ* transcription could highlight signal transduction systems that normally regulate the expression of the *degP* locus.

The results presented in this chapter indicate that *degP* transcription is not only influenced by σ^E, but it is also controlled by a parallel system, the CpxA/CpxR signal transduction pathway. Like σ^E, the Cpx pathway can be activated by perturbations in the bacterial envelope.

RESULTS

The *degP-lacZ* operon fusion accurately reflects *degP* transcription

A *degP-lacZ* operon fusion was constructed to study the transcriptional regulation of the *degP* locus in *E. coli*. The fusion was recombined onto a λ phage and was placed in single-copy at the *attB* locus on the *E. coli* chromosome (see Chapter Two for details). Like the wild-type *degP* locus, transcription from this fusion is altered by genetic backgrounds that alter the activity of $E\sigma^E$, the RNA polymerase responsible for transcriptional-initiation at the *degP* locus [Erickson and Gross 1989, Wang and Kaguni 1989]. $E\sigma^E$ activity is modulated in response to the synthesis of outer membrane proteins. For example, high-level synthesis of outer membrane proteins such as OmpF or OmpC increases $E\sigma^E$ activity. Conversely, mutations which decrease the number of proteins found in the outer membrane decrease $E\sigma^E$ activity [Mecsas *et al.* 1993]. Figure 3.1 shows that high-level synthesis of the outer membrane proteins OmpF or OmpC stimulates transcription of the *degP-lacZ* fusion. In contrast, the *ompR*::Tn*10* mutation, which decreases $E\sigma^E$ activity by eliminating expression of *ompF* and *ompC* [Slauch *et al.* 1988], decreases transcription from the *degP-lacZ* fusion (Figure 3.1). Thus, the *degP-lacZ* fusion is an accurate reporter of transcription at the *degP* locus.

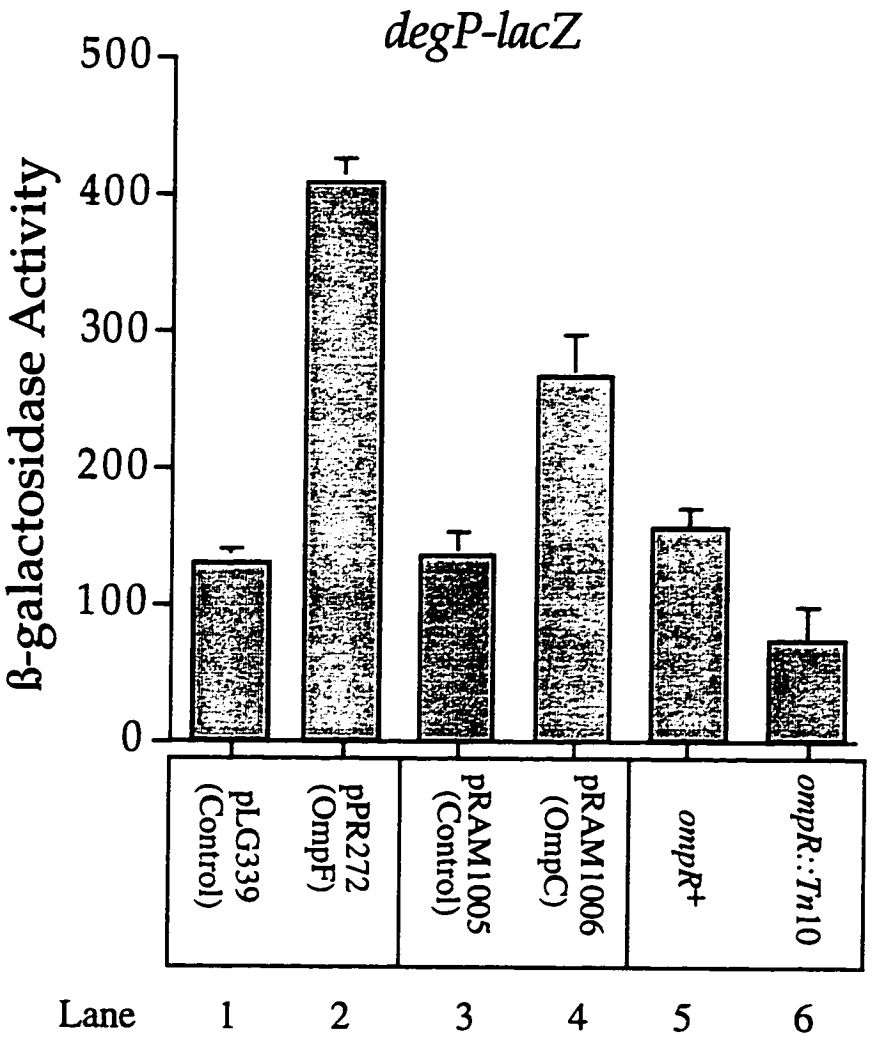


Figure 3.1. Transcription of a *degP-lacZ* fusion is modulated in response to outer-membrane protein levels. Lanes 1-4 display the ß-galactosidase activities of PND2000 (MC4100, λRS88[*degP-lacZ*]) transformed with: Lane 1, pLG339 (control for pPR272); Lane 2, pPR272 (overexpresses *ompF*); Lane 3, pRAM1005 (control for pRAM1006); Lane 4, pRAM1006 (overexpresses *ompC*). Lanes 5 and 6 show the ß-galactosidase activities of PND2000 and PND257 (PND2000, *ompR*::Tn*10*), respectively. Strains were grown in Luria broth with the appropriate antibiotic to select for plasmids when necessary. See Chapter Two for details.

Mutations in *cpxA* stimulate transcription of *degP*

To identify genes involved in transcriptional regulation of *degP*, we sought mutations that altered transcription of the *degP-lacZ* fusion. Our first candidate for such a gene arose from concurrent studies of two novel envelope proteins in our laboratory. The LamB-LacZ-PhoA fusion protein [Snyder and Silhavy 1995] and the signal-sequence processing-defective maltoporin, LamBA23D [Carlson and Silhavy 1993], are both targeted to the bacterial envelope and both damage the envelope when their genes are highly expressed. Such high-level synthesis of LamB-LacZ-PhoA causes cell lysis due to an undefined perturbation in the bacterial envelope [Snyder and Silhavy 1995]. LamBA23D contains a mutation which renders signal-sequence cleavage of this protein inefficient. High-level synthesis of this protein confers hypersensitivity to detergents, suggesting that LamBA23D perturbs the integrity of the outer membrane [Carlson and Silhavy 1993].

Independent attempts to identify extragenic suppressor mutations of the lethality conferred by either LamB-LacZ-PhoA or LamBA23D yielded gain of function mutations in a previously identified locus, *cpxA* [Cosma *et al.* 1995]. *cpxA* specifies an inner-membrane sensor homolog in the two-component family of regulatory proteins [Weber and Silverman 1988]. *cpxR*, the gene specifying CpxA's putative cognate response regulator, lies immediately upstream of the *cpxA* locus and shares homology with other two-component response regulators [Dong *et al.* 1993].

Based on homology between *cpxA* and this family of regulatory proteins, it seemed likely that the *cpxA* suppressor mutations played a regulatory function in relieving the toxicity conferred by LamB-LacZ-PhoA and LamBA23D. Indeed, through biochemical analysis, we have shown that the *cpxA* suppressors enhance the rate of proteolysis of LamB-LacZ-PhoA [Cosma *et al.* 1995]. This result prompted us to examine the effects of the *cpxA* suppressor mutations on transcription from our *degP-lacZ* fusion. Each *cpxA* suppressor allele was transduced into the *degP-lacZ* fusion strain and the resulting ß-galactosidase activity was

determined. Figure 3.2 shows that the *cpxA* suppressor alleles increase transcription from the *degP-lacZ* fusion from three- to ten-fold more than that of a wild-type strain.

It is interesting to note that we initially characterized the *cpxA24*, *cpxA17* and *cpxA41* alleles as stronger suppressors of the lethality conferred by LamB-LacZ-PhoA than *cpxA723*, *cpxA741* and *cpxA744* [Cosma *et al.* 1995]. Figure 3.2 shows that *cpxA24*, *cpxA17* and *cpxA41* also increase transcription from *degP* to a higher level than *cpxA723*, *cpxA741* and *cpxA744*. Thus, the *cpxA* alleles that most strongly stimulate transcription of *degP* are also the strongest *cpxA* suppressor alleles.

Analysis of *cpxA* and *cpxR* null mutations

Previous work has demonstrated that *degP* transcription is modulated in response to the amount of outer membrane proteins localized to the bacterial envelope [Mecsas *et al.* 1993]. This effect is mediated by modulating the activity of $E\sigma^E$, and presently the mechanism of this regulation is unknown. Since CpxA is an inner membrane sensor homolog and since the *cpxA* suppressors stimulate *degP* transcription, it seemed possible that CpxA and CpxR were responsible for sensing the extracytoplasmic parameter which influences $E\sigma^E$ activity. If the Cpx proteins were the sole sensory circuit for modulating $E\sigma^E$ activity, *cpx* null strains should no longer alter $E\sigma^E$ activity in response to fluctuations in the number of outer membrane proteins found in the bacterial envelope.

cpxA and *cpxR* were each inactivated by insertion of antibiotic-resistance cassettes within their respective open reading frames (see Materials and Methods). The insertion within *cpxR* is polar and strains containing this insertion are *cpxR*$^-$ and *cpxA*$^-$ (see Chapter Two for details). When introduced into the *degP-lacZ* fusion strain, the *cpxA* and *cpxR* null alleles both conferred a decrease in transcription from this fusion (For example, compare lanes 1, 3 and 5 in Figure 3.3a). However, the *cpx* null mutations were not epistatic to transcriptional induction of the *degP-lacZ* fusion by any factors known to alter $E\sigma^E$ activity. Specifically, overexpression of *ompF* or *ompC* still caused the same magnitude increase in transcription from

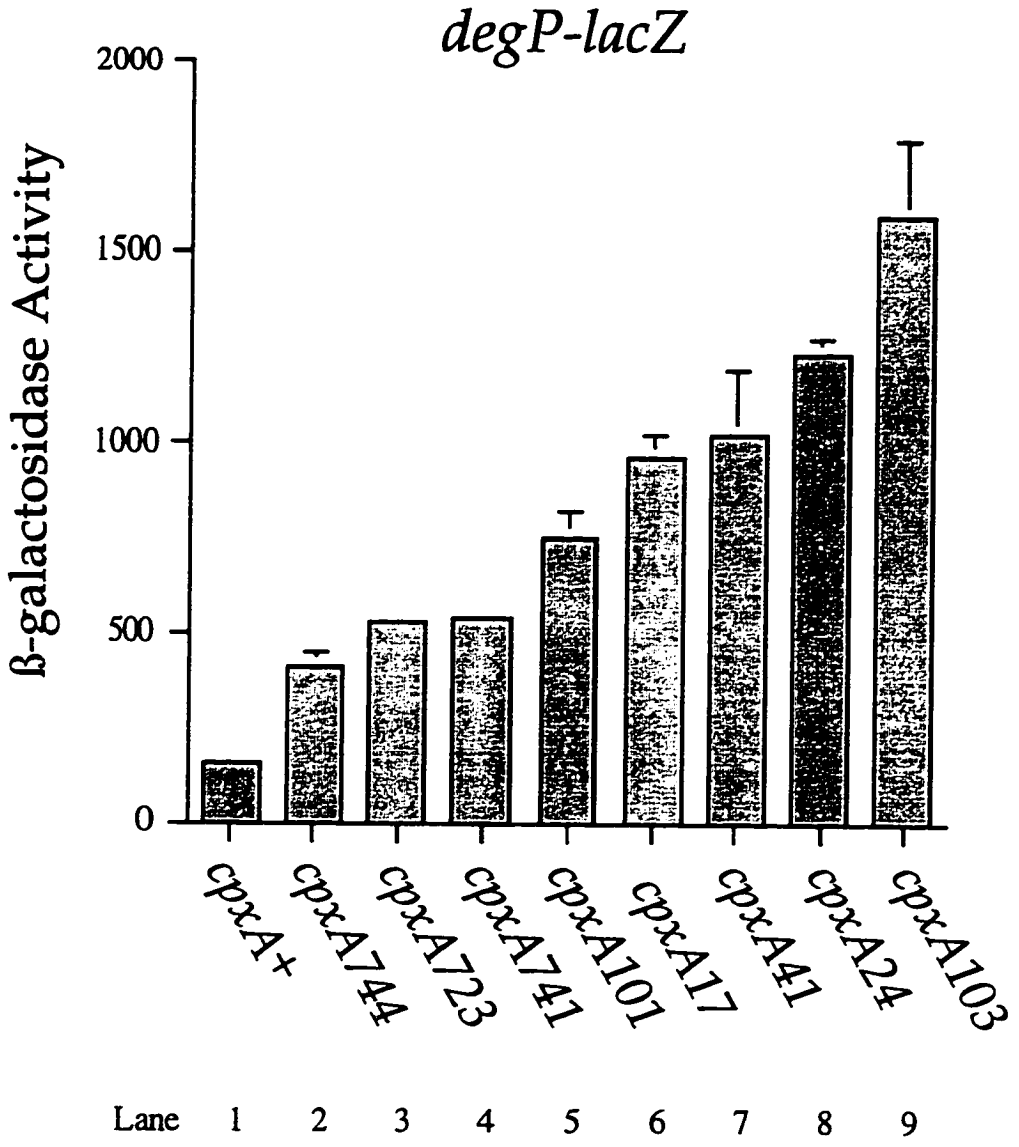


Figure 3.2. *cpxA* suppressor mutations activate transcription of *degP-lacZ*. ß-galactosidase activities of PND2000 (MC4100, λRS88[*degP-lacZ*]) (Lane 1) and PND2000 containing the various mutant *cpxA* alleles indicated (Lanes 2 through 9) were assayed. The mutant *cpxA* alleles stimulate transcription of *degP-lacZ* ~ 3- to 10-fold over that of the wild-type strain. Strains were grown in Luria broth as described in Chapter Two.

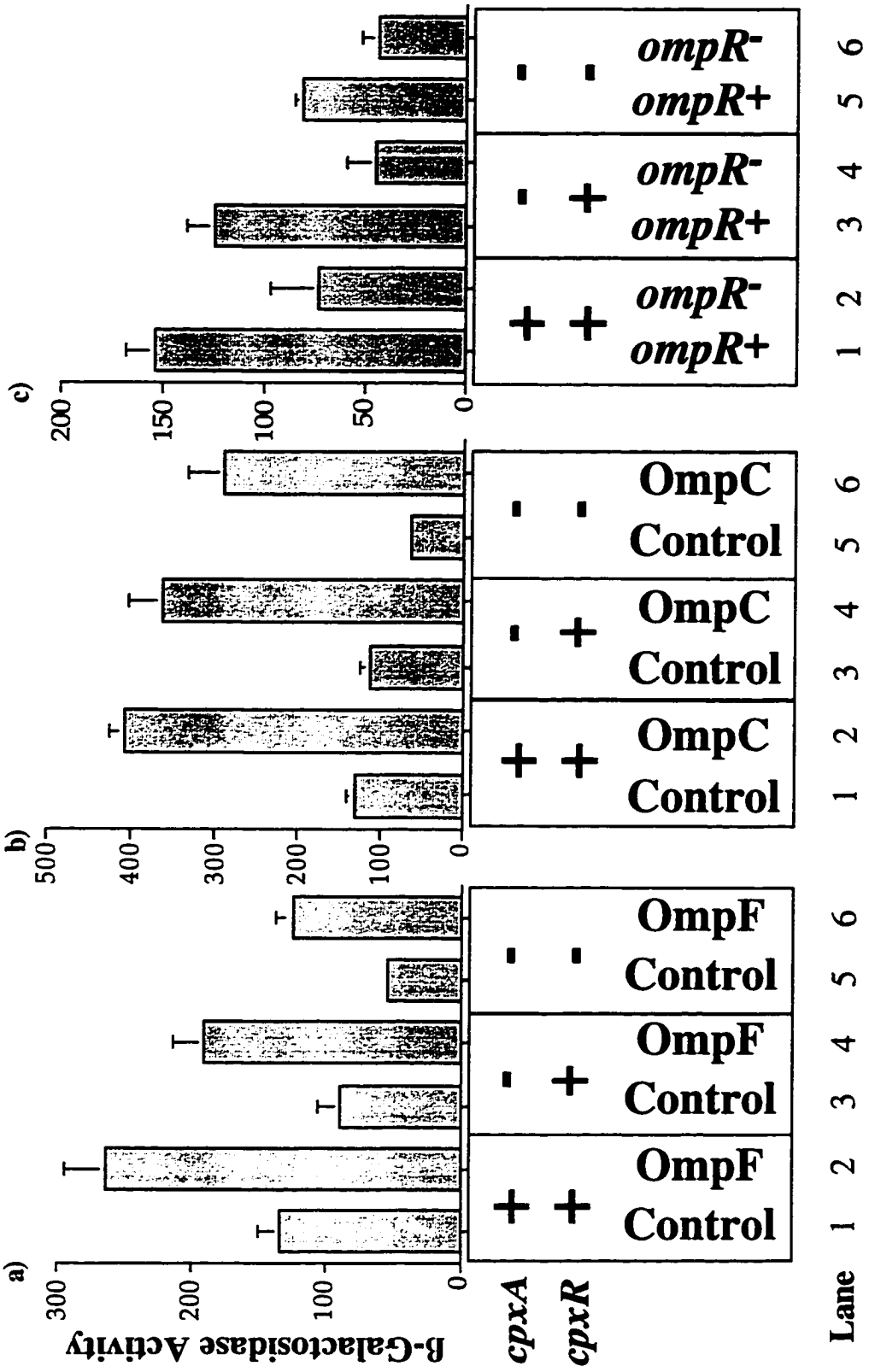
the *degP-lacZ* fusion with or without functional Cpx proteins (Figure 3.3a, b). Also, the *ompR*::Tn*10* mutation decreases *degP* transcription in the *cpxA*$^-$ and *cpxA*$^-$*R*$^-$ strains (Figure 3.3c). Thus, *cpxA* is not required for modulating $E\sigma^E$ activity under these circumstances.

Acetyl-phosphate can stimulate *degP* transcription through CpxR

Most two-component sensors are responsible for phosphorylating and dephosphorylating their cognate response regulators in order to elicit appropriate internal responses to a particular external input. The phosphorylated species of the response regulator is typically the species that actively elicits the internal responses [Stock *et al.* 1990, Parkinson 1993]. Many response regulators can also be phosphorylated by the low molecular-weight compound, acetyl-phosphate (Ac~P) [Lukat *et al.* 1993, McCleary *et al.* 1993]. In wild-type cells, the effect of phosphorylation of response-regulators by Ac~P is negligible. However, when a given sensor molecule is lost to mutation, the effect of phosphorylation of the cognate response regulator by Ac~P can become significant. With the sensor molecule rendered non-functional, there is no phosphatase activity to counteract the phosphorylation of the response regulator by Ac~P [McCleary *et al.* 1993]. Since Ac~P can be synthesized from acetyl-CoA and P_i [McCleary *et al.* 1993], the intracellular concentration of Ac~P rises with the use of the glycolytic pathway. Hence, carbon sources utilized in glycolysis increase the intracellular concentration of Ac~P and this ultimately leads to an increase in the phosphorylation of certain "orphaned" response regulators.

Our analysis of the *cpxA* and *cpxR* null mutations indicates that CpxR can be phosphorylated by Ac~P under certain conditions. When grown in Luria broth, the *cpxA*$^-$ strain displays a decrease in *degP* transcription compared with an isogenic *cpxA*$^+$ strain (Compare lanes 1 and 3 in Figure 3.3a, b and c). However, when *cpxA*$^+$ and *cpxA*$^-$ strains are grown in the presence of carbon sources such as D-glucose, maltose or lactose, the *cpxA*$^-$ strain displays a dramatic increase in *degP* transcription (Figure 3.4, compare lanes 1 and 2 and data not shown). In contrast, the *cpxR*$^-$*A*$^-$ double mutant does not alter *degP* transcription when grown in these

Figure 3.3. The Cpx proteins do not modulate *degP-lacZ* transcription in response to outer-membrane protein levels. Panel a) Lanes 1, 3 and 5 show ß-galactosidase levels of strains transformed with pLG339 (control for pPR272). Lanes 2, 4 and 6 show ß-galactosidase levels of strains transformed with pPR272 (overexpresses *ompF*). Panel b) Lanes 1, 3 and 5 show ß-galactosidase levels of strains transformed with pRAM1005 (control for pRAM1006). Lanes 2, 4 and 6 show ß-galactosidase levels of strains transformed with pRAM1006 (overexpresses *ompC*). Panel c) The Cpx proteins do not mediate the decrease in transcription from *degP-lacZ* conferred by the *ompR*::Tn*10* mutation. Lanes 1, 3 and 5 are *ompR*$^+$ while Lanes 2, 4 and 6 are *ompR*::Tn*10*. In all Panels, Lanes 1 and 2 display ß-galactosidase levels of PND2000 (MC4100, λRS88[*degP-lacZ*], *cpxA*$^+$, *cpxR*$^+$); Lanes 3 and 4 display ß-galactosidase levels of PND242 (PND2000, *cpxA::cam*); Lanes 5 and 6 display ß-galactosidase levels of PND325 (PND2000, *cpxR::Ω*). All strains were grown in Luria broth and the appropriate antibiotic for plasmid selection when necessary as described in the Chapter Two.



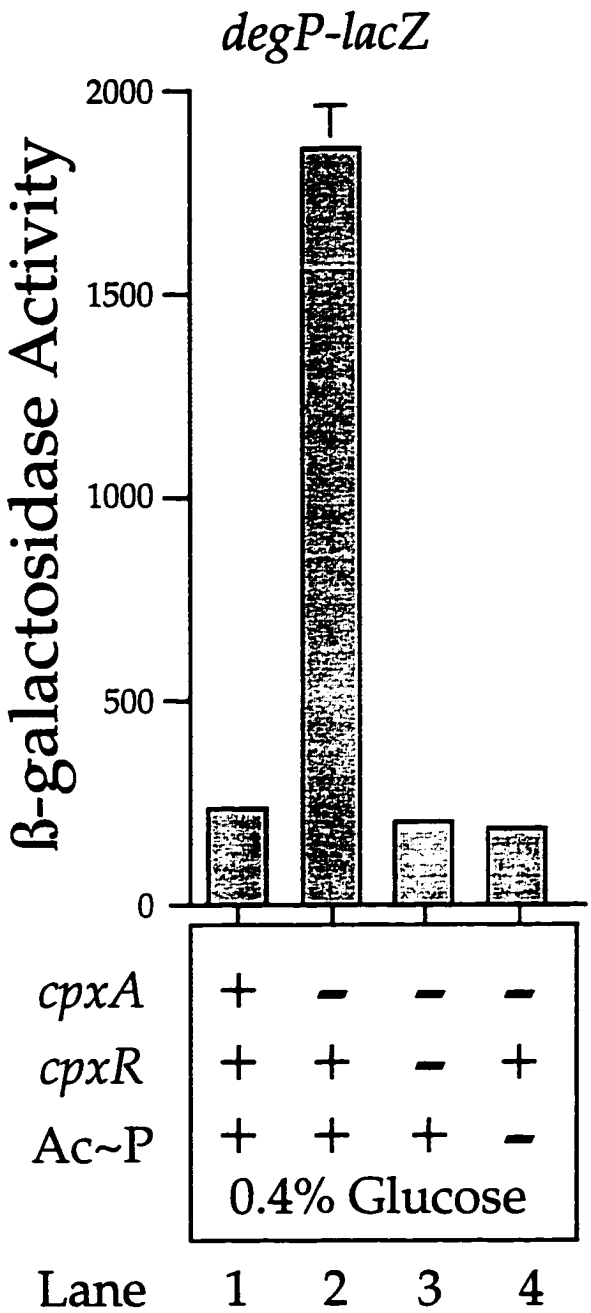


Figure 3.4. Ac~P mediates transcriptional induction of *degP-lacZ* by phosphorylating CpxR in the absence of CpxA. (Lane 1) PND495 (PND2000, *zej*::Tn*10*); (lane 2) PND422 (PND2000, *cpxA*::*cam*, *zej*::Tn*10*); (lane 3) PND496 (PND2000, *cpxR*::Ω, *zej*::Tn*10*); (lane 4) PND421 (PND2000, *cpxA*::*cam*, *zej*::Tn*10* Δ[*pta*, *ackA*, *hisQ*, *hisP*]). Strains were grown in M63 minimal medium with 0.4% glucose as a carbon source (see Chapter Two for details).

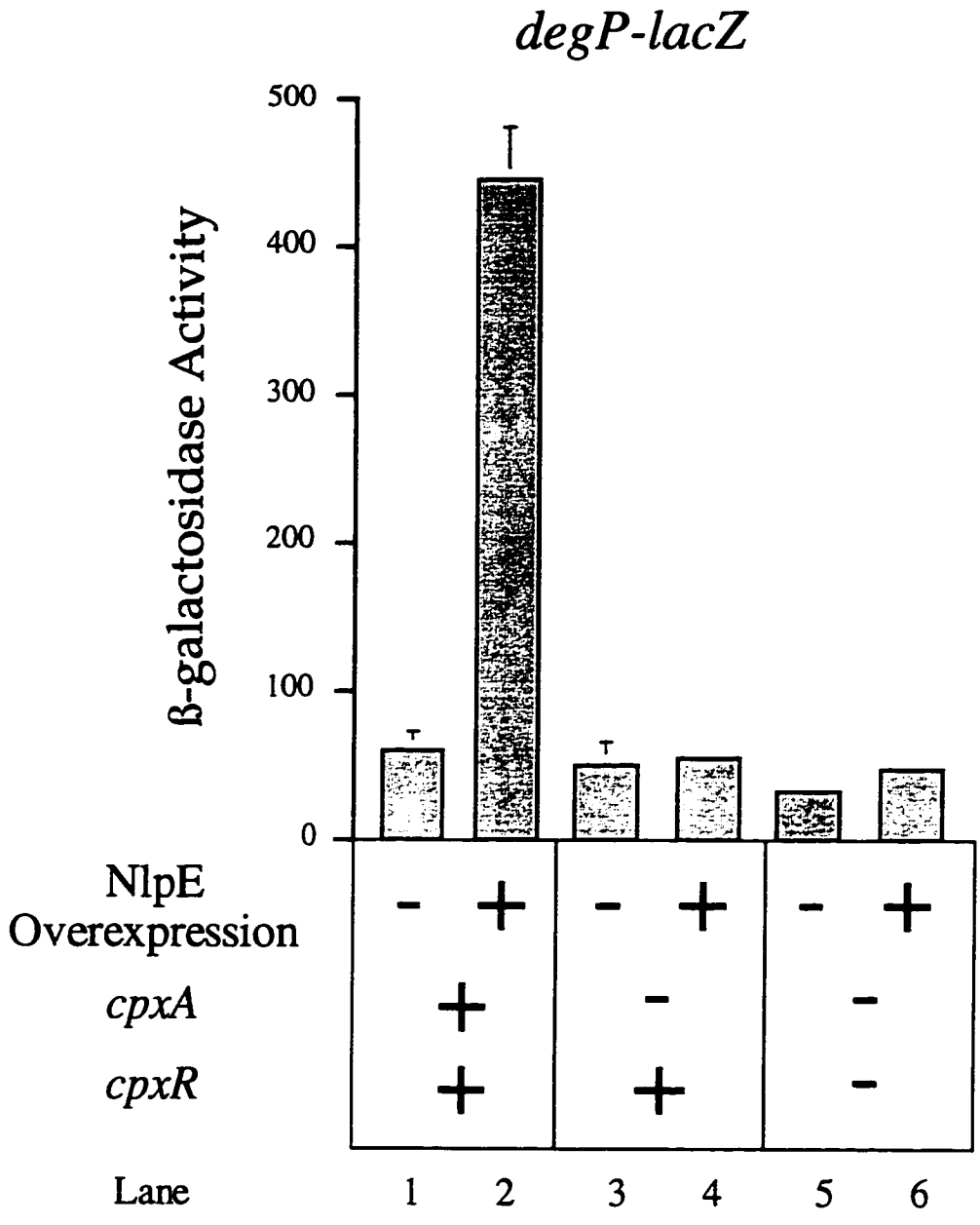
same carbon sources (Figure 3.4, compare lanes 2 and 3), indicating that the transcriptional induction of *degP* observed in the absence of CpxA proceeds through CpxR *via* another factor. This other factor is Ac~P. Deletion of *pta* and *ackA*, the two genes responsible for Ac~P synthesis [McCleary *et al.* 1993], is epistatic to the effect of glucose on *degP* transcription in a *cpxA*$^-$ background (Figure 3.4, compare lanes 2 and 4). These results further imply that CpxR-phosphate (CpxR-P) can direct transcriptional induction of *degP* and they also provide indirect evidence that CpxA can dephosphorylate CpxR-P.

The Cpx pathway mediates transcriptional induction of *degP* in response to high-level synthesis of an envelope lipoprotein

The observed induction of *degP* transcription by Ac~P in a *cpxA*$^-$ background is clearly not a reflection of wild-type regulation. Indeed, from the data presented thus far, several models can explain the Cpx-mediated transcriptional regulation of *degP*. For example, it is possible that the Cpx proteins are normally involved in transcriptional induction of *degP* in response to an unknown extracytoplasmic parameter. If so, the observed induction of *degP* synthesis by Ac~P or *cpxA* mutations has simply exaggerated a normal function of the Cpx system by perturbing this pathway. It is also possible that the Cpx proteins normally regulate the synthesis of an outer membrane protein which, when overproduced by activation of the Cpx pathway, increases $E\sigma^E$ activity, ultimately stimulating transcription of *degP*. Alternatively, the induction of *degP* transcription by Ac~P in the *cpxA* null strain or by the *cpxA* mutations could reflect situations whereby a hyper-activated Cpx pathway would alter the transcription of genes outside of its normal regulon. Evidence presented below favors the first model.

In an independent attempt to identify high-copy suppressors of the lethality conferred by LamB-LacZ-PhoA, we identified a new envelope lipoprotein, NlpE [Snyder *et al.* 1995]. High-level synthesis of NlpE increases transcription of *degP* ten-fold (Figure 3.5 lanes 1 and 2) and suppresses the lethality conferred by LamB-LacZ-PhoA [Snyder *et al.* 1995]. In this case, transcriptional induction of *degP* is dependent on CpxA. There is no induction of *degP* transcription by overproduction of NlpE in either the *cpxA*$^-$ or *cpxR*$^-$*A*$^-$ backgrounds (Figure 3.5)

Figure 3.5. Overexpression of *nlpE* induces transcription of *degP-lacZ* through the Cpx signal transduction pathway. Lanes 1, 3 and 5 show ß-galactosidase activity of strains transformed with pBAD18 (control for pND18). Lanes 2, 4 and 6 show the ß-galactosidase activity of strains transformed with pND18 (overexpresses *nlpE*). Lanes 1 and 2: PND497 (PND2000, *zej::*Tn*10* Δ[*pta, ackA, hisQ, hisP*], *ara*$^+$). Lanes 3 and 4: PND498 (PND497, *cpxA::cam*). Lanes 5 and 6: PND499 (PND497, *cpxR::Ω*). All strains were grown in Luria broth containing 0.4% L-arabinose and 50μg/ml ampicillin (See Chapter Two for details). The experiments in Figure 3.5 were performed with strains deleted for *pta* and *ackA*. Since NlpE synthesis is driven from the *araB* promoter [Guzman *et al.* 1995] in this experiment, full transcriptional induction requires growth in arabinose. Hence, Ac~P synthesis must be eliminated to prevent hyper-phosphorylation of CpxR in the *cpxR*$^+$*A*$^-$ background.



and the synthesis of NlpE itself is unaffected by the *cpx* null mutations (data not shown). The Cpx-dependent transcriptional induction of *degP* highlights two points. First, the wild-type Cpx proteins can activate *degP* transcription in response to an extracytoplasmic stimulus. Second, the observed transcriptional induction of *degP* by Ac~P and the *cpxA* suppressor mutations likely reflects a normal function of the Cpx pathway even though the observed induction occurs by perturbing this pathway.

The Cpx pathway is not a general monitor of lipoprotein synthesis

Since overproduction of NlpE activates *degP* transcription via the Cpx pathway, it seemed possible that the Cpx proteins were responsible for modulating *degP* transcription in response to the high-level synthesis of lipoproteins in general. In this case, the Cpx pathway would alter *degP* transcription in response to the level of lipoproteins, just as *degP* transcription is modulated in response to the levels of outer membrane proteins [Mecsas *et al.* 1993]. However, high-level synthesis of a variety of lipoproteins, including OsmB [Jung *et al.* 1989], Lpp [Nakamura *et al.* 1982], Pal [Chen and Henning 1987], lipoprotein-28 [Yu *et al.* 1986], NlpD [Ichikawa *et al.* 1994], and TraT [Ogata *et al.* 1982], does not induce transcription of *degP-lacZ* (data not shown). Thus, the Cpx pathway does not appear to monitor lipoprotein synthesis in general, implying that the enhanced transcription observed by overexpression of *nlpE* is related to the actual function of NlpE.

The Cpx pathway activates transcription from *degP* but not transcription from a minimal *rpoHp3* promoter

At the time of this study, only two promoters in *E. coli* were known to be utilized by $E\sigma^E$: the *degP* promoter and the *P3* promoter of *rpoH* [Lipinska *et al.* 1988, Erickson and Gross, 1989, Wang and Kaguni, 1989]. Using a *rpoHp3-lacZ* operon fusion, Mecsas *et al.* [1993] have shown that the minimal *P3* promoter is regulated by $E\sigma^E$ in a fashion analogous to that seen with the *degP* promoter.

We wished to determine whether activation of the Cpx pathway directly altered σ^E levels. If this were true, activation of the Cpx pathway would affect transcription of the

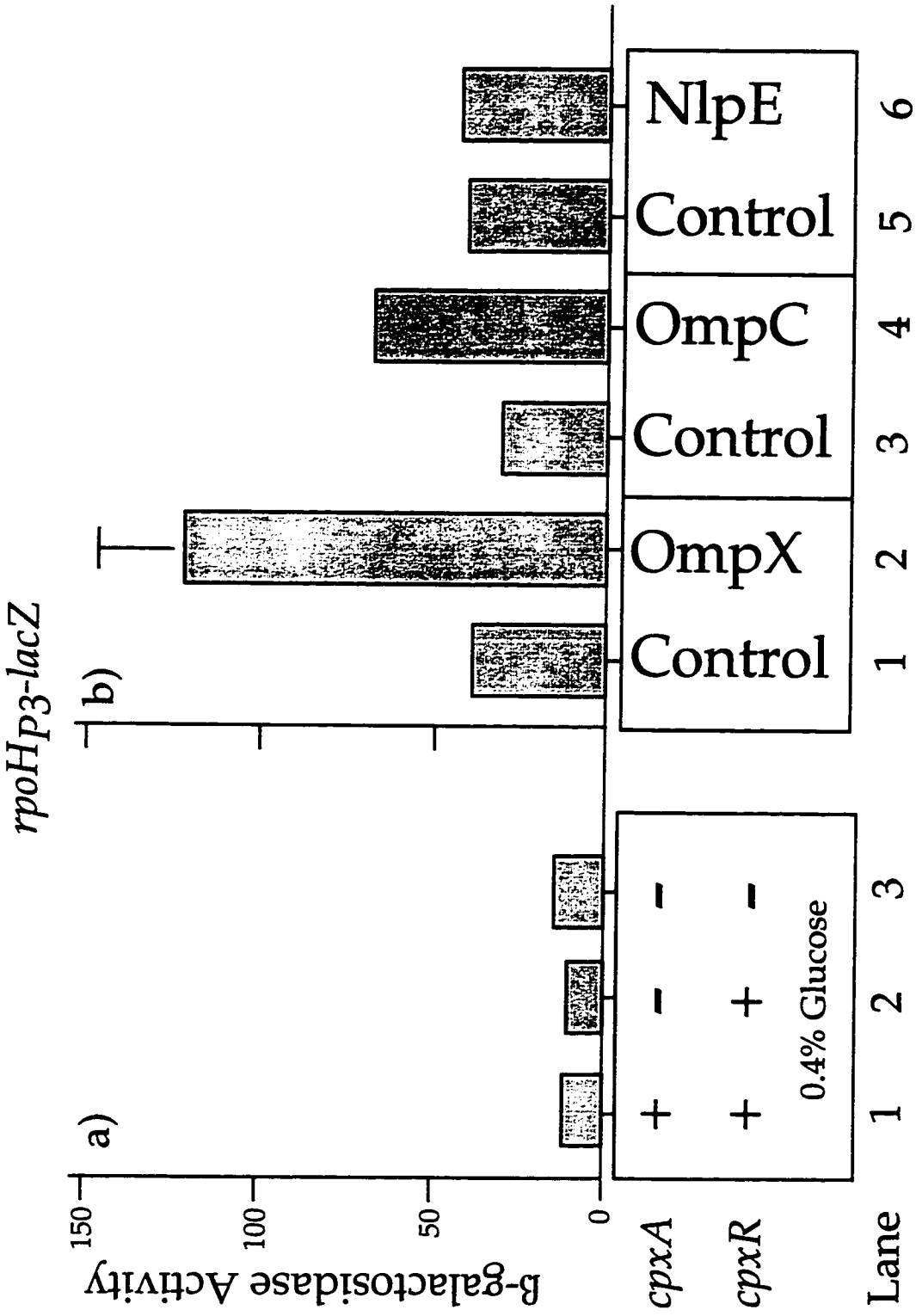
rpoHp3-lacZ fusion in a similar fashion to that observed with *degP*. However, activation of the Cpx pathway had no effect on transcription from the *rpoHp3-lacZ* operon fusion. Figure 3.6a shows that in a *cpxA*⁻ background, carbon sources such as glucose do not affect transcription from the *rpoHp3* promoter (compare Lanes 1 and 2). This is in contrast to the ten-fold stimulation of *degP* transcription observed under the same conditions (see Figure 3.4). Also, while overexpression of *ompX* or *ompC* stimulates transcription from both *rpoHp3* and *degP* (Figure 3.6b, lanes 2 and 4 and Mecsas *et al.* [1993]), overexpression of *nlpE* has no effect on *rpoHp3* transcription (Figure 3.6b, lane 6). These results reveal a new layer in the regulation of *degP* transcription. The Cpx pathway does not directly alter σ^E levels. Rather, this pathway affects *degP* transcription by working in parallel with $E\sigma^E$.

Activation of *degP* transcription by the Cpx pathway is dependent on $E\sigma^E$ activity

The fact that activation of the Cpx pathway stimulated transcription of *degP* and not of *rpoHp3-lacZ*, suggested that the Cpx pathway functions specifically at the *degP* promoter, perhaps working in concert with $E\sigma^E$ to activate *degP* transcription. If this were true, activation of *degP* transcription by the Cpx pathway would be dependent on a functional copy of the *rpoE* gene, which encodes σ^E [Hiratsu *et al.* 1995, Raina *et al.* 1995, Rouvière *et al.* 1995]. Figure 3.7 indicates that activation of *degP* transcription by the Cpx pathway is partially dependent on the activity of $E\sigma^E$. Inactivation of *rpoE* decreases the transcriptional induction of *degP* by overexpression of *nlpE* (Figure 3.7, compare lanes 2 and 4). The results illustrated in Figure 3.7 suggest that the Cpx pathway can function in concert with $E\sigma^E$ to induce *degP* transcription. However, the *rpoE* null mutation is not completely epistatic to the transcriptional induction of *degP* by activation of the Cpx pathway. This indicates that the Cpx pathway can function in concert with at least one other RNA polymerase to drive *degP* transcription.

It should be noted that the experiments performed to generate the data illustrated in Figure 3.7 utilized the *degP-lacZ* fusion in the MC1061 [Silhavy *et al.* 1984] strain background.

Figure 3.6. Activation of the Cpx signal transduction pathway does not stimulate transcription from *rpoHp3-lacZ*. Panel a) Acetyl-phosphate does not stimulate transcription of *rpoHp3-lacZ* in the absence of CpxA. Lane 1: PND381 (MC4100, λRS45[*rpoHp3-lacZ*]). Lane 2: PND394 (PND381, *cpxA::cam*). Lane 3: PND393 (PND381, *cpxR::Ω*). All strains in Panel a were grown in M63 minimal media with 0.4% glucose. Panel b) Overexpression of *nlpE* does not stimulate transcription of *rpoHp3-lacZ*. All lanes show the ß-galactosidase activity of PND500 (PND381, *ara*$^+$) transformed with: Lane 1, pBR322 (control for pJE100); Lane 2, pJE100 (overexpresses *ompX*); Lane 3, pRAM1005 (control for pRAM1006); Lane 4, pRAM1006 (overexpresses *ompC*); Lane 5, pBAD18 (control for pND18); Lane 6, pND18 (overexpresses *nlpE*). Strains in Lanes 1 through 4 were grown in Luria broth with the appropriate antibiotic to select for plasmids. Strains in Lanes 5 and 6 were grown in Luria broth with 0.4% L-arabinose to stimulate transcription from the *araB* promoter.



This was necessitated because of the instability of the *rpoE::cam* mutation in the MC4100 background (data not shown). Importantly, this change in strain background is not problematic since it does not affect the observed transcriptional regulation of *degP* in response to activation of the Cpx pathway (compare lanes 1 and 2 in Figure 3.7 with lanes 1 and 2 in Figure 3.5).

Since the transcriptional induction of *degP* observed by activation of the Cpx pathway was not entirely dependent on $E\sigma^E$, we were interested in determining the transcriptional-initiation site(s) for the Cpx-mediated induction. S1 nuclease mapping of the transcription start sites of *degP* was performed with RNA prepared from *rpoE*$^+$ strains which contained either an *nlpE* overexpressing plasmid (pND18) or a control plasmid (pBAD18). Figure 3.8 shows the transcripts induced by overexpression of *nlpE*. These transcripts begin between nucleotides 147 and 165 of the published *degP* sequence [Lipinska *et al.* 1988]. Figure 3.8a shows that transcription initiated at position 159, which is attributed to $E\sigma^E$ [Lipinska *et al.* 1988, Erickson and Gross 1989], can be induced by the Cpx pathway. This is consistent with the results presented in Figure 3.7 which indicate that the Cpx pathway can function in concert with $E\sigma^E$ to activate *degP* transcription. Figure 3.8a also shows the induction of other transcripts that initiate near position 159. Presently, we do not know which (if any) of these other transcripts is also a product of $E\sigma^E$-directed transcription. However, we note that activation of the Cpx pathway generates novel protected fragments initiating at positions 147 and 148 which are not present in the uninduced strain (compare lanes 1 and 2 of Figure 3.8a). These fragments may represent the source of the residual transcriptional induction of *degP* by the Cpx pathway in the absence of $E\sigma^E$.

Discussion

The molecular nature of the pleiotropic *cpxA* mutations

cpxA was first identified by mutations which prevented efficient transfer of F plasmids from donor to recipient strains (conjugative plasmid expression) [McEwen and Silverman 1980a]. Subsequent analyses have shown that these alleles, as well as the *cpxA* suppressor mutations

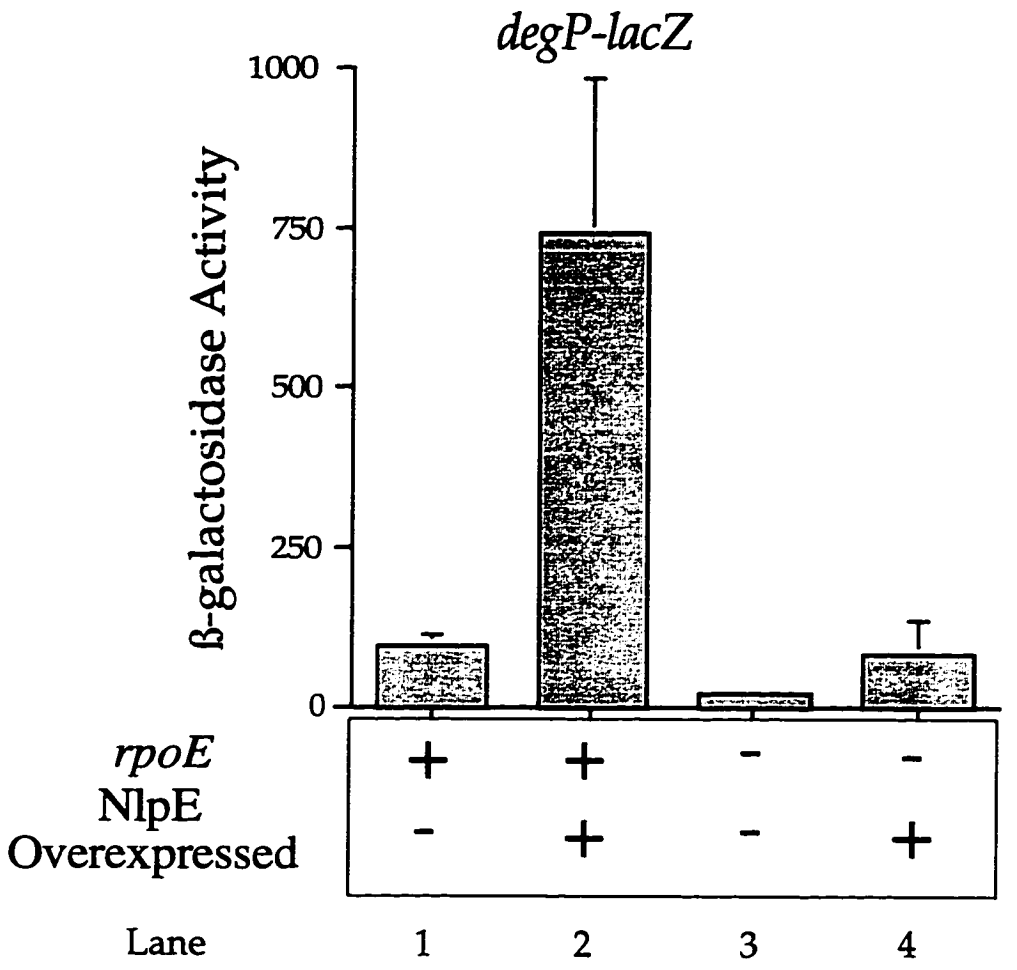
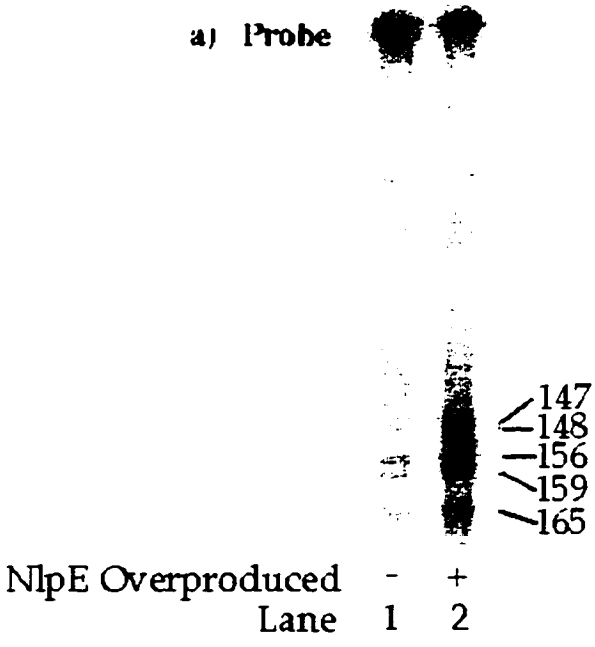


Figure 3.7. Cpx-mediated stimulation of *degP* transcription is partially-dependent on the activity of $E\sigma^E$. Lanes 1 and 3 show ß-galactosidase activity of strains transformed with pBAD18 (control for pND18). Lanes 2 and 4 show ß-galactosidase activity of strains transformed with pND18 (overexpresses *nlpE*). Lanes 1 and 2: PND819 (MC1061 λRS88[*degP-lacZ*]). Lanes 3 and 4: PND818 (MC1061, λRS88[*degP-lacZ*] *rpoE::cam*). All strains were grown at 30°C in Luria broth containing 0.4% L-arabinose and 50µg/ml ampicillin (see Chapter Two for details). Although the standard deviation for the ß-galactosidase activity shown in lane 4 is relatively large, Student's t test for comparison of two means indicates that the difference between the values shown in lanes 3 and 4 is statistically significant at a confidence level greater than 0.99 (Harris 1987).

Figure 3.8. Activation of the Cpx pathway stimulates *degP* transcription at the σ^E promoter and nearby sites. Panel a): Lanes 1 and 2 show the *degP* transcription start sites for strain PND2000 *ara*$^+$ transformed with either a control plasmid, pBAD18 (lane 1), or the *nlpE* overexpressing plasmid, pND18 (lane 2). The undigested probe (340 nucleotides in length) is shown in the figure. The DNA fragments corresponding to transcripts which initiate at positions 147, 148, 156, 159 and 165 of the *degP* sequence (Lipinska *et al.* 1988) are indicated on the right-hand side of the figure. RNA was prepared from strains grown at 30°C in Luria broth containing 0.4% L-arabinose and 50μg/ml ampicillin. See Chapter Two for details. Panel b): The transcription start sites are designated with asterisks (*). The sequence illustrated in Panel b spans from nucleotide 106 to 201 of the published sequence (Lipinska *et al.* 1988). The -10 and -35 sites of the σ^E promoter are underlined and the first codon of the *degP* open reading frame is shown in boldface. Periods (.) are spaced every 10 nucleotides for reference.



b)

5' TTTTGCAGAAACTTTAGTTCGGAACTTCAGGCTATAAAACGAATCTGAAC
AACACAGCAATTTTGCGTTATCTGTTAATCGACACCCAAATACATG 3'

identified in our laboratory (see Figure 3.2), are highly pleiotropic. For example, these *cpxA* mutations confer many (but not necessarily all) of the following phenotypes: decreased stability of inner- and outer membrane proteins, including Lpp and OmpF; low-level resistance to aminoglycosides; impairment of ion-driven transport systems such as those for lactose and proline; inability to grow on non-fermentable carbon sources such as succinate; the ability to utilize L-serine as a carbon source; and isoleucine/valine auxotrophy [McEwen and Silverman 1980b, 1982, Rainwater and Silverman 1990, Dong *et al.* 1993].

Our analysis indicates that the pleiotropic *cpxA* mutations are not null mutations. While the pleiotropic *cpxA* suppressors increase *degP* transcription, a defined *cpxA* null mutation actually decreases *degP* transcription in the absence of cross-phosphorylation by Ac~P. Moreover, the *cpxA* null also unveils the basis for the observed pleiotropy of the *cpxA* missense mutations. These mutant CpxA proteins phosphorylate CpxR in an unregulated manner and ultimately cause CpxR-P to accumulate to high levels. Under these conditions, transcription from genes normally regulated by CpxR is altered and the regulation of genes not normally found in the *cpx* regulon is affected as well. Thus, the pleiotropic mutations probably disrupt the phosphatase activity and/or enhance the kinase activity of CpxA. This is not unprecedented behavior for a two-component sensor. The *E. coli* osmosensor, EnvZ, can be altered by mutation to a phosphatase$^-$ kinase$^+$ species, and this mutant EnvZ hyper-phosphorylates its cognate response regulator, OmpR, ultimately altering transcription from its normal downstream targets, *ompF* and *ompC*, as well as other loci [Slauch *et al.* 1988].

This model also clarifies another observation in the *cpx* literature. Many of the previous phenotypic characterizations of the pleiotropic *cpxA* mutations noted that a large chromosomal deletion encompassing *cpxA* and spanning from *rha* to *pfkA* reverted the pleiotropic phenotypes [Dong *et al.* 1993]. However, the precise meaning of the results obtained with this deletion is confounded by its sheer size. The deletion removes several genes and at least 10 kilobases of DNA [Miller 1992]. From our analysis, it is clear that deletion of both *cpxA* and *cpxR* prevents activation of the Cpx pathway altogether.

The function of the Cpx proteins

While there are a variety of phenotypes conferred by the pleiotropic *cpxA* mutations (see above), it is unclear whether these phenotypes reflect a normal function of the Cpx proteins or whether they are due to aberrant properties of the hyper-activated Cpx pathway. For example, Silverman has examined the effects of *cpxA* mutations on F plasmid transmission. These studies have shown that the pleiotropic *cpxA* mutations decrease the steady-state levels of the cytoplasmic TraJ protein [Silverman *et al.* 1993], providing an explanation for the inefficient transfer. However, deletion of the *cpx* genes reverts the F plasmid transfer phenotypes to a wild-type state [Silverman *et al.* 1993], suggesting that the effects observed on F plasmid transfer may reflect aberrant properties of the hyper-activated Cpx pathway.

In contrast, the data presented here suggest that the Cpx proteins are normally involved in regulating *degP* transcription. First, the *cpxR*$^-$*A*$^-$ double mutant decreases *degP* transcription two-fold, indicating that the Cpx proteins normally contribute to *degP* transcription in a wild-type cell. Second, activation of the Cpx pathway (either by Ac~P or by mutation of *cpxA*) increases *degP* transcription three- to ten-fold. Third, an extracytoplasmic stimulus (*nlpE* overexpression) activates *degP* transcription through the wild-type Cpx proteins, arguing that transcriptional induction of *degP* is a normal function of the Cpx pathway.

While the precise molecular basis for many of the other phenotypes conferred by the pleiotropic *cpxA* mutations remains to be determined, at least a subset of these phenotypes may be attributable to the activation of *degP* transcription. For example, Silverman and colleagues noted that both inner- and outer membrane proteins, including Lpp and OmpF, were destabilized in a *cpxA2* background [McEwen and Silverman 1982, McEwen *et al.* 1983]. It seems likely that this phenotype is due to activation of *degP* transcription. Other phenotypes such as low-level resistance to aminoglycosides and impairment of ion-driven transport systems, also originate from alterations in the physiology of the bacterial envelope [Rainwater and

Silverman 1990]. It is possible that these phenotypes are also due to the destabilization of certain envelope proteins by increased levels of DegP.

The Cpx regulon

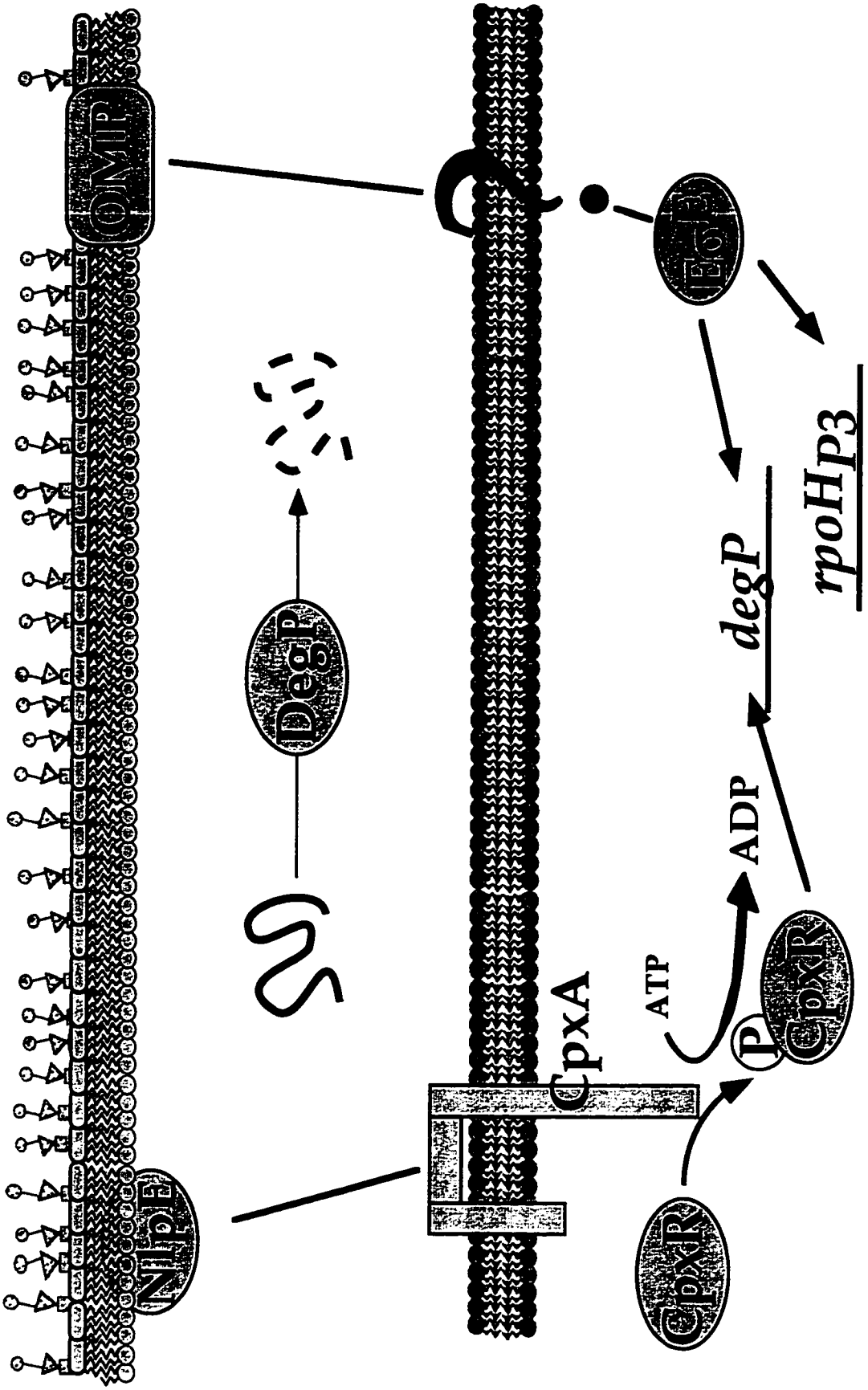
The Cpx pathway stimulates transcription at sites upstream of the *degP* open reading frame, including the site utilized by $E\sigma^E$ (Figures 3.7, 3.8). The simplest explanation for this result is that CpxR binds to a site upstream of the *degP* open reading frame and works in concert with $E\sigma^E$ and perhaps other RNA polymerases to drive *degP* transcription (Figure 3.9). Indeed, CpxR shares homology with other two-component DNA binding proteins, including OmpR and ArcA [Dong *et al.* 1993]. There are greater than 100 nucleotides upstream of the -35 site of *degP* in our *degP-lacZ* operon fusion which could support binding of CpxR. However, direct biochemical analysis is required to test this model.

While activation of the Cpx pathway does not increase transcription from *rpoHp3-lacZ*, it is still possible that the Cpx proteins might influence transcription from *rpoHp3* at its wild-type chromosomal locus. In the *rpoHp3-lacZ* fusion, only the minimal promoter containing the -10 and -35 sites of *rpoHp3* is used to drive transcription [Mecsas *et al.* 1993]. There are no upstream sequences present in this fusion that could potentially support binding of transcription factors. Thus, we do not know whether the Cpx regulon stimulates transcription at other σ^E promoters or whether the Cpx and σ^E regulons simply intersect at *degP*. Indeed, there is reason to believe that *degP* is only one of a group of genes regulated by the Cpx pathway. Although the toxicity conferred by induction of either LamB-LacZ-PhoA or LamBA23D is suppressed by activation of the Cpx pathway, removal of a functional *degP* gene is only partially epistatic to the suppression, indicating that other gene products participate in the observed suppression [Cosma *et al.* 1995, Snyder *et al.* 1995].

What does CpxA sense?

The Cpx proteins regulate *degP* transcription in response to the high-level synthesis of the envelope lipoprotein, NlpE. However, increased levels of lipoproteins in general does not

Figure 3.9. A model for the action of the Cpx signal transduction pathway on *degP* transcription. The CpxA inner-membrane sensor, responds to the level of the NlpE lipoprotein present in the bacterial envelope. CpxA communicates this information to its cognate response regulator, CpxR, which in turn activates *degP* transcription by working in concert with $E\sigma^E$ and perhaps other RNA polymerases. The activity of $E\sigma^E$ is modulated by an unknown signal transduction system in response to the expression level of outer membrane proteins.



induce transcription of *degP-lacZ*, implying that the enhanced transcription of *degP* caused by overexpression of *nlpE* is probably related to the actual function of NlpE. Presently, this function is unknown, but we suggest that NlpE may be regulated in response to some physiological parameter associated with the outer membrane. Alteration of the levels and/or activity of NlpE would be communicated to the Cpx pathway to appropriately alter envelope physiology. We note that NlpE contains a serine protease inhibitor motif [Snyder *et al.* 1995]. If NlpE is indeed a protease inhibitor, CpxA may sense the need for increased expression of *degP* and other gene products when the function of another envelope protease is inhibited.

The results described in this chapter have been published:

Danese, P.N., W.B. Snyder, C.L. Cosma, L.J.B. Davis and T.J. Silhavy. 1995. The Cpx Two-component signal transduction pathway of *Escherichia coli* regulates transcription of the gene specifying the stress-inducible periplasmic protease, DegP. *Genes & Development*, **9**: 387-398.

Snyder, W.B., L.J.B. Davis, P.N. Danese, C.L. Cosma and T.J. Silhavy. 1995. Overproduction of NlpE, a new outer membrane lipoprotein, suppresses the toxicity of periplasmic LacZ by activation of the Cpx signal transduction pathway. *J. Bacteriology*, **177**: 4216-4223.

Cosma, C.L., P.N. Danese, J.H. Carlson, T.J. Silhavy and W.B. Snyder, . 1995. Activation of the Cpx two-component signal transduction pathway in *Escherichia coli* suppresses envelope associated stresses. *Molecular Microbiology*, **18**: 491-505.

Four. *cpxP*, a pH-regulated Member of the Cpx Regulon

INTRODUCTION

The results presented in the previous chapter demonstrate that the Cpx two-component signal transduction pathway controls the transcription of the *degP* locus. In conjunction with this study, we have shown that the Cpx pathway can combat various extracytoplasmic protein-mediated stresses. For example, when highly produced, the LamB-LacZ-PhoA fusion protein forms a massive disulfide-bonded aggregate in the bacterial envelope, ultimately causing cell lysis. Activation of the Cpx pathway suppresses this lethal phenotype [Cosma *et al.* 1995, Snyder *et al.* 1995].

High-level synthesis of the mutated maltoporin, LamBA23D, is also toxic. Specifically, LamBA23D confers upon *E. coli* a hypersensitivity to detergents such as SDS, implying that this protein perturbs the structure of the outer membrane [Carlson and Silhavy 1993]. Activation of the Cpx pathway also suppresses this SDS-hypersensitive phenotype [Cosma *et al.* 1995].

In both of these cases, the Cpx-mediated suppression is partly due to the increased synthesis of the periplasmic protease, DegP. However, tests of epistasis indicate that the activated Cpx system can partially ameliorate these extacytoplasmic toxicities even in the absence of DegP [Cosma *et al.* 1995, Snyder *et al.* 1995]. Thus, the Cpx pathway controls at least one other factor that can combat extractyoplasmic protein-mediated stresses.

The objective of this study was to identify missing members of the Cpx regulon in an effort to further define its stress-combative function. In this chapter, the identification of a new Cpx-regulated locus, *cpxP* is described. *cpxP* is a pH-regulated locus that encodes a periplasmic protein that aids in combating extracytoplasmic protein-mediated toxicity.

RESULTS

Rationale

Since the Cpx proteins control the expression of *degP* at the transcriptional level, and since CpxR is homologous to the OmpR transcription factor, I reasoned that the Cpx signal transduction system would likely control its other regulatory targets at the transcriptional

level as well. Given this reasoning, I screened a collection of *lacZ* operon fusions for those fusions whose transcription could be induced upon activation of the Cpx pathway. Specifically, I generated *lacZ* operon fusions throughout the chromosome of strain PND541 (MC4100, *ara*$^+$, *nadA*::Tn*10*, *Δ[gal-att-bio]*). PND541 also harbors the pND18 plasmid, which expresses the *nlpE* locus from the arabinose promoter, *pBAD* (see Chapters Two and Three for details on pND18). Since overproduction of NlpE activates the Cpx pathway [Chapter Three, Snyder *et al.* 1995], this signal transduction system can be conditionally activated in PND541 simply by growing the strain in the presence of arabinose.

The screen for Cpx-regulated loci

I used λp*lac*Mu53 [see Chapter Two and Bremer *et al.* 1985 for details] to generate *lacZ* operon fusions throughout the chromosome of PND541. λp*lac*Mu53 carries a kanamycin-resistance determinant, allowing a direct selection for the creation of *lacZ* fusions [Bremer *et al.* 1985]. A total of 13,213 colonies harboring stable integrants of λp*lac*Mu53 were individually streaked onto two types of media: 1) Luria agar containing X-Gal, and 2) Luria agar containing X-Gal supplemented with 0.4% L-arabinose [see Chapter Two for details]. Of these 13,213 colonies, 107 displayed a qualitative increase in Lac activity when grown on Luria agar in the presence of L-arabinose. In general, colonies displaying increased Lac activity under these conditions should fall into two classes. 1) The colonies could harbor a *lacZ* fusion that is transcriptionally regulated by an arabinose-inducible promoter. 2) Alternatively, the *lacZ* fusion could be under the control of an NlpE-inducible promoter.

To distinguish between these possibilities I transferred each λp*lac*Mu53-generated *lacZ* fusion from the 107 strains described above, to strain PND900 (MC4100, *ara*$^+$). These 107 strains were then transformed with either pBR322 (control for pLD404) or pLD404. pLD404 constitutively overproduces NlpE and thus, it provides an arabinose-independent means of activating the Cpx pathway [Snyder *et al.* 1995]. Of the 107 strains transformed with pLD404, one harbored a *lacZ* fusion whose transcription was induced by this plasmid. The *lacZ* fusion carried by this strain was renamed *cpxP-lacZ* (*cpx*-pH regulated locus). Figure 4.1 shows that

overproduction of NlpE stimulates *cpxP-lacZ* transcription approximately five-fold (compare lanes 1 and 2). Figure 4.1 also illustrates two other aspects of *cpxP-lacZ* transcription. First, The Cpx system is the major contributor to *cpxP-lacZ* transcription, because the *cpxA* null mutation nearly abolishes transcription of this fusion (compare lanes 1 and 3 in Figure 4.1). Second, in the absence of CpxA, overproduction of NlpE does not stimulate *cpxP-lacZ* transcription (compare lanes 1 and 2 with lanes 3 and 4). Thus, *cpxP-lacZ* transcription is activated by overproduction of NlpE in a CpxA-dependent fashion.

In the absence of CpxA, acetyl-phosphate can mediate the transcriptional induction of *cpxP-lacZ*

As mentioned in Chapter Three, *degP* transcription can be stimulated in the absence of CpxA when acetyl-phosphate (Ac~P) levels are increased by growth in the presence of glucose. This effect is most likely mediated by hyperphosphorylation of CpxR *via* acetyl-phosphate. This phenomenon is also observed with *cpxP*.

Specifically, Figure 4.2 shows that growth in the presence of glucose has little effect on *cpxP* transcription in a wild-type background (compare lanes 1 and 2). In addition, growth in the presence of glucose has little effect on *cpxP* transcription in a *cpxA*$^+$ background that is deleted for the genes responsible for Ac~P synthesis (compare lanes 3 and 4). Lane 5 of Figure 4.2 again shows that the *cpxA*$^-$ mutation drastically reduces *cpxP-lacZ* transcription. However, when this same *cpxA*$^-$ strain is grown in the presence of glucose, *cpxP-lacZ* transcription is stimulated more than 100-fold (compare lanes 5 and 6). This induction is completely eliminated by a mutation that abolishes Ac~P synthesis (Figure 4.2, compare lanes 5 and 6 with lanes 7 and 8). Thus, acetyl-phosphate can mediate the transcriptional induction of *cpxP-lacZ* in the absence of CpxA. This result has two implications. First, it implies that the transcriptional activation of *degP* and *cpxP* by the Cpx system proceeds *via* the same mechanism. Second, it supports the hypothesis presented in Chapter Three that CpxR-phosphate (CpxR-P) is the activating species for the Cpx signal transduction system.

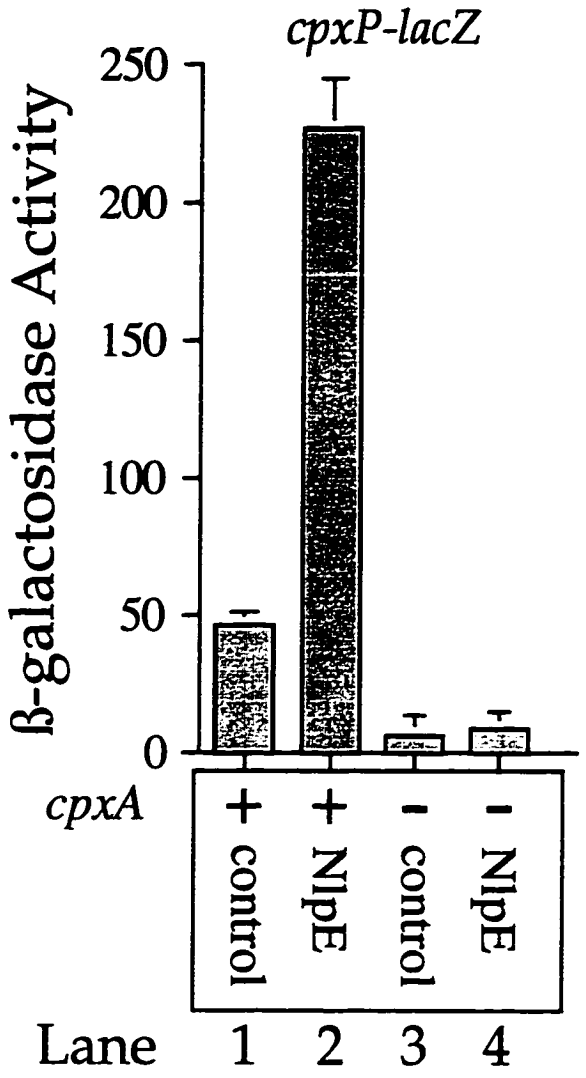
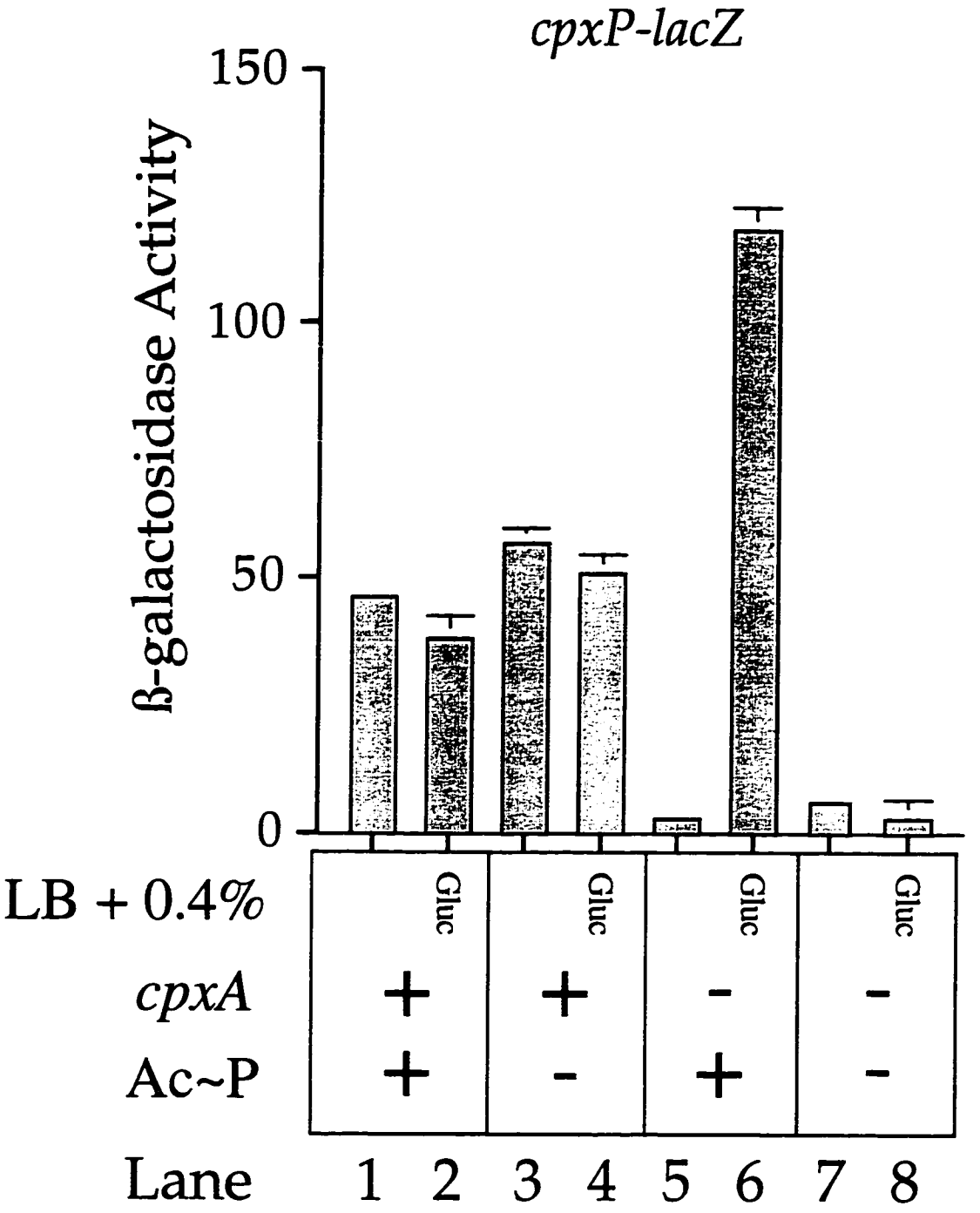


Figure 4.1. *cpxP-lacZ* transcription is induced in a CpxA-dependent fashion by overproduction of the outer-membrane lipoprotein, NlpE. ß-galactosidase activities were determined for SP1 (MC4100, *ara*$^+$, λ*plac*Mu53[*cpxP-lacZ*]) (lanes 1 and 2) and SP7 (SP1, *cpxA::cam*) (lanes 3 and 4). The strains whose ß-galactosidase activities are depicted in lanes 1 and 3 were transformed with the pBR322 plasmid (control for pLD404). The strains whose ß-galactosidase activities are depicted in lanes 2 and 4 were transformed with pLD404 (overproduces NlpE). *cpxP-lacZ* transcription is induced ~ 4.6-fold when NlpE is overproduced in the presence of CpxA (compare lanes 1 and 2). All strains were grown in Luria broth containing 50μg/ml ampicillin as described in Chapter Two.

Figure 4.2. Acetyl phosphate can mediate the transcriptional induction of *cpxP-lacZ* in the absence of CpxA. ß-galactosidase activities were determined for the following strains: SP34 (MC4100, *ara*$^+$, λp*lac*Mu53[*cpxP-lacZ*], *zej*::Tn*10*) (lanes 1 and 2), SP35 (SP34, Δ[*pta, ackA, hisQ, hisP*]) (lanes 3 and 4), SP36 (SP34, *cpxA::cam*) (lanes 5 and 6), SP37 (SP34, *cpxA::cam*, Δ[*pta, ackA, hisQ, hisP*]) (lanes 7 and 8). Strains whose ß-galactosidase activities are depicted in odd-numbered lanes were grown in Luria broth. Strains whose ß-galactosidase activities are depicted in even-numbered lanes were grown in Luria broth supplemented with 0.4% glucose to stimulate acetyl-phosphate production. Growth in the presence of glucose stimulates *cpxP-lacZ* transcription ~ 100-fold in the absence of CpxA (compare lanes 5 and 6).



σ^E does not regulate *cpxP* transcription

The results described in Chapter Three indicate that the Cpx and σ^E regulatory systems both control transcription of the *degP* locus. Based on this result alone, it is not clear whether all members of the Cpx regulon are also controlled by σ^E or whether these systems share only a subset of common regulatory targets. Accordingly, I was interested in determining if *cpxP* transcription was also regulated by σ^E.

To address this issue, SP1 (MC4100, *ara*$^+$, λp*lac*Mu53[*cpxP-lacZ*]) was transformed with plasmids that overproduce the outer membrane porin proteins, OmpF and OmpC. Mecsas *et al.* [1993] have shown that overproduction of these outer membrane proteins will stimulate σ^E activity. However, these plasmids have no effect on *cpxP* transcription when compared with their respective control plasmids (data not shown).

In a complementary analysis, I also introduced an *rpoE* null mutation, which abolishes σ^E synthesis [Hiratsu *et al.* 1995, Raina *et al.* 1995, Rouvière *et al.* 1995], into SP1, and assayed its effect on *cpxP* transcription. The *rpoE* null mutation did not decrease *cpxP* transcription (data not shown), indicating that *cpxP* transcription is not regulated by σ^E. Thus, the σ^E and Cpx regulons intersect, but do not completely overlap.

Identification of the *cpxP* locus

Since the *cpxP* locus is regulated by the Cpx signal transduction system, I was interested in identifying the *cpxP* open reading frame. I mapped the location of the *cpxP-lacZ* fusion to the 88 to 89 minute region of the *E. coli* chromosome. Surprisingly, I found that the *cpxP-lacZ* fusion was tightly linked to the *cpxR*::Ω insertion (> 99.5% linkage by P1 transduction).

Because of the tight linkage between the *cpxP-lacZ* fusion and the *cpx* operon, I directly sequenced the region surrounding *cpxR* and found that the *cpxP-lacZ* fusion is inserted within the open reading frame of the gene immediately upstream of *cpxR*. This open reading frame was previously referred to as ORF_*o167* [Plunkett *et al.* 1993].

cpxP specifies a 19.1 kDa protein with no homology to any other sequence presently in the databases. Figure 4.3 shows the *cpxP* DNA sequence, the corresponding predicted amino acid sequence and other salient features of this locus. For example, the fusion joint that creates *cpxP-lacZ* is positioned within the 13th codon of the *cpxP* open reading frame (see Figure 4.3), disrupting the final 93% of this open reading frame. Hence, strains containing this λp*lac*Mu53-generated fusion are also *cpxP*$^-$.

Moreover, the first 23 codons of the *cpxP* open reading frame appear to specify a signal sequence. Specifically, the predicted amino acid sequence of this region begins with a basic residue (R), followed by a hydrophobic stretch of 20 residues ending in an alanine, which is immediately followed by an acidic residue (E). These features are the hallmark of a signal sequence, and they imply that *cpxP* encodes an exported protein [Murphy and Beckwith 1996].

cpxP transcription is regulated in _trans_ by the Cpx proteins

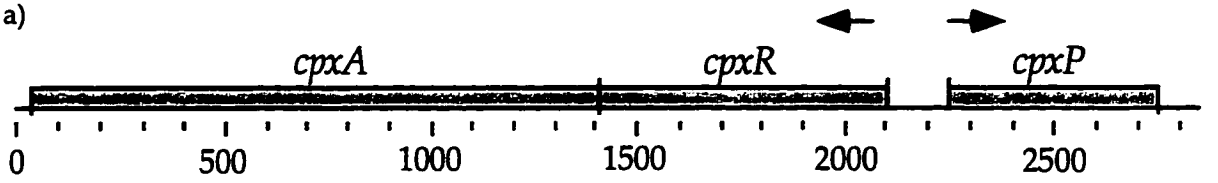
While it is clear that the *cpxA::cam* mutation drastically reduces *cpxP-lacZ* transcription, it remained formally possible that this reduction was due to a *cis*-dominant effect conferred by the nearby *cpxA::cam* mutation. However, this is not the case. Transcription from the *cpxP-lacZ* fusion is restored in a *cpxA::cam, recA*$^-$ strain when a complementing copy of *cpxA* is provided in *trans* from a low-copy number plasmid (data not shown).

In addition, I generated a new *cpxP-lacZ* fusion *de novo* (see Chapter Two for details) and situated it at the λ*att* site and away from the *cpx* operon. This fusion behaves in a qualitatively similar manner to the λp*lac*Mu53-generated *cpxP-lacZ* fusion (Figure 4.4).

For example, Figure 4.4a shows that the *cpxR::Ω* mutation abolishes transcription of the *cpxP-lacZ* fusion situated at the λ*att* site (compare lanes 1 and 3). Note that because of the tight linkage between *cpxP* and *cpxR*, I was unable to create a strain harboring both λp*lac*Mu53[*cpxP-lacZ*] and the *cpxR::Ω* mutation. The *Ω* cassette is inserted within the 21st codon of the *cpxR* open reading frame, leaving only 246 nucleotides between the *Ω* cassette and the *cpxP-lacZ* fusion (Figure 4.3b). Figure 4.4b also shows that overproduction of NlpE stimulates transcription of the *cpxP-lacZ* fusion situated at λ*att* in a CpxA-dependent fashion

Figure 4.3. The *cpxP* locus. a) the *cpxP* open reading frame is shown in relation to the *cpx* operon. The *cpx* operon and *cpxP* are divergently transcribed as shown by the arrows. The size of this genomic region is also shown in nucleotides. b) The nucleotide and deduced amino acid sequence of the *cpxP* open reading frame. The site of the *cpxR::Ω* insertion is marked with the *Ω* symbol. The start codon of *cpxR* (which is shown in reversed typeface) is depicted by a leftward-pointing arrow. The deduced primary amino acid sequence of *cpxP* is shown below the nucleotide sequence. A putative Shine-Dalgarno sequence (GGGAG) is enclosed within a box. The residues comprising the putative CpxP signal sequence are shown in boldface. The position of the λp*lac*Mu53[*cpxP-lacZ*] fusion joint is marked by a downward-pointing arrow within the 13th codon of the *cpxP* open reading frame. An asterisk (*) marks the stop codon of the *cpxP* open reading frame. A putative ρ-independent transcriptional terminator stem loop is underlined with inverted arrows. The adjacent sequence of eight consecutive thymine nucleotides in this putative ρ-independent transcriptional terminator is underlined. The nucleotide sequence shown in part b corresponds to position 67200 to position 68039 of the published DNA sequence for the *E. coli* chromosomal region from 87.2 to 89.2 minutes. The accession number for this sequence is L19201.

a)



b)

Ω

1
TCCCCATCGTGGGCAACAATCACGTTGAAGCCTTCCATCTCGAGCAGCTCCTTTAATAGG

Яхφ

GAAGTCAGCTCTCGGTCATCATCAACTAACAGGATTTTATTCATTGTTTAAATACCTCCG

AGGCAGAAATTACGTCATCAGACGTCGCTAATCCATGACTTTACGTTGTTTTACACCCCC

TGACGCATGTTTGCAGCCTGAATCGTAAACTCTCTATCGTTGAATCGCGACAGAAAGATT

'trp''lacZYA

TTGGGAGCAAATGATGCGCATAGTTACCGCTGCCGTCATGGCCTCAACGCTGGCAGTCAG
M M R I V T A A V M A S T L A V S

TTCATTAAGCCACGCTGCTGAAGTCGGTTCAGGCGATAACTGGCATCCGGGTGAAGAACT
S L S H A A E V G S G D N W H P G E E L

TACGCAGCGCAGTACGCAGAGCCATATGTTCGACGGCATAAGTTTAACCGAACATCAGCG
T Q R S T Q S H M F D G I S L T E H Q R

TCAGCAGATGCGAGATCTTATGCAACAGGCCCGGCACGAACAGCCTCCTGTTAATGTTAG
Q Q M R D L M Q Q A R H E Q P P V N V S

CGAACTGGAGACAATGCATCGCCTTGTCACCGCAGAAAATTTTGATGAAAACGCTGTGCG
E L E T M H R L V T A E N F D E N A V R

CGCACAGGCAGAAAAAATGGCGAATGAGCAAATTGCTCGTCAGGTTGAGATGGCAAAAGT
A Q A E K M A N E Q I A R Q V E M A K V

CCGCAACCAAATGTATCGCCTGTTAACGCCGGAGCAGCAAGCGGTTTTAAACGAGAAACA
R N Q M Y R L L T P E Q Q A V L N E K H

TCAACAACGAATGGAGCAGTTGCGTGACGTGACGCAATGGCAAAAAAGTTCATCGTTGAA
Q Q R M E Q L R D V T Q W Q K S S S L K

GCTATTGAGTAGTAGCAACTCACGTTCCCAGTAGTAAACCCTGTTTTCCTTGCCATAGAC
L L S S S N S R S Q *

840

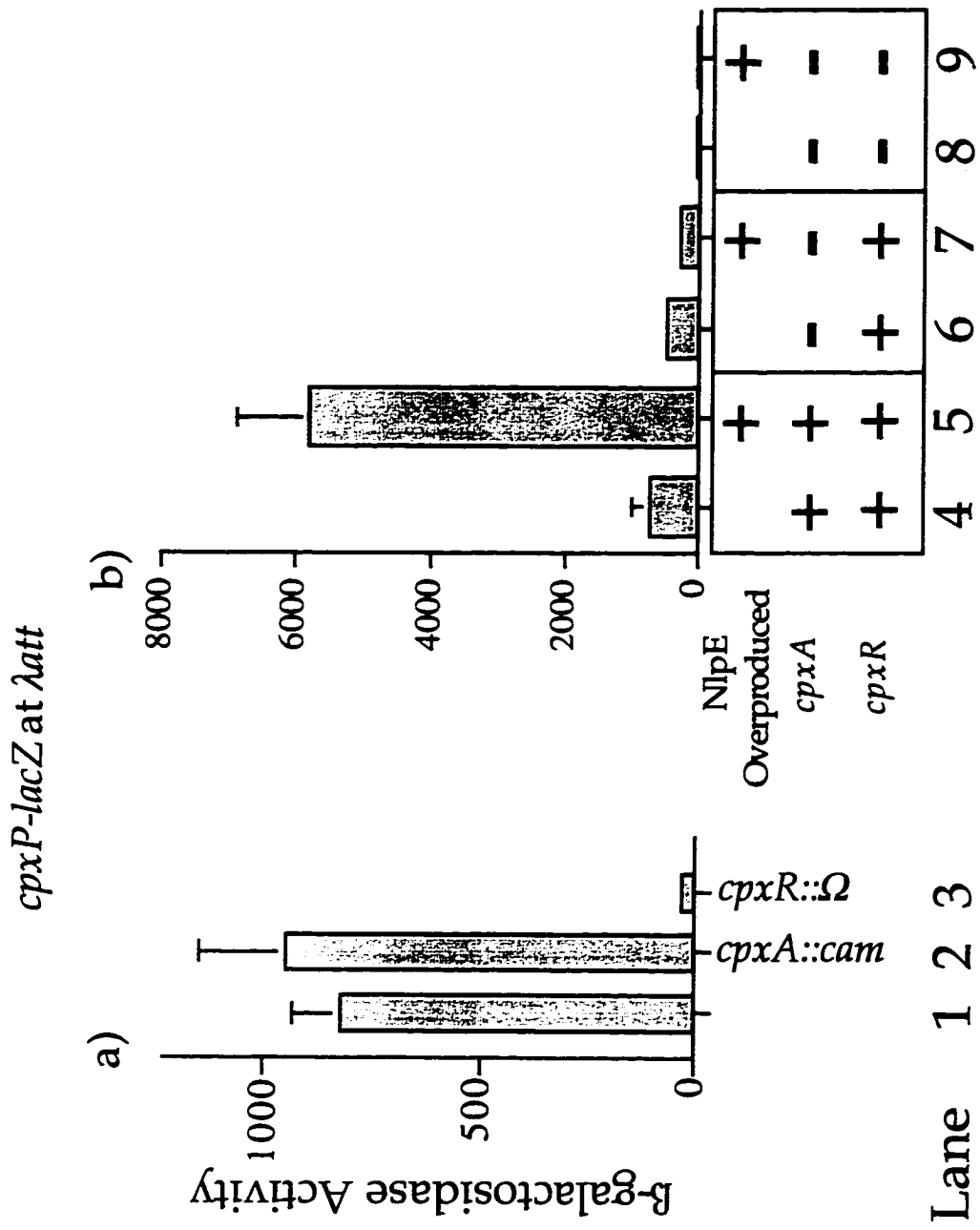
ACCATCCCTGTCTTCCCCCACATGCTGTGGGGGTTTTTTTTATCCTCAATTTGCCTGCTG

(compare lanes 4 and 5 with 6 and 7; also compare Figure 4.4b with Figure 3.5). Thus, the *cpxP-lacZ* fusion is controlled by the Cpx system in *trans*.

There is one quantitative difference between the *cpxP-lacZ* fusion situated within the *cpxP* open reading frame (λp*lac*Mu53[*cpxP-lacZ*]) and the *cpxP-lacZ* fusion at λ*att* (λRS88[*cpxP-lacZ*]). Specifically, while the *cpxA* null mutation reduces transcription from λp*lac*Mu53[*cpxP-lacZ*] (Figure 4.1, compare lanes 1 and 3), it does not reduce transcription from λRS88[*cpxP-lacZ*] (Figure 4.4a compare lanes 1 and 2). There are several possible explanations for this discrepancy. First, since λRS88[*cpxP-lacZ*] does not disrupt the *cpxP* open reading frame, it is possible that the *cpxP* gene product can stimulate *cpxP* transcription in the absence of *cpxA*. A prediction of this model is that transcription from λRS88[*cpxP-lacZ*] would be reduced in a *cpxA*$^-$ *cpxP*$^-$ background. Alternatively, the *cpxA*-independent transcription of λRS88[*cpxP-lacZ*] may result from the altered position of the *cpxP-lacZ* fusion within the *E. coli* genome (λ*att* vs. *cpxP*) and/or the different λ phages used to generate λp*lac*Mu53[*cpxP-lacZ*] and λRS88[*cpxP-lacZ*]. For example, λp*lac*Mu53 contains a two kilobase region of the *trp* operon between its *lac* DNA sequence and the fusion joint. This large portion of DNA could conceivably modify the succeptibility of λp*lac*Mu53[*cpxP-lacZ*] to regulation by CpxA. In addition, because the λp*lac*Mu53[*cpxP-lacZ*] fusion is situated at the *cpxP* locus, this fusion contains all of the upstream *cis*-acting regulatory DNA sequences that normally regulate transcription of *cpxP*. This is not true for λRS88[*cpxP-lacZ*], which contains DNA up to nucleotide -410 with respect to the *cpxP* translational initiation site [see Chapter Two for details].

To help distinguish amongst these possibilites, I generated a *cpxP::kan* deletion/insertion allele (see Chapter Two for details) and introduced this mutation into SP594 (MC4100, λRS88[*cpxP-lacZ*]) and SP619 (SP594, *cpxA::cam*). In both of these backgrounds, the *cpxP::kan* mutation does not alter transcription from λRS88[*cpxP-lacZ*] (data not shown). Thus, it seems more likely that *cpxP* transcription is altered at λ*att* because of a chromosomal position effect and/or because of the different λ phages used to generate each *lacZ* fusion. For

Figure 4.4. The Cpx signal transduction system regulates transcription of a *cpxP-lacZ* fusion situated at the *λatt* site on the *E. coli* chromosome. a) The *cpxR* null mutation abolishes transcription of a *cpxP-lacZ* fusion situated at the *λatt* site on the *E. coli* chromosome. ß-galactosidase activities of the following strains were determined: SP594 (MC4100, λRS88[*cpxP-lacZ*]) (lane 1), SP619 (SP594, *cpxA::cam*) (lane 2), SP620 (SP594, *cpxR::Ω).* b) Overproduction of NlpE induces transcription of a *cpxP-lacZ* fusion situated at the *λatt* site on the *E. coli* chromosome. Lanes 4, 6 and 8 show ß-galactosidase activities of strains transformed with pBAD18 (control for pND18). Lane 5, 7 and 9 show ß-galactosidase activities of strains transformed with pND18 (overexpresses *nlpE*). Lanes 4 and 5: SP702 (MC4100, *ara*$^+$, *zej*::Tn*10*, Δ[*pta, ackA, hisQ, hisP*], λRS88[*cpxP-lacZ*]). Lanes 6 and 7: SP704 (SP702, *cpxA::cam*). Lanes 8 and 9: SP706 (SP702, *cpxR::Ω*). All strains were grown in Luria broth containing 0.4% L-arabinose and 50μg/ml ampicillin (see Chapter Two for details). The experiments whose results are depicted in Figure 5.3b were performed with strains deleted for *pta* and *ackA*. Since NlpE synthesis is driven from the *araB* promoter (Guzman *et al.* 1992) in this experiment, full transcriptional induction requires growth in arabinose. Hence, Ac~P synthesis must be eliminated to prevent hyper-phosphorylation of CpxR in the *cpxR*$^+$*A*$^-$ background.



example, it could be that at this position in the chromosome (*λatt*), less CpxR-phosphate is required to stimulate *cpxP* transcription. In this scenario, the basal level of CpxR-phosphate present in a *cpxA* null background would be sufficient to direct *cpxP* transcription. In this scenario, of course, *cpxP-lacZ* transcription would still be abolished in a *cpxR*$^-$ background. Note, however, that despite this one difference, analysis of *cpxP* transcription generated from λRS88[*cpxP-lacZ*] demonstrates that CpxA regulates *cpxP* transcription in *trans*.

CpxP is an Exported Protein

Since the deduced amino acid sequence suggested that the CpxP protein contained a signal sequence, I wanted to determine the subcellular location of this protein. To this end, I fused the first 71 codons of the *cpxP* open reading frame to the alkaline phosphatase coding sequence (lacking a signal-sequence coding region), thus generating a CpxP-AP protein fusion (see Chapter Two for details). I then determined: 1) the amount of alkaline phosphatase activity generated by this fusion, and 2) the subcellular location of the CpxP-AP protein fusion. Since alkaline phosphatase is only active in relatively oxidizing subcellular compartments like the periplasm, the alkaline phosphatase activity generated by the CpxP-AP fusion provides an indirect assessment of the subcellular location of CpxP.

Figure 4.5 shows the results of alkaline phosphatase assays performed on SP627a (MC4100, *ara74::cam*) harboring: 1) control plasmids that do not produce alkaline phosphatase fusion proteins, 2) plasmid pND24 which produces the CpxP-AP fusion, or 3) pCH215. pCH215 produces a SecY-AP fusion protein, in which the alkaline phosphatase moiety is fused to cytoplasmic loop 5 of the inner-membrane protein, SecY [Dr. Chris Harris, unpublished plasmid]. The SecY-AP fusion serves as an example of an AP protein fusion that is not situated in an oxidizing compartment. Accordingly, this fusion should display little to no alkaline phosphatase activity.

Figure 4.5a shows that the only strain displaying an appreciable amount of alkaline phosphatase activity is the CpxP-AP fusion (lane 2). Note that the alkaline phosphatase assays used to generate the data illustrated in Figure 4.5 were performed in the presence of 5mM

Figure 4.5. The CpxP-alkaline phosphatase fusion protein (CpxP-AP) possesses relatively high alkaline phosphatase activity. Strain SP627a (MC4100 *ara74::cam*) was transformed with pBR322 (control for pND24) (lanes 1 and 5), pND24 (produces CpxP-AP) (lanes 2 and 6), pBAD18 (control for pCH215) (lanes 3 and 7) and pCH215 (produces a SecY-alkaline phosphatase fusion protein, SecY-AP) (lanes 4 and 8). The transformants were grown in Luria broth containing 50μg/ml ampicillin supplemented with 0.4% L-arabinose to induce the synthesis of the SecY-AP from pCH215. a) The CpxP-AP fusion protein displays relatively high alkaline phosphatase activity. The alkaline phosphatase activities of each of these four transformant strains were determined in the presence of 5mM iodoacetamide (lanes 1 to 4). Iodoacetamide covalently modifies free cysteine sulfhydryl groups, thus preventing spontaneous activation of non-exported alkaline phosphatase molecules after cell lysis [Derman and Beckwith 1991] (see Chapter Two for details). b) The amount of alkaline phosphatase cross-reacting species generated by the CpxP-AP and SecY-AP fusion proteins is comparable. Immunoblot analysis was performed on whole-cell protein extracts generated from SP627a transformed with pBR322, pND24, pBAD18 and pCH215 (lanes 5 to 8). The whole-cell extracts were separated by SDS-polyacrylamide gel electrophoresis (equivalent O.D.$_{600}$ units were loaded in each lane) and subjected to immunoblot analysis with anti-alkaline phosphatase and anti-OmpR antisera. OmpR serves as an additional loading control.

iodoacetamide (IAA). IAA covalently bonds to free sulfhydryl groups and as a consequence, it prevents the spontaneous activation of reduced (cytoplasmic) alkaline phosphatase molecules after cell lysis [Derman *et al.* 1991]. Thus, all of the alkaline phosphatase activity observed in this experiment is due to exported alkaline phosphatase molecules.

The data of Figure 4.5a imply that the CpxP-AP fusion protein is exported to an oxidizing compartment. However, when performing such alkaline phosphatase assays, it is important to correct the observed amount of alkaline phosphatase activity for the amount of fusion protein that is responsible for generating this activity. Figure 4.5b shows the results of an immunoblot of whole cell protein extracts from the four strains whose alkaline phosphatase activities were determined in Figure 4.5a. The whole cell extracts were probed with anti-alkaline phosphatase and anti-OmpR antisera (The OmpR protein serves as an internal loading control). Figure 4.5b shows that the amount of alkaline phosphatase cross-reacting material generated in the CpxP-AP producing strain is less than that generated in the SecY-AP producing strain (compare lanes 6 and 8). This result substantiates the hypothesis that, unlike the SecY-AP fusion protein, CpxP-AP is exported to an extracytoplasmic oxidizing compartment.

The CpxP-AP protein fusion is localized to the periplasm

To determine the precise subcellular location of CpxP-AP, I fractionated proteins from a strain producing this fusion protein into whole-cell, spheroplast and periplasmic fractions. Figure 4.6 lane 1 shows an immunoblot of protein fractions prepared from SP627a transformed with: 1) pBR322 (control for pND24, lane 1) and 2) pND24 (produces CpxP-AP, lanes 2 through 4). The immunoblot was probed with anti-alkaline phosphatase, anti-MalE, and anti-OmpR antisera. MalE and OmpR serve as model periplasmic and cytoplasmic proteins, respectively.

Lane 1 of Figure 4.6 shows the endogenously encoded wild-type alkaline phosphatase, MalE and OmpR proteins from whole-cell protein extracts of SP627a transformed with pBR322 (control for pND24). Lane 2 of Figure 4.6 shows the CpxP-AP fusion, MalE and OmpR proteins from whole-cell protein extracts. Note that there are two bands generated by the CpxP-AP

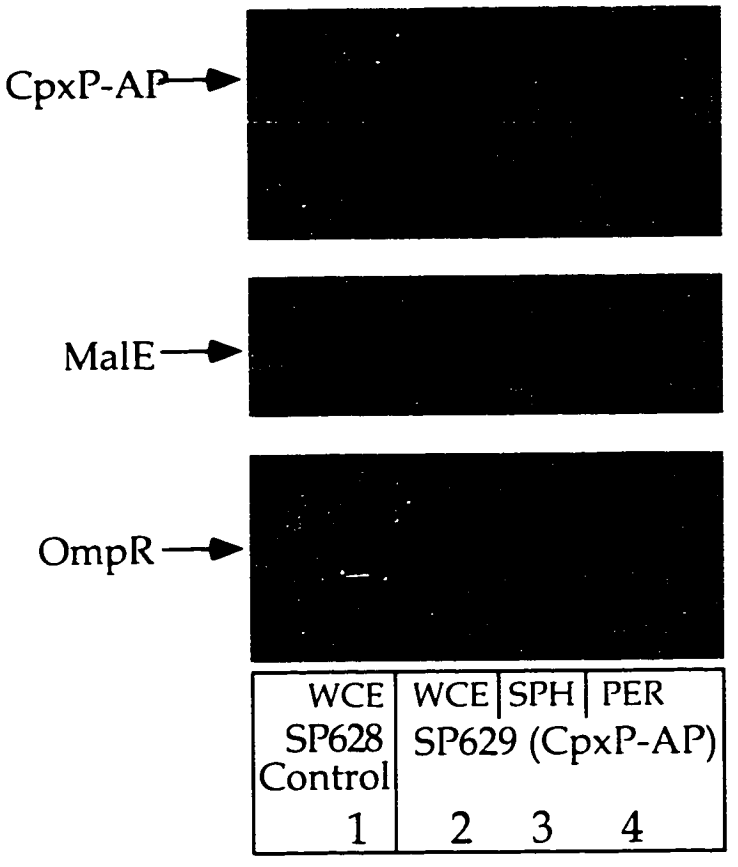


Figure 4.6. Subcellular fractionation of the CpxP-alkaline phosphatase fusion protein (FinE-AP). Whole-cell protein extracts were prepared from strain SP627a (MC4100, *ara74::cam*) transformed with plasmid pBR322 (control for pND24) (lane 1). Whole-cell-, spheroplast- and periplasmic extracts were prepared from SP627a transformed with pND24 (lanes 2 to 4). The protein extracts were separated by SDS polyacrylamide gel electrophoresis and subjected to immunoblot analysis with anti-alkaline phosphatase, anti-MalE and anti-OmpR antisera. Abbreviations: WCE, whole-cell extract; SPH, spheroplast extract; PER, periplasmic extract. MalE and OmpR serve as model periplasmic and cytoplasmic proteins, respectively. Both strains were grown to late-log phase in M63 minimal medium supplemented with 0.4% maltose and 50μg/ml ampicillin. Protein extracts were then generated as described in Chapter Two.

fusion. It seems most likely that the lower band represents a degradation product of the CpxP-AP protein fusion. Nevertheless, the majority of the upper band, and the entire portion of the lower band is found in the periplasmic protein fraction, indicating that CpxP-AP is predominantly a periplasmic protein (compare lanes 3 and 4 of Figure 4.6). As expected, MalE is found within the periplasmic protein fraction, while OmpR is predominantly found in spheroplast protein fractions (compare lanes 3 and 4 of Figure 4.6).

The periplasmic location of CpxP is consistent with our proposed function for the Cpx signal transduction system. Specifically, if the Cpx regulon is involved in combating protein-mediated toxicities in the bacterial envelope, I would expect the members of the Cpx regulon to be found within the envelope. This prediction is clearly supported by the subcellular location of both DegP and CpxP.

CpxP is a stress-combative member of the Cpx regulon

Since CpxP encodes a periplasmic protein, and since *cpxP* transcription is controlled by the Cpx system, I was interested in determining if CpxP was an extracytoplasmic-stress-combative factor. To address this issue, I created three derivatives of WBS164 (MC4100, Φ(*lamB-lacZX90*) Hyb10-1[λp1(209)]): SP9 (WBS164, *degP*::Tn*10*), SP10 (WBS164, λp*lac*Mu53[*cpxP-lacZ*]) and SP24 (WBS164, *degP*::Tn*10*, λp*lac*Mu53[*cpxP-lacZ*]). Recall that λp*lac*Mu53[*cpxP-lacZ*] disrupts the *cpxP* open reading frame, and thus, strains carrying this fusion are also *cpxP*$^-$.

LamB-LacZX90 is a derivative of the LamB-LacZ hybrid fusion that contains a late nonsense mutation near its carboxy-terminus [Snyder and Silhavy 1995]. Much like the LamB-LacZ-PhoA tribrid fusion protein, LamB-LacZX90 forms a disulfide bonded aggregate in the bacterial envelope and causes cell lysis as a result of its export to the envelope [Snyder and Silhavy 1995]. Activation of the Cpx pathway suppresses this lethal phenotype [Snyder 1995].

To determine if CpxP functions to ameliorate the toxicity associated with LamB-LacZX90, I transformed WBS164, SP9, SP10 and SP24 with either pBR322 (control for pLD404)

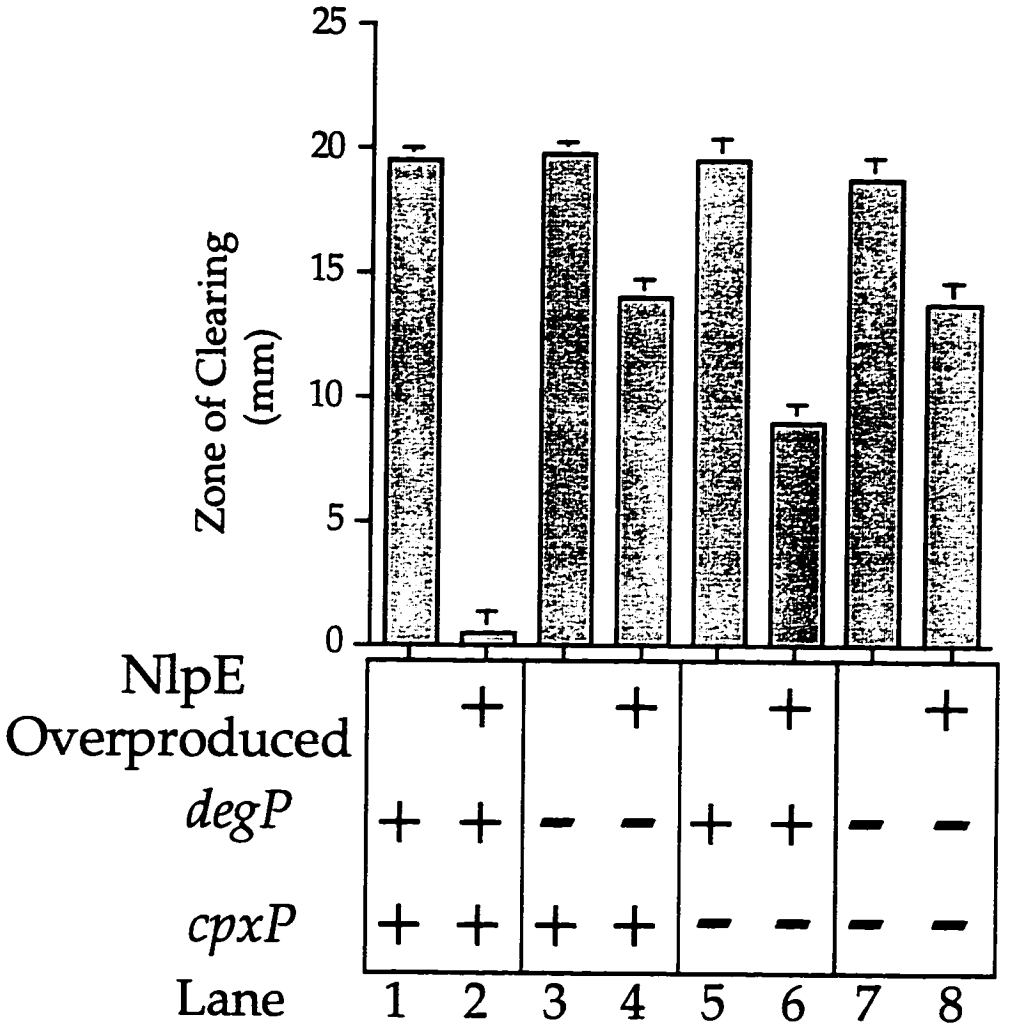
or pLD404 (overproduces the envelope lipoprotein NlpE). Previous studies have shown that overproduction of NlpE alleviates the toxicity associated with LamB-LacZX90 by activating the Cpx pathway [Snyder 1995].

I then quantified the susceptibility of each of the eight transformed strains to growth in the presence of maltose, which induces synthesis of LamB-LacZX90. Figure 4.7 shows the zone of growth inhibition caused by the addition of 10µl of 40% maltose to a filter disc that was placed on a lawn of each of the eight transformed strains. The larger the zone of inhibition, the more susceptible the strain is to the expression of LamB-LacZX90.

Lane 1 of Figure 4.7 shows that the parent strain, WBS164, transformed with the control vector, pBR322, is exquisitely maltose sensitive. Lane 2 shows that WBS164 transformed with the NlpE-overproducing plasmid (pLD404) is protected from the synthesis of LamB-LacZX90. Lane 4 shows that SP9 (WBS164, *degP*::Tn*10*) transformed with the NlpE-overproducing plasmid is more maltose-sensitive than the isogenic *degP*$^+$ strain (compare lanes 2 and 4). However, this strain is still less maltose-sensitive than the parent strain (WBS164) transformed with the pBR322 control vector. Thus, as previously described [Cosma *et al.* 1995, Snyder *et al.* 1995], the Cpx signal transduction system utilizes DegP (as well as other factors) to combat the extracytoplasmic toxicity conferred by LamB-LacZX90.

Lane 6 of Figure 4.7 shows that SP10 (WBS164, λp*lac*Mu53[*cpxP-lacZ*]) transformed with the NlpE-overproducing plasmid is also more maltose-sensitive than WBS164 transformed with the same NlpE-overproducing plasmid (compare lanes 2 and 6). Thus, *cpxP* is also utilized by the Cpx signal transduction system to combat the toxicity associated with LamB-LacZX90. Finally, when SP24 (WBS164, *degP*::Tn*10*, λp*lac*Mu53[*cpxP-lacZ*]) is transformed with the NlpE-overproducing plasmid, the strain is no more maltose sensitive than SP9 (WBS164, *degP*::Tn*10*) transformed with the same plasmid (compare lanes 4, 6 and 8). This final result has two implications. First, it suggests that CpxP and DegP may function within the same pathway to combat the toxicity associated with LamB-LacZX90. Second, since the *degP*$^-$, *cpxP*$^-$ double mutation does not completely abolish the ability of NlpE

Figure 4.7. CpxP combats extracytoplasmic stress. 10µl of 40% maltose was added to filter discs that had been placed on lawns of the following strains: WBS164 (MC4100 Φ(*lamB-lacZX90*) Hyb10-1[λp1(209)]) (lanes 1 and 2), SP9 (WBS164, *degP*::Tn*10*) (lanes 3 and 4), SP10 (WBS164, λp*lac*Mu53[*cpxP-lacZ*]) (lane 5 and 6), SP24 (WBS164, *degP*::Tn*10*, λp*lac*Mu53[*cpxP-lacZ*]) (lanes 7 and 8). Strains in odd-numbered lanes were transformed with pBR322 (control for pLD404). Strains in even-numbered lanes were transformed with pLD404 (overproduces NlpE and activates the Cpx signal transduction pathway). The values displayed along the Y-axis (zone of clearing) represent the amount of growth inhibition caused by the addition of maltose. The zone of clearing value is defined as the diameter of growth inhibition around the maltose-saturated filter disc, minus the diameter of the filter disc itself (7mm). The maltose disc assays were performed on M63 minimal agar containing 50µg/ml ampicillin and 0.2% glycerol as a carbon source.



overproduction to ameliorate the LamB-LacZX90-associated toxicity, the Cpx signal transduction system must control still other factors that can combat this toxicity.

cpxP transcription is stimulated by extracellular alkaline pH

Recently, Nakayama and Watanabe [1995] demonstrated that the *cpx* locus altered the pH-regulation of the *Shigella sonnei* virulence locus, *virF*. When introduced into *E. coli*, *virF* transcription is repressed at pH 6.0 and induced at pH 7.4. However, in a *cpxA* null mutant strain, *virF* transcription is derepressed at pH 6.0. Interestingly, a *cpxR* null mutation abolishes *virF* transcription altogether at both pH 6.0 and 7.4. Taken together, these results suggest that the activity of the Cpx signal transduction system may be affected (either directly or indirectly) by extracellular pH.

This study prompted us to examine the transcriptional regulation of *cpxP* as a function of pH. To this end, I determined the ß-galactosidase activity of SP1 (MC4100, *ara*$^+$, λp*lac*Mu53[*cpxP-lacZ*]) and SP7 (SP1, *cpxA::cam*) as a function of pH. Specifically, SP1 and SP7 were grown in Luria broth buffered with 100 mM sodium phosphate ranging in pH from 5.3 to 8.4, and the resulting ß-galactosidase activities of these strains were determined. From this analysis, I found that the amount of *cpxP* transcription rises almost 50-fold from pH 5.3 to 8.4 (squares of Figure 4.8). This transcriptional induction is not observed in the *cpxA::cam* background (circles of Figure 4.8), indicating that this phenomenon is dependent on CpxA. Note that the λRS88[*cpxP-lacZ*] fusion is induced by alkaline pH in a qualitatively similar fashion as is observed with λp*lac*Mu53[*cpxP-lacZ*]. However, in the case of λRS88[*cpxP-lacZ*], the induction of *cpxP* transcription is approximately seven fold when comparing the amount of transcription generated at pH 6.0 to the amount of transcription generated at pH 8.5 (data not shown).

The cytoplasmic pH of *E. coli* is maintained at an approximate value of 7.7 irrespective of the external pH [Harold and Maloney 1996]. As a consequence of this homeostatic phenomenon, the ΔpH component of the proton-motive force (PMF) diminishes as the external pH rises. Because of this effect, it was formally possible that the alkaline-pH-mediated

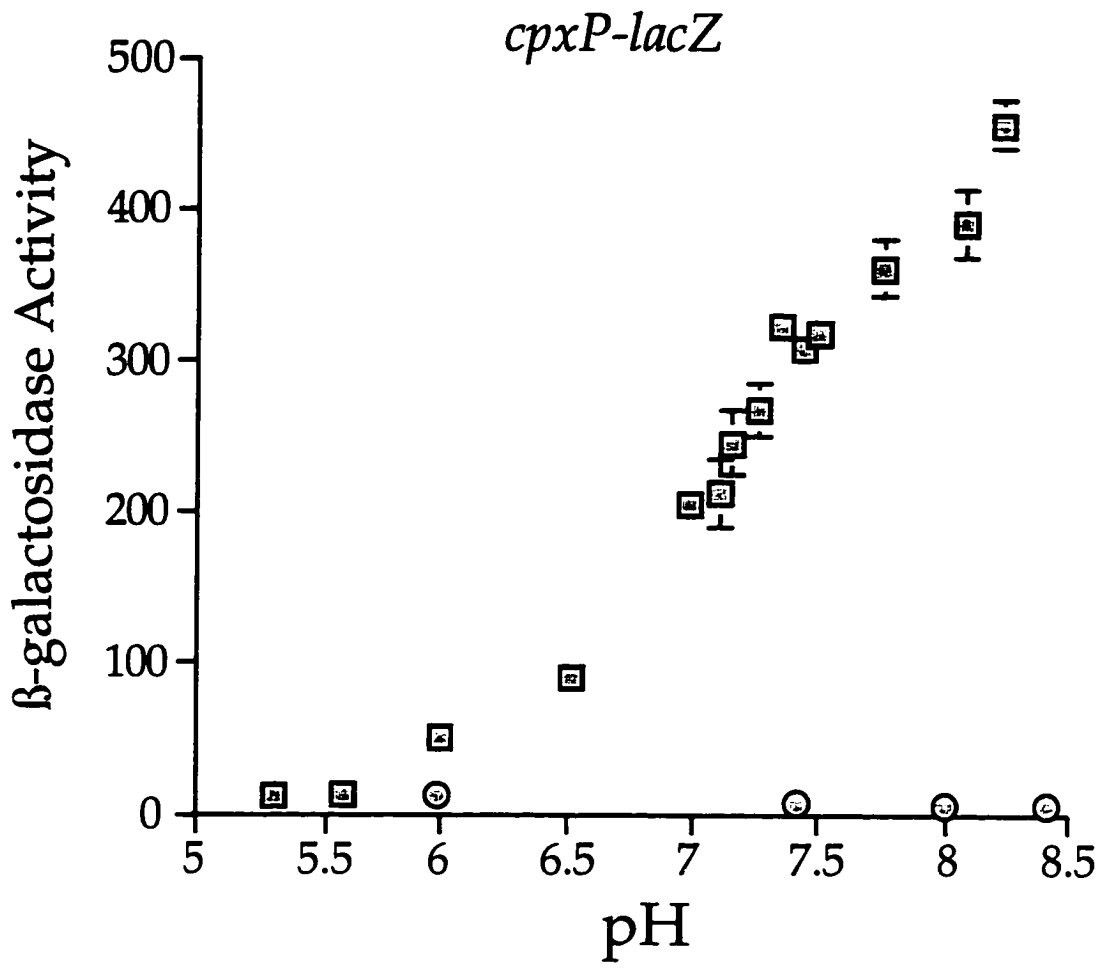


Figure 4.8. Transcription of the *cpxP-lacZ* fusion is induced by alkaline pH. The ß-galactosidase activities of SP1 (MC4100, *ara*$^+$, λ*plac*Mu53[*cpxP-lacZ*]) (squares) and SP7 (SP1, *cpxA::cam*) (circles) were determined after strains had been grown at the indicated pH values. Strains were grown in buffered Luria broth ranging from pH 5.3 to 8.4. Luria broth was buffered with 100mM sodium phosphate as desribed in Chapter Two.

induction of *cpxP* transcription was due to a collapse of the ΔpH gradient across the inner membrane. This model predicts that *cpxP* transcription should also be induced by protonophore uncouplers such as CCCP [Lewis *et al.* 1994]. To test this model, I determined the amount of *cpxP* transcription from SP569 (MC4100, *ara*$^+$, *ilv*::Tn*10* *ΔuncBC*, λp*lac*Mu53[*cpxP-lacZ*]) before and after growth in the presence of CCCP, an uncoupler of the PMF. The *ΔuncBC* mutation in SP569 is required to prevent the strain from depleting its ATP reserves in an effort to buttress a collapsing PMF in the presence of CCCP.

Samples of SP569 were grown in Luria broth (buffered at pH 6.1 with 100mM sodium phosphate) and then incubated in the presence of 125μM CCCP for a period of time ranging from 0 to 135 minutes. The ß-galactosidase activities of each of these samples was then determined. Figure 4.9 shows that *cpxP-lacZ* transcription does not rise under these conditions, indicating that *cpxP* transcription is not stimulated by a collapse of the ΔpH gradient across the inner membrane. Rather, *cpxP* transcription appears to be induced by the alkalinity of the external environment.

Since the Cpx system is also involved in regulating *degP* transcription [Chapter Three, Raina *et al.* 1995], I was also interested in determining if transcription from a *degP-lacZ* operon fusion could be induced by alkaline pH. By quantifying the amount of *degP* transcription generated as a function of pH ranging from 5.5 to 8.5, I have found that *degP* transcription is induced by both acidic and alkaline pH. However, in the case of *degP*, the pH-mediated stimulation is conferred by an increase in σ^E activity. This conclusion is based on two results. First, induction of *degP* transcription by alkaline or acidic pH is not dependent upon the integrity of the Cpx pathway. Second, transcription from an *rpoHp3-lacZ* operon fusion (which is solely controlled by σ^E, is also induced by both acidic and alkaline conditions in a qualitatively similar fashion to that observed with *degP* transcription (data not shown).

cpxP and _cpx_ null mutations confer a hypersensitivity upon _E. coli_ to alkaline pH

In light of the alkaline pH regulation of *cpxP*, I wanted to determine if the *cpxP* or *cpx* null mutations conferred upon *E. coli* any growth defects under conditions of extreme alkaline

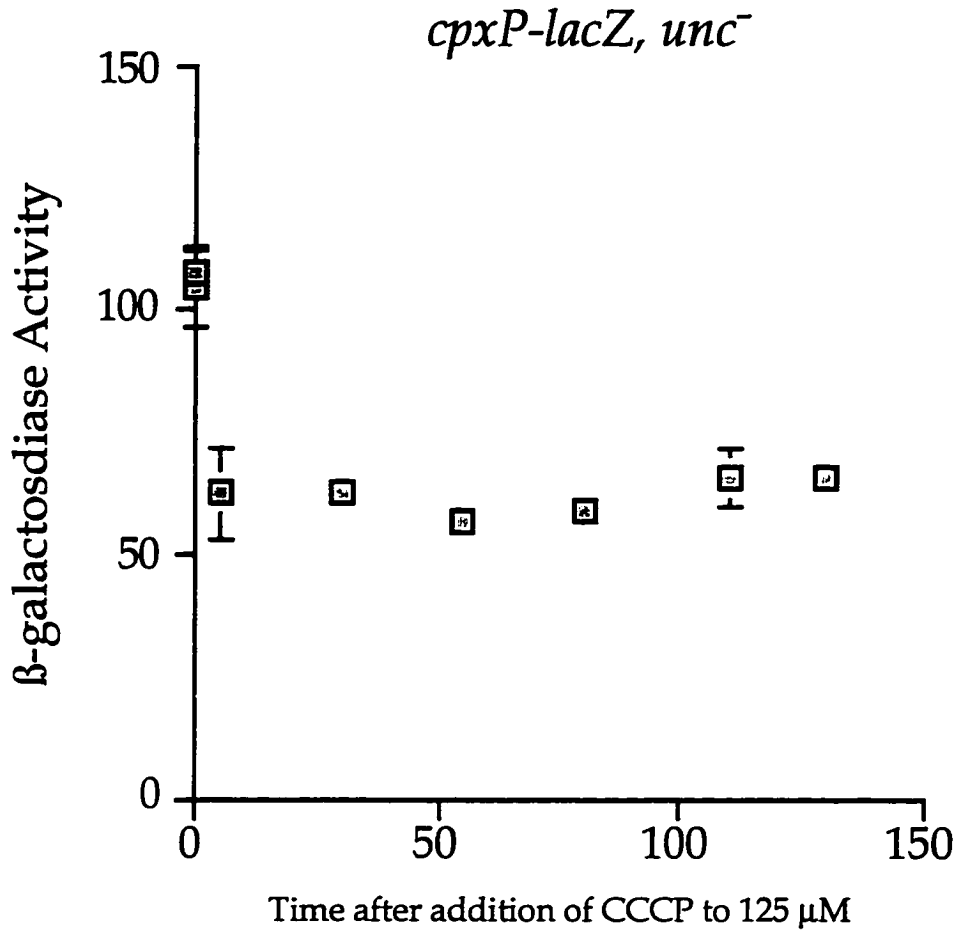


Figure 4.9. Transcription of the *cpxP-lacZ* fusion is not induced by depeletion of the proton-motive force. Eight sets of cultures of strain SP569 (MC4100, *ara*$^+$, *ilv*::Tn*10* Δ*uncBC*, λ*plac*Mu53[*cpxP-lacZ*]) were grown to mid-log phase in Luria broth buffered with 100mM sodium phosphate (pH 6.1). CCCP was then added to six sets of cultures to a final concentration of 125µM, and these cultures were reincubated for 5 to 135 minutes before ß-galactosidase activities were determined. The zero-minute timepoints were generated from cultures grown in the absence of CCCP. See Chapter Two for details.

pH. Strains SP744 (MC4100), SP754 (SP744, *cpxA::cam*), SP762 (SP744, *cpxR::Ω*) and SP774 (SP744, λp*lac*Mu53[*cpxP-lacZ*]) were grown to saturation in Luria broth. Serial dilutions of each strain were then plated on Luria broth containing 100 mM Tris-HCl (ranging from pH 7.0 to 9.4) and plates were incubated at 37°C for 48 hours. After this incubation period, the number of colony forming units/milliliter (CFU/ml) was determined for each strain at each pH value. Figure 4.10 shows the Logarithm_{10} of the CFU/ml value of each of these strains when grown from pH 7.0 to 9.4. In contrast to the parental strain (squares of Figure 4.10), the *cpxA*$^-$, *cpxR*$^-$ and *cpxP*$^-$ strains each display an inability to form colonies in the pH range from 8.8 to 9.4 The *cpxR*$^-$ strain (circles of Figure 4.10) is the most alkaline-hypersensitive strain, as it does not form any colonies at pH 9.0 and above. The *cpxP*$^-$ strain (triangles of Figure 4.10) is the second most alkaline-hypersensitive strain, while the *cpxA*$^-$ strain (diamonds of Figure 4.10) is the least hypersensitive to alkaline pH. Since the *cpxR* null mutation confers a higher degree of alkaline hypersensitivity than the *cpxP* null mutation, it implies that CpxR controls other factor(s), in addition to CpxP, that is(are) also needed for growth under alkaline conditions.

DISCUSSION

Studies by Cosma *et al.* [1995] and Snyder *et al.* [1995] have shown that activation of the Cpx signal transduction system is capable of combating extracytoplasmic protein-mediated toxicities. Since activation of the Cpx pathway can combat toxicities conferred by at least two different types of envelope proteins, it argues that the members of the Cpx regulon most likely ameliorate these toxicities by performing general and fundamental functions within the envelope. Indeed, the identification of the *degP* protease gene as a regulatory target of the Cpx system supports this notion.

However, Cosma *et al.* [1995] and Snyder *et al.* [1995] have shown DegP is not the only factor that is regulated by the Cpx system to combat extracytoplasmic stresses. This information provided the impetus to search for novel regulatory targets of the Cpx signal transduction system. Through this search, I have identified CpxP, a periplasmic protein whose

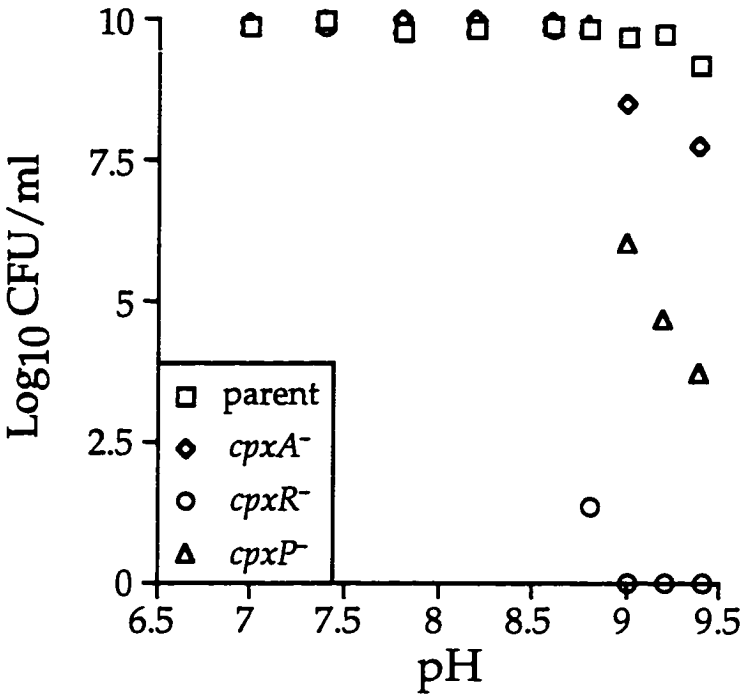


Figure 4.10. *cpxP* and *cpx* null strains are hypersensitive to alkaline pH. Strain SP744 (MC4100) (squares), SP754 (SP744, *cpxA::cam*) (diamonds), SP762 (SP744, *cpxR::Ω*) (circles), SP774 (SP744, λp*lac*Mu53[*cpxP-lacZ*]) (triangles) were grown to saturation in Luria broth. Serial dilutions of each culture were then plated on Luria broth buffered with 100 mM Tris-HCl (ranging from pH 7.0 to 9.5). Strains were incubated at 37°C for 48 hours and the number of colony forming units per milliliter (CFU/ml) of each culture at each pH value was determined as described in Chapter Two. The results in this figure are plotted as the Log_{10} of the CFU/ml values.

synthesis is increased at the transcriptional level by the Cpx system. Surprisingly, the *cpxP* locus is adjacent to the *cpx* operon, intimating a fundamental link between the functions of these two loci.

The Cpx and σ^E regulons

Presently, *degP* and *cpxP* are the only two loci whose transcription is known to be regulated by the Cpx pathway. In the case of *degP*, the Cpx pathway functions in concert with σ^E to stimulate transcription from this locus [Chapter Three]. Interestingly, the activities of σ^E and Cpx are each attuned to the physiology of the bacterial envelope. For example, σ^E activity rises in response to the overproduction of various outer membrane proteins, while Cpx activity rises when the outer membrane lipoprotein, NlpE is overproduced [Chapter Three, Mecsas *et al.* 1993, Snyder *et al.* 1995]. The joint regulation of *degP* by Cpx and σ^E prompted us to determine if all members of the Cpx regulon are also controlled by σ^E. However, this is not the case. *cpxP* transcription is not stimulated by activators of σ^E, nor is *cpxP* transcription reduced by a null mutation in the σ^E structural gene. This information implies that the Cpx and σ^E regulons intersect, but do not completely overlap.

Unidentified members of the Cpx regulon

There are several reasons to suspect that the Cpx regulon contains other, as yet unidentified, members in addition to DegP and CpxP. For example, the *cpxP*$^-$, *degP*$^-$ double mutation does not completely block the ability of the activated Cpx system to suppress the toxicity conferred by LamB-LacZX90 (Figure 4.7). This results implies that there are other envelope stress-combative factors controlled by the Cpx pathway. In addition, the *cpxR* null mutation is more hypersensitive to alkaline pH than the *cpxP*$^-$ strain, again implying that CpxR controls other stress-combative factors.

Finally, I note that in this study, I did not perform an exhaustive search of the genome for *lacZ* fusions whose transcription could be stimulated by the Cpx pathway. Indeed, a *degP*-

lacZ fusion was not identified among the 13,213 *lacZ* fusions that were screened. Accordingly, this screen should be repeated in an effort to identify other Cpx regulatory targets.

The extracytoplasmic stress-combative function of CpxP

Tests of epistasis indicate that both CpxP and DegP are utilized by the activated Cpx pathway to suppress the toxic effects associated with the exported LamB-LacZX90 protein fusion. Interestingly, when the Cpx pathway is activated in the *cpxP*$^-$, *degP*$^-$ double mutant strain, the strain is no more sensitive to the effects of LamB-LacZX90 than the *cpxP*$^+$, *degP*$^-$ strain. This result implies that CpxP and DegP function within the same pathway to suppress the toxic effects of LamB-LacZX90. For example, CpxP may function upstream of DegP, perhaps preparing substrates for this protease, or CpxP may function downstream of DegP, perhaps further processing DegP's proteolytic products. Alternatively, CpxP may directly alter the activity of DegP. However, since the precise biochemical function of CpxP not known, I cannot presently distinguish among such possibilities.

Activation of the Cpx pathway by alkaline pH

In addition to the stress-combative function of *cpxP*, I have found that the transcription of this locus is strongly induced by alkaline conditions in a CpxA-dependent fashion. It is presently unclear as to whether CpxA directly senses extracellular pH or whether it senses an indirect consequence that high pH imposes upon *E. coli*.

However, I favor the latter model for the following reasons. First, the Cpx pathway can be activated by overproduction of various envelope proteins, including NlpE and the P-pilus subunits, PapG and PapE [Chapters Three and Six, Snyder *et al.* 1995, C.H. Jones, P.N. Danese, T.J. Silhavy and S.J. Hultgren, unpublished observations]. It seems unlikely that overproduction of these proteins activates the Cpx pathway by radically altering the pH of the bacterial envelope. Second, since the *cpxP* and *cpxR* null strains are hypersensitive to alkaline pH, it implies that these genes are needed to specifically combat toxic effects caused by growth in extreme alkaline conditions. Thus, it seems more likely that CpxA would sense indirect consequences that alkaline pH confers upon the bacterial envelope.

I note that other stress-combative envelope proteins are also required to protect *E. coli* from alkaline conditions. For example, *surA* null strains, which are defective for the efficient assembly of outer membrane porins, are also hypersensitive to alkaline pH [Lazar and Kolter 1995]. Similarly, strains that do not produce the stress-inducible envelope protein, PspA are hypersensitive to alkaline pH in stationary phase [Weiner and Model 1994]. Taken together, these results suggest that structural damage may be inflicted upon the bacterial envelope by alkaline pH, and *E. coli* may utilize proteins such as SurA, PspA and the regulatory targets of CpxR to weather such onslaughts.

Five. Accumulation of the Enterobacterial Common Antigen Lipid II Biosynthetic Intermediate Stimulates *degP* transcription in *Escherichia coli*

INTRODUCTION

The results described in this chapter were initiated by George "Holt" Oliver, a senior undergraduate conducting thesis research in the Silhavy laboratory.

As mentioned in Chapter Three, *degP* transcription is modulated by at least two signal transduction systems that function in parallel with respect to each other. The Cpx signal transduction pathway and the σ^E regulatory system each control *degP* transcription in response to extracytoplasmic signals [Mecsas *et al.* 1993, Chapter Three]. To further characterize the transcriptional regulation of *degP* by Cpx and σ^E, Holt screened for negative regulators of *degP* transcription. Through this approach, Holt identified three genes in the *rff/rfe* gene cluster that, when inactivated, stimulate *degP* transcription.

The *rff/rfe* gene cluster is required for the synthesis of the enterobacterial common antigen (ECA), a glycolipid found in the outer-leaflet of the outer membrane in nearly all species of the *Enterobacteriaciae* [reviewed in Mäkelä and Mayer 1976, Kuhn *et al.* 1988, Rick and Silver 1996]. The polysaccharide portion of ECA consists of three sugar moieties: N-acetyl-D-glucosamine (GlcNAc), N-acetyl-D-mannosaminuronic acid (ManNAcA) and 4-acetamido-4,6-dideoxy-D-galactose (Fuc4NAc). These sugars form a linear trimeric repeat with the following structure: –> 3) α-D-Fuc4NAc-(1–>4)-ß-D-ManNAcA-(1–>4)-α-D-GlcNAc-(1–>) (Figure 5.1) [Männel and Mayer 1978]. Each polysaccharide chain is ultimately linked to phosphatidic acid *via* a phosphodiester linkage.

The ECA polysaccharide trimer is synthesized in stepwise fashion at the inner membrane by attaching each sugar to an undecaprenol-phosphate carrier molecule (Figure 5.2a). The first step in ECA synthesis is the conversion of undecaprenol-phosphate (C_{55}~P) and UDP-GlcNAc to C_{55}~PP-GlcNAc, also known as Lipid I. UDP-ManNAcA is then used to convert Lipid I into C_{55}~PP-GlcNAc-ManNAcA (Lipid II). The complete trimer is then synthesized from TDP-Fuc4NAc and Lipid II, creating C_{55}~PP-GlcNAc-ManNAcA-Fuc4NAc (Lipid III) [Rick *et al.* 1985, Barr and Rick 1987, Meier-Dieter *et al.* 1990]. Finally, Lipid III is

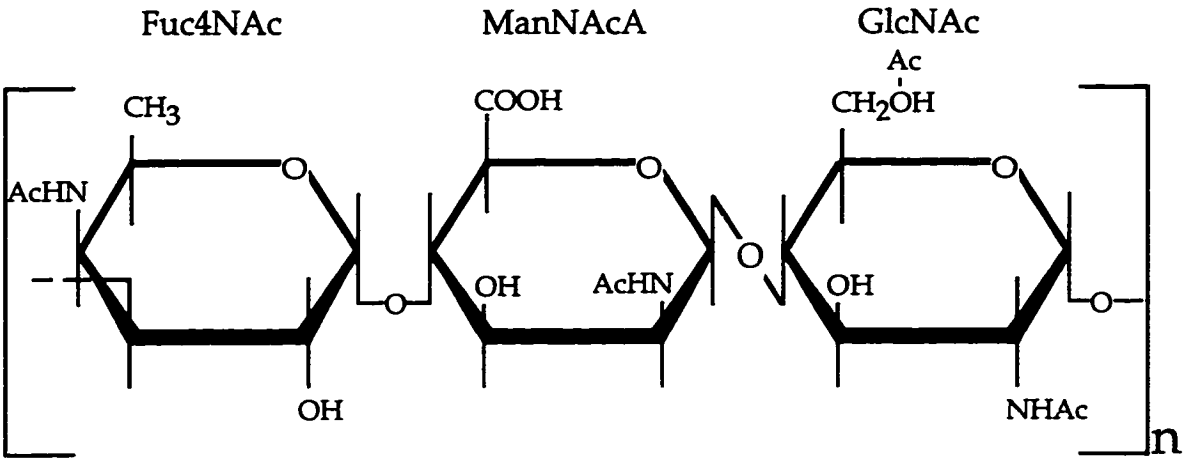
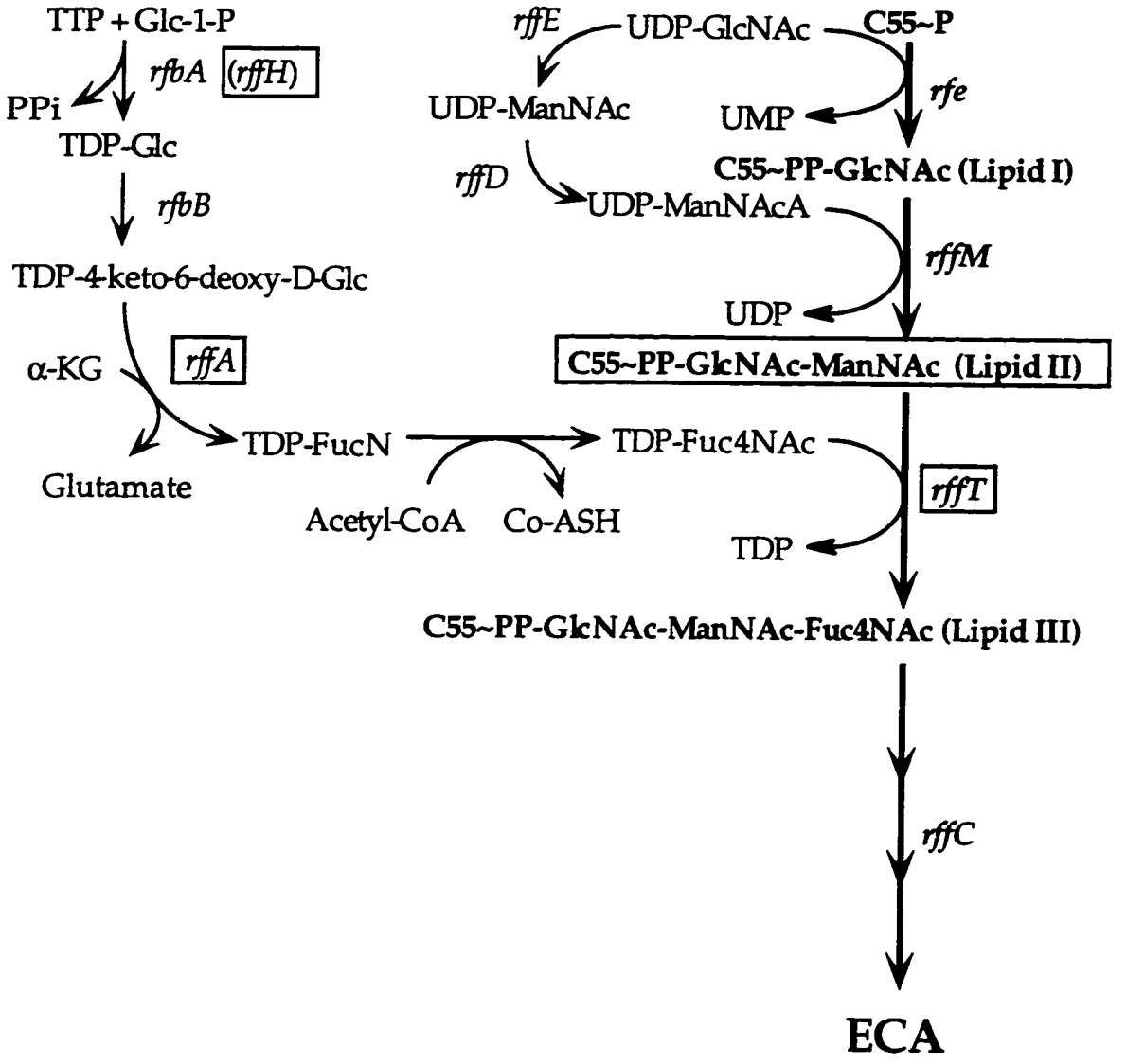


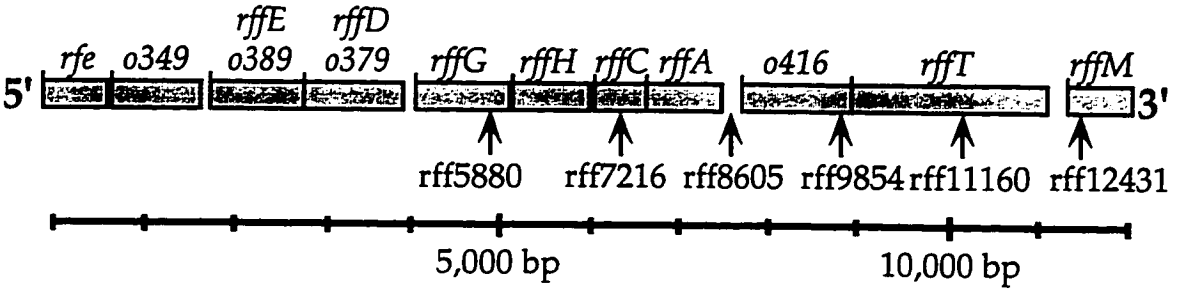
Figure 5.1. The trisaccharide repeat unit of the enterobacterial common antigen polysaccharide moiety. This repeat unit, [α-D-Fuc4NAc-(1-->4)-ß-D-ManNAcA-(1-->4)-α-D-GlcNAc], constitutes the aminosugar polymer of ECA. N-acetylglucosamine (GlcNAc) also serves as the attachment site for the lipid anchor. N-acetyl-D-mannosaminuronic acid and 4-acetamido-4,6-dideoxy-D-galactose are abbreviated ManNAcA and Fuc4NAc, respectively.

Figure 5.2. The ECA polysaccharide biosynthetic pathway and the *rff/rfe* gene cluster. a) The ECA polysaccharide biosynthetic pathway. Gene names are listed in italics adjacent to the reactions that their products catalyze. The genes affected by the mutational analysis employed in this study are enclosed within rectangles. Abbreviations: C55~P (undecaprenol-phosphate); GlcNAc (N-acetyl glucosamine); ManNAcA (N-acetyl-D-mannosaminuronic acid); Fuc4NAc (4-acetamido-4,6-dideoxy-D-galactose); TTP (thymidine triphosphate); Glc-1-P (glucose-1-phosphate); PP_i (pyrophosphate); TDP-Glc (thymidine diphosphate-glucose); TDP-4-keto-6-deoxy-D-Glc (thymidine diphosphate-4-keto-6-deoxy-D-glucose); α-KG (α-ketoglutarate); TDP-FucN (thymidine diphosphate-fucosamine); Acetyl-CoA (acetyl-coenzyme A); Co-ASH (coenzyme A); UDP (uridine diphosphate); ManNAc (N-acetyl mannose); UMP (uridine monophosphate). b) The *rff/rfe* gene cluster. The open reading frames of this cluster are shown as shaded boxes. All open reading frames are transcribed from 5' to 3' as shown. The primers used to map the transposon-generated mutations are named below each open reading frame, and the positions of these primers are indicated by arrows. A 12,000 base pair (bp) scale is depicted below the gene cluster for reference.

a)



b) The *rff/rfe* gene cluster



polymerized, attached to phosphatidic acid and transported to the outer membrane. The nature and chronology of these final three events is unknown.

The mutations identified in this study all interfere with the conversion of Lipid II to Lipid III (see Figure 5.2). Indeed, the results described here indicate that the accumulation of Lipid II stimulates *degP* transcription. Specifically, the Lipid II-accumulating mutants can i) confer upon *E. coli* a hypersensitivity to bile-salts, ii) confer a hypersensitivity to the synthesis of the outer membrane protein, LamB and iii) stimulate both the Cpx pathway and σ^E activity. These phenotypes suggest the *rff* mutations perturb the structure of the bacterial outer membrane. Moreover, analysis of these mutations suggests that although the Cpx and σ^E systems function in parallel to regulate *degP* transcription, they can be simultaneously activated by the same perturbation.

RESULTS

Rationale and mutant isolation

We sought to identify negative regulators of *degP* transcription by generating null mutations that increased transcription from a *degP-lacZ* reporter fusion. To facilitate this analysis, we created a *degP-lacZ* fusion strain that also carried the *ompR*::Tn*10* null mutation, PND257 (MC4100, *ompR*::Tn*10*, λRS88[*degP-lacZ*]). *ompR* null strains fail to produce the major outer membrane proteins, OmpC and OmpF [Slauch *et al.* 1988], and consequently, σ^E activity is reduced in such strains. Because of the reduction in σ^E activity, PND257 is phenotypically Lac$^-$ (white) on Luria agar containing 1.4μg/ml X-Gal. This Lac$^-$ phenotype provides a simple screen for mutants with increased *degP* transcription. By generating null mutations throughout the chromosome of PND257, we hoped to identify mutants with increased Lac activity, and by extension, mutations that impaired the function of negative regulators of *degP* transcription.

We used λNK1324 (contains Tn*10cam*, see Chapter Two for details) to perform transposon mutagenesis on strain PND257. The chloramphenicol-resistant colonies generated by this procedure were screened for those with increased *degP* transcription. Approximately

15,000 chloramphenicol-resistant colonies were screened and 11 colonies with increased *degP* transcription were analyzed. Of these 11 isolates, nine grew poorly on lactose-MacConkey agar, while the remaining two grew as well as the parent (PND257) on this medium. Since hypersensitivity to MacConkey agar is often an indicator of a structural perturbation in the outer membrane [Nikaido and Vaara 1985], and since *degP* transcription is attuned to such perturbations [Mecsas *et al.* 1993, Raina *et al.* 1995, Rouvière *et al.* 1995], we chose to analyze the nine MacConkey-sensitive mutants.

Figure 5.3 shows that each transposon-generated insertion increases *degP* transcription approximately three to six-fold when compared to the parent strain. For example, insertions #5 and #31 increase *degP* transcription approximately six-fold (Figure 5.3, compare lanes 2 and 4 with lane 1), while insertion #22 increases *degP* transcription approximately three-fold (Figure 5.3, compare lanes 1 and 3). The six remaining transposon-generated mutations increase *degP* transcription to the same extent as that observed with insertions #5 and 31 (data not shown).

Null mutations within the *rff/rfe* gene cluster stimulate *degP* transcription

The transposon insertions in each of the MacConkey-sensitive isolates were all tightly linked to the *rff/rfe* gene cluster located at approximately 85 minutes on the *Escherichia coli* chromosome. Since the *rff/rfe* gene cluster is involved in the biosynthesis of the enterobacterial common antigen (ECA), we were interested in determining whether the Tn*cam* insertions affected the biosynthesis of ECA. At this point, we enlisted the help of Dr. Paul Rick and Dr. Kathleen Barr (Uniformed Services University of the Health Sciences), who are experts in the study of ECA. Using a passive hemagglutination assay for ECA biosynthesis [Rick *et al.* 1985], Drs. Rick and Barr determined that eight of the nine insertions did not produce ECA (ECA^-). Only one insertion (#22) remained ECA^+. Interestingly, insertion #22 causes the weakest induction of *degP* transcription (*e.g.*, see Figure 5.3).

Fine structure mapping of each insertion using Tn*10* insertions within the *rfe/rff* gene cluster indicated that five of the nine Tn*cam* insertions (numbers 5, 41, 45, 47 and 51) were

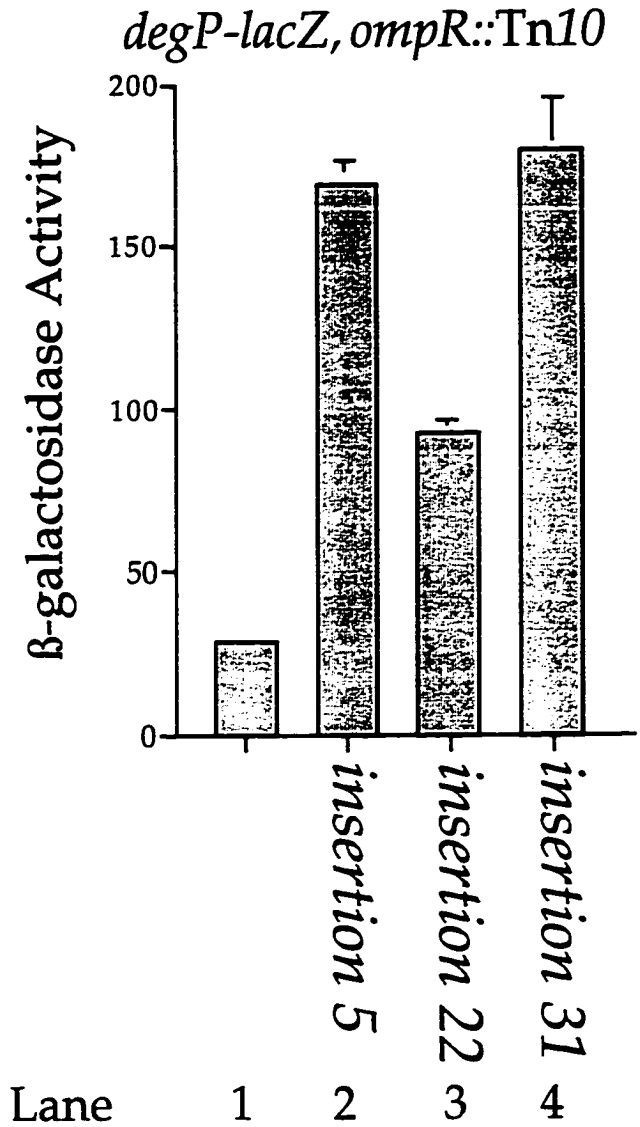


Figure 5.3. The Tn*cam* insertions stimulate *degP-lacZ* transcription. The ß-galactosidase activities of PND257 (MC4100, *ompR*::Tn*10*, λRS88[*degP-lacZ*]) (lane 1), PND788 (PND257, Tn*cam* insertion#5) (lane 2), PND789 (PND257, Tn*cam* insertion#22) (lane 3) and PND790 (PND257, Tn*cam* insertion#31) (lane 4) were determined. Tn*cam* insertions#5 and #31 each stimulate *degP-lacZ* transcription ~ 6-fold, while insertion#22 stimulates *degP-lacZ* transcription ~ 3-fold when compared to the parent strain. All strains were grown in Luria broth as described in Chapter Two.

tightly linked to the *rffT* locus (see Figure 5.2b). Mapping of each insertion by PCR confirmed the transductional mapping data. Each of these transposon insertions is located within the *rffT* open reading frame (data not shown). Three of the nine Tn*cam* insertions were tightly linked to the *rffA* locus (numbers 31, 32, 33) (see Figure 5.2b) and PCR analysis confirmed that these insertions are indeed situated within the *rffA* open reading frame (data not shown).

The remaining insertion (#22) was tightly linked to the *rffC* locus. However, PCR mapping of the *rffH::cam* insertion indicated that the transposon was located within the *rffH* open reading frame, immediately upstream of the *rffC* locus (data not shown, and see Figure 5.2b). Thus, our screen identified three distinct loci in the *rff* gene cluster, *rffT*, *rffA* and *rffH*. Accordingly, Tn*cam* insertions # 5, 22 and 31 will now be referred to as *rffT::cam*, *rffH::cam* and *rffA::cam*, respectively.

The *rffH::cam* mutation

The *rffH::cam* mutation was anomalous in two major regards. First, unlike the other eight mutations, the *rffH::cam* mutation did not confer an ECA$^-$ phenotype although it mapped within the *rff/rfe* gene cluster. Second, the *rffH::cam* mutation conferred a relatively weak increase in *degP* transcription (approximately 50% of that observed with the other Tn*cam* insertions).

A potential explanation of the phenotypes conferred by the *rffH::cam* mutation was provided by previous biochemical analysis of the *rffH* gene product [Marolda and Valvano 1995]. Specifically, the predicted *rffH* gene product shares 65% identity with RfbA (Figure 5.4), a glucose-1-phosphate thymidylylase that plays a role in the initial stages of Fuc4NAc biosynthesis [Stevenson *et al.* 1994]. Marolda and Valvano [1995] have demonstrated that the *rffH* gene product does indeed display glucose-1-phosphate thymidylylase activity, thus confirming what the sequence homology between RffH and RfbA had suggested. Given the activity of the *rffH* gene product, we predicted that a lesion in this locus would reduce production of TDP-glucose and hence, slow Fuc4NAc synthesis (see Figure 5.2a). As a consequence, the *rffH::cam* mutation would slow the production of ECA by impeding the

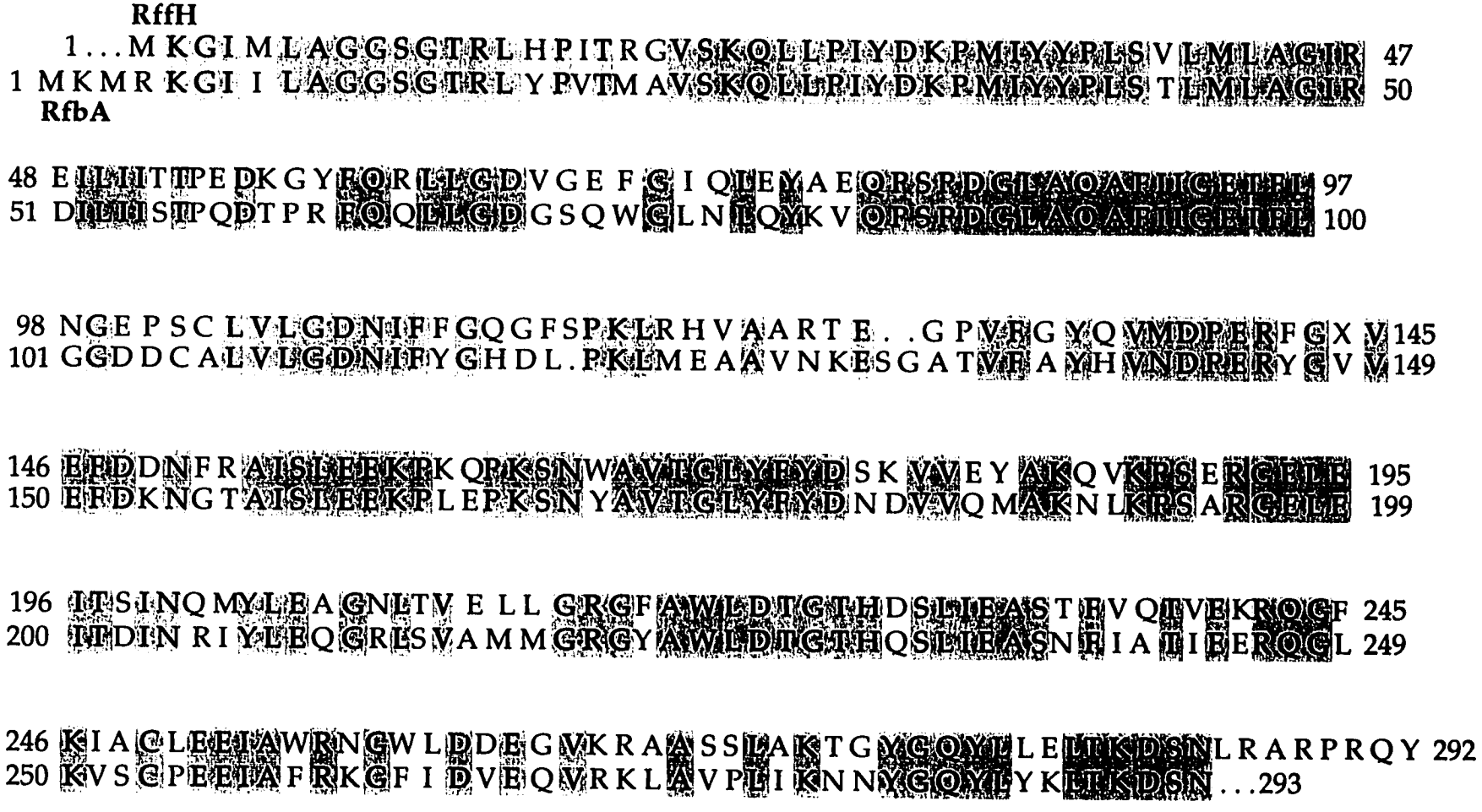


Figure 5.4. Homology between the RfbA polypeptide and the predicted product of the *rffH* gene. The sequences, which were aligned using the GAP program from the GCG software package [Devereux *et al.* 1984], share 64.9% sequence identity. Regions of identity between the two sequences are shaded.

conversion of Lipid II to Lipid III. However, since a functional *rfbA* gene product is still presentin the *E. coli* chromosome [Stevenson *et al.* 1994], Fuc4NAc synthesis and ECA synthesis would be reduced but not abolished. According to this model, a lesion in *rffH* would i) not confer an ECA$^-$ phenotype, and ii) display an attenuated increase in *degP* transcription compared with the *rffT* and *rffA* mutations that completely abolish the synthesis of Lipid III. The results presented in the next section support this model.

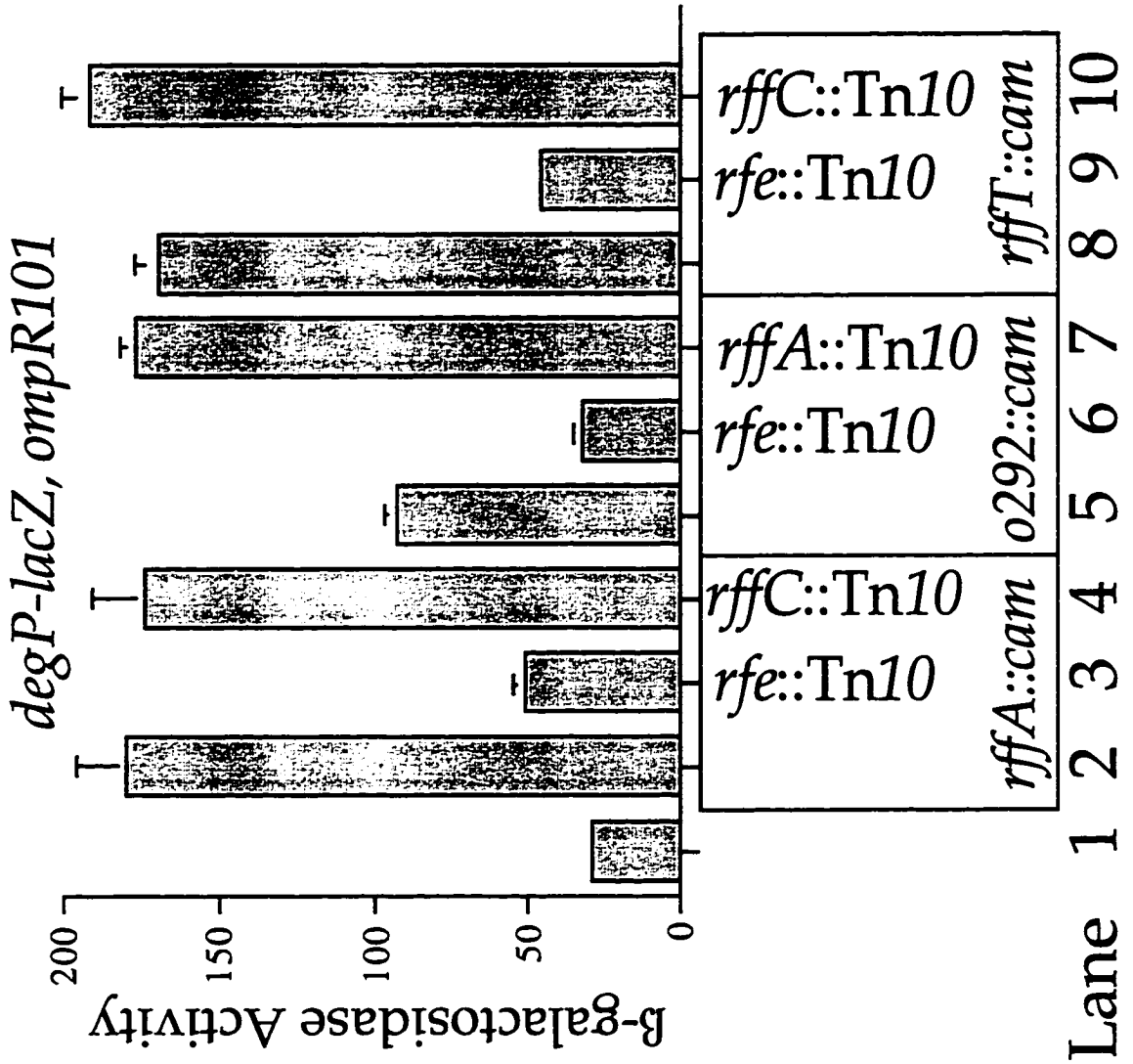
The Accumulation of the Lipid II ECA biosynthetic Intermediate increases *degP* transcription and confers a hypersensitivity to bile salts

From the data presented thus far, it is clear that all of the mutations identified in our screen affect the same general step in the biosynthesis of ECA. Specifically, the insertions within *rffT* and *rffA* cause the accumulation of Lipid II while the *rffH::cam* mutation most likely impedes the conversion of Lipid II to Lipid III (see Figure 5.2a).

What is not clear, however, is whether it is the absence of ECA or whether it is the accumulation of the Lipid II intermediate that stimulates *degP* transcription and confers sensitivity to MacConkey agar. Various tests of epistasis were performed to distinguish between these two possibilities. For example, the *rfe*::Tn*10* mutation was introduced into the *rffT::cam*, *rffA::cam* and *rffH::cam* strains, and the resulting amounts of *degP* transcription were determined (Figure 5.5). The *rfe*::Tn*10* mutation blocks ECA synthesis at its earliest step, preventing the addition of GlcNAc-phosphate to undecaprenol-phosphate (see Figure 5.2a). If the lack of ECA is the cause of the observed phenotypes, the *rfe*::Tn*10*, *rffA::cam* double mutant strain should display at least the same degree of *degP* transcriptional induction and MacConkey sensitivity as observed with the *rffA::cam* mutation alone. If the accumulation of Lipid II is responsible for the transcriptional induction of *degP*, then the double mutant strain should not display the transcriptional induction observed with the single chloramphenicol-resistant insertion mutations. Of course, these predictions also hold true for the *rffT::cam* and *rffH::cam* mutant strains.

Figure 5.5 indicates that the accumulation of Lipid II is responsible for the observed phenotypes. The *rfe*::Tn*10*, *rffA::cam* double mutant strain shows only a minimal induction of

Figure 5.5. The accumulation of Lipid II stimulates transcription of *degP-lacZ*. ß-galactosidase activities were determined for the following strains: PND423 (MC4100, *ompR101*, λRS88[*degP-lacZ*]) (lane 1), SP173 (PND423, *rffA::cam*) (lane 2), SP190 (PND423, *rffA::cam*, *rfe*::Tn*10*) (lane 3), SP193 (PND423, *rffA::cam*, *rff*C::Tn*10*) (lane 4), SP172 (PND423, *rffH::cam*) (lane 5), SP185 (PND423, *rffH::cam*, *rfe*::Tn*10*) (lane 6), SP188 (PND423, *rffH::cam*, *rffA*::Tn*10*) (lane 7), SP171 (PND423, *rffT::cam*) (lane 8), SP179 (PND423, *rffT::cam*, *rfe*::Tn*10*) (lane 9), SP182 (PND423, *rffT::cam*, *rff*C::Tn*10*) (lane 10). The *rfe*::Tn*10* mutation blocks the transcriptional induction of *degP-lacZ* that is conferred by the *rffA::cam*, *rffH::cam* and *rffT::cam* insertions (compare lanes 2 and 3, 5 and 6, 8 and 9). The *rff*C::Tn*10* mutation does not block the transcriptional induction of *degP-lacZ* conferred by the *rffA::cam* and *rffT::cam* mutations (compare lanes 2 and 4, 8 and 10). The weak transcriptional induction of *degP-lacZ* conferred by the *rffH::cam* mutation can be increased by the *rffA*::Tn*10* mutation (compare lanes 5 and 7). The *ompR101* allele is a deletion within the *ompR* open reading frame, rendering the strains described in this figure *ompR*$^-$. All strains were grown in Luria broth as described in Chapter Two.



degP transcription (Figure 5.5, compare lanes 2 and 3; 5 and 6; 8 and 9) and these strains are no longer MacConkey hypersensitive.

The *rffC*::Tn*10* mutation was also introduced into the *rffT::cam*, and *rffA::cam* strains. The *rffC*::Tn*10* mutation blocks ECA synthesis at a step after the synthesis of Lipid III (see Figure 5.2a). If the accumulation of Lipid II is the cause of the increase in *degP* transcription, the *rffC*::Tn*10* mutation should have no effect on the transcriptional induction of *degP* caused by the *rffT::cam*, and *rffA::cam* mutations. Figure 5.5 verifies this prediction: the *rffC* null mutation does not alter the transcriptional induction of *degP* caused by the *rffT* and *rffA* null mutations (compare lanes 2 and 4; 8 and 10).

Finally, based on the biochemical analysis of RffH [Marolda and Valvano 1995], and on the homology between the *rffH* gene product and the RfbA protein, we have suggested that the *rffH::cam* mutation attenuates, but does not abolish, the conversion of Lipid II to Lipid III. This notion explains the weak induction of *degP* transcription conferred by this mutation, and it also explains why the *rffH::cam* lesion does not confer an ECA$^-$ phenotype. If this model is correct, we predict that introducing the *rffA::Tn10* mutation into a strain that contains the *rffH::cam* insertion should raise *degP* transcription to the level observed with the *rffA*$^-$ mutation alone. Again, this prediction is verified. Specifically, the *rffH*$^-$, *rffA*$^-$ double-mutant strain displays the same degree of transcriptional induction of *degP* as the *rffA*$^-$ mutation does in isolation (compare lanes 2, 5 and 7 in Figure 5.5).

There are two general conclusions that can be drawn from Figure 5.5. First, the accumulation of Lipid II stimulates *degP* transcription and confers MacConkey sensitivity in these strains. Second, the *rffH::cam* mutation impedes, but does not abolish, the conversion of Lipid II to Lipid III in the biosynthesis of ECA.

The induction of *degP* transcription by accumulation of the Lipid II Intermediate is decreased in an *ompR*$^+$ background

Since the experiments described above have all utilized strains that were *ompR*$^-$, we were also interested in determining the effects of Lipid II accumulation in an *ompR*$^+$

background. Figure 5.6 shows that while the *rff* mutations still stimulate *degP* transcription in the *ompR*$^+$ background, the magnitude of the induction of *degP* transcription conferred by these mutations is significantly reduced compared to the analogous *ompR*$^-$ strains. These *ompR*$^+$, Lipid II-accumulating strains are also no longer as MacConkey-sensitive as their *ompR*$^-$ counterparts.

Since the major proteins whose synthesis is controlled by OmpR are the outer membrane proteins, OmpF and OmpC, it seemed likely that the enhanced *degP* transcriptional induction observed in the *ompR*$^-$ background was due to the lack of these proteins. Consistent with this observation, production of OmpC in an *ompR*$^-$, Lipid II-accumulating background, reduced the fold-induction of *degP* transcription (data not shown). Thus, at a minimum, it is the lack of OmpC that heightens the MacConkey sensitivity and the increase in *degP* transcription that is observed in the Lipid II-accumulating strains.

Accumulation of the Lipid II intermediate can confer a hypersensitivity to high-level synthesis of the outer membrane protein, LamB

During the course of this study, Gregory Bowman (a rotation student in the Silhavy laboratory) noted that the Lipid II-accumulating mutant strains were exquisitely sensitive to growth on maltose and maltodextrins. As a consequence, we wanted to determine if these strains had difficulty growing in the presence of all types of sugars, or whether their hypersensitivity was restricted to growth in the presence of maltose and its oligomers. To this end, we assayed the growth of the Lipid II-accumulating strains on Luria agar in the presence of high concentrations of maltose, lactose and glucose. The Lipid II-accumulating strains were only hypersensitive to maltose in the *ompR*-minus background, indicating that this hypersensitivity is not simply a sugar-mediated effect (data not shown).

Since the various phenotypes described for these strains appear to be associated with perturbations in the outer membrane, we thought that the observed maltose-sensitivity might be due to high-level synthesis and export of the outer membrane porin, LamB (LamB synthesis is induced in the presence of maltose). To address this issue, the *lamBΔ60* mutation, which

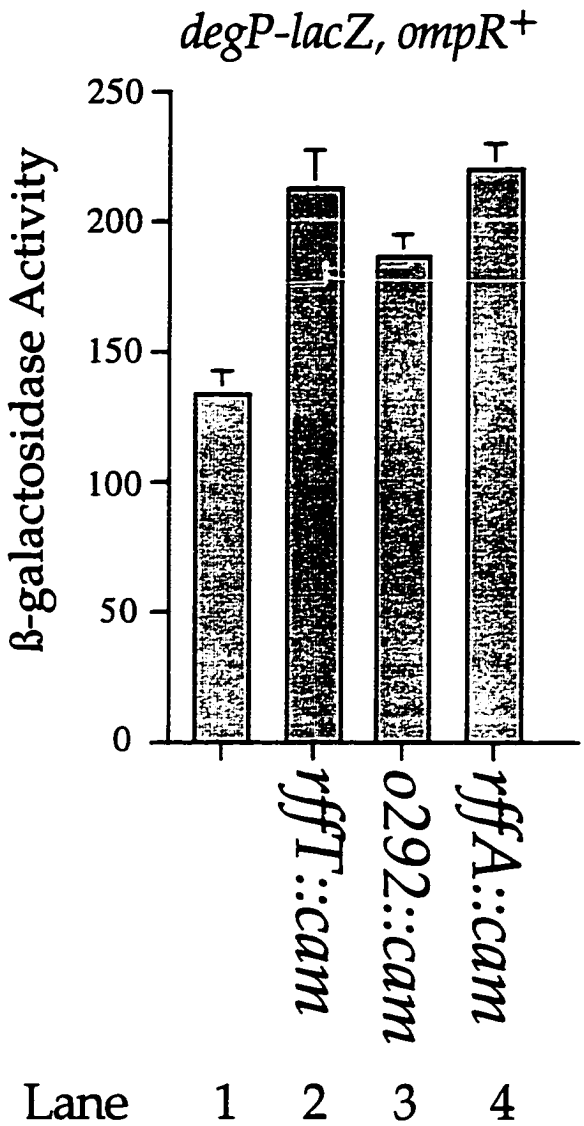


Figure 5.6. Accumulation of Lipid II stimulates transcription of *degP-lacZ* in the *ompR*$^+$ background. ß-galactosidase activities were determined for the following strains: PND2000 (MC4100, λRS88[*degP-lacZ*]) (lane 1), SP332 (PND2000, *rffT::cam*) (lane 2), SP333 (PND2000, *rffH::cam*) (lane 3), SP334 (PND2000, *rffA::cam*) (lane 4). The *rffT::cam* and *rffA::cam* mutations stimulate *degP-lacZ* transcription ~ 1.6-fold. The *rffH::cam* mutation stimulates *degP-lacZ* transcription ~ 1.4-fold when compared to wild-type. All strains were grown in Luria broth as described in Chapter Two.

deletes a portion of the LamB signal sequence (and prevents its export across the innermembrane [Emr and Silhavy 1980]), was introduced into the Lipid II-accumulating strains. We then determined if these *lamBΔ60* strains were also hypersensitive to high levels of maltose. Figure 5.7 indicates that the *lamBΔ60* mutation abolishes the hypersensitivity of the Lipid-II accumulating strains to high concentrations of maltose (compare lanes 2 and 6; 3 and 7; 4 and 8). Thus, the maltose-sensitivity observed with these strains is due to high-level export of wild-type LamB, further suggesting that the accumulation of Lipid II perturbs the physiology of the outer membrane.

Accumulation of Lipid II does not interfere with incorporation or assembly of LamB in the outer membrane

Because of the observed toxicity associated with high-level synthesis of LamB in the Lipid II-accumulating mutants, we wanted to determine if these strains displayed defects in the incorporation and assembly of LamB into the outer membrane. To address this issue, we performed two experiments: 1) We examined the amount of LamB protein associated with membrane fractions of a parental strain, PND257 (MC4100, *ompR*::Tn*10* λRS88[*degP-lacZ*]) as well as derivative strains that accumulate Lipid II, PND788 (PND257 *rffT::cam*), PND789 (PND257, *rffH::cam*), PND790 (PND257, *rffA::cam*). 2) We also examined the kinetics of LamB trimerization in PND257, PND788, PND789 and PND790. None of the Lipid II-accumulating strains display defects in the incorporation LamB into membrane fractions, nor do these mutants display defects in the trimerization of LamB (data not shown). Thus, the toxicity associated with high-level synthesis of LamB in Lipid II-accumulating mutants is not the result of a gross structural defect in LamB assembly.

The *rffT::cam, rffA::cam and rffH::cam* mutations increase σ^E activity

Finally, we were interested in the mechanism(s) by which degP transcription was being stimulated as a result of Lipid II-accumulation. Two regulatory pathways are known to modulate *degP* transcription. As described in Chapter One, *degP* transcription can be increased by increasing σ^E activity, through an as yet undefined signal transduction pathway [Mecsas *et al.* 1993]. *degP* transcription is also regulated by the Cpx two-component signal transduction

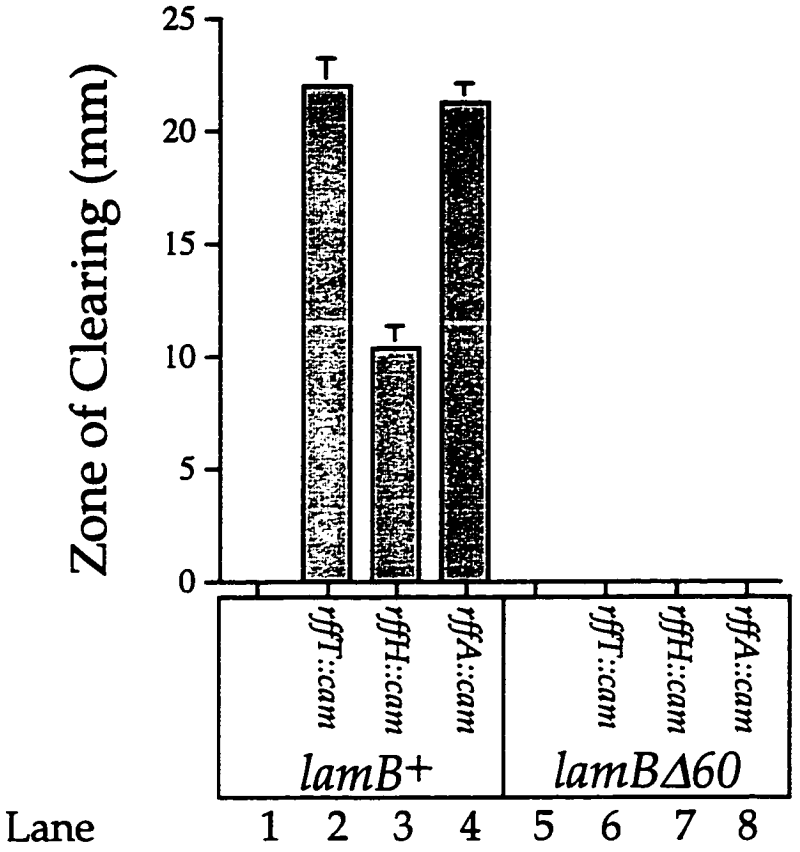


Figure 5.7. High-level export of the LamB maltoporin in Lipid II-accumulating strains is toxic. 10µl of 20% maltose was added to filter discs that had been placed on lawns of the following strains: SP299 (MC4100, *ompR101*, *zja*::Tn*10* *lamB*$^+$, λRS88[*degP-lacZ*]) (lane 1), SP301 (SP299, *rffT::cam*) (lane 2), SP303 (SP299, *rffH::cam*) (lane 3), SP305 (SP299, *rffA::cam*) (lane 4), SP298 (MC4100, *ompR101*, Tn*10* linked to *lamBΔ60*, λRS88[*degP-lacZ*]) (lane 5), SP300 (SP298, *rffT::cam*) (lane 6), SP302 (SP298, *rffH::cam*) (lane 7), SP304 (SP298, *rffA::cam*) (lane 8). The *ompR101* allele is a deletion within the *ompR* open reading frame, rendering the strains described in this figure *ompR*$^-$. The *lamBΔ60* mutation deletes part of the coding sequence for the hydrophobic core of the LamB signal sequence [Emr and Silhavy 1980]. Thus, strains containing the *lamBΔ60* mutation cannot export LamB across the inner membrane. The values displayed along the Y-axis (zone of clearing) represent the amount of growth inhibition caused by the addition of maltose. The zone of clearing value is defined as the diameter of growth inhibition around the maltose-saturated filter disc, minus the diameter of the filter disc itself (7mm). All strains were grown in Luria broth at 37°C as described in Chapter Two.

pathway [Chapter Two, Raina *et al.* 1995], which functions in parallel with the σ^E signal transduction pathway [see Figure 3.9]. To determine the specific route by which the *rffT::cam rffH::cam* and *rffA::cam* mutations functioned to increase *degP* transcription, we introduced each insertion into SP245 (MC4100, *ompR*::Tn*10 rpoHp3-lacZ*). The *rpoHp3* promoter is recognized solely by RNA polymerases containing the σ^E subunit. Hence, this fusion provides an assay for only σ^E activity [Mecsas *et al.* 1993, Chapter Three]. Figure 5.8 shows that *rpoHp3-lacZ* transcription is induced by the Tn*cam* insertions in a qualitatively similar fashion to that observed with the induction of *degP* transcription. For example, the *rffT::cam* and *rffA::cam* insertions stimulate *rpoHp3-lacZ* transcription approximately 2.6-fold (Figure 5.8, compare lane 1 with lanes 2 and 4). The *rffH::cam* insertion stimulates *rpoHp3*-mediated transcription approximately 2.3-fold (compare lanes 1 and 3 of Figure 5.8).

The *rffT, rffA and rffH* mutations activate the Cpx signal transduction pathway

The results presented in Figure 5.8 clearly demonstrate that the *rffT, rffH* and *rffA* mutations activate *degP* transcription, at least in part, by stimulating σ^E activity. However, we were also interested in determining if these mutations stimulated *degP* transcription *via* the Cpx pathway as well. Accordingly, the *cpxR* null mutation was introduced into the parent strain (PND257) as well as the *rffT, rffH* and *rffA* mutant strains (PND788, PND789, PND790), and the amount of *degP-lacZ* transcription generated from these strains was quantified. Interestingly, elimination of the Cpx pathway by a *cpxR* null mutation decreases, but does not abolish the transcriptional induction of *degP* conferred by the *rffT, rffH* and *rffA* mutations (Figure 5.9). For example, in a *cpxR*$^-$ background, the *rffT::cam* and *rffA::cam* mutations only stimulate *degP* transcription 1.6 to 1.7-fold (Figure 5.9, compare lane 1 with lanes 2 and 4). Similarly, the *rffH::cam* mutation stimulates *degP* transcription approximately 1.4-fold in the *cpxR*$^-$ background (Figure 5.9, compares lanes 1 and 3). Although the transcriptional induction of *degP* is qualitatively similar in the *cpxR*$^+$ and *cpxR*$^-$ background, CpxR is clearly responsible for the majority of the *degP*'s transcriptional induction in the parental background (PND257).

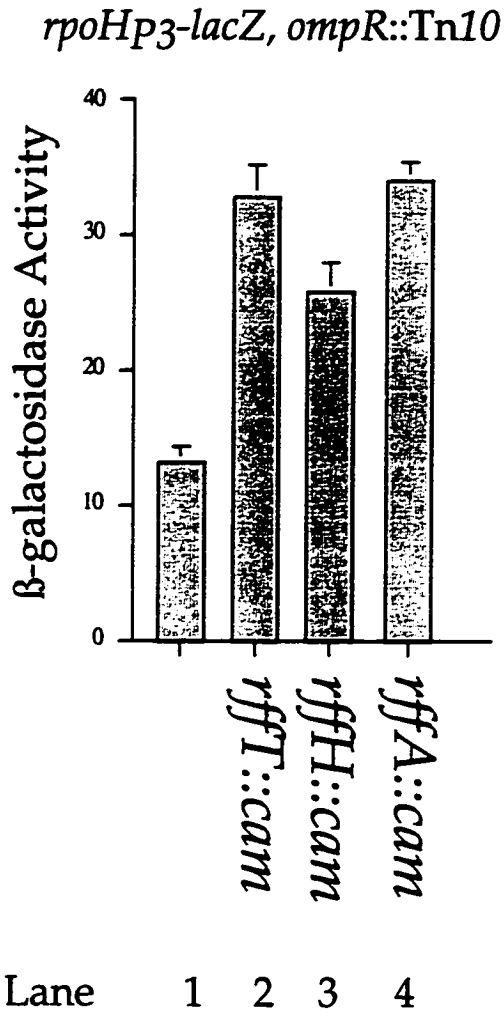


Figure 5.8. The *rffT::cam, rffH::cam and rffA::cam* mutations stimulate σ^E activity. The ß-galactosidase activities of SP245 (MC4100, *ompR*::Tn*10*, λRS45[*rpoHP3-lacZ*]) (lane 1), SP282 (SP245, *rffT::cam*) (lane 2), SP283 (SP245, *rffH::cam*) (lane 3) and SP284 (SP245, *rffA::cam*) (lane 4) were determined. The *rffT::cam* and *rffA::cam* mutations stimulate *rpoHp3-lacZ* transcription ~ 2.6-fold. The *rffH::cam* mutation stimulates *rpoHp3-lacZ* transcription ~ 2.3 fold. All strains were grown in Luria broth as described in Chapter Two.

For instance, the *rffT::cam* mutation stimulates *degP* transcription approximately 6-fold in the *cpxR*$^+$ background, but only 1.7-fold in the *cpxR*$^-$ background (compare lane 2 of Figure 5.3 with lane 2 of Figure 5.9). Note that the values in Figures 5.3 and 5.9 are directly comparable because the ß-galactosidase assays utilized to quantify *degP* transcription in these figures were performed using isogenic strains in the same experiment.

Since the transcriptional induction of *degP* is partially blocked by the *cpxR* null mutation, and since the *rffT*, *rffH* and *rffA* mutations also increase transcription of *rpoHp3-lacZ*, these mutations must activate both signal transduction pathways that control *degP* transcription.

Discussion

By searching for negative regulators of *degP* transcription, we have shown that mutations that cause the accumulation of the Lipid II intermediate in the pathway for ECA biosynthesis cause several envelope-associated perturbations. First, these mutations confer a hypersensitivity to growth in the presence of bile salt detergents, which is a classic indicator of an outer membrane permeability defect [Nikaido and Vaara 1985]. Second, these mutations confer a hypersensitivity to high-level export of the wild-type LamB porin, also suggesting a perturbation in outer membrane physiology. Third, these mutations increase *degP* transcription by stimulating both the σ^E modulatory signal transduction system [Mecsas *et al.* 1993] and also the Cpx signal transduction system [Chapter Three]. Since *degP* encodes a periplasmic protease that destroys misfolded extracytoplasmic proteins [*e.g.*, see Misra *et al.* 1991], this is also an indicator of a perturbation in the physiology of periplasmic and/or outer membrane proteins. Taken together, these results imply that Lipid II accumulation perturbs the physiology of envelope proteins, thus causing an increase in *degP* transcription.

Accumulation of Lipid II

The results presented in Figure 5.5 clearly demonstrate that it is the accumulation of Lipid II that stimulates *degP* transcription. Moreover, previous studies have also noted a toxicity associated with the accumulation of Lipid II. For example, Rick *et al.* [1988] have

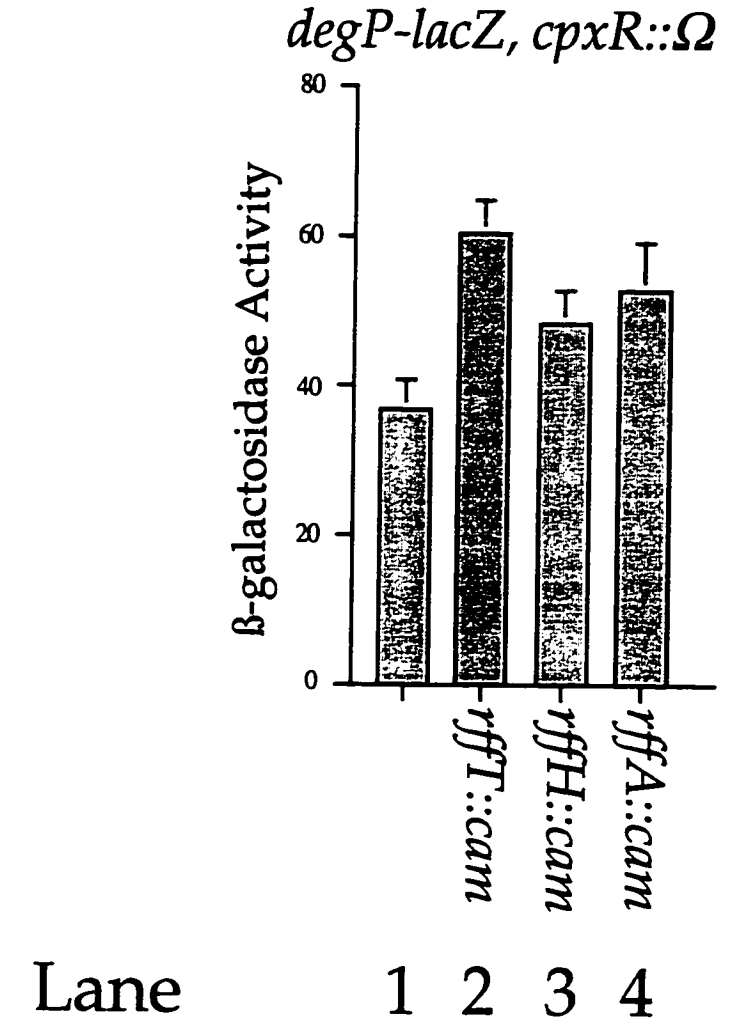


Figure 5.9. The *rffT::cam, rffH::cam and rffA::cam* mutations activate the Cpx signal transduction pathway. The ß-galactosidase activities of SP150 (MC4100, *ompR*::Tn*10*, *cpxR::Ω*, λRS88[*degP-lacZ*]) (lane 1), SP231 (SP150, *rffT::cam*) (lane 2), SP232 (SP150, *rffH::cam*) (lane 3) and SP233 (SP150, *rffA::cam*) (lane 4) were determined. The *rffT::cam* and *rffA::cam* mutations stimulate *degP-lacZ* transcription 1.6 to 1.7-fold in the absence of CpxR. Similarly, the *rffH::cam* mutation stimulates *degP-lacZ* transcription ~ 1.4-fold in the absence of CpxR. This is in contrast to the 3 to 6-fold stimulation conferred by these mutations in the presence of CpxR. All strains were grown in Luria broth as described in Chapter Two.

observed that *Salmonella typhimurium* strains containing a lesion in *rfbA* (which encodes a homolog of RffH) accumulate the Lipid II intermediate and display hypersensitivity to sodium dodecyl sulfate (SDS). This SDS-hypersensitive phenotype can be suppressed by mutations that block the accumulation of Lipid II.

Despite the body of evidence presented here, indicating that Lipid II accumulation confers a host of envelope-associated defects, it is unclear as to why Lipid II accumulation exerts these effects. While ECA is not essential for viability of *E. coli*, the undecaprenol carrier lipid that is used to synthesize the ECA trisaccharide *is* essential. It is possible that accumulation of Lipid II indirectly impedes the synthesis of the peptidoglycan layer by sequesting undecaprenol-phosphate (C55-P). An alternative model posits that the partially completed ECA trisaccharide may directly interfere with the biogenesis of envelope proteins, thus altering the permeability of the outer membrane and signalling for increased levels of the envelope protease, DegP. An analysis of the peptidoglycan structure, and an analysis of a variety of periplasmic and outer membrane proteins may ultimately be informative in distinguishing between these hypotheses.

rffH

This chapter describes the first mutational analysis of *rffH*. Previous studies which have sought mutations in genes involved in ECA biosynthesis have uncovered several loci in the *rff/rfe* and *rfb* gene clusters. For example, mutations in *rffE*, *rffD*, *rffC*, *rffA*, *rffT* and *rffM* have all been identified because of their abolition of ECA biosynthesis (Figure 5.2) [Meier-Dieter *et al.* 1990].

Indeed, *rfbA* mutations (Figure 5.2a) have also been shown to radically reduce (but not abolish) ECA biosynthesis in *S. typhimurium* [Rick *et al.* 1988]. However, despite the homology between RffH and RfbA, no mutations were ever identified in *rffH*. Based on the previous analyses and on the results presented in this chapter, we suggest that RffH and RfbA perform redundant functions for the biosynthesis of ECA. The reasons for suggesting this are

three-fold. First, RffH and RfbA share 65% amino acid sequence identity and they each display glucose-1-phosphate thymidylylase activity [Figure 5.4 and Marolda and Valvano 1995]. Second, *rfbA* null strains do not completely abolish ECA biosynthesis in *S. typhimurium.* We suggest that this residual production of ECA is due to the activity of an RffH homolog in *S. typhimurium.* Finally, Figure 5.5 clearly demonstrates that the accumulation of Lipid II stimulates *degP* transcription. If RffH partially contributes to the conversion of Lipid II tc Lipid III, then inactivation of this locus should display an attenuated increase in *degP* transcription and should remain ECA$^+$. Moreover, when the *rffH* null mutation is combined with a second mutation that completely abolishes the conversion of Lipid II to Lipid III (*i.e.*, *rffA*$^-$), the second mutation should raise the transcriptional induction of *degP* from the attenuated response observed with only the *rffH* mutation to the strong induction observed with the *rffA* mutation. All of these predictions are verified by the results presented in this chapter.

Thus, although the *rffH* locus appears to be involved in the biosynthesis of ECA, its identification in this role has been lacking because mutational inactivation of this locus is phenotypically masked by the redundant function of the *rfbA* gene.

The lack of porin enhances *E. coli*'s susceptibility to Lipid II accumulation

From the results of Figures 5.3 and 5.6, it is clear that strains lacking the outer membrane porins, OmpF and OmpC are more susceptible to the toxic effects of Lipid II accumulation. The reasons for this enhanced susceptibility are presently unclear. It is possible that the lack of OmpF and OmpC directly alters the structure of the outer membrane, perhaps making this membrane more susceptible to the perturbations caused by the accumulation of Lipid II. For example, Lipid II accumulation may increase the ability of the outer membrane to be solubilized by bile salts when OmpF and OmpC are absent.

Alternatively, We note that the lack of porins in an *ompR*$^-$ background decreases the expression of the σ^E regulon (including *degP*) by approximately 4-fold [Mecsas *et al.* 1993].

Since σ^E regulation is involved in responding to extracytoplasmic protein stresses, *ompR*$^-$ strains (*i.e.*, σ^E attenuated) may not be equipped to properly handle large scale perturbations such as those caused by the accumulation of Lipid II. However, since the precise biochemical effect(s) of Lipid II accumulation are not known, we cannot presently distinguish among these possibilities.

The export-associated toxicity of LamB

To our knowledge, this chapter describes the first instance in which export of the wild-type LamB protein is toxic. The requirement for export of LamB is specific, as expression of the non-exportable LamBΔ60 mutant is not toxic. Interestingly, this toxicity is not due to a gross structural defect in the assembly of LamB. However, we cannot exclude the possibility that a minor structural alteration in LamB assembly (not detectable by membrane fractionation or trimerization studies) confers this toxicity. Alternatively, the transit of large amounts of LamB protein *en route* to the outer membrane may confer the toxicity observed in the Lipid II-accumulating strains. Suppressor analysis may ultimately be informative in identifying the precise molecular nature of this toxicity.

Transcriptional induction of *degP*

Finally, we note that the Lipid II-mediated induction of *degP* transcription represents the first instance in which *degP* transcription has been shown to be simultaneously stimulated by increases in both Cpx and σ^E activity (Figures 5.8 and 5.9).

While Lipid II accumulation manifests several phenotypes, all of these phenotypes intimate a perturbation in the structure of the outer membrane (*e.g.*, SDS hypersensitivity, bile-salt hypersensitivity, toxicity to export of LamB and increased transcription of *degP*). The fact that Lipid II accumulation stimulates both signal transduction pathways that control *degP* transcription further supports the hypothesis that both of these signal transduction pathways monitor extracytoplasmic stress [Mecsas *et al.* 1993, Cosma *et al.* 1995, Snyder *et al.* 1995, Chapter Three]. Moreover, the activation of both pathways indicates

that under some circumstances both Cpx and σ^E are affected by the same types of extracytoplasmic stresses.

Six. Parallel Pathways Perceive Periplasmic Problems

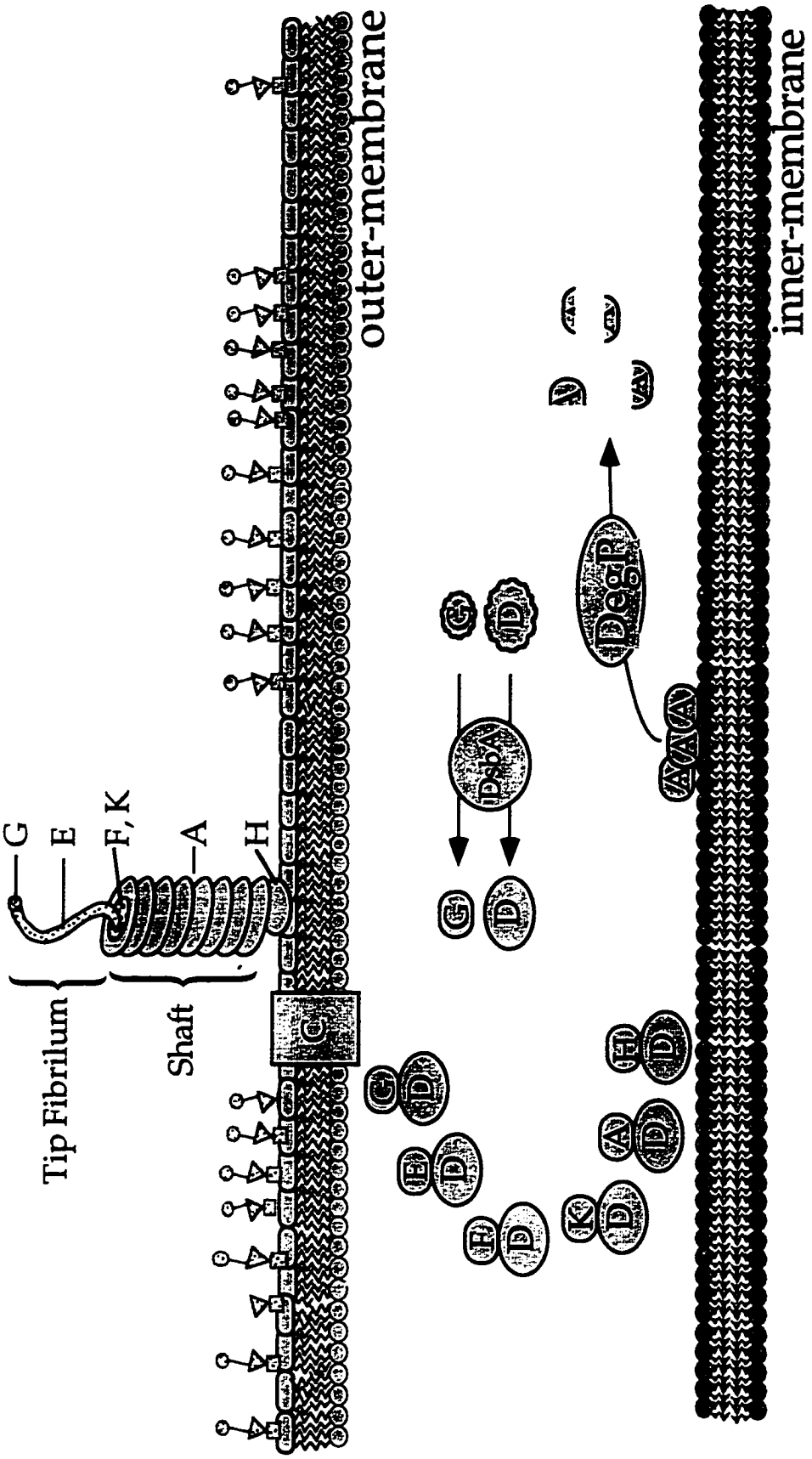
INTRODUCTION

Uropathogenic strains of *E. coli* often express adhesive P pili that attach these pathogens to the human urogenital tract. These P pili are specialized adhesive organelles that emanate from the outer membrane of *E. coli* into the surrounding medium. The P pilus consists of a massive oligomer of six different proteins, PapA, PapH, PapK, PapE, PapF and PapG (see Figure 6.1 for reference). PapH stands at the base of the pilus and anchors the organelle to the outer membrane. PapA is the major component of the pilus, forming the pilus shaft region that is attached to PapH. Emanating from the shaft region is the tip fibrilum which is comprised of PapE. PapK and PapF both serve to bridge the main shaft of the P pilus to the tip fibrilum. Finally, PapG is found at the end of the tip fibrilum. PapG is the P pilus adhesin, and this protein binds to Galα(1–>4)Gal moieties found at the surface of uroepithelial cells [for a thorough review, see Hultgren *et al.* 1996].

P pilus assembly is a tightly regulated process, and presently three major proteins are known to be required for the assembly of this organelle. Each P pilus subunit (Figure 6.1) transiently associates with the periplasmic chaperone, PapD, which pilots the subunit to the outer membrane usher protein, PapC. PapC is required to dissociate the PapD/pilus subunit interactions, and this dissociation ultimately leads to an ordered assembly of the P pilus at the surface of the outer membrane [Hultgren *et al.* 1996]. The periplasmic disulfide bond oxidoreductase, DsbA is also required for P pilus assembly. Specifically, DsbA is required for the proper folding of PapD and PapG [Jacob-Dubuisson *et al.* 1994].

Inactivation of any of these three assembly factors (PapC, PapD or DsbA) abrogates P pilus assembly. For example, mutational inactivation of PapC causes all P pilin subunits to remain associated with PapD in the periplasm. In contrast, if PapD is inactivated, the P-pilin subunits irreversibly aggregate with the inner membrane, and some of these subunits (*e.g.*, PapA) are then rapidly degraded by DegP [Lindberg *et al.* 1989]. As expected, *dsbA* null

Figure 6.1. Biogenesis of a P pilus. A completed P pilus is shown at the surface of the outer membrane. The order of pilus subunit assembly is depicted by the proximity of each subunit to the outer membrane usher, PapC. The subunit nearest the membrane (PapG) is added first. PapE is added second, etc. DsbA is shown catalyzing the formation of functional PapD and PapG proteins. DegP is shown proteolyzing aggregated PapA subunits. PapA, PapC, PapD, PapE, PapF, PapG, PapH and PapK are represented by A, C, D, E, F, G, H and K, respectively.



mutations, which inactivate PapD, confer a similar phenotype to that observed with the *papD* null mutation.

RESULTS

Overproduction of PapG activates the Cpx pathway and stimulates *degP-lacZ* transcription

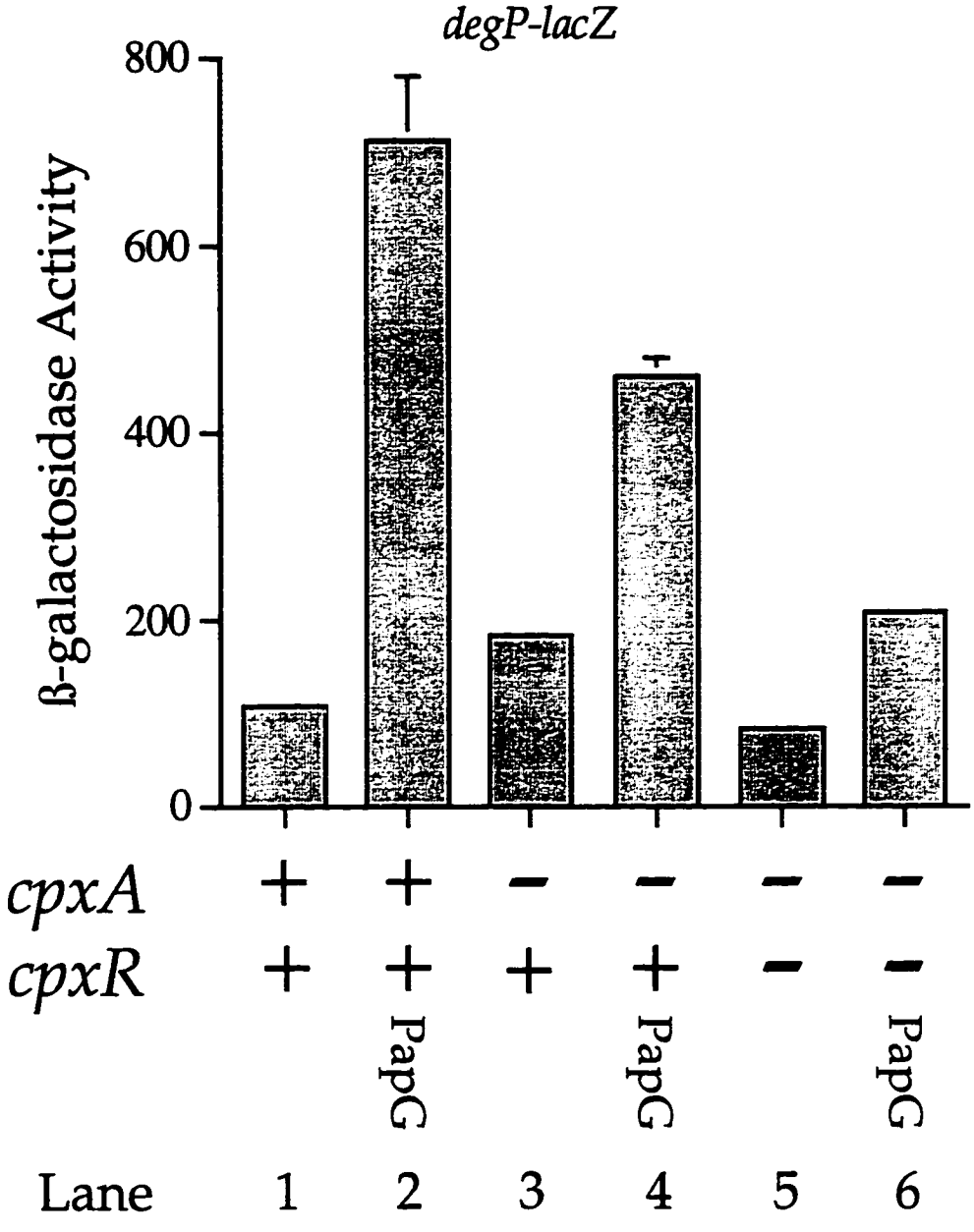
The results presented in this chapter stemmed from initial studies performed by C. Hal Jones, a post-doctoral fellow in Dr. Scott Hultgren's laboratory at Washington University (Saint Louis). Dr. Jones showed that in the absence of PapD, overproduction of PapG produces a toxic aggregate within the bacterial envelope. This aggregation stimulates *degP* transcription (Figure 6.2). Interestingly, Figure 6.2 also shown that this stimulation is heavily (but not entirely) dependent on the integrity of the Cpx system.

I transformed strains PND2000 (MC4100, λRS88[*degP-lacZ*]), SP558 (PND2000, *cpxA::cam*) and SP559 (PND2000, *cpxR::Ω*) with pMMB66 (control for pHJ8) and pHJ8 (overproduces PapG), and then determined the amount of *degP-lacZ* transcription generated from these transformants. Lanes 1 and 2 of Figure 6.2 show that overproduction of PapG stimulates *degP-lacZ* transcription approximately 6.7-fold. In contrast, PapG overproduction only stimulates *degP-lacZ* transcription approximately 2.4-fold in the absence of CpxA (compare lanes 3 and 4 of Figure 6.2). Finally, in the *cpxR*$^-$, *cpxA*$^-$ background, the stimulatory effect of PapG overproduction on *degP-lacZ* transcription is only approximately 2.2-fold (compare lanes 5 and 6). Thus, overproduction of PapG stimulates *degP* transcription, in part, by activation of the Cpx signal transduction system.

Overproduction of PapG activates σ^E

Despite the stimulation of *degP* transcription by the Cpx pathway, Figure 6.2 clearly shows that there is residual transcriptional induction of *degP* by overproduction of PapG even in the *cpx*$^-$ background. Therefore, a second signal transduction system must also stimulate *degP-lacZ* transcription in response to overproduction of PapG. Presently, two signal transduction systems are known to regulate *degP* transcription: the Cpx system and the σ^E modulatory system [Chapter Three, Mecsas *et al.* 1993].

Figure 6.2. Overproduction of pilus subunit PapG stimulates transcription of *degP-lacZ*. PND2000 (MC4100 λRS88[*degP-lacZ*]) (lanes 1 and 2), SP558 (PND2000, *cpxA::cam*) (lanes 3 and 4) and SP559 (PND2000, *cpxR::Ω*) (lanes 5 and 6) were each transformed with pMMB66 (control for pHJ8) and pHJ8 (overproduces PapG). The ß-galactosidase activities of these six transformants were then determined. In the $cpxR^{+}A^{+}$ background, PapG overproduction stimulates *degP-lacZ* transcription ~ 6.7-fold. In the $cpxR^{+}A^{-}$ background, PapG overproduction stimulates *degP-lacZ* transcription ~ 2.4-fold. In the $cpxR^{-}A^{-}$ background, PapG overproduction stimulates *degP-lacZ* transcription ~ 2.2-fold. All strains were grown to early-log phase in Luria broth containing 50μg/ml ampicillin. Upon reaching early-log phase, IPTG was added to each culture to a final concentration of 0.5mM to induce synthesis of PapG. After the addition of IPTG, cultures were incubated for an additional 90 minutes before ß-galactosidase activites were determined (see Chapter Two for details).



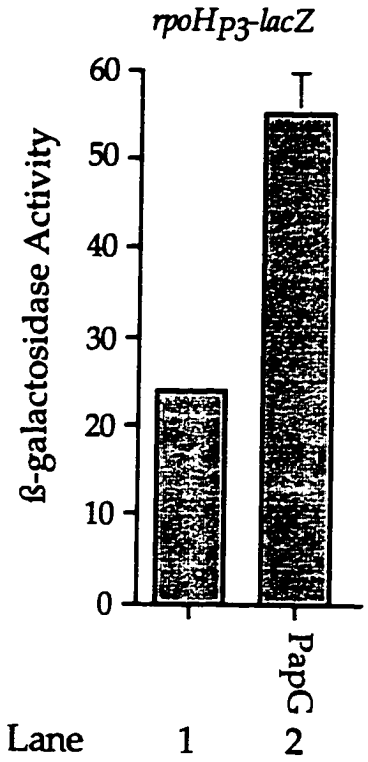


Figure 6.3. Overproduction of PapG increases σ^E activity. Strain SP616 (MC4100, λRS45[*rpoHp3-lacZ*]) was transformed with either pMMB66 (control for pHJ8) (lane 1) or pHJ8 (overproduces PapG) (lane 2), and the resulting ß-galactosidase activities were determined. PapG overproduction stimulates σ^E activity ~ 2.4-fold. All strains were grown to early-log phase in Luria broth containing 50μg/ml ampicillin. Upon reaching early-log phase, IPTG was added to each culture to a final concentration of 0.5mM to induce synthesis of PapG. After the addition of IPTG, cultures were grown for an additional 90 minutes before ß-galactosidase activities were determined (See Chapter Two for details).

The activity of σ^E is modulated by the expression levels of outer membrane proteins. For example, when outer membrane porins are overproduced, σ^E activity (and hence *degP* transcription) rise. We were interested in determining if the residual stimulation of *degP* transcription by overproduction of PapG in the *cpx*⁻ background was due to activation of σ^E. To address this issue, we determined the ß-galactosidase activity of SP616 (MC4100, λRS45[*rpoHp3-lacZ*]) transformed with either: pMMB66 (control for pHJ8) or pHJ8 (overproduces PapG). The *rpoHp3* promoter is solely regulated by σ^E, and thus, this promoter provides a Cpx-independent assay for σ^E activity [Chapter Three, Mecsas *et al.* 1993].

Figure 6.3 shows that overproduction of PapG stimulates *rpoHp3-lacZ* transcription approximately 2.4 fold (compare lanes 1 and 2), indicating that overproduction of PapG stimulates σ^E activity. Interestingly, the degree of stimulation (~ 2.4-fold) closely agrees with the approximate 2.2-fold stimulation of *degP* transcription in the absence of the Cpx proteins (see Figure 6.2, lanes 5 and 6).

Overproduction of PapG stimulates transcription of *cpxP-lacZ*

In an effort to further substantiate the hypothesis that overproduction of PapG stimulates the Cpx pathway, we also wanted to assay a Cpx-regulated locus that was not regulated by σ^E. To this end, we determined the ß-galactosidase activities of strains SP594 (MC4100, λRS88[*cpxP-lacZ*]), SP619 (SP594, *cpxA::cam*) and SP620 (SP594, *cpxR::Ω*) after these strains had been transformed with either: pMMB66 (control for pHJ8) or pHJ8 (overproduces PapG). *cpxP* is a Cpx-regulated locus whose transcriptional regulation is wholly dependent on CpxR and is independent of σ^E (Chapter Four).

Figure 6.4 shows that overproduction of PapG in the parental strain (SP594) stimulates *cpxP-lacZ* transcription approximately 10.3-fold (compare lanes 1 and 2). In the *cpxA*⁻ background (SP619), PapG overproduction only stimulates *cpxP-lacZ* transcription approximately 2.0-fold (compare lanes 3 and 4 of Figure 6.4). Finally, in the *cpxR*⁻, *cpxA*⁻

background (SP620), *cpxP-lacZ* transcription is not induced by overproduction of PapG (compare lanes 5 and 6 of Figure 6.4).

There are two conclusions that can be drawn from the results presented in Figure 6.4. First, overproduction of PapG clearly activates the Cpx pathway to stimulate *cpxP-lacZ* transcription. However, even in the *cpxA*$^-$ background, there is a residual (~ 2.0-fold) induction of *cpxP-lacZ* transcription caused by overproduction of PapG. Since all induction is abolished by the *cpxR*$^-$ mutation, this result implies that CpxA and at least one other factor contribute to the activation of CpxR by overproduction of PapG. The identity of this other factor is presently unknown.

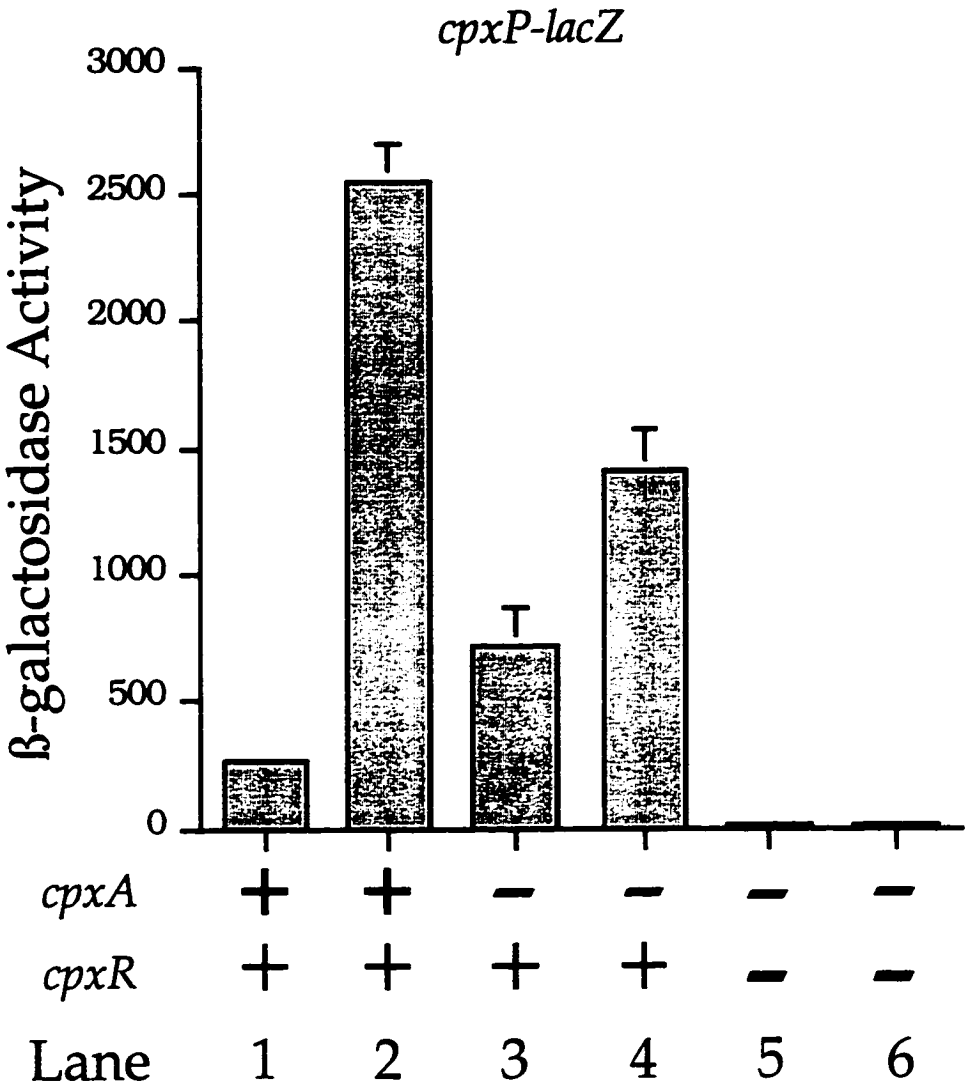
DISCUSSION

Taken together, the results presented in this chapter represent the first instance in which the overproduction of one protein has been shown to simultaneously activate both the Cpx signal transduction pathway and the σ^E modulatory system. This result is important for several reasons.

First, overproduction of PapD, the chaperone that prevents PapG aggregation, prevents the activation of the Cpx and σ^E systems when PapG is overproduced [C.H. Jones and S.J. Hultgren, personal communication]. This result implies that it is the aggregation of PapG that activates σ^E and the Cpx pathway.

Second, it has already been established that σ^E can be activated in response to protein misfolding and misassembly [Mecsas *et al.* 1993, P. Rouvière and C. Gross, personal communication]. In contrast, prior to this study, only two signals were known to activate the Cpx pathway: overproduction of the outer membrane lipoprotein, NlpE [Chapter Three, Snyder *et al.* 1995] and the accumulation of Lipid II [Chapter Four]. While both of these latter stimuli perturb the physiology of the bacterial envelope, the precise mechanism of their activation of the Cpx system is unclear. The results presented in this chapter imply a

Figure 6.4. Overproduction of pilus subunit PapG stimulates transcription of *cpxP-lacZ*. SP594 (MC4100 λRS88[*cpxP-lacZ*]) (lanes 1 and 2), SP619 (PND2000, *cpxA::cam*) (lanes 3 and 4) and SP620 (PND2000, *cpxR::Ω*) (lanes 5 and 6) were each transformed with pMMB66 (control for pHJ8) and pHJ8 (overproduces PapG). The ß-galactosidase activities of these six transformants were then determined. In the $cpxR^+A^+$ background, PapG overproduction stimulates *cpxP-lacZ* transcription ~ 10.3-fold. In the $cpxR^+A^-$ background, PapG overproduction stimulates *cpxP-lacZ* transcription ~ 2.0-fold. In the $cpxR^-A^-$ background, PapG overproduction stimulates *cpxP-lacZ* transcription ~ 1.1-fold. All strains were grown to early-log phase in Luria broth containing 50μg/ml ampicillin. Upon reaching mid-log phase, IPTG was added to each culture to a final concentration of 0.5mM to induce synthesis of PapG. After the addition of IPTG, cultures were incubated for an additional 90 minutes before ß-galactosidase activites were determined (see Chapter Two for details).

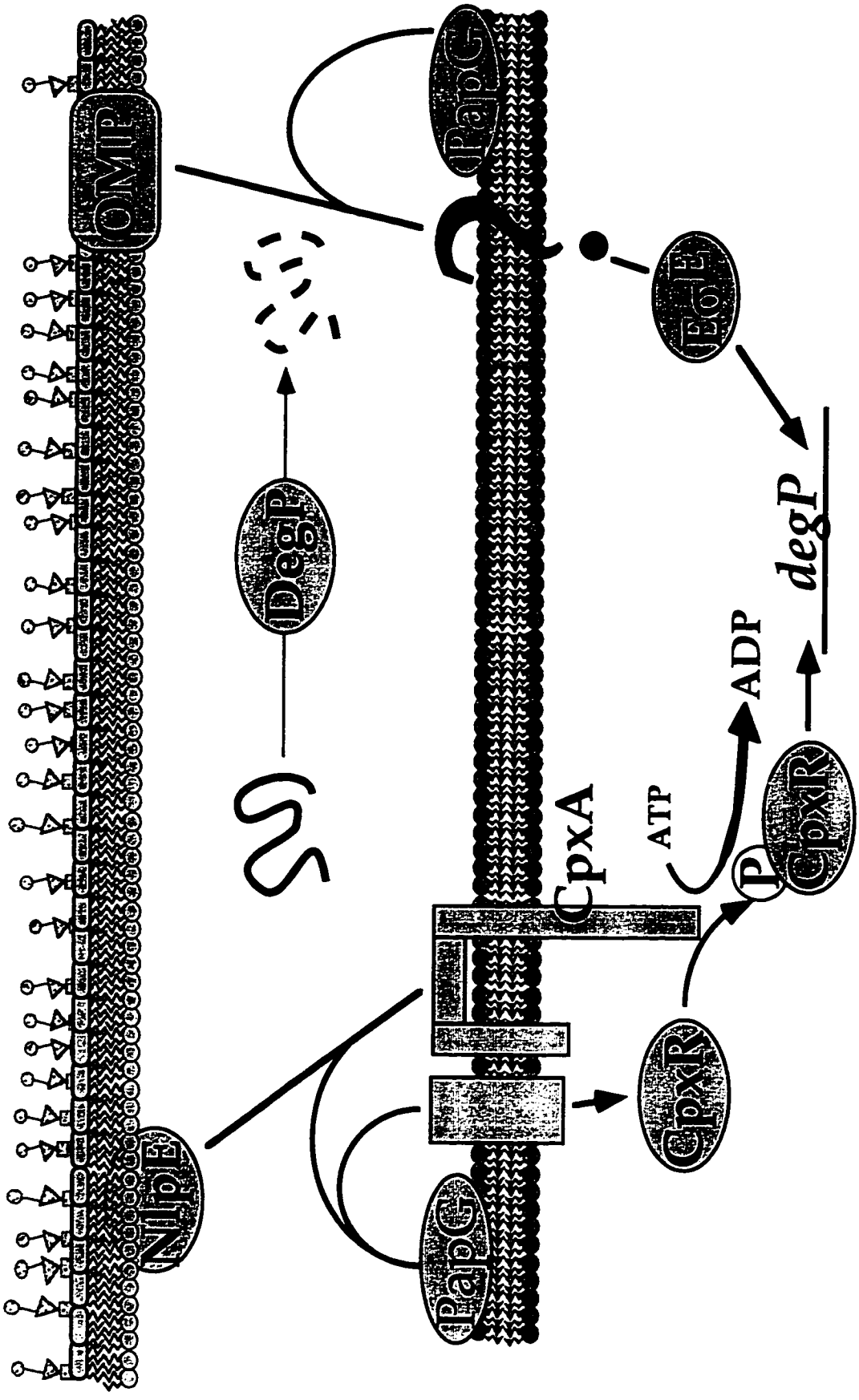


physiological role for the Cpx pathway in monitoring the assembly state of certain extracytoplasmic proteins.

Third, the results presented in the following chapter (Chapter Seven) indicate that the Cpx pathway can stimulate the synthesis of DsbA, which is required for the proper folding of PapG and PapD. Taken together, these results imply that the Cpx pathway not only monitors the assembly state of certain extracytoplasmic proteins, but it attempts to ameliorate perturbations in certain assembly processes by increasing the synthesis of 1) folding agents that can rectify extracytoplasmic protein folding defects and 2) proteases that can destroy misfolded extracytoplasmic proteins.

There is now a collection of outer membrane proteins that, when overproduced will activate CpxA, σ^E and/or other extracytoplasmic sensory systems. For example, overproduction of outer membrane porins stimulates only σ^E activity [Mecsas *et al.* 1993, Chapter Three], whereas overproduction of the outer membrane lipoprotein, NlpE stimulates only CpxA [Chapters Three and Four, Snyder *et al.* 1995]. The results presented in this chapter show that overproduction of PapG stimulates CpxA, σ^E and at least one other sensor [Figure 6.4]. Taken together, these results imply that *E. coli* employs multiple signal transduction systems (Cpx, σ^E and others) to monitor the physiological state of its extracytoplasmic proteins. In some instances these sensory systems monitor the state of the same protein (*e.g.*, PapG), and in other cases each system monitors the physiological state of its own specific set of proteins (Figure 6.5).

Figure 6.5. A model showing the activation of Cpx, σ^E and other sensory systems that monitor the physiological state of extracytoplasmic proteins. σ^E is activated by the level of outer membrane porins as well as the P pilus subunit PapG. CpxA is activated by the level of the outer-membrane lipoprotein, NlpE as well as PapG. PapG also stimulates an unknown sensor (depicted as a rectangle in the inner membrane) that can also activate CpxR.



Seven. The σ^E and the Cpx Signal Transduction Systems Control the Synthesis of Periplasmic Protein-Folding Enzymes in *Escherichia coli*.

INTRODUCTION

σ^E, the second heat-shock inducible σ factor identified in *E. coli*, commands a regulon of at least 11 proteins [Rouvière *et al.* 1995]. Although the σ^E regulon is large, only three of its members have been positively identified: 1) σ^E itself; 2) σ^{32}, the classical heat-shock σ factor; and 3) the periplasmic protease, DegP [Lipinska *et al.* 1988, Erickson and Gross 1989, Raina *et al.* 1995, Rouvière *et al.* 1995]. With this information alone, the primary function of σ^E is not immediately clear. Does σ^E primarily control the synthesis of various σ factors, or does its regulation of DegP more accurately reflect its *raison d'être*?

Fortunately, there is more information regarding the function of σ^E and its other prokaryotic homologs. For instance, σ^E is most homologous to a family of σ factors that is primarily involved in regulating extracytoplasmic and extracellular functions [Lonetto *et al.* 1994, Rouvière *et al.* 1995]. Additionally, strains lacking σ^E (*rpoE*$^-$) display hypersensitivity to detergents and hydrophobic agents, suggesting an outer membrane-permeability defect [Raina *et al.* 1995, Rouvière *et al.* 1995]. Furthermore, Mecsas and coworkers [1993] have shown that σ^E activity is modulated in response to the level of outer membrane proteins found in the outer membrane. Taken together, these results intimate a connection between σ^E and *E. coli*'s extracytoplasmic environs.

The fact that σ^E is a heat-shock inducible σ factor also provides an intriguing hint concerning its function. σ^{32}, the classical heat-shock σ factor, controls the synthesis of at least 25 heat-shock proteins [Rouvière *et al.* 1995, Gross 1996]. The best characterized members of the σ^{32} regulon are cytoplasmic proteases (*e.g.*, Lon) and cytoplasmic molecular chaperones (*e.g.*, DnaK, GroEL/ES) [Gross 1996]. Since σ^E activity is also heat-shock inducible, and since its activity is also modulated by extracytoplasmic events, it seems possible that σ^E serves a complementary function to that performed by σ^{32}. Specifically, σ^E might control the synthesis

of a host of extracytoplasmic proteases and molecular chaperones, just as σ^{32} controls the synthesis of such proteins for the cytoplasm.

Certainly, σ^E's regulation of the periplasmic protease, DegP, supports this notion. Yet, DegP is presently the only extracytoplasmic protein whose synthesis is known to be controlled by this σ factor. To clarify the primary function of σ^E, more members of its regulon must be identified.

In a similar fashion to σ^E, the activity of the Cpx two-component signal transduction system is linked to the physiology of the bacterial envelope. For example, the activity of the two-component inner-membrane sensor, CpxA, can be stimulated by overproduction of the envelope lipoprotein, NlpE, as well as the P pilus subunit, PapG. CpxA responds to these stimuli by communicating with its cognate response regulator, CpxR, which ultimately increases transcription of the *degP* and *cpxP* genes [Chapters Three, Five, Six Cosma *et al.* 1995, Raina *et al.* 1995, Snyder *et al.* 1995].

In addition to being activated by these extracytoplasmic protein-mediated signals, we have shown that activation of the Cpx system can combat the toxicities conferred by various mutant envelope proteins [Cosma *et al.* 1995, Snyder *et al.* 1995]. The Cpx system performs this stress-combative function, in part, by stimulating the synthesis of the periplasmic proteins, DegP and CpxP (See Chapters Three and Five). However, tests of epistasis indicate that the Cpx system can partially combat these extracytoplasmic protein stresses even without DegP and CpxP [Cosma *et al.* 1995, Snyder *et al.* 1995, Chapter Five]. Therefore, the Cpx pathway must control at least one other factor that can combat envelope protein toxicities.

The results described above indicate that although σ^E and the Cpx proteins are influenced by, and affect extracytoplasmic events, the precise functions of the σ^E and Cpx systems are not firmly established. To further our understanding of the roles of both σ^E and Cpx, I sought to identify and characterize new members of each of these regulons.

Through this effort, I have found that the Cpx and σ^E systems each control the synthesis of periplasmic proteins that can aid in protein folding. Since the Cpx and σ^E systems also control the synthesis of the periplasmic protease, DegP, the results described in this chapter suggest that the primary functions of these two regulons may be to mediate protein folding and protein turnover within the bacterial envelope.

RESULTS

Activation of the σ^E and Cpx regulons alters the profile of periplasmic proteins.

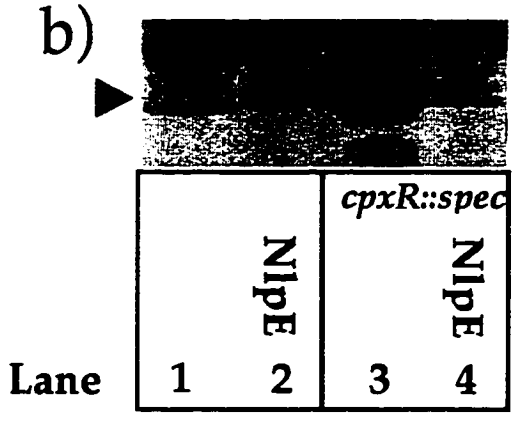
Since the σ^E and Cpx systems exert their effects on the bacterial envelope, it seemed reasonable to assume that some members of their respective regulons would be found in the periplasm of *E. coli*. Under this assumption, I decided to screen for periplasmic proteins whose synthesis could be activated by σ^E or by the Cpx signal transduction system.

Figure 7.1a shows the profile of periplasmic proteins from a strain CLC198 (MC4100, *degP*::Tn*10*) transformed with either: 1) a control plasmid (pBR322, lane 1), or 2) a plasmid that overproduces σ^E (pND12, lane 2). There are some minor alterations in the protein profiles between lanes 1 and 2. However, the most striking difference between these two lanes is the increased intensity of a band that migrates in the 32 kDa size range (Figure 7.1a, compare lanes 1 and 2).

Note that one would expect to see an increased level of DegP in lane 2 as well. However, CLC198 contains a *degP*::Tn*10* mutation, which was utilized in an effort to mitigate any effects that increased proteolysis would have on the profile of periplasmic proteins during these experiments.

In a similar fashion, Figure 7.1b shows the profile of periplasmic proteins from strains SP779 (MC4100, λRS88[*degP-lacZ*], *ara74*::*cam*, *zab*::Tn*10*) and SP781 (SP779, *cpxR*::Ω) transformed with either: 1) a control plasmid (pBAD18, lanes 1 and 3), or 2) a plasmid that overproduces the outer membrane lipoprotein, NlpE (pND18, lanes 2 and 4). Since overproduction of NlpE activates the Cpx signal transduction system [Snyder *et al.* 1995,

Figure 7.1 Activation of the σ^E and Cpx regulons alters the profile of periplasmic proteins. a) Activation of the σ^E regulon increases the intensity of a band migrating in the 32 kDa size range. Periplasmic extracts were prepared from CLC198 (MC4100, *degP*::Tn*10*) transformed with pBR322 (control for pND12) (lane 1), or pND12 (overexpresses *rpoE*) (lane 2). The 32 kDa band whose intensity rises from lane 1 to lane 2 is marked with an arrow. b) Activation of the Cpx two-component signal transduction pathway increases the intensity of a band migrating in the 23 kDa size range. Periplasmic extracts in lanes 1 and 2 were prepared from SP779 (MC4100, λRS88[*degP-lacZ*], *ara74*::*cam*, *zab*::Tn*10*) transformed with either pBAD18 (control for pND18) (lane 1) or pND18 (overexpresses *nlpE*) (lane 2). Periplasmic extracts in lanes 3 and 4 were prepared from SP781 (SP779, *cpxR*::Ω) transformed with either pBAD18 (lane 3) or pND18 (lane 4). The 23 kDa band whose intensity rises from lane 1 to lane 2 is marked with an arrow. Strains used to generate the extracts depicted in panel a) were grown in Luria broth with ampicillin. Strains used to generate extracts depicted in panel b) were grown in Luria broth containing ampicillin and 0.4% L-arabinose. See Chapter Two for details.



Chapter Three], the strain used to prepare the periplasmic extract shown in lane 2 of Figure 7.1b possess an activated Cpx signal transduction system. Lanes 3 and 4 of Figure 7.1b show extracts from transformant derivatives of SP781, which contains a *cpxR* null mutation. Thus, these lanes serve as controls to help in determining if any changes between the protein profiles of lanes 1 and 2 are actually dependent on the Cpx pathway. Figure 7.1b shows a band of approximately 23 kDa in size whose intensity rises from lane 1 to lane 2. No change in the intensity of this band is observed when comparing lanes 3 and 4, indicating that the increased intensity of this band is dependent on CpxR.

Amino-terminal sequencing of the 32 kDa and 23 kDa bands.

The 32 kDa and 23 kDa bands shown in Figure 7.1 were promising candidates for proteins that could be regulated by σ^E and Cpx, respectively. Accordingly, the identity of the first 11 amino acid residues from the amino terminus of each protein was determined by Edman degradation (see Chapter Two for details). The sequence determined for the 32 kDa band (AEAAKPATAAD) corresponds to residues 26 through 36 of FkpA, a peptidyl-prolyl *cis/trans* isomerase identified by Horne and Young [1995]. Missiakas *et al.* [1996] have provided evidence suggesting that FkpA performs a protein folding function within the extracytoplasmic compartments of *E. coli*. The sequence determined for the 23 kDa band (AQYEDGKQYTT) corresponds to residues 20 through 30 of DsbA, a periplasmic protein required for efficient disulfide bond formation in *E. coli* [Bardwell *et al.* 1991, Kamitani *et al.* 1992].

Previous characterization of FkpA had not determined its subcellular location [Horne and Young 1995]. In this study, the amino-terminal sequence determined for FkpA corresponds to the predicted amino terminus that would be generated after a signal-sequence-cleavage event. Taken together, the sequencing and fractionation of FkpA demonstrate that it is a periplasmic protein with a functional signal sequence. Note that the sequence determined for DsbA also corresponds to the mature (signal-sequence processed) form of the protein.

Transcriptional regulation of *fkpA*.

There are several possible explanations for the increased amount of FkpA found in periplasmic extracts of strains overproducing σ^E. For example, FkpA synthesis could be induced at the transcriptional or translational levels. The stability of FkpA could also be affected in these strains. However, since σ^E is involved in transcriptional initiation [Erickson and Gross 1989], the simplest model posits that increased levels of σ^E concomitantly increase *fkpA* transcription.

Accordingly, I constructed an *fkpA-lacZ* operon fusion to determine if the σ^E-overproducing plasmid (pND12) affected *fkpA* transcription. This fusion was recombined onto a λ phage and was placed in single-copy at the *attB* locus on the *E. coli* chromosome (see Chapter Two for details). ß-galactosidase activities were determined from derivatives of SP887 (MC4100, λRS88[*fkpA-lacZ*]) that were transformed with either: 1) the pBR322 control plasmid (Figure 7.2, lane 1), or 2) pND12, which overproduces σ^E (Figure 7.2, lane 2). Figure 7.2 illustrates that the σ^E-overproducing plasmid stimulates *fkpA* transcription approximately 7-fold when compared to its control strain. Note that this amount of transcriptional induction accounts for the increased amount of FkpA shown in Figure 7.1a. Thus, σ^E can stimulate *fkpA* transcription.

***fkpA* transcription is affected by fluctuations in σ^E activity**

To determine the extent of σ^E's influence on *fkpA*, I quantified the amount of *fkpA* transcription that is generated during situations in which σ^E activity is altered by extracytoplasmic events.

For example, Mecsas and coworkers [1993] demonstrated that overproduction of the outer membrane protein, OmpX stimulates σ^E activity approximately 4-fold. Accordingly, I was interested in determining if *fkpA* transcription would also be induced by overproduction of OmpX. I measured the amount of *fkpA-lacZ* transcription generated from SP887 (MC4100, λRS88[*fkpA-lacZ*]) that contained either: 1) a control plasmid, pBR322 (Figure 7.3a lane 1) or

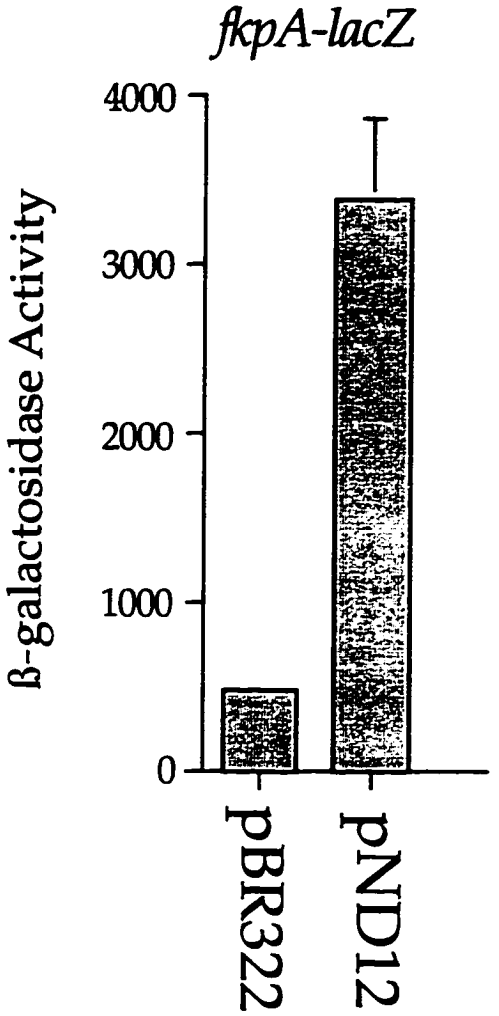


Figure 7.2. Overproduction of σ^E stimulates transcription of *fkpA-lacZ*. ß-galactosidase activities of SP887 (MC4100, λRS88[*fkpA-lacZ*]) transformed with pBR322 (control for pND12) (lane 1) or pND12 (overexpresses *rpoE*) (lane 2) were assayed. The *rpoE* overexpressing strain stimulates transcription of *fkpA-lacZ* ~ 7-fold over that of the control strain. Strains were grown in Luria broth with ampicillin as described in Chapter Two.

2) pJE100, a plasmid that overproduces the outer membrane protein, OmpX [Mecsas *et al.* 1993]. Comparison of lanes 1 and 2 of Figure 7.3a shows that overproduction of OmpX stimulates *fkpA-lacZ* transcription approximately 2-fold when compared to a control strain. Thus, overproduction of OmpX, which stimulates σ^E activity, also stimulates *fkpA* transcription, albeit to a lesser extent than is observed with *degP* transcription.

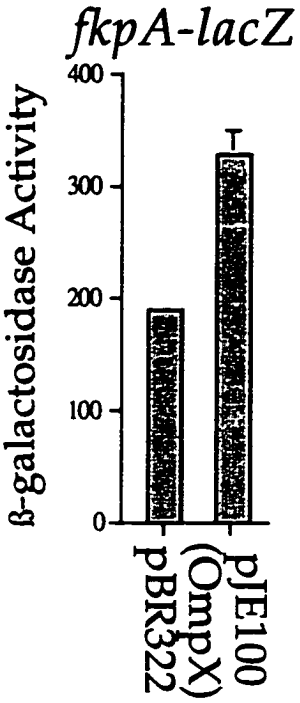
The *surA* null mutation impairs the assembly of the porins, OmpF and OmpC, and as a result of this assembly defect, the mutation also increases σ^E activity approximately 5-fold [Lazar and Kolter 1995, Missiakas *et al.* 1996, Rouvière and Gross, personal communication, data not shown]. *surA* specifies an abundant periplasmic peptidyl prolyl *cis/trans* isomerase, and it is believed that the SurA protein directly catalyzes a step(s) in the folding of the porins mentioned above [Lazar and Kolter 1995, Missiakas *et al.* 1996, Rouvière and Gross, personal communication].

The porin assembly defect conferred by the *surA* null mutation is aggravated by the absence of the DegP protease. Although the *degP* null mutation does not stimulate σ^E activity on its own, when it is introduced into a *surA* null strain, σ^E activity rises 10-fold [data not shown, Rouvière and Gross, personal communication].

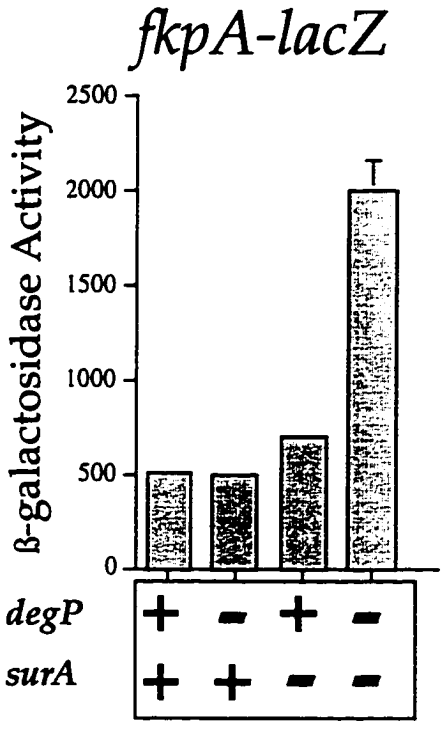
I was interested in determining the extent of *fkpA* transcriptional induction under these circumstances as well. Specifically, I quantified the transcription generated from the *fkpA-lacZ* fusion in either: 1) a wild-type background (Figure 7.3b lane 1); 2) a *degP*$^-$ background (Figure 7.3b lane 2); 3) a *surA*$^-$ background (Figure 7.3b lane 3) or 4) a *surA*$^-$ *degP*$^-$ double-mutant background (Figure 7.3b lane 4). As expected, the *degP* null mutation has no effect on *fkpA* transcription. In contrast, the *surA* null increases *fkpA* transcription approximately 1.5-fold when compared to the wild-type strain (compare lanes 1 and 3 of Figure 7.3b). The *surA*$^-$ *degP*$^-$ double mutant displays the largest induction of *fkpA* transcription at 4-fold over that of the wild-type strain (compare lanes 1 and 4 of Figure 7.3b). Again, these results indicate that extracytoplasmic defects that increase σ^E activity, also stimulate *fkpA* transcription.

Figure 7.3. *fkpA* transcription is affected by fluctuations in $E\sigma^E$ activity. a) Overproduction of the outer-membrane protein, OmpX, stimulates *fkpA-lacZ* transcription. ß-galactosidase activities of SP887 (MC4100, λRS88[*fkpA-lacZ*]) transformed with either pBR322 (control for pJE100) (lane 1), or pJE100 (overproduces OmpX) (lane 2) were determined. The OmpX overproducing plasmid increases *fkpA-lacZ* transcription ~ 1.8 fold when compared to the control strain. b) The *surA*⁻ *degP*⁻ double mutant stimulates *fkpA-lacZ* transcription. ß-galactosidase activities of SP887 (lane 1), SP940 (SP887, *degP*::Tn*10*) (lane 2), SP921 (SP887, *surA::kan*) (lane 3) and SP942 (SP921, *degP*::Tn*10*) (lane 4) were determined. The *degP*::Tn*10* mutation alone does not stimulate *fkpA* transcription (compare lanes 1 and 2). In contrast, the *surA* null mutation alone stimulates *fkpA* transcription ~ 1.4-fold, while the *surA*⁻ *degP*⁻ double mutant stimulates *fkpA-lacZ* transcription ~ 4-fold when compared to the control strain. Strains were grown in Luria broth and all procedures were performed as described in Chapter Two.

a)



b)



A σ^E promoter stimulates *fkpA* transcription

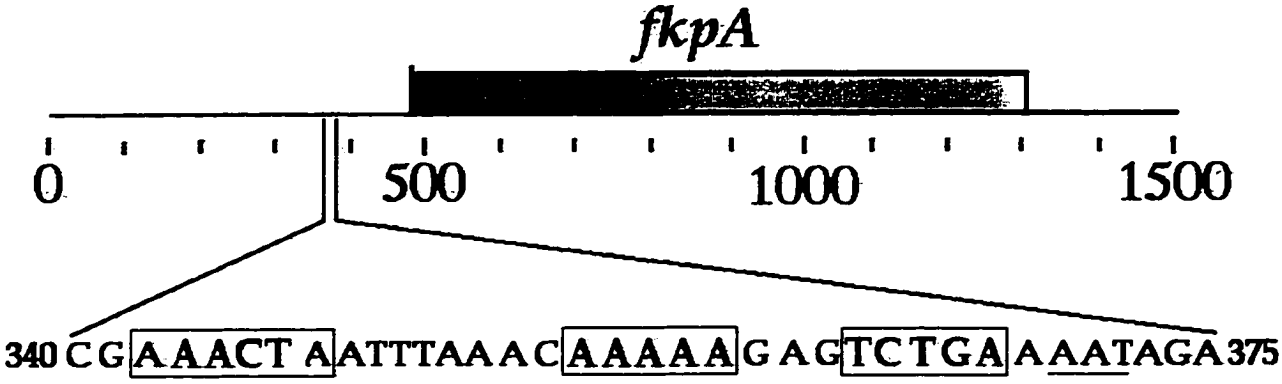
The transcriptional induction of *fkpA* by overproduction of σ^E (and by stimuli that activate σ^E) suggests that *fkpA* transcription is controlled, at least in part, by a σ^E promoter. Indeed, an analysis of the noncoding upstream sequence of *fkpA* highlights a putative σ^E promoter (Figure 7.4a). Nucleotides 342 to 347 of the published *fkpA* sequence [Horne and Young 1995] contain 4 of the 6 consensus nucleotides for a σ^E -35 promoter site (aAACTa). This site is followed by a consensus 16 nucleotide-long spacer region that contains a string of five adenine nucleotides that are also characteristic of σ^E promoters [Lipinska *et al.* 1988, Erickson and Gross 1989, Raina *et al.* 1995, Rouvière *et al.* 1995]. This spacer region is followed by a -10 site that possesses five of five consensus nucleotides (TCTGA).

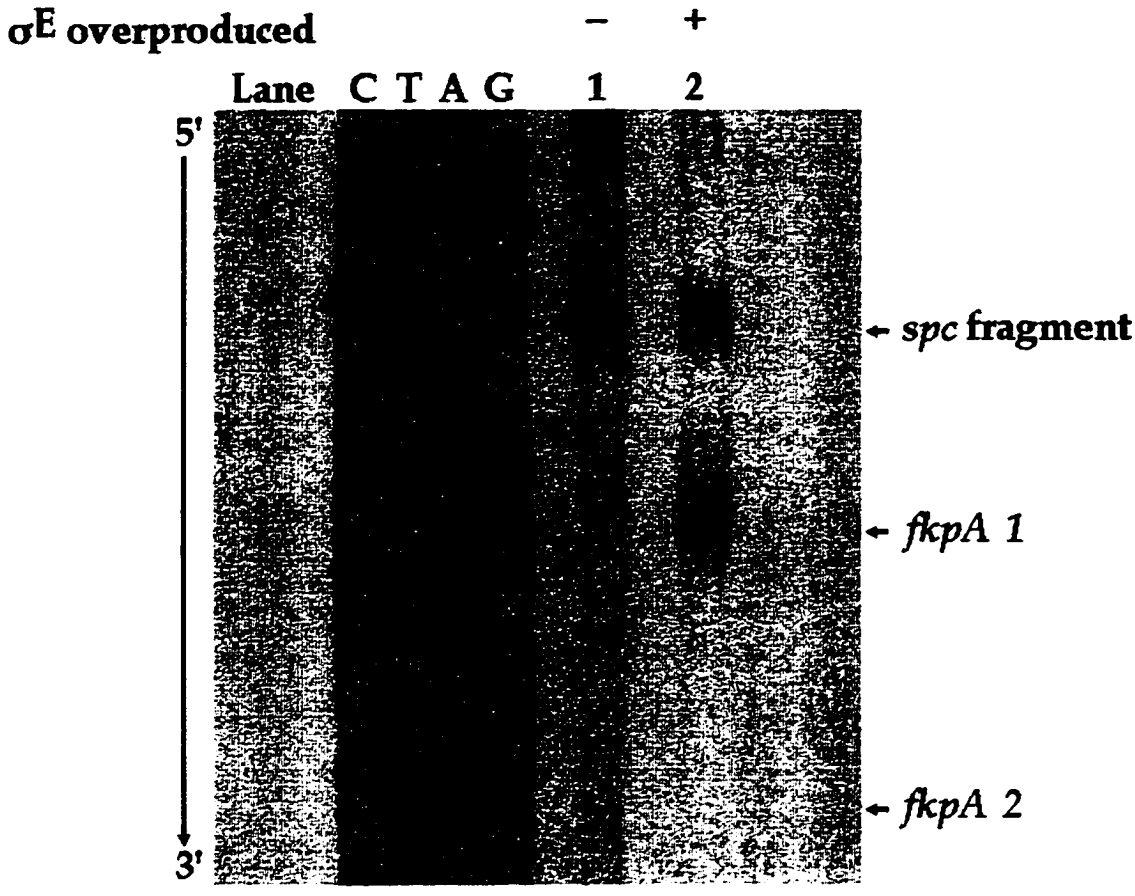
In light of this putative σ^E promoter, I wanted to determine the start site of the *fkpA* transcript(s) that was(were) induced upon overproduction of σ^E. To this end, I performed S1 nuclease protection assays utilizing RNA prepared from a strain that was transformed with either 1) pBR322 (control for pND12) or 2) pND12 (overproduces σ^E). Figure 7.4b shows that the transcripts induced by overproduction of σ^E begin at nucleotides 370, 371 and 372 of the published *fkpA* sequence, which correspond to positions of +4, 5 and 6 with respect to the σ^E -10 promoter site (see Figure 7.4a) [Horne and Young 1995].

Although this initiation site is relatively close to the σ^E -10 site (the strongest σ^E transcripts begin at positions +8 and +9 [Raina *et al.* 1995, Rouvière *et al.* 1995]), other *E. coli* promoters have also been shown to initiate transcription at such early sites [Harley and Reynolds 1987]. Moreover, the results presented in Figure 7.4b also help to explain the attenuated induction of *fkpA* transcription that is observed upon activation of σ^E.

Recall that situations in which σ^E activity is induced 4-fold will only stimulate *fkpA* transcription 2-fold (Figure 7.3). There are at least three possible explanations for this observation. First, the position of the transcriptional initiation sites relative to the σ^E -10 site

Figure 7.4. Overproduction of σ^E stimulates transcription from a site downstream of a σ^E-like promoter. Panel a); The *fkpA* coding sequence is shown as a shaded rectangle with a scale (in nucleotides) below. A portion of the upstream non-coding sequence of *fkpA* is shown below the scale. This nucleotide sequence corresponds to positions 340 to 375 in the published sequence of Horne and Young [1995]. The regions comprising the components of the σ^E-like promoter are enclosed in rectangles and consensus nucleotides are shown in bold-face. The initiation sites of transcripts induced by overproduction of σ^E (see panel b) are underlined. Panel b): Lanes 1 and 2 show the *fkpA* transcription start sites from strain SP887 (MC4100, λRS88[*fkpA-lacZ*]) transformed with either a control plasmid, pBR322 (lane 1), or the σ^E overproducing plasmid, pND12 (lane 2). A digested fragment corresponding to the 5′ end of the *spc* operon is also noted by the arrow marked "*spc* fragment." This fragment serves as an internal loading control. Two groups of *fkpA* transcripts are shown (*fkpA*-1, and *fkpA*-2). The *fkpA*-1 arrow shows protected DNA fragments that correspond to transcripts that initiate at positions 371, 372 and 373 of the *fkpA* sequence [Horne and Young 1995]. These positions are underlined in panel a) and they correspond to positions +4, 5 and 6 relative to the σ^E -10 site shown in panel a). Note that the *fkpA* 1 fragments are only present in lane 2 (σ^E overproduced). The *fkpA*-2 arrow shows a protected DNA fragment that corresponds to a transcript that initiates at position 401 of the *fkpA* sequence [Horne and Young 1995]. Note that the *fkpA*-2 fragment is only present in lane 1 (σ^E not overproduced). The lanes labeled C, T, A and G indicate dideoxy sequencing reactions initiated by the same oligonucleotide used to label the S1 probe (Fkpalac3). The 5′ to 3′ direction of the non-template nucleotide sequence of *fkpA* is from the top of the panel b) to the bottom, as shown by the 5′——>3′ arrow. RNA was prepared from strains grown at 37°C in Luria broth containing 125μg/ml ampicillin. See Chapter Two for details.





may diminish the ability of RNA polymerases containing σ^E to transcribe this promoter. Second, the σ^E -35 site of *fkpA* does not possess 100% identity with the consensus -35 site (see Figures 7.4a and 7.5). The σ^E-regulated *rpoEP2* promoter, which contains a non-consensus -10 site and a transcriptional initiation site of +10, also displays an attenuated response to activation by σ^E [Rouvière *et al.* 1995]. Third, lane 1 of Figure 7.4b shows that when σ^E is not overproduced, a transcriptional initiation site is located at position 401 of the *fkpA* sequence. When σ^E is overproduced, this transcript disappears (lane 2 of Figure 7.4b). Thus, the total amount of transcriptional induction that is observed with the *fkpA-lacZ* fusion is attenuated because although σ^E overproduction stimulates transcriptional initiation at nucleotides 370 to 372, it also concomitantly decreases transcription initiating at nucleotide 401.

I have also noted that the *rpoE* null mutation (which eliminates σ^E synthesis) does not radically reduce transcription of *fkpA-lacZ* (data not shown). However, this is not surprising in light of the results presented in Figure 7.4b. When σ^E is not activated (*i.e.*, non-stressing conditions), the majority of transcription generated from *fkpA* initiates at position 401. It is only upon *activation* of σ^E that I observe transcriptional initiation at sites 370 to 372.

Taken together, the results of Figure 7.4b show that *fkpA* possesses a σ^E-activatable promoter that has the standard features recognized by σ^E. Figure 7.5 shows this *fkpA* promoter aligned with the three previously characterized σ^E promoters of *E. coli*.

Transcriptional regulation of *dsbA*

There are several possible explanations for the increased amount of DsbA found in periplasmic extracts of strains possessing an activated Cpx signal transduction system. For example, DsbA synthesis could be induced at the transcriptional or translational levels. The stability of DsbA could also be affected in these strains. However, since The Cpx pathway controls the transcription of *degP*, and since CpxR is homologous to the OmpR subfamily of two-component transcription factors [Dong *et al.* 1993], the simplest interpretation of the results presented in Figure 7.1b is that activation of the Cpx pathway stimulates *dsbA* transcription.

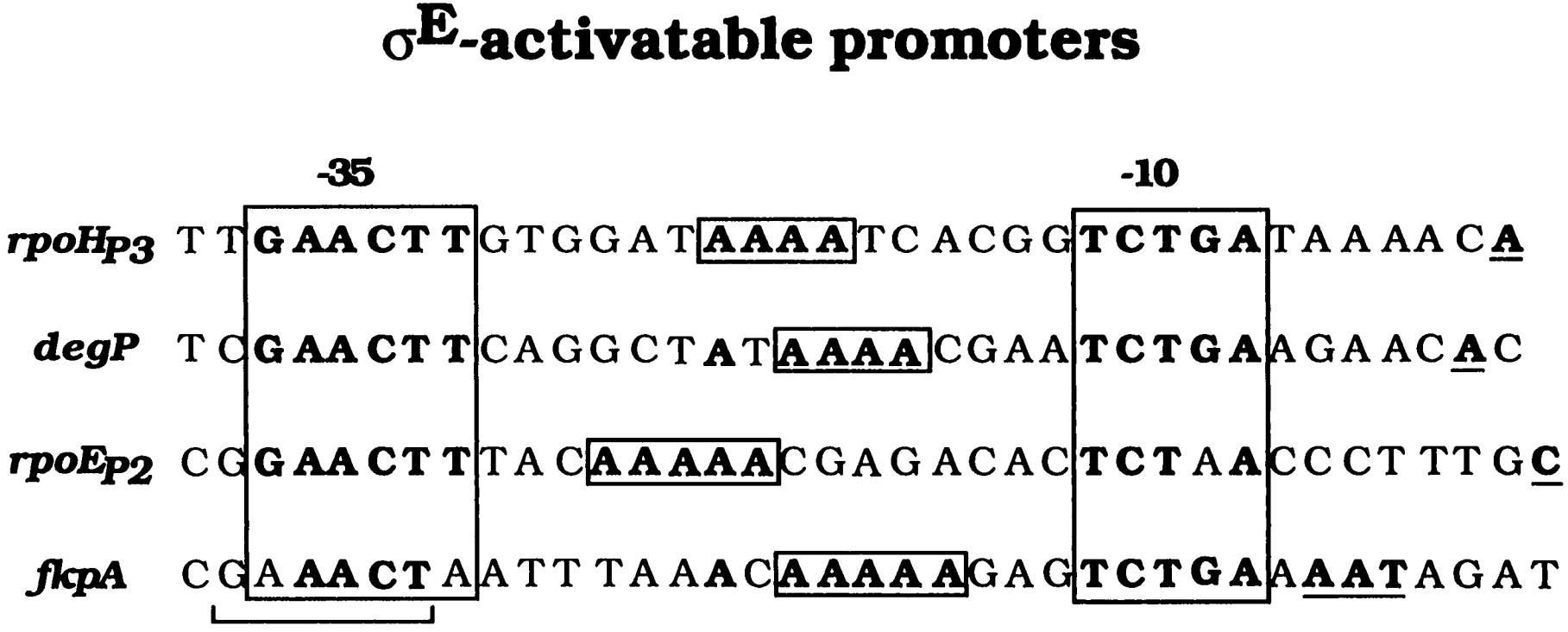


Figure 7.5. Alignment of σ^E-activatable promoters. The -35, -10 and polyA regions are boxed. The transcription start sites are underlined. See text for details.

To test the possibility that the Cpx pathway could activate transcription from the *dsbA* locus, I created an *orfA-dsbA-lacZ* operon fusion. This fusion was recombined onto a λ phage and was placed in single-copy at the *attB* locus on the *E. coli* chromosome (see Chapter Two for details).

The incorporation of *orfA* into the operon fusion was necessitated because the *dsbA* gene is situated in an operon with an upstream gene, *orfA*, of unknown function (Figure 7.6). Transcription of *dsbA* is directed from two promoters, each of which contributes to approximately one-half of DsbA synthesis. The first promoter is situated within the *orfA* (p*dsbA*) coding sequence, while the second is positioned upstream of *orfA* (p*orfA-dsbA*). This latter promoter cotranscribes both *orfA* and *dsbA* (Figure 7.6) [Belin and Boquet 1994].

Thus, to ensure that all of the transcripts used to synthesize DsbA were represented in the operon fusion, I fused the promoter region of *orfA*, the entire *orfA* coding sequence (which contains the first *dsbA* promoter) and the first 86 nucleotides of the *dsbA* coding sequence to the *lac* operon, creating p*orfA-dsbA-lacZYA* (Figure 7.6).

I first wanted to determine if *dsbA* transcription could be stimulated by the *cpxA24* allele, which possesses a mutation that hyperactivates the Cpx pathway and stimulates *degP* transcription approximately 8-fold [Chapter Three]. To assess the effect of this mutation on *dsbA* transcription, I introduced the *cpxA24* mutation into strain SP969 (MC4100, λRS88[p*orfA-dsbA-lacZ*]), thus generating strain SP971 (SP969, *cpxA24*). Comparison of lanes 1 and 2 in Figure 7.7 shows that the *cpxA24* mutation stimulates p*orfA-dsbA-lacZ* transcription 6-fold, indicating that activation of the Cpx pathway stimulates *dsbA* transcription.

The transcriptional induction of *dsbA* can also be observed when the outer membrane lipoprotein, NlpE is overproduced. Overproduction of NlpE has previously been shown to activate the wild-type Cpx pathway and stimulate *degP* transcription [Chapter Three, Snyder *et al.* 1995]. Figure 7.8 shows the results of ß-galactosidase assays performed on strains SP994 (MC4100, λRS88[p*orfA-dsbA-lacZ*], *ara74::cam zab*::Tn*10*), SP995 (SP994, *cpxA::cam*) and SP996 (SP994, *cpxR::Ω*) transformed with either: 1) pBAD18 (control for pND18) or pND18

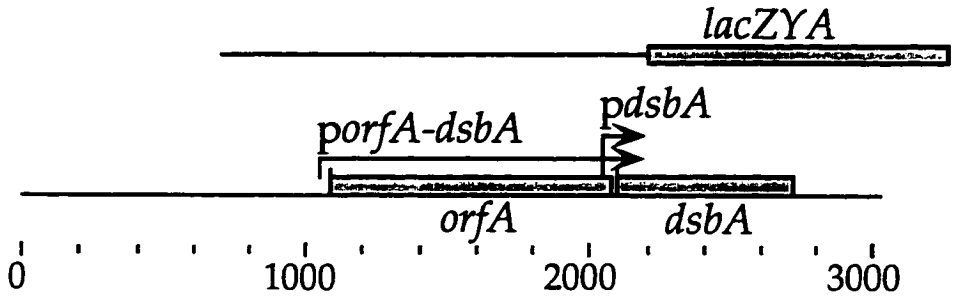


Figure 7.6. The *orfA-dsbA* operon and the p*orfA-dsbA-lacZ* operon fusion. The *orfA* and *dsbA* open reading frames are shown as shaded rectangles. The two promoters that transcribe *dsbA* (p*orfA-dsbA* and p*dsbA*) are depicted as arrows emanating from their respective initiation sites. The genomic DNA used to create the p*orfA-dsbA-lacZ* operon fusion is shown as a thin line fused to the *lacZ* operon above the *orfA* and *dsbA* coding sequences. A 3000 nucleotide-long scale is shown below the operon for reference.

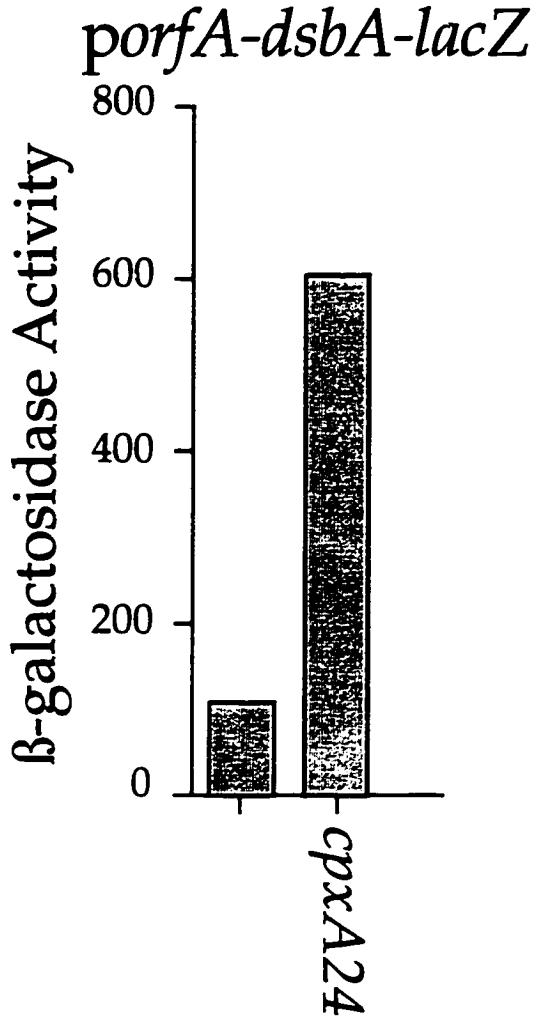


Figure 7.7. A hyperactive *cpxA* allele stimulates transcription of p*orfA-dsbA-lacZ*. ß-galactosidase activities from SP969 (MC4100, λRS88[p*orfA-dsbA-lacZ*]) and SP971 (SP969, *cpxA24*) were determined. The *cpxA24* mutation stimulates *dsbA-lacZ* transcription ~ 6-fold.

(overproduces NlpE). Comparison of lanes 1 and 2 in Figure 7.8 shows that NlpE overproduction stimulates p*orfA-dsbA-lacZ* transcription 5.3-fold. This stimulatory effect is not observed in the *cpxA*⁻ and *cpxR*⁻ *cpxA*⁻ backgrounds (compare lanes 1 and 2 with lanes 2 and 4; 5 and 6 of Figure 7.8). Thus, overproduction of NlpE stimulates *dsbA* transcription by activating the wild-type CpxA protein.

Activation of the Cpx pathway stimulates *dsbA* transcription from the promoter upstream of the *orfA* locus

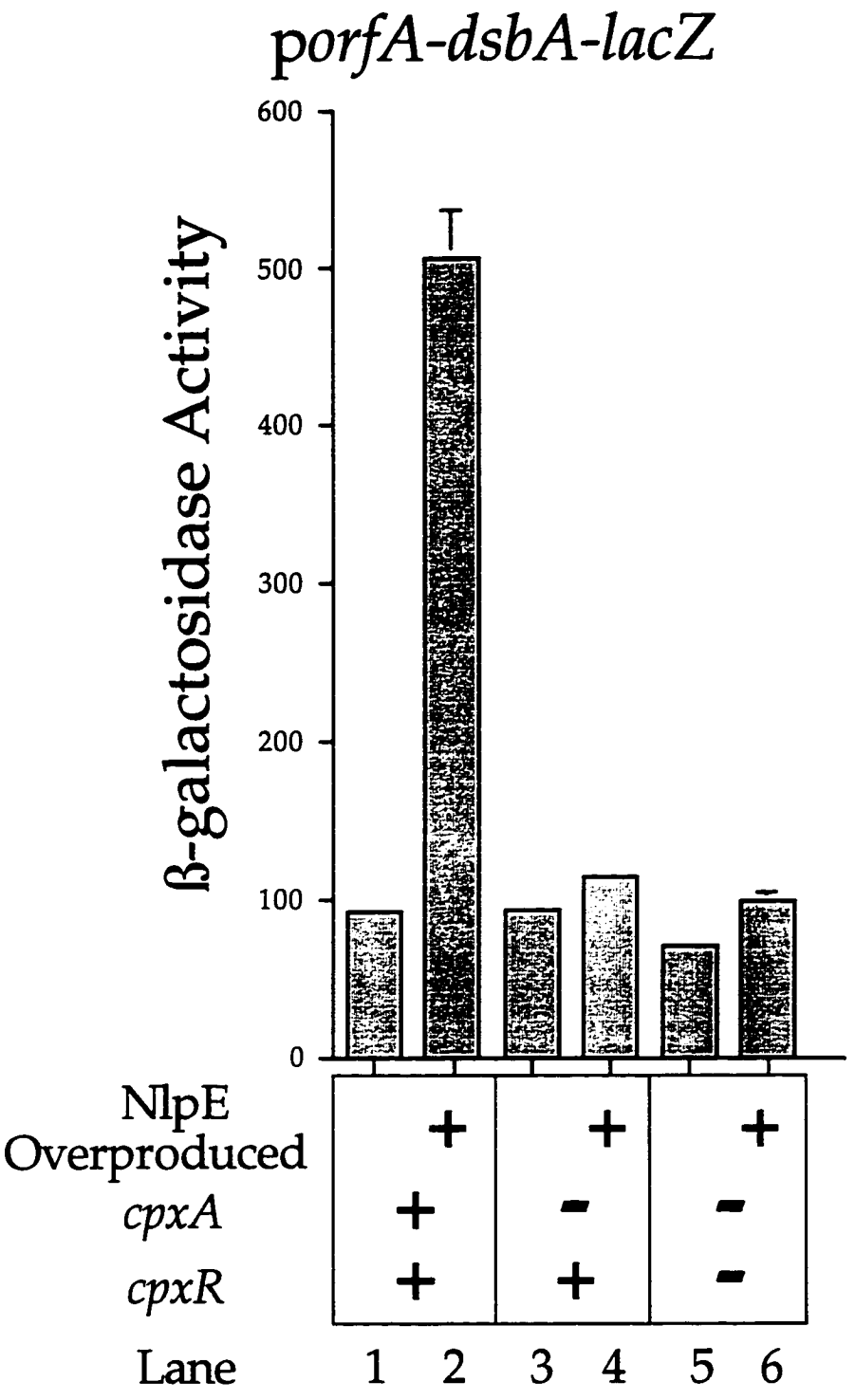
Since *dsbA* transcription has been shown to originate from two promoters, one within the *orfA* coding sequence and one which also contranscribes *orfA* (see Figure 7.6), I was interested in determining which of these two promoters was activated by the activated Cpx pathway. To address this issue, I used S1 nuclease protection assays to quantify the amount of transcription generated from the p*orfA-dsbA* promoter and the p*dsbA* promoter using RNA prepared from strains that contain either an *nlpE* overexpressing plasmid (pND18) or a control plasmid (pBAD18). Recall that overproduction of NlpE activates *dsbA* transcription in a CpxA-dependent fashion (Figure 7.8).

The p*dsbA* promoter is unaffected by overproduction of NlpE (data not shown). In contrast, Figure 7.9a shows that transcription from the p*orfA-dsbA* promoter is stimulated overproduction of NlpE. Also, the primary transcriptional initiation site induced by overproduction of NlpE corresponds to the +1 site described for p*orfA-dsbA* by Belin and Pascal [1994] (see Figure 7.9a, b). Thus, overproduction of NlpE increases the synthesis of DsbA by stimulating transcription from the p*orfA-dsbA* promoter.

σ^E and Cpx do not coregulate transcription of *fkpA* and *dsbA*

Previous studies have indicated that the Cpx signal transduction system and σ^E jointly control the transcription of the *degP* locus [Chapter Three, Raina *et al.* 1995]. Accordingly, I was interested in determining if the transcription of any of the other members of the Cpx and σ^E regulons were also jointly regulated. To address this issue in this study, I determined if the overproduction of σ^E could activate *dsbA* transcription, and I also determined if activation of the Cpx pathway (by overproduction of NlpE) could activate *fkpA* transcription. In the former

Figure 7.8. Overproduction of NlpE stimulates p*orfA-dsbA-lacZ* transcription *via* the Cpx pathway. Lanes 1, 3 and 5 show ß-galactosidase activity of strains transformed with pBAD18 (control for pND18). Lanes 2, 4 and 6 show the ß-galactosidase activity of strains transformed with pND18 (overexpresses *nlpE*). Lanes *1* and 2: SP994 (MC4100, λRS88[p*orfA-dsbA-lacZ*], *ara74::cam zab*::Tn*10*). Lanes 3 and 4: SP995 (SP994, *cpxA::cam*). Lanes 5 and 6: SP996 (SP994, *cpxR::Ω*). All strains were grown in Luria broth containing 0.4% L-arabinose and 50µg/ml ampicillin (See Chapter Two for details).



case, overproduction of σ^E does not activate *dsbA* transcription, and in the latter case, overproduction of NlpE does not stimulate *fkpA* transcription (data not shown). Thus, while Cpx and σ^E intersect in their regulation of *degP*, their regulons do not completely overlap.

DISCUSSION

The control of protein folding agents within the bacterial envelope

The results presented in this chapter provide the first evidence that the σ^E and Cpx regulons include not only proteases like DegP, but that they also contain periplasmic enzymes that can engage in protein-folding activities. This result is important because it implies that there is a second envelope-protein stress-combative tool at the disposal of *E. coli* - specifically, protein folding agents. Thus, much like σ^{32} controls the synthesis of a host of cytoplasmic proteases and molecular chaperones [Gross 1996], the σ^E and Cpx regulons may perform a complementary function, mediating protein folding and protein turnover within the bacterial envelope.

FkpA and the σ^E regulon

The initial identification of *fkpA* noted that this protein was homologous to the Mip-family of peptidyl-prolyl *cis/trans* isomerases [Horne and Young 1995]. The Mip proteins are found in prokaryotic pathogens such as *Legionella pneumophila* [Cianciotto *et al.* 1989, 1990, Fischer *et al.* 1992], where they are required for macrophage infection (*m*acrophage *i*nfectivity *p*otentiator).

Missiakas *et al.* [1996] have recently provided evidence that high-level synthesis of FkpA can suppress extracytoplasmic stresses, such as the accumulation of unfolded periplasmic and outer membrane proteins. These authors have also demonstrated that FkpA is a peptidyl-prolyl *cis/trans* isomerase, suggesting that the function of this protein is to facilitate the folding of other extracytoplasmic proteins.

The results presented in this chapter provide a satisfying complementary analysis to that performed by Missiakas *et al.* [1996]. Specifically, I have shown that both the

overproduction of σ^E and the creation of extracytoplasmic stresses that stimulate σ^E activity will increase the synthesis of FkpA. This increased synthesis is mediated by a σ^E-activatable promoter that shares the common features of the other known σ^E-regulated promoters of *E. coli* (Figure 7.5). Taken together, these results suggest that *fkpA* is the newest member of the σ^E regulon.

Cpx and DsbA

In addition, the results presented in this chapter suggest that the Cpx pathway is also involved in mediating protein folding functions within the bacterial envelope. Specifically, DsbA synthesis is increased by activation of the Cpx pathway. Interestingly, this study indicates that the Cpx pathway also stimulates transcription of *orfA*, the gene upstream of *dsbA* (Figure 7.6). However, the predicted *orfA* protein product possesses no similarity to any other sequence currently in the sequence databases, and the function of this gene is unknown.

The Cpx-mediated stimulation of DsbA synthesis is intriguing for several reasons:

First, Chapter Six has shown that aggregation of the P pilus subunit, PapG will activate the Cpx pathway. PapG and the P pilus-specific chaperone, PapD, both require DsbA for proper folding [Jacob-Dubuisson *et al.* 1994]. Taken together, these results suggest that *E. coli* may utilize the Cpx pathway to monitor the physiological state of extracytoplasmic structures such as P pili. By monitoring such events, the Cpx pathway could potentially ameliorate perturbations in protein folding and assembly by increasing the synthesis of DegP and DsbA, which could destroy or refold damaged proteins.

Second, we have previously shown that activation of the Cpx pathway can combat extracytoplasmic protein-mediated toxicities [Cosma *et al.* 1995, Snyder *et al.* 1995, Snyder 1995, Chapter Four]. The activated Cpx system performs this function, in part, by stimulating the synthesis of CpxP and DegP. However, tests of epistasis indicate that the activated Cpx system can still partially suppress these stresses even in the absence of DegP and CpxP, implying that there are other stress-combative members of the Cpx regulon. DsbA is a promising candidate for such a factor. However, Snyder and Silhavy [1995] have demonstrated

that the *dsbA* null mutation does not appreciably impede the ability of the Cpx pathway to ameliorate the periplasmic toxicity associated with the LamB-LacZ-PhoA fusion protein. While this does not preclude the possibility that the Cpx pathway can utilize DsbA in other stress-combative situations, the result does imply that there is still at least one other unidentified factor utilized by the Cpx regulon to combat extracytoplasmic protein-mediated toxicities.

Third, from the results presented in this chapter, it is not clear as to whether transcription of any of the other Dsb proteins (DsbB, C, D) can also be stimulated by the Cpx pathway. Since this study has only searched for periplasmic Cpx-inducible proteins, the inner-membrane Dsb proteins (DsbB and DsbD) would not have been identified by this approach. Accordingly, an analysis of membrane proteins in Cpx-activated strains could be informative in identifying the remaining members of the Cpx regulon.

Three classes of σ^E and Cpx regulatory targets

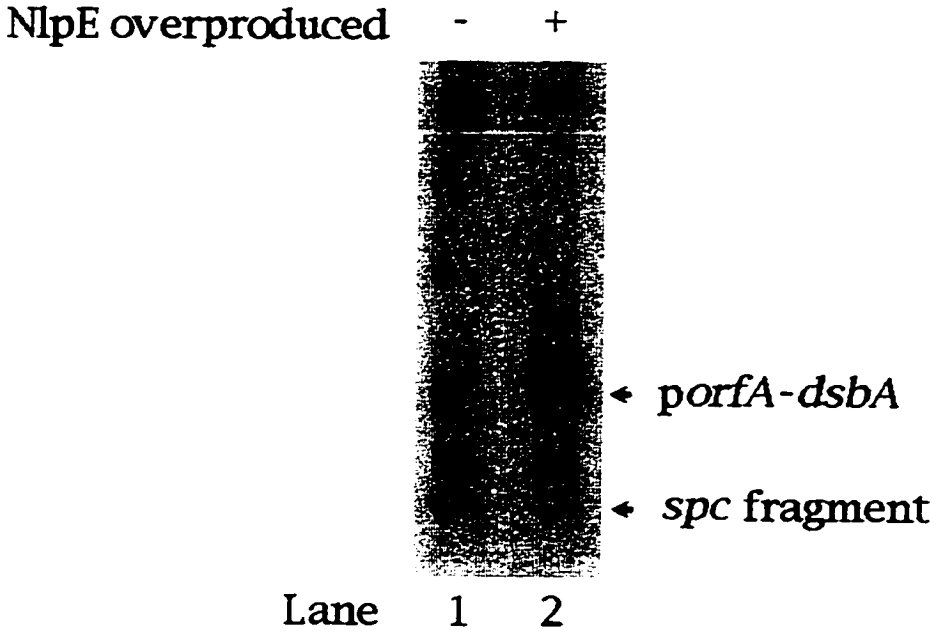
Figure 7.10 shows an updated list of Cpx-regulated and σ^E-regulated loci. As the figure illustrates, there are now three types of genes that are controlled by either the Cpx or σ^E regulons: 1) Those that are controlled solely by Cpx (*dsbA, cpxP*), 2) those that are controlled solely by σ^E (*rpoHp3, rpoEp2, fkpA*) and 3) those that are jointly controlled by both Cpx and σ^E (*degP*) [Chapter Three, Lipinska *et al.* 1988, Erickson and Gross, 1989, Raina *et al.* 1995]

The simplest explanation for the interactions depicted in Figure 7.10 is that they are all direct. For example, CpxR would bind upstream of its regulatory targets (*degP, cpxP, orfA-dsbA*) and stimulate transcription from the promoters of these genes. Indeed, Joseph Pogliano and Simon Lynch (Harvard Medical School) have recently demonstrated footprinting of CpxR at the *degP* and *dsbA* loci, lending support to this model.

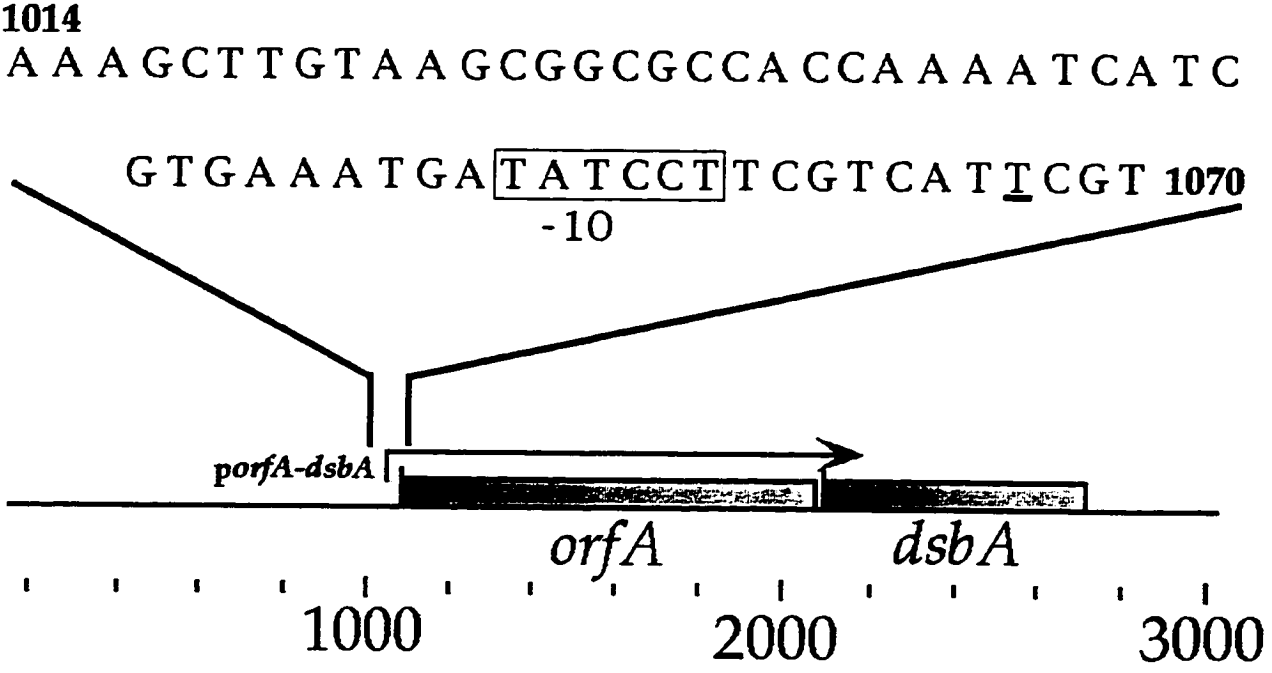
In sum, the results presented in this chapter and in previous studies indicate that the Cpx two-component signal transduction system and the heat-shock inducible σ factor, σ^E, both control the synthesis of a periplasmic protease (DegP) and periplasmic enzymes that are

Figure 7.9. Activation of the Cpx pathway by overproduction of NlpE stimulates transcription from the p*orfA-dsbA* promoter. Panel a): Lanes 1 and 2 show major p*orfA-dsbA* transcription start site as well as minor, nearby start sites from strain SP994 (MC4100, λRS88[p*orfA-dsbA-lacZ*], *ara*$^-$/^{R}*::cam* with linked Tn*10*) transformed with either a control plasmid, pND18 (lane 1), or the *nlpE* overexpressing plasmid, pND18 (lane 2). An S1 nuclease-protected fragment corresponding to the 5′ end of the *spc* operon is also noted by the arrow marked "*spc* fragment." This fragment serves as an internal loading control. The arrow marked "p*orfA-dsbA*" shows protected DNA fragments that correspond to transcripts that initiate at positions 1066 and 1067 of the published *orfA-dsbA* sequence [Belin and Boquet 1994]. Position 1067 is the transcription initiation site first described by Belin and Boquet [1994], and this position is underlined in panel b). Panel b): The p*orfA-dsbA* promoter. The nucleotide sequence surrounding the p*orfA-dsbA* promoter is shown, spanning from nucleotide 1014 to 1070 of the published sequence [Belin and Boquet 1994]. The putative -10 site of this promoter is boxed, and the major transcription initiation site is underlined. The *orfA-dsbA* operon is shown below the nucleotide sequence and a 3000 nucleotide-long scale is also shown for reference.

a)

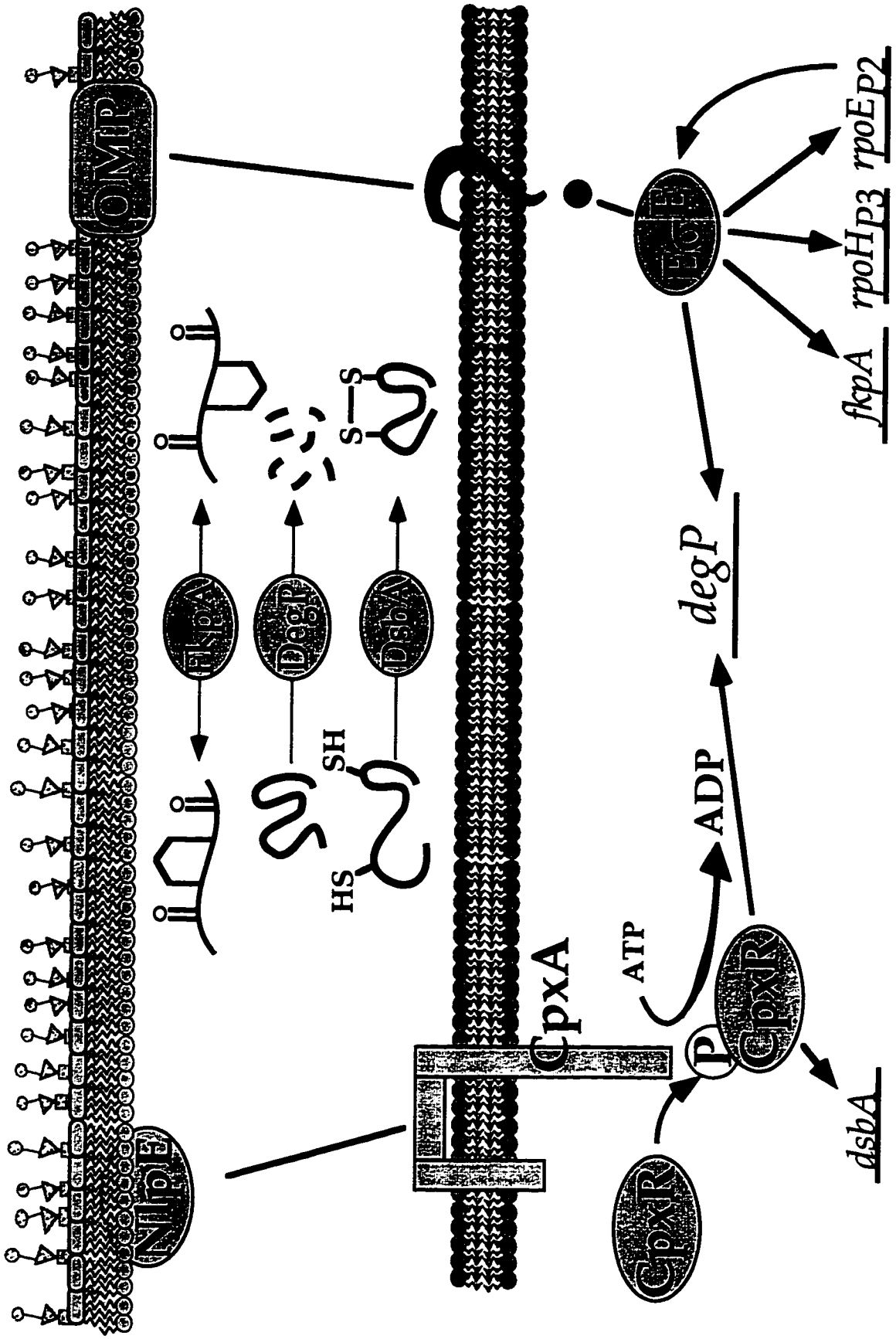


b)



capable of performing protein folding functions [Chapter Three, Lipinska *et al.* 1988, Erickson and Gross 1989, Raina *et al.* 1995]. Given these two classes of proteins, it seems likely that the primary functions of the Cpx and σ^E systems are to monitor and mediate protein folding and protein turnover functions within the extracytoplasmic compartments of E. coli. Indeed, I would not be surprised if the many of the remaining unidentified members of the Cpx and σ^E regulons fall into these two classes of proteins.

Figure 7.10. A model of the σ^E and Cpx regulons. The Cpx two-component signal transduction system is shown as being activated by the outer-membrane lipoprotein, NlpE. The inner-membrane sensor, CpxA is shown phosphorylating its cognate response regulator, CpxR, whereupon the activated CpxR-phosphate molecule stimulates transcription of the *degP* and *dsbA* genes. The DegP and DsbA proteins are shown in the periplasm, performing proteolytic and protein folding functions, respectively. An unknown signal transduction pathway (depicted as a question-mark) is shown monitoring the level of an outer membrane protein and stimulating σ^E activity. σ^E is shown activating transcription of *fkpA*, *degP*, *rpoHP3* and *rpoEP2*. The FkpA protein is also shown in the periplasm, facilitating the interconversion of *cis* and *trans* isomers of prolyl-containing peptides.



Eight. Discussion

At the inception of this study, my chief aims were 1) to understand how σ^E activity was regulated, and 2) to identify σ^E's regulatory targets. My interest in σ^E did not stem from a deep-seated desire to study *E. coli*'s alternate σ factors. Rather, I was operating under the assumption that σ^E was a master regulator of protein folding and protein turnover within the bacterial envelope. I reasoned that by analyzing σ^E activity, I would ultimately address two fundamental questions regarding the biology of *E. coli*: 1) How does *E. coli* mediate the biogenesis of its extracytoplasmic proteins? and 2) If envelope protein biogenesis is perturbed, how does *E. coli* respond?

Fortunately, the σ^E structural gene had not been identified at the inception of this study. As a consequence, I turned to *degP* transcription as an indirect reporter for σ^E activity. With the help of Christine Cosma, Bill Snyder and Laura Davis, I was able to show that σ^E was not the sole regulator of *degP* transcription. Specifically, we showed that the Cpx two-component signal transduction system regulated *degP* transcription in parallel with the signal transduction system that controls σ^E activity [Chapter Three]. As a result of this discovery, my studies veered away from σ^E and delved more heavily into the Cpx system. This is not to say that I gave up on σ^E entirely. Indeed, I developed several projects that were designed to probe σ^E function by perturbing the biogenesis of envelope proteins. However, like an uninvited guest that turns out to be the life of the party, the *cpx* locus forced its way into these projects as well.

Does this mean that CpxA and CpxR are employed to regulate aspects of protein biogenesis and turnover within the bacterial envelope? Christine, Bill and I have each provided evidence to support this view; and ultimately, I believe that the answer to this question will be an unqualified "yes." However, at the present time, there is still room for debate.

THE FUNCTION OF THE CPX SIGNAL TRANSDUCTION SYSTEM (CPXA/R)

Most of the early characterization of the *cpx* operon analyzed hyperactivated alleles of this locus - the so-called *cpx** alleles. As a result, these studies obscured the precise function

of the *cpx* locus by cataloguing a large number of seemingly unrelated *cpx**-mediated phenotypes. In retrospect, it seems more reasonable to elucidate the function of the Cpx proteins by asking two specific questions: 1) What types of stimuli does CpxA sense? and 2) What responses do the Cpx proteins elicit as a result of these stimuli?

What does CpxA sense?

This first question is one of the most difficult to answer. As an example of this difficulty, I point to one of the best characterized two-component signal transduction systems, the EnvZ/OmpR system. For years, we have known that EnvZ is an osmolarity sensor. However, despite this knowledge, we still do not know the precise molecular signal(s) that is(are) actually monitored by EnvZ.

In comparison with EnvZ, our knowledge of the CpxA sensory system is situated at an even more rudimentary level. Presently, there are six phenomena that are known to stimulate CpxA activity. The three simplest stimulatory phenomena may ultimately prove to be the most informative with respect to CpxA's sensory function. Specifically, C. Hal Jones and I have shown that overproduction of either NlpE, PapG or PapE will stimulate CpxA activity [Chapters Three, Six, Snyder *et al.* 1995, C.H. Jones, personal communication]. NlpE is an outer membrane lipoprotein, while PapG and PapE are each structural P pilus subunits. In each of these cases, the overproduced protein aggregates and associates with the inner membrane [Snyder 1995, C.H. Jones, personal communication]. Accordingly, the simplest model to explain these results suggests that CpxA monitors the assembly of a specific subset of envelope proteins in *E. coli*.

Three other phenomena are also known to stimulate CpxA activity: 1) inactivation of the *pss* gene, which is required for biosynthesis of phosphatidylethanolamine, a major constituent of both the inner and outer membranes (W. Dowhan, U. Texas Medical School, Houston); 2) alkaline pH in the surrounding growth medium [Chapter Four], and 3) accumulation of the ECA biosynthetic intermediate, Lipid II [Chapter Five]. The effects of

these latter three phenomena on envelope physiology are less clear than the effects conferred by the three overproduced proteins described above.

However, in each of these latter cases, it also seems likely that envelope protein physiology is significantly perturbed. For example, Bill Dowhan's laboratory has shown that inactivation of *pss* causes the misfolding of certain membrane proteins, including lacy [Bogdanov *et al.* 1996]. In addition, Lazar and Kolter [1995] have shown that alkaline pH intensifies the porin assembly defect associated with the *surA* null mutation. Thus, alkaline pH may also stimulate CpxA activity by perturbing envelope protein physiology. Finally, many of the phenotypes associated with Lipid II accumulation (*e.g.*, hypersensitivity to bile salt detergents, hypersensitivity to export of LamB) also suggest a perturbation in the physiology of outer membrane proteins that could potentially activate the Cpx pathway.

While the results described above suggest that CpxA may monitor the assembly of envelope proteins, it is also clear that CpxA can only monitor a specific subset of these proteins. I emphasize this point because the Cpx pathway is *not* stimulated by 1) overproduction of an array of outer membrane porin proteins [Chapter Three, unpublished observations], 2) overproduction of various envelope lipoproteins [Chapter Three], and 3) the *surA* null mutation, which impairs the assembly of certain outer membrane porins [unpublished observations]. Thus, Cpx sensory function is limited to a subset of envelope proteins.

What common features do the members of this subset share? With a collection of only three proteins, it is difficult to generate a meaningful theme. However, I note that of these three proteins, two (PapE and PapG) are *normally* synthesized only by certain pathogenic strains of *E. coli*; and one (NlpE) is synthesized at very low levels under standard laboratory conditions [Snyder 1995].

In contrast, envelope proteins that are normally and abundantly synthesized by *E. coli* (OmpF, OmpC *etc.*) stimulate only σ^E activity and not CpxA [Chapters Three and Four, unpublished observations]. Thus, I suggest that CpxA may monitor the assembly of proteins and specialized protein structures that are only synthesized under specific environmental

conditions. As an example, I suggest that the Cpx pathway may be intimately tied to monitoring the assembly status of extracellular pathogenic structures such as P pili.

I do not wish to simply suggest that CpxA monitors the assembly of envelope proteins without providing alternative hypotheses. One possible alternative posits that CpxA is an extracellular alkaline pH sensor. According to this model, the other stimulators of Cpx activity would indirectly create alkaline conditions within the envelope. However, I consider a strict interpretation of this model unlikely because overproduction of NlpE will activate the Cpx pathway even in a buffered acidic growth medium [unpublished observation].

Note that these two hypotheses for CpxA's sensory function are not mutually exclusive. For example, CpxA could perform the economical task of acting as both a protein assembly sensor and an alkaline pH sensor. Regardless of which of these models is a more accurate representation of the truth, we will ultimately learn the precise sensory function of CpxA by direct biochemical analysis. If CpxA functions like its close cousin, EnvZ, it will possess an autokinase activity, a CpxR kinase activity and a CpxR-phosphate phosphatase activity. If true, we may then ask if any of these activities can be altered (enhanced?) by the addition of NlpE or PapG, or by alterations in pH. Such tests will ultimately clarify the sensory role of CpxA.

What are the regulatory targets of the Cpx proteins?

The second question that must be addressed in order to clarify the function of the Cpx pathway is "How does the Cpx pathway respond to factors that stimulate CpxA activity?" That is, from a teleological perspective, do the Cpx-mediated responses agree with the stimuli that elicit these responses? Presently, there are two known effects that can be elicited by activation of the wild-type Cpx pathway. First, activation of the wild-type Cpx pathway can alleviate the toxic effects of various protein-mediated extractyoplasmic toxicities, including those conferred by LamB-LacZ-PhoA, LamB-LacZX90 and LamBA23D [Cosma *et al.* 1995, Snyder *et al.* 1995]. Second, activation of the wild-type Cpx pathway performs this stress-combative function (in part) by stimulating synthesis of the periplasmic proteins DegP,

CpxP and DsbA [Chapters Three, Four and Seven]. I emphasize the *wild-type* Cpx pathway because there are myriad phenotypes associated with the *cpx** alleles, and it is unclear as to which of these phenotypes reflect normal functions of the Cpx system and which do not. Thus, I believe it is more informative to restrict my discussion to only those effects that are observed with the unmutated Cpx proteins.

Taken as a whole, the extracytoplasmic protein-stress combative functions performed by the regulatory targets of the Cpx pathway are consistent with the notion that CpxA is involved in monitoring the assembly of certain envelope proteins. Furthermore, the Cpx regulatory targets suggest that *E. coli*'s principal method of coping with extractyoplasmic protein-mediated stress is to increase the synthesis of enzymes that can destroy or refold damaged and misassembled proteins (*i.e.*, DegP and DsbA). This theme of protein folding and proteolysis is also observed within the σ^E regulon (see below).

It is interesting to note that misassembled forms of certain P pilus subunits (*e.g.*, PapA) are destroyed by DegP [Lindberg *et al.* 1989]. In addition, DsbA is required at several steps in the P pilus assembly process [Jacob-Dubuisson *et al.* 1994]. In my opinion, it is not a coincidence that overproduction of P pilus subunits can activate a signal transduction system that stimulates the synthesis of enzymes that are involved in the biogenesis and turnover of this structure.

THE FUNCTION OF THE σ^E REGULON

At the inception of this study, we knew that σ^E was a heat-shock inducible σ factor that directed transcription from two promoters: the *degP* promoter and the *P3* promoter of the *rpoH* locus [Lipinska *et al.* 1988, Erickson and Gross 1989, Wang and Kaguni 1989]. Given this information, I speculated that σ^E was the heat-shock σ factor for the bacterial envelope. That is, I suspected that σ^E was primarily involved in directing protein folding and protein turnover functions within the extracytoplasmic environs of *E. coli*.

This notion has ultimately been supported with a wealth of information. For example, Joan Mecsas and coworkers [1993] were the first to show that σ^E activity is modulated by the level of outer membrane proteins within the envelope, thus providing a link between σ^E and extracytoplasmic events. In addition, σ^E activity is induced by the *surA* null mutation, which impairs the assembly of outer membrane porins [Lazar and Kolter 1995, P. Rouvière and C.A. Gross, personal communication]. In conjunction with these studies, Lonetto *et al.* [1994] showed that σ^E is a member of a family of σ factors that are each involved in controlling various extracytoplasmic functions. Taken together, these results provide strong circumstantial evidence that σ^E is primarily involved in controlling extracytoplasmic activities in *E. coli*.

The phenotypes associated with *rpoE* null strains also provide a link between σ^E activity and the bacterial envelope. Specifically, strains that are null for *rpoE* display a severe growth defect at temperatures above 37°C [Hiratsu *et al.* 1995, Raina *et al.* 1995, Rouvière *et al.* 1995]. Although this result is not strictly informative with regard to the precise function of σ^E, it is important because it provides a link back to the Cpx system (see below). In addition to the temperature-sensitive growth defect, *rpoE* null strains are hypersensitive to growth in the presence of hydrophobic dyes and bile salt detergents, implying that σ^E is needed to maintain the structural integrity of the outer membrane [Raina *et al.* 1995, Rouvière *et al.* 1995]. Furthermore, this study has shown that σ^E controls the synthesis of the periplasmic peptidyl-prolyl *cis/trans* isomerase, FkpA, which can alleviate certain extracytoplasmic protein-folding defects [Chapter Seven, Missiakas *et al.* 1996].

Taken together, these results imply that σ^E is important for maintaining the integrity of the outer membrane. σ^E is most likely needed in this capacity to facilitate the biogenesis of outer membrane proteins. Interestingly, much like the Cpx system, the two extracytoplasmic proteins whose synthesis is known to be controlled by σ^E are a protease (DegP) and a protein folding enzyme (FkpA). This observation further underscores the view that *E. coli* responds to

stresses that perturb protein physiology (*e.g.*, heat-shock) by increasing the synthesis of proteolytic and protein folding enzymes.

A link between Cpx and σ^E

As mentioned above, *rpoE* null strains display a temperature-sensitive growth defect. In an effort to further characterize σ^E function, the Gross laboratory sought multicopy suppressors of this *rpoE*-associated phenotype. Interestingly, one of the multicopy suppressors identified by this approach was NlpE, implying that the activated Cpx system can functionally substitute for the σ^E activity [C.A. Gross, personal communication]. How can the Cpx system substitute for σ^E function? The Cpx pathway must either control the synthesis of proteins that are also jointly controlled by σ^E, and/or the Cpx pathway must control the synthesis of proteins that are functionally redundant with those controlled by σ^E. Certainly, DegP is a protein whose synthesis is jointly controlled by σ^E and Cpx. However, it is the absence of DegP as well as *other* factors that confers the severe temperature-sensitive growth defect associated with the *rpoE* null mutation [Rouvière *et al.* 1995]. Thus, the Cpx pathway must control other proteins (aside from DegP) that are either regulated by σ^E or functionally related to the σ^E regulatory targets. Accordingly, it is important to identify the remaining members of the Cpx and σ^E regulons. Such identifications should ultimately help to highlight the common functions of these two systems.

Although Cpx can substitute for σ^E function, there is a subtle, but informative distinction between these two regulatory systems. When σ^E is inactivated by null mutation, the phenotypes that are associated with this lesion are severe (*e.g.*, temperature-sensitive growth inhibition, hypersensitivity to detergents, *etc.*). In contrast, there are no observed growth defects or hypersensitivities associated with unperturbed *cpx* null strains. The implications of these results are two-fold: First, σ^E must regulate extracytoplasmic processes that are important for the function of unstressed cells. In contrast, the Cpx proteins appear to perform a more specialized stress-responsive function.

I emphasize the fact that *cpx* null strains display no growth defects in *unperturbed* cells because this is not necessarily true in situations where envelope protein physiology is altered. For example, overproduction of NlpE in a *cpx* null strain causes a severe growth defect, implying that the Cpx pathway is needed to relieve the toxic effect(s) associated with the overproduced NlpE protein [unpublished observation].

Regulation of σ^E activity

Before I discuss some of the loose ends associated with this study, I leave the reader with Figure 8.1, a model summarizing our knowledge of the σ^E and Cpx systems. As can be seen, there are three types of stimuli that activate σ^E and/or CpxA: 1) those that stimulate both Cpx and σ^E (PapG), 2) those that stimulate only σ^E (*e.g.*, overproduction of OmpF) and 3) those that stimulate only Cpx (*e.g.*, overproduction of NlpE).

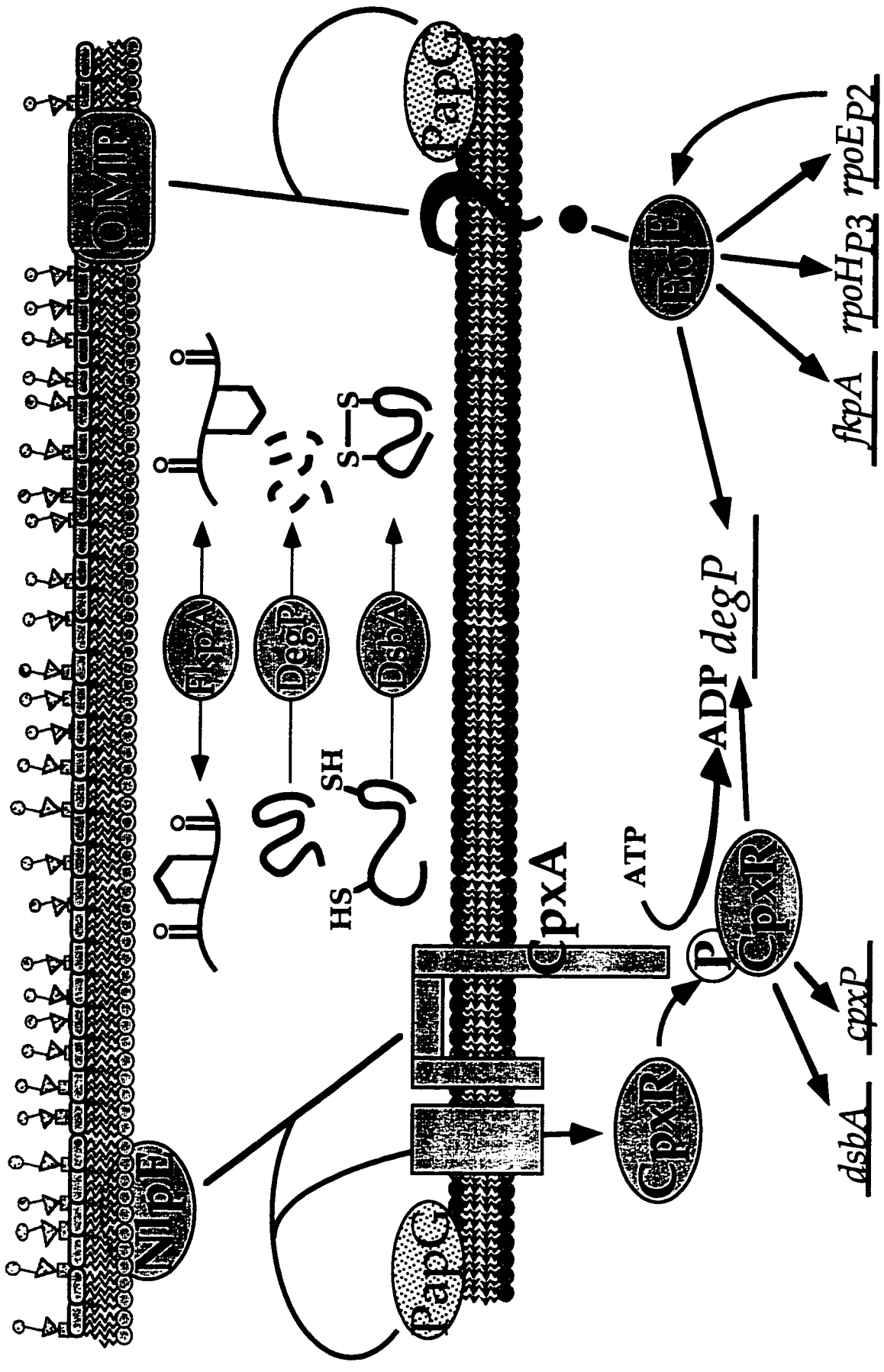
Similarly, there are also three types of regulatory targets within the σ^E and Cpx regulons: 1) those targets that are jointly controlled by σ^E and Cpx (*degP*), 2) those targets that are solely controlled by σ^E (*rpoHP3*, *fkpA*), and 3) those targets that are solely controlled by Cpx (*cpxP*, *dsbA*). In each of these regulons, we find a protease (DegP) and a protein-folding enzyme (DsbA and FkpA). I believe that many of the remaining unidentified Cpx and σ^E regulatory targets will also specify proteins that fall into these two classes.

LOOSE ENDS AND POTENTIAL AVENUES OF STUDY

Unidentified regulatory targets of Cpx and σ^E

Rouvière and colleagues [1995] estimate that the σ^E regulon consists of at least 11 proteins. Presently, only four of these proteins have been positively identified [Lipinska *et al.* 1988, Erickson and Gross 1989, Wang and Kaguni 1989, Raina *et al.* 1995, Rouvière *et al.* 1995, Chapter Seven]. In a similar fashion, I provide evidence in Chapters Four and Seven that at least one other Cpx regulatory target remains at large. The identification of the remaining σ^E and Cpx regulatory targets will probably provide the most information regarding the functions

Figure 8.1. A model of the σ^E and Cpx regulons. The Cpx two-component signal transduction system is shown as being activated by the outer-membrane lipoprotein, NlpE and the P pilus subunit, PapG. PapG is also shown stimulating CpxR activity through an unknown signal transduction system (depicted as a shaded rectangle). The inner-membrane sensor, CpxA is shown phosphorylating its cognate response regulator, CpxR, whereupon the activated CpxR-phosphate molecule stimulates transcription of the *degP*, *cpxP* and *dsbA* genes. The DegP and DsbA proteins are shown in the periplasm, performing proteolytic and protein folding functions, respectively. An unknown signal transduction pathway (depicted as a question-mark) is shown monitoring the level of both an outer membrane protein as well as the P pilus subunit, PapG. This signal transduction pathway is also shown stimulating σ^E activity. σ^E is shown activating transcription of *fkpA*, *degP*, *rpoHp3* and *rpoEp2*. The FkpA protein is also shown in the periplasm, facilitating the interconversion of *cis* and *trans* isomers of prolyl-containing peptides.



of the Cpx and σ^E proteins. Accordingly, I suggest two approaches to identifying these remaining targets.

First, the screen for *lacZ* operon fusions whose transcription is altered by overproduction of NlpE should be repeated [see Chapter Four]. In the study described in Chapter Four, I did not perform an exhaustive search of the genome, and thus, other Cpx-regulated targets may await identification.

Furthermore, in addition to their control of certain periplasmic proteins, it is distinctly possible that the Cpx and σ^E systems may control the synthesis of membrane proteins. The study described in Chapter Seven only analyzed the profiles of periplasmic proteins for those proteins that were regulated by Cpx and σ^E. Accordingly, a similar analysis of membrane proteins may identify new members of the Cpx and σ^E regulons.

Note that in the study described in Chapter Seven, I also identified a 16 kDa protein that was both *activated* by σ^E and *repressed* by CpxR [unpublished observation]. Because of its novel regulation, I determined the identity of the first 10 residues from the amino terminus of this protein (DKSHDVNHGS). However, this sequence does not yet match any proteins present in the sequence databases. I suggest that this sequence be rechecked against the updated sequence databases periodically, as its potential dual regulation by σ^E and Cpx may provide more information on the link between these two regulatory systems.

Cpx biochemistry

Finally, I note that it is important to analyze the biochemical activities of CpxA and CpxR (Tracy Raivio is now performing these analyses). Specifically, we must determine if CpxA and CpxR communicate *via* the classical phosphorylation/dephosphorylation interactions that are commonly observed in two-component signal transduction systems. In addition, it will be important to determine if CpxR directly binds to DNA sequences upstream of the *degP*, *cpxP* and *orfA-dsbA* promoters. This information would ultimately confirm or refute the view that CpxR is a transcription factor that directly regulates these loci.

Appendix. Multicopy Suppression of Cold-Sensitive *sec* Mutations in *Escherichia coli*

The results presented in this chapter stemmed from my initial thesis project that was pursued during my first three years as a graduate student. I present the results obtained from this study as an appendix, so as not to detract from the coherence of the previous eight chapters. Some of the results described in this appendix were performed by Christopher K. Murphy, who was a postdoctoral fellow in Jon Beckwith's laboratory at Harvard Medical School during the time of this study. Specifically, Chris performed the pulse-chase experiment shown in Figure A.1 and he also demonstrated that the multicopy suppressors described below did not obviate the requirement for the Sec pathway. This study would be incomplete without these results, and I am grateful for his help.

ABSTRACT

Mutations in the secretory (*sec*) genes in *Escherichia coli* compromise protein translocation across the inner membrane and often confer conditional-lethal phenotypes. We have found that overproduction of the chaperonins GroES and GroEL from a multicopy plasmid suppresses a wide array of cold-sensitive *sec* mutations in *E. coli*. Suppression is accompanied by a stimulation of precursor protein translocation. This multicopy suppression does not bypass the Sec pathway because a deletion of *secE* is not suppressed under these conditions. Surprisingly, progressive deletion of the *groE* operon does not completely abolish the ability to suppress, indicating that the multicopy suppression of cold-sensitive *sec* mutations is not dependent on a functional *groE* operon. Indeed, overproduction of proteins unrelated to the process of protein export suppresses the *secE501* cold-sensitive mutation, suggesting that protein overproduction, in and of itself, can confer suppression. This multicopy suppression is reminiscent of the previously characterized suppressors of *sec* mutations which compromise protein synthesis and the observation that low levels of protein synthesis inhibitors can suppress as well. In all cases, the mechanism of suppression is unrelated to the process of protein export. We suggest that the multicopy plasmids also suppress the *sec* mutations by compromising protein synthesis.

INTRODUCTION

Genetic analyses have identified six *sec* genes (*secA, B, D, E, F* and *Y*), whose products are required for efficient protein translocation across the cytoplasmic membrane of *Escherichia coli* [Murphy and Beckwith 1996]. Because protein translocation is an essential process, only conditional-lethal loss of function *sec* mutations are obtainable by genetic means. At the non-permissive temperature, strains containing either cold-sensitive (Cs) or temperature-sensitive (Ts) mutations in *secA, D, E, F* or *Y* accumulate cytoplasmic precursor proteins and eventually die [Murphy and Beckwith 1996]. However, even at such non-permissive temperatures, the conditional-lethal alleles only partially block translocation, indicating that they are not complete null mutations (For examples, see Oliver and Beckwith 1981, Gardel *et al.* 1987, Riggs *et al.* 1988, Schatz *et al.* 1989, Baba *et al.* 1990). The nature of these partial defects is best illustrated by the Cs alleles of the *secE* locus. The Cs *secE15* and the *secE501* mutations do not lie within the *secE* coding region. Instead, they alter the untranslated leader of the *secE* mRNA and reduce the steady-state level of wild-type SecE [Schatz *et al.* 1991]. Hence, the cold-sensitivity conferred by these *secE* mutations implies that the cell requires wild-type levels of the secretory apparatus for viability at low temperatures. Consistent with this notion, Pogliano and Beckwith [1993] have demonstrated that protein translocation is itself an inherently cold-sensitive process. Although the Cs mutations in *secD, F* and *Y* have not been as thoroughly characterized, it has been suggested that they act by decreasing the overall amount of functional secretory apparatus, in similar fashion to those in *secE* [Bieker and Silhavy 1990].

Several studies have sought to identify extragenic suppressors of the conditional-lethal *sec* mutations with the hope that the suppressor mutations would identify genes encoding interacting proteins involved in protein secretion. Instead, suppressor analysis has yielded secondary mutations that act by compromising protein synthesis [Brickman *et al.* 1984, Ferro-Novick *et al.* 1984, Oliver 1985, Lee and Beckwith 1986, Riggs *et al.* 1988]. Indeed, low levels of chloramphenicol and other protein synthesis inhibitors suppress the lethality and secretion defects associated with the *sec*-conditional mutants. Although the precise

mechanism of this suppression is unknown, it has been suggested that decreased synthesis of precursor proteins relieves a lethal burden placed on the mutant Sec machinery [Lee and Beckwith 1986].

In addition, previous studies have shown that high-level synthesis of the chaperonins GroES and GroEL suppresses the *secA51* and *secY24* temperature sensitive (Ts) mutations [Van Dyk *et al.* 1989, Ueguchi and Ito 1992]. Based on the folding activities of the GroE proteins, it was suggested that the suppression was the result of an increased frequency of proper folding of thermolabile SecA and SecY proteins [Van Dyk *et al.* 1989]. Here we have analyzed the ability of different genes cloned in multicopy vectors, including *groES* and *groEL*, to suppress cold-sensitive *sec* mutations. Our results reveal that functional chaperonin genes are not required for suppression. Rather, it is the actual process of protein overproduction which functions to suppress the cold-sensitive *sec* mutations.

MATERIALS AND METHODS

Media and reagents.

Media were prepared as described by Silhavy *et al.* [1984]. Standard microbiological techniques were used for strain construction and bacterial growth [Silhavy *et al.* 1984].

Strains.

All strains used in this study are derivatives of MC4100 or MC1000 [Silhavy *et al.* 1984]. The conditional-lethal alleles listed in Table A.1 have been described: *secD1*, [Gardel *et al.* 1987]; *secE15, 501*, [Schatz *et al.* 1991]; *secF62*, [Gardel *et al.* 1990]; *secY39, 40*, [Baba *et al.* 1990]; *secA51*, [Schmidt *et al.* 1988]; *secY24*, [Shiba *et al.* 1984]; *prlF1*, [Kiino and Silhavy 1984]; *hisH8606*, [Van Dyk *et al.* 1989]. Each conditional allele was introduced into the appropriate strain background by co-transduction with a linked Tn*10* insertion mutation. The *secD* and *secF* alleles were co-transduced with the *zaj*::Tn*10* mutation [Singer *et al.* 1989]. The *secE* alleles were co-transduced with either the *argE*::Tn*10* mutation [Singer *et al.* 1989] or *zijRK498*(Tn*5*) [Pogliano and Beckwith 1993]. The *secY* mutations were co-transduced with a linked Tn*10* described by Bieker and Silhavy [1990]. The *secA* alleles were co-transduced with a *leu*::Tn*10*

mutation. The *prlF1* allele was co-transduced with an *argG*::Tn*10* mutation. Each mutation was transduced into the appropriate strain background at the permissive temperature (30°C for temperature-sensitive strains and 37°C for cold-sensitive strains) and subsequently scored for the conditional-allele at the non-permissive temperature (42°C for temperature-sensitive strains and 23°C for cold-sensitive strains). All mutant strains were compared to isogenic strains containing the appropriate Tn*10* insertion linked to a wild-type allele of the gene in question.

Analysis of multicopy suppression of *sec* cold-sensitive and temperature-sensitive mutations.

Strains were grown on Luria agar containing 125 μg/ml ampicillin at 23°C for cold-sensitive mutations and 42°C for temperature-sensitive mutations. Suppression was measured by colony formation after 48 hours. A score of "+" in Tables A.1 indicates that the colonies of the experimental strain were as large as the isogenic wild-type control strain. A score of "+/-" indicates that the colonies of the experimental strain were smaller than the isogenic wild-type control strain. A score of "-" indicates that no colonies formed with the experimental strain, whereas the isogenic wild-type control strain formed colonies. "+/-" colonies were no more than 50% of the size of colonies scored as "+."

Analysis of multicopy suppression of the *secE* deletion.

Suppression of *ΔsecE* by the various plasmids (See Table A.1 and Results section for details) was tested in the strain CM100 [MC4100, *ΔsecE19-111*, pCM10)]. The *ΔsecE19-111* allele results in a deletion of codons 19 to 111 of the chromosomal *secE* open reading frame [Schatz *et al.* 1991]. This deletion is complemented by plasmid pCM10 [Murphy and Beckwith 1994], an ampicillin-resistant derivative of pBR322 containing a complementing copy of *secE* flanked by the counter-selectable genes *lacY* and *rpsL*, which confer tONPG [Berman and Beckwith 1990] and streptomycin sensitivity, respectively. CM100 was transformed to tetracycline (2μg/ml) resistance with pBR322 or pOF39, and then growth (*i.e.*, indicative of the loss of pCM10, and, thus, *secE*) was tested on minimal succinate plates containing 1.5 mg/ml streptomycin and 1mM tONPG [Murphy *et al.* 1995]. Growth of these strains was not observed

after 10 days on the selective plates, whereas CM100 transformed with plasmid pJS51 (*secE*$^+$, Schatz *et al.* 1989) plasmids grew after two days on the same plates.

Radiolabelling and immunoprecipitation.

Strain KJ184 (MC1000, *phoR, araD139, Δ(ara-leu)7679, ΔlacX74, galE, galK, rpsL, thi, secF62 zaj*::Tn*10*; Pogliano and Beckwith 1993) was transformed with pBR322, pCGSH1 (*secDF*$^+$; Gardel *et al.* 1990), or pOF39. Overnight cultures grown at 37°C in M63 minimal medium plus thiamine (1μg/ml), amino acids (50μg/ml, except methionine), and maltose (0.4%) were passaged at 1:50 into the same medium. After reaching an O.D.$_{600}$ of 0.3, these cultures were placed at the non-permissive temperature of 23°C for 30 min. 3 ml of cells were added to pre-equilibrated tubes containing 150μCi of [^{35}S]methionine. After 30s, 300μl of 0.5% methionine was added; a 1 ml aliquot of cells was removed and placed in an ice-water bath immediately, and at 1 and 4 min after labelling. Samples were immunoprecipitated with antisera to MBP (prepared by Kathy Strauch) and OmpA (a generous gift from Carol Kumamoto) as described previously [Pogliano and Beckwith 1994]. Precursor and mature forms of MBP and OmpA were analyzed by 10% SDS-PAGE and autoradiography.

Plasmids.

pOF39 carries a 2.2 kb sized fragment containing the *groE* operon inserted into the *Eco*RI/*Hind*III site of plasmid pBR325. pOF39 generates high-level synthesis of the chaperonins GroES and GroEL [Fayet *et al.* 1986]. Plasmids pBR322 and pBR325 have been described [Bolivar 1978]. pND1 was created by deleting the small *Eco*RV fragment of pOF39 and religating the large fragment. pND1 contains the entire *groES* gene and approximately 60% of the 5' end of the *groEL* open reading frame. The following single-stranded DNA primers were used to create plasmids pND5 and pND6: GroE5': 5' CAC ATT CTT GCC CGC CTG ATG AAT GC 3'; Mopdelc: 5' CGT CTT TAG CTG CCA TAA GCT TTA TTC CTT AAA TTC 3'; Deltas1: 5' CAT TGT CGA TCT TCT AAG CTT TCA CAC CGT AGC 3'. pND5 was created by amplifying DNA from pOF39 by the polymerase chain reaction (PCR) [Sambrook *et al.* 1989] using the GroE5' and Mopdelc primers. The resulting PCR product and pOF39 were each digested with

*Eco*RI and *Hind*III, and the PCR product was ligated with the large fragment generated by the pOF39 digestion. This resulting plasmid, pND5, contains the *groES* gene and deletes the entire *groEL* open reading frame. The *groE* chromosomal DNA contained in pND5 spans from nucleotide 1 to 467 in the published sequence [Hemmingsen *et al.* 1988]. Nucleotide 467 is located in the intergenic region between the *groES* and *groEL* open reading frames, 40 nucleotides downstream of the *groES* stop codon. pND6 was created by amplifying pOF39 DNA by PCR using the GroE5' and Deltas1 primers. The resulting PCR product and pOF39 were each digested with *Eco*RI and *Hind*III, and the PCR product was ligated with the large fragment generated by the pOF39 digestion. The resulting plasmid, pND6, deletes the entire *groEL* open reading frame and the final 22 codons of the *groES* open reading frame. The first 74 codons (approximately 76%) of the *groES* open reading frame remain.

Plasmids that overproduced various cytoplasmic proteins were analyzed for their ability to suppress the conditional growth defects conferred by the *secE501* mutation. pNF2 is a P_{rm}-driven derivative of the LacZ-encoding plasmid p1109 that expresses 1500U of ß-galactosidase [Kuldell and Hochschild 1994; N. Kuldell, personal communication). This plasmid was tested in strain KJ188 (isogenic to strain KJ184, except that it contains *secE501 zijRK498*(Tn*5*) instead of *secF62 zaj*::Tn*10*) at 23°C. pSPER1-E is a derivative of pSPER1 [Huisman and Kolter 1994] that is deleted for part of the gene encoding RspA, thus inactivating its overexpression phenotype of stationary phase gene repression. The RspA fragment generated from this plasmid is highly overproduced (G. Huisman, personal communication; data not shown). This plasmid was tested in strain PS265 (MC4100, *secE501, zijRk498*[Tn*5*]) at 23°C.

RESULTS

A GroES/EL overproducing plasmid suppresses *sec* Cs mutations.

We initially observed that plasmid pOF39, which overexpresses the chaperonin genes *groES* and *groEL* [Fayet *et al.* 1986], suppressed the cold-sensitive *sec* mutations listed in Table A.1. This suppression of cold-sensitivity appears to be specific to *sec* mutations since cold-

Table A.1. Multicopy suppression of cold-sensitive *sec* mutations by the *groE* operon

Mutation	1) pOF39 (GroES$^+$L$^+$)	2) pND1 (GroES$^+$L')	3) pND5 (GroES$^+$L$^-$)	4) pND6 (GroES'L$^-$)	5) pBR325 (control)	6) pBR322 (control)	Reference
secA51(Ts)	+/-	-	-	-	-	-	Van Dyk *et al.* 1989, This study
secY24(Ts)	+	-	-	-	-	-	Van Dyk *et al.* 1989, This study
secY39(Cs)	+/-	+/-	+/-	+/-	-	-	This study
secY40(Cs)	+	+	+	+	+/-	-	This study
secE15(Cs)	+/-	+/-	+/-	+/-	-	-	This study
secD1(Cs)	+	+	+	+	+/-	-	This study
secE501(Cs)	+	+	+	+	+/-	-	This study
secF62(Cs)	+	+	+	+	+/-	-	This study
prlF1(Cs)	-						This study
hisH8606(Cs)	-						Van Dyk *et al.* 1989
ΔsecE	-						This study

+ indicates growth comparable to a wild-type control strain
+/- indicates growth that is significantly slower than a wild-type control strain
- indicates no growth

sensitive mutations in two non-*sec* genes (*prlF*, *hisH*) are not suppressed (Table A.1). Although the observed suppression is specific to mutations in the *sec* pathway, we were surprised by the large array of mutations within this pathway which were suppressed by overexpression of *groE*. Indeed, overexpression of *groE* suppresses mutations in all *sec* genes in which cold-sensitive mutations have been found. Because high-level synthesis of the GroE proteins suppressed this large class of *sec* mutations, it seemed that an analysis of this suppression could provide insights into the actions of the GroE proteins and/or the nature of the lethality conferred by the cold-sensitive *sec* mutations.

Overproduction of GroES/EL enhances the rate of protein export in *sec* Cs mutants.

The mechanism of GroE-mediated suppression was examined by analyzing the effects of GroES/L overproduction on the secretion defects associated with the *sec* mutants. Analysis of the kinetics of signal-sequence cleavage of precursor maltose-binding protein (pMBP) and pOmpA in a *secF62Cs* background indicated that secretion was enhanced significantly when the GroES/EL overexpressing plasmid was present. Figure A.1 shows that in a *secF62Cs* strain transformed with pBR322, the majority of newly synthesized maltose-binding protein and OmpA exists as an unprocessed precursor species. Transformation of the same strain with a complementing *secF* plasmid (pCGSH1) completely relieves the secretion defect observed in the pBR322-transformed strain. Finally, transformation of the *secF62* mutant with pOF39 significantly enhances translocation over the pBR322 control strain, even though suppression of the processing defect is not as strong as observed with the complementing pCGSH1 plasmid. A similar effect has also been observed when the *groE* operon was overexpressed in cells lacking SecD and SecF (J. Pogliano and J. Beckwith, personal communication). This result suggests that overproduction of GroES/EL suppresses the lethality of the *sec* mutants by enhancing the rate of protein translocation.

There are a number of possible mechanisms by which high-level synthesis of the GroE proteins could suppress these *sec* mutations and enhance protein export. For example, it is possible that overexpression of *groE* bypasses the need for the Sec pathway altogether.

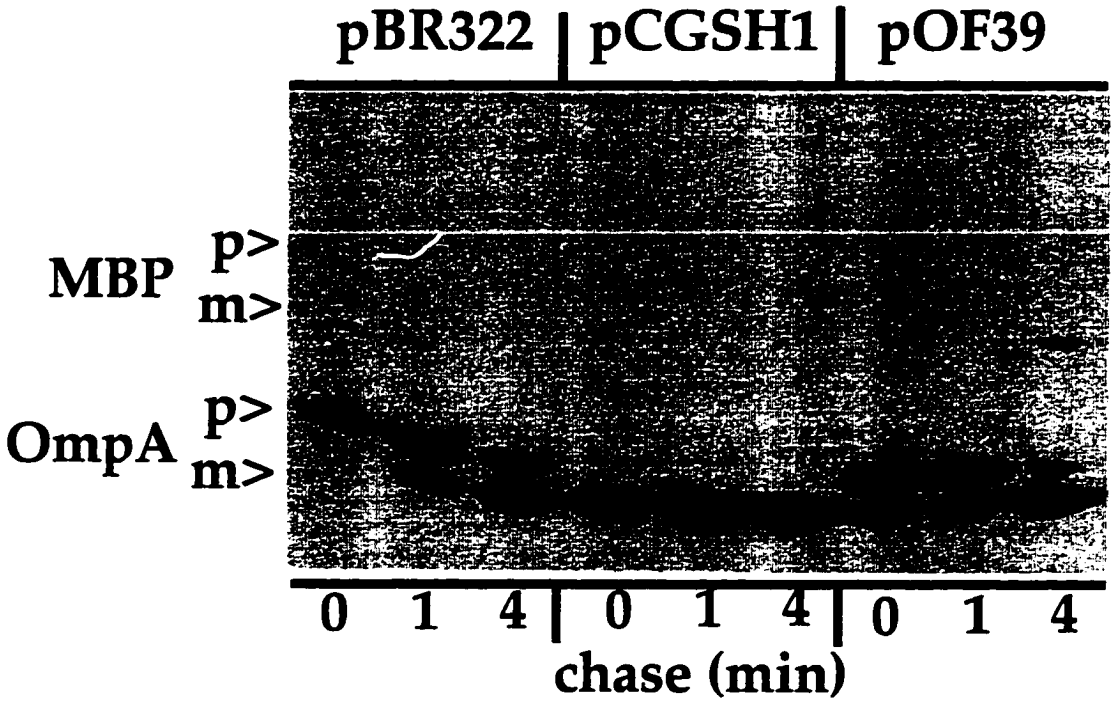


Figure A.1. Plasmids expressing SecD and SecF or GroES/L suppress the secretion defect conferred by the *secF62* cold sensitive allele. Strain KJ184 containing plasmid pBR322, pCGSH1, or pOF39 was grown at 23°C for 30 min and then subjected to pulse-chase and SDS-PAGE/autoradiography analysis as described in Materials and Methods to examine signal sequence processing of MBP and OmpA. m, mature species; p, precursor species; chase times (min) are indicated below the lanes. The percent mature protein (corrected for the number of methionines in the precursor and mature forms) at the pulse, 1 min. and 4 min. times points are: for KJ184/pBR322 mMBP, 0%, 0% and 52%; for mOmpA, 32%, 45%, and 74%; for KJ184/pCGSH1 mMBP and mOmpA, all points 100%. For KJ184/pOF39 mMBP, 49%, 50% and 74%. For mOmpA 45%, 59% and 88%.

However, overexpression of the *groE* operon does not suppress a deletion of the *secE* gene (Table A.1), indicating that this is not the case. Alternatively, it is possible that overexpression of the *groE* operon increases the frequency of proper folding of cold-sensitive mutant Sec proteins. This model has been used to explain the suppression of the temperature-sensitive *sec* mutations by high-level synthesis of GroES/L [Van Dyk *et al.* 1989]. However, this model fails to explain the suppression of the *secE* cold-sensitive mutations, because these mutations confer cold-sensitivity not by altering the primary amino acid sequence of SecE, but by reducing the levels of SecE protein [Schatz *et al.* 1991]. Thus, these models cannot account for the GroE-mediated suppression of the *sec* mutations. Another model posits that overproduction of GroES/EL suppresses the *sec* mutations by enhancing the rate of translocation of an essential protein(s) which is present in insufficient quantities in the bacterial envelope in a *sec* mutant. Such a stimulation in the translocation of an essential protein(s) could be accomplished via a protein unfolding activity of GroES/EL. Indeed, this explanation seems likely given the ability of the GroE proteins to suppress aggregation of unfolded proteins *in vitro* [Zeilstra-Ryalls *et al.* 1991]. However, evidence presented below indicates that the observed suppression is not related to the specific functions of the GroE proteins. Rather, the suppression is related to protein overproduction *per se*.

Deletion-derivatives of a GroES/EL overproducing plasmid suppress *sec* Cs mutations.

In an attempt to determine whether the suppression of the cold-sensitive *sec* mutations was dependent on overproduction of GroEL, a plasmid was constructed that contains only *groES* and 60% of the 5' end of the *groEL* open reading frame (see Materials and Methods for details). This plasmid, pND1, suppressed the cold-sensitive *sec* mutations as well as pOF39 (Table A.1, Figure A.2). There are a number of explanations for the observed suppression by pND1: 1) suppression may be solely dependent on GroES, 2) the amino-terminal fragment of GroEL produced by pND1 may retain the function(s) required for suppression or 3) suppression is not related to the functions of the GroE proteins, but is instead dependent on the protein overproduction itself. Plasmid pND5 was created to further distinguish between these

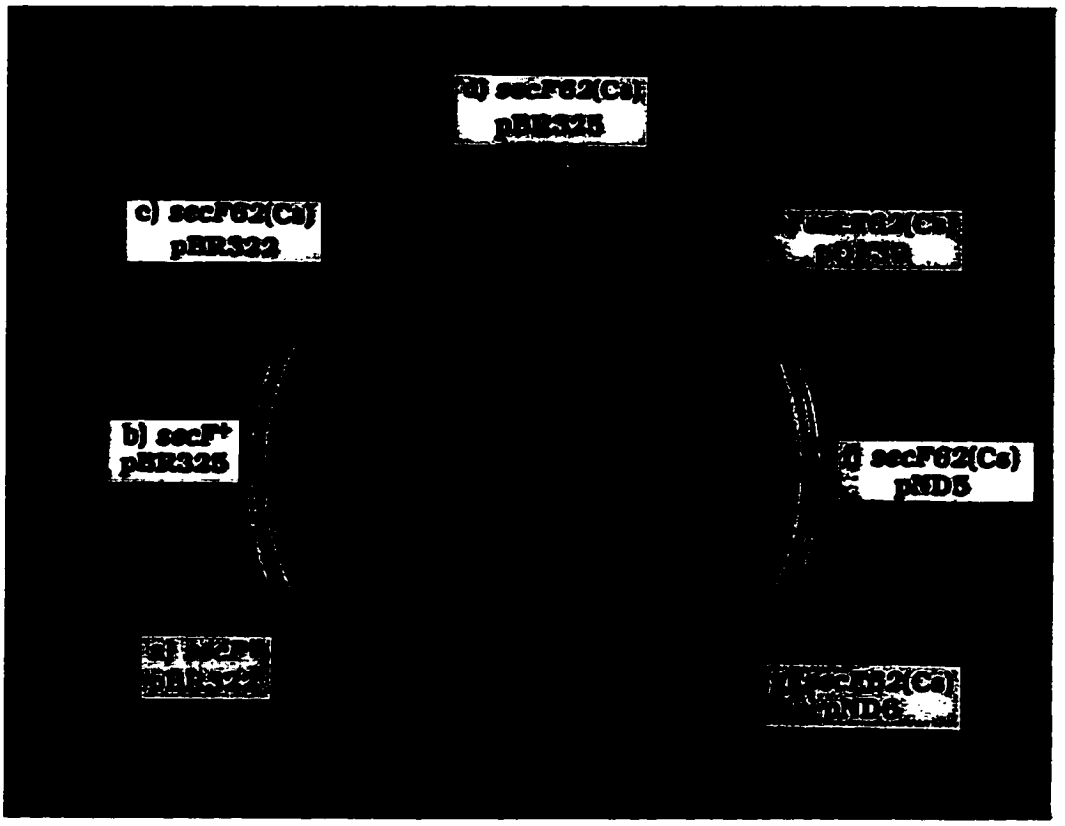


Figure A.2. Multicopy suppression of the *secF62*(Cs) cold-sensitive mutation. Regions a and b show strain PND11 [MC4100, *secF*$^{+}$ *zaj*::Tn*10*]. Regions c through g show strain PND10 [MC4100, *secF62*, *zaj*::Tn*10*]. The strains were transformed with the following plasmids: a) pBR322 (control for pBR325); b) pBR325 (control for pOF39, pND5, pND6); c) pBR322; d) pBR325; e) pOF39 (overproduces GroES and GroEL); f) pND5 (Overproduces GroES); g) pND6 (overproduces a truncated form of GroES). Strains were grown on Luria agar containing 125 µg/ml ampicillin for 48 hours at 23°C.

possibilities. pND5, which overexpresses *groES* alone, was sufficient to suppress the Cs *sec* mutations (Table A.1, Figure A.2, panel f), albeit to a lesser extent than observed with pOF39 and pND1. This result could indicate that the multicopy suppression of the Cs mutations was mediated primarily by GroES. To test this, we deleted approximately 25% of the 3' end of the *groES* coding region from plasmid pND5, creating pND6. pND6 produces a non-functional GroES protein as assayed by its inability to complement the temperature-sensitivity and phage λ growth defect of the chromosomal *groES619* mutation (data not shown). Surprisingly, pND6 was able to suppress a number of the *sec* Cs mutations (Table A.1, Figure A.2, panel g). Even more striking was the finding that pBR325, the parent plasmid for pOF39, pND5 and pND6, weakly suppresses certain *sec* mutations (Table A.1, Figure A.2, panel d). pBR322, which is the parent plasmid of pBR325, does not suppress any *sec* mutation tested (Table A.1, Figure A.2 panel c). pBR325 differs from pBR322 in that it encodes the chloramphenicol acetyl transferase (*cat*) gene [Bolivar 1978]. We suggest that pBR325 has partial suppressor activity due to expression of this *cat* gene. Importantly, the *cat* gene itself is not specifically responsible for suppression in the pOF39, pND5 and pND6 derivative plasmids because in each of these plasmids the *groE* operon is inserted within the *cat* gene [Fayet *et al.* 1986].

The results presented above indicated that the suppression observed with pOF39 was not dependent on the actual functions of the overproduced chaperonins. Rather, it appeared that protein overproduction, *per se*, was mediating suppression. This model makes a clear prediction. If such a method of suppression were occurring, then overproduction of any cytoplasmic protein could potentially suppress *sec* Cs mutations.

Overexpression of proteins unrelated to the protein export process suppress the *secE501* Cs mutation.

Two proteins that have no known role in protein secretion were analyzed for their ability to suppress the *secE501* mutation when overproduced: 1) a non-functional derivative of RspA (RspA-E), a protein involved in the expression of stationary-phase genes [Huisman and Kolter 1994] and 2) ß-galactosidase, a protein involved in lactose metabolism [Kuldell and

Hochschild 1994, Miller 1984]. Both plasmids pSPER1-E (which overproduces RspA-E) and pNF2 (which overproduces ß-galactosidase) suppressed the cold-sensitive phenotype of the *secE501* allele (data not shown).

DISCUSSION

Our data support the notion that overexpression of genes unrelated to the process of protein export suppresses the cold-sensitive *sec* mutations. This multicopy suppression is similar to the suppression of *sec* mutations by ribosomal protein mutations and protein synthesis inhibitors [Lee and Beckwith 1986]. In all cases, suppression is not directly related to the process of protein export. We suggest that the multicopy suppressors act by inhibiting translation, and in this way, indirectly suppress the *sec* mutations. For example, large amounts of transcript produced from a gene on a multicopy vector could compete with host chromosomal transcripts for the translational apparatus. This would create a situation similar to that observed when protein synthesis is compromised. This model predicts that decreasing the length of the suppressing gene would reduce the strength of suppression. Consistent with this prediction, the progressive deletion derivatives of the *groE* plasmid pOF39 are also progressively weaker multicopy suppressors of the *sec* mutations (See Table A.1 and Figure A.2).

Presently, the mechanism by which translational inhibitors suppress *sec* mutations is unknown. Lee and Beckwith [1986] have suggested that the *sec* mutations are suppressed when translation is compromised because the synthesis of presecretory proteins is reduced. This reduction allows the defective Sec machinery to better manage the demands for secretion. Alternatively, physiological states which compromise translation in *E. coli* could increase the relative levels of Sec proteins, thus conferring suppression. For example, Dennis [1976] has noted that transcription of the *spc* operon, which includes *secY*, is induced when translation is compromised. In either case, it is clear that *sec* suppressor analysis often does identify genes whose products are not directly involved in protein translocation.

An important point is raised from these findings. Certain *sec* conditional alleles can be suppressed effectively by overproduction of a variety of proteins, whereas other alleles are not suppressed at all. This may be explained by considering that the mutations are not of equal strength [Pogliano and Beckwith 1993]. For instance, the *secE501*Cs allele is relatively weak compared to *secE15;* strains containing the former mutation can grow at 30°C, whereas the latter cannot grow at this temperature [Schatz *et al.* 1991]. Thus, the *secE501* mutation is partially suppressed by the relatively weak multicopy suppressor, pBR325, whereas the *secE15* mutation is not (Table A.1). Analyzing suppression of stronger alleles at an intermediate, or semi-permissive temperature might also lead to observable suppression. Alternatively, it is possible that suppression of stronger alleles by the functional GroES/EL overproducing plasmid may actually be due to the functions of these chaperonins.

For example, studies by Van Dyk *et al.* [1989] and Ueguchi and Ito [1992] have shown that overproduction of the GroE proteins suppresses *sec* Ts mutations. Our analysis confirms this observation. Moreover, the deletion derivatives of the *groE* plasmids do not suppress the *sec* Ts mutations (Table A.1). Van Dyk and colleagues [1989] and Ueguchi and Ito [1992] have suggested that overproduction of the GroE proteins suppresses these *sec* Ts mutations by enhancing the frequency of proper folding of the thermolabile mutant Sec proteins. However, it is also possible that the suppression of the *sec* Ts mutations is not dependent on the function of the *groE* gene products. In this case, the dependence of suppression on the entire length of the *groE* operon would be due to the quantity of overexpressed protein needed for suppression. That is, the amount of overproduced protein required for suppression is only achieved when both GroES and GroEL are overexpressed. Consistent with this possibility, Ueguchi and Ito [1992] have found other multicopy suppressors of the *secY24* Ts mutation. These loci specify products that do not appear to function in protein translocation (see below). This observation is qualitatively similar to the results described in this study and is consistent with the model that the suppression of the *sec* Ts mutations by overproduction of the GroE proteins is not

dependent on the actual functions of the GroE proteins. A more detailed analysis is required to determine the precise mechanism of GroE-mediated multicopy suppression in this case.

Several other studies have utilized multicopy suppression in an attempt to identify other proteins involved in protein secretion. Esnault and co-workers [1993] have examined a yeast *secY* homolog, *sec61*. The *sec61-2* temperature-sensitive mutation confers a translocation defect for proteins entering the lumen of the endoplasmic reticulum. The temperature-sensitivity conferred by *sec61*-2 can be partially suppressed by multicopy vectors expressing either the *SSS1* or *SSS2* genes. *SSS1* encodes an essential protein which is required for protein translocation. The *SSS2* suppressor, however, is allelic with *TDH3*, encoding glyceraldehyde-3-phosphate dehydrogenase [Esnault *et al.* 1993]. *TDH3* is not involved in protein translocation. Rather, its normal function is in intermediary metabolism. Esnault and colleagues [1993] suggested that the high-level expression of *TDH3* from a multicopy plasmid indirectly suppresses *sec61-2* in a manner similar to that observed for multicopy suppression of the *sec* cold-sensitive mutations.

In *E. coli*, several genes have been identified as multicopy suppressors of *sec* conditional mutations. These include: *ssyG*, which is allelic with the *infB* gene and encodes translation initiation factor 2 [Shiba *et al.* 1986]; *htpG*, which encodes a heat-shock protein; *hns*, which encodes a highly expressed histone-like protein involved in chromosome compaction; *msyB*, which specifies a highly acidic protein of unknown function [Ueguchi and Ito 1992]; *YSY6*, a yeast gene of unknown function [Sakaguchi *et al.* 1991]; *ydr*, a suppressor of a dominant lethal *secY* allele; and *yajC* (also referred to as *ORF3* or *ORF12*) an open reading frame of unknown function located in the *secDF* operon [Taura *et al.* 1994]. In several of these studies, deletion or mutation of the gene responsible for multicopy suppressor activity did not lead to defects in secretion, suggesting that they are not directly involved in the export process. In light of our findings, we propose that high-level synthesis of these proteins may compromise translation by competition for factors required for protein synthesis.

Although we do not dispute the overall utility of multicopy suppression, our analysis highlights some of the pitfalls which may be encountered in this type of analysis. Ideally, before embarking on multicopy suppressor analysis, the starting mutation must be carefully characterized, and the precise nature of the phenotype conferred by this mutation should be determined. Also, any physiological change brought about by overexpressing proteins in the cell that could indirectly affect the process being studied must be carefully ruled out as the cause for suppression. Ultimately, one must demonstrate that the multicopy suppressor specifies a product which is directly involved in the process in question.

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