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Characterization of the Functions of the Sigma E Regulon in Escherichia coli

by

Christina A. Onufryk

DISSERTATION

Submitted in partial satisfaction of the requirements for the degree of

DOCTOR OF PHILOSOPHY

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Abstract

Upon exposure to heat stress or other conditions that denature proteins, all cells respond by inducing the activity of proteins that counteract that damage. *Escherichia coli*, a Gram-negative bacterium, has two cellular compartments: The cytoplasm and the envelope. Each of these compartments has its own response(s) to combat the stress of unfolded proteins. The *E. coli* transcription factor σ^E responds to envelope stress by inducing expression of many genes, the majority of which function in the envelope. In addition, σ^E is an essential transcription factor; however, its essential function(s) are unknown. In this thesis, I dissect the function of the six lipoproteins in the σ^E regulon, *yeaY, yfgL, nlpB, yfeY, yraP*, and *yifO*, by examining deletion phenotyes of each gene. One lipoprotein, YfiO, is essential for cellular viability. Nevertheless, overexpression of this protein is not sufficient to alleviate the requirement of σ^E for viability, suggesting that the σ^E regulon provides more than one essential function. The remaining five lipoproteins in the σ^E regulon are nonessential; in fact, cells are viable even when all five are removed simultaneously. Three of these nonessential lipoproteins exhibit phenotypes that suggest they are important for maintenance of the integrity of the cell envelope: Cells lacking NlpB are selectively sensitive to rifampicin, and those lacking YraP are selectively sensitive to SDS. In addition, both Δ*yraP* strains and Δ*nlpB* strains are synthetically lethal with *surA::Cm*, which encodes a periplasmic chaperone/PPIase. This observation suggests that NlpB and YraP play roles in a periplasmic folding pathway that functions in parallel with that of SurA. Finally, the Δ*yfgL* mutant exhibits a broad range of envelope defects, including sensitivity to several membrane-impermeable agents, an

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altered OMP profile, synthetic lethality with both *surA::Cm* strains and Δ*fkpA::Cm* strains, and sensitivity to BPI. We suggest that this lipoprotein performs an important, but yet unknown role in the barrier function of the cell envelope. I have also characterized five additional σ^E regulon members of unknown function, *yggN*, *ybfG*, *ygiM*, *yidQ*, and *yicK*, using the same assays that I employed in characterizing the lipoprotein genes. *ΔyggN, ΔybfG, ΔygiM, ΔyidQ, and ΔyicK* mutants do not exhibit any phenotypes under the conditions tested.

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Chapter One: General Introduction

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OVERVIEW

Upon exposure to heat stress or other conditions that denature or unfold proteins, all cells respond by inducing proteins that counteract that damage. *Escherichia coli*, a Gram-negative bacterium, has two subcellular compartments: The cytoplasmic compartment and the envelope. Each compartment can mount independent response(s) to combat the stress of unfolded proteins. The *E. coli* transcription factor σ^E responds to envelope stress by inducing expression of many genes, the majority of which either affect or function in the envelope (reviewed in: Alba and Gross 2004). In addition, σ^E is essential for viability in *E. coli*; however, its essential function(s) are unknown (De Las Peñas 1997).

The goal of my graduate work was to gain a greater understanding of the essential functions of σ^E. I pursued this goal using two strategies: The first approach relied on the fact that strains in which *rpoE*, the gene encoding σ^E, has been disrupted acquire an extragenic suppressor which permits survival in the absence of σ^E. I tried to map this suppressor mutation in order to elucidate the essential function(s) that obviate an *E. coli* cell's need for σ^E. This strategy was ultimately unsuccessful for technical reasons. The results of this study are summarized in Appendix C. The second approach was to study the functions provided by members of the σ^E regulon. I focused on understanding the role of the six σ^E-transcribed lipoproteins. These studies are presented in Chapter 2.

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The purpose of this introduction is to provide context for my research. First, I describe the organization of the envelope compartment of a Gram-negative bacterial cell. I then provide a more detailed introduction to the biogenesis and function of lipoproteins. Finally, I discuss the three signal-transduction pathways known to respond to envelope stress: The CpxAR two-component signaling pathway, the BaeSR two-component signaling pathway, and the σ^E signaling pathway.

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THE ENVELOPE COMPARTMENT OF THE CELL

The hallmark of the Gram-negative bacterial cell is the existence of two membrane layers, the inner cytoplasmic membrane and the outer membrane, which, in turn, form the boundaries of two aqueous subcellular compartments, the cytoplasm and the envelope (Figure 1-1). The cytoplasm is an energy rich, highly regulated environment which houses the nucleoid, and it is in this compartment that basic cellular processes such as transcription, DNA replication, and translation are performed. In addition, the synthesis of many outer membrane components, such as lipopolysaccharide (LPS) precursors, takes place in the cytoplasm. Proteins in the cytoplasm are maintained in a reduced state, primarily by the action of thioredoxin and gluataredoxin (reviewed in: Ritz and Beckwith 2001). In stark contrast to the cytoplasmic compartment, the envelope contains no ATP and proteins are maintained in the oxidized state by the action of the Dsb proteins (reviewed in: Ritz and Beckwith 2001). The envelope of the Gram-negative

bacterial cell is tripartite: It is comprised of the inner membrane (IM), the periplasmic space, and the outer membrane (OM) (Figure 1-2), and it constitutes about 15 – 30% of the volume of the cell (reviewed in: Oliver 1996). The conditions of the envelope vary with those of the external environment due to the existence of pores in the outer membrane, which allow the free exchange of small molecules and some specific substrates (reviewed in: Nikaido, 1996).

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The inner membrane

The composition of inner membrane is that of a typical lipid bilayer. It contains three major phospholipids: Phosphatidyl glycerol, cardiolipin (diphosphatidyl glycerol) and phosphatidyl ethanolamine (Cronin 1996). The IM is protein rich; about 5.5% of *E. coli* proteins are integral membrane proteins whose membrane spanning portions are usually α-helical and very hydrophobic (Kadner 1996; Duong, Eichler et al. 1997; Serres 2004). In addition, many other proteins are peripherally associated with the inner membrane. The IM is responsible for selective transfer of nutrients into the cytosol, for energy generation, for intercompartmental signaling, and for translocation of proteins, carbohydrates and lipids that are to be assembled in the periplasm and OM (Kadner 1996; Duong, Eichler et al. 1997). Folded proteins that are destined to the periplasm or outer membrane are translocated across the inner membrane by the TAT (twin arginine translocation) system (Berks, Palmer et al. 2003).

However, unfolded proteins require an entirely different cellular targeting machinery.

Unfolded proteins are translocated across the inner membrane via the SecYEG translocon either post-translationally or co-translationally, and they are targeted to the SecYEG translocon by one of two pathways (Manting and Driessen 2000; Driessen, Manting et al. 2001). Post-translational translocation is accomplished via the Sec system, which translocates the OM porins and most periplasmic proteins (Manting and Driessen 2000; Driessen, Manting et al. 2001). In this system, SecB binds to an unfolded protein and delivers it to SecA, which transfers it to the translocon for export into the periplasm through the inner membrane (Randall and Hardy 2002). Co-translational translocation is accomplished via the SRP particle, which is universally conserved among all organisms (Poritz, Strub et al. 1988; Poritz, Bernstein et al. 1990; Bernstein, Zopf et al. 1993; Miller, Bernstein et al. 1994). In this system, SRP, which is comprised of 4.5S RNA and the Ffh protein, binds to a nascent polypeptide chain and delivers it to the membrane-localized receptor FtsY. FtsY then transfers these proteins to the form of the Sec YEG translocon associated with YidC (Driessen, Manting et al. 2001). The SRP system is used for integral membrane proteins. Interestingly, the few lipoproteins that have been examined also use the SRP system, raising the possibility that this entire class of proteins is translocated by SRP (Kusters, Lentzen et al. 1995; De Gier, Valent et al. 1997; Froderberg, Houben et al. 2004; Yi, Celebi et al. 2004).

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The outer membrane

The outer membrane is a structure unique to Gram-negative bacteria, and it provides these bacteria with high resistance to hydrophobic antibiotics, detergents, and hydrophobic dyes (reviewed in: Nikaido 1996; Nikaido 2003). It consists of an asymmetric lipid bilayer punctuated by porins, which provide access to small molecules. Larger specific molecules gain access through TonB-dependent receptors. Each of these constituents is described below. I end this section with a discussion of the features of the envelope contributing to the barrier function of Gram-negative bacteria.

The asymmetric lipid bilayer of the OM consists of an inner leaflet formed by phospholipids enriched for phosphatidylethanolamine (Osborn, Gander et al. 1972) and an outer leaflet composed of LPS, a constituent unique to the outer membrane (Kamio and Nikaido 1976). LPS has three distinct portions (Raetz 1996); Lipid A, the core region, and the O antigen. Lipid A is the cell proximal portion of LPS. It has a β-1, 6-linked glucosamine disaccharide backbone that is acylated with (R)-3-hydroxymyristic acid residues at 4 positions. Two of these chains are further acylated so that each Lipid A moiety carries 6 or 7 saturated fatty acid residues. In addition, both ends of this molecule are phosphorylated. Lipid A provides the hydrophobic anchor for the LPS in the outer membrane and is the active constituent in endotoxin, which can induce septic shock in a host organism. The core region of LPS is covalently linked to Lipid A and is a non-repeating oligosaccharide of approximately 6 to 10 sugars in length. Finally, O

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antigen, which is covalently linked to the core region, is a repeating oligosaccharide consisting of approimately 1 to 40 repeat units. The number of units is highly variable from strain to strain. O antigen is absent in the K-12 laboratory strain of *E. coli* because its synthesis pathway has been mutationally inactivated (Stevenson, Neal et al. 1994).

Porins, channel forming proteins in the OM, comprise approximately 25% of the surface area of *E. coli*. The high number of porin molecules per cell ($\sim 10^5$) is necessary to support sufficient influx of glucose and the other small molecules to maintain maximal growth (Nikaido and Vaara 1985). There exist two types of porins: The predominant trimeric porins, OmpF, OmpC, PhoE, and LamB; and the monomeric porin OmpA. The structure of OmpF reveals that each monomer consists of 16 anti-parallel β-strands, and each of these β-strands traverses the outer membrane (Cowan, Garavito et al. 1995). Together, these β-strands comprise a β-barrel structure, which surrounds an inner channel (Cowan, Schirmer et al. 1992; Cowan, Garavito et al. 1995) (Figure 1-3). Thus, each trimeric porin has three channels connecting the periplasmic space to the external environment. OmpC, OmpF and PhoE porins are non-specific channels that permit small aqueous molecules access to the periplasm. However, these porins exclude lipophilic solutes (Nikaido 1996). In contrast, LamB is a specific channel which permits maltose and maltodextrin to enter the cell (Luckey and Nikaido 1980). OmpC has a smaller channel than OmpF, and it is most prevalent under high osmotic conditions; the converse is true in media of low osmolarity. This

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distinction helps regulate the flow of solutes into the cell (Nikaido and Vaara 1985). OmpA, a monomeric porin, is also comprised of a β-barrel structure surrounding an inner channel; however, the conductance of this channel is sufficiently low such that OmpA probably does not contribute significantly to the permeability of *E. coli* (Nikaido 1996).

Omps undergo a complex series of folding and oligomerization steps prior to insertion into the outer membrane (Pugsley 1993). In the periplasm, Omp polypeptides transition first to a folded monomer state (Rouvière 1996), then convert to a meta-stable trimer state, and finally form stable trimers which are inserted in the outer membrane (Vos-Scheperkeuter and Witholt 1984). In addition to requiring the activity of periplasmic chaperones (minimally SurA, Skp, FkpA and DegP; see Periplasm section below), the porin assembly pathway is also accelerated by various lipid components, including both phospholipids and LPS, which function at discrete stages in the assembly process (de Cock, Pasveer et al. 2001). Mutations in the porin assembly pathway lead to the accumulation of unfolded or unassembled porin intermediates, which are potent inducers of σ^E activity (Mecsas, Rouviere et al. 1993; Rouviere and Gross 1996; Walsh, Alba et al. 2003).

Bulky nutrients, such as Vitamin B12 and iron siderophores, are too large to diffuse through the trimeric porins and are imported across the OM via TonB-dependent transport systems. The TonB system is comprised of several proteins that span the periplasm from the IM to the OM. This system requires the proton-

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motive force (PMF) of the IM as an energy source: The energy derived from the cytoplasmic membrane is transferred via the integral membrane proteins ExbB and ExbD from TonB to outer membrane receptors, which bind the molecules of interest with very high affinity (reviewed in: Nikaido 2003). In contrast to the porins, through which solutes passively diffuse, the TonB system uses the energy of the PMF to maintain concentrations of substrates in the cell that vastly exceed those in the external milieu (Reynolds, Mottur et al. 1980). TonB transport systems are also used to export toxins from the *E. coli* cell.

Several features of the envelope contribute to the exceptional barrier function of the outer membrane of Gram-negative bacteria. The impermeability of the OM results from efficient extrusion of toxins via TonB mediated efflux pumps (see above) and from the chemical and physical features of LPS itself that limit diffusion through this membrane. A membrane comprised of LPS has very low fluidity, much lower than that of a membrane derived from typical phospholipids (reviewed in: Nikaido 2003). This phenomenon is a consequence of the fact that Lipid A has many more fatty acid constituents than other phospholipids and that all of these fatty acids are saturated (Labischinski, Barnickel et al. 1985; Labischinski, Naumann et al. 1989). The high density of fatty acid chains in Lipid A increases the potential for H^+ binding between adjacent LPS molecules in the OM (reviewed in: Nikaido 2003). In fact, islands of LPS can maintain their integrity in a sea of phospholipids for days (Takeuchi and Nikaido 1981). X-ray diffraction studies of isolated LPS show that these hydrocarbon chains are highly

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ordered in a nearly crystalline arrangement (Ueki 1979). Strong lateral interactions between LPS molecules are also made possible because LPS binds the divalent cations Mg^{++} and Ca^{++} to neutralize electrostatic repulsion. As a consequence of the low fluidity the OM and strong lateral interactions between LPS molecules, diffusion of hydrophobic antibiotics through the outer membrane is about two orders of magnitude slower than through a typical lipid bilayer. In addition to these two features, outer membrane integrity is actively maintained via the TolA-Pal system, which is described in the Function of Lipoproteins section below.

<u>The Periplasm</u>

The periplasmic space lies between the inner and outer membranes of Gram-negative bacteria. This compartment is a dynamic space, capable of regulation to accommodate changes in the external and internal environments that surround it. The constituents of the periplasm include the peptidoglycan layer, which maintains the shape and rigidity of the bacterial cell, membrane-derived oligosaccharides (MDOs), which are thought to buffer the cell from osmotic stress, and proteins that perform a variety of functions (Kennedy 1996). Some periplasmic proteins are freely diffusible; however, their mobility is approximately 100-fold lower than proteins in the cytoplasm, possibly due to the high viscosity of the periplasm itself or to the sieving effect of peptidoglycan (Brass, Higgins et al. 1986). Other lipid-modified proteins, or lipoproteins, are anchored via their lipid

moiety either to the outer leaflet of the IM or to the inner leaflet of the OM (Wu 1996). Lipoproteins will be discussed in detail in the next section of the introduction, because they are directly relevant to the research report in Chapter 2.

Many periplasmic proteins fulfill important functions in the detection and processing of essential nutrients and in their transport into the cell (Oliver 1996). The nutrient binding proteins form a predominant group of periplasmic proteins; in fact, approximately 30-40 binding proteins have been characterized in *E. coli.* These proteins concentrate a variety of molecules, including amino acids, ions, sugars, peptides and vitamins in the periplasm to facilitate their transfer to the cytoplasmic compartment. Several periplasmic detoxifying enzymes and a number of periplasmic catabolic enzymes have also been characterized (reviewed in: Oliver 1996). Other periplasmic proteins promote the biogenesis of proteins entering this compartment and of components destined for incorporation into the peptidoglycan, IM, or OM. Those proteins that facilitate protein maturation do so by forming and shuffling disulfide bonds, performing cis-trans isomerization of prolyl residues, and performing chaperone functions. Several of the periplasmic chaperones and peptidyl-prolyl isomerases (PPIases) have been implicated in the biogenesis of outer membrane porins (Missiakas 1996; Rouviere and Gross 1996; Behrens, Maier et al. 2001). Because the σ^E signal-transduction pathway monitors the accumulation of porin precursors (see next section), I will describe the proteins that promote porin assembly in further detail.

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SurA is required for the folding of porin monomers, an early step in porin biogenesis (Rouviere and Gross 1996). In the absence of SurA protein, assembly of the OmpC, OmpF and LamB porins is impaired, and these porins are present at only about 10% of their WT amount in the outer membrane (Rouvière and Gross1996). In addition, the activity of σ^E is increased 10 to 15-fold as a consequence of the accumulation of unfolded porin precursors (Mecsas, Rouviere et al. 1993; Rouviere and Gross 1996; Walsh, Alba et al. 2003). SurA is both a chaperone (Behrens, Maier et al. 2001) and member of the parvulin family of PPIases (Rouviere and Gross 1996). However, it is the chaperone function of SurA that is required for OMP assembly *in vivo* (Behrens, Maier et al. 2001). Two pieces of experimental evidence implicate SurA in a direct role in porin maturation. First, SurA has been shown to interact preferentially (>50-fold) with *in vitro* synthesized porins over other similarly sized proteins (Behrens, Maier et al. 2001). Second, a heptameric phage display library identified peptide sequences that are overrepresented in porins as the best SurA binding partners (Bitto and McKay 2003). SurA has been reported to be a member of the σ^E regulon (Dartigalongue, Missiakas et al. 2001).

FkpA, like SurA, also exhibits both PPIase (Horne and Young 1995; Bothmann and Pluckthun 2000; Ramm and Pluckthun 2000) and chaperone activities (Bothmann and Pluckthun 1998; Bothmann and Pluckthun 2000; Ramm and Pluckthun 2000). Like SurA, the chaperone activity of FkpA is independent of its PPIase activity (Bothmann and Pluckthun 2000; Ramm and Pluckthun

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2000). FkpA has been verified to be a member of the σ^E regulon (Danese 1997). Whereas SurA encodes two tandem Parvulin-like PPIase domains, the PPIase domain of FkpA exhibits great similarity to PPIases of the FKBP family (Horne and Young 1995). The precise role of FkpA in promoting porin assembly is unknown. However, FkpA is implicated in porin biogenesis because deletion of *fkpA* induces the activity of σ^E at least 5-fold. In addition, overexpression of FkpA partially relieves defects in porin folding brought about by mutational inactivation of *rfaD*, a gene encoding a factor that promotes porin assembly (Missiakas, Betton et al. 1996).

Skp, a presumed periplasmic chaperone, was first isolated via affinity chromatography as the predominant periplasmic protein that bound to the unfolded outer membrane porin OmpF (Chen and Henning 1996). Skp was also identified in a screen for periplasmic proteins that improved phage display of an antibody fragment, suggesting that Skp exhibits chaperone activity (Bothmann and Pluckthun 1998). That Skp actually plays a role in porin assembly was shown by the fact that strains deleted for *skp* exhibit reduced levels of porins in their outer membranes (Chen and Henning 1996). *In vitro* studies indicate that one form of Skp can insert in phospholipid membranes, suggesting that this protein may be required for the efficient targeting of porins to the OM (De Cock, Schafer et al. 1999).

DegP is a periplasmic stress protein that is a member of both the σ^E and Cpx regulons (see next section). DegP was identified in a screen for genes whose

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functions were required for the survival of *E. coli* only at high temperature. DegP was then shown to be a periplasmic protease (Lipinska, Sharma et al. 1988; Lipinska, Fayet et al. 1989; Strauch, Johnson et al. 1989; Lipinska, Zylicz et al. 1990; Raina, Missiakas et al. 1995). Interestingly, this protease also exhibits a chaperone activity, which is most pronounced at low temperatures (Spiess, Beil et al. 1999). The precise role of DegP in the porin maturation pathway is unclear; however, both Skp and DegP are synthetically lethal with SurA, yet not with each other. This observation has led Silhavy and collaborators to hypothesize that SurA and Skp/DegP comprise parallel folding pathways that support the maturation of porins (Rizzitello, Harper et al. 2001).

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A BRIEF SURVEY OF LIPOPROTEINS

Overview

A lipoprotein is an envelope protein whose N-terminal cysteine is covalently modified with a lipid moiety (reviewed in: Wu 1996; Narita, Matsuyama et al. 2004). Proteins targeted for lipid modification each have a lipobox motif encoded in their signal sequences. The consensus sequence of the lipobox is: Leu (Ala, Val)$_{-4}$- Leu$_{-3}$ -Ala (Ser)$_{-2}$- Gly (Ala)$_{-1}$ -Cys$_{+1}$, where Cys$_{+1}$ designates the cysteine to be modified (Wu 1996). The *E. coli* genome is predicted to encode 100 lipoproteins, of which 90 have been experimentally verified (Miyadai, Tanaka-Masuda et al. 2004). Very few lipoproteins have been extensively investigated, and as a consequence, little is known about the

physiological roles of lipoproteins in *E. coli*. Because σ^E is essential and plays a pivotal role in maintaining the integrity of the envelope, I investigated the possibility that the six lipoproteins in the σ^E regulon play important cellular roles in this process. The phenotypes of strains deleted for each of these σ^E–transcribed lipoproteins are described in Chapter 2.

Synthesis of lipoproteins

Lpp, also known as Murein or Braun's lipoprotein, is the most abundant lipoprotein in *E. coli*. It was the first lipoprotein to be discovered because of its covalent attachment to peptidoglycan (Braun and Rehn, 1969). Analysis of the isolated lipoprotein indicated that the amide-linked fatty acid was palmitate and that fatty acids in thioester linked diacyl glycerol

portion were phospholipids (Hantke and Braun 1973). These findings prompted efforts to elucidate a biosynthetic pathway for the assembly of lipoproteins.

Using a partially *in vitro* system, Inouye determined that Lpp was synthesized as a prolipoprotein with a 20 amino acid signal sequence at its N-terminus (Inouye 1977). This prolipoprotein was not processed by the first signal peptidase isolated (Signal Peptidase I). Moreover, processing of prolipoprotein was inhibited by globomycin, which does not affect the activity of Signal Peptidase I. Additionally, several other prolipoproteins accumulated following globomycin treatment, indicating that the *E. coli* genome encoded more than one

lipoprotein. Together, these experiments suggested that globomycin was likely to inhibit a specific signal peptidase whose activity was required for the processing of lipoproteins. Signal peptidase II (encoded by *lsp*) was soon isolated (Wu 1996).

Interestingly, the prolipoproteins that accumulated in the presence of globomycin were diacyl substituted, leading to the suggestion that this substitution not only preceded signal peptide cleavage, but that it might also be required for such cleavage (Hussain, Ichihara et al. 1980; Ichihara, Hussain et al. 1981; Hussain, Ichihara et al. 1982). In fact, the first committed step in the synthesis of lipoproteins is recognition of the lipobox by phosphatidylglycerol:prolipoprotein diacylglycerol transferase, with the subsequent addition of diacylglycerol to sulfur of the cysteine residue (diagrammed in Figure 1-4). Signal peptidase II then cleaves the prolipoprotein immediately N-terminal to the modified cysteine. Finally, apolipoprotein N-transferaseacylase N-acylates this cysteine, usually with palmitate, to create the mature lipoprotein.

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Localization of lipoproteins

The protein and lipid moieties of lipoproteins are assembled on the periplasmic face of the inner membrane; however, most lipoproteins are localized to the inner leaflet of the OM. The Lol system plays a critical role in the membrane specificity of lipoprotein targeting (reviewed in: Narita, Matsuyama et al. 2004). An ABC transporter (LolCDE) releases lipoproteins from the inner membrane (Yakushi, Masuda et al. 2000), allowing them to interact with LolA, a

lipoprotein-specific periplasmic chaperone (Matsuyama, Tajima et al. 1995; Tajima, Yokota et al. 1998; Miyamoto, Matsuyama et al. 2001). The lipoprotein to be targeted is then transferred from LolA to LolB, a lipoprotein anchored to the outer membrane (Matsuyama, Yokota et al. 1997; Tanaka, Matsuyama et al. 2001). LolB facilitates incorporation of lipoproteins into the outer membrane by an unknown mechanism. The lipoproteins associated with the inner membrane usually have an Asp residue immediately following the modified Cys_{+1} (Yamaguchi, Yu et al. 1988), and an Asp, Glu, Gln or Asn at position +3 (reviewed in: Narita, Matsuyama et al. 2004). These amino acids prevent a lipoprotein that is to be retained in the inner membrane from interacting with LolCDE ("lol avoidance") (Yamaguchi, Yu et al. 1988; Matsuyama, Tajima et al. 1995; Yakushi, Masuda et al. 2000).

Functions of lipoproteins

Lpp is the most abundant and best characterized lipoprotein in *E. coli*. The N-terminus of the protein moiety of Lpp is anchored in the outer membrane via its lipid-modified cysteine, and about 1/3 of the Lpp molecules are also covalently attached to the peptidoglycan layer via the ε-amino group of its C-terminal lysine. Approximately 10% of the peptidoglycan monomers in *E. coli* are linked to Lpp. The anchoring of the outer membrane to the peptidoglycan via Lpp is important for the maintenance envelope integrity (reviewed in: Nikaido 1994; Wu 1996). *lpp* mutants exhibit envelope defects, as is indicated by leakage of periplasmic

proteins and by hypersensitivity to both EDTA and low osmotic strength media (Yem and Wu 1977; Yem and Wu 1978).

The OM lipoprotein Pal also functions in an important, yet poorly understood process that helps maintain the barrier function of the OM. Like Lpp, Pal anchors the OM to the peptidoglycan via the ε-amino group of its C-terminal lysine. However, the functions of Pal require the PMF of the IM in a manner that is similar to that of the TonB transport systems (Lloubes, Cascales et al. 2001). With the help of the integral membrane proteins TolR and TolQ, the energy derived from the cytoplasmic membrane is transferred from TolA to the periplasmic protein TolB and to the OM lipoprotein Pal. Cells lacking one of the components of this system show a variety of envelope defects, some of which are similar to those of cells lacking Lpp. These phenotypes include hypersensitivity to various drugs, leakage of periplasmic proteins, and dramatically increased formation of outer membrane vesicles (Lloubes, Cascales et al. 2001; Lazzaroni, Dubuisson et al. 2002). Interestingly, *pal* mutants exhibit increases in permeability not only to lipophilic agents (Lazzaroni, Dubuisson et al. 2002), but also to large, hydrophilic molecules like vancomycin (Cascales, Lloubes et al. 2001; Lloubes, Cascales et al. 2001). The molecular basis of these phenotypes is not known. However, TolB exhibits homology to oligopeptide peptidases and to β-lactamase. Taken together with the fact that Pal binds non-covalently to peptidogylcan, these observations suggest that Pal may direct TolB in the

hydrolysis of peptidoglycan and may allow remodeling of the connections between peptidoglycan and the outer membrane (Abergel, Bouveret et al. 1999).

Three inner membrane localized lipoproteins, AcrA (Ma, Cook et al. 1993), AcrE (Ma, Cook et al. 1993), and EmrA (Lomovskaya and Lewis 1992; Lomovskaya, Lewis et al. 1995; Tegos, Stermitz et al. 2002), function together with an RND (metal resistance, nodulation, cell division) permease to form an efflux pump that transfers its substrates across both the IM and the OM to remove them from the cell. These three lipoproteins are members of the membrane fusion (MFP) protein family and have homology to the membrane fusion protein of simian virus 5 (Dinh, Paulsen et al. 1994). Substrates of the permease are transported across the cytoplasmic membrane, possibly into a channel created by the MFP, which permits their exit to the extracellular milieu (Dinh, Paulsen et al. 1994).

Several lipoproteins, including NlpE (Raivio and Silhavy 2001) and YafY (Miyadai, Tanaka-Masuda et al. 2004), are potent inducers of the CpxAR two-component system, which, like σ^E, also monitors envelope integrity (see below; reviewed in: Raivio and Silhavy 2001). To date, LolB, which, as mentioned previously, functions in the lipoprotein localization process, is the only essential lipoprotein to have been identified (Tanaka, Matsuyama et al. 2001; Narita, Tanaka et al. 2002). As reported in Chapter 2, I have identified the second essential lipoprotein in *E. coli*.

TWO-COMPONENT SYSTEMS THAT COMMUNICATE ENVELOPE STRESS

Overview

In bacteria, adaptive responses to changing environmental conditions are mediated by two component systems, which involve modular protein domains (Stock, Robinson et al. 2000). These systems minimally consist of a sensor kinase, which is usually membrane-localized, and a cytoplasmic response-regulator. Upon detection of an activating signal, the sensor kinase autophosphorylates a histidine residue. This high-energy phosphoryl group is then transferred to an aspartic residue in the response-regulator in a reaction catalyzed by the response-regulator itself. The phosphorylated response-regulator then usually functions as an activator in transcription. The phospho-asp residue is intrinsically unstable, and the phosphoryl group is eventually transferred to a water molecule, thereby deactivating the transcriptional activator. The half-life of the phospho-asp residue can vary from seconds to hours, depending upon the phosphatase activity of a particular sensor kinase. The *E. coli* genome encodes 36 two-component systems (Oshima, Aiba et al. 2002). Of these, the role of the CpxAR system in sensing and communicating envelope stress has been studied in detail (Raivio and Silhavy 2001); there is also some evidence that the BaeSR system functions in envelope stress (Raffa and Raivio 2002).

Discovery that CpxAR functions in envelope stress

That CpxAR is involved in envelope stress became apparent through a study of two toxic proteins: The LamB-LacZ-PhoA fusion protein and LamBA23D, which has a defective signal sequence. Both proteins are targeted to the envelope and both cause cell death upon overexpression (Carlson 1993; Snyder 1995). A search for extragenic suppressors of the toxicity of these proteins identified gain of function alleles in the CpxA sensor histidine kinase ORF, and these alleles are referred to as *cpxA** (Cosma 1995). Additional studies then showed that *cpxA** alleles activated the transcription of the chaperone/protease DegP. This finding strongly suggested that CpxAR promoted transcription of stress-combative genes (Danese 1995). Interestingly, a second extragenic suppressor of the two toxic proteins, NlpE, functions by activating CpxAR through an unknown mechanism (Danese 1995; Snyder, Davis et al. 1995).

Inducers of the CpxAR regulon

Several different stimuli have been reported to induce the CpxAR regulon; however, in no case has the mechanism between inducing signal and response been established. For example, CpxAR activity is induced by alkaline pH, and the functions that are transcribed by CpxAR induction are necessary for survival under those conditions (Danese and Silhavy 1998). However, neither the molecular basis for alkaline pH induction of CpxAR nor the Cpx functions required for survival in these conditions have been elucidated. CpxAR-controlled

genes are also induced in mature biofilms (Beloin, Valle et al. 2004). Several insults to the plasma membrane, including inactivation of FtsH, a membrane localized protease that functions in membrane quality control (Shimohata, Chiba et al. 2002); overexpression of several plasma membrane proteins (Shimohata, Chiba et al. 2002); and alteration in the lipid content of the inner membrane (Mileykovskaya and Dowhan 1997; Danese, Oliver et al. 1998) activate the CpxAR pathway. Interestingly, in a genomic survey of the effects of overexpression of each *E. coli* lipoprotein on CpxAR activity, YafY, a lipoprotein anchored to the inner membrane, was the most potent inducer of CpxAR (Miyadai, Tanaka-Masuda et al. 2004). Together, these observations raise the possibility that CpxAR may also sense the status of the inner membrane. Finally, the Cpx system is induced by accumulation of P-pili intermediates (Hung, Raivio et al. 2001; Lee, DiGiuseppe et al. 2004). The specific inducing agent has been determined to be the N-terminal extension of PapE (Hung, Raivio et al. 2001; Lee, DiGiuseppe et al. 2004). Now that a specific inducer of this regulon has been identified, it is likely that a mechanism for induction will be forthcoming.

The signal-transduction pathway

As just described, many molecules and conditions activate CpxA, the sensor kinase. However, a precise molecular mechanism for activation has not yet been determined. One intriguing component in the system is CpxP, which is transcribed divergently from CpxAR and may play a regulatory role in the

response. CpxAR activates transcription of both CpxP and CpxAR. Overexpression of CpxP downregulates the expression of CpxAR even in the presence of inducing stimuli (Raivio, Popkin et al. 1999). Moreover, induction of the Cpx regulon via spheroplasting is abrogated when a membrane-tethered derivative of CpxP is overexpressed (Raivio, Laird et al. 2000). Together, these experiments have led Silhavy and colleagues to suggest that CpxP binds to CpxA to hold it in an inactive conformation. They also posit that the inducing signal functions, in part, to titrate CpxP from CpxA (Raivio, Popkin et al. 1999; Raivio, Laird et al. 2000; Raivio and Silhavy 2001). However, a direct interaction between CpxP and CpxA has not been demonstrated. In addition, deletion of CpxP has only a small effect on CpxAR expression and does not prevent activation (Raivio, Popkin et al. 1999). Therefore, additional mechanisms that activate CpxA must exist.

Functions of the CpxAR regulon members

Several genomic techniques, including screening a library of *lacZ* operon fusions for those upregulated in a *cpxA** background, have allowed researchers to identify a number of genes in the Cpx regulon. The *cpxP* gene, described above, was identified from such a screen and shown to be important in combating stress (Danese and Silhavy 1998). Cells lacking CpxP are more sensitive to toxicity induced by fusion proteins and to growth under alkaline conditions (Danese and

Silhavy 1998). However, the mechanism by which CpxP allows cells to resist the toxicity of fusion proteins is not understood.

Several of the genes controlled by CpxAR encode proteases, chaperones or protein folding agents (Raivio and Silhavy 2001). These include DegP, which, as mentioned previously, functions predominantly as a chaperone at low temperature and predominantly as a protease at high temperature (Spiess, Beil et al. 1999); DsbA, which forms disulfide bonds in periplasmic proteins (Bardwell, McGovern et al. 1991); and PpiA, a periplasmic peptidyl-prolyl isomerase of unknown function. Indeed, overexpression of the CpxAR regulon restored the ability of cells lacking σ^E to grow at high temperatures, indicating that the functions of one regulon can substitute for the other under this condition (Connolly, De Las Penas et al. 1997).

The BaeSR envelope stress response

The BaeS sensor kinase and BaeR response regulator are also implicated in the physiological response to envelope stress. BaeSR is suspected to respond to envelope stress for the following reasons: First, BaeSR promotes transcription of *spy*, a gene that is also transcribed by CpxR. Second, strains lacking BaeSR, like strains lacking CpxAR, are more sensitive to indole and to accumulation of the misfolded pilus protein PapG. Finally, strains deficient in both CpxR and BaeR show synergistic phenotypes (Raffa and Raivio 2002). These studies are in their infancy.

THE σ^E ENVELOPE STRESS RESPONSE

Overview of the ECF (extracytoplasmic function) σ's

σ^E is a member of the ECF Family σ factors, which is also known as the Group 4 Family. This family contains the largest number of members, many of which function in some aspect of regulating the cell envelope (Lonetto, Brown et al. 1994). Two of *E. coli*'s 6 σ's are members of the ECF family, although only one, σ^E, monitors envelope stress. Seven of the eighteen σ's in *B. subtilis* are members of the ECF family, and three of these seven ECF σ's, σ^W, σ^X, and σ^M, control overlapping sets of genes which are likely to be responsive to functionally related signals (Gruber and Gross 2003). For example, all three σ's are induced by antibiotics acting on the cell wall (Gruber and Gross 2003; Mascher, Margulis et al. 2003). The vast majority of the 63 σ's in *S. coelicolor* are ECF family members (reviewed in: Gruber and Gross 2003), but few have been studied in detail. Those whose functions have been investigated were found to play a role in combating oxidative stress (Gruber and Gross 2003). In the remainder of this section, I review the discovery, signal transduction pathway, and functions of the σ^E envelope stress response.

Discovery of the σ^E envelope stress response

σ^E was discovered in an investigation aimed at understanding how σ^{32} was transcribed at lethal temperatures. σ^{32}, encoded by the gene *rpoH*, is responsible

for transcribing the cytoplasmic heat shock genes, which are the predominant cellular transcripts when the cell is subjected to lethal temperatures. However, at the time of this investigation, σ^{70}, the housekeeping sigma factor in *E. coli*, was the only σ factor known to transcribe *rpoH*. The purification of an activity that allowed the transcription of σ^{32} at 50°C led to the identification of σ^{E}. σ^{E} was not only active at these high temperatures, but it also utilized a distinct promoter to transcribe *rpoH* (Erickson, Vaughn et al. 1987; Erickson and Gross 1989). This new σ factor was also shown to transcribe *degP*, which, as described previously, is essential only at high temperatures (Erickson and Gross 1989). In addition, σ^{E} was identified independently as a heat resistant contaminant in a standard preparation of RNA polymerase (Wang and Kaguni 1989). Taken together, these early studies suggested that σ^{E} played a role in the adaptation of *E. coli* to survival at extremely high temperatures. *RpoE*, the gene encoding σ^{E}, was then cloned based on its homology to other ECF σ's (Rouvière 1995). *RpoE* was also cloned independently by a second group in a promoter fusion screen (Raina, Missiakas et al. 1995).

A more precise understanding of the cellular role of σ^{E} came from studies identifying inducers of σ^{E} activity. These studies revealed that overexpression of porin monomers induced σ^{E} activity and that induction was dependent on transit of the porins across the inner membrane to the envelope compartment of the cell (Mecsas, Rouviere et al. 1993). Moreover, a deficit of porins, generated by

inactivating the transcription factor required for high level expression of the OmpF and OmpC porins, decreased the basal rate of σ^E activity (Mecsas, Rouviere et al. 1993). Taken together, these results suggested that the level of porin precursors in the *E. coli* envelope influenced the basal σ^E activity of the cell. Additionally, a variety of mutant strains, each lacking a single envelope chaperone or folding catalyst, such as SurA, DsbA, FkpA, exhibited increased σ^E activity (Missiakas, Betton et al. 1996; Rouvière 1996). Such strains were hypothesized to accumulate higher levels of unfolded or misfolded envelope proteins, suggesting that σ^E responded to folding stress in the envelope.

<u>σ^E is also required for viability</u>

The inability to cotransduce a null allele of *rpoE*, the gene encoding σ^E, with a linked drug marker revealed that σ^E was essential at all temperatures tested (18°C to 42°C) in both minimal and rich media (De Las Peñas 1997). However, a frequently arising unlinked suppressor mutation, *sup*$^+$, allowed *rpoE::Cm* cells to continue growing at low temperatures (De Las Peñas 1997). Understanding of the mechanism of suppression would further our understanding of the essential function(s) of σ^E. Appendix A recounts my unsuccessful attempts to map the suppressor locus. As discussed above, induction of the Cpx regulon permitted Δ*rpoE sup*$^+$ cells to grow at high temperature (Connolly, De Las Penas et al. 1997). However, high overexpression of the Cpx regulon does not provide the

function(s) supplied by *sup*$^+$ that permit growth of a Δ*rpoE* strain at low temperature (Connolly, De Las Penas et al. 1997).

The signal-transduction pathway governing σ^E activity

The *rpoE* gene is located in an operon that contains *rseA* and *rseB*, which encode two negative regulators of σ^E activity (De Las Penas, Connolly et al. 1997; Missiakas, Mayer et al. 1997). RseA spans the inner membrane, and its cytoplasmic domain is a σ^E-specific anti-sigma factor which binds extremely tightly to σ^E (De Las Penas, Connolly et al. 1997; Missiakas, Mayer et al. 1997). Thus, under non-inducing conditions, σ^E is held in an inactive form at the cytoplasmic face of the inner membrane by virtue of its tight association with RseA. The periplasmic side of RseA binds to RseB (De Las Penas, Connolly et al. 1997; Missiakas, Mayer et al. 1997). As described below, RseB enhances the sensitivity of this signal transduction cascade.

The challenge of this signal-transduction system is to communicate envelope stress across the inner membrane to the cytoplasmic compartment of the cell. The signal is transduced by the regulated proteolysis of RseA, which releases active σ^E to carry out transcription (Ades, Connolly et al. 1999). A proteolytic cascade degrades RseA: RseA is initially cleaved by DegS, a membrane tethered protease whose active site is in the periplasm (Alba, Leeds et al. 2002; Kanehara, Ito et al. 2002). YaeL, a membrane embedded protease and an orthologue of

mammalian Site-2 protease, then cleaves RseA at its cytoplasmic face, releasing a complex consisting of σ^E and the cytoplasmic fragment of RseA (Alba, Leeds et al. 2002; Kanehara, Ito et al. 2002). This complex is then disassembled by ClpXP, an ATP-dependent cytoplasmic protease that both degrades the RseA fragment and releases σ^E (Flynn 2004).

The proteolytic cascade that degrades RseA is initiated when the C-termini of porins bind to the PDZ domain of DegS (Walsh, Alba et al. 2003). This event activates DegS, resulting in the degradation of RseA (Walsh, Alba et al. 2003; Wilken, Kitzing et al. 2004). C-termini of Omps are normally buried in the trimer interface of mature porins (Cowan, Garavito et al. 1995). An increase in exposed porin C-termini indicates the accumulation of unassembled porin precursors. As mentioned previously, porins have a complex assembly pathway that involves LPS, inner membrane lipids, and many periplasmic chaperones. Therefore, an increase in porin assembly intermediates may reflect the titration of chaperones by misfolded or unassembled proteins. The accumulation of these porin intermediates may also signal disturbances in lipid processing or increases in porin expression in response to environmental stimuli. Thus, fluctuations in the level of porin intermediates in the periplasm are an excellent indicator of envelope homeostasis.

The activity of σ^E is responsive to the levels of porin intermediates over a wide range and is insensitive to the concentrations of DegS and YaeL (Mecsas, Rouviere et al. 1993). This phenomenon results from the fact that degradation of

RseA is initiated only by DegS (Alba, Leeds et al. 2002). Because DegS is the only element in the system responsive to porin intermediates, the fraction of activated DegS accurately reflects the amount of unassembled porins (Grigorova et al, G & D, in press). At least two mechanisms ensure that YaeL does not cleave DegS. First, the periplasmic face of RseA contains motifs that inhibit YaeL cleavage (Kanehara, Ito et al. 2003). These inhibitory motifs are removed by DegS cleavage (Kanehara, Ito et al. 2003; Walsh, Alba et al. 2003), thereby releasing YaeL inhibition. That inhibitory mechanism is dependent both upon the PDZ domain of YaeL (Kanehara, Ito et al. 2003) and the presence of RseB (Grigorova et al, G & D, in press). In a second mechanism, DegS itself inhibits cleavage by YaeL in a reaction that requires neither RseB nor the PDZ domain of YaeL (Grigorova et al, G & D, in press). The nature of these inhibitory reactions has yet to be determined. Together, these mechanisms ensure that RseA cleavage is initiated only when DegS is activated by binding to unassembled OMP intermediates, thereby ensuring the sensitivity of the response to signal.

<u>The functions of the σ^E regulon</u>

The identities of the first known members of the σ^E regulon suggested that the role of this regulon was to facilitate the folding of polypeptides in the bacterial envelope. These genes included DegP, a periplasmic chaperone/protease (Lipinska, Sharma et al. 1988; Erickson 1989), and FkpA, a periplasmic peptidyl prolyl *cis/trans* isomerase that assists in porin maturation (Danese 1997). σ^E also

plays a regulatory role as it is solely responsible for transcribing *rpoH* (encoding σ^{32}) at lethal temperatures (Erickson, Vaughn et al. 1987). σ^E also auto-regulates the expression of its own operon, (*rpoErseABC*) (Rouviere, De Las Penas et al. 1995). Recently, three genomic strategies have been utilized to identify additional members of the σ^E regulon. First, both (Dartigalongue, Missiakas et al. 2001) and (Rezuchova, Miticka et al. 2003) searched for σ^E-dependent promoters by screening for increased expression of genomic DNA sequences fused to *lacZ* upon increased expression of σ^E. Second, whole-genome expression analysis identified genes that were up-regulated in response to σ^E overexpression during exponential growth (Rhodius, ms. in prep.). Finally, transcription start site mapping and bioinformatic analysis identified additional σ^E -dependent promoters (Rhodius, ms. in prep). Taken together, approximately 48 σ^E -dependent promoters, which control the expression of nearly 100 genes, have been identified.

The majority of the σ^E-dependent promoters drive production of envelope proteins, including chaperones and proteases that act on misfolded periplasmic proteins. Several phospholipid and lipopolysaccharide biosynthesis and transport proteins, as well as a variety of inner and outer membrane proteins are also members of the σ^E regulon. σ^E also transcriptionally upregulates six genes known to encode lipoproteins. Given the importance of the σ^E regulon in maintaining envelope integrity, we thought it probable that the six σ^E–transcribed lipoproteins

might play important roles in this process. The phenotypes associated with deletion of each of these lipoproteins are the subject of this report.

These genes are the subject of the next chapter. Several envelope proteins, including 4 outer membrane porins, OmpA, OmpC, OmpF, and OmPA, are strongly down-regulated upon increased expression of σ^E. Finally, some of the promoters regulate cytoplasmic proteins including proteases and transcription factors. Interestingly, two of the cytoplasmic proteases in the regulon are important in degrading cytoplasmic RseA (Flynn 2004).

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Figure Legends, Chapter One

Figure 1-1: The hallmark of the Gram-negative bacterial cell is the existence of two aqueous subcellular compartments: The cytoplasm and the envelope. The cytoplasm, indicated by the arrow and large bracket, is an energy rich, highly regulated environment which houses the nucleoid. The envelope, which is indicated by the arrow and small bracket, surrounds the cytoplasm and is bounded by the IM and the OM.

Figure 1-2: Schematic representation of the E. coli envelope and its components: The outer membrane, the inner membrane, and the periplasm. The inner leaflet of the outer membrane is comprised of typical phospholipids, whereas the outer leaflet is comprised of lipopolysaccharides (LPS), highly-charged sugar molecules that are anchored to the outer membrane via lipid A. The OM is also punctuated by porins which place the periplasm in direct contact with the extracellular environment. The constituents of the periplasm include the peptidoglycan layer, which maintains the shape and rigidity of the bacterial cell, membrane-derived oligosaccharides (MDOs), which are thought to buffer the cell from osmotic stress, lipoproteins, which are anchored via their lipid moiety either to the outer leaflet of the IM or to the inner leaflet of the OM. The inner membrane is a typical phospholipid bilayer that is associated with many integral and peripheral membrane proteins.

Figure 1-3: The structure of trimeric porins. (A) A ribbon diagram showing an OmpF momoner, which consists of 16 anti-parallel transmembrane b-strands. (B) A view of the OmpF trimer down the threefold axis. Both images have been reproduced from Cowan, et al (1995).

Figure 1-4: The proposed pathway for the biosynthesis of lipoproteins in E. coli.

Figure 1-1: The hallmark of the Gram-negative bacterial cell is the existence of two subcellular compartments: The cytoplasm and the envelope

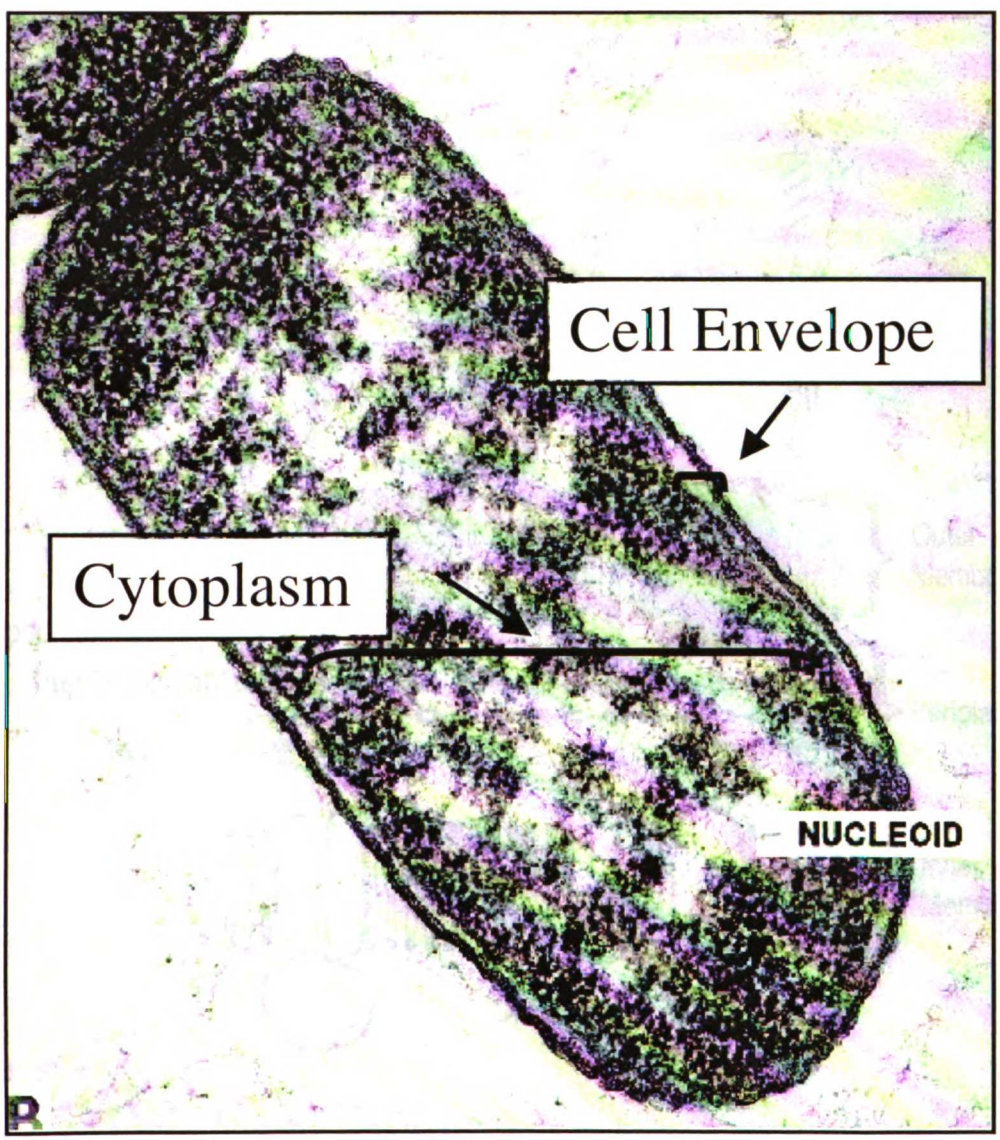

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Figure 1-2: Schematic representation of the E. coli envelope and its components: The outer membrane, the inner membrane, and the periplasm.

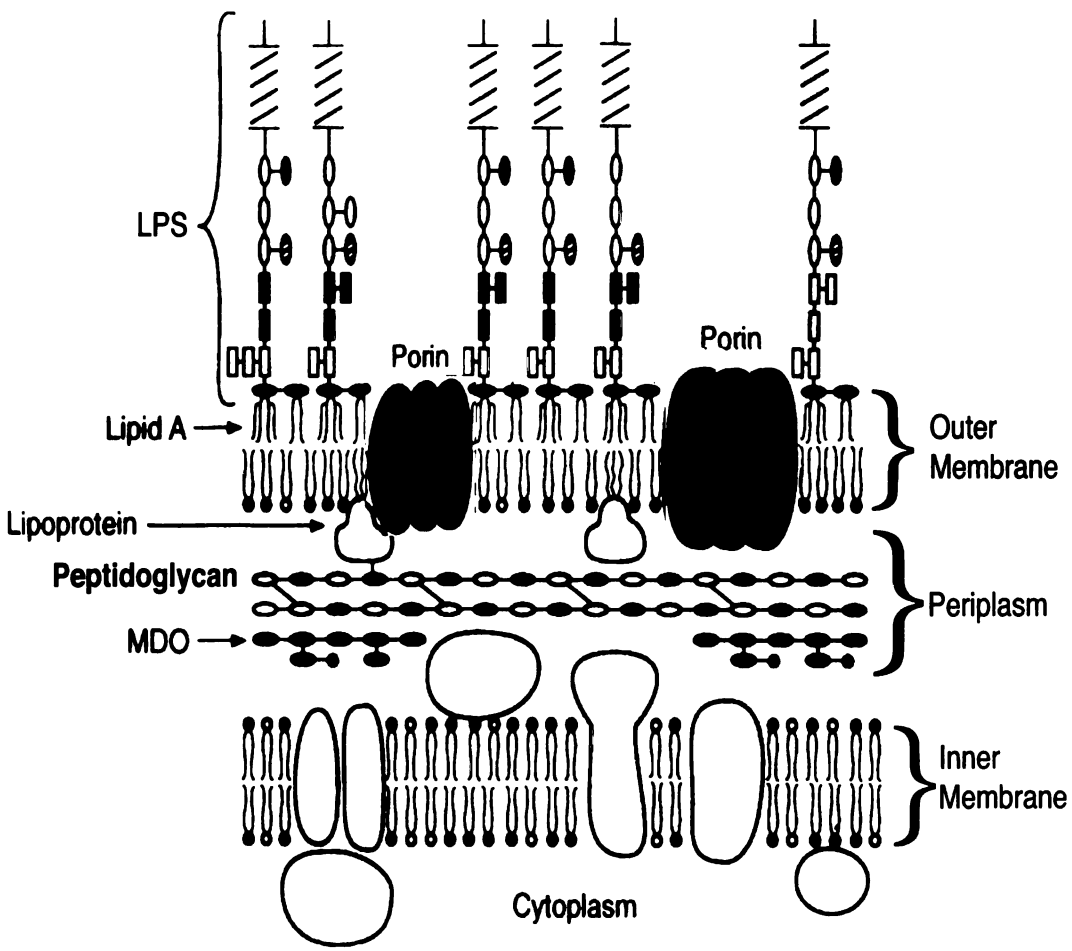

Figure 1-3: The structure of trimeric porins

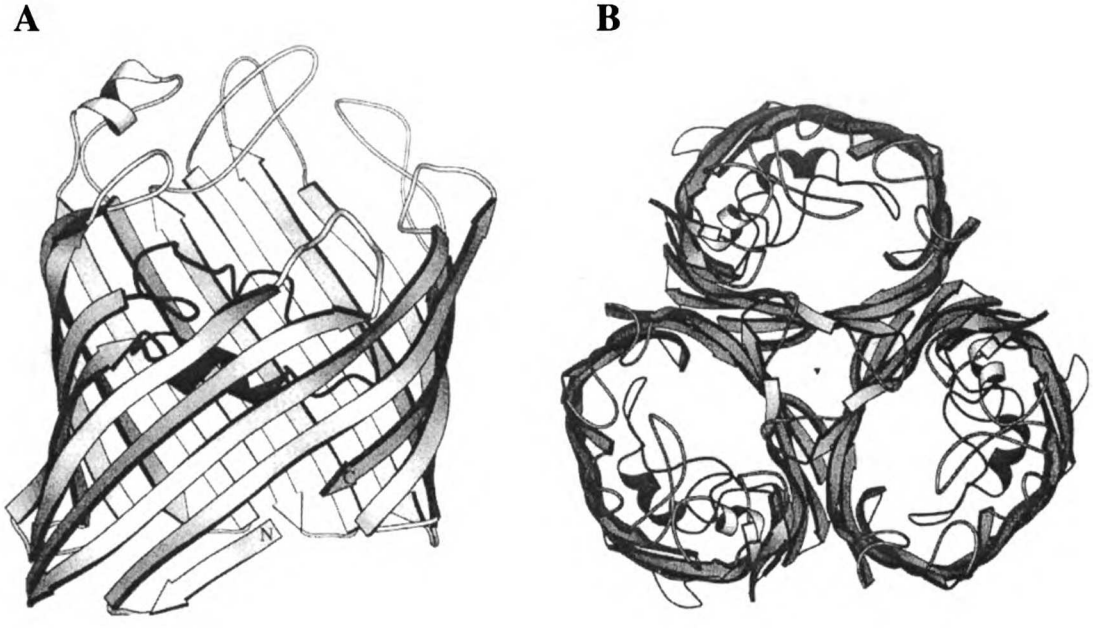

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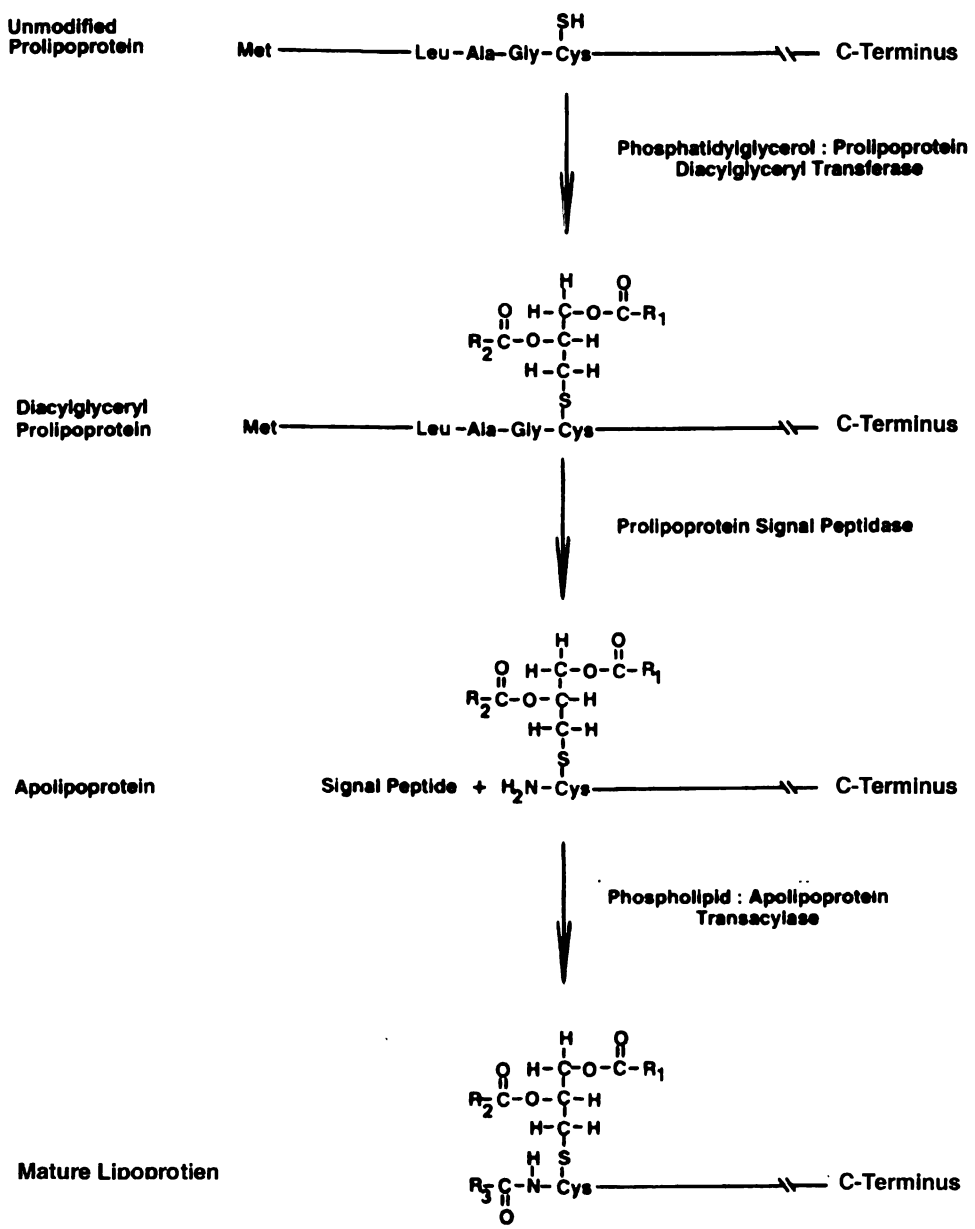
Figure 1-4: The proposed pathway for the biosynthesis of lipoproteins in *E. coli.*



Chapter Two: A Characterization of Six σ^E-Transcribed Lipoproteins

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Abstract

In *Escherichia coli*, the functions of the σ^E regulon are required for envelope homeostasis during stress and are essential for viability under all growth conditions. The σ^E regulon encodes six lipoproteins, and we have examined the deletion phenotypes of each. One lipoprotein, YfiO, is essential for cellular viability. However, overexpression of this protein is not sufficient to alleviate the requirement of σ^E for viability, suggesting that the σ^E regulon provides more than one essential function. The remaining five lipoproteins in the σ^E regulon are nonessential; in fact, cells are viable even when all five are removed simultaneously. Three of these nonessential lipoproteins exhibit phenotypes that suggest they are important for maintenance of the integrity of the cell envelope: Cells lacking NlpB are selectively sensitive to rifampicin, and those lacking YraP are selectively sensitive to SDS. In addition, both Δ*yraP* strains and Δ*nlpB* strains are synthetically lethal with *surA::Cm*, which encodes a periplasmic chaperone and PPIase. This observation may suggest that NlpB and YraP play roles in a periplasmic folding pathway that functions in parallel with that of SurA. Finally, the Δ*yfgL* mutant exhibits a broad range of envelope defects, including sensitivity to several membrane-impermeable agents, an altered OMP profile, synthetic lethality with both *surA::Cm* strains and Δ*fkpA::Cm* strains, and sensitivity to BPI. We suggest that this lipoprotein performs a very important but yet unknown function in maintaining the integrity of the cell envelope.

INTRODUCTION

When exposed to denaturants such as heat or ethanol, all cells induce stress responses to restore protein homeostasis. In *Eschericia coli*, the alternative sigma factor σ^{32} responds to the accumulation of misfolded and unfolded cytoplasmic proteins (Yura and Nakahigashi 1999). Two well characterized stress response pathways, the two-component CpxAR system (reviewed in: Raivio and Silhavy 2001), and the σ^E signal transduction cascade (reviewed in: Alba and Gross 2004) respond to stress in the envelope. In addition to mitigating envelope stress, the σ^E regulon provides an essential function to the cells during normal cell growth, as σ^E is essential under all conditions tested (De Las Peñas 1997). In this report, we investigate the physiological role of the σ^E regulon.

Recently, three genomic strategies have been employed to identify members of the σ^E regulon. First, two groups (Dartigalongue, Missiakas et al. 2001; Rezuchova, Miticka et al. 2003) searched for σ^E-dependent promoters by screening for increased expression of genomic DNA sequences fused to *lacZ* upon increased expression of σ^E. Second, whole-genome expression analysis identified genes that were up-regulated in response to σ^E overexpression during exponential growth (Rhodius, ms. in prep.). Finally, transcription start site mapping and bioinformatic analysis identified additional σ^E -dependent promoters (Rhodius, ms. in prep). Taken together, approximately 48 σ^E -dependent promoters, which control the expression of approximately 100 genes, have been identified. The

majority of the promoters drive production of envelope proteins such as chaperones and proteases that act on misfolded periplasmic proteins, phospholipid and lipopolysaccharide biosynthesis and transport proteins, and a variety of inner and outer membrane proteins, including six genes predicted or known to encode lipoproteins. These six lipoproteins are the subject of the present work.

A lipoprotein is an envelope protein whose N-terminal cysteine is covalently modified with a lipid moiety (reviewed in: Wu 1996; Narita, Matsuyama et al. 2004). Proteins targeted for lipid modification each have a lipobox motif encoded in their signal sequences. The consensus sequence of the lipobox is: Leu (Ala, Val)$_{-4}$- Leu$_{-3}$ -Ala (Ser)$_{-2}$- Gly (Ala)$_{-1}$ -Cys$_{+1}$, where Cys$_{+1}$ designates the cysteine to be modified. A transacetylase reaction occurs at the periplasmic face of the inner membrane, wherein the sulfhydryl group of Cys$_{+1}$ is initially modified by addition of diacylglycerol. The signal sequence is then cleaved after the Gly$_{-1}$ residue by signal peptidase II, and Cys$_{+1}$ becomes the N-terminal residue of the mature lipoprotein. Finally, the amino group of Cys$_{+1}$ is substituted by a fatty acid residue, usually palmitate (Sankaran and Wu 1994). These steps take place on the periplasmic face of the inner membrane.

Lipoproteins are inserted into either the inner membrane (IM) or outer membrane (OM) via their lipid modification; most are localized to the inner leaflet of the outer membrane; the remainder reside on the periplasmic face of the inner membrane. The Lol system plays a critical role in the membrane specificity of lipoprotein targeting. (reviewed in: Narita, Matsuyama et al. 2004). An ABC

transporter (LolCDE) releases newly-assembled lipoproteins from the inner membrane (Yakushi, Masuda et al. 2000), allowing them to interact with LolA, a periplasmic chaperone specific for lipoproteins (Matsuyama, Tajima et al. 1995; Tajima, Yokota et al. 1998; Miyamoto, Matsuyama et al. 2001). The lipoprotein is then transferred from LolA to LolB, a lipoprotein anchored to the outer membrane (Matsuyama, Yokota et al. 1997; Tanaka, Matsuyama et al. 2001). LolB facilitates incorporation of lipoproteins into the outer membrane by an unknown mechanism. The lipoproteins associated with the inner membrane usually have an Asp residue immediately following the modified Cys_{+1} (Yamaguchi, Yu et al. 1988), and an Asp, Glu, Gln or Asn at position +3 (reviewed in: Narita, Matsuyama et al. 2004). These amino acids prevent a lipoprotein that is to be retained in the inner membrane from interacting with LolCDE ("lol avoidance") (Yamaguchi, Yu et al. 1988; Matsuyama, Tajima et al. 1995; Yakushi, Masuda et al. 2000).

The *E. coli* chromosome is predicted to encode approximately 100 lipoproteins, about 90 of which have been experimentally confirmed (Miyadai, Tanaka-Masuda et al. 2004). However, few of these have been extensively characterized. Lpp, the most abundant lipoprotein in *E. coli*, anchors the OM to the peptidoglycan (reviewed in: Nikaido 1994; Wu 1996). *lpp* mutants exhibit decreased envelope integrity, as indicated by leakage of periplasmic proteins and by hypersensitivity to both EDTA and low osmotic strength media (Yem and Wu 1977; Yem and Wu 1978). The OM lipoprotein Pal also functions in a very

important, yet poorly understood process that helps to maintain the barrier function of the OM. Like Lpp, Pal anchors the OM to the peptidoglycan. However, the functions of Pal require the PMF of the IM in a manner that is similar to that of the TonB transport systems (Lloubes, Cascales et al. 2001). Mutations in *pal* result in membrane alterations similar to those observed in *lpp* mutants (Bernadac, Gavioli et al. 1998). Other lipoproteins, such as PulS and PrsA, have been shown to assist in the secretion of proteins across the outer membrane and in the release of bacteriocins (Nikaido 1996). Three inner membrane localized lipoproteins, AcrA (Ma, Cook et al. 1993), AcrE (Ma, Cook et al. 1993) and EmrA (Lomovskaya and Lewis 1992; Lomovskaya, Lewis et al. 1995; Tegos, Stermitz et al. 2002), function together with an RND (metal resistance, nodulation, cell division) permease to form an efflux pump that transfers its substrates across the IM and the OM to remove them from the *E. coli* cell (Dinh, Paulsen et al. 1994). Several lipoproteins, including NlpE (Raivio and Silhavy 2001) and YafY (Miyadai, Tanaka-Masuda et al. 2004), are potent inducers of the CpxAR two-component system, which, like σ^E, also monitors envelope integrity (reviewed in: Raivio and Silhavy 2001). To date, LolB is the only essential lipoprotein to have been identified (Tanaka, Matsuyama et al. 2001; Narita, Tanaka et al. 2002). Given the importance of the σ^E regulon in maintaining envelope integrity, we thought it probable that the six σ^E–transcribed lipoproteins might play important roles in this process. The phenotypes associated with deletion of each of these lipoproteins are the subject of this report.

Experimental Procedures

Media

Luria-Bertani broth (LB), LB agar, M9 minimal medium, M9 agar, and TSB were prepared as described in (Miller 1972). M9 minimal medium and M9 agar were supplemented with 0.2% glucose, 1 mM MgSO4, vitamins and all amino acids (40 μg/ml). These media are referred to as M9 complete medium or M9 complete agar. When necessary, media were supplemented with 100 μg/ml ampicillin, 10μg/ml tetracycline, 30 μg/ml kanamycin, or 20 μg/ml chloramphenicol.

Bacterial Strains

The bacterial strains and plasmids used in this study are listed in Table 2-4, and their construction is described briefly below: The chromosomal disruptions of the *yeaY*, *yfgL*, *nlpB*, *yfeY*, and *yraP* ORFs were generated according to the procedure described in (Datsenko and Wanner 2000) as follows: PCR products were generated by using several pairs of 56- to 70-nt-long primers that included 36- to 50-nt homology extensions to the gene of interest and 20-nt priming sequences for pKD3 or pKD4 as template. The 1.1 kb PCR products were purified, treated with *Dpn*I, and then transformed into CAG 49001. Strain CAG 49001 was grown in 500 ml SOB supplemented with 100 μg/ml ampicillin and 0.2% L-arabinose at 30°C to an OD600 of 0.6 and then made electrocompetent by

concentrating 100-fold and washing three times with ice-cold 10% glycerol. PCR products were gel-purified, digested with *Dpn*I, repurified, and suspended in elution buffer (10 mM Tris, pH 8.0). 50 ul of cells and 10–100 ng of PCR product were used in each electroporation. Electroporations were performed with BioRad *E. coli* Pulser according to the manufacturer's instructions. 1 ml SOC was added to shocked cells, which were then incubated 1 h at 37°C. The cells were then spread onto LB agar supplemented with 20 μg/ml chloramphenicol to select Cm^R transformants, or with 30 μg/ml kanamycin to select Km^R transformants. After primary selection, mutants were maintained on medium without an antibiotic. They were colony purified once non-selectively at 37°C and then tested for ampicillin sensitivity to test for loss of the pKD46 plasmid. If it was not lost, then a few isolates of each mutant were colony-purified once at 43°C and similarly tested. Each knockout was confirmed via PCR.

pCP20 is an Amp^R and Cm^R plasmid that shows temperature-sensitive replication and thermal induction of FLP synthesis. Cm^R and Km^R mutants were transformed with pCP20, and Amp^R transformants were selected at 30°C, after which a few were colony purified once non-selectively at 43°C. The transformants were then tested for loss of all antibiotic resistances. The majority of the mutants lost the FRT-flanked Cm^R gene and the FLP helper plasmid simultaneously.

To generate a strain carrying a targeted disruption of *yfiO* linked to the cotransducible marker *pheA18::Tn10*, strain KM44 was transduced with a P1 lysate grown on CAG12158 to generate CAG41264. A second copy of *yfiO* was inserted into the chromosomal *lacZ* gene of CAG41264 via the targeted insertion

of a 2.26 kb PCR fragment encoding the promoter Ptrc, *yfiO*, and β-lactamase with its endogenous promoter. This PCR fragment was amplified from pCO110 using primers

5'ttatttttgacaccagaccaactggtaatggtagcgaccggcgctcagctCTGACAGTTACCAATGC TTAATCA3'

and

5'atgaccatgattacggattcactggccgtcgttttacaacgtcgtgactgTGAGCTGTTGACAATTA ATCATCCGG3', each of which contain 40 bp of homology to *lacZ* at their 5' ends.

The targeted disruption of *lacZ* was performed using methods described in (Murphy 1998), and transformants were selected on LB plates containing 30μg/ml ampicillin. The resultant strain CAG41439 was confirmed to carry 2 WT alleles of *yfiO* via PCR performed with primers of sequences flanking each gene. The endogenous chromosomal copy of *yfiO* in CAG41439 was then disrupted, using methods described in (Murphy 1998) by a 1.1 kb PCR product amplified from pKD3 using primers

5'aaacggcagctcaagggctgccgttttgtgtttcaggtttctgCATATGAATATCCTCCTTA3'

and

5'gagaatctcccgtattacattttgaggaaagtcaaaacgtcTGTGTAGGCTGGAGCTGCTTC3'.

Transformants were selected on LB plates containing 20 μg/ml chloramphenicol and 1mM IPTG, generating CAG 41356 The chromosomal disruption of endogenous *yfiO* in CAG 41356 was confirmed via PCR. Lysates were prepared

from CAG 41356 and used in linked marker cotransduction experiments to determine whether *yfiO* is essential.

Motif Search

The lipobox motifs at the N-termini of yeaY, yfgL, yfiO, yfeY and yraP polypeptides were identified using the program Motif included in version 10.3 of the Genetics Computer Group, Inc. (GCG) sequence analysis software package.

Linked Marker Cotransductions

The *yfiO::Cm* allele was linked to *pheA18::Tn10* from strain CAG12158 as described above. These two genes are 95% linked. CAG45114 and CAG41351 were infected with P1 lysate grown on CAG41356 using a standard P1 transduction protocol. Tetracycline resistant transductants were selected at 30°C on LB agar plates + 10 μg/ml tetracycline, 10mM sodium citrate. After approximately 16 hours, tetracycline resistant transductants were streak-purified and then screened for chloramphenicol resistance at 30°C on LB agar plates supplemented 20 μg/ml chloramphenicol. The frequency of cotransduction of *yfiO::Cm* with *pheA18::Tn10* was calculated by dividing the number of *yfiO::Cm pheA18::Tn10* transductants by the total number of *pheA18::Tn10* transductants. This experiment was repeated twice and yielded similar results. The linked marker cotransduction assays into CAG45114 and CAG 41351 using P1(*rpoE::Cm*

nadB::Tn10) were performed as described above. P1(*rpoE::Cm nadB::Tn10*) was prepared from CAG 37125.

Phenotype Assays

To identify the phenotypes of the Δ*yeaY*, Δ*yfgL*, Δ*nlpB*, Δ*yfeY*, or Δ*yraP* strains, efficiencies of plating (EOP) were performed as follows: Overnight cultures of strains of CAG 41241, CAG 41434, CAG41254, CAG41408, CAG41245, the *surA::Cm* strain 41265, and WT strain CAG45114 were grown in LB at 30°C. The cultures were serially diluted 10-fold to 10^{-7}. One hundred microliters of each dilution of each culture were spread on LB agar and LB agar plates supplemented with one of the following agents: 1.5 μg/ml crystal violet, 0.15 μg/ml polymixin-B, 3.5% SDS, 1mM EDTA, 3.5%SDS + 1mM EDTA, 150 μg/ml novobiocin, 5 μg/ml rifampicin, and 0.1% phenethyl alcohol. The EOPs were calculated by dividing the number of colony-forming units (cfus) of a given mutant on supplemented LB plates by the number of cfus of that same mutant on LB plates. Where phenotypes were observed, EOPs for each phenotype for each strain were performed three times with three independent isolates.

To determine whether the phenotypes of the Δ*nlpB*, Δ*yraP*, and Δ*yfgL* strains could be complemented, the EOP experiments were repeated as described above using strains CAG 41350 and CAG 41517, which carry pCO109; CAG 41534 and CAG 41536, which carry pCO122; CAG 41511 and CAG 41513,

which carry pCO123; and CAG 41510, CAG41512, CAG41516, and CAG 41535, which carry pBA169.

Sensitivity to growth on LB agar at 18°, 30°, 37°, and 42°C and on M9 complete agar at 18°, 30°, 37°, and 42°C were performed by diluting overnight cultures of strains CAG 41241, CAG 41434, CAG41254, CAG41408, CAG41245, the *surA::Cm* strain 41265, and WT strain CAG45114 10-fold to 10^{-5}, and titering 10μl of each dilution of each strain on LB agar and M9 complete agar plates. Plates were placed at 18°C for 72h or at 30°, 37° or 42°C for 16h. The mutant strains' growth under these conditions was compared to WT, and no differences were observed. The sensitivity of these strains to growth on MacConkey agar at 30°C was also tested in this manner. Again, no differences in between WT and the mutants were observed.

Sensitivities to 1M each of CuCl2, NiCl2, CrCl2, CoCl2, FeCl2 and CdCl2, and DTT were tested as well. The solutions listed above were spotted onto sterile Whatman paper discs that were allowed to dry before being placed onto lawns of CAG 41241, CAG 41434, CAG41254, CAG41408, and CAG41245 on LB. Zones of clearing on mutant strains' lawns were compared to that of WT strain CAG45114, and no differences were observed.

Motility of the null mutants was also tested. Overnight cultures strains were grown in LB at 30°C. The next day, the cultures were diluted 1:10 in LB, the OD600 of each culture was recorded, and the number of cells/μl of each culture was calculated. A volume of each culture equivalent to 10^6 cells was then

inoculated into the centers of LB soft agar plates (4 mg/ml agar in LB broth). After 24 h of incubation at 30°C and 37°C, the diameter of the swarm was measured and compared to WT. No differences were observed.

Sensitivity of the mutants to osmotic shock was tested in the following manner: Mutant strains were grown at 30°C in low osmolarity medium, described in (Lacroix, Loubens et al. 1991) and in low osmolarity medium supplemented with 0.1M NaCl, 0.2M NaCl, 0.3M NaCl, and 0.4M NaCl. The OD600 for each culture was measured every 60 minutes for 600 minutes. The slopes of $\log_{10}$OD600 vs. time plots for each strain grown in each medium were compared to that of WT to determine whether NaCl concentration affected the mutants' growth rates. No differences were found.

σ^E activity assay

σ^E activity was assayed by monitoring β–galactosidase activity from a chromosomal σ^E-dependent lacZ reporter gene Φ λ[rpoHP3-lacZ] as described previously (Ades, Connolly et al. 1999). Cells to be assayed were grown at 30°C in M9 complete medium (100μg/ml ampicillin and 1mM IPTG were added when necessary), and single point determinations were made at the indicated optical densities. The rate of β-galactosidase synthesis from the σ^E-dependent reporter gene increases as the cells enter mid-log phase when grown in LB (J. Mecsas and C.A. Gross, unpublished). Due to this growth phase effect, differences in σ^E

activity were determined by comparing the slopes of the initial linear portion of the plots of β-galactosidase activity/0.5 ml cells versus OD450. Slopes were determined by linear regression analysis. The value of each slope was normalized to the WT value, taken as 1.0

Preparation and Analysis of Outer Membrane Proteins

Outer membrane proteins were prepared from strains grown in LB broth at 37°C (100μg/ml ampicillin and 1mM IPTG were added when necessary). Cultures were grown to an OD600 of 0.5-1.0, whereupon10 mls of cells were harvested by centrifugation at 5000 rpm for 10 minutes. The OD600 of each culture was recorded at time of harvest. Pellets were resuspended in 100mM Tris-HCl pH=8, 20% glucose and incubated on ice for 10 minutes. The cells were spun again at 5000 rpm for 10 minutes, and pellets were resuspended in 100mM Tris-HCl pH=8, 20% glucose supplemented with 10mM EDTA. Cell walls were digested with lysozyme (10 μg/ml) on ice for 30 minutes. Each sample was adjusted to 10mM MgCl2 and was then lysed by the addition of 1 ml of ice-cold water followed by three freeze-thaw cycles. 5μg/ml each of DNAse I and RNAse A were added to each sample, and the cell extracts were incubated on ice until they became completely fluid, indicating that the nucleic acids in the extracts had been digested. The extracts were then centrifuged for 15 minutes at 15,000 rpm, and the pellets were washed with 20mM NaPO4,m pH 7.0. Cytoplasmic membranes were solubilized with 1 ml of 0.5% sarcosyl in 20mM NaPO4 at room

temperature for 30 minutes. The insoluble outer membranes were pelleted by centrifugation at 15,000 rpm for 15 minutes, washed twice with 1ml sarcosyl solution, and resuspended in 40 μl of SDS sample buffer (100mM Tris-HCl, 200mM DTT, 4% SDS, 0.2% bromophenol blue, 20% glycerol). Cells/μl for each sample were calculated from the OD600 recorded at harvest. A volume of each sample equivalent to 10^9 cells was loaded and resolved on a 12% polyacrylamide SDS gel containing 50% w/v urea. (The addition of urea is necessary to resolve OmpC and OmpF.)

Assay of Susceptibility to BPI-derived Peptide P2

Peptide P2 as described by (Barker HC 2000) containing residues 86-104 of BPI flanked by cysteines (SKISGKWKAQKRFLKMSGNFGC) was synthesized by the Macromolecular Resources Facility at Colorado State University (Fort Collins, CO). Size and purity of the peptide were confirmed by MALDI-Mass spectroscopy and analytical HPLC. The method of (Barker HC 2000) was modified to determine the P2 susceptibility of strains CAG 45114, CAG 41241, CAG 41434, CAG41254, CAG41408, and CAG41245. Bacteria were cultured to stationary phase without agitation in 5ml tryptic soy broth (TSB), washed twice in 0.9% NaCl and diluted to the desired cfu ml-1 in 50ml of 20mM sodium

phosphate buffer pH=6.0. Samples were incubated with 4mg ml-1 P2 peptide for 90min at 37°C without agitation. Aliquots (20 ml) were serially diluted, plated on LB agar plates and allowed to grow for 18h at 37°C for enumeration of surviving cfu.

Results

Identification of lipoproteins in the σ^E regulon

A computational motif search of the 96 members of the σ^E regulon revealed that the amino acid sequences of six of these genes encode lipobox motifs embedded in signal sequences (Figure 2-1). The proteins encoded by these genes have each been confirmed to carry lipid modifications (Miyadai, Tanaka-Masuda et al. 2004). Because these lipoproteins lack an Asp at position +2, they are all likely to interact with the lol pathway and to be localized to the outer membrane. To determine whether any of these lipoproteins is essential, we generated chromosomal deletions of the genes encoding each lipoprotein using the methods described in (Datsenko and Wanner 2000). We were able to delete *yfgL*, *yraP*, *yeaY*, *nlpB* and *yfeY*, indicating that these genes were not essential. The results for *nlpB* and *yfgL* validate previous reports wherein null alleles of these genes were generated via insertional mutagenesis (Bouvier, Pugsley et al. 1991; Eggert, Ruiz et al. 2001). We considered the possibility that these five genes were not essential because their encoded lipoproteins perform redundant functions. Were this so, a quintuple knock out strain would be inviable. We constructed such a strain using methods described in (Datsenko and Wanner 2000) and found this strain to be viable at temperatures ranging from 18°C to 42°C in both standard LB medium and M9 medium supplemented with all the amino acids. Thus, if these lipoproteins indeed do perform the same function, there must exist additional

cellular proteins that can compensate for this function in their absence. Because YraP and OsmY exhibit 29% identity and 50% similarity, we postulated that these two genes perform redundant functions; however, a strain carrying both the Δ*yraP* and Δ*osmY* null alleles is still viable. We concluded that the five nonessential lipoproteins as a group do not perform an essential function in *E. coli.*

Because we were unable to delete *yfiO* using the strategy by which we constructed the other null mutants, we considered that *yfiO* might be essential. To test this possibility, we first constructed a null allele of *yfiO* in a strain carrying a second copy of this gene inserted in the *lac* operon (see Materials and Methods). We then performed linked marker cotransduction to assess whether *yfiO* was essential. Whereas Δ*yfiO::Cm* was >95% cotransducible with *pheA18:: Tn10* in a WT strain carrying a plasmid-encoded copy of *yfiO* (Table 2-1A, line 1), Δ*yfiO::Cm* could not be cotransduced with *phe18:: Tn10* into the WT strain alone, where successful cotransduction would result in lack of YfiO (Table 2-1A, line 2). Due to the close linkage of Δ*yfiO::Cm* and *pheA18:: Tn10*, we obtained only very few Tet^R transductants in the WT strain, even though both recipient strains are equally transducible by *pheA18:: Tn10* when it is not linked to Δ*yfiO::Cm* (Table 2-1B). We conclude that *yfiO* is essential.

The essentiality of *yfiO* raised the possibility that σ^E is essential solely because it provides YfiO. We tested this idea by determining whether *rpoE::Cm* could be transduced into WT cells carrying a plasmid encoding *yfiO* transcribed from a P_{trc} promoter. Linked marker cotransduction experiments demonstrated

that such cells were not able to accept the *rpoE::Cm* marker (Table 2-1B) even when P_{trc} expression of YfiO was induced by addition of IPTG. Additional controls were performed to ensure that the P1(*rpoE::Cm nadB::Tn10*) lysate used in these experiments was of a sufficient titer to generate transductants (data not shown). Thus, the σ^E regulon must provide two or more functions that are essential for cell viability.

Phenotypes of strains deleted for non-essential lipoproteins

We assessed whether any of the individual nonessential lipoprotein deletion mutants altered the growth properties of cells under a variety of conditions (see Materials and Methods for a detailed description of all conditions tested). We first tested whether we could identify a nonpermissive growth temperature, as this would enable us to examine the function of each lipoprotein using depletion strains. However, all strains were able to grow at temperatures ranging from 18°C to 42°C both in standard LB medium and in minimal M9 medium supplemented with all of the amino acids, indicating that none of these lipoproteins was essential for growth at temperature extremes. Because the σ^E regulon is induced under hypo-osmotic conditions (data not shown), we next tested whether the mutants were altered in their osmotic sensitivity. This phenotype is also general indicator of envelope integrity (reviewed in: Nikaido 1994; Wu 1996). However, all strains grew equally well in hypo-osmotic medium (described in: Lacroix, Loubens et al. 1991) alone as in hypo-osmotic medium supplemented with 0.1M, 0.2M, 0.3M or

0.4M NaCl (data not shown). All deletion strains were also unimpaired in their sensitivity to a variety of metals and in their motility. Finally, given that the σ^E regulon lipoproteins are all targeted to the outer membrane, we tested the lipoprotein deletion mutants for their sensitivity to a variety of agents that are normally excluded by a WT outer membrane. These agents included amphipathic antibiotics (rifampicin, novobiocin), detergents (SDS, bile salts), hydrophobic dyes (crystal violet) and EDTA, which destabilizes the outer membrane by chelating divalent cations bound to LPS (reviewed in: Nikaido 1996). Three of the mutants exhibited EOP phenotypes (Table 2-2A), each of which could be reversed by supplying the missing lipoprotein on a plasmid (Table 2-2B). Δ*yfgL* exhibited the broadest phenotypes: Plating was eliminated on rifampicin, and although cells grew on SDS and novobiocin, colony growth was dramatically slowed. This phenotype was reversed in Δ*yfgL* strains carrying pyfgL (data not shown). In contrast, Δ*nlpB* and Δ*yraP* exhibited highly specific phenotypes, with the former exhibiting sensitivity only to rifampicin and the latter exhibiting sensitivity only to SDS. In contrast, a *surA::Cm* mutant strain, which interferes with OMP assembly and destabilizes the outer membrane, exhibits general sensitivity to hydrophobic dyes, hydrophobic amphipathic antibiotics and detergents (Rouvière 1996) (Table 2-2A).

Effect of lipoprotein deletion on σ^E activity

The activity of σ^E is a sensitive indicator of the integrity of the outer membrane (reviewed in: Alba and Gross 2004). The assembly status of β-barrel porins is sensed directly by the signal transduction pathway controlling σ^E activity (Walsh, Alba et al. 2003). Additionally, this pathway indirectly senses the status of LPS assembly and protein folding (Alba and Gross 2004). Because several of the lipoprotein deletion strains exhibit sensitivities to agents normally excluded by the outer membrane, we thought it probable that these mutants would also exhibit increased σ^E activity (Figure 2-2). Δ*yfgL*, which is sensitive to the broadest range of agents tested in Table 2-1, exhibited a 10-fold increase in σ^E activity, which is only slightly less than the induction mediated by the *surA::Cm* mutant, a potent inducer (Rouvière 1996). Δ*yraP* also exhibited slight induction of the response. The induction phenotype of the Δ*yfgL* mutant was relieved by expression of YfgL from a plasmid (Figure 2-2).

Many mutations that increase σ^E activity also affect OMP profiles. For example, the levels porins OmpF, OmpC, OmpA and LamB are significantly reduced in the *surA::Cm* mutant, in which σ^E activity is highly induced (Rouvière 1996). We determined the OmpF, OmpC, OmpA, and LamB levels in outer membrane preparations purified from each lipoprotein deletion mutant, using the *surA::Cm* mutant and WT MG1655 as a basis for comparison. Δ*yfgL* strains were deficient in the content of the four porins tested, exhibiting almost as severe a

phenotype as *surA::Cm* (Figure 2-3A, 2-3B). This phenotype was complemented when YfgL was expressed from a plasmid (Figure 2-3C). No other lipoprotein deletion mutant showed any OMP profile defects.

Do these lipoproteins contribute to the protein folding capacity of the envelope?

A number of periplasmic chaperones and periplasmic peptidy prolyl isomerases (PPIases) facilitate the folding, assembly, and insertion of trimeric porins into the outer membrane. SurA has both a chaperone and PPIase activity and is required for the proper assembly of porins (Rouvière 1995; Behrens, Maier et al. 2001); DegP is predominantly a chaperone at low temperatures and has both chaperone and protease activity at elevated temperatures (Spiess, Beil et al. 1999). Skp also exhibits chaperone activity and has been found to bind to outer membrane porin precursors (Chen and Henning 1996; Bothmann and Pluckthun 1998; De Cock, Schafer et al. 1999). Deletion of the gene encoding any one of these three folding agents is not lethal. However Δ*skp surA::Cm* and *degP::Km surA::Cm* double mutants are synthetically lethal whereas the Δ*skp degP::Km* double mutant is not (Rizzitello, Harper et al. 2001). This finding led the Silhavy group to propose the existence of two parallel periplasmic folding pathways: One involving SurA and the other involving Skp and DegP (Rizzitello, Harper et al. 2001). We have expanded this study to include our lipoprotein deletion strains, as several confer envelope phenotypes. We have also included FkpA, a periplasmic

PPIase and chaperone in this analysis, because mutations in *fkpA* exhibit phenotypes similar to those reported for *surA* (Missiakas, Betton et al. 1996).

Our studies were performed in MG1655, rather than in the MC4100 strain utilized by Rizzitello et al (Rizzitello, Harper et al. 2001). Our first experiments were to determine whether it is possible to observe the previously reported double mutant phenotypes in MG1655. We found that although the synthetic phenotypes were maintained, they were less severe in MG1655 than MC4100 (Table 2-3A). The Δ*skp surA::Cm* and *degP::Km surA::Cm* double mutants were distinctly impaired as they formed very small colonies that could be visualized only after 48 hours at 30°C. We refer to this phenotype as "very small" (vs). In contrast, the single mutant strains formed colonies after about 18 hours at 30°C. Moreover, we found that Δ*fkpA* is not synthetically lethal with Δ*skp*, *degP::Km* or *surA::Cm*.

Two of the lipoprotein deletion strains, Δ*nlpB* and Δ*yraP*, exhibited a synthetic phenotype with *surA::Cm*, forming vs colonies on LB after 48 hours but not with any of the other three chaperones (Table 2-3B). Thus, Δ*nlpB* and Δ*yraP* exhibit genetic interactions very similar to those shown for Δ*skp* and *degP::Km*. The simplest interpretation of these results is that the NlpB and YraP lipoproteins are part of the DegP/Skp folding pathway. A third lipoprotein deletion strain, Δ*yfgL*, exhibited a novel synthetic phenotype: When a Δ*yfgL* strain was transduced with either P1(*surA::Cm*) or P1(Δ*fkpA::Cm*), the resulting double mutants formed vs colonies. These phenotypes would be explained if YfgL functioned in more than one periplasmic protein folding pathway.

The BPI phenotype of lipoprotein deletion mutants

The human bactericidal/permeability inducing (BPI) protein has potent antimicrobial activity against gram negative bacteria (reviewed in: Weiss 2003). The P2 peptide derived from BPI retains much of the anitmicrobial properties of BPI itself. BPI and P2 increase outer membrane permeability and decrease cellular O_2 consumption (Weiss 2003) The cytotoxic mechanism, though not well understood, may result from membrane rupture. We tested whether the lipoprotein deletion mutants survived more poorly than WT strains in the presence of the P2 peptide. We find that the Δ*yfgL* strain is significantly more sensitive to P2 than WT cells (Figure 2-4). In addition, Δ*yraP* is marginally more sensitive than WT cells although the difference may not be statistically significant. We cannot distinguish whether the increased sensitivity of Δ*yfgL* cells to P2 is simply a consequence of envelope defects or whether it results instead from loss of a specific mechanism conferring resistance to the effects of the peptide. We favor the former idea because these cells have multiple alterations in their envelope.

Discussion

The cellular roles of most lipoproteins are very poorly understood. The *E. coli* genome is predicted to encode approximately 100 lipoproteins, making it a daunting task to characterize this entire group. In addition, very few previously characterized lipoproteins proved to have significant phenotypes. Because σ^E is essential for viability and plays a major role in envelope homeostasis, we hoped that choosing to study lipoproteins encoded in the σ^E regulon would enrich for those with roles in the process. Indeed, four of the six lipoproteins in the regulon confer distinct envelope phenotypes when they are absent from the cell.

LPS rough mutants, and other mutations that alter the porin content of the outer membrane, are known to destabilize the cell envelope and increase its permeability to a wide variety of detergents, dyes, and antibiotics (Nikaido 1996). Both NlpB and YraP appear to be involved in maintaining envelope integrity, as well; deleting either gene disrupts the barrier function of the outer membrane. However, each mutant strain is affected in its permeability only to a specific class of molecules. Cells lacking YraP become selectively sensitive to SDS but not to rifampicin, whereas those lacking NlpB are sensitive to rifampicin but exhibit unaltered sensitivity to SDS. Such specific sensitivity has not been previously reported. The NlpB and YraP lipoproteins may be involved in the function of highly specific efflux pumps, although pumps with such specificity have not previously been identified. Alternatively, these lipoproteins may be required for processes that impart very subtle alterations to the outer membrane. In turn, these

alterations are what might confer the selective permeability we observe. This latter possibility would be consistent with the fact that each selectively permeable mutant also exhibits a synthetic phenotype with *surA::Cm*. These data, together with the previous observations by Rizzitello et al (Rizzitello, Harper et al. 2001), lead us to posit that both NlpB and YraP work in the DegP/Skp pathway to facilitate assembly of porins and other outer membrane proteins.

Cells lacking YfgL exhibit a number of different phenotypes, all of which indicate a disturbance in the barrier function of the cell envelope. Δ*yfgL* cells are modestly pleiotropic in their sensitivities to agents normally excluded by the outer membrane. Additionally, they exhibit a large increase in σ^E activity, significant sensitivity to BPI, and synthetic phenotypes not only with *surA::Cm* but also with Δ*fkpA::Cm*. Eggert et al (Eggert, Ruiz et al. 2001) have found that Δ*yfgL* strains are resistant to killing by glycolipid derivatives of vancomycin, which block the transglycosylation step of peptidoglycan synthesis. They proposed that inhibiting this step of peptidoglycan synthesis might normally activate lytic transglycosylases (autolysins) and suggest that Δ*yfgL* cells are resistant to carbohydrate-modified vancomycin compounds because YfgL might be required for activation of these potentially suicidal eneymes (Eggert, Ruiz et al. 2001). In this context, it is interesting to find that three lytic transglycosylases, MltA, MltB, and MltC, are also lipoproteins that reside in the outer membrane of the cell envelope (Engel, Smink et al. 1992; Ursinus and Holtje 1994; Ehlert, Holtje et al. 1995; Dijkstra and Keck 1996), However, given the broad range of phenotypes

exhibited by Δ*yfgL* cells, we favor the idea that resistance to glycolipid derivatives of vancomycin is yet another indication of the extensive changes in the outer membrane of such cells. Understanding the molecular basis of these phenotypes will undoubtedly provide insight into a fundamental process in the envelope.

Finally, we have demonstrated that YfiO is an essential lipoprotein. Unfortunately, the YfiO depletion strains that have been constructed thus far remain viable under the conditions tested. This result may suggest that cells need very little of this particular lipoprotein for survival. Our future efforts will be directed towards constructing a YfiO depletion strain in which depletion leads to cell death. Such a strain would be an invaluable tool in allowing us to gain greater insight into identifying the essential function(s) of YfiO.

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Figure Legends, Chapter Two

Figure 2-1: The Lipoboxes of the six σ^E-transcribed lipoproteins. Displayed are the N-terminal sequences of each lipoproteinin the σ^E regulon. The signal sequence is shown in small italic type, and the lipobox motif is depicted in large bold type. The conserved cysteine residue in each lipobox, which is destined for lipid modification, is shaded.

Table 2-1A: P1 transduction experiments demonstrate that yfiO is essential.

Table 2-1B: P1 transduction experiments demonstrate that providing YfiO is not sufficient to relieve σ^E essentiality.

Table 2-2A: Efficiency of plating (EOP) of various deletion mutant strains in the presence vs. absence of selected agents. "*" indicates that colonies were not apparent until 48 hr at 30°C. For comparison, all other strains formed colonies after 18 hr at 30°C.

Table 2-2B: The EOP phenotypes observed in the ΔyfgL, ΔnlpB, and ΔyraP mutants can be complemented by the presence of pyfgL, pnlpB, and pyraP, respectively, The complementing plasmids are derivatives of pTrc99A wherein YfgL, NlpB, or YraP are expressed from a promoter that is not fully repressed in the absence of inducer (IPTG). As a result of this phenomenon, partial complementation of the *ΔyfgL* and *ΔyraP* phenotypes are observed in strains carrying pyfgL and pyraP, respectively, in the absence of IPTG. The phenotypes are not complemented when *ΔyfgL, ΔnlpB,* and *ΔyraP mutants* that carry only the vector. *ΔyfgL* strain on SDS and novobiocin were also complemented, "NT" indicates that a phenotype was not tested.

Figure 2-2: σ^E activity of strains in which each of the non-essential lipoproteins has been deleted Relative σ^E activity in WT (CAG 45114), *ΔyeaY* (CAG 41241), *ΔyraP* (CAG 41251), *ΔnlpB* (CAG 41258), *ΔyfeY* (CAG 41413), *ΔyfgL* (CAG 41445), and *surA::Cm* (CAG 41265) strains growing at 30° in LB was assayed by monitoring β-galactosidase activity from a single copy ΦΛ[rpoHP3-lacZ] fusion as described in Materials and Methods. The increased σ^E activity observed in the *ΔyfgL* mutant can be complemented by pyfgL.

Figure 2-3: The OMP profiles of wild type, ΔyfgL, and ΔsurA::Cm strains. A) Outer membrane fractions were prepared from cells that have been growing exponentially at 37°C in LB + 0.2% maltose, as described in Materials and Methods. The fractions were analyzed on a 12% SDS-polyacrylamide gel with 50% urea and stained with Coomassie. The positions of LamB, OmpC, OmpF and OmpA are indicated. B) The levels of LamB, OmpC, OmpF, and OmpA were quantified via spot densitometry as described in Materials and Methods. C) The OMP profile defect in the *ΔyfgL* mutant can be complemented by pyfgL

Table 2-3A: The synthetic pheonotypes of strains lacking various periplasmic chaperones. "vs" indicates that colonies appeared after 48 hr on LB at 30°; "+"indicates that the phenotype of the double mutant was the same as that of the single mutant; "nt" indicates not tested.

Table 2-3B: ΔnlpB, ΔyraP and ΔyfgL exhibit synthetic phenotypes with periplasmic chaperones. "vs" indicates that colonies appeared after 48 hr on LB at 30°. For comparison, the single mutant strains formed colonies after 18 hr at 30°C. "+" indicates that the phenotype of the double mutant was the same as that of the single mutant.

Figure 2-4: Effects of deleting non-essential lipoprotein genes on sensitivity to BPI derived P2 peptide. Stationary phase bacteria grown without agitation in 5ml TSB were diluted to ~2 x 10^7cfu ml-1 in 20mM sodium phosphate buffer pH=6.0 in a final volume of 50ml. Samples were incubated with 4mg ml-1 for 90min at 37°C. Data represent the mean colony forming units per ml from serial dilutions of triplicate samples plated on LB agar and incubated for 18h at 37°C.

Table 2-4: Strains and plasmids used in the experiments described in Chapter Two

Figure 2-1: The Lipoboxes of the six σ^E-transcribed lipoproteins

yfgL	(1)	*MQLRKLLLPGLLSVT***LLSG**C	(20)
yraP	(1)	*MKALSPIAVLISAL***LLQG**C	(19)
yfiO	(1)	*MTRMKYLVAAATLSL***FLAG**C	(20)
yeaY	(1)	*MAVQKNVIKGILAGTFAL***MLSG**C	(23)
nlpB	(1)	*MAYSVQKSRLAKVAGVSLVL***LLAA**C	(25)
yfeY	(1)	*MKSLRLMLCAMPL***MLTG**C	(18)

Table 2-1A: P1 transduction experiments demonstrate that *yfiO* is essential.

	Donor P1(Δ*yfiO*::Cm *pheA18*::Tn10)			Donor P1(*pheA18*::Tn10)
Recipient	**Number of Tet^R transductants**	**Number of Tet^R Cm^R cotransductants**	**Linkage**	**Number of Tet^R transductants**
MG1655	**7**	**0**	**0%**	**>100**
MG1655 + pCO110 (p*yfiO*)	**100**	**97**	**97%**	**>100**

Table 2-1B: P1 transduction experiments demonstrate that providing YfiO is not sufficient to relieve σ^E essentiality.

	Donor P1(*rpoE*::Cm *nadB*::Tn10)			Donor P1(*nadB*::Tn10)
Recipient	**Number of Tet^R transductants**	**Number of Tet^R Cm^R cotransductants**	**Linkage**	**Number of Tet^R transductants**
MG1655	**5**	**0**	**0%**	**>100**
MG1655 + pCO110 (p*yfiO*)	**3**	**0**	**0%**	**>100**

Table 2-2A: Efficiency of plating (EOP) of various deletion mutant strains in the presence vs. absence of selected agents.

	EOP		
Strain	**3.5% SDS**	**5 ug/ml rifampicin**	**150 ug/ml novobiocin**
WT	**0.9 +/- 0.2**	**0.1 +/- 0.03**	**0.7 +/- 0.2**
ΔyfgL	**0.4 +/- 0.1***	**6×10^{-9} +/- 3×10^{-9}**	**0.3 +/- 0.1***
ΔnlpB	**0.8 +/- 0.1**	**4×10^{-5} +/- 1×10^{-5}**	**0.7 +/- 0.1**
ΔyraP	**4×10^{-7} +/- 2×10^{-7}**	**0.6 +/- 0.1**	**0.8 +/- 0.1**
surA::Cm	**2×10^{-6} +/- 1×10^{-6}**	**1×10^{-8} +/- 1×10^{-9}**	**6×10^{-5} +/- 4×10^{-5}**

Table 2-2B: The EOP defects observed in the *ΔyfgL*, *ΔnlpB*, and *ΔyraP* mutants can be complemented by the presence of pyfgL, pnlpB, and pyraP, respectively

	EOP			
Strain	**3.5% SDS**	**3.5% SDS + 1mM IPTG**	**5 μg/ml rif**	**5 μg/ml Rif + 1mM IPTG**
***ΔyfgL* + vector**			1.52×10^{-8} +/- 1.26×10^{-8}	4×10^{-8} +/- 3×10^{-8}
***ΔyfgL* + pyfgL**	NT	NT	2.5×10^{-2} +/- 4×10^{-3}	1.23 +/- 0.18
***ΔnlpB* + vector**	NT	NT	1.5×10^{-3} +/- 1×10^{-4}	8×10^{-4} +/- 3×10^{-4}
***ΔnlpB* + pnlpB**	NT	NT	3×10^{-3} +/- 3×10^{-4}	0.4 +/- 0.02
***ΔyraP* + vector**	1×10^{-7} +/- 1.3×10^{-8}	1×10^{-7} +/- 1.2×10^{-8}	NT	NT
***ΔyraP* + pyraP**	0.65 +/- 0.084	1.45 +/- 0.22	NT	NT

Figure 2-2: σ^E activity of strains in which each of the non-essential lipoproteins have been deleted

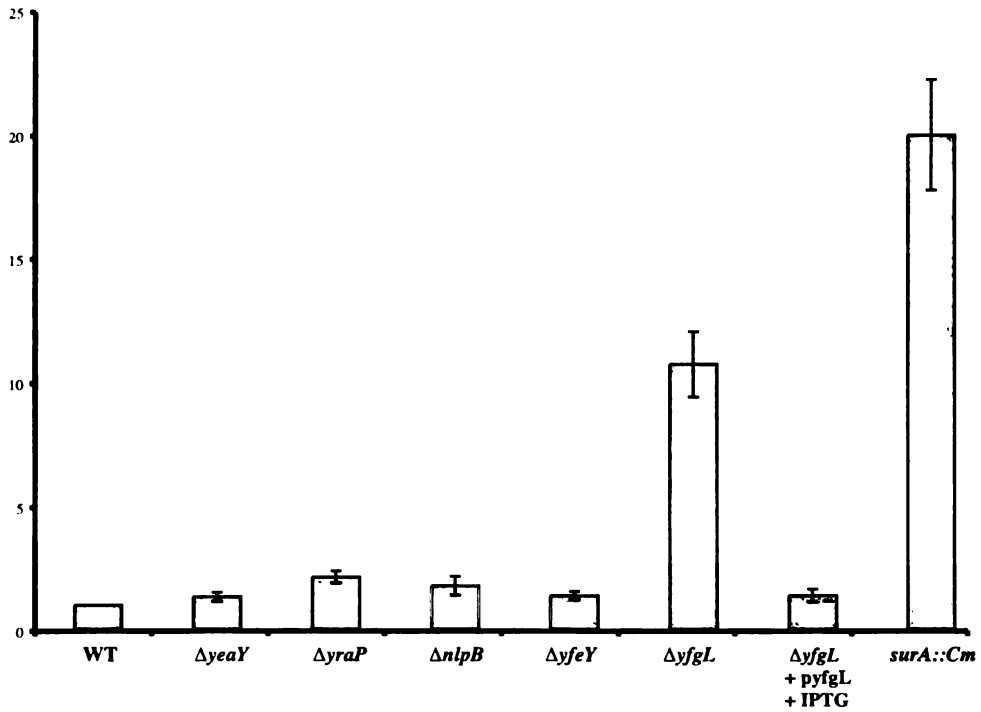

Figure 2-3A: The Omp profiles of WT, Δ*yfgL*, and Δ*surA::Cm* strains

Figure 2-3B: The levels of LamB, OmpC, OmpF, and OmpA were quantified via spot densitometry

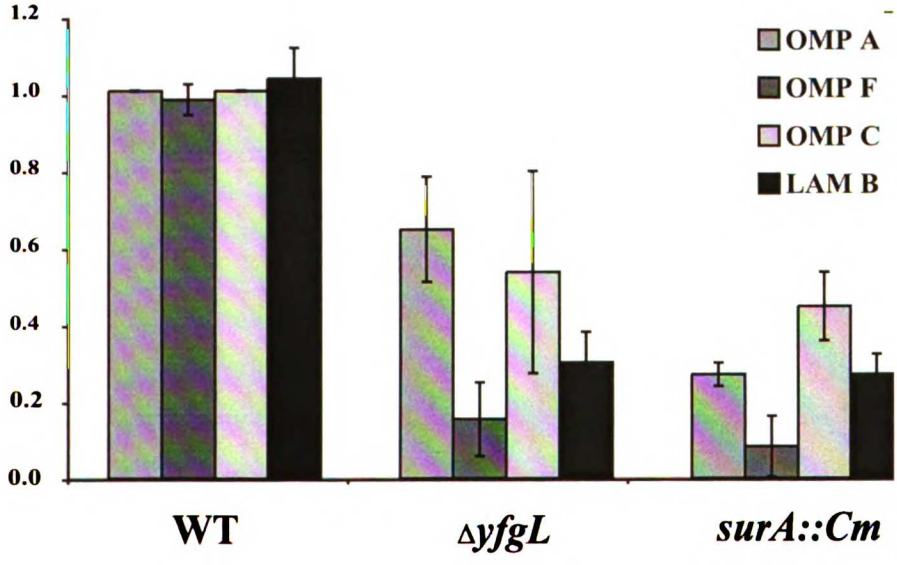

Figure 2-3C: The Omp profile defects observed in the Δ*yfgL* strain can be complemented by pyfgL

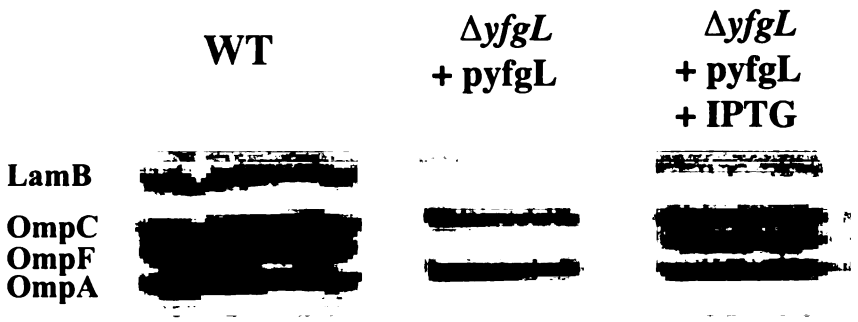

Figure 2-4: Effects of deleting non-essential lipoprotein genes on sensitivity to BPI-derived P2 peptide

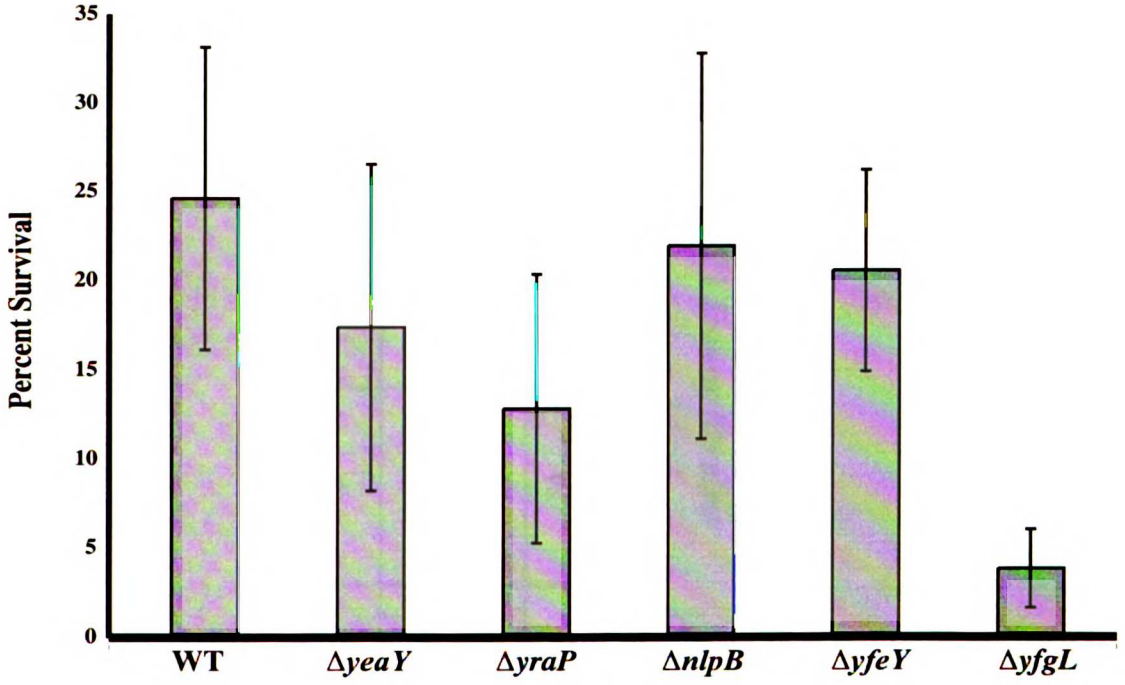

Table 2-3A: The synthetic pheonotypes of strains lacking various periplasmic chaperones

Transduction Recipients	P1 Donor Strains: *surA::Cm*	*degP::Km*	*Δskp, zae::Tn10*	*ΔfkpA::Cm*
surA::Cm	nt	vs	vs	nt
degP::Km	vs	nt	+	+
Δskp, zae::Tn10	vs	+	nt	+
ΔfkpA	+	+	+	nt

Table 2-3B: Δ*nlpB*, Δ*yraP* and Δ*yfgL* exhibit synthetic phenotypes with periplasmic chaperones

Transduction Recipients	P1 Donor Strains			
	surA::Cm	*degP::Km*	*Δskp, zae::Tn10*	*ΔfkpA::Cm*
ΔnlpB	vs	+	+	+
ΔyraP	vs	+	+	+
ΔyfgL	vs	+	+	vs

Table 2-4: Strains and plasmids used in the experiments described in Chapter 2

Strains	Relevant Genotype	Source or Reference
BW25141	*Δ(araD-araB)567* Δ*lacZ4787 lacIp-4000(lacI^q) Δ(phoB-phoR)580 galU95 ΔuidA3::pir+ rpoS396(Am) Δend:: FRT rph-1 Δ(rhaD-rhaB)568 rnD3* pKD4, Ap^R Kan^R	Datsenko (2000) PNAS 97(12): 6640-6645
BW25141	*Δ(araD-araB)567 ΔlacZ4787 lacIp-4000(lacI^q) Δ(phoB-phoR)580 galU95 ΔuidA3::pir+ rpoS396(Am) Δend:: FRT rph-1 Δ(rhaD-rhaB)568 rnD3* pKD3, Ap^R Cm^R	Datsenko (2000) PNAS 97(12): 6640-6645
BW25113	*Δ(araD-araB)567 ΔlacZ4787 lacIp-4000(lacI^q) Δ(phoB-phoR)580 rpoS396(Am) rph-1 Δ(rhaD-rhaB)568 rnD3 hsdR514* pKD46 Ap^R	Datsenko (2000) PNAS 97(12): 6640-6645
BT340	*supE44 DlacU169 (f80 lacZDM15) hsdR17 recA1 endA1 gyrA96 thi-1 relA1* pCP20 Ap^R	Cherepanov (1995) Gene 158(1), 9-14
KM44	*argE3 his4 leuB6 proA2 thr1 ara14 galK2 lacY1 mtl1 xyl5 thi1 rpsL31 tsx33 D(recC ptr recB recD)::Ptac-bet exo kan*	Murphy (1998) J. Bact 180(8): 2063-2071
CAG 12158	MG1655 *pheA18::Tn10*, Tet^R	Singer, et al, 1989
CAG 37125	*ΔlacX74, galK, galU, Δ (araABC-leu)7679, araD139, hsdR, rpsL, mcrB rpoE::ΩCm, nadB51::Tn10,* Cm^R, Tet^R	Alejandro De Las Peñas
CAG 41241	CAG 45114 *ΔyeaY::Cm*, Cm^R	This study
CAG 41245	CAG 45114 *ΔyraP::Cm*, Cm^R	This study
CAG 41247	CAG 45114 *ΔyeaY*	This study
CAG 41251	CAG 45115 *ΔyraP*	This study

Strains	Relevant Genotype	Source or Reference
CAG 41254	CAG 45114 *ΔnlpB::Cm*, Cm^R	This study
CAG 41258	CAG 45114 *ΔnlpB*	This study
CAG 41264	KM44 *pheA18::Tn10*, Tet^R	This study
CAG 41265	CAG 45114 *surA::Cm*, Cm^R	This study
CAG 41292	CAG 45114 *degP::Km*, Km^R	This study
CAG 41293	CAG 45114 *ΔyraP, degP::Km*, Km^R	This study
CAG 41295	CAG 45114 *ΔnlpB, degP::Km*, Km^R	This study
CAG 41311	CAG 45114 *ΔyraP, ΔfkpA::Cm*, Cm^R	This study
CAG 41313	CAG 45114 *ΔnlpB, ΔfkpA::Cm*, Cm^R	This study
CAG 41318	CAG 45114 *ΔfkpA::Cm*, Cm^R	This study
CAG 41323	CAG 45114 *Δskp zae502::Tn10*, Tet^R	This study
CAG 41324	CAG 45114 *ΔyraP, Δskp zae502::Tn10*, Tet^R	This study
CAG 41326	CAG 45114 *ΔnlpB, Δskp zae502::Tn10*, Tet^R	This study
CAG 41349	CAG 41264 *lacZ::ptrc-b2595-bla*, Ap^R Tet^R	This study
CAG 41350	CAG 45114 pCO109, Ap^R	This study
CAG 41351	CAG 45114 pCO10, Ap^R	This study
CAG 41356	CAG 41349 *ΔyfiO::Cm*, Cm^R Ap^R Tet^R	This study
CAG 41408	CAG 45114 *ΔyfeY::Cm*, Cm^R	This study
CAG 41413	CAG 45114 *ΔyfeY*	This study
CAG 41434	CAG 45114 *ΔyfgL::Km*, Cm^R	This study
CAG 41445	CAG 45114 *ΔyfgL*	This study

Strains	Relevant Genotype	Source or Reference
CAG 41510	CAG 45114 pBA169, Ap^R	This study
CAG 41511	CAG 45114 pCO123, Ap^R	This study
CAG 41512	CAG 41251 pBA169, Ap^R	This study
CAG 41513	CAG 41251 pCO123, Ap^R	This study
CAG 41516	CAG 41445 pBA169, Ap^R	This study
CAG 41517	CAG 41445 pCO109, Ap^R	This study
CAG 41534	CAG 41258 pCO122, Ap^R	This study
CAG 41535	CAG 41258 pBA169, Ap^R	This study
CAG 41536	CAG 45114 pCO122, Ap^R	This study
CAG 41541	CAG 45114 *ΔyraP, ΔnlpB, ΔyeaY, yfeY::Cm, yfgL::Km*, Cm^R, Km^R	This study
CAG 41543	CAG 45114 *ΔyfgL, degP::Km*, Km^R	This study
CAG 41544	CAG 45114 *ΔyfgL, Δskp zae502::Tn10*, Tet^R	This study
CAG 41551	CAG 45114 *ΔfkpA*	This study
CAG 41552	CAG 45114 *ΔfkpA::Cm, Δskp zae502::Tn0*, Cm^R, Tet^R	This study
CAG 41553	CAG 45114 *ΔfkpA::Cm, degP::Km*, Cm^R, Km^R	This study
CAG 41554	CAG 45114 *ΔfkpA , surA::Cm*, Cm^R	This study
CAG 41560	CAG 45114 *Δskp, degP::Km* , Cm^R, Km^R	This study
CAG 49001	CAG 45114 pKD46, Ap^R, Cm^R	This study

Plasmids	Insert/Construction	Source/Reference
pTrc99A	Expression vector, pBR322 ori, pTrc promoter, Ap^R	Amersham Pharmacia Biotech
pBA169	pTrc99A ΔNcoI; eliminates vector ATG, Ap^R	Benjamin Alba
pCO109	*yfgL* in pBA169, Ap^R	This study
pCO110	*yfiO* in pBA169, Ap^R	This study
pCO122	nlpB in pBA169, Ap^R	This study
pCO123	yraP in pBA169, Ap^R	This study
pCP20	FLP^+, $\lambda cI857^+$, λP_R Rep^{ts} Cm^R, Ap^R	Cherepanov (1995) Gene 158(1), 9-14
pKD3	FRT-flanked Cm^R gene from pSC140 cloned into pANTSγ, Cm^R	Datsenko (2000) PNAS 97(12): 6640-6645
pKD4	FRT-flanked Km^R gene from pCP15 cloned into pANTSγ, Km^R	Datsenko (2000) PNAS 97(12): 6640-6645
pKD46	*araC*, P*araB* and *Red γ, β, and exo* cloned into pINT-ts, Ap^R	Datsenko (2000) PNAS 97(12): 6640-6645

Appendix A: Characterization of four additional newly-identified members of the σ^E regulon

In *Escherichia coli*, the functions of the σ^E regulon are required for envelope homeostasis during stress and are essential for viability under all growth conditions. By characterizing the novel members of this regulon, I hoped to gain further insight into the physiological role of σ^E. Here, I describe the phenotypic analysis of *yggN*, *ybfG*, *ygiM*, *yidQ*, and *yicK*. These genes encode additional novel members of the σ^E regulon and were not included in Chapter 1.

Whereas the gene *yicK* is reported to be a member of the sugar efflux transporter (SET) family in *E. coli* (Liu JY 1999), the amino acid sequences of *yggN*, *ybfG*, *ygiM*, and *yidQ* do not encode any recognizable functional motifs. To characterize these genes further, I generated chromosomal deletions of *yggN*, *ybfG*, *ygiM*, *yidQ*, and *yicK* using the methods described in (Datsenko 2000). I was able to delete each gene, indicating that none were essential for viability. Each mutant strain was able to grow at temperatures ranging from 18°C to 42°C both in standard LB medium and in minimal M9 medium supplemented with all of the amino acids, indicating that none of these genes was essential for growth at temperature extremes. Because the σ^E regulon is induced under hypo-osmotic conditions (see Appendix B), I next tested whether the mutants were altered in their osmotic sensitivity. However, each mutant grew equally well in hypo-osmotic medium (described in: Lacroix, Loubens et al. 1991) alone and in hypo-osmotic medium supplemented with 0.1M, 0.2M, 0.3M or 0.4M NaCl (data not shown). These deletion strains were also unimpaired in their sensitivity to a

variety of metals and in their motility. I also tested the deletion mutants for their sensitivity to a variety of agents that are normally excluded by a WT outer membrane. These agents included amphipathic antibiotics (rifampicin, novobiocin), detergents (SDS, bile salts), hydrophobic dyes (crystal violet) and EDTA, which destabilizes the outer membrane by chelating divalent cations bound to LPS. None of the mutants exhibited any sensitivity to any of these agents. In addition, neither the Δ*yggN*, Δ*ybfG*, Δ*ygiM*, Δ*yidQ*, nor Δ*yicK* alleles had any effect on steady state σ^E activity. The OMP profiles of these mutants did not exhibit any defects. All the phenotypic assays performed with the Δ*yggN*, Δ*ybfG*, Δ*ygiM*, Δ*yidQ*, and Δ*yicK* strains were carried out as described in Chapter 1.

A number of periplasmic chaperones and periplasmic peptidy prolyl isomerases (PPIases), such as SurA, Skp, FkpA, and DegP, facilitate the folding, assembly, and insertion of trimeric porins into the outer membrane. (Chen and Henning 1996; Bothmann and Pluckthun 1998; De Cock, Schafer et al. 1999). Deletion of the gene encoding any one of these four folding agents is not lethal. However Δ*skp surA::Cm* and *degP::Km surA::Cm* double mutants are synthetically lethal whereas Δ*skp degP*::Km double mutants are not (Rizzitello, Harper et al. 2001). This finding led the Silhavy group to propose the existence of two parallel periplasmic folding pathways: one involving surA and the other involving Skp and DegP (Rizzitello, Harper et al. 2001). To determine whether

yggN, *ybfG*, *ygiM*, *yidQ*, or *yicK* also contribute to the protein folding capacity of the envelope, I performed P1 transductions to analyze the genetic interactions of *yggN*, *ybfG*, *ygiM*, *yidQ*, and *yicK* with Δ*skp*, *degP::Km*, Δ*fkpA::Cm*, or *surA::Cm*. None of these novel genes exhibited any synthetic phenotypes with Δ*skp*, *degP::Km*, Δ*fkpA::Cm*, or *surA::Cm*.

References, Appendix A

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Appendix B: The activity of σ^E decreases in media of increasing osmotic strength

In *E. coli*, the *mdoGH* operon encodes functions that are required for the biosynthesis of MDOs, a class of soluble periplasmic glucans. Mutations in *mdoG* or *mdoH* not only block the synthesis of these highly branched sugars, but they also suggest that MDOs function as effectors in osmotic adaptation (Bohin 2000). In fact, the synthesis of MDOs and their accumulation in the periplasm are inversely proportional to the osmotic strength of a cell's environment (Kennedy 1982). For cells grown in medium of low osmolarity, it appears that MDOs are the principal source of fixed anion in the periplasmic space and, thus, they act to maintain the high osmotic pressure and Donnan membrane potential of the periplasmic compartment. However, mutations in the *mdoGH* operon also have pleiotropic effects on cell motility, on the levels of OmpF in the outer membrane, and on the regulation of capsule production (Fiedler 1988).

The *mdoGH* operon was recently found to be under the transcriptional control of σ^E (Rhodius, in prep). Because the accumulation of high levels of MDOs may greatly modify the physical properties of the cell envelope, I tested to see whether or not the activity of σ^E itself responded to changes in osmotic strength. As shown in Figure A-1, steady state σ^E activity decreases as the osmotic strength of the media increases. This result suggests that the σ^E regulon may encode additional functions that are necessary for the survival of *E. coli* in

response to changes in osmolarity. This experiment has only been performed once and should be repeated by those who are interested in pursuing the questions that this result raises.

References, Appendix B

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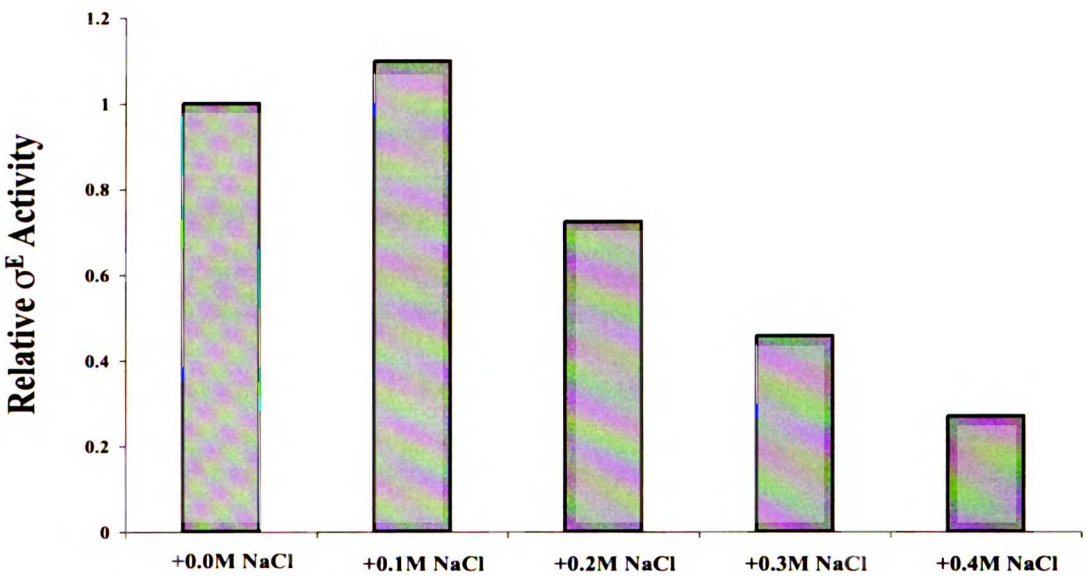
Rhodius, et al. Manuscript in prep.

Figure Legends, Appendix B

Figure B-1: The activity of σ^E decreases when WT cells are grown in media of increasing osmotic strength. The activity of σ^E decreases when WT cells are grown in media of increasing osmotic strength. σ^E activity was assayed by monitoring β-galactosidase activity from a chromosomal σ^E-dependent lacZ reporter gene Φ λ[rpoHP3-lacZ] as described previously in Chapter 1.

Figure B-1: The activity of σ^E decreases when WT cells are grown in media of increasing osmotic strength.



Appendix C: Mapping a suppressor of *rpoE*$^-$ lethality

In *Escherichia coli*, the functions of σ^E are required for envelope homeostasis under conditions that induce extracytoplasmic stress. However, σ^E is also essential for viability under all growth conditions. Strains in which *rpoE*, the gene encoding σ^E, has been disrupted acquire an extragenic suppressor which permits survival in the absence of σ^E (De Las Peñas 1997). By mapping the suppressor mutation and characterizing the mechanism by which it prevents lethality in strains lacking σ^E, I hoped to identify the essential cellular functions that are governed by σ^E.

Strains carrying the suppressor of *rpoE*$^-$ lethality (*sup*$^+$) have two verified phenotypes: First, *sup*$^+$ strains are sensitive to growth on LB agar containing 1% SDS and 1mM EDTA (ie: SDS/EDTA^S), whereas WT strains are resistant to these agents (ie: SDS/EDTA^R). Second, *sup*$^+$ strains are resistant to growth inhibition due to the overexpression of RseA and RseB, two negative regulators of σ^E activity (RseAB^R) (De Las Peñas 1997). In contrast, WT strains are sensitive to the overexpression of these proteins (RseAB^S) (De Las Peñas 1997). I used these two distinguishing phenotypes to determine whether the *sup*$^+$ locus was removed by genetic exchange, thereby allowing me to localize the region on the chromosome to which this mutation maps.

Initially, I used Hfrs that carry Tet^R markers approximately 20' downstream of their origins of transfer to localize the suppressor mutation to an interval of 10'or less on the 100'*E. coli* chromosome (Figure C-1). Before performing the

Hfr matings, I tested each Hfr for its SDS/EDTA and RseAB overexpression phenotypes (Table C-1). Because Hfrs #6 and #3 were SDS/EDTA^S, they were not used in any further experiments.

Next, SDS^R Tet^R Hfr "donor" strains #1, 2, 4, 5, and 7 (Fig. 1) were each mated to the Kan^R Str^R Tet^S *sup*$^+$ F- "recipient" strain CAG 37187. The matings were performed as described in (Miller 1972). Str^R Tet^R exconjugants were selected on LB agar plates supplemented with 100 μg/ml streptomycin and 10 μg/ml tetracycline. Streptomycin was used to counterselect against donor strains. Only the recipients into which at least 20'of Hfr DNA was transferred would be both Str^R and Tet^R.

The Str^R Tet^R exconjugants were then screened for SDS/EDTA^R, the phenotype which indicates that the exconjugants had presumably lost the suppressor locus which confers SDS/EDTA sensitivity. In general, about 50% of the markers between an Hfr's origin of transfer and its selected marker are incorporated into the recipient (Hayes 1953). Therefore, it is necessary to score as many as 10 exconjugants to establish whether a particular Hfr strain transfers the region in which the mutation is located. However, incorporation of markers on the Hfr chromosome following the selected marker on an is usually very low (Hayes 1953). Only matings using Hfrs #2 and #7 produced SDS^R exconjugants, indicating that the suppressor mutation was located between 67' and 6.75' (going in a clockwise direction) on the *E. coli* chromosome (Table C-2).

I then constructed two additional *sup*$^+$ recipients that allowed me to limit the extent of transfer past the Tet^R marker of the Hfr. The first *sup*$^+$ recipient, CAG 41449, carried a Kan^R marker located at 87.5' and was used as a recipient in matings with Hfr #2. The other *sup*$^+$ recipient, CAG 41039, carried a Kan^R marker located at 93.75 and was used in matings with Hfr#7 (Figure C-2). By selecting for Tet^R Kan^R exconjugants, genetic material from the Hfr could not be recombined into the recipient's chromosome beyond the Kan^R marker.

90% of the exconjugants obtained from mating CAG 41449 with Hfr#2 lost the *sup*$^+$ locus, even though transfer of genetic material below 87.5' was selected against (Table C-3). This result strongly suggested that the *sup*$^+$ locus was clockwise from 87.5'. Consistent with this location, no exconjugants obtained from the mating of CAG 41039 with Hfr #7 lost the *sup*$^+$ allele when transfer of genetic material above 93.75' was restricted. The conclusion from these experiments was that *sup*$^+$ was located between 90.3' and 6.75' in a clockwise direction. Furthermore, mating CAG 37187 and Hfr #1, which transfers clockwise from its oriT at 95.4', did not produce exconjugants that lost the *sup*$^+$ locus. Because loci near the oriT of an Hfr are inefficiently transferred into the recipient, the *sup*$^+$ allele is therefore likely to be counterclockwise from 96'. Taken together, these experiments map the *sup*$^+$ locus to region between 90.3' and 96' on the *E. coli* chromosome.

Next, I used the mapping strains described in (Singer, Baker et al. 1989) to more accurately localize the suppressor mutation. Each of these isogenic mapping

strains carries a single Kan^R or Tet^R marker at a defined position; in the complete set of strains, alternating antibiotic resistance markers are spaced approximately 1 minute apart along the entire *E. coli* chromosome. These alternating selectable markers were introduced into the suppressor strain CAG 41001 via multiple sequential P1 transductions. By selecting for the presence of one antibiotic resistance marker and scoring for the loss of the other, I was able to substitute DNA segments in the suppressor strain's chromosome with segments from the corresponding chromosomal regions in the mapping strains. The transductants carrying such "replacements" were then scored for the presence or the absence of suppressor phenotypes as described above. By walking the region of the chromosome to which I previously localized the suppressor (ie: 90.3' and 96'), I hoped to map the suppressor mutation to a 50 kb interval. The results from these transductions are summarized in Table C-4. None of the "replacement" transductions along that chromosomal interval removed the suppressor mutation.

Though many have successfully used Hfrs have to map mutations in *E. coli*, this strategy is not without its caveats. First, the strains which I used to map the suppressor of *rpoE*$^-$ lethality were neither isogenic to the strain carrying the suppressor, nor were they isogenic to one another. Each of the Hfrs had been exposed to a different set mutagens during its construction, and each Hfr has undoubtedly accumulated a large number of unique, unreported genetic lesions. As a result, none of the Hfrs can truly be said to have a "WT" genotype. Though I

tested the Hfrs' SDS/EDTA and RseAB phenotypes to ensure that these strains did not carry a suppressor of *rpoE*$^-$ lethality, I could never be unequivocally sure that Hfr matings didn't introduce an anti-suppressor mutation into the *sup*$^+$ strain's chromosome, rather than remove the remove the suppressor mutation itself. Because the results I obtained from my Hfr mating experiments are internally consistent, I am disinclined to doubt them. However, if the suppressor were the result of a number of closely spaced point mutations or of a short chromosomal inversion, I would never be able to map its chromosomal position using the transduction strategy that I described above. Were this to be the case, an alternate strategy must be employed to identify the suppressor locus.

References, Appendix C

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Figure Legends, Appendix C

Figure C-1: Hfr mapping set described in Singer, et al, (1989). 1. HfrH; 2. KL227; 3. KL208; 4. KL96; 5. KL16; 6. KL228; 7. KL14. Arrowheads indicate the origin and direction of transfer of each Hfr. The bars indicate positions of the Tet^R insertions used as selective markers in matings. This figure has been reproduced from Singer, et al, (1989).

Table C-1: The SDS/EDTA phenotypes and RseAB overexpression phenotypes for each Hfr described in (Singer, Baker et al. 1989) were assayed. Only those Hfrs that were SDS/EDTA^R and RseAB^S were used in the matings depicted in Table 1B.

Table C-2: The results of matings performed with the Hfrs depicted in Figure C-1 and the F- recipient strain CAG 37187.

Figure C-2: A diagram of two Hfrs and the F- recipient strains with which they were crossed in order to localize the suppressor of rpoE- lethality to a 5'-10' interval on the E. coli chromosome.

Table C-3: The results of mating Hfr #2 to F- recipient strain CAG 41449 and of mating Hfr #7 to F- recipient strain CAG 41039. Diagrams of these strains are depicted in Figure 2.

Table C-4: Results of "replacement" transductions along the chromosomal interval between 90.25' and 97. Alternating Tet^R and Kan^R markers from the mapping strains described in (Singer, et al, 1989) were introduced into the suppressor strain CAG 41001 via multiple sequential P1 transductions. By selecting for the presence of one antibiotic resistance marker and scoring for the loss of the other, I was able to substitute DNA segments in the suppressor strain's chromosome with segments from the corresponding chromosomal regions in the mapping strains. The transductants carrying such "replacements" were then scored for the presence or the absence of suppressor phenotypes. Asterisks "*" indicate the intervals of the sup^+ chromosome that I was unable to via P1 transduction.

Figure C-1: Hfr mapping set described in Singer, et al, (1989). 1. HfrH; 2. KL227; 3. KL208; 4. KL96; 5. KL16; 6. KL228; 7. KL14.

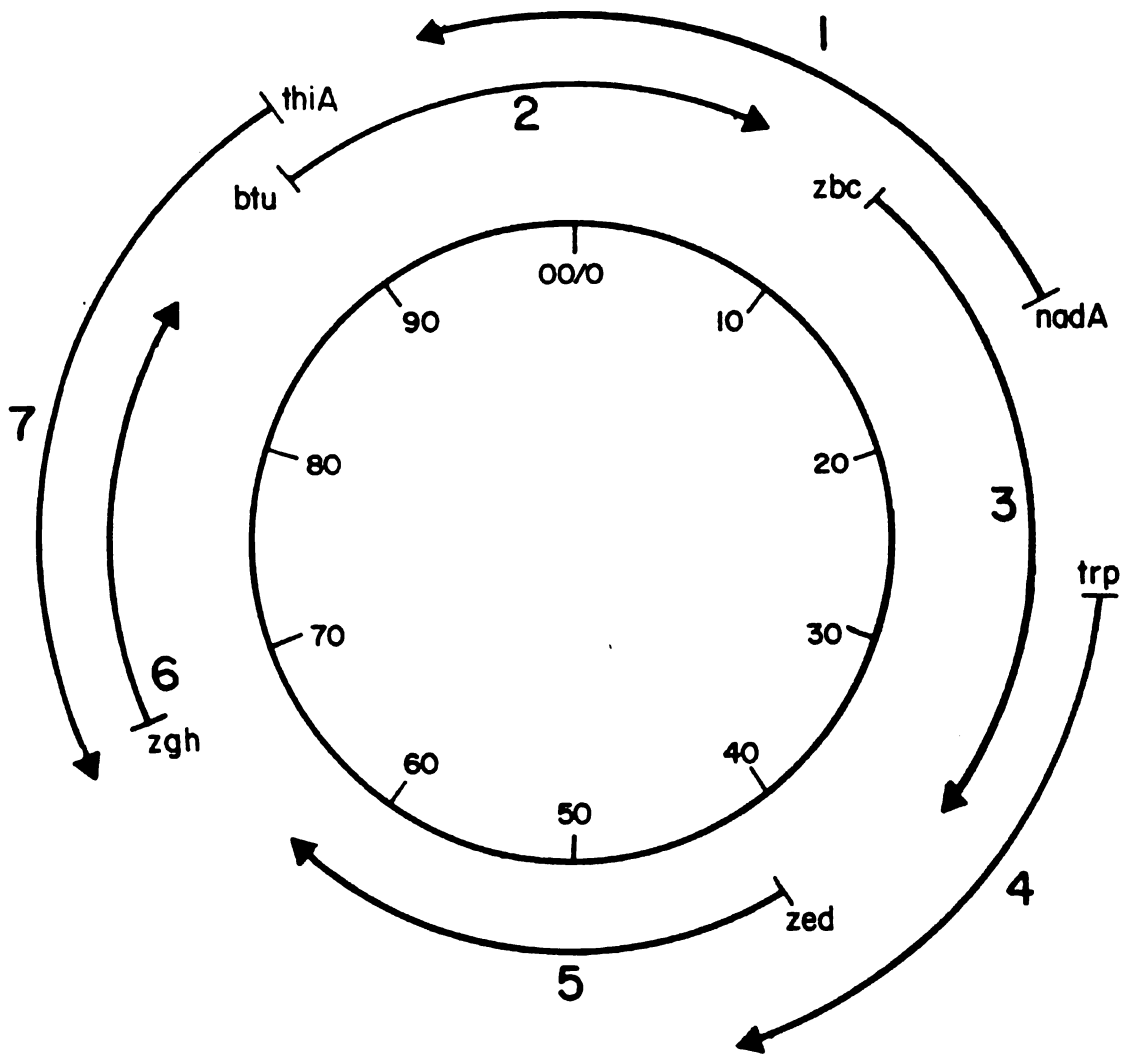

Table C-1: The SDS/EDTA phenotypes and RseAB overexpression phenotypes for each Hfr described in (Singer, Baker et al. 1989) were assayed. Only those Hfrs that were SDS/EDTA^R and RseAB^S were used in the matings depicted in Table 1B

	WT (CAG 16037)	*sup*$^+$ (CAG 37187)	Hfr #1 (CAG 5051)	Hfr #2 (CAG 5052)	Hfr #3 (CAG 5053)	Hfr #4 (CAG 5054)	Hfr #5 (CAG 5055)	Hfr #6 (CAG 8209)	Hfr #7 (CAG 8160)
1% SDS/ 1mM EDTA	R	S	R	R	S	R	R	S	R
RseAB Overexpression	S	R	S	S	Not Tested	S	S	S	S

Table C-2: The results of matings performed with the Hfrs depicted in Figure C-1 and the F^- recipient strain CAG 37187.

Mating Cross	Duration	Selection	SDS/EDTA Phenotype	RseAB Phenotype
CAG 37187 x Hfr #1	30'	Str, Tet	SDS S (10/10)	R
CAG 37187 x Hfr #2	15'	Str, Tet	SDS R (9/10)	SDS/EDTA^R excongjugants were RseAB^S
CAG 37187 x Hfr #2	30'	Str, Tet	SDS R (16/20)	SDS/EDTA^R excongjugants were RseAB^S
CAG 37187 x Hfr #4	30'	Str, Tet	SDS S (10/10)	R
CAG 37187 x Hfr #5	30'	Str, Tet	SDS S (10/10)	R
CAG 37187 x Hfr #7	15'	Str, Tet	SDS R (3/10)	SDS/EDTA^R excongjugants were RseAB^S
CAG 37187 x Hfr #7	30'	Str, Tet	SDS R (13/40)	SDS/EDTA^R excongjugants were RseAB^S
CAG 37187 x Hfr #7	30'	Kan, Tet	SDS R (11/19)	SDS/EDTA^R excongjugants were RseAB^S

Figure C-2: A diagram of two Hfrs and the F- recipient strains with which they were crossed in order to localize the suppressor of *rpoE*- lethality to a 5'-10' interval on the *E. coli* chromosome.

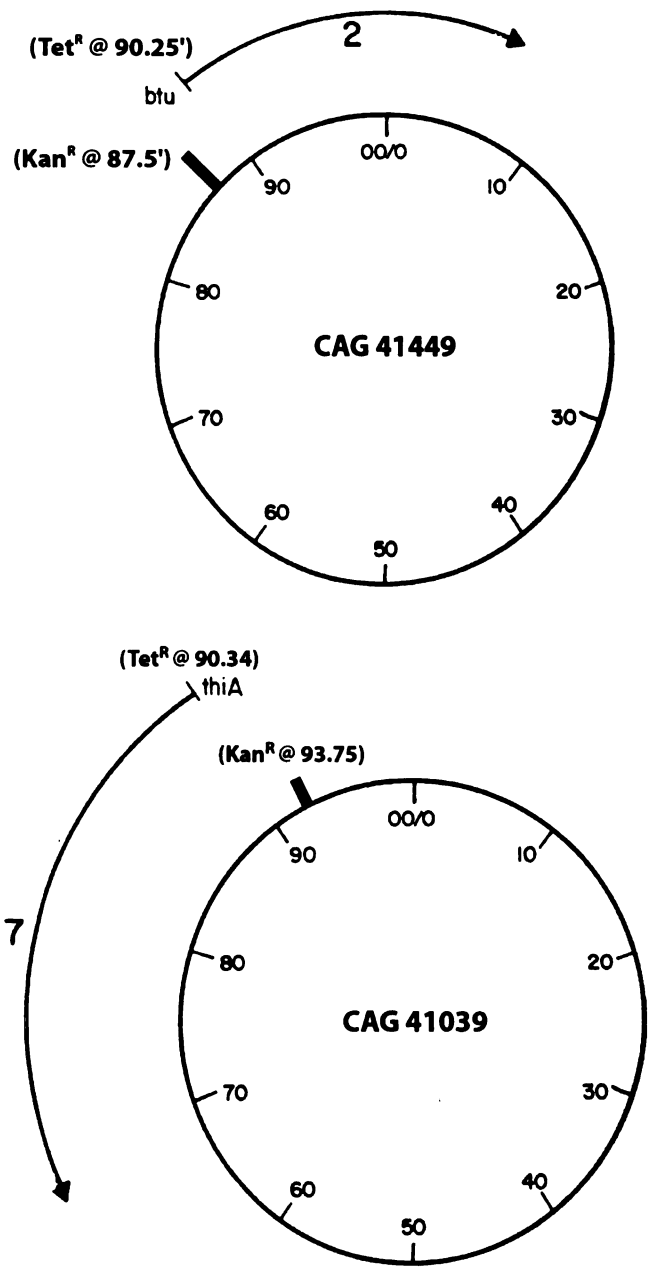

Table C-3: The results of mating Hfr #2 to F- recipient strain CAG 41449 and of mating Hfr #7 to F- recipient strain CAG 41039. Diagrams of these strains are depicted in Figure 2.

Mating Cross	Duration	Selection	SDS/EDTA Phenotype	RseAB Phenotype
CAG 41449 x Hfr #2	30'	Kan, Tet	SDS R (9/10)	SDS/EDTA^R excongjugants were RseAB^S
CAG 41039 x Hfr #7	30'	Kan Tet	SDS S (18/18)	R

Table C-4: Results of "replacement" transductions along the chromosomal interval between 90.25' and 97'

Interval in *sup*$^+$ strain replaced via transduction	SDS/EDTA Phenotype	RseAB Phenotype
90.25' – 90.75'	S	R
90.75' – 91.5'	S	R
91.4' – 92.5' *		
92.5' – 93.75'	S	R
93.75' – 94.2'	S	R
94.2' – 94.8' *		
94.8' – 95.4' *		
95.4' – 95.8'	S	R
95.8' – 97' *		

Appendix D: Physiological characteristics of an *E. coli* strain carrying a suppressor of *rpoE*$^{-}$ lethality

σ^E is required for survival under all growth conditions (De Las Peñas 1997). Strains in which *rpoE*, the gene encoding σ^E, has been disrupted acquire an extragenic suppressor which permits survival in the absence of σ^E (De Las Peñas 1997). To gain a greater understanding of how the suppressor of *rpoE*$^-$ lethality (*sup*$^+$) affects an *E. coli* cell's physiology, I characterized the function of σ^E in a strain carrying the *sup*$^+$ allele.

rpoE$^+$ *sup*$^+$ strains exhibit a 10-fold decrease in σ^E activity compared to WT strains at 30°C (Figure D-1). Because the basal rate of σ^E activity is modulated by the level of unassembled OMPs, (Mecsas, Rouviere et al. 1993), (Walsh NP 2003), this observation suggests that the levels of OMP precursors are lower in a *sup*$^+$ strain than in a WT strain. The suppressor of *rpoE*$^-$ lethality may reduce a cell's physiological requirement for the functions of σ^E by lowering the levels of OMP synthesis. To test this possibility, I determined whether σ^E is essential in a *ΔompR* strain, in which the levels porins OmpF and OmpC expression are dramatically reduced. However, overexpression of RseA and RseB is still toxic in a *ΔompR* strain (Figure D-2), suggesting that the removal of porin precursors is not sufficient to suppress *rpoE*$^-$ lethality.

References, Appendix D

De Las Peñas, A., L. Connolly, and C. A. Gross. (1997). "σ^E is an essential sigma factor in *E. coli*." *Journal of Bacteriology* **179**(21): 6862-6864.

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Figure Legends, Appendix D

Figure D-1: sup$^+$ strains exhibit a 10-fold decrease in σ^E activity compared to WT strains at 30°C. σ^E activity was assayed by monitoring β-galactosidase activity from a chromosomal σ^E-dependent lacZ reporter gene Φ λ[rpoHP3-lacZ] as described previously in Chapter 1. Cells to be assayed were grown at 30°C in M9 complete, and single point determinations were made at the indicated optical densities. The rate of β -galactosidase synthesis from the σ^E-dependent reporter gene increases as the cells enter mid-log phase when grown in LB (J. Mecsas and C.A. Gross, unpublished). Due to this growth phase effect, differences in σ^E activity were determined by comparing the slopes of the initial linear portion of the plots of β -galactosidase activity/0.5 ml cells versus OD450.

Table D-2: Overexpression of RseA and RseB is still toxic in a ΔompR strain, suggesting that the removal of porin precursors is not sufficient to suppress rpoE$^-$ lethality. Wild Type, rpoE$^+$ sup$^+$, or ΔompR strains carrying either pTrc99A or pRseA RseB were grown in LB medium (described in Chapter 1) containing 100μg/ml of ampicillin and 0.2% glucose at 30°C until saturation. The cultures were serially diluted 10-fold to 10^{-7}. One hundred microliters of each dilution of each culture were spread on LB agar plates supplemented with 100μg/ml of ampicillin + 0.2% glucose to determine viable counts. The dilutions were also plated on LB plates containing 100μg/ml of ampicillin + 1mM IPTG to induce the

Trc99A promoter. The plates were incubated at 30°C for 18 hours. The efficiency of plating was calculated by dividing the number of colony-forming units (cfus) of a given strain grown on LB +Amp +IPTG plates by the number of cfus of that same strain on LB +Amp +Glu plates.

Figure D-1: *sup*$^+$ strains exhibit a 10-fold decrease in σ^E activity compared to WT strains at 30°C

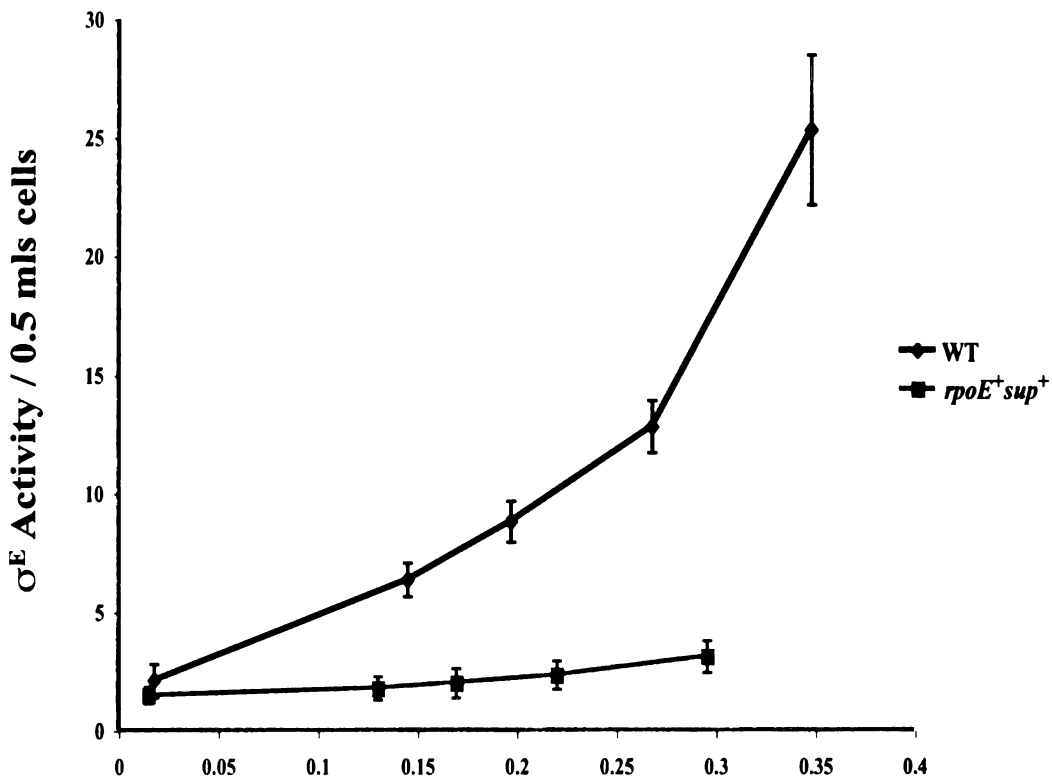

Table D-2: Overexpression of RseA and RseB is still toxic in a *ΔompR* strain, suggesting that the removal of porin precursors is not sufficient to suppress *rpoE*$^-$ lethality.

Strain	Plasmid	Efficiency of plating at 30°C
Wild Type	pTrc99A	1.0
Wild Type	p*RseA RseB*	3.1×10^{-4}
rpoE$^+$ *sup*$^+$	pTrc99A	1.0
rpoE$^+$ *sup*$^+$	p*RseA RseB*	0.8
ΔompR	pTrc99A	1.0
ΔompR	p*RseA RseB*	4.1×10^{-4}

Chapter Three: Conclusions and Future Directions

Conclusions and Future Directions

In *Escherichia coli*, the functions of the σ^E regulon are required for envelope homeostasis during stress and are essential for viability under all growth conditions; however, at the time during which this work was begun, very little was known about the kinds of functions that are regulated by the activity of this transcription factor. As described previously, genetic, genomic, and bioinformatic strategies have been employed to identify additional members of this regulon. The results of these efforts revealed that the genes in the σ^E regulon encode not only periplasmic chaperones and proteases that act on misfolded periplasmic proteins, but also enzymes required for the biosynthesis of phospholipids, LPS, and other components of the periplasm and OM. However, the majority of the members of this regulon are novel genes of unknown function, and my goal was to discover additional functions of the σ^E regulon by characterizing these novel genes. I also hoped that such analysis might also lead me to a better understanding of why σ^E is essential for viability.

The major portion of my work is specifically concerned with characterizing the functions of the lipoproteins encoded in the σ^E regulon. I chose to investigate this group of proteins, not only because six are represented in this regulon, but also because many lipoproteins, including Lpp and Pal, play roles in maintaining the integrity of the *E. coli* envelope. This is also the principal function of σ^E. I performed a variety of phenotype assays and genetic experiments using the

lipoprotein null mutants I generated, the results of which I will summarize here: Five of the six lipoproteins in the σ^E regulon are non-essential; in fact, cells are viable even when all five are removed simultaneously. Three of these nonessential lipoproteins exhibit phenotypes that suggest they are important for maintenance of the integrity of the cell envelope: Cells lacking NlpB are selectively sensitive to rifampicin, whereas those lacking YraP are selectively sensitive to SDS. In addition, both Δ*yraP* strains and Δ*nlpB* strains are synthetically lethal with *surA::Cm*, which encodes a periplasmic chaperone and PPIase. This observation may suggest that NlpB and YraP play roles in a periplasmic folding pathway that functions in parallel with that of SurA. Finally, the Δ*yfgL* mutant exhibits a broad range of envelope defects, including sensitivity to several membrane-impermeable agents, an altered OMP profile, synthetic lethality with both *surA::Cm* strains and Δ*fkpA::Cm* strains, and sensitivity to BPI. Taken together, these results suggest that the lipoprotein YfgL plays an important but as yet unknown function in maintaining envelope integrity in *E. coli.*

The most interesting observation I made during the course of this work is that the σ^E-transcribed lipoprotein YfiO is essential. Of the approximately 100 lipoproteins encoded in the *E. coli* genome, only one other, LolB, which plays a critical role in the membrane specificity of lipoprotein targeting, has previously been shown to be essential. Strikingly, YfiO is highly conserved in Gram-negative bacteria (V. Rhodius, pers. comm), including *Salmonella typhimurium*,

Yersinia pestis, and *Pseudomonas aeruginosa*. This observation suggests that YfiO may play an important role in a function specific for the survivial of Gram-negative bacteria or in the biogenesis of structures unique to Gram-negative bacterial cells. Nevertheless, overexpression of YfiO is not sufficient to alleviate a cell's requirement for σ^E for viability, indicating that the σ^E regulon provides more than one essential function.

It will be necessary to perform additional experiments to elucidate the function(s) of YfiO. Because YfiO is localized to outer membrane, I presume that this protein performs in an essential function associated with this structure. YfiO may participate in functions related to the insertion of porins into the OM, to the delivery of LPS to the OM, to the spatial regulation of peptidoglycan assembly, of outer membrane biogenesis, or to the co-ordination of any of these processes with cell division. To investigate these possibilities, it would be imperative to construct a YfiO depletion strain to enable us to monitor various phenotypes during the course of YfiO depletion. First, it would be important to monitor the integrity of the outer membrane during the course of YfiO depletion, as assessed by the permeability of the strain to various dyes that are normally excluded by an intact OM. Other phenotypes that should be monitored in a YfiO depletion strain include cell morphology, Omp profiles, membrane protein profiles, and peptidoglycan composition. Attempts should be made to generate partial function alleles of *yfiO*. Strains carrying such alleles could be used in subsequent genetic analyses, such as suppressor or enhancer screens, to identify potentially

informative genetic interactions. By performing two-hybrid screens and formaldehyde cross-linking experiments, it might be possible to determine whether YfiO interacts with the peptidoglycan or with other proteins in the periplasm or OM. Additional reagents, such as anti-YfiO antibodies should be generated in order to make it possible to perform experiments such as quantitative Westerns to determine the cellular concentration of YfiO.

I have also examined the phenotypes of five additional newly-identified non-lipoprotein genes in the σ^E regulon. To date, I have not succeeded in identifying any phenotypes for these genes that might give me insight into their functions. My efforts to this end are made even more difficult by the fact that none of these genes encode any recognizable motifs or exhibit any homologies that might assist me in their characterization. To learn more about the functions of these genes, it would behoove me to analyze these mutants for their ability to grow under conditions that I have not yet tested, such as the following: Anaerobiasis, extreme pH, transition into stationary phase, and OMP overexpression. It may also be informative to investigate the genetic interactions that these mutants may exhibit with Lipid A mutants, LPS rough mutants, and peptidogycan biosynthesis mutants.

I also took a genetic approach in my attempts to understand the essential function(s) of the σ^E regulon. Strains in which *rpoE*, the gene encoding σ^E, has been disrupted acquire an extragenic suppressor which permits survival at 30°C in the absence of σ^E. By mapping the suppressor mutation and characterizing the

mechanism by which it prevents lethality in strains lacking σ^E, I hoped to identify the essential cellular functions that are governed by σ^E. I used Hfr strains to localize the suppressor of *rpoE*$^-$ lethality to a 7-minute interval of the *E. coli* chromosome (90.3'- 96'). Unfortunately, I was unable to map the suppressor mutation to smaller interval using the transduction technique that I described in Appendix C.

Ideally, the Hfr mapping experiments should be repeated, and a phenotype other than SDS/EDTA^S should be monitored to determine whether the *sup*$^+$ locus has been removed by genetic exchange with Hfr DNA. A strain in which *rpoE* is placed under the transcriptional control of a conditional promoter would be a valuable tool in future Hfr mapping experiments. Such a strain would be used as a recipient in Hfr matings, and the chromosomal position of the suppressor mutation could then be mapped by determining whether σ^E is still essential in the resulting exconjugants. If such experiments were to map the suppressor to an entirely different chromosomal interval than indicated by my previous results, I would again perform replacement transductions to narrow the locus to which this mutation maps. However, if, using this new strategy, I were to find the suppressor mapped to the same chromosomal location as before, it would be necessary for me to consider the possibility that the interval to which the suppressor maps contains either a chromosomal inversion or carries more than one mutation.

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