Outer Membrane Biogenesis and Stress Response in *Escherichia coli*

by

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ABSTRACT

Protein folding is essential in all cells, and misfolded proteins cause many diseases. In the Gram-negative bacterium *Escherichia coli*, protein folding must be carefully controlled during envelope biogenesis to maintain an effective permeability barrier between the cell and its environment. This study explores the relationship between envelope biogenesis and cell stress, and the return to homeostasis during envelope stress.

A major player in envelope biogenesis and stress response is the periplasmic protease DegP. Work presented here explores the growth phenotypes of cells lacking *degP*, including temperature sensitivity and lowered cell viability. Intriguingly, these cells also accumulate novel cytosolic proteins in their envelope not present in wild-type. Association of novel proteins was found to be growth time- and temperature-dependent, and was reversible, suggesting a dynamic nature of the envelope stress response. Two-dimensional gel electrophoresis of envelopes followed by mass spectrometry identified numerous cytoplasmic proteins, including the elongation factor/chaperone TufA, illuminating a novel cytoplasmic response to envelope stress.

A suppressor of temperature sensitivity was characterized which corrects the defect caused by the lack of *degP*. Through random Tn*10* insertion analysis, aribitrarily-primed polymerase chain reaction and three-factor cross, the suppressor was identified as a novel duplication-

truncation of *rpoE*, here called *rpoE'*. *rpoE'* serves to subtly increase RpoE levels in the cell, resulting in a slight elevation of the σ^E stress response. It does so without significantly affecting steady-state levels of outer membrane proteins, but rather by increasing proteolysis in the envelope independently of DegP.

A multicopy suppressor of temperature sensitivity in strains lacking *degP* and expressing mutant OmpC proteins, *yfgC*, was characterized. Bioinformatics suggests that YfgC is a metalloprotease, and mutation of conserved domains resulted in mislocalization of the protein. *yfgC*-null mutants displayed additive antibiotic sensitivity and growth defects when combined with null mutation in another periplasmic chaperone, *surA*, suggesting that the two act in separate pathways during envelope biogenesis. Overexpression of $YfgC_{6his}$ altered steady-state levels of mutant OmpC in the envelope, showing a direct relationship between it and a major constituent of the envelope. Curiously, purified $YfgC_{6his}$ showed an increased propensity for crosslinking in mutant, but not in a wild-type, OmpC background.

DEDICATION

To God, through whom all things are possible

To Jessica, for constant encouragement and support

To my parents, for instilling in me the love of learning

To Hope, for opening my eyes to science

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INTRODUCTION

Escherichia coli (*E. coli*) is a common microorganism belonging to the family *Enterobacteriaceae*. These bacteria are characterized by their rod shape and Gram-negative staining properties. Members of this family naturally reside in the intestinal tract of humans and other animals. Although they are a part of the normal intestinal flora, members of *Enterobacteriaceae* can cause serious disease in humans, including colitis, bubonic/pneumonic plague and bacterial septicemia (Wells *et al,* 1983; Darfeuille-Michaud *et al,* 1998; Parkhill *et al,* 2001).

E. coli was discovered in 1885 by Theodore Escherich and originally named *Bacillus coli*. It has an extremely rapid growth rate and is amenable to genetic manipulation; because of this, and the fact that it can be rendered harmless without seriously compromising its basic cellular functions, it has historically been a model organism of choice for genetic and physiological studies. Nonetheless, although the bacterium has been studied for over a century, the nature of much of its inner workings remains a mystery. In particular, the interaction between the cell and its environment is a topic of intense interest, especially considering the essential cellular need for separation of the two. Any advances in understanding of the mechanisms by which *E. coli* assembles and maintains the membranous barrier between itself and the environment could potentially be exploited in a clinical setting.

Structure of the *Escherichia coli* cell envelope

E. coli is a Gram-negative organism, possessing both an inner and an outer membrane. These membranes are separated by an aqueous compartment known as the periplasm. Together, these three compartments comprise what is known as the *E. coli* cell envelope (Fig. 1). The envelope is responsible for mediating interaction between the cell and its external environment. Some components of the envelope respond to extracellular signals, such as medium pH and osmolarity, while others respond to intracellular signals, such as misfolded proteins. The envelope also mediates the entry of nutrients and other small molecules, and the expulsion of waste and other toxic compounds. The cell must therefore maintain a healthy cell envelope as a part of its survival strategy.

Inner membrane

The inner membrane (IM) of *E. coli* is a symmetrical bilayer comprised of phospholipids and proteins. Its phospholipids are arranged such that the polar head groups sandwich the opposing leaflet's hydrophobic lipid tails, forming a barrier to small molecules. The primary phospholipids comprising the membrane of *E. coli* are phosphotidylethanolamine (70-80%), phosphatidylglycerol (15-25%), and cardiolipin (5-10%) (Kadner, 1996). Bacterial cells can modulate the fluidity of the membrane by altering the composition and percentage of each component (Morein *et al,* 1996; Matsumoto *et al,* 2006). The IM has a substantial proteinaceous component, possessing integral (embedded,

membrane-spanning) and peripheral (loosely associated) membrane proteins. Integral membrane proteins residing in the IM tend to have hydrophobic stretches of 15-30 amino acids flanked by charged amino acids, generally following the so-called “positive inside rule,” in which positively charged amino acids are found in higher abundance at the cytoplasmic (rather than the periplasmic) side of the membrane-spanning helix (von Heijne, 1994). It is thought that this topology helps to anchor the protein by making it thermodynamically unfavorable for it to slide either way out of the membrane. It may also serve as a signal to the IM translocation machinery to stop translocating a protein and instead insert it laterally into the lipid bilayer (von Heijne, 1986). In contrast, peripheral membrane proteins are held at the membrane through protein-protein interactions, or as is the case with lipoproteins, by a lipid moiety, which can be anchored to the peptidoglycan layer.

The IM is responsible for many cellular metabolic functions, including cell replication, protein secretion, DNA replication and maintaining the chemical gradient required for energy production in the cell. As in higher organisms, the latter is accomplished through the electron transport chain and the ATP synthase complex (Haddock and Jones, 1977; Deckers-Hebestreit and Altendorf, 1996). Many inhibitors, such as carbonyl cyanide m-chlorophenylhydrazone (CCCP), act by depolarizing the membrane, crippling the cell (Lewis *et al,* 1994). Thus, *E. coli* and other bacteria have evolved various efflux systems (such as the

AcrAB/TolC complex) to protect themselves against agents which might harm their membrane potential or otherwise penetrate the IM and do cellular damage (Bavro *et al,* 2008; Misra and Bavro, 2009).

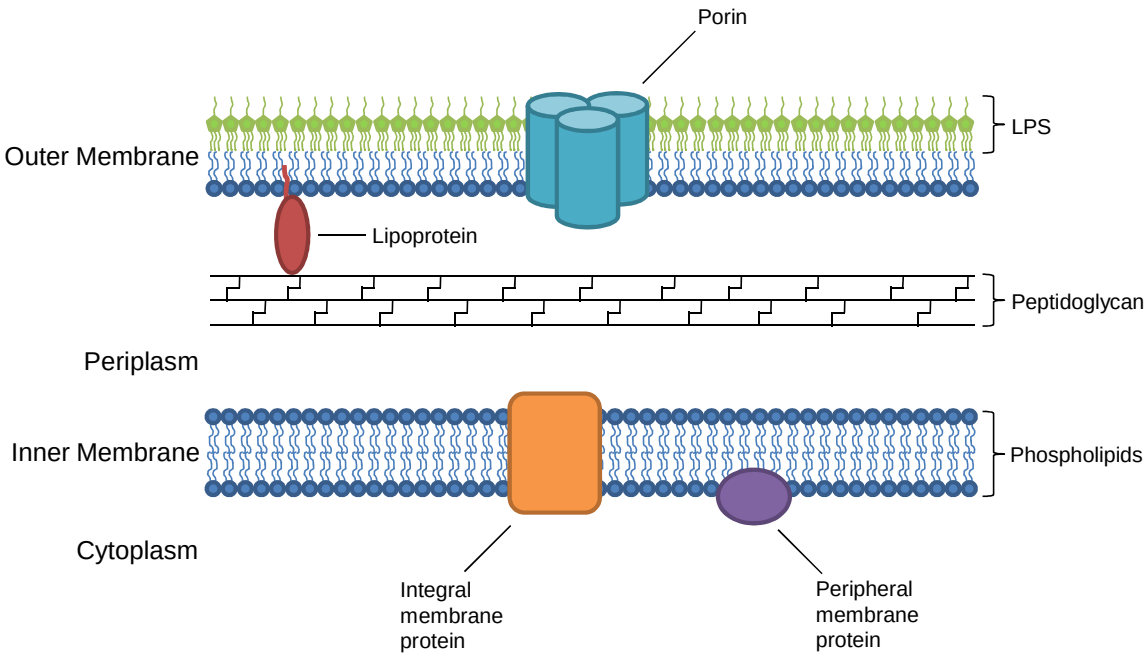


Fig. 1. Schematic representation of the *E. coli* cell envelope.

Periplasm

Between the inner and outer membranes of *E. coli* lies the aqueous environment known as the periplasm, or periplasmic space. It can be thought of as a "buffer zone" between the cell and its environment, and is comprised of soluble saccharides and proteins. When measured by cryo-fixation transmission electron microscopy, the periplasm ranges from ~18

to ~22 nm in width (Matias, 2003). Indeed, the crystal structure of the periplasm-spanning outer membrane channel TolC reveals its length to be ~17 nm (Murakami *et al,* 2002).

The periplasm plays a crucial role in cellular metabolism and signaling. For example, soluble sugars must be transported by proteins across the periplasm to the inner membrane for further transport into the cytoplasm. Maltose binding protein (MalE) is an example of such a protein. It is responsible for carrying maltose and related sugars across the periplasm for metabolism (Duplay *et al,* 1984). Protein systems such as the EnvZ/OmpR and Cpx two component regulatory systems are responsible for transducing signals across the inner membrane in order for the cell to adapt to changing environmental or cellular conditions (Cai and Inouye, 2002; Raivio and Silhavy, 1999).

Many chaperones, proteases and other folding factors (discussed in detail below) operate in the periplasm. These proteins must necessarily derive energy in order to function, yet there is no evidence that the periplasm contains ATP or any other obvious chemical energy source. Although addressing this apparent paradox is an area of active research, the precise nature of the energy source in the periplasm is not known at this time. However, it is possible that energy is released to the chaperones during substrate folding by promoting protein conformations of a lower-energy state.

Peptidoglycan

The periplasm is home to the thin peptidoglycan layer (also known as the murien sacculus) characteristic of Gram-negative bacteria. It is this layer of peptidoglycan that gives *E. coli* its rod shape and protects it from distortion by turgor pressure. Peptidoglycan is assembled in the periplasm and consists of alternating *N*-acetylglucosamine (NAG) and *N*-acetylmuramic acid (NAM) residues connected by a β-1,4 linkage (Schleifer and Kandler, 1972). The NAG-NAM strands are crosslinked by pentapeptide linkers capped by a D-alanine repeat. Drugs such as vancomycin bind directly to this D-alanine repeat, inhibiting crosslinking of peptidoglycan strands, and thus, cell wall synthesis (Reynolds, 1989). Other drugs, such as penicillin or ampicillin interfere with the enzymes responsible for cell wall synthesis, but do not bind directly to the peptidoglycan itself (Matsuhashi *et al,* 1974). Peptidoglycan strands are crosslinked in such a fashion as to allow the free diffusion of water and other molecules. Despite the large openings in the lattice, peptidoglycan remains rigid, and is able to serve as an anchor point for the major structural lipoprotein in the cell, murien lipoprotein (Lpp; Braun and Sieglin, 1970).

Outer membrane

The outermost layer of the *E. coli* cell envelope is the outer membrane (OM). The OM is an asymmetric bilayer consisting of an inner leaflet of phospholipids and an outer leaflet of lipopolysaccharide (LPS).

Its phospholipid content is roughly similar to that of the IM, although it is enriched somewhat for phosphatidylethanolamine (Nikaido, 1996). It is in constant direct contact with the cell's environment, and as such serves as the primary barrier between the cell and any noxious compounds found in the extracellular milieu. However, this presents a problem for the cell, as it must allow selective passage of nutrients in and waste products out. To accomplish this conflicting goal, *E. coli* has evolved several OM porins, chief among them OmpC and OmpF, to selectively allow the diffusion of nutrients into the cell. In addition, it possesses specific transporters such as the maltoporin LamB (responsible for the specific import of maltose and maltodextrins). Waste export is also achieved through the use of channel proteins such as TolC. Large hydrophobic molecules are excluded by the net negative charge conferred by the presence of LPS in the outer leaflet of the OM.

Lipopolysaccharide

LPS is found exclusively in the outer leaflet of the *E. coli* outer membrane. LPS is essential in *E. coli* (but not in some other Gram-negatives, such as *Neisseria meningitidis*) and is the immunodominant antigen in *E. coli* and most other gram-negative bacteria (Schnaitman and Klena, 1993). LPS is assembled sequentially from a group of phospholipids based upon glucosamine, beginning with the synthesis of Lipid A in the cytoplasm and later at the inner membrane (Trent, 2004, Doerrler and Raetz, 2002). Lipid A consists of a β-1'-6-linked disaccharide

of glucosamine, further modified by phosphorylation at the 1 and 4' positions, and acylated at the 2, 3, 2' and 3' positions. The 2'- and 3'-linked fatty acyl moieties are then esterified with laurate and myristate.

The next step in LPS synthesis is the assembly of the core oligosaccharide (OS). This is accomplished through a stepwise addition of sugars to Lipid A, and occurs at the cytoplasmic face of the IM. The core oligosaccharide can be subdivided into an proximal inner and distal outer core. To the 6' position of Lipid A, two molecules of 3-deoxy-D-*manno*-octulosonic acid (also known as Kdo) are added. This Lipid A-Kdo macromolecule constitutes the minimum required for *E. coli* survival; cells with the minimal LPS molecule exhibit a "deep-rough" phenotype, and are highly sensitive to hydrophobic antibiotics (Smit *et al,* 1975; Nikaido and Vaara, 1985). The next distal sugars of the inner and core are several heptose moieties. The composition of the outer core varies between strains, but in *E. coli* K-12 (used in this study) includes D-rhammose, D-glucose and D-galactose (Müller-Loennies, 2003). As with the assimilation of Kdo residues onto Lipid A, addition of the sugars of the core OS occurs at the cytoplasmic face of the IM.

In wild-type strains, the final step in LPS synthesis is the addition of the O-antigen. The O-antigen is transported across the IM along with Lipid A-core OS by the flippases Wzx and MsbA, respectively (Wang and Quinn, 2010). Evidence suggests that the protein LptB interacts with MsbA to provide the energy required to flip LPS from the cytoplasmic to

periplasmic side of the IM (Sperandeo *et al,* 2007). Two additional proteins, LptF and LptG, have recently been identified and proposed to be a part of a larger IM complex (including MsbA, LptB and LptC) required to flip LPS across the membrane (Ruiz *et al,* 2008; Sperandeo *et al,* 2008).

Once at the periplasmic face of the IM, the O-antigen is polymerized by the Wzy polymerase and ligated to the Lipid A-core OS molecule by WaaL, resulting in a nascent LPS molecule (Raetz and Whitfield, 2002). It is important to note that in *E. coli* K-12,van IS*5* insertion element disrupts O-antigen synthesis, and thus the K-12 LPS molecule terminates at the core OS (Liu and Reeves, 1994). In any case, LPS is carried from the IM through to the inner face of the OM by a process that is not yet fully understood. It is thought that the periplasmic protein LptA carries the newly assembled LPS molecule to the OM, where it could interact with the LptD (formerly Imp) and LptE (formerly RlpB) protein complex (Bos and Tommassen*,* 2004; Wu *et al,* 2006). In some manner, the LptDE complex facilitates the flip of LPS onto the outer surface of the cell. Interestingly, depletion of any of the proteins involved in LPS transport to the surface of the cell results in the accumulation of LPS molecules in non-native compartments of the cell (Sperandeo *et al,* 2006; Sperandeo *et al,* 2008), as well as novel modifications to the LPS (Ruiz *et al,* 2008), indicating that synthesis of LPS is uncoupled from its transport and that the proteins must function in a coordinated manner to transport LPS to the cell surface.

Proteins of the envelope

β-barrel OMPs

Most of the proteins resident in the OM assume a β-barrel conformation in their native structure. A notable exception is TolC, which has a unique α/β structure that is only manifest in its trimeric native conformation (Koronakis *et al,* 2000). β-barrel OMPs are comprised of between eight and twenty-two alternating anti-parallel β-sheets, arranged such that hydrophobic amino acids form the exterior of the mature barrel (in order to anchor securely in the OM), with mostly polar amino acids facing the interior (Tamm *et al,* 2001; Wimley, 2003). The end result of this conformation is an aqueous channel. At the interface of each side of the OM, OMPs tend to have aromatic amino acids, again to ensure secure anchoring in the OM. In general, OMPs have large, highly variable loops facing the exterior of the cell, with smaller loops facing the periplasm (Tamm *et al,* 2004). The external loops of OMPs often serve as receptors for various bacteriophages, such as Lambda (LamB), K20 (OmpF) and Tu1B (OmpC) (Randall-Hazelbauer and Schwartz, 1973; Traurig and Misra, 1999; Vakharia and Misra, 1996).

OMPs can be classified according to their function. For example, structural OMPs such as OmpA serve to stabilize the envelope (Henning *et al,* 1978), in part by anchoring the OM to the PG layer (Koebnik, 1995). Others, such as LamB and BtuB, serve as specific transporters for their ligands, in this case maltose/maltodextrins and vitamin B_{12} respectively

(Schirmer *et al,* 1995; Chimento *et al,* 2003). Some specific transporters actively transport their substrates across the OM. For example, FecA, FhuA and FepA are responsible for the transport of iron chelators (ferric citrate, ferrichrome and enterobactin, respectively) back into the cell from the environment (Andrews *et al,* 2003). As can be expected, active transporters require energy to function, which is transduced from the IM via the TonB system to the OM**.** OMPs such as OmpT function as enzymes in the outer membrane; OmpT is known to degrade antimicrobial peptides and serves as an effector of pathogenesis (Stumpe *et al,* 1998).

As mentioned above, TolC is a unique OMP, possessing a single β-barrel realized by the trimerization of three constituent monomers (as opposed to other β-barrel OMPs in which each monomer is its own barrel). Additionally, it possesses a large periplasmic channel domain comprised of long α-helices; again, three trimers must come together to form the mature protein. Once formed, this channel spans the periplasm, connecting to various efflux machineries to rid the cell of antiobiotics and also as a channel for the secretion of hemolytic enzymes (Koronakis *et al,* 2003).

Still other OMPs, exemplified by the porins OmpC, OmpF and PhoE, serve as general transporters between the external environment and the cell itself (Misra and Benson, 1988; Cowan *et al,* 1992; Overbeeke and Lugtenberg, 1980). The term porin is a generic one used to describe β-barrel OMPs with only semi-specific transport properties. Porins are the

dominant OMPs in *E. coli*, and of these the most abundant are OmpC and OmpF, which share approximately 60% homology (Mizuno *et al,* 1983). Three β-barrel monomers come together to form a complete porin trimer in the OM (Fig. 2). Biogenesis of OMPs is discussed below.

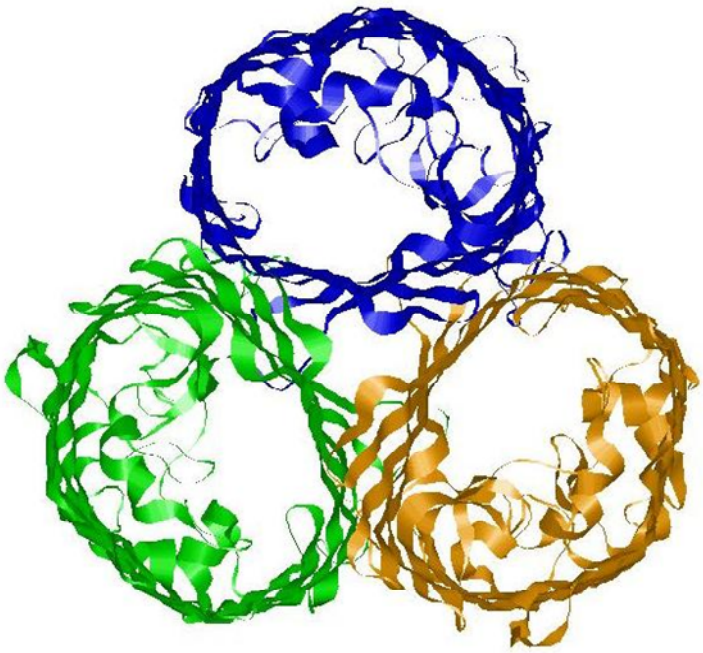


Fig. 2. Crystal structure of an OmpC trimer. PDB file 2J1N.

Porin synthesis must be carefully regulated in order to maintain a permeability profile appropriate to the environment the cell finds itself in. One aspect of this profile is mediated by the different properties of the two major OMPs, OmpC and OmpF. Although these porins are very similar, OmpF has a larger pore size (on the order of 1.2 nm), while OmpC is slightly smaller (around 1.1 nm; Nikaido, 2003). Under conditions of low osmolarity (such as could be found in waterways or soil) OmpF is

preferentially expressed, in order to maximize the diffusion of nutrients into the cell (Kawaji *et al,* 1979). However, OmpC is preferentially expressed under conditions of high osmolarity, such as is found in the intestinal tract of humans and other animals (Forst *et al,* 1989; Forst and Inouye, 1988). In this case, the cell needs to limit the amount of influx due not only to high salt concentrations, but also due to the presence of potentially harmful agents such as bile salts and other detergents. Regulation in this manner is primarily accomplished by the EnvZ/OmpR two-component signal transduction system, in which the IM-localized sensor kinase, EnvZ, responds to extracellular signals by autophosphorylating and transferring its phosphate to the cytoplasmic response regulator, OmpR. OmpR-P then binds directly to the promoter regions of *ompF* and *ompC*, modulating their expression as needed (Slauch and Silhavy, 1991).

Lipoproteins

Lipoproteins are defined as proteins anchored to either the IM or OM by a fatty-acylated cysteine residue at their amino-terminus. *E. coli* possesses over 90 lipoproteins of diverse functions, with most of them residing at the periplasmic face of the OM (Narita *et al,* 2004). Many lipoproteins are essential for cellular function, including BamD (Malinverni *et al,* 2006), LptE (Wu *et al,* 2005), and LolB (discussed below; Tanaka *et al,* 2001).

As with β-barrel OMPs, lipoproteins are synthesized in the cytoplasm and transported across the IM by the Sec machinery (Hayashi

and Wu, 1990). Unlike β-barrel OMPs, however, the signal peptide of lipoproteins is recognized and cleaved by LspA (signal peptidase II), which is specific to lipoproteins (Hussain *et al,* 1982; Yu *et al,* 1984). The signal sequence of lipoproteins has a consensus sequence near the cleavage site consisting of Leu-Ala/Ser-Gly/Ala-Cys (Hayashi and Wu, 1990).

After translocation and subsequent modification, lipoproteins are bound by the IM ATP-binding lipoprotein release complex consisting of LolCDE (Yakushi *et al,* 2000). LolCDE is responsible for segregating lipoproteins based on their ultimate destination, either in the IM or OM. Segregation is determined by the amino-terminal sequence of amino acids. Critically, this sequence adheres to the so-called "+2 rule," in which the amino acid directly after the fatty-acylated cysteine determines the localization of the lipoprotein. Lipoproteins with an aspartate residue in the +2 position are not released from LolCDE and segregate to the IM (Narita *et al,* 2002), while those with other amino acids in the same position localize to the OM (Gennity and Inouye 1991; Gennity *et al,* 1992).

Binding of lipoproteins destined for the OM to LolCDE increases the affinity of LolD for ATP, which is required for lipoprotein release from the complex (Yakushi *et al,* 1998). Interestingly, ATP binding causes a conformational change in LolD, decreasing the strength of the interaction between it and the bound lipoprotein (Ito *et al,* 2006). Because lipoproteins possess a hydrophobic domain in addition to their soluble

periplasmic domain, they cannot freely diffuse across the periplasm. The periplasmic lipoprotein transporter LolA must bind them for release from LolCDE and subsequent transport to the OM (Matsuyama *et al,* 1995; Tajima *et al,* 1998; Yakushi *et al,* 1998). Based on the crystal structure of LolA, it is thought that it binds lipoproteins using a binding pocket consisting of an open β-barrel capped by a lid comprised of three α-helices (Takeda *et al,* 2003).

After transport through the periplasm, the lipoprotein is transferred to the OM lipoprotein LolB, which mediates the proper insertion of lipoprotein into the OM (Matsuyama *et al,* 1997; Yokota *et al,* 1999). Cells cannot tolerate the absence of LolB; depletion of LolB results in the accumulation of lipoproteins in the periplasm, even in the absence of the major envelope lipoprotein, Lpp (Tanaka *et al,* 2001).

Protein secretion across the inner membrane

Protein translocation: the Sec translocon

As with all cellular proteins, the synthesis of OMPs begins in the cytoplasm. Nascent OMP polypeptides are targeted to the inner membrane secretory complex by their amino-terminal signal sequence, which consists of a positively charged segment, followed by a long hydrophobic series and ending with a short polar sequence capped by the signal peptidase recognition sequence Ala-X-Ala, where X is any amino acid (von Heijne, 1990). The nascent proteins are escorted to the IM by SecB, which holds them in a translocation-competent state (Weiss *et al,*

1988). At the IM, SecB transfers the polypeptide to the Sec translocation complex, consisting of SecYEG, of which the channel protein SecY and SecE are indespensible (Hanada *et al*, 1994). The accessory protein SecA hydrolyzes ATP from the cytoplasm and provides the energy needed to drive the OMP through the translocon (Karamanou *et al,* 1999). Once the signal sequence of the nascent OMP has been translocated across the IM, it is cleaved by the leader peptidase LepB at the periplasmic face of the IM (Dalbey, 1991). The OMP is then escorted to the OM by an as yet not fully understood mechanism.

Protein translocation: the SRP pathway

Proteins destined for residence in the IM generally target to the Sec translocon via the signal recognition particle (SRP) pathway. The SRP consists of a small protein, Ffh, and a 4.5S RNA (Luirink and Dobberstein, 1994). As they emerge from the ribosome, proteins with a highly hydrophobic signal sequence preferentially bind to the SRP, which in turn directs the proteins to a co-translational translocation pathway (Lee and Bernstein, 2001). After binding to the ribosome and newly translating protein, SRP interacts with the FtsY receptor protein to provide the GTPase activity necessary to transfer the protein to the SecYEG translocon (Powers and Walter, 1995). In this way, co-translational translocation proceeds and IM proteins are inserted laterally into the membrane (Urbanus *et al,* 2001; Rehling *et al,* 2003). In some cases, inserted proteins require the YidC protein for final assembly. It is thought

that YidC has two major functions: assisting in final folding of the transmembrane regions of substrate proteins (Dalbey and Kuhn, 2004) and clearing the channel of the translocon after an IM protein has been properly inserted (Samuelson *et al,* 2001).

Protein translocation: the TAT pathway

The twin arginine transport (TAT) translocon is utilized by proteins that must traverse the IM in a folded state. The system gets its name from the presence of two consecutive and conserved arginine residues in the signal sequence of translocated proteins. These arginines can be substituted with lysine (first residue) or with glutamine, asparagine or lysine (second residue), but efficiency of export is highly reduced (Stanley *et al,* 2000). Substitution of both arginine residues prevents export by the TAT system entirely (Stanley *et al,* 2000). Generally speaking, the TAT system is required for binding of redox substrates in the periplasm (Robinson and Bolhuis, 2001) although the TAT system can also be utilized for transporting substrates with cytoplasmic folding kinetics which are simply too rapid for the Sec system to accommodate (Lee *et al,* 2006).

The core TAT system consists of three IM proteins, TatABC. *E. coli* also possesses a fourth subunit, TatE, although it appears to have its origins as a genetic duplication of *tatA*, and seems to play only a minor role in the transport function of this system (Mangels *et al,* 2005). It has been shown that there are at least two states of the TAT complex; a resting state consisting of TatBC, and a post-substrate binding activated

state, which consists of TatBC and a recruited homooligomer of TatA. It is thought that TatC is the central factor driving assembly of the TatABC complex, while TatA functions to specifically stabilize TatB (Mangels *et al,* 2005). The role of TatB is not entirely clear, although it has been shown to bind to both the signal sequence and the amino-terminus of substrate proteins (Alami *et al,* 2003).

Proteins exported across the IM bind first to TatC in an energy-independent manner, and subsequently to TatB (Alami *et al,* 2003). After substrate binding, the TatBC-substrate complex binds to TatA homooligomer in an energy-dependent manner (the energy in this case being derived from proton motive force), and the substrate is translocated across the IM (Mori and Cline, 2002). Finally, the signal peptidase LepB (the same signal peptidase utilized by the Sec system) cleaves the signal peptide and the substrate is released into the periplasm (Yahr and Wickner, 2001).

Assembly of outer membrane proteins

After translocation into the periplasm, OMPs cannot simply traverse the aqueous environment on their own due to their composition of β-sheets. The exposure of large hydrophobic sections before membrane insertion would result in rapid aggregation as water was excluded from areas of hydrophobic interactions between nascent monomers. In order to combat this, cells have evolved numerous proteins to escort OMPs to their final destination in the OM. The process of escort and insertion is

depicted in Fig. 3.

Skp

The periplasmic chaperone Skp is a non-essential protein that, while a minor player, has been implicated in OMP biogenesis by several lines of evidence. Skp was discovered as a protein that was retained by sepharose-bound OmpF in an affinity column, and subsequently demonstrated that its absence conferred a general defect in OMP levels (Chen and Henning, 1996). Since then, focus has largely been on Skp's role on OmpA biogenesis as a model protein, although there is evidence that it acts on PhoE (Harms *et al,* 2001), OmpF/C and LamB (Chen and Henning, 1996), and the primary member of the OM assembly complex BamA (Qu *et al,* 2007). It is thought that Skp works by bringing OmpA protomers and LPS together just prior to OmpA insertion into the OM (Bulieris *et al,* 2003). Crystal structure analysis shows that Skp forms a trimer in solution (Schlapschy *et al,* 2004) resembling a jellyfish, with three "tentacles" (one from each monomer) forming a substrate binding pocket (Walton and Sousa, 2004; Korndorfer *et al,* 2004).

Skp is thought to bind unfolded OMP substrates in its central cavity through electrostatic and hydrophobic interactions (Qu *et al,* 2007). It is thought that hydrophobic interactions between Skp and its substrate are not enough for tight binding, and thus Skp must rely on an electrostatic mechanism to tightly bind substrate (Qu *et al,* 2009). Skp begins its interaction with OMP substrate soon after it emerges from the Sec

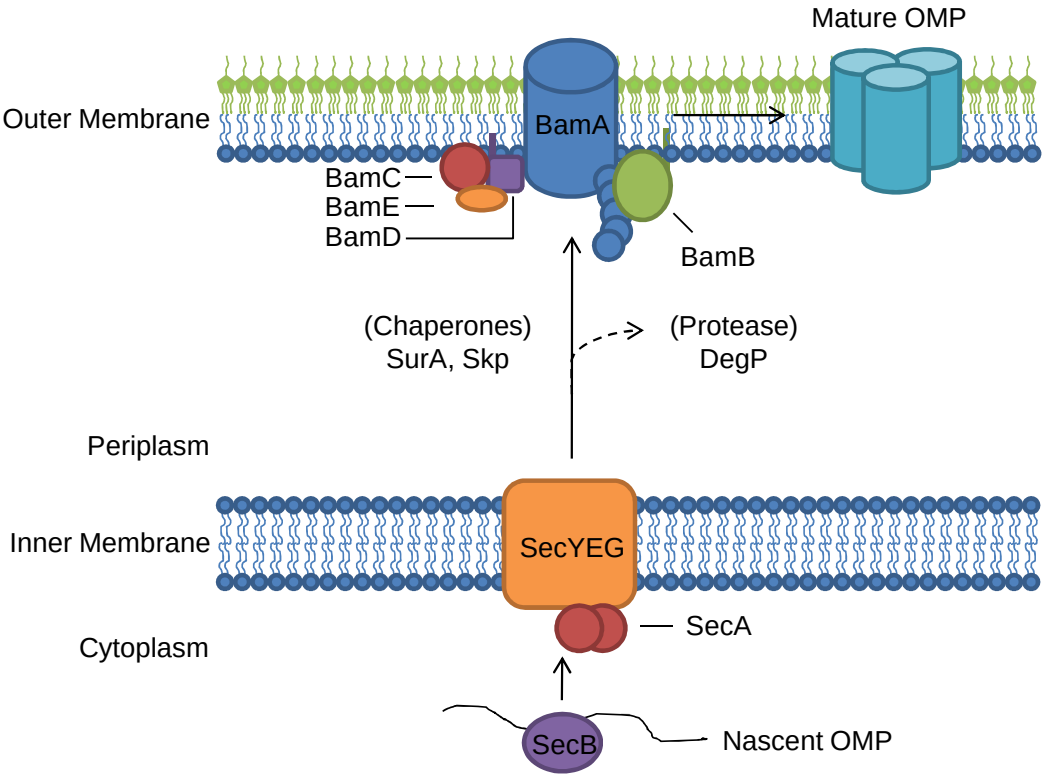


Fig. 3. Schematic depiction of OMP assembly.

translocon (Harms *et al,* 2001), and in the case of OmpA is required for the formation of a soluble folding intermediate after release from Sec (Schäfer *et al,* 1999).

SurA

Survival protein A (SurA) is one of the most studied periplasmic chaperones in *E. coli*. It is a member of the Parvulin peptidyl-prolyl *cis*/*trans* isomerase family (Rouviere and Gross, 1996), meaning it catalyzes the isomerization of an X-Proline peptide bond, where X is any amino acid, from *cis* to *trans* orientation. SurA binds to a consensus sequence of amino acids that is characteristic of OMPs, Aro-X-Aro-X-Pro,

where X is any amino acid and Aro is any aromatic amino acid (Bitto and McKay, 2003), and shows a binding preference for aromatic amino acids in general (Xu *et al,* 2007). However, the PPIase activity has been shown to be only a minor factor in SurA's role in envelope biogenesis, as its two parvulin-like domains can be removed without significantly affecting its activity (Behrens *et al,* 2001)

In addition to isomerase activity, the main function of SurA has been shown to be its chaperone activity in the biogenesis of the OM (Behrens *et al,* 2001). A disruption of *surA* results in decreased levels of the maltoporin LamB, OmpC and OmpA in the outer membrane (Lazar and Kolter 1996; Rouviere and Gross, 1996). However, this reduction in OMP levels is also due in part to downregulation at the post-transcriptional level by small RNAs during the σ^E envelope stress response (Rouviere and Gross, 1996; discussed below), which is turned on in *surA* mutants. Due to its central role in the proper folding of OMPs, cells lacking *surA* have increased sensitivity to compounds normally excluded from an intact OM, including bile salts, sodium dodecyl sulfate (SDS) and hydrophobic antibiotics (Lazar and Kolter, 1996). Recently, SurA has been shown to interact with the outer membrane assembly protein BamA, presumably to pass substrate to the assembly machinery (Bennion *et al,* 2010, Vuong *et al,* 2008). The precise nature of this interaction is an area of intense interest.

DegP

DegP is the primary protease resident in the periplasm, responsible for degradation of aberrant proteins in the cell envelope. It is a serine protease, utilizing an active catalytic site triad consisting of His105, Asp135 and Ser210 (Lipinska *et al,* 1989). Following the amino-terminal protease domain, it possesses two PDZ domains, often used to bind carboxy-terminal substrate residues (Iwanczyk *et al,* 2007). It is generally believed that the PDZ domains function as gates to direct substrates to the active site (Krojer *et al,* 2002). Recently, the crystal structure of DegP has been solved, and the data suggest that it exists in an inactive form as a hexamer, oligomerizing into two active forms, a 12-mer and a 24-mer (Krojer *et al,* 2008). The active forms of DegP posess a large inner cavity. Amazingly, entire monomers of OMPs have been found inside this cavity in crystal structure (Krojer *et al,* 2008). It is not entirely clear whether these monomeric OMPs are in the process of degradation or if they are being sequestered by the chaperone function of DegP. Nevertheless, the finding lends strong support to the idea that DegP is directly involved in OMP biogenesis.

DegP has long been known to be required for *E. coli* growth at elevated temperatures, presumably because of the increased need for processing of envelope protein intermediates (Strauch *et al,* 1989). Interestingly, at low temperatures DegP has been shown to behave as a molecular chaperone, but at elevated temperatures exhibits almost

exclusively protease activity (Spiess *et al,* 1999). In that study, only a protease-deficient mutant of DegP carrying an S210A mutation was able to refold substrate protein at high temperatures. Later studies showed that $DegP_{S210A}$ alleviates envelope stress caused by misfolded OmpC and OmpF by capturing, rather than degrading the proteins (Misra *et al,* 2000; Castillo-Keller and Misra, 2003).

Evidence suggests that DegP works in concert with Skp in a pathway separate from the main, SurA driven, OMP assembly pathway. In such a model, DegP serves a quality control function in the envelope by attempting to refold OMPs which have fallen off the main assembly pathway, degrading those that cannot be refolded and assembled properly (Rizzitello *et al,* 2001; Sklar *et al,* 2007). In addition to its quality control function, DegP has been shown to participate in the activation of the CpxAR stress response (discussed below) by degrading the negative regulator, CpxP, when CpxP binds unfolded substrate in the periplasm (Isaac *et al,* 2005; Buelow and Raivio, 2005).

Other assembly factors

In addition to Skp, SurA, and DegP, other proteins residing in the periplasm have been implicated in the process of envelope biogensis. For example, the PPIase FkpA was isolated as a multicopy suppressor of OMP assembly defects caused by the simultaneous lack of *degP* and *dsbC*, a protein responsible for disulfide bond formation in the periplasm (Missiakas *et al,* 1996). It was later shown that FkpA possesses

chaperone activity on the non-native substrate citrate synthase (normally residing in the cytoplasm; Buchner *et al,* 1998), and later still that it can overcome aggregation of MalE31, a mutant form of periplasmic MalE (Arié *et al,* 2001).

Other folding factors such as PpiA and PpiD have been implicated in the assembly of OMPs, although their exact roles are not entirely clear. PpiA has been shown to be positively regulated by the Cpx system (Pogliano *et al,* 1997) and its PPIase activity *in vitro* is beyond doubt (Liu and Walsh 1990; Hayano *et al,* 1991), yet *in vivo* its effects seem to be negligible (Kleerebezem *et al,* 1995). PpiD was isolated as a multicopy suppressor of Δ*surA* mutants, and as with *surA* mutations confers a reduction in OMPs in the OM (Dartigalongue and Raina, 1998). It was shown to have somewhat overlapping substrate recognition sites with SurA, but with a somewhat larger set of predicted substrate sequences (Stymest and Klappa, 2008). Interestingly, it was very recently shown that, although PpiD contains a parvulin-like domain, its PPIase activity *in vitro* is undetectable despite the fact that it readily binds to substrate (Weininger *et al,* 2010). This is in direct contradiction to an earlier report (Dartigalongue, and Raina 1998) that showed high PPIase activity. The exact reason for this discrepancy is not known at this time.

Finally, the periplasm is home to many proteins which require disulfide bond formation in order to assume their correct native structure. To accomplish this, cells utilize DsbA, responsible for catalyzing disulfide

bonds between the sulfhydryl groups of pairs of cysteine residues (Bardwell *et al,* 1991). DsbA is a member of the thioredoxin family of proteins, possessing a Cys-X-X-Cys motif, where X is any amino acid; the sequence in DsbA consists of Cys-Pro-His-Cys (Akiyama *et al,* 1992). The structure of the active site is critical to the function of DsbA, as mutations in either proline or histidine result in a decrease in oxidative activity (Grauschopf *et al,* 1995). After transferring electrons from its active site cysteines to those of the substrate (thus oxidizing it), DsbA must be re-oxidized from the reduced state to catalyze another reaction. The IM protein DsbB accomplishes this through the reduction of quinines (Bardwell *et al,* 1993; Missiakas *et al,* 1993; Kobayashi *et al,* 1997).

Catalysis of disulfide bonds by DsbA does not always occur between the correct cysteine residues in the substrate protein, and some bonds may thus require isomerization to correct (Rietsch *et al,* 1996). The periplasmic protein DsbC is another member of the thioredoxin family, and recognizes misfolding caused by incorrectly oxidized cysteine pairs (Missiakas *et al,* 1994). In addition to its capability to isomerize these bonds, it also possesses general chaperone activity for misfolded proteins (Chen *et al,* 1999; Denoncin *et al,* 2010). DsbC's cysteine pair must be kept in a reduced state in order to be oxidized by the incorrectly bonded substrate cysteines. It is kept in this state by the IM protein DsbD, which functions to shuttle electrons from the cytoplasm to DsbC and other substrate proteins in the periplasm (Rietsch *et al,* 1997; Katzen and

Beckwith, 2000).

Assembly and insertion at the outer membrane

The final phase of OMP assembly is the process by which a nascent OMP is folded and inserted in the OM in its native tertiary or quaternary structure. After transit through the periplasm, nascent OMPs are transferred to the Beta-barrel Assembly Machinery (BAM), comprised of BamABCDE (Wu *et al,* 2005). The exact nature of this process is still mysterious, with several competing models for BAM assembly/insertion in the literature. However, much has been learned recently regarding the five BAM members discovered to date.

The core of the BAM complex is the integral OMP BamA (formerly YaeT). Originally identified as Omp85 in *Neisseria*, it is highly conserved in Gram-negative bacteria, and is essential in *E.* coli (Volhoux *et al,* 2004). BamA homologs are found in widely varying organisms, including human mitochondria (Kutik *et al,* 2009; Tommassen 2010). Although BamA has not been crystallized, the crystal structure of its distant homolog in *Bordatella pertussis*, FhaC has been solved, and suggests that BamA assumes a β-barrel conformation in the OM (Clantin *et al,* 2007). BamA possesses five tandem polypeptide transport associated (POTRA) motifs, which are thought to bind substrate protein through β-augmentation (Knowles *et al,* 2009). The amino-terminal POTRA domain (POTRA 1) is not required for *E. coli* viability, and while such mutants have severely depleted levels of LamB and OmpF, levels of TolC in the OM remain

unaffected, suggesting a divergent assembly pathway for the two types of OMPs (Bennion *et al,* 2010). The exact mechanism of interaction between OMPs and the POTRA domain of BamA is not known, although there is evidence that SurA may play a role in at least some cases (Bodelón *et al,* 2009).

The other four proteins comprising the BAM complex are all lipoproteins. Of these, BamB (formerly YfgL) has been the most studied to date. BamB interacts directly with BamA, and this interaction is independent of BamCDE (Vuong *et al,* 2008). While not crystallized, analysis of related proteins suggests that it takes an eight-bladed β-propellor conformation (Gatsos *et al,* 2008). This class of proteins has widely varying functions, among them scaffolding, protein-protein interaction and ligand binding (Jawad and Paoli 2002). It is predicted that the BamA-BamB interaction takes place between BamA's POTRA domain and a β-propeller fold in BamB.

BamB plays a critical role in the proper assembly of OMPs, and strains lacking *bamB* display pleiotropic phenotypes. For example, *bamB* null mutants have an assembly defect in OMP biogenesis (Ruiz *et al,* 2005; Charlson *et al,* 2006), which is exacerbated when combined with a *surA* deletion. Similarly, a *bamB degP* double mutant is synthetically lethal at elevated temperatures and rich media (Charlson *et al,* 2006). These two observations can be reconciled by envisioning a system wherein SurA and BamB function to properly fold and insert OMPs. Under

normal circumstances, DegP degrades accumulating misfolded OMPs in a *bamB* or *surA* background, but when *degP* is also lacking folding intermediates build up and are deleterious to the cell, possibly by interfering with normal BamA function. Recently, it was found that reconstituted BAM is much more efficient at *in vitro* refolding of OmpT substrate in the presence of BamB than without it, showing BamB's importance in the function of the complex (Hagan *et al,* 2010).

Cells lacking *bamB* are hypersensitive to antibiotics, reflecting a defect in the permeability barrier function of the OM (Ruiz *et al,* 2005). In the pathogenic *E. coli* strain LF82 (a contributor to Chron's disease), when *bamB* is deleted from its chromosome the bacterium is highly attenuated with respect to its ability to invade intestinal epithelial cells (Rolhion *et al,* 2005). When cells are simultaneously deleted for *bamB* and *degP*, they become extremely temperature sensitive (TS) as well as hypersensitive to antibiotics (Charlson *et al,* 2006). Paradoxically, BamB has been reported to be involved in homologous recombination as a kinase (Khairnar *et al,* 2007), although this is difficult to reconcile with 1) BamB's cellular localization and 2) the lack of nucleoside triphosphates in the periplasm of *E. coli*. Furthermore, cells lacking *bamB* show no reduction in recombination over their wild-type counterparts when measured by efficiency of P1 transduction (Vuong *et al,* 2008).

BamC (formerly NlpB) is the third member of the BAM complex. Its function in the complex is not well-established, and indeed is not

conserved throughout Gram-negative bacteria. It is known that BamC interacts with BamA, and that this interaction is dependent upon BamD (Malinverni *et al,* 2006). Cells lacking BamC have minor defects in cell permeability (specifically to the antibiotic rifampin), but are fully viable and their ability to assemble OMPs is not compromised (Onufryk *et al,* 2005; Wu *et al,* 2005). Interestingly, a synthetic phenotype is observed in *bamC surA* double mutants, similar to the phenotype observed in *degP surA*, suggesting that BamC and DegP operate in the same or overlapping pathway(s) (Onufryk *et al,* 2005).

The only other essential protein in the BAM complex besides BamA is the lipoprotein BamD (formerly YfiO; Onufryk *et al,* 2005). It is required for BAM complex stability, mediating contact between BamA and Bam C, and it is possible that this explains why it is essential in *E. coli* (Malinverni *et al,* 2006). Cells harboring an insertion in the 3'-region of *bamD*, which does not completely disrupt its function, display similar phenotypes to *bamB* null mutants, implying that the two proteins are functionally related (Wu *et al,* 2005). In agreement with this, *bamD bamB* double mutants cannot be created. BamD is predicted to have a series of tetratricopeptide repeat (TPR) motifs, which are generally associated with protein-protein interactions (D'Andrea and Regan, 2003; Gatsos *et al,* 2008). Although *bamD* is an essential gene in *E. coli*, it can be depleted from the cell, resulting in a significant reduction in both LPS-dependent and -independent OMPs as a function of time until eventual cell death

(Malinverni *et al,* 2006). Furthermore, the depletion of BamD from the cell envelope causes an increase in DegP levels in the periplasm, suggesting that the stress in the system triggers the σ^E envelope stress response system.

The final protein of the BAM complex was only recently discovered. BamE (formerly SmpA) is not essential in *E. coli*, and interacts directly with BamA (Sklar *et al,* 2007). Cells lacking *bamE* display only moderate reductions in steady-state LamB and OmpA levels, antibiotic sensitivity, and induction of the σ^E response. However, BamE does affect the stability of the complex, and synthetic phenotypes manifest themselves in double mutants between *bamE* and any other member of the complex (Sklar *et al,* 2007). It also appears as though it is required for stable BamD-BamA interaction. The BamE homolog, Om1A, in *Xanthomonas axonopodis* has been crystallized, revealing a POTRA-like structure which suggests the ability to bind to nascent OMPs as they interact with the BAM complex (Vanini *et al,* 2008).

Envelope stress response

Under normal physiological circumstances, a small fraction of OMPs falls off the assembly pathway(s) discussed above, and is quickly dealt with through refolding by chaperones or degradation by DegP. However, there are times in which the mechanisms normally in place to deal with these events are unable to cope with the stress. For example, cells may lack critical components such as DegP or SurA, they may

express mutant forms of OMPs, LPS, or both. In this case, *E. coli* has evolved several response systems to alleviate the envelope stress.

Cpx

The Cpx response is mediated by the two-component system consisting of the histidine kinase CpxA residing in the IM and the periplasmic response regulator CpxR (Weber and Silverman, 1988; Jianming *et al,* 1993). The Cpx system is activated in response to a diverse set of envelope stressors, including high pH (Nakayama and Watanabe, 1995), misfolded pilin subunits (Jones *et al,* 1997), overexpression of certain lipoproteins (Snyder *et al,* 1995; Miyadai *et al,* 2004), perturbation of phospholipids in the OM (Mileykovskaya and Dowhan, 1997), attachment to surfaces (Otto and Silhavy, 2002), or misfolded OMPs (Gerken *et al,* 2010).

CpxA possesses a periplasmic sensing domain, connected to a cytoplasmic catalytic domain by two transmembrane helices (Weber and Silverman, 1988). In response to stress, it autophosphorylates at H249, the conserved histidine residue common to histidine kinases (Raivio and Silhavy, 1997). CpxA's phosphotransferase activity then phosphorylates CpxR at a conserved aspartate residue (Raivio and Silhavy, 1997). CpxR-P is the active form of the protein, and drives transcription of a regulon of over 50 genes, including genes involved in OMP synthesis and assembly, motility and chemotaxis, adhesion, and virulence (Price and Raivio, 2009). Notably, CpxR-P increases the transcription of *degP*, *ppiA*, *ppiD*, and

dsbA (Buelow and Raivio, 2005), and indeed increased transcription of *degP* appears to be one of the chief functions of the Cpx response (Gerken *et al,* 2010).

Autophosphorylation of CpxA can occur by one of two pathways. The first pathway is mediated by direct binding of misfolded protein to CpxA (Keller and Hunke, 2002). Alternatively, under conditions of envelope stress, the periplasmic inhibitor protein CpxP is titrated away from CpxA by substrate protein, and is degraded along with the substrate by DegP (Isaac *et al,* 2005). However, CpxP is not required for full activation of the Cpx pathway; in the absence of *cpxP* the regulon can still be fully induced by CpxAR (DiGiuseppe and Silhavy, 2003). The transcription of *cpxAR* is autoregulated, and is increased under conditions of elevated CpxR-P (Raivio *et al,* 1999). As an added level of control, transcription of *cpxP* is increased during the Cpx stress response (Danese and Silhavy, 1998; Raivio *et al,* 1999; Buelow and Raivio, 2005).

Sigma E

The other major response to envelope stress is mediated by the alternate sigma factor, σ^E (gene name *rpoE*; Erickson and Gross, 1989). Sigma E has classically been recognized to respond to misfolded outer membrane proteins (Walsh *et al,* 2003), but has also been shown to be activated by the accumulation of lipid A intermediates and structurally modified LPS molecules (Tam and Missiakas, 2005), as well as by entry into stationary phase (Kabir *et al,* 2005) through the signaling molecule

guanosine 3',5'-bispyrophosphate (ppGpp; Costanzo and Ades, 2006; Costanzo *et al,* 2008). In *Salmonella typhimurium* the σ^E response can be activated by acid pH in the extracellular environment (Muller *et al,* 2009); this is corroborated by the fact that in *E. coli* the pathway is involved in adhesion and invasion of the human intestinal lining, a normally acidic environment (Rolhion *et al,* 2007).

σ^E is essential for viability in *E. coli*. The reason for this is not clear, but is likely because of the critical barrier function of the envelope (Hayden and Ades, 2008). For example, members of the BAM complex (including the essential *bamA*) are positively regulated by the response, presumably in an attempt to assemble as many OMPs as possible to restore the integrity of the OM (Rhodius *et al,* 2006).

The σ^E regulon includes a diverse set of genes responsible for envelope maintenance, and often overlaps with that of the Cpx pathway (Raivio and Silhavy, 1999). One of the critical similarities is that both pathways drive transcription of *degP*, in order to lower the levels of misfolded OMPs through refolding or, failing that, through degradation (Lipinska *et al,* 1988; Danese *et al,* 1995). Despite the similarities between the Cpx and the σ^E responses, the diversity in activating signals indicates that the systems respond to envelope stressors in at least some distinct ways. Indeed, the Cpx system is known to downregulate the σ^E response, suggesting an independent role for each (de Wulf *et al,* 2002).

Other important regulon members are sRNAs, which function in a

post-transcriptional manner to bind the 5'-untranslated region of OMP mRNA, often occluding the Shin-Delgarno sequence, and trigger sRNA-mRNA complex degradation (Massé *et al,* 2003). Members of this group include *rybB*, *micA*, *rseX,* and *omrAB* (Papenfort *et al,* 2006; Johansen *et al,* 2006; Douchin *et al,* 2006; Guillier and Gottesman, 2008; Thompson *et al,* 2007; Bossi and Figueroa-Bossi, 2007; Overgaard *et al,* 2009). As is the case with many RNA-RNA interactions, regulation of OMP mRNA by sRNAs requires the presence of the RNA chaperone Hfq (Figueroa-Bossi *et al,* 2006; Guisbert *et al,* 2007).

Under normal physiological conditions, σ^E is sequestered at the cytoplasmic face of the IM by the anti-sigma factor RseA (De Las Peñas *et al,* 1997; Campbell *et al,* 2003). Two other proteins, RseB and RseC, reside near RseA in the IM, although their exact physiological roles are unclear and in dispute (Missiakas *et al,* 1997; De Las Peñas *et al,* 1997; Collinet *et al,* 2000; Dartigalongue *et al,* 2001). The response is triggered by the presence of unfolded OMPs, specifically the sequence Tyr-Gln-Phe located at the carboxy-terminus and which is normally sequestered in the folded structure of the protein (Walsh *et al,* 2003). These are bound by the PDZ domain of the essential protein DegS at the periplasmic face of the IM (Alba *et al,* 2001).

Substrate binding by DegS' PDZ domain causes a conformational change in the protein, and triggers a proteolytic cascade termed regulated intramembrane proteolysis (RIP; Alba *et al,* 2002). DegS cleaves RseA in

the periplasm near the IM, which subsequently allows another protease, RseP, to cleave RseA further in the IM, releasing an RseA-RpoE complex into the cytoplasm (Alba *et al,* 2002). Once in the cytoplasm, the remaining RseA is fully degraded by the Clp protease, liberating σ^E and allowing it to drive transcription of its target regulon (Flynn *et al,* 2003).

Like the Cpx system, the σ^E-mediated envelope stress response is controlled by an autofeedback loop. In the short-term, the signal is amplified because *rpoE* is positively regulated during the response. However, the anti-sigma factor *rseA* is also positively regulated, providing a necessary long-term shutoff mechanism for when the cell needs to return to homeostasis (Rhodius *et al,* 2006). Cells lacking *rseA* are able to overcome lethality normally associated with the lack of either *rseP* or *degS* by uncoupling σ^E activity from any regulation (Alba *et al,* 2001).

Vesiculation

It has been known for some time that Gram-negative cells produce outer membrane vesicles (OMVs; Sonntag *et al,* 1978; Bernadac *et al,* 1998). OMVs range in size from 10 to 200 nm in diameter, and in addition to the constituents of the OM they contain periplasmic proteins (Mashburn-Warren *et al,* 2008; Deatherage *et al,* 2009). They are utilized by bacteria in signaling (Mashburn and Whitelay, 2005; Mashburn-Warren *et al,* 2008; Mashburn-Warren *et al,* 2009) and as effectors of virulence (Wai *et al,* 2003; Balsalobre *et al,* 2006; Kesty *et al,* 2004; Chitcholtan *et al,* 2008). OMVs have also been of interest as vaccine delivery vehicles

and as a tool for antibiotic therapy (Kadurugamuwa and Beveridge 1996; Henry *et al,* 2004; Kim *et al,* 2008).

Recently, it was discovered that cells possess the ability to respond to stress through the release OMVs (McBroom and Kuehn, 2007). This stress response is independent of other known stress responses, and functions as a bulk removal process of stress-causing envelope components. Vesiculation in Gram-negative bacteria can be induced in cells lacking members of the structural Tol-Pal complex (Bernadac *et al,* 1998), in cells lacking *degS* or *degP* (McBroom and Kuehn 2007), and in the presence of misfolded proteins or modified LPS (Kadurugamuwa and Beveridge 1997; Nguyen *et al,* 2003; McBroom and Kuehn 2007). Interestingly, the level to which cells vesiculate is not correlated with the level of stress in the cell as measured by the instability of the OM (McBroom *et al,* 2006). Indeed, even wild-type cells produce OMVs, albeit at very low levels.

It appears as though vesiculation does not occur by the random flow of envelope proteins into OMVs. Cells which overexpress periplasmic cytochrome b_{562} fused to the carboxy-terminus of OmpC and ending in the sequence Tyr-Tyr-Phe are known to have an upregulated σ^E response, because the fusion mimics a misfolded OMP intermediate (Walsh *et al,* 2003). OMVs from these cells are enriched for the construct over another periplasmic protein, MalE (McBroom and Kuehn 2007), suggesting a specific packaging mechanism. However, no mechanism has yet been

elucidated to explain this preferential packaging.

Rationale and goals of this work

The periplasmic protease *degP* is essential for growth at high temperatures and/or in the presence of intrinsically misfolding OMPs (Strauch *et al,* 1989; Misra *et al,* 2000). Despite this, little is known about the exact effects of *ΔdegP* on the cell envelope. This work describes the effects on the cell envelope when grown under nonpermissive conditions, including the striking association of cytoplasmic chaperones with the envelope under conditions of stress.

Cells can overcome lethality associated with the lack of *degP* by several ways. Studies showing this have helped to elucidate the inner workings of *E. coli* in terms of envelope biogenesis and stress response. For example, induction of the Cpx and σ^E stress response systems by the loss of *cyaA* and subsequent slowing of growth rate have provided clues as to not only the interplay between metabolite signaling and the envelope, but also the kinetic nature of stress (Strozen *et al,* 2005). Another group has shown that a disruption in the major murien lipoprotein, *lpp*, disrupts the interaction between peptidoglycan and the OM, allowing periplasmic contents to leak out into the environment, thus suppressing the defect caused by the lack of *degP* (Strauch *et al,* 1989). In a general manner, cells can overcome the *degP* defect by constitutively activating either the Cpx (Danese *et al,* 1995) or the σ^E (Waller and Sauer, 1996) pathways by a gain of function *cpxA** mutation or through an activated

DegS protein, respectively. Finally, SohA was isolated as a suppressor of *degP*-mediated lethality at high temperatures, although as a side effect cells are rendered cold-sensitive (Baird and Georgopoulos, 1990).

Previous students in the laboratory have utilized the TS phenotype of Δ*degP* cells to isolate suppressors of lethality. For example, cells harboring the lethal combination of Δ*degP* and $ompC_{501}$ can be rescued by overexpression of truncated *ompR* or by the sRNA *ipeX* (Castillo-Keller *et al,* 2006). In the same study, the novel protein *yfgC* was also isolated as a multicopy suppressor of lethality. Work presented here characterizes YfgC and its mechanism of action. Single-copy mutations in the chromosome can also abrogate the need for *degP*. Another student isolated such suppressors, some of which mapped in *rseA* and *envZ* (Gerken, 2009). One of these suppressors possessed the unique quality of overcoming lethality without a significant reduction of major OMPs. This study presents the characterization of the mutation and its effects on envelope stress response.

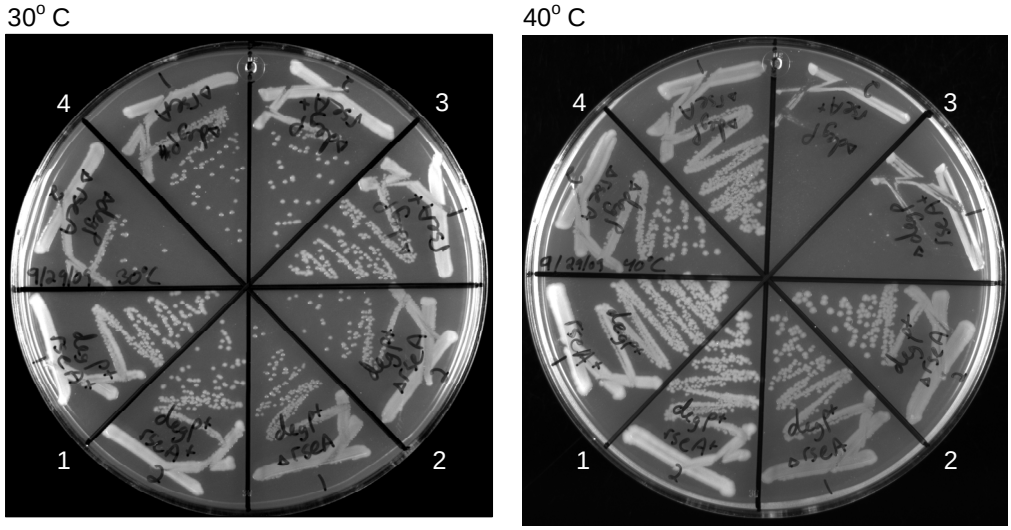
Chapter 2

RESULTS I: ENVELOPE STRESS CAUSED BY THE LACK OF DEGP

Phenotype of cells lacking *degP*

The Misra laboratory investigates the various mechanisms by which *E. coli* generates and maintains its cell envelope. Outer membrane protein (OMP) assembly can follow one of two basic pathways: a nascent OMP may fold properly and be inserted into the outer membrane (OM), or in some cases may fall off the assembly pathway and be subject to degradation.

The primary means by which the cell degrades aberrantly folded proteins is through the DegP protease. In cells lacking *degP*, misfolded proteins accumulate and cause significant cellular stress, including the observation that at 40°C, these cells are unable to form single co lonies (Fig. 4, right panel). Constitutively activating the σ^E stress response by removing its negative regulator, RseA, restores growth through the global downregulation of OMP synthesis. In this context, it has been demonstrated that the temperature sensitive (ts) phenotype in cells lacking *degP* can be overcome simply by removing *ompC* (Gerken, 2009). The reasons for such a severe *ts* phenotype in Δ*degP* cells are not clear at this time, although it is speculated that the cells are unable to cope with accumulated misfolded proteins because all available chaperones are titrated away from their normal functions, or that the accumulation of intermediates interferes with assembly at the BAM complex.



1. WT
2. Δ*rseA*
3. Δ*degP*
4. Δ*rseA* Δ*degP*

Fig. 4. Growth defect of *ΔdegP* cells on solid media. Cells lacking *degP* were streaked at 30°(left panel) and 40°C (right pa nel) and grown overnight. *TS* defect is rescued in *rseA degP* double mutants.

To further investigate the phenotype caused by the lack of *degP,* cells harboring an in-frame chromosomal deletion of *degP* were grown at 39°C. This temperature for the assay was chosen becau se it is just below the nonpermissive temperature for these cells – they are able to grow, yet still display a growth defect (Fig 5A), growth at a slower rate and to a lower cell density than wild-type. Additionally, there is an approximately five-fold reduction in viability when *ΔdegP* cells grown at 39°C were plated on solid medium at the permissive t emperature of 30°C (Fig. 5B).

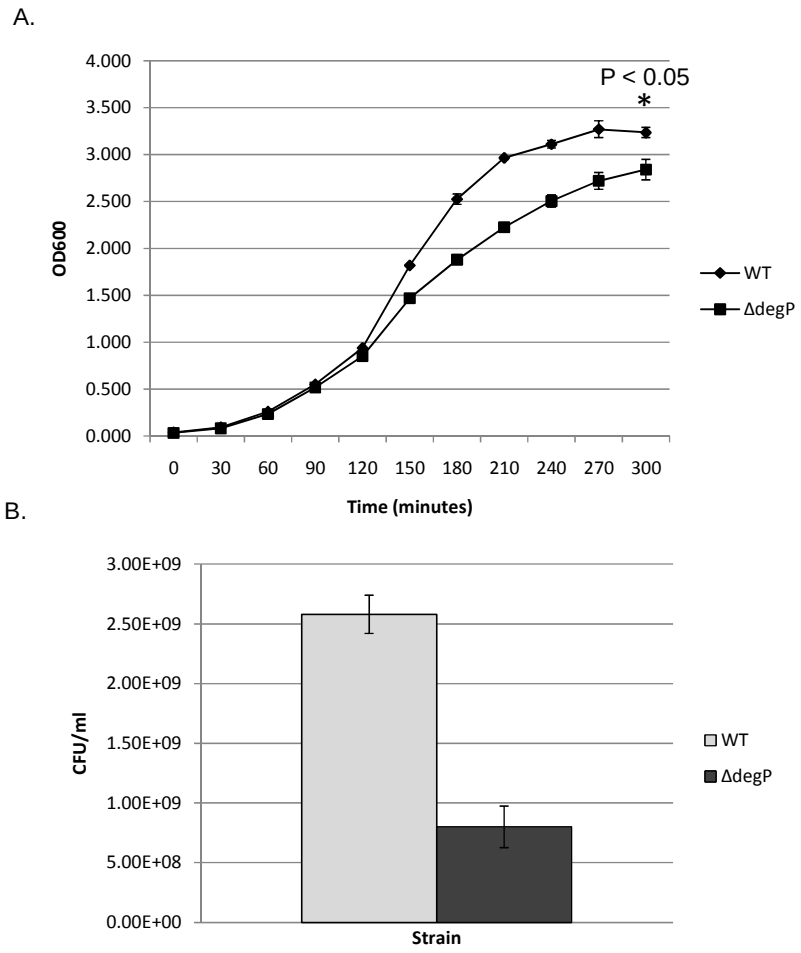


Fig. 5. Growth defect in Δ*degP* cells in liquid media. A. Cells lacking *degP* display a growth defect over time in LB at 39°C. B. *ΔdegP* cells plated at final time point in A assayed for viability after subsequent plating onto LBA at 30°C.

It was then asked whether this growth defect in liquid media could be rescued by returning Δ*degP* cells to a permissive temperature. Cells were subcultured from overnight cultures to a starting OD_{600} of 0.025 in prewarmed LB and grown at the non-permissive temperature for 2.5 hours. At this point, Δ*degP* cells had begun to show the expected growth defect (Fig. 6). Cells were then diluted to OD_{600} of 0.025 under one of two

conditions: LB prewarmed to either 33°C or 39°C. Growth was resumed for another 2.5 hours. At either temperature tested, wild-type cells were able to resume normal growth. However, cells lacking *degP* were only able to recover growth when diluted into media at the permissive temperature. At the non-permissive temperature, Δ*degP* cells displayed a severe defect in resuming growth. Thus, the growth defect of Δ*degP* cells is not a permanent state; when the thermal stress is removed cells are capable of recovery.

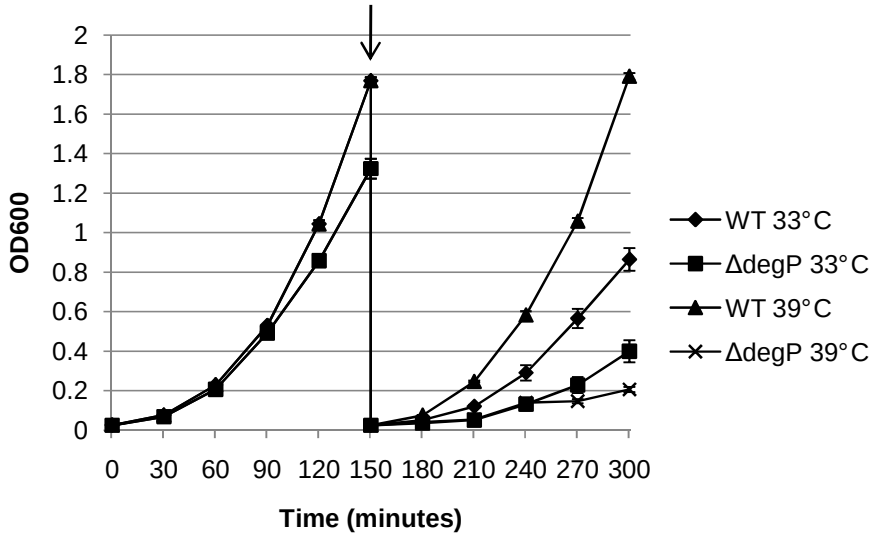


Fig. 6. *DegP* growth defect can be rescued by lowering temperature. Wild-type and Δ*degP* cells were grown at 39°C until the time indicated by the arrow. Cultures were split and growth continued at either elevated (39°C) or recovery (33°C) temperatures.

Analysis of cell envelopes from Δ*degP* cells

Appearance of novel proteins in the envelope

Since the ultimate effect of the absence of DegP in the cell envelope is

manifested in the envelope, cell envelopes were isolated and examined for any differences. Cells were grown at 39°C for five hours, pelleted and lysed by the French Press method. Extracted membranes were finally subjected to extraction with cold 100 mM sodium carbonate. This treatment relies on the high alkalinity of sodium carbonate to strip proteins which are not firmly rooted in a membrane; that is, those that are not integral membrane proteins (Molloy *et al,* 2000). While the process does not specifically enrich for OMPs, it does preferentially strip proteins with high α-helical content (the majority of IM proteins and the minority of OM proteins), thus providing a convenient method to clarify crude membrane preparations to make them more amenable to analysis. Interestingly, even though cells lack the chief player in envelope protein degradation, the steady-state levels of the major OMPs, OmpC and OmpA remained largely unchanged (Fig 7).

Consistently, carbonate-treated membranes from *ΔdegP* cells contained considerable background when stained with coomassie blue after SDS polyacrylamide gel electrophoresis (SDS-PAGE), even though loading was normalized to cell density, suggesting that there was more overall protein present in the membranes of these cells. Several unique protein bands were present in envelopes isolated from Δ*degP* cells. In particular, two prominent bands with molecular mass slightly greater than OmpC (molecular weight 39 kD) strongly associated with the carbonate-insoluble fraction of the membranes in *ΔdegP* cells. The two proteins had

a molecular mass of ~55 kD and ~45 kD (Fig. 7, bands 1 and 2).

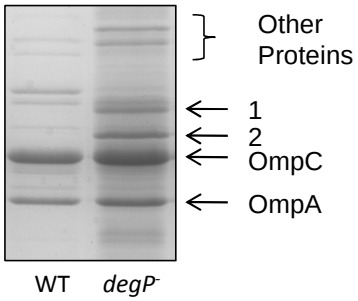


Fig. 7. Examination of envelope proteins in Δ*degP* cells. Membranes were extracted with sodium carbonate, analyzed by SDS-PAGE and stained with coomassie blue. Novel protein bands are indicated by numbered arrows.

Novel proteins are sarcosyl soluble

Membrane proteins can have differential solubility properties when treated with different types of chemicals. Using the anionic detergent sarcosyl, the extent to which the novel proteins had associated with the membrane was investigated. Membranes were incubated with sarcosyl (final concentration 2%) or 10 mM Tris pH 7.5 for thirty minutes at room temperature. Under these conditions, integral OMPs remain insoluble, while inner membrane proteins and peripheral proteins are extracted. After incubation, samples were centrifuged at 100,000 x g to separate soluble and insoluble fractions. As before, membranes prepared from *degP*$^{+}$ cells did not contain significant amounts of the novel protein bands (Fig. 8, lanes 2 and 4). When incubated with non-extracting Tris, membranes from cells lacking *degP* still contained the unknown associating proteins in the insoluble fraction (Fig. 8, lane 6). Conversely,

when extracted with sarcosyl, the proteins primarily localized to the soluble fraction (Fig. 8, lane 7) indicating that they had not fully integrated into the membrane, whereas OmpA and OmpC (as expected) remained insoluble. These results show that, unlike typical OMPs, the novel proteins were not integrated into the membrane.

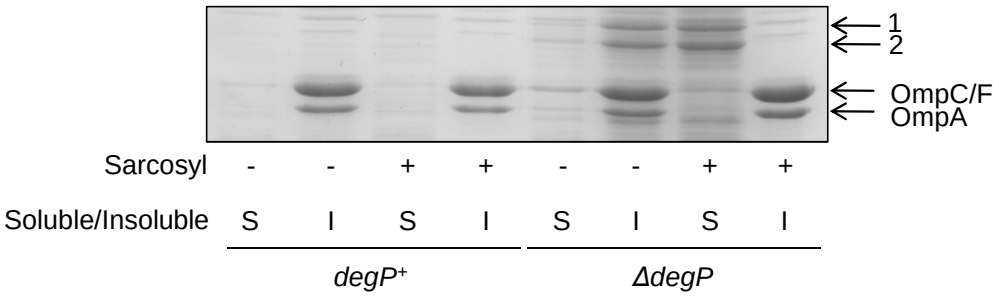


Fig. 8. Novel proteins are sarcosyl soluble. Total membranes from wild-type and *ΔdegP* cells were treated with 2% sarcosyl and fractionated. Soluble (S) and Insoluble (I) fractions were analyzed by SDS-PAGE and stained with coomassie blue.

Identification of Novel Proteins

Because the unknown proteins associated with the membranes, there was the possibility that they were involved in envelope biogenesis or stress response. To begin the process of identification, carbonate-treated membranes were analyzed by SDS-PAGE and stained with Coomassie Blue R-240. The bands in question were excised from the gel and subjected to nano-LC-MS/MS analysis. Initial attempts to identify these proteins via mass spectrometry were unsuccessful, most likely due to the sensitivity of the MS instrument coupled with contamination by the

complex protein mixture present around the excised bands.

Two-dimensional gel electrophoresis

In order to conclusively identify the two proteins visible in this fraction in cells lacking *degP,* the carbonate-insoluble membrane fraction was subjected to two-dimensional gel electrophoresis (2D-GE). The purpose of this approach was to achieve better separation between the proteins of interest and any neighboring proteins. The principle of 2D-GE is simple: protein mixtures are separated out in the first dimension according to isoelectric focusing point, and subsequently separated in the second dimension by size. Instead of protein bands, this technique ultimately yields protein spots or teardrops. 2D-GE analysis was carried out by Kendrick Labs (Madison, WI). Examination of the 2D gels revealed many more protein spots enriched in the carbonate-insoluble membrane fraction of Δ*degP* cells than initially observed in 1D analysis (Fig. 9, second panel). As seen in 1D analysis, the steady-state levels of the major OMPs did not change dramatically in cells lacking *degP*. However, many other protein spots associated with the carbonate-insoluble fraction of Δ*degP* cells which were not present in the *degP*$^{+}$ membranes.

Cytoplasmic chaperones are present in membranes of ΔdegP cells

Twelve protein spots highly enriched in *ΔdegP* membranes (Fig. 8, numbered) were excised from the dried gels, rehydrated and sent to the proteomics consortium at the University of Arizona (Tucson, AZ) for nano-LC-MS/MS analysis. Results of the analysis are summarized in Table 1.

An intriguing subset of the proteins enriched in *ΔdegP* membranes

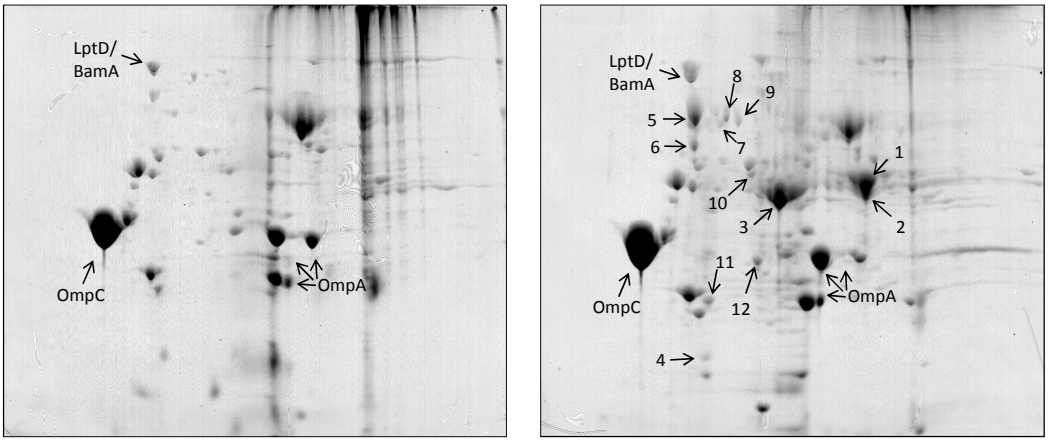


Fig. 9. 2D analysis of carbonate treated envelopes. Cells lacking *degP* accumulate numerous proteins not present in wild-type cells. Numbered spots were excised and analyzed by MS.

consisted of several which normally reside in the cytoplasm and participate in protein synthesis, including the small ribosomal protein S1 (RpsA). We also identified HtpG, a heat shock chaperone of the HSP90 family (Spence and Georgopoulos, 1989; Thomas and Baneyx, 2000), trigger factor (Tig), which is involved in nascent polypeptide binding at the ribosome and possesses PPIase activity (Crooke and Wickner, 1987; Hesterkamp *et al,* 1996), and elongation factor Ts (Tsf), responsible for catalyzing the release of GDP from TufA so that it can bind another GTP and aminoacyl-tRNA during protein synthesis (Hoogvliet *et al,* 1999; Manchester, 2003).

The larger of the two prominent bands in 1D analysis appeared to correspond with two large protein spots of approximately 55 kD. The higher molecular weight species was identified as cytoplasmic

tryptophanase (TnaA; MW 53 kD), responsible for the reversible conversion of tryptophan to indole (Watanabe and Snell, 1972; Snell, 1975). It is not clear how TnaA might be involved in stress caused by misfolding proteins. However, since *tnaA* insertion mutants have been shown to have reduced fitness (Junker *et al,* 2006) in competitive culture increased amounts of steady-state TnaA may confer a fitness advantage. Additionally, it was recently shown that oxidative stress caused by the lack of the small HSPs IbpA and IbpB results in the overexpression of TnaA, therefore raising the level of the stress alarmone indole and suppressing biofilm formation (Kuczyńska-Wiśnik *et al,* 2010). Further, a recent paper showed that increased indole production conferred community-wide resistance to cells under antibiotic stress conditions (Lee *et al*, 2010).

Identification of TufA in membrane extracts

The second major protein spot clarified by 2D-GE was subsequently identified as elongation factor Tu (TufA), running at approximately 45 kD. This is consistent with its published molecular weight of 43 kD, especially considering the large size of the spot in the gel (Fig. 9, second panel). TufA is present in many organisms, where it functions primarily to charge the ribosome with aminoacyl-tRNA during protein synthesis. It is therefore not surprising that Tsf was detected in our analysis (see above), because Tsf and TufA are known to form a complex localized at the ribosome (Kawashima *et al,* 1996). Additionally, TufA homologs are widespread, and have been shown to possess

chaperone activity during conditions of heat shock (Caldas *et al,* 1998; Ristic *et al,* 2007; Fu *et al,* 2008). Because of this, TufA was chosen as a characteristic cytoplasmic protein for investigation under these stress conditions.

Membrane association of TufA is growth temperature-dependent

The focus of the investigation was turned to charactering the nature of association with the membranes. It was asked whether TufA association was the direct result of high temperatures by asking whether the association of these proteins was dependent upon the temperature at which the cells were grown. Parallel cultures of cells lacking *degP* were grown at 30°C and 39°C, and membranes were extracte d with sodium carbonate. As seen in Fig. 10, the protein was present primarily in the carbonate-insoluble fraction when cells were grown at the elevated temperature, indicating that their association was a result of thermal stress in the envelope.

Membrane association of TufA is growth time-dependent.

Similarly, it was asked whether the protein's association with the carbonate-insoluble membrane fraction was a growth time-dependent phenomenon; that is, whether the proteins associated with the fraction based on a continuing stress over time. Indeed, it was found that the proteins' association with the carbonate-insoluble membrane fraction began to occur at around two hours of growth, with maximal association

Table 1. Envelope proteins identified by MS.

Spot	Gene	Name	No. Peptides
1	*tnaA*	Tryptophanase	31
2	*tnaA*	Tryptophanase	5
3	*tufA*	Elongation Factor Tu	38
4	*ahpC*	Alkyl Hydroperoxide Reductase	1
5	*rpsA*	30S Ribosomal Protein S1	1
6	*tig*	Trigger Factor	48
7	*btuB*	Vitamin B12 Receptor	32
8	*htpG*	Heat Shock Protein 90	1
9	*proS*	Prolyl-tRNA Synthase	38
10	*asnS*	Asparaginyl-tRNA Synthase	2
11	*purC*	Phosphoribosylaminoimidazole-succinocarboxamide Synthase	12
12	*tsf*	Elongation Factor Ts	16

occurring at around three hours (Fig. 11). This suggests that the proteins' association is an accumulative phenomenon, meaning that continuing thermal stress in Δ*degP* cells leads to a continued associated of TufA with the envelope.

Heterologous expression of degP rescues growth defect and prevents association of TufA with membranes

DegP has been shown to possess both chaperone and protease activity (Spiess *et al,* 1999). To determine whether the lack of chaperone or protease activity (or both) is responsible for the growth defect of Δ*degP*,

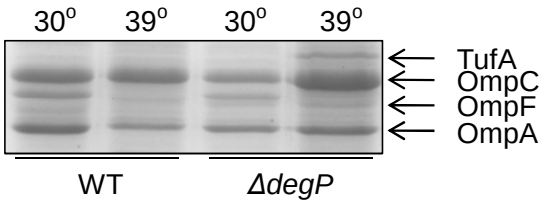


Fig. 10. Association of novel bands with Δ*degP* membranes is growth temperature-dependent. Membranes were extracted with sodium carbonate, run analyzed by urea SDS-PAGE and stained with coomassie blue. Note that OmpF is absent at high temperatures, as its synthesis is shut down at the transcriptional level.

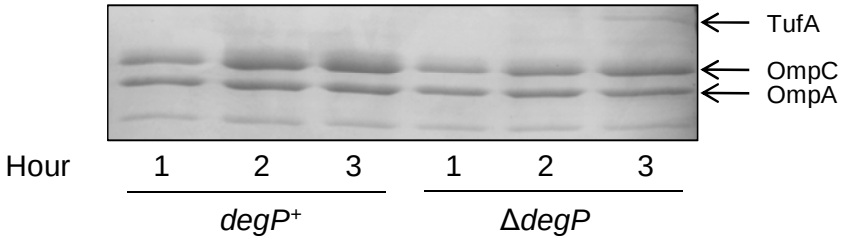


Fig. 11. Association of novel proteins is growth time-dependent. Cells were grown at 39°C, and samples taken at the indicate d times. Membranes were extracted with sodium carbonate, analyzed by SDS-PAGE and stained with coomassie blue.

cells were transformed with the low copy number plasmid pACYC184 harboring either wild-type *degP* or a protease-deficient mutant, $degP_{S210A}$. In this mutant, the active site serine has been mutated to alanine, preventing DegP's catalytic triad from forming and thus rendering it unable to degrade substrate proteins, although it is still capable of binding and sequestering them. Wild-type and Δ*degP* cells harboring pACYC184 empty vector, pACYC184-*degP* or pACYC184-$degP_{S210A}$ were diluted to a starting OD_{600} of 0.025 in prewarmed LB broth at 39°C and grown w ith vigorous agitation. Cell density was tracked for five hours of growth.

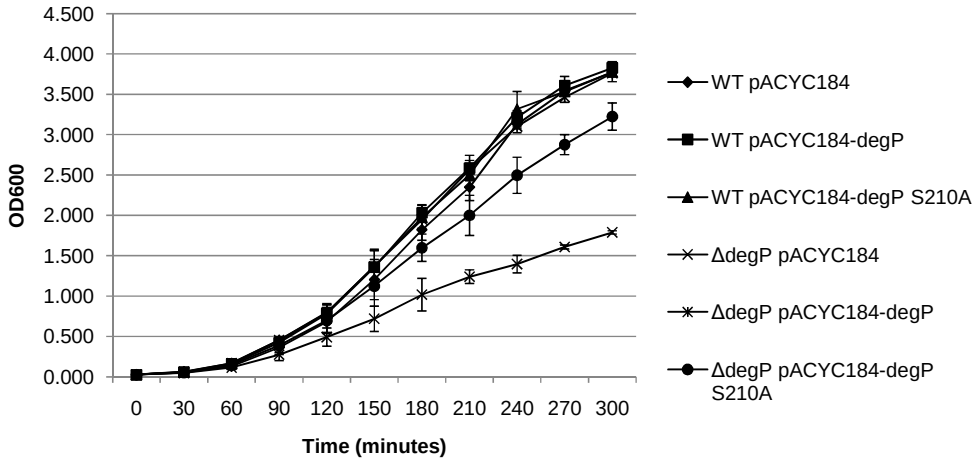


Fig. 12. Proteolytic activity of DegP is required for complete rescue of temperature sensitivity. Cells harboring pACYC184, pACYC184-*degP* and pACYC184-$degP_{S210A}$ were cultured in LB at 39°C and cell density measured every thirty minutes.

In all backgrounds tested, $degP^+$ cells had no growth defect (Fig. 12). Cells lacking *degP* which harbor the empty vector alone were severely impaired in growth. When $degP_{S201A}$ was expressed from the plasmid, growth was improved, but not to the level of wild-type. Only when a fully active copy of *degP* was expressed from the plasmid was the growth defect of Δ*degP* cells abrogated and growth restored to the same level as chromosomally $degP^+$ cells. These data show that, while the lack of chaperone activity of DegP plays a role in the Δ*degP* growth defect, the primary cause of the defect is the lack of proteolytic activity supplied by wild-type DegP.

It was asked whether a reduction in TufA association with membranes would correspond to a restoration of growth in *ΔdegP* cells expressing *degP* or $degP_{S210A}$. Cells were harvested from the final time

point in Fig. 12, lysed by French Press and run on SDS-PAGE. Indeed, a reduction of TufA-membrane association was observed in cells expressing either allele of *degP*. This reduction correlated with the level of growth improvement, with *degP*$^{+}$ expression reducing TufA levels to 16% of vector control (Fig. 13, lane 5).

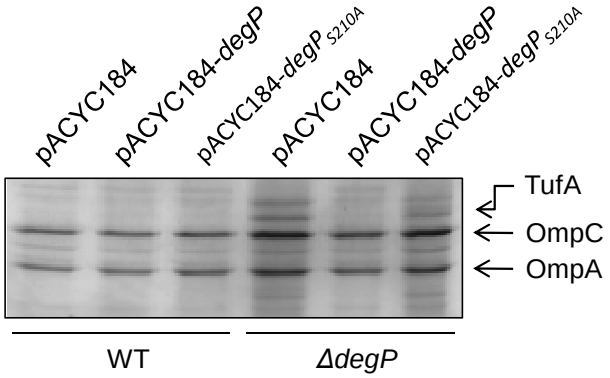


Fig. 13. Heterologous *degP* expression reduces TufA association with membranes. Cells were harvested from the teriminal time point in Fig. 11. Carbonate-extracted membranes were analyzed by SDS-PAGE and stained with coomassie blue.

DegP$_{S210A}$ expression did not restore growth to the same level as wild-type, and accordingly only reduced TufA-membrane association to 60% of vector (Fig. 13, lane 6). Nevertheless, these results indicated that reducing overall stress in the cell envelope reduced the propensity for TufA to associate with the membranes in a proportional manner.

TufA localizes to the membranes in ΔdegP cells

To confirm that the characteristic protein under examination is in fact TufA the subcellular localization was examined using α-TufA

antibodies. Wild-type and *ΔdegP* cells were grown for five hours at 39°C, pelleted and washed once with 10 mM Tris pH 7.5. Periplasm was isolated by gentle osmotic shock, and cells again pelleted and lysed by French Press. After centrifugation, the soluble fraction representing primarily cytoplasm was saved, and membranes resuspended in 10 mM Tris pH 7.5. These three fractions were analyzed by SDS-PAGE, transferred to PVDF membrane and probed with α-TufA antibodies. As expected, TufA was present in large amounts in the cytoplasm of both strains (Fig. 14A). It was also present in the periplasmic fraction of both strains, although this is likely an artifact caused by the periplasmic extraction method, since osmotic shock is known to cause periplasmic localization of TufA (Berrier *et al,* 2000). However, only the membranes of *ΔdegP* cells contained TufA. This was not the result of soluble fraction contamination, as the same samples probed with α-MalE antibodies showed no residual MalE in the membrane fractions (Fig. 14B). It was confirmed that *degP*$^{+}$ membranes were present by probing with α-TolC antibodies (Fig. 14C).

Analysis of outer membrane vesicles

It is known that Gram-negative bacteria utilize the release of OMVs as an envelope stress response (McBroom and Kuehn 2007). Indeed, cells lacking *degP* are known to vesiculate (McBroom *et al,* 2006), and this is thought to relieve stress by promoting the mass exodus of misfolded or otherwise harmful envelope proteins. However, to date no group has

investigated the contents of vesicles released during extreme thermal stress in *ΔdegP* cells.

OMVs are produced by ΔdegP cells

Wild-type cells and those lacking *degP* were grown for five hours at 39°C and centrifuged to pellet cells. Culture supernatants were then filtered by a 0.45 μM syringe filter and centrifuged at 100,000x g to pellet OMVs, which were in turn resuspended in 10 mM Tris pH 7.5. OMVs were analyzed by SDS-PAGE and stained with coomassie blue. It was noted that *ΔdegP* cells produced large amounts of OMVs during growth at high temperature (Fig. 15), while their wild-type counterparts did not produce OMVs at a level detectable by coomassie staining (data not shown). Wild-type cells were therefore excluded from further analysis.

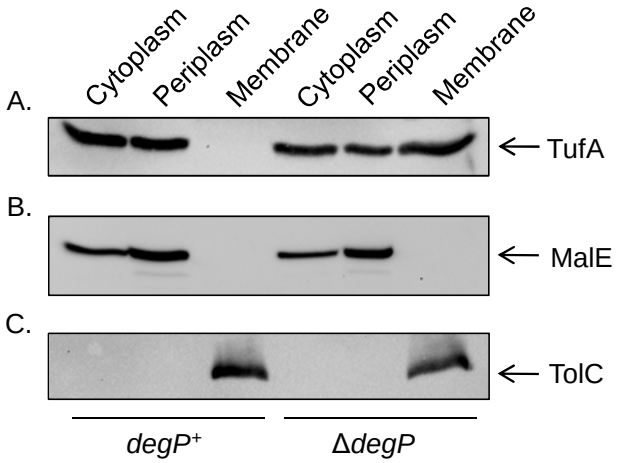


Fig. 14. TufA preferentially associates with membranes in Δ*degP* cells. A. Cell fractions were probed with polyclonal antibodies raised against LamB and TufA. B. The same fractions as in A were probed with polyconal antibodies raised against MalE to check for soluble contamination of the envelope. C. Fractions were probed with antibodies raised against TolC to ensure presence of envelope. Note that cytoplasmic preparation is still expected to contain small amounts of periplasmic proteins.

OMVs contain multiple compartments of the cell

OMVs prepared from *ΔdegP* cells were run on SDS-PAGE gels and probed with antibodies specific to markers of various cellular compartments. The proteins DnaK, MalE, AcrB and LamB were used as markers of cytoplasm, periplasm, inner membrane and outer membrane, respectively. OMVs contained detectable levels of LamB, MalE, and a small amount of DnaK, indicating that they in fact packaged even cytoplasmic contents (Fig. 16).

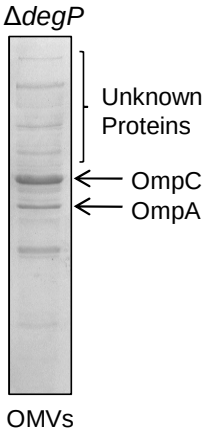


Fig. 15. *ΔdegP* cells produce large amounts of OMVs. Cells grown for five hours at 39°C were pelleted, and the supernatant subsequently filtered and centrifuged to isolate OMVs. OMVs were analyzed by SDS-PAGE and stained with coomassie blue.

Although the OMVs contained DnaK, a cytoplasmic protein, no AcrB was detected as a representative IM protein. The data show that OMVs released during our stress conditions contained representative proteins from three out of four compartments of the cell. Previous studies have not reliably detected cytoplasmic proteins in OMVs, possibly because the conditions tested were not as severe as those tested here.

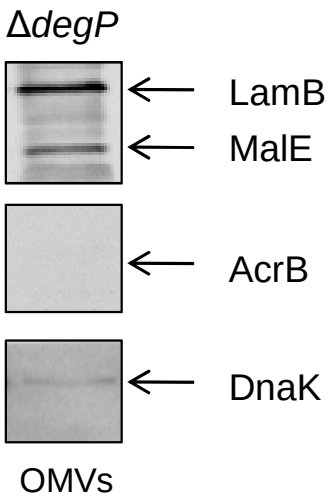


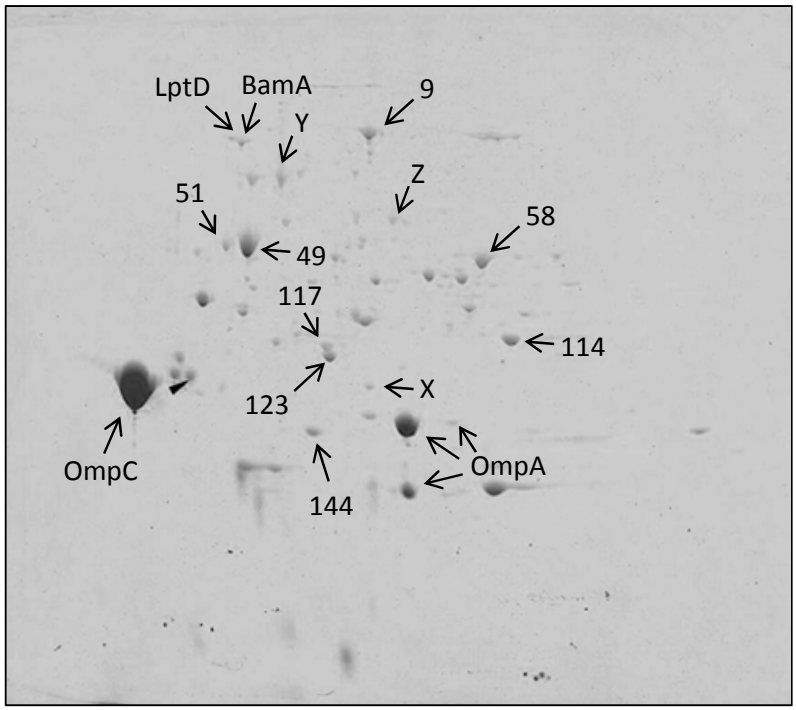
Fig. 16. OMVs contain representatives from different cellular compartments. OMVs and membranes were probed with polyclonal antibodies raised against LamB, MalE, AcrB and DnaK.

Proteomic analysis of OMV contents

Outer membrane vesicles isolated from *ΔdegP* cells were separated by 2D-GE (Kendrick Labs, Madison WI). The gels were examined for prominent spots in OMVs using the computerized analysis and numbering provided. Nine such spots were excised from the dried vesicle gels (Fig. 17) and sent for nano-LC-MS/MS analysis at the proteomics consortium at University of Arizona (Tucson, AZ). It was noted that the computerized analysis did not detect all the proteins with high abundance in OMVs, and so three additional spots were excised after manual inspection. Certain spots, such as OmpA and OmpC, were consciously avoided during this analysis since we had already identified them in 2D gels using this method.

Interestingly, using this analysis, several periplasmic and

cytoplasmic proteins were identified (Table 2). To date, no other group has identified cytoplasmic proteins in outer membrane vesicles. The presence of cytoplasmic proteins indicates the severity of the *ΔdegP* phenotype at high temperatures.



OMVs

Fig. 17. 2D analysis of Δ*degP* outer membrane vesicles. Numbered spots correspond to computerized analysis, while lettered spots refer to manual analysis.

Table 2. Proteins identified in vesicle analysis by MS.

Spot	Gene	Name/Function	Peptides
9	*aceE*	Pyruvate Dehydrogenase Subunit	24
49	*groL*	GroESL Large Subunit	12
51	*grol*	GroESL Large Subunit	12
58	*oppA*	Periplasmic Oligopeptide Transporter	11
71	*lpdA*	Lipoamide Dehydrogenase	25
114	*tolB*	Colicin Import; Structural	8
117	*glpQ*	Periplasmic Glycerophosphodiester Phosphodiesterase	14
123	*malE*	Periplasmic Maltose Binding Protein	12
144	*mglB*	Galactose/Glucose Binding Protein	23
X	*ompT*	OM Protease	10
Y	*aceF*	Pyruvate Dehydrogenase Subunit	25
Z	*aceF*	Pyruvate Dehydrogenase Subunit	14

Chapter 3

RESULTS II: NOVEL SUPPRESSOR OF THE TEMPERATURE SENSITIVITY PHENOTYPE IN A DEGP MUTANT

Chromosomal suppressors of Δ*degP* temperature sensitivity

One of the ways to understand the consequences of mutations in important genes is to isolate revertants (so-called “suppressor mutations”) which suppress the harmful effects. For example, cells lacking *lamB* are unable to grow on media containing maltodextrins as the sole carbon source unless compensatory mutations in another porin, *ompF,* widen the pore enough for the sugars to enter (Misra, 1993). In this manner, the cell can find a way around the selective pressures it encounters.

Double mutants in *degP* and *bamB* display a synthetic lethal phenotype at elevated temperatures (at or above 37° C), growing poorly even at 30°C. They are also hypersensitive to vancomyci n, a large antibiotic normally excluded by the OM. The synthetic phenotype is thought to arise from an inability to properly assemble OMPs (the *bamB* defect) coupled with an inability to degrade misfolded OMP intermediates (the *degP* defect). Because of this, the double mutant provides an ideal background in which to isolate suppressor mutations which improve the assembly environment and/or the envelope stress response, thus rescuing the cell from lethality.

Isolation of a suppressor of ΔdegP temperature sensitivity

A previous student in the laboratory conducted a selection to isolate

suppressors of the extreme temperature sensitivity of a *bamB degP* double mutant. Liquid cultures of the double mutant were grown in LB at 30°C, diluted and spread onto LB agar plates, and incubated at 37°C until colonies appeared. Temperature resistant colonies were then purified at 30°C and suppressor mutants categorized based on the strength of suppression and OMP phenotype, as cells lacking *bamB* display lower steady-state levels of major OMPs in the envelope (Charlson *et al,* 2006). Using this method, many suppressors were isolated, including a mutation in *rseA*, which altered the RseA-RpoE binding pocket, abrogating RseA's ability to sequester RpoE at the IM and ultimately driving the σ^E envelope stress response. The *rseA* mutation suppressed *ΔbamB ΔdegP* stress by lowering the load of OMPs in the envelope. Another suppressor was isolated in *envZ*, which constitutively activated EnvZ by altering its balance kinase/phosphatase activity in favor of kinase (hyperphosphorylation). Again, this mutant suppressed envelope stress in part by lowering OmpF and LamB levels in the envelope.

A third suppressor was isolated at high temperature, and was noted to suppress temperature sensitivity without lowering the steady-state levels of OMPs. Through marker replacement studies, it was determined that the mutant suppressed temperature sensitivity caused by the lack of *degP.* Because of the unusual property regarding OMPs, it was selected for further investigation.

Suppression of ΔdegP temperature sensitivity

As the suppressor mutation was shown to correct the defect caused by a lack of *degP*, improvement in growth in those cells was tested. To that end, cells harboring the suppressor mutation as well as a *ΔdegP* null allele were grown on solid media at 30°, 37°, and 40°C. At 30°C Δ*degP* cells have no discernable growth defect, reflecting the slower growth and thus reduced demand for and accumulation of OMP intermediates in the envelope (Fig. 18).

At elevated temperatures, however, *ΔdegP* cells displayed increased temperature sensitivity, corresponding with increasing temperatures; at 37°C *ΔdegP* cells are smaller than their wild-type parental cells, while at 40°C they are unable to form uniform colonies (Fig. 18). At both elevated temperatures tested, the suppressor was able to restore temperature resistance, although not entirely to the level of wild-type, indicating an almost complete correction of the *degP* defect. Interestingly, $degP^{+}$ cells harboring the suppressor showed the same growth pattern as wild-type.

Because *ΔdegP* cells were found to have a growth defect in liquid culture, it was asked whether the suppressor mutation rescued temperature sensitivity there also. The above cells were cultured in LB at 39°C for five hours, with cell density tracked every thirty minutes. Cells lacking *degP* displayed a negative growth phenotype at the elevated temperature (Fig. 19). However, *ΔdegP* cells also expressing the

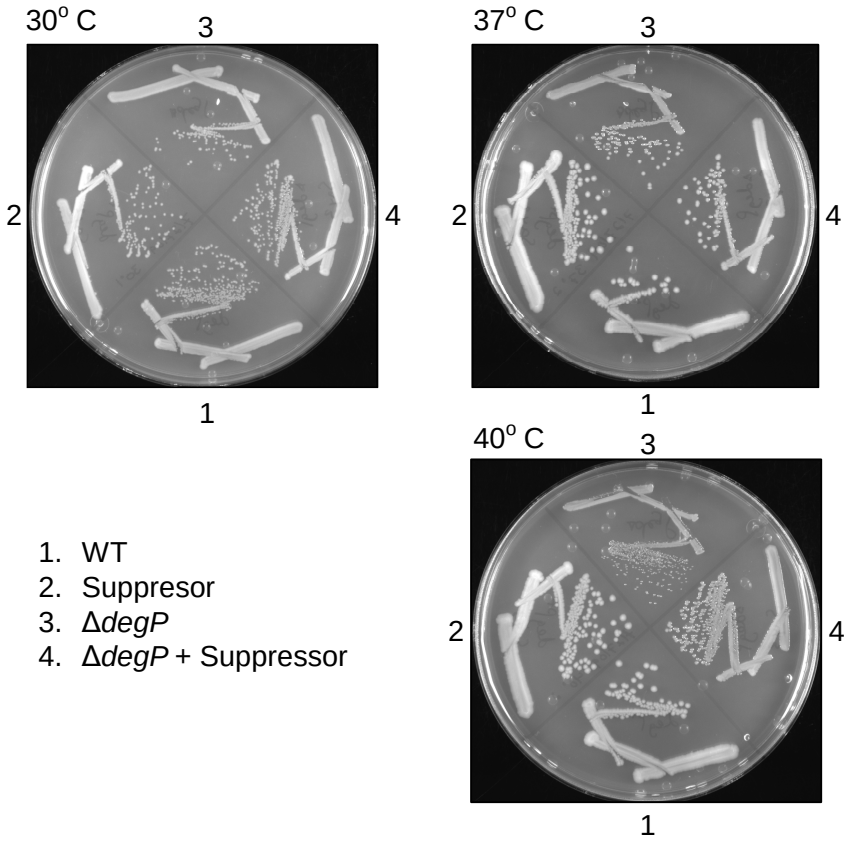


Fig. 18. Suppressor rescues growth defect of Δ*degP* cells at 40°C. Strains were streaked on LBA and incubated at the indicated temperatures overnight.

suppressor grew at a rate and to a final cell density indistinguishable from wild-type. As on solid media, *degP*$^{+}$ cells expressing the suppressor did not grow to any differently than wild-type.

Suppressor reduces stress phenotypes

Stress caused by the lack of *degP* at high temperatures is manifested in the cell envelope. To investigate the suppressor's effect on the envelope, membranes were isolated, treated with sodium carbonate and analyzed by SDS-PAGE. Consistent with the near-complete rescue

of the *degP* growth defect, *ΔdegP* cells containing the suppressor had vastly reduced levels of TufA and TnaA in the envelope (Fig. 20, lane 4). Indeed, 2D-GE analysis of carbonate-treated membrane extracts from these cells shows a significant reduction in the level of the cytoplasmic proteins identified in *ΔdegP* cells (Fig. 21).

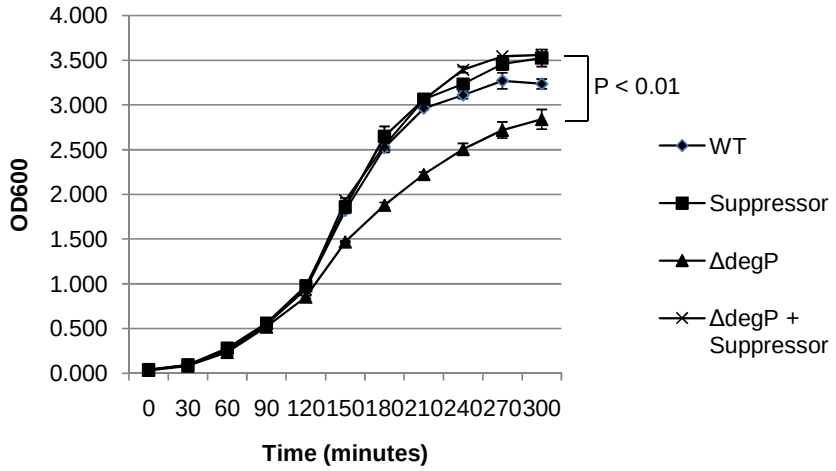


Fig. 19. Suppressor rescues growth defect of Δ*degP* cells in liquid culture. Cells were grown for five hours at 39°C, with cell de nsity tracked every thirty minutes.

Similarly, *ΔdegP* cells containing the suppressor mutation showed a reduction in vesiculation compared to those containing *ΔdegP* alone (Fig. 22, lane 8), to a level below detection by the isolation and visualization methods used. This level of vesiculation reflects the complete reduction in envelope stress, and is as low as wild-type. As vesiculation itself is a stress response, it is believed that the reduction seen is not caused by the

suppressor *per se*, but is rather a consequence of the reduction in envelope stress caused by the suppressor.

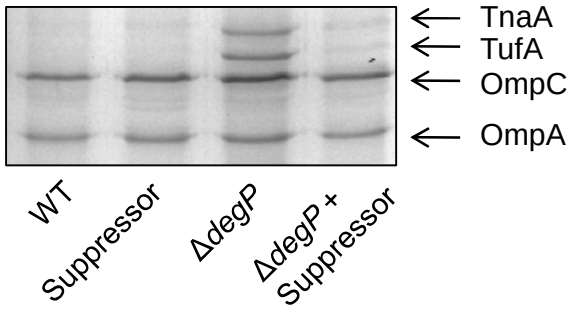


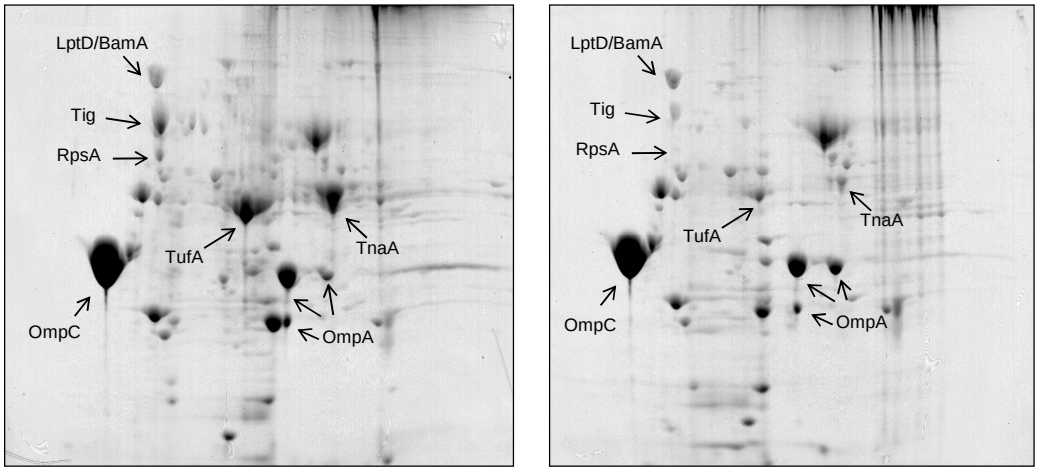
Fig. 20. Suppressor mutation reduces association of cytoplasmic proteins with membrane. Cells were harvested at the terminal time point of Fig. 18. Carbonate-extracted membranes were analyzed by SDS-PAGE and stained with coomassie blue.

Locating the suppressor mutation

The ultimate goal of the suppressor analysis was to determine, mechanistically, how the mutant functions to correct the *degP* defect in the envelope. To that end, it was set out to identify the gene or genes affected in the suppressor strain.

P1 transductional mapping of the chromosome

In an attempt to narrow down the chromosomal region of interest, physical mapping of the chromosome was undertaken by a series of P1 transductions. A series of known insertion mutations (Kan^r or Cml^r) from the CAG (Singer *et al,* 1989) and Keio (Baba *et al,* 2006) collections, as well as those from our laboratory, were introduced into a strain containing



Δ*degP* Δ*degP* + Suppressor

Fig. 21. Total cytoplasmic protein content of membranes is reduced by suppressor. Carbonate-treated membranes were analyzed using 2D-GE. Representative cytoplasmic proteins are indicated along with three OMPs.

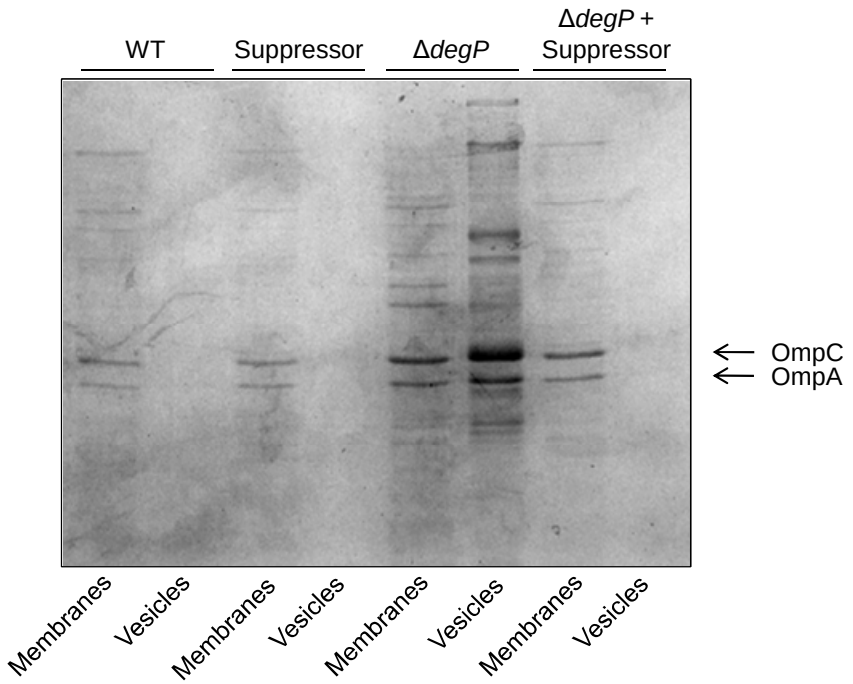


Fig. 22. Vesiculation is reduced in suppressor. Cells were grown at 39°C for five hours, followed by vesicle and membrane isolation. Proteins were visualized by coomassie blue.

the unknown suppressor mutation, and selected for the appropriate antibiotic (kanamycin or chloramphenicol). Antibiotic resistant transductant were screened for temperature sensitivity by replica plating on LBA plates and grown at 40°and 30°C. Only whe n the insertion mutation is within approximately 90 kb will cells become TS. Forty such insertions, spaced on average 1.5' apart in the chromosome, were introduced into a *ΔdegP* strain containing the suppressor linked to Tn*10* (Appendix D). None of the markers tested gave rise to TS colonies, which was curious considering the thorough nature of coverage provided.

Random transposon insertion analysis links suppressor to Tn10

To overcome the inconclusive data obtained from P1 transductional mapping, it was decided to take an alternative genetic approach in order to accurately determine the location of the suppressor. A random Tn*10* transposon insertion library was introduced into a *ΔdegP* suppressor strain by P1 transduction, selecting for tetracycline resistant (Tet^R) colonies. Over 1000 resulting Tet^R transductant colonies were screened for temperature sensitivity by replica plating at 40°and 3 0°C. One of two outcomes was anticipated: either the transposon could insert itself into the mutated gene directly, or it could insert near to the mutation. In either case, transductants displaying temperature sensitivity would have lost the suppressor through the recombination of chromosomal DNA near the transposon. Because of size limitations of the P1 phage, this could only occur if the suppressor was within approximately 2 minutes (90 kb) of the

Tn*10*.

One such temperature-sensitive transductant was obtained, and was purified at 30°C. Its TS phenotype was confirmed by re-streaking purified colonies at 40°. To determine linkage between the suppressor and Tn*10*, fresh P1 lysate prepared on this strain was reintroduced into *ΔdegP* cells without the suppressor. The resulting transductant colonies were replica plated at 40° and 30°C to determine the ratio of temperature-sensitive to temperature-resistant colonies. Of the 455 transductants screened, 225 (49.5%) were temperature-sensitive, indicating that the two were reasonably close together in the chromosome, but that the *Tn10* was not inserted directly in the gene where the suppressor resided. However, the exact locations of both the suppressor and the Tn*10* remained unknown at this point.

Identification of yfiF::Tn10 using AP-PCR

In order to conclusively determine the chromosomal location of the Tn*10*, a technique known as arbitrarily primed polymerase chain reaction (AP-PCR) was utilized, as it is capable of giving the location of the insertion at the nucleotide level.

The principle of AP-PCR is simple, and has been used successfully by another group to identify the location of an unknown Tn*10* insert (Fontaine *et al,* 2008). Using wild-type and *Tn10* strains, a preliminary, low-stringency PCR was carried out using two primers each containing a unique 22-bp 5'-sequence and a 3'-sequence consisting of ten random

nucleotides followed by either GATAT or ACGCC (hence, *arbitrarily* primed PCR). Under low-stringency conditions, with a low primer annealing temperature of 40°C, these primers can lan d virtually anywhere in the chromosome due to the random nature of their nucleotide sequence. However, a third primer specific to either the right or left insertion sequence of Tn*10* was utilized in order to produce PCR products unique to the strain containing the insertion. Using purified first round PCR product as a template, a second round of PCR was carried out, this time at high stringency, with an annealing temperature of 55°C. The appropriate Tn*10* primer and a primer consisting solely of the unique portion of the arbitrary primers were used to enrich for the PCR products unique to Tn*10*.

Due to the small size of secondary PCR fragments (<1000 bp), reactions were analyzed on a 4% w/v agarose gel in order to tighten the bands, and the banding patterns compared (Fig. 23). Bands unique to the Tn*10* strain were excised from the gel, purified and sequenced using the Tn*10*-specific primers. Using this method, the transposon was found to disrupt *yfiF* at nucleotide 883.

yfiF is located at 58.5' on the *E. coli* chromosome. Upon closer inspection of the markers used for physical mapping, it was discovered that this region (52.8'-60.6') was not represented by the known collection of kan^r or cm^r inserts used earlier. Since the maximum distance mappable by P1 phage is 2', this narrows the window to roughly 54.8'-58.6', which is

still not inclusive of *yfiF*. These data explain why inital P1 mapping was unsuccessful in locating the transposon and the suppressor mutation.

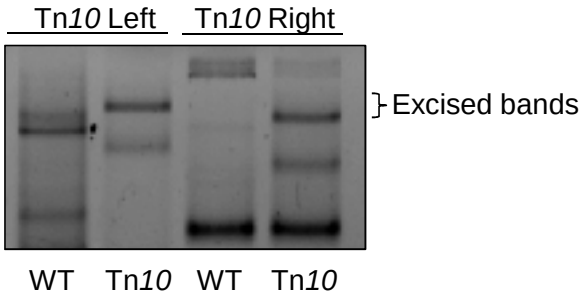


Fig. 23. Unique bands in AP-PCR products from Tn*10* strain. Secondary PCR products from Tn*10* left and right primer sets were run on 0.4% agarose gel. Unique bands enriched in Tn*10* were excised and sequenced.

Fine mapping of the 58' region

In order to determine which side of *yfiF*::Tn*10* the suppressor was located, fine mapping was employed using known markers in the 58' region of the chromosome. The insertions *sseA*::kan^r (57.1'), *yfhR*::kan^r (57.3'), *csiE*::kan^r (57.4') and *glrK*::kan^r (57.9') were introduced by P1 transduction into *ΔdegP* cells containing the suppressor linked to *yfiF*::Tn*10*. Resulting kan^r transductant colonies were screened by replica plating on LBA plates containing tetracycline or kanamycin to test the frequency at which they became Tet^S. As the markers became more proximal to *yfiF*::Tn*10*, the frequency at which the cells became Tet^S increased, indicating that the kanamycin and tetracycline markers were

located closer together. Tet^R and Tet^S colonies were then tested for temperature sensitivity, in order to map the suppressor mutation in a three-factor cross.

In a three-factor cross, if the suppressor mutation is located on the proximal side of *yfiF*::Tn*10* relative to the kanamycin marker, it would be expected that very few of the Tet^S colonies (those that have lost the Tn*10*) would remain temperature-resistant, because the likelihood that one marker will be recombined out of the chromosome without the other decreases as more crosses are required (in this case, a quadruple recombination event; Fig. 24A). Conversely, if the suppressor is located to the distal side, most of the Tet^S colonies will also be temperature-resistant, because only a double recombination event would be required to recombine one marker out of the chromosome during transduction while leaving the other behind (Fig. 24B).

Using this method it was determined that the two most prevalent classes of kan^r transductants were either Tet^S *ts* or Tet^R *tr*. For example, using *glrK*::kan^r 37.5% and 40.6% of transductants were Tet^S *ts* and Tet^R *tr*, respectively. A smaller number of colonies (21.9%) were Tet^R *ts*, while no Tet^S *tr* colonies were isolated. Together, these data indicated that the suppressor mutation was located between *glrK* and *yfiF*, a total chromosomal distance of approximately 27,000 bp.

Direct sequencing of the rpoE-nadB region yields a novel PCR binding pattern

PCR primers were designed to directly sequence the region between *glrK* and *yfiF*, including the intergenic regions between genes (in order to identify any promoter mutations). All PCR reactions were carried out in duplicate in strains harboring the suppressor, as well as their wild-type counterpart. Direct sequencing of this region failed to uncover any mutations in any genes upstream of *rseA*. Approximately 18.4 kb DNA was sequenced in total before finding any indication of a mutation. When sequencing in the *rpoE-nadB* region, it was noted that novel, high molecular weight bands frequently arose. These bands were at first ignored as an artifact of PCR. Consistently, however, a band with a molecular weight of approximately 1000 bp higher than the desired PCR product and was present when the primers used in the reactions included portions of the *rpoE-nadB* intergenic region. An example of this phenomenon is seen in Fig. 25, in which the primer set amplified all of *rpoE*, the 5'-134 nucleotides of *nadA* and the intervening intergenic sequence (1961 bp in the native sequence). An additional band, running at approximately 3000 bp, is present in the reactions amplified from suppressor strain.

Because the novel band arose frequently and consistently in the reactions, it was decided to sequence it to determine the identity of the amplified DNA. PCR was performed in wild-type and suppressor cells

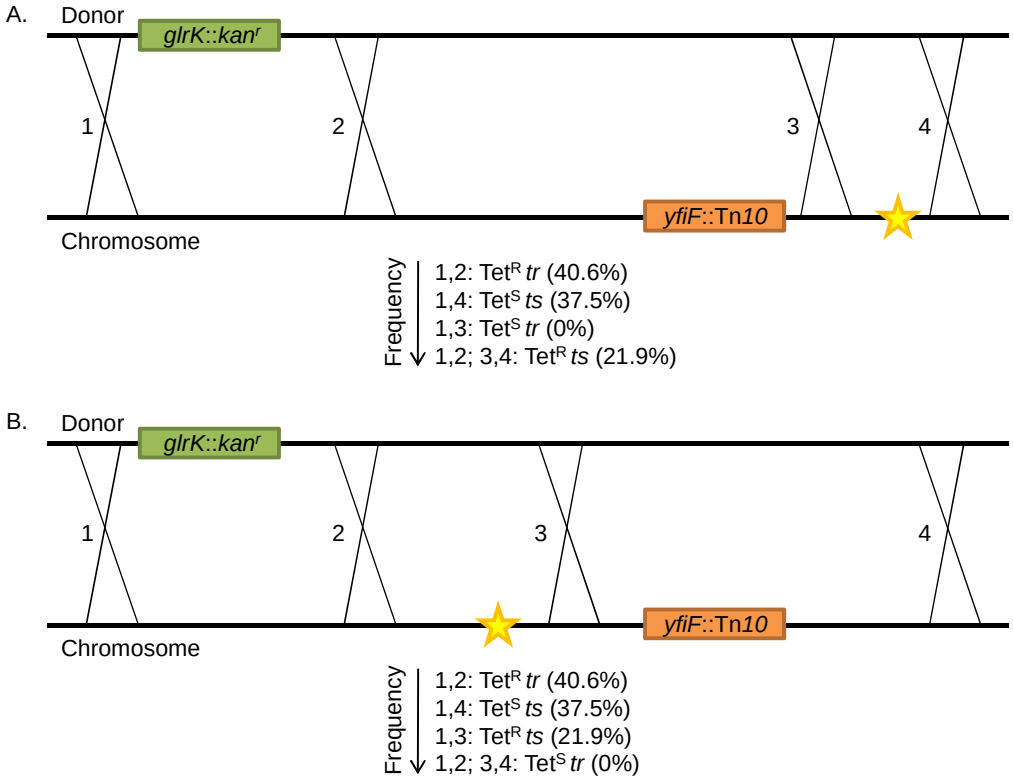


Fig. 24. Schematic of recombination in three-factor crosses. A. Expected frequency of possible crosses if suppressor is on distal side of *yfiF* from *glrK* B. Expected frequency of possible crosses if suppressor is on proximal side of *yfiF* from *glrK*. Frequency of recombinant classes only fits with possibilities in B.

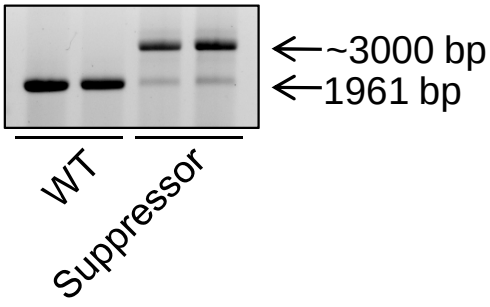


Fig. 25. Example of novel banding pattern. Primers amplify an additional product approximately 1000 bp larger than the intended PCR product.

using primers specific for the *rpoE-nadB* intergenic region. This primer set was expected to yield a PCR fragment of 1207 bp. When PCR products were run on a 0.8% w/v agarose gel, the expected fragment at the correct weight was present (Fig. 26). Additionally, in cells containing the suppressor the higher molecular weight band with a weight of approximately 2200 bp was present. The lower molecular weight bands from both cell types as well as the higher molecular weight band from the suppressor strain were excised from the gel, purified and sequenced using the same primers used for amplification.

Upon sequencing, it was discovered that the sequences from all three types of excised bands were identical to both each other and the published sequence for this region, suggesting that a duplication event had taken place between *rpoE* and *nadB*.

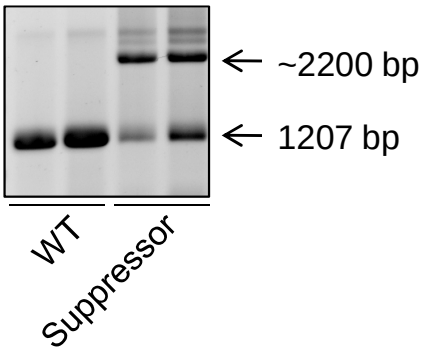


Figure 26. *rpoE-nadB* intergenic region contains extra sequence. Primers amplify a 1207 bp native fragment and a ~2200 bp novel fragment. Both band types were excised from the gel and sequenced.

Suppressor is a duplication-truncation of rpoE

Attempts to directly sequence the PCR product in Figure 24 was unsuccessful, because multiple sequences were returned for each reaction (data not shown). Attempts to use gel-purified PCR product were also unsuccessful due to the short length of readable sequence obtained, although the sequences did suggest that the mutation occurred very close to the 5'-end of *nadB* in the intergenic region (-21 bp from translation start), and consisted of at least part of a 5'-truncation of *rpoE*. However, in order to conclusively identify the mutation by PCR and subsequent sequencing, a clean homogeneous product needed to be obtained. To this end, a primer set consisting of one primer landing upstream of *rpoE* (reading into *rpoE*) and one primer landing upstream of *nadB* (reading into *nadB*) were utilized. Because *rpoE* and *nadB* are divergently transcribed, the only way to obtain PCR product was if there was truly a duplication of *rpoE* or *nadB* such that the two "forward" primers are able to prime the reaction by facing each other in the chromosome (Fig. 27), at which point they will amplify a 651 bp fragment.

Indeed, when the PCR products amplified from the divergent primer set were run on an agarose gel, the only instance where any bands were present was when the suppressor mutation was also present (Fig. 28B), indicating that the interpretation of the above sequencing data was correct. As a control reaction, the same strains were subjected to PCR amplification of the region consisting of the 157 bases upstream of *nadB*

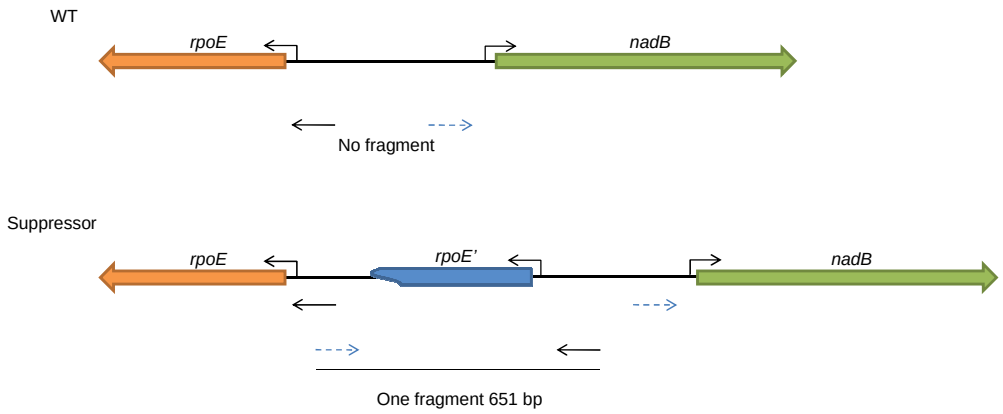


Fig. 27. Schematic description of divergently primed PCR. In wild-type cells, divergent primers are unable to amplify product. In the case of duplication in the *rpoE-nadB* intergenic region, additional primer binding sites will align and product will form.

and the first 124 bases of *nadB* itself (Fig. 28A). In this case, PCR products were amplified in both strain backgrounds, with the suppressor background containing the novel fragment. The PCR product produced from divergent primers was purified and sequenced using the same primers as were used for amplification. The sequences revealed a novel duplication-truncation in *rpoE*. The mutation consisted of *rpoE* truncated at nucleotide C396, corresponding with amino acid F122. After F122, the amino acid sequence continued with MVWYA before a stop codon (UAG) was reached. In addition to coding for additional, non-native amino acids, the region immediately after F122 was identical to the DNA sequence beginning at 21 bases upstream of the *nadB* translation start. The sequence then continued until the translation start site of the native copy of *rpoE* was reached (Fig. 29).

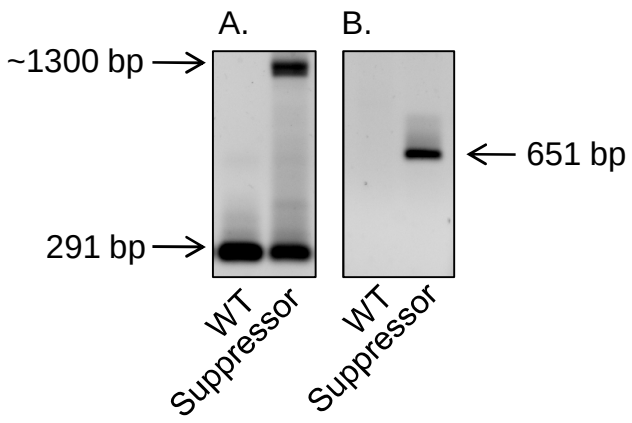


Fig. 28. Divergent primer set amplifies PCR product unique to suppressor. A. Normally oriented primers give rise to multiple PCR products. B. Using divergent primers, PCR product is only amplified in suppressor cells.

To confirm the location of the mutation, a *nadB*::Tn*10kan* insertion was introduced into a *ΔdegP rpoE'-yfiF*::Tn*10* strain by P1 transduction. When screened by replica plating, it was found cells remained Tet^R at a high frequency, 74%. Interestingly, although *nadB* is directly adjacent to the *rpoE'* mutation, transductant cells only became *ts* 43% of the time. We speculate that the low number of *ts* recombinants is most likely due to a selective disadvantage to losing the suppressor, which biases the number of *ts* colonies obtained by this method downward. No colonies were isolated which were Tet^S *ts*, further confirming that the suppressor laid on the upstream side of *nadB*.

As a final confirmation of the suppressor location, a Δ*rseC*::kan^r insertion was introduced into a *ΔdegP rpoE'-yfiF*::Tn*10* strain by P1 transduction. This insertion was chosen due to its close proximity to *rpoE,*

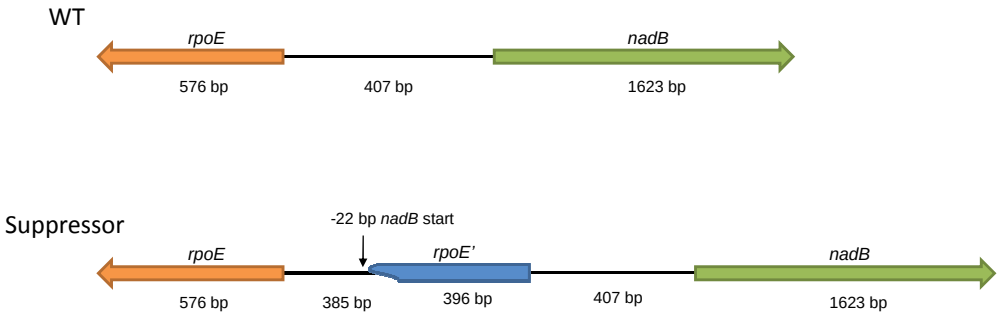


Fig. 29. Chromosomal arrangement of suppressor. Sequencing reveals suppressor to be a novel duplication-truncation of *rpoE* at nucleotide 396. The *nadB* upstream region is also truncated 22 bases upstream of *nadB* start.

coupled with the fact that it does not appear to have a role in σ^E activation or repression. In this case, cells became Tet^S 25%, but became temperature-sensitive 82.5% of the time, indicating that they lost the suppressor at a high frequency. Using the same divergent primer set as above, the four different recombinant types (Tet^R/*tr*, Tet^R/*ts*, Tet^S/*tr* and Tet^S/*ts*) along with the parental Kan^S strains were subjected to PCR analysis and their banding patterns analyzed (Fig. 30A). To ensure that the strains' DNA could be amplified using PCR, a parallel reaction was carried out using primers specific for amplification of *mscL,* a mechanical pressure-sensitive IM channel (Sukharev *et al,* 1994) distal in the chromosome from the region of interest (Fig. 30B). Indeed, the PCR fragment was only seen when amplified from temperature-resistant colonies (Fig. 31), i.e. those who retained the suppressor during recombination.

Mechanism of suppressor action

RpoE' suppressor does not exhibit classical σ^E OMP phenotype

To confirm the observation that OMP levels are unchanged in the suppressor background, *degP*$^+$ cells harboring *rpoE'*-Δ*rseC*::kan^r as well as Δ*rseC*::kan^r alone were grown in LB broth at 37°C until late exponential phase and lysed by French Press. Carbonate-extracted membranes were analyzed by urea SDS-PAGE and stained with coomassie blue. In this analysis, cells containing *rpoE'* had no difference in steady-state OMP levels as measured by coomassie blue staining (Fig. 32), indicating that this classical measure of elevated σ^E activity was inadequate to explain suppression in this case.

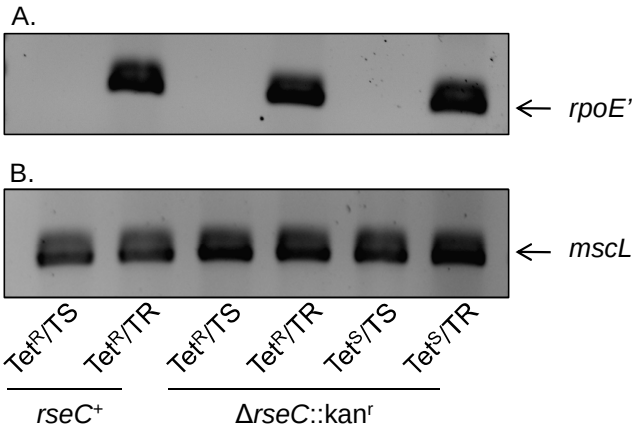


Fig. 30. Confirmation of *rpoE'* mutation in recombinant strains. A. Recombinant strains were subjected to divergently primed PCR, and product was amplified only in TR strains. B. DNA from strains used in A were amplified using standard PCR with primers specific to *mscL* as an assay control.

RpoE levels are elevated in the rpoE' background

The suppressor mutation was found to be a novel *rpoE* duplication-truncation. Because of the duplication in *rpoE*, it was asked if steady-state RpoE levels were elevated in cells harboring *rpoE'*. Both *degP*$^+$ and *ΔdegP* cells containing *rpoE'* were grown until late exponential phase, and whole-cell samples were taken to analyze RpoE levels. Cells were spun down and resuspended in SDS-PAGE buffer containing β-mercaptoethanol and boiled for five minutes. After boiling the samples were analyzed by SDS-PAGE, transferred to PVDF membrane, and probed with α-RpoE and α-MalE antibodies. In strains containing the *rpoE'* suppressor, RpoE levels were four-fold (*degP*$^+$) to five-fold (*ΔdegP*) higher than in their *rpoE*$^+$ partners (Fig. 33A). Yet, the steady state levels of OMPs are only slightly reduced in the *rpoE*' mutant (Fig. 32). It is noteworthy that, despite the *rpoE'* open reading frame, no smaller molecular weight bands reacted with the α-RpoE antibodies.
RpoE must be released into the cytoplasm in order to become active. One possible explanation for the increase in RpoE levels without a concomitant reduction in OMPs is that, since *rseA* is itself a member of the RpoE regulon, the *rpoE'* mutation functions to increase the level of membrane-bound rather than soluble RpoE, thus limiting the σ^E response. To test this, cells harboring *rpoE'* in both *degP*$^+$ and *ΔdegP* backgrounds were grown at 37°C until late exponential phase, ly sed by French Press and separated into soluble and membrane fractions. The fractions were

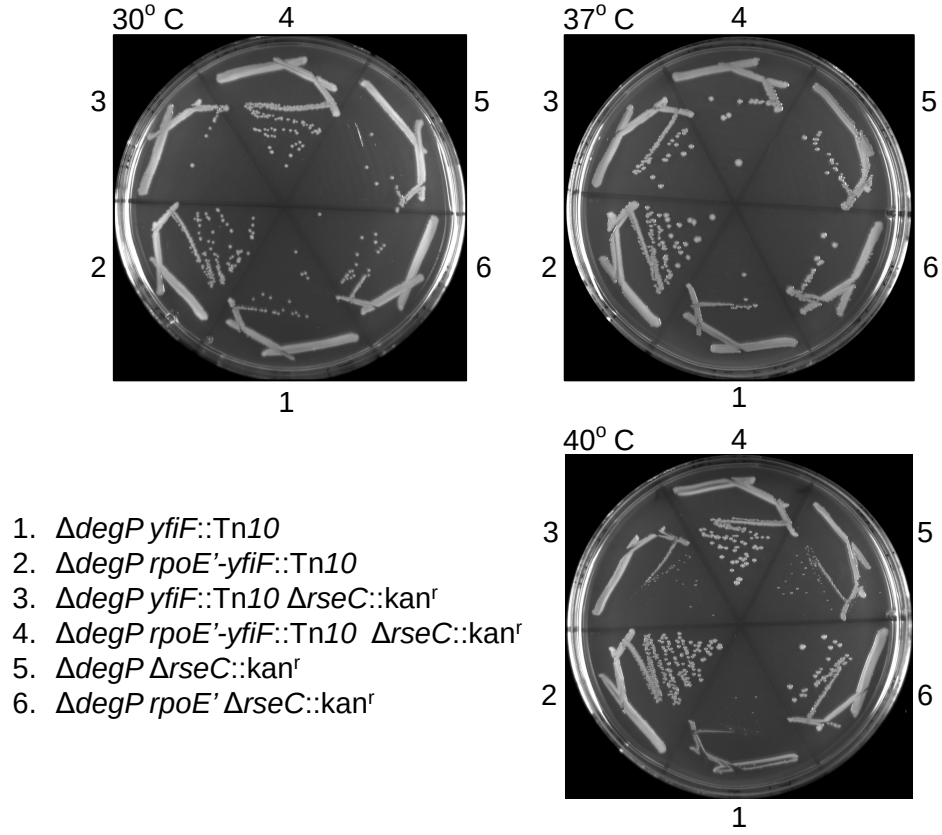


Fig. 31. Growth defect in recombinant strains. As a confirmation of the proximity of the suppressor to the *rpoE* region, recombinant Δ*rseC*::kan^r strain types were streaked on LBA and incubated overnight at the indicated temperatures.

analyzed by SDS-PAGE, transferred to PVDF membrane and probed with α-RpoE antibodies, as well as α-TolC and α-MalE antibodies to detect membrane and soluble fractions, respectively. Surprisingly, RpoE levels increased in both fractions (Fig. 33B and C), and so the increase in total levels cannot be attributed to an increase in either active or inactive RpoE. As with whole-cell lysates, no smaller RpoE' proteins were detected, suggesting either that the truncated protein is not actually produced in the cell, or that the antibodies used did not recognize it.

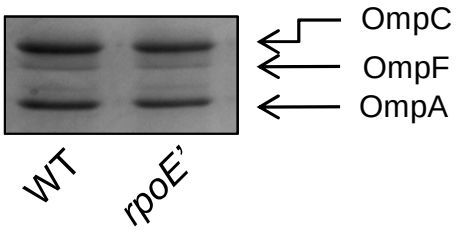


Fig. 32. *rpoE'* mutation has no effect on steady-state OMP levels. Carbonate-extracted membranes from wild-type and *rpoE'* were run on SDS-PAGE and stained with coomassie blue.

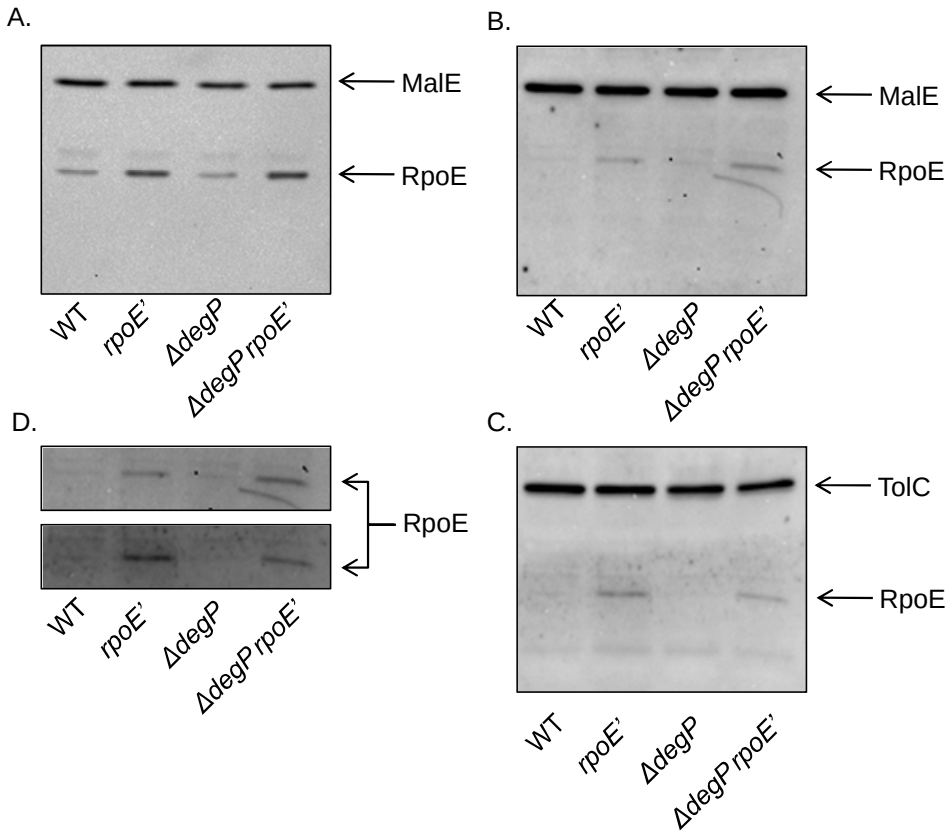


Fig. 33. Steady-state RpoE levels increase in *rpoE'* strains. Whole-cell lysates (A) and soluble fraction (cytosol and periplasm; B) were analyzed by SDS-PAGE and probed with α-MalE and α-RpoE antibodies. C. Membranes were analyzed by SDS-PAGE and probed with α-TolC and α-RpoE antibodies. D. Darkened images from B and C to show faint detection of RpoE in strains lacking *rpoE'*.

Overexpressed RpoE and RpoE'$_{6his}$ do not rescue ΔdegP temperature sensitivity

Because no chromosomally-encoded RpoE' was detected using Western blotting, it was postulated that the mutation could be *cis* acting rather than *trans*. It was reasoned that the suppressor could act by providing the cell with an additional copy of the native *rpoE* transcriptionstart site, rather than through the production of *rpoE' per se*. To test this, wild-type and truncated *rpoE* were cloned into the pBAD24 vector, in which expression is controlled by the arabinose-inducible *araBAD* promoter. In order to facilitate visualization of the truncated protein in the event the α-RpoE antibodies failed to recognize it, RpoE' was cloned with a carboxy-terminal hexahistidine tag (6his). The plasmids, along with empty vector control, were transformed into wild-type and *degP*::Tn*10* cells.

The six resulting strains were tested by streaking colonies on LBA plates containing ampicillin, with or without 0.2% arabinose to induce expression from the plasmid. The strains were grown overnight at 40° and 30° C. Neither *rpoE*$^+$ nor *rpoE'$_{6his}$* overexpression rescued *ΔdegP* temperature-sensitivity, and in fact overexpression from pBAD24-*rpoE*$^+$ had a deleterious effect on *degP*$^+$ strains (Fig. 34). Even at the low temperature, leaky expression of wild-type rpoE had a slightly deleterious effect in the cells, indicating that even the leaky expression of *rpoE* in an *rpoE*$^+$ background is too much for the cell to cope with.

Overexpressed RpoE and RpoE'$_{6his}$ localize to soluble and insoluble fractions

The fact that neither overexpressed *rpoE* nor *rpoE'$_{6his}$* rescued temperature sensitivity could reflect (a) mislocalization or aggregation during overexpression, (b) a consequence of overexpression of a critical regulator, or (c) that the expressed protein does not function *in vivo*.

To dissect these possibilities, cells were cultured in LB broth containing ampicillin at 30°C and grown until mid-exponential phase, when the cultures were induced with 0.2% arabinose. Cells were grown for two additional hours and membranes isolated by French Press lysis. After centrifugation, the soluble fraction was saved for further analysis. Soluble and membrane fractions from the six strains were analyzed by SDS- PAGE, transferred to PVDF membrane and probed with antibodies specific to RpoE, MalE and TolC (Fig. 35). The α-RpoE antibodies used recognized RpoE', and so probing specifically for the his-tag was not necessary. In a pattern reminiscent of chromosomal *rpoE'*, both wild-type and truncated *rpoE* localized to the membrane and soluble fractions. Chromosomal RpoE is visible running at its native molecular weight in the membrane fraction (Fig. 35B) but not in the soluble fraction (Fig. 35A), indicating that the σ^E response was not triggered under these conditions.

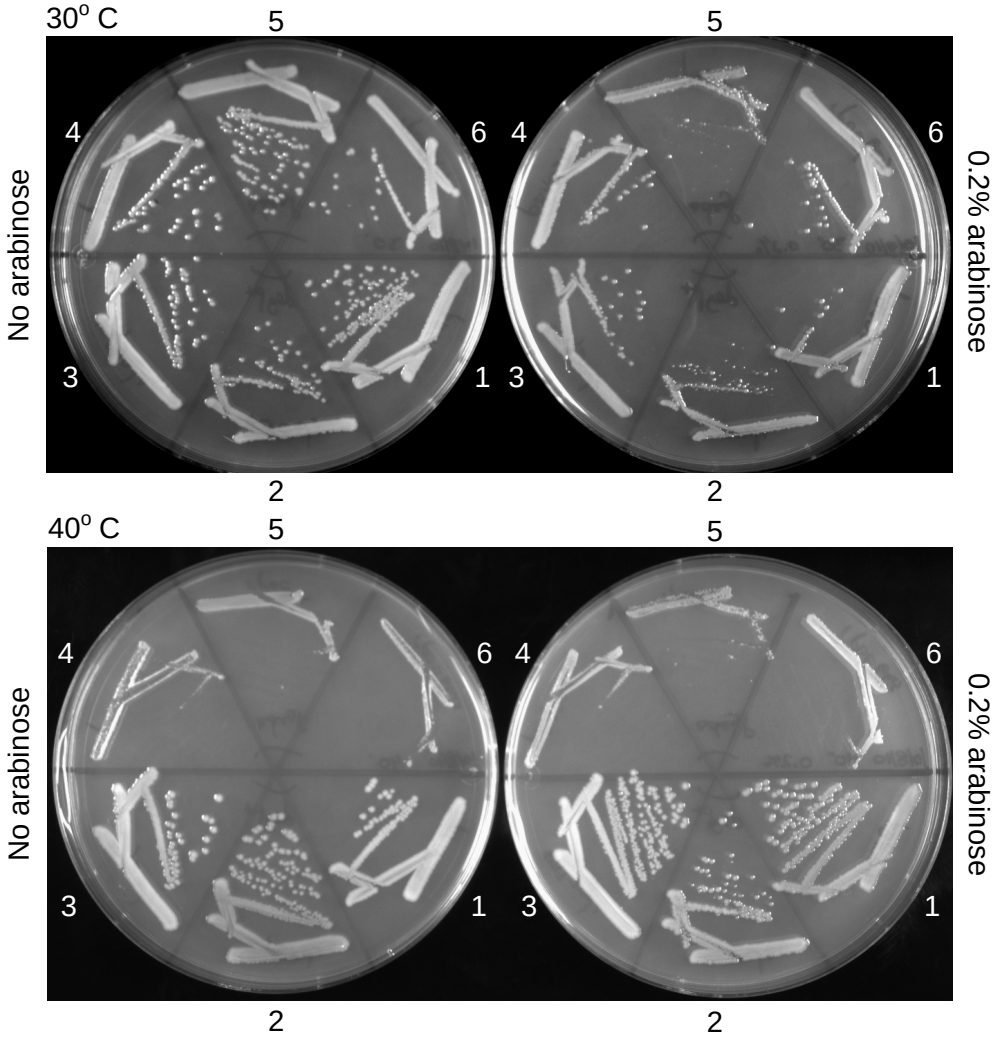
Since the truncated protein localized to the soluble fraction, yet failed to rescue the temperature sensitivity of *ΔdegP* cells, these data indicate that the effect of the suppressor is realized in *cis* through a slight

increase in wild-type *rpoE* transcription levels. Overexpressed RpoE must be at least somewhat functional, as steady-state MalE levels are decreased in this background (Fig. 35A). Additionally, membranes analyzed by urea SDS-PAGE and stained with coomassie blue showed that overexpressed *rpoE* resulted in a reduction in OmpC, OmpF and OmpA, a classical marker of increased σ^E activity (Fig. 35C).

Upregulation of the σ^E response in rpoE' cells

Since the suppressor did not exert its effect through the expression of truncated *rpoE'*, but rather appeared to act by allowing slightly more transcription/translation of wild-type *rpoE*, we sought to uncover subtle variations in σ^E response. To accomplish this, *rpoE'* was introduced into cells containing a Δ*rybB*::*lacZ* fusion. *RybB* is an sRNA under the sole control of σ^E, and is responsible for downregulation of *ompC* through binding and subsequent degradation of *ompC* mRNA (Johansen *et al,* 2006).

Cells were tested by growing cultures at 37°C until m id- to late-exponential phase and subjecting them to analysis by β-galactosidase assay. All assays were performed in at least quadruplicate. In cells harboring the *rpoE'* suppressor, Δ*rybB*::*lacZ* activity increased 1.3-fold (Fig. 36), indicating a very slight activation of σ^E response. This activation is the same as in cells lacking *degP* alone, indicating that the suppressor mutation serves to counteract the level of stress caused by the lack of this critical protein. In the *rpoE'* Δ*degP* double mutant, an additive effect was



1. *degP* + pBAD24
2. *degP* + pBAD24-*rpoE*
3. *degP* + pBAD24-$rpoE_{6his}$
4. Δ*degP* pBAD24
5. Δ*degP* pBAD24-*rpoE*
6. Δ*degP* pBAD24-$rpoE_{6his}$

Figure 34. Overexpression of *rpoE* does not rescue Δ*degP* temperature sensitivity. Wild-type and *ΔdegP* cells containing plasmid-borne *rpoE* and *rpoE'*$_{6his}$ were plated without and with 0.2% arabinose at the indicated temperatures and grown overnight.

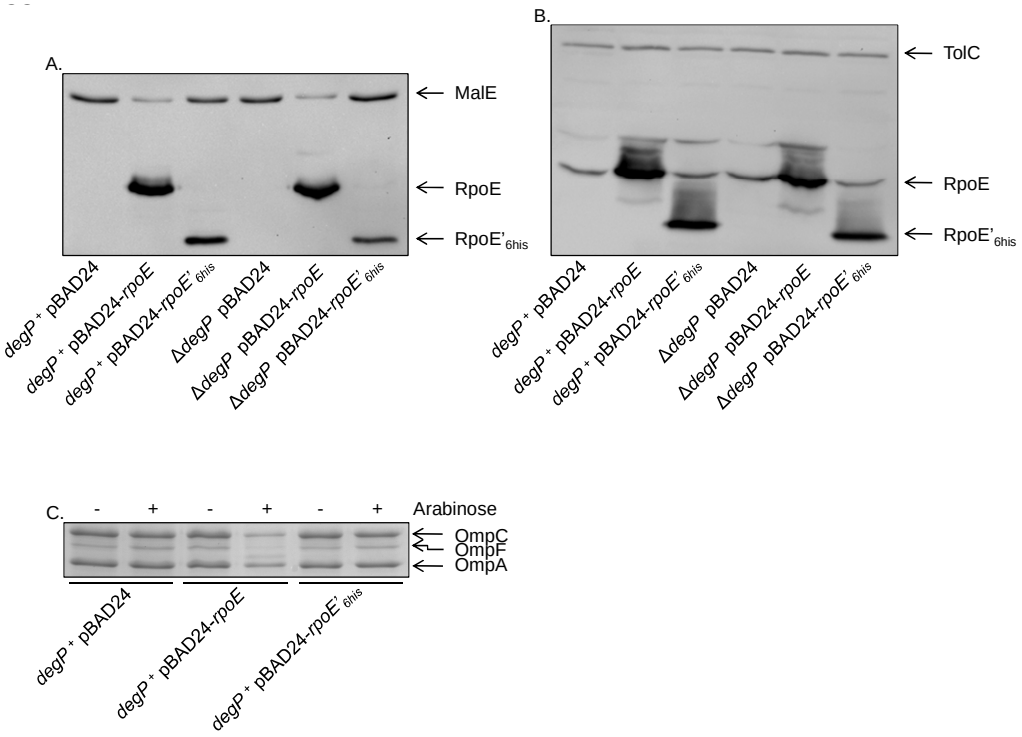


Fig. 35. Overexpressed RpoE localizes to membrane and soluble fractions. After induction, cells containing plasmid-borne *rpoE* and *rpoE'*$_{6his}$ were lysed and separated into membrane and soluble fractions. A. Soluble fraction was analyzed by SDS-PAGE and probed with α-MalE and α-RpoE antibodies. B. Membrane fraction was analyzed by SDS-PAGE and probed with α-TolC and α-RpoE antibodies. C. Membranes from wild-type cells induced for *rpoE*$^{+}$ or *rpoE'*$_{6his}$ expression were analyzed by urea SDS-PAGE and stained with coomassie blue.

observed, with *rybB*::*lacZ* activity increasing 1.7-fold over wild-type, indicating that the suppressor increases *rpoE* expression over that of *degP* alone. This result is not interpreted to be a cumulative response, because the increase in *rybB* expression in Δ*degP* cells is caused by stress, while the increase caused by *rpoE'* is a response to that stress. *RpoE'* acts in part by increasing proteolysis in the envelope. However, this data does explain why OMP levels are not significantly lowered in

these strains: while the increase in *rybB* expression is significant relative to wild-type, it is not sufficient to trigger the destruction of OMP mRNA.

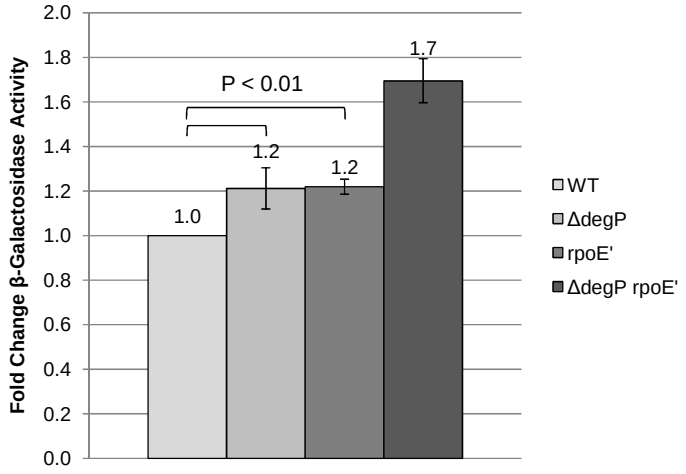


Fig. 36. *rpoE'* increases σ^E activity. Cells harboring *rybB*::*lacZ* were grown to late-log phase and assayed by β-galactosidase assay.

RpoE' acts in part by increasing proteolysis in the envelope

In addition to lowering the expression of OMPs, the σ^E envelope stress response functions to increase proteolysis in the envelope through the periplasmic protease DegP. Yet, the su ppressor functions to abolish temperature sensitivity caused by the lack of *degP*, and so cannot increase its expression to compensate for the increased stress. It was therefore asked whether the suppressor caused the upregulation of another protease in the envelope.

$AcrA_{L222Q}$ is an unstable mutant of AcrA, the membrane fusion protein in the TolC-AcrAB efflux complex. In a Δ*tolC* background, the absence of DegP, or growth at 30°C stabilize $AcrA_{L222Q}$ (Gerken and Misra, 2004). Because of its differential stability properties it makes an

ideal substrate to assay envelope proteolysis activity *in vivo*. $AcrA_{L222Q}$ was introduced into cells harboring *rpoE'* and lacking *tolC*. In order to test for proteolysis in this background, *degP* was also removed by a *degP*::kan^r insertion.

Cells were grown overnight in LB broth at 37°and 3 0°C, at which point whole cell samples were taken according to final cell density and boiled in SDS-PAGE buffer containing β-mercaptoethanol. Samples were analyzed by SDS-PAGE, transferred to PVDF membrane and probed with antibodies specific to AcrA and TolC-trimer. TolC-trimer antibodies cross-react with BamA, providing a loading control for the gels. As expected, $AcrA_{L222Q}$ is stable at 30°C (Fig. 37, lanes 1-4). At 37°C, i t was degraded *degP*$^+$ cells, but stabilized in *ΔdegP* cells (Fig. 37, lanes 5, 6 and 7). However, in cells lacking *degP* but containing *rpoE'*, $AcrA_{L222Q}$ levels fell to 20% of the level of *ΔdegP* alone (Fig. 37, lane 8), reflecting a decrease in the stability of the protein and thus an increase in proteolysis in the envelope.

Envelope proteolysis is increased in an rseA degP double mutant

Since the *rpoE'* mutant shows increased σ^E response, and since envelope proteolysis is increased in strains containing the suppressor, we reasoned that there could be a proteolytic pathway directly controlled by σ^E in addition to DegP. Using the $AcrA_{L222Q}$ mutant background, Δ*rseA*::kan^r and *degP*::Tn*10* deletions were introduced by P1 transduction. Cells were grown overnight at 37°C, p repared as above,

and probed with α-AcrA and α-LamB antibodies. In *rseA*$^{+}$ *degP*$^{+}$ cells, AcrA$_{L222Q}$ is readily degraded (Fig. 38), while in *rseA*$^{+}$ *ΔdegP* cells the protein is stabilized. When *rseA* is removed from *degP*$^{+}$ cells, no additional reduction is noted over wild-type, although LamB levels are reduced due to increased σ^{E} response. However, in *ΔrseA ΔdegP* cells the steady-state levels of AcrA$_{L222Q}$ remained low, indicating that there was DegP-independent protease activity responsible for degradation.

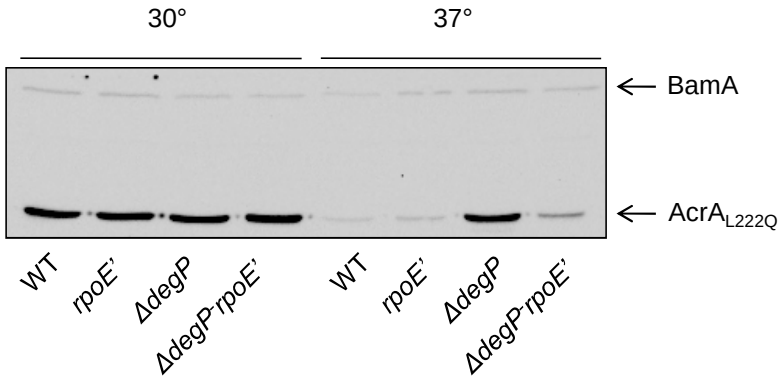


Fig. 37. *rpoE'* increases proteolysis in the envelope. Whole-cell samples from overnight cultures grown at the indicated temperatures were run on SDS-PAGE and probed with α-TolC trimer and α-AcrA antibodies. Note that α-TolC trimer antibodies cross-react with BamA.

acrA is not normally a part of the σ^{E} regulon; however it is possible that it could be affected by the extreme induction of σ^{E} in an *rseA*$^{-}$ background. To eliminate this as a factor in reduction of AcrA$_{L222Q}$ levels, *acrA$_{L222Q}$* expression was directly measured by quantitative reverse-transcriptase PCR (qRT-PCR). Total RNA was extracted from overnight

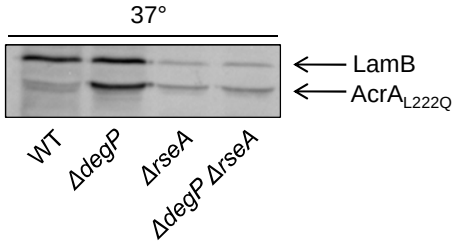
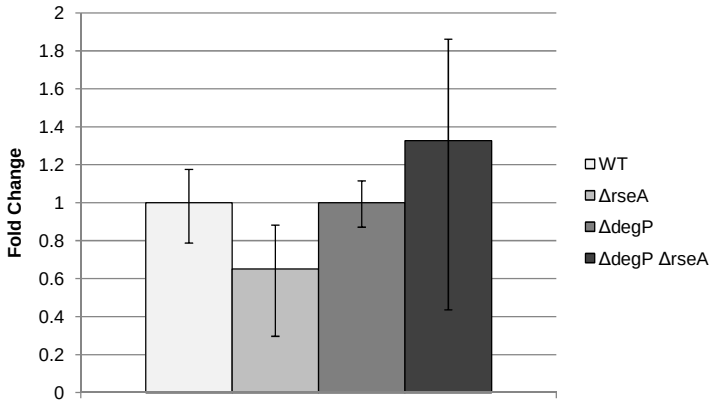


Fig. 38. Δ*rseA* cells degrade AcrA$_{L222Q}$ in the absence of *degP*. Whole-cell samples from overnight cultures were run on SDS-PAGE and probed with α-LamB and α-AcrA antibodies.

cultures and converted it into cDNA. Expression levels quantified using the ΔΔ*ct* method with the housekeeping gene *purC* as a control. No significant difference was found in *acrA$_{L222Q}$* expression in any of the strains tested (Fig. 39). These data supported the notion that there is a *bona fide* proteolytic member of the σ^E regulon in the envelope responsible for the observed destabilization/degradation. Further, the data show that in an *rpoE'* background this activity is upregulated, and that this at least in part constitutes the mechanism of suppression.



Fig, 39. AcrA$_{L222Q}$ transcription is unchanged in Δ*rseA* strains. cDNA made from overnight culture total RNA was subjected to qRT-PCR and analyzed using the ΔΔ*ct* method.

Chapter 4

RESULTS III: THE MULTICOPY SUPPRESSOR YFGC

Isolation and identification of yfgC as a multicopy suppressor

Cells expressing OmpF mutants which intrinsically misfold require the presence of DegP in order to degrade or capture those mutants as they accumulate in the envelope (Misra *et al,* 2000). Previous work exploited this fact to isolate multicopy plasmid suppressors of lethality caused by the lack of *degP* in a background expressing a mutant OmpC protein, $OmpC_{2cys}$ (Castillo-Keller *et al,* 2006). This OmpC mutant contains two non-native cysteine residues which spontaneously form a disulfide bond in the periplasm, preventing the protein from folding and inserting into the OM properly. It is possible to relieve lethality simply by removing *dsbA*, which encodes for a periplasmic disulfide bond isomerase, thus preventing the disulfide bond from forming (Castillo-Keller *et al,* 2006).

Double mutant cells in *degP*::kan^r and $ompC_{2cys}$ are dependent on heterologous expression of plasmid-borne $degP_{S210A}$ for survival. Cells expressing the protease-deficient $DegP_{S210A}$ from the Ispropyl β-D-thiogalactopyranoside (IPTG)-inducible pACYC184 plasmid were transformed with a plasmid library consisting of Sau3A-digested chromosomal DNA ligated into BamH1-digested pBR322 vector (Castillo-Keller *et al,* 2006). The resulting transformants were selected on media lacking IPTG to prevent $degP_{S210A}$ expression and therefore demand that

the plasmid-borne DNA rescue lethality. Two multicopy suppressors, expressing *ompR'* and the novel sRNA *ipeX* were isolated, which function to lower the expression of *ompC* at the transcriptional and post-transcriptional level, respectively (Castillo-Keller *et al,* 2006).

A third multicopy suppressor, *yfgC*, was isolated using this method, but was not characterized at that time. *yfgC* is located at 56.3' on the *E. coli* chromosome. Bioinformatics suggests that it is a part of the M40 family of zinc-metalloproteases (Fig. 40). YfgC possesses an amino-terminal 27 amino acid signal sequence, culminating in a canonical Ala-X-Ala sequence, where X is any amino acid, and which in YfgC is Ala-Phe-Ala. This signal sequence was shown to be cleaved through sequencing the amino-terminus of mature Yfgc by Edman degradation. YfgC is thought to be a zinc-metalloprotease due to a conserved zinc-binding motif with the consensus sequence His-Glu-X-X-His, where X is any amino acid. In *E. coli*, this sequence is $_{136}$His-Glu-Ile-Ser-His$_{140}$. The motif is conserved throughout the domains of life, including humans. Finally, YfgC possesses a carboxy-terminal tetratricopeptide repeat (TPR) domain, consisting of four structurally conserved TPR motifs. TPR motifs are commonly found in substrate binding domains (D'Andrea and Regan, 2003).

YfgC is positively regulated by σ^E

The level of *yfgC* mRNA was shown to be increased 3.3-fold in cells overexpressing *rpoE* by microarray analysis (Rhodius *et al,* 2006). To

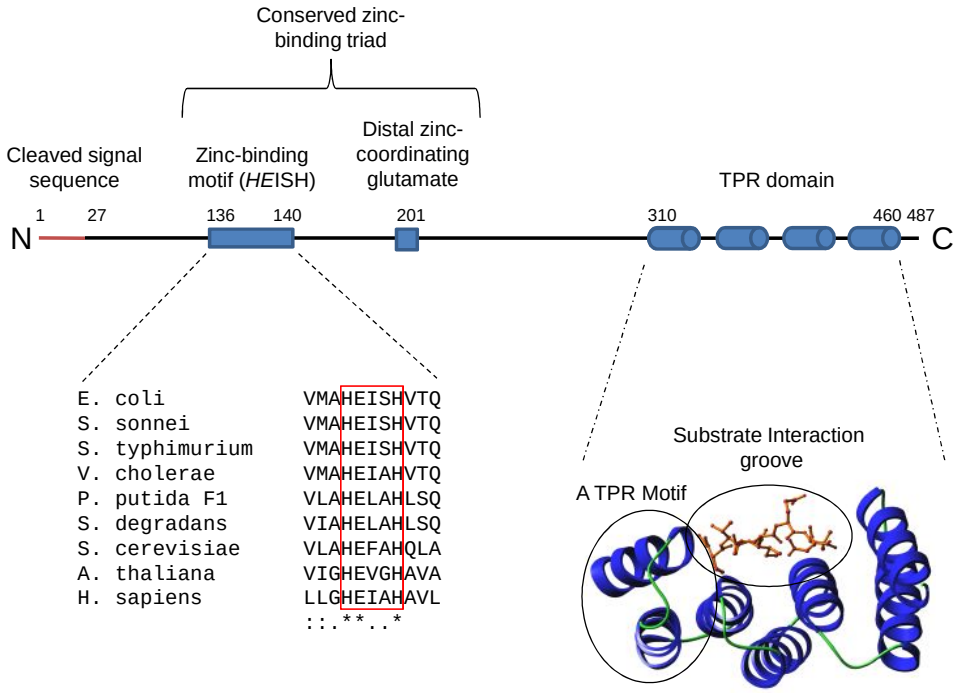


Fig. 40. Domain structure of YfgC. YfgC contains an amino-terminal cleaved signal sequence, a conserved zinc-binding triad, and a carboxy-terminal tetratricopeptide repeat (TPR) domain. Example of TPR structure taken from Scheufler *et al*, 2000.

confirm this in the strains used in this study, a Δ*yfgC*::*lacZ* transcriptional fusion was constructed at the native *yfgC* locus. The fusion was then introduced into wild-type and Δ*rseA*::scar backgrounds by P1 transduction. The resulting strains were grown at 37°C until late e xponential phase and subjected to β-galactosidase assay. In the Δ*rseA* strain Δ*yfgC*::*lacZ* activity was 10-fold higher than in the wild-type background, indicating that *yfgC* is a *bona fide* member of the σ^E stress response (Fig. 41).

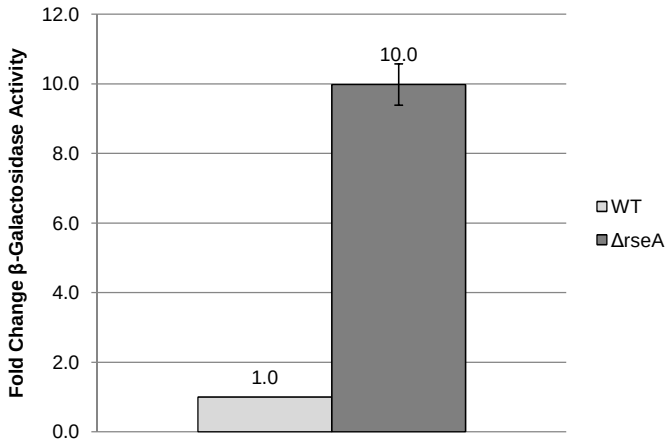


Fig. 41. *yfgC* is positively regulated by σ^E. Expression of y*fgC*::*lacZ* transcriptional fusion was assayed by β-galactosidase activity in the absence of the anti-sigma factor *rseA*.

Since an elevated σ^E response is reflected in increased Δ*yfgC*::*lacZ* activity, then downregulation of the σ^E response through overexpression of the anti-sigma factor *rseA* should have the opposite effect. Indeed, when $rseA_{6his}$ cloned into the arabinose-inducible plasmid pBAD24 was transformed into Δ*rseA*::scar cells and overexpressed, β-galactosidase activity from the Δ*yfgC*::*lacZ* fusion was reduced six-fold from the level without induction (Fig. 42). Induction of the empty vector in the same background had no effect. Presumably, the ~25% drop in fusion activity between uninduced vector and uninduced pBAD24-*rseA* is due to leaky expression from the plasmid.

Phenotypes of cells lacking *yfgC*

Lack of yfgC confers OM permeability defect

One of the hallmarks of a disruption in the permeability function of

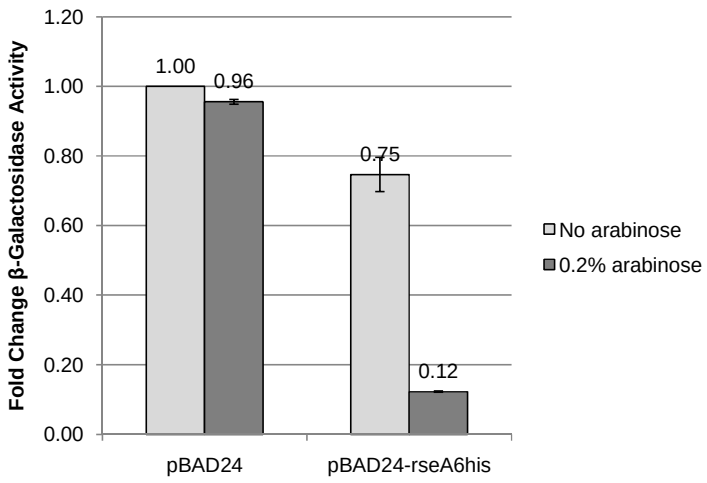


Fig. 42. Overexpression of *rseA* reduces *yfgC* transcription. Sequestration of σ^E by overexpressed RseA causes reduced transcription of *yfgC*::*lacZ* as measured by β-galactosidase activity.

the OM is sensitivity to antibiotics, which are normally excluded from the cell. Antibiotic sensitivity can be induced by removing key genes involved in envelope biogenesis (for example, *bamB* or *surA).* To test whether Δy*fgC* cells were sensitive to antibiotics, overnight cultures were mixed into molten LBA and overlaid on solid LBA plates. Antibiotic discs impregnated with rifampin or erythromycin were placed on the cooled agar and a lawn was allowed to grow overnight. Sensitivity was measured by comparing the diameter of the zones of inhibition surrounding each disc. Cells lacking *yfgC* were significantly more sensitive to both antibiotics tested than wild-type cells (Fig. 43) indicating that the permeability barrier function of the OM was compromised in these cells. Sensitivity to rifampin was increased by 2.3 mm in *yfgC*$^-$ cells, while sensitivity to erythromycin

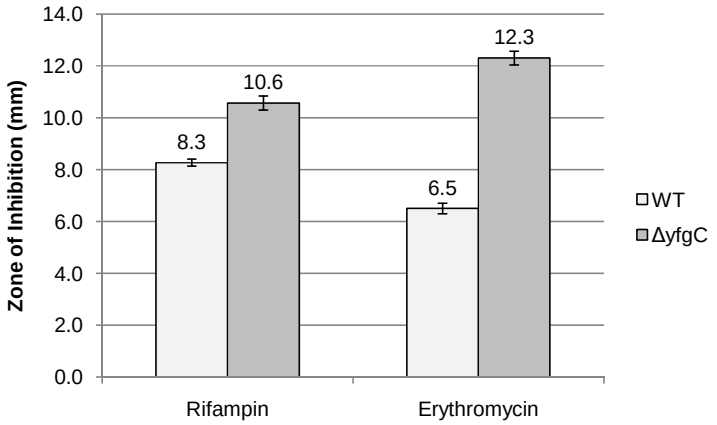


Fig. 43. Cells lacking *yfgC* are sensitive to antibiotics. Antibiotic discs were placed on a growing lawn of wild-type and *yfgC*$^{-}$ cells and grown overnight. Diameter of discs was 6.1 mm.

was increased by 5.8 mm. This sensitivity could be reversed to nearly wild-type levels through the heterologous expression of *yfgC* from the arabinose-inducible plasmid pBAD24 (Fig. 44).

YfgC and SurA confer additive sensitivity defects

Since YfgC has been implicated in OMP assembly, in addition to testing *yfgC* alone we chose to also construct isogenic Δ*surA*::kan^{r} and double mutant strains, in order to determine whether YfgC acted in the same assembly pathway as SurA. Cells lacking *surA* were significantly more sensitive to antibiotics (Fig. 45), with an increase of 8 mm (rifampin) and 7.4 mm (erythromycin) over wild-type. An additive effect was observed in double mutants, which were more sensitive to rifampin and erythromycin, with significant increases in zones of inhibition over either single mutant. This additive effect suggests that YfgC and SurA act in different, but possibly overlapping, assembly pathways in the envelope.

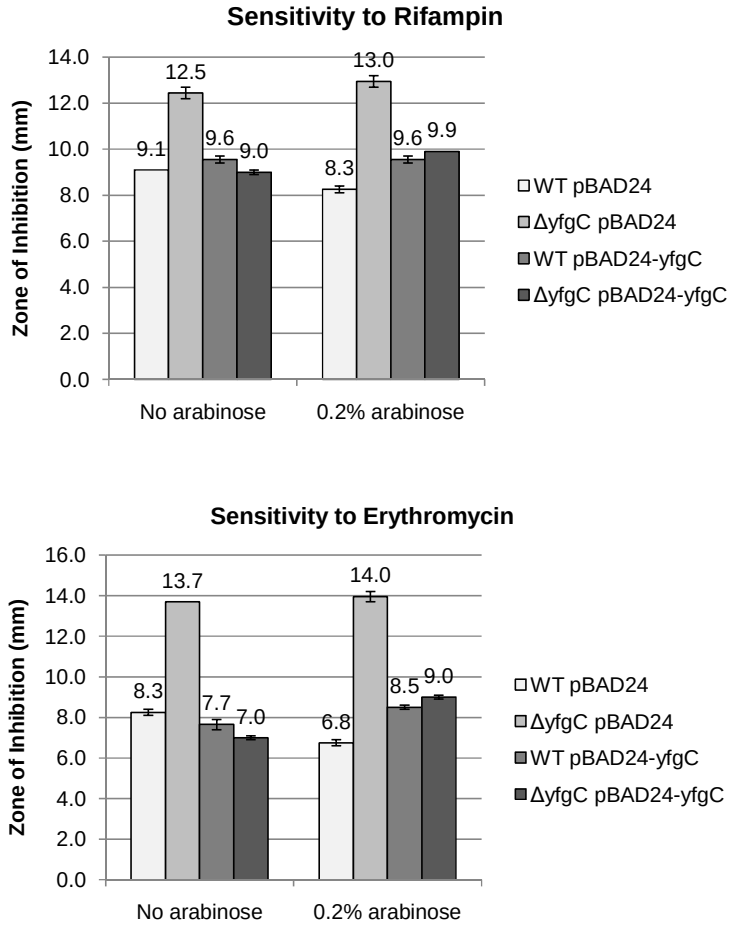


Fig. 44. Plasmid-borne *yfgC* complements antibiotic sensitivity. Wild-type and *yfgC*⁻ cells harboring pBAD24-*yfgC* were grown overnight on media containing 0.2% arabinose to induce expression of *yfgC*. Diameter of antibiotic discs was 6.1 mm.

Lack of yfgC exacerbates surA growth defect

Since YfgC and SurA appear to act in different envelope assembly pathways, it was asked whether any individual growth defects in liquid medium would have an additive effect in a double mutant. Cells were subcultured from overnights into fresh LB broth and grown for three hours at 37°C with vigorous shaking. Cell density was tracked every thirty

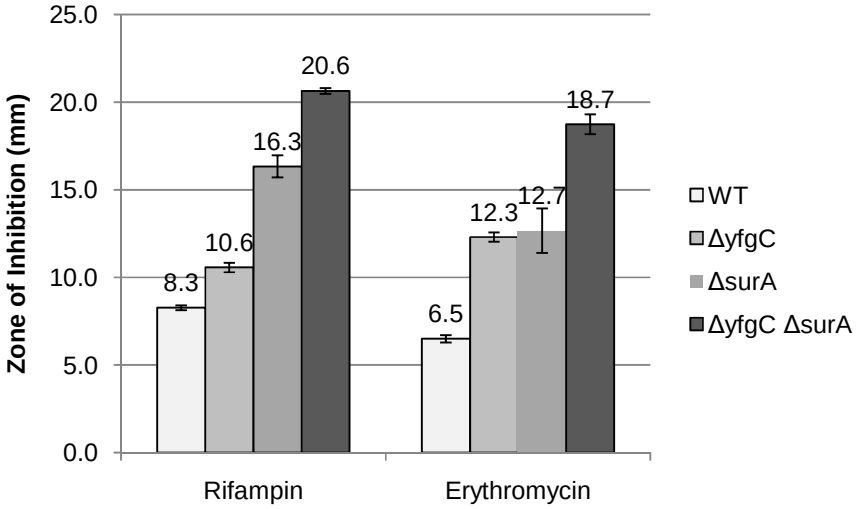


Fig. 45. Δ*yfgC* exacerbates Δ*surA* antibiotic sensitivity. Antibiotic discs were placed on a growing lawn of cells and incubated overnight. Diameter of discs was 6.1 mm.

minutes. Cells lacking *yfgC* showed no significant growth defect over wild-type (Fig. 46), while Δ*surA* cells grew more poorly than wild-type, and to a lower cell density. Strikingly, the double mutant took a significantly longer time to exit lag phase, and did not grow to nearly the same cell density by the end of the experiment. This synthetic phenotype in liquid media strengthens the supposition that YfgC and SurA provide different, but possibly overlapping functions necessary for proper envelope biogenesis.

YfgC overexpression rescues Δ*degP ompC*$_{501}$ lethality

YfgC rescues OmpC$_{501}$ *ΔdegP temperature sensitivity on solid media*

Because YfgC is known to overcome lethality resulting from $OmpC_{2cys}$ expression in a Δ*degP* background, we sought to investigate its activity in other mutant OmpC backgrounds. Besides $OmpC_{2cys}$, other

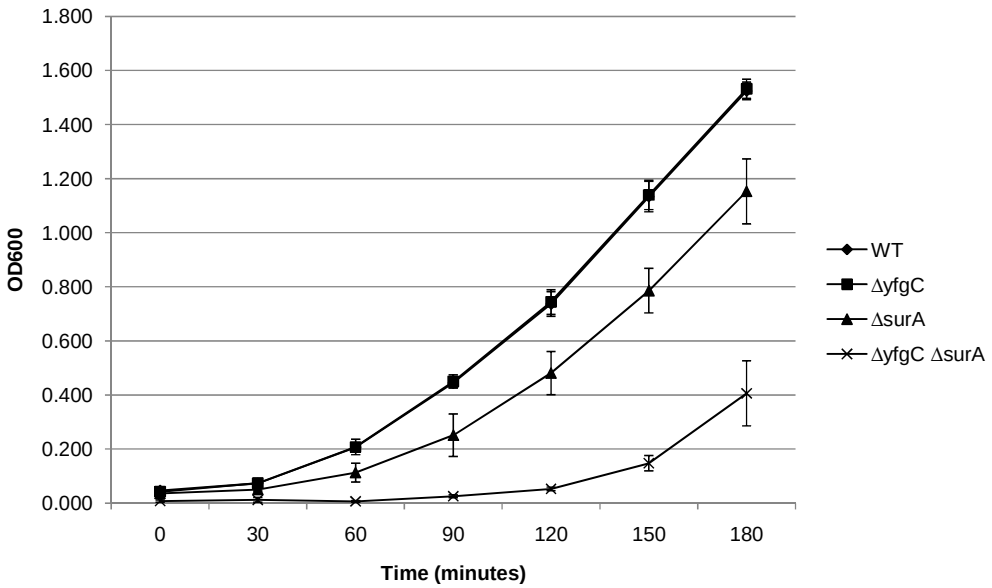


Fig. 46. Δy*fgC* exacerbates Δ*surA* growth phenotype. Cells were incubated at 37°C in LB, and cell density tracked ever y thirty minutes.

OmpC mutants are known to misfold in the envelope. $OmpC_{501}$ is one such mutant. It was isolated as a spontaneous SDS-resistant mutant of another *ompC* allele, the dex^+ mutant $OmpC_{124}$ (Xiong *et al,* 1996). $OmpC_{501}$ possesses a deletion in loop two of the interior of the barrel, rendering it Dex^+, but also carries the compensatory mutation G42D. An additional mutant isolated in the screen, $OmpC_{509}$, contained a mutation in another glycine residue, G224D. The G42D and G224D mutations render the interior of the barrel more negatively charged, thus preventing the entry of SDS.

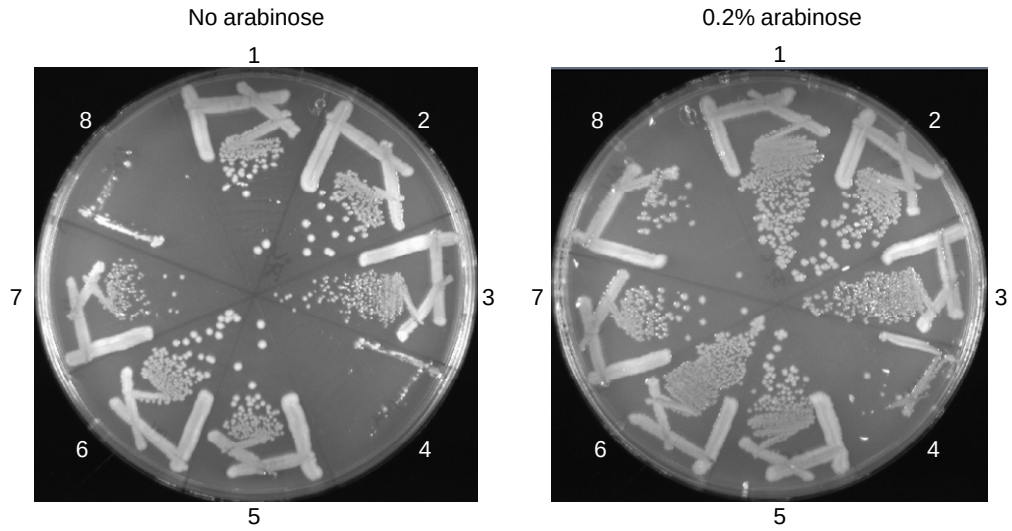
Cells harboring wild-type *ompC*, parental $ompC_{124}$ and the terminal mutants $ompC_{501}$ and $ompC_{509}$ were transformed with pBAD24 vector or pBAD24-were transformed with pBAD24 vector or pBAD24-*yfgC*. Since these strains were chromosomally $degP^+$, they did not exhibit a growth

defect when routinely grown on LBA ampicillin plates at 30°C. In order to remove *degP* from *ompC*$_{501}$ cells, it was required to grow the cells on M63-salt based minimal media. The requirement for this is two-fold: not only does growth slow sufficiently on minimal media so as to reduce the demand for OMPs in the envelope, but *ompC* transcription is also repressed under conditions of low osmolarity (Forst *et al,* 1989).

The resulting *degP*$^{-}$ strains were streaked at 30°C overnight onto LBA ampicillin plates with and without 0.2% arabinose to induce *yfgC* transcription (Fig. 47). Prior to the experiment, cells were grown on M63 minimal medium containing ampicillin but no arabinose, in order to avoid preloading them with YfgC. Wild-type and *ompC*$_{124}$ cells grew with no defect regardless of plasmid or induction. As expected, both ompC$_{501}$ pBAD24 and pBAD24-*yfgC* cells were unable to grow without inducer (Fig. 47, left panel). On media containing arabinose, growth of *ompC*$_{501}$ pBAD24 cells was also completely inhibited (Fig. 47, right panel). Overexpression of *yfgC*, however, restored growth in these cells. This experiment indicated that YfgC acts in diverse *ompC* mutant backgrounds. Since no severe defect was uncovered in Δ*degP ompC*$_{509}$ background, it was excluded from further analysis.

YfgC rescues OmpC$_{501}$ *growth defect in liquid media*

Because overexpression of *yfgC* rescues such a severe defect in a Δ*degP ompC*$_{501}$ background, the degree to which it improved growth in liquid media was determined. *degP*$^{+}$ cells harboring the *ompC*$_{501}$ allele



1. *ompC*$^{+}$ pBAD24
2. *ompC*$_{124}$ pBAD24
3. o*mpC*$_{509}$ pBAD24
4. o*mpC*$_{501}$ pBAD24
5. o*mpC*$^{+}$ pBAD24-*yfgC*
6. o*mpC*$_{124}$ pBAD24-*yfgC*
7. o*mpC*$_{509}$ pBAD24-*yfgC*
8. o*mpC*$_{501}$ pBAD24-*yfgC*

Fig. 47. Overexpression of *yfgC* rescues *ompC*$_{501}$ Δ*degP* lethality. Strains were streaked on LBA plates containing ampicillin and 0.2% arabinose (right panel only) and grown overnight at 30°C.

and a wild-type copy of *yfgC* under the control of the arabinose-inducible *araBAD* promoter were grown to mid-log phase at 30°C and 37°C, with cell density measured every thirty minutes. At mid-log phase the cultures were split, with half receiving 0.2% arabinose. Growth was continued for two more hours post-induction.

At the permissive temperature of 30°C, cells grow no rmally in the presence or absence of inducer, suggesting that the OmpC folding defect is not as severe (Fig. 48). However, at 37°C cells wit hout arabinose began to experience slower growth than their induced counterparts shortly after splitting, and failed to grow to the same final cell density, indicating

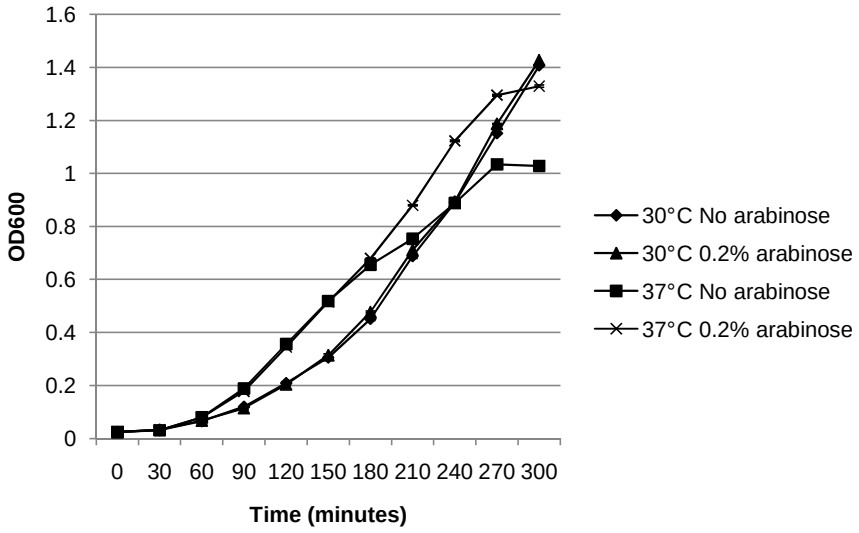


Figure 48. *yfgC* overexpression rescues $ompC_{501}$ growth defect. Cells expressing $ompC_{501}$ and containing pBAD24-*yfgC* were grown in LB supplemented with ampicillin, with cell density tracked every thirty minutes. Where indicated, cultures were induced with 0.2% arabinose at 150 minutes.

that YfgC exerts a positive effect in these cells.

YfgC restores cell viability in $OmpC_{501}$ cells

Cells were sampled from the final time point in Fig. 47 and plated on LBA containing ampicillin at the permissive temperature to determine cell viability. Consistent with growth data, at 30°t hese cells had no difference in cell viability (Fig. 49). However, at 37°C cells without induction of *yfgC* showed a five-fold reduction in cell viability relative to those induced, indicating that YfgC plays a significant role in combating the stress caused in a $ompC_{501}$ background at high themperatures. Curiously, overexpression of *yfgC* at 30°C increased cell viability two-fold, although it did not seem to improve growth in liquid media.

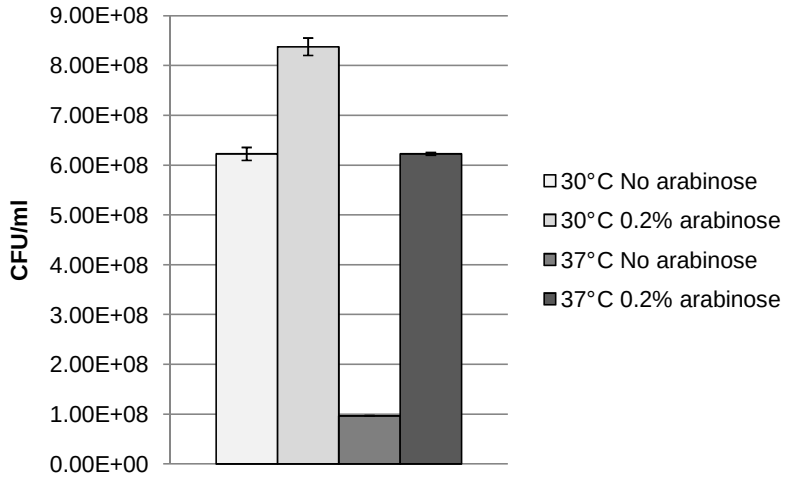


Fig. 49. *yfgC* overexpression increases viability of $ompC_{501}$ cells. Cells were taken from the final time point in Fig. 46 and plated at 30°C. After overnight growth, colonies were counted and CFU/ml calculated.

yfgC transcription responds to envelope stress

Because YfgC is capable of rescuing cells from *degP*-mediated lethality in an $ompC_{501}$ background, it was asked if *yfgC* transcription increases in the presence of mutant *ompC*. Wild-type, $ompC_{124}$ and $ompC_{501}$ alleles were introduced into a strain harboring Δ*yfgC*::*lacZ*. Additionally, another strain was constructed in which Δ*ompC*::cm^r was introduced to test for reduced *yfgC* transcription in the complete absence of *ompC*. The resulting strains were subjected to β-galactosidase assay (Fig. 50). In the presence of $ompC_{124}$, *yfgC* transcription is increased 1.8-fold, reflecting an increase in envelope stress in this mutant. $OmpC_{501}$ expression increases *yfgC* transcription even higher, to 3.0-fold above wild-type, which is consistent with an even increased level of envelope

stress. In the absence of *ompC,* envelope stress is significantly reduced, and *yfgC* transcription reflects this with a 40% reduction relative to wild-type. These data indicate that *yfgC* transcription is responsive to envelope stress caused by misfolded OmpC.

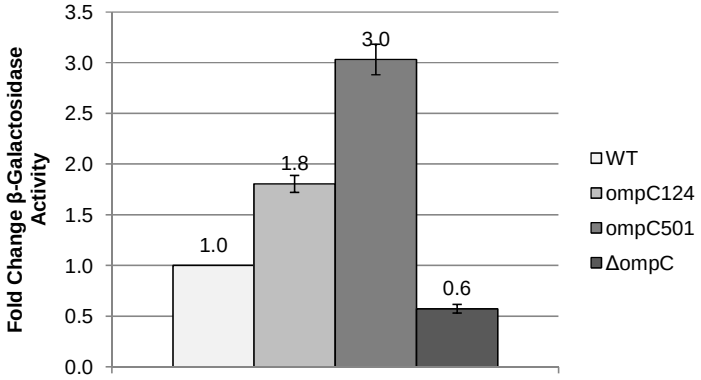


Fig. 50. *yfgC* transcription is regulated in response to envelope stress. Cells were grown until late-log phase and *yfgC*::*lacZ* activity determined by β-galactosidase assay.

Determining YfgC's mechanism of action

Ultimately, YfgC must exert its effect somewhere in the envelope for it to rescue cells from temperature sensitivity. Normally, we would utilize mutant forms of YfgC in order to investigate the ways and degree to which they abrogate wild-type function. However, during the course of this study it was discovered that the mutations [H136F, E137G] and E201A (both affecting the zinc-coordinating triad), as well as a deletion of the fourth TPR motif (ΔTPR4) rendered the protein insoluble. Since these alleles mislocalize to the insoluble fraction *in vivo*, and therefore could not exert any effects on the envelope, the investigation of function was limited

to wild-type YfgC.

To enable future biochemical studies of *YfgC*, it was cloned into pBAD24 using a primer which incorporated a hexahistidine tag at the carboxy-terminus of the protein. It was determined by rescue of $ompC_{501}$ temperature sensitivity that $YfgC_{6his}$ is a functional protein *in vivo* (Table 3).

Table 3. $YfgC_{6his}$ rescues *ompC*-mediated lethality.

	30°C LBA No arabinose	30°C LBA 0.2% arabinose	30°C M63 No arabinose
$ompC^+$ pBAD24	++	++	++
$ompC^+$ pBAD24-$yfgC_{6his}$	++	++	++
$ompC_{124}$ pBAD24	++	++	++
$ompC_{124}$ pBAD24-$yfgC_{6his}$	++	++	++
$ompC_{501}$ pBAD24	-+	-	++
$ompC_{501}$ pBAD24-$yfgC_{6his}$	-+	++	++

YfgC reduces mutant OmpC levels in the envelope

YfgC possesses two conserved motifs which make it an ideal candidate protease/chaperone activity in the envelope. First, it contains the conserved zinc-coordinating motif HEISH, common to many metalloproteases. Second, it contains a structurally conserved TPR domain, which is often thought to be involved in substrate interaction. Mutations in either region affect the proper folding of the protein. Because YfgC has been shown to have an effect on cells expressing $ompC_{501}$, and

because it was isolated in an *ompC*$_{2Cys}$ background, we investigated its effect on mutant OmpC levels in the envelope.

Cells expressing wild-type *ompC*, *ompC*$_{501}$, *ompC*$_{2Cys}$, and the intermediate *ompC* alleles *ompC*$_{124}$ and *ompC*$_{1Cys}$ were transformed with pBAD24 and pBAD24-*yfgC*$_{6his}$. The resulting ten strains were cultured in LB at 37°C until mid-log phase. At that time they were induced with 0.2% arabinose and grown for another two hours. Membranes were extracted, analyzed by SDS-PAGE and stained with coomassie blue to visualize the major OMPs.

It was immediately obvious that YfgC$_{6his}$ expression had a significant effect on the levels of OmpC$_{501}$ and OmpC$_{2Cys}$ (Fig. 51A, lanes 6 and 10). OmpC$_{501}$ and OmpC$_{2Cys}$ levels were reduced in cells expressing YfgC$_{6his}$, concomitant with an increase in OmpF. To quantify the relative change in OMP levels, the ratio of OmpC to OmpF was calculated (Fig. 51B). Cells expressing wild-type and the two parental *ompC* alleles had virtually unchanged ratios of OmpC:OmpF regardless of the presence pBAD24 or pBAD24-*yfgC*$_{6his}$, with OmpC levels being approximately 80% of OmpF. In cells expressing *ompC*$_{501}$ or *ompC*$_{2Cys}$, however, this ratio changed to 135% and 161% of OmpF, respectively, for empty vector control. Expression of *yfgC*$_{6his}$ altered the OmpC:OmpF ratio further, but in the opposite direction. In the *ompC*$_{501}$ background YfgC$_{6his}$ expression brought OmpC to 28% of OmpF, and in *ompC*$_{2Cys}$ the ratio was even lower, at 19% of OmpF.

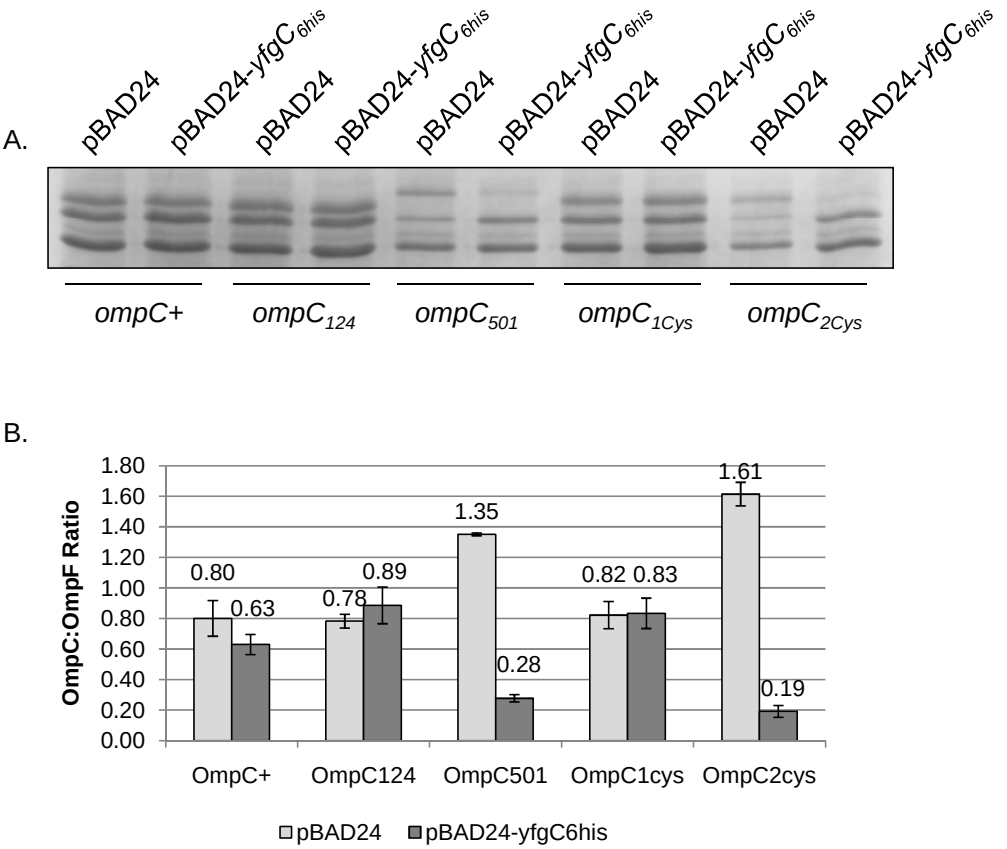


Fig. 51. *YfgC$_{6his}$* alters the OMP profile in the envelope. A. Membranes prepared from cells expressing *yfgC$_{6his}$* were run on SDS-PAGE and stained with coomassie blue. B. Ratio of OmpC to OmpF in cell membranes was calculated by comparing the two proteins' relative abundance.

Reduction of OmpC levels is not due to transcriptional repression

One argument for why OmpC levels are reduced in cells expressing *yfgC$_{6his}$* is that the overexpression triggers an envelope stress response in itself, resulting in a repression of *ompC* synthesis. To address this, the activity of an *ompC*::*lacZ* transcriptional fusion located at the native *ompC* locus was assayed by β-galactosidase assay. The *ompC*::*lacZ* strain was transformed with pBAD24 and pBAD24-*yfgC$_{6his}$*. Cells were grown until mid-log phase and either induced with 0.2% arabinose or treated with the

same amount of sterile water. Growth was continued for two more hours, and β-galactosidase activity measured. In *yfgC*$_{6his}$-expressing cells, *ompC* transcription levels were reduced 20% in when cells were treated with arabinose (Fig. 52). However, the same reduction was observed in cells harboring the empty vector alone, and so this phenomenon could not be attributed to expression of *yfgC* by itself. Thus, the observed change in OmpC:OmpF ratios in mutant *ompC* backgrounds was not due to inhibition of *ompC* transcription by overexpressed *yfgC*.

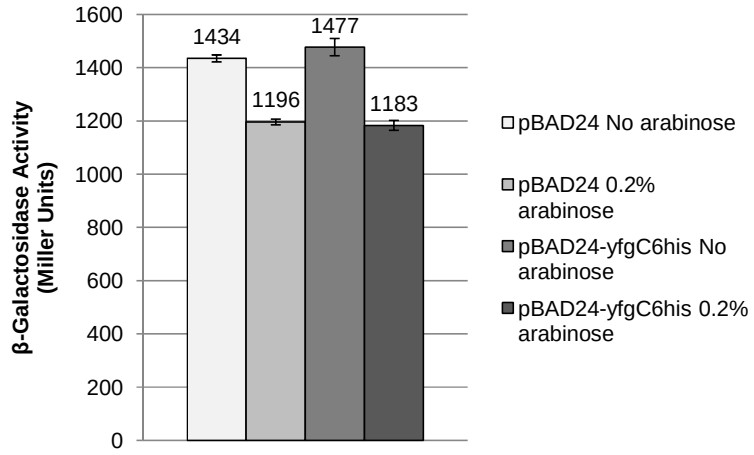


Fig. 52. *yfgC* overexpression does not affect *ompC* transcription. An *ompC*::*lacZ* transcriptional fusion was assayed by β-galactosidase assay in the presence and absence of induced *yfgC* expression.

Purified YfgC$_{6his}$ *forms crosslinked complexes in mutant OmpC backgrounds*

Since YfgC$_{6his}$ was observed to alter OmpC$_{501}$ levels, YfgC-OmpC

interaction was investigated through copurification using $YfgC_{6his}$ as bait on a Nickel-Nitriloacetic acid (Ni-NTA) liquid chromatography column. If YfgC interacts with OmpC, the two proteins should co-elute from the column. Purification was initially attempted by growing cells expressing $ompC_{501}$ and $yfgC_{6his}$, lysing the cells by French Press and passing the filtered soluble fraction through an Ni-NTA column. Unfortunately, this method failed to pull down detectable levels of OmpC with $YfgC_{6his}$.

An explanation for this result is that YfgC may act only transiently with its substrate proteins, and/or that this interaction is not strong enough to withstand the purification process. To circumvent this problem, the experiment was repeated using the *in vivo* crosslinker Dithiobis[succimidyl propionate] (DSP). Because it contains a central disulfide bond, DSP can be cleaved by reducing agents such as DTT or β-mercaptoethanol. DSP has a linker arm length of 12.0 Å and is specific to free amine groups, such as lysines and the amino-terminal methionine. YfgC contains several lysine residues in its putative TPR domain: two in TPR1, two in TPR2, one in TPR3 and one in TPR4. It is therefore possible to crosslink bound substrates with YfgC using this crosslinker.

Cells were grown at 30°C until mid-log phase, at which point $yfgC_{6his}$ expression was induced with 0.2% arabinose and growth continued for two hours. After growth, cells were kept warm, and subsequent DSP crosslinking was carried out according to the manufacturer's instructions. Crosslinked cells were pelleted and lysed by

French Press. The soluble fraction was passed through a 0.45 µM low-protein binding filter and immediately loaded onto an Ni-NTA column for purification. $YfgC_{6his}$ was eluted with imadazole, and fraction peaks visualized by coomassie staining after SDS-PAGE.

It was noted that, after boiling in SDS-PAGE buffer containing β-mercaptoethanol, $YfgC_{6his}$ ran at its normal position of 51 kD in all three *ompC* backgrounds (Fig. 53A, lanes 2, 4 and 6). However, when samples were prepared in non-reducing SDS-PAGE buffer, only $YfgC_{6his}$ expressed in wild-type *ompC* background ran normally (Fig. 53A, lane 1). In either $ompC_{124}$ or $ompC_{501}$ backgrounds' oxidized samples, only a minor portion of $YfgC_{6his}$ ran at its denatured position, and was not detected in a higher molecular weight form (Fig. 53A, lanes 3 and 5). The ratio of reduced to oxidized $YfgC_{6his}$ was quantified, and is shown in Fig. 53B.

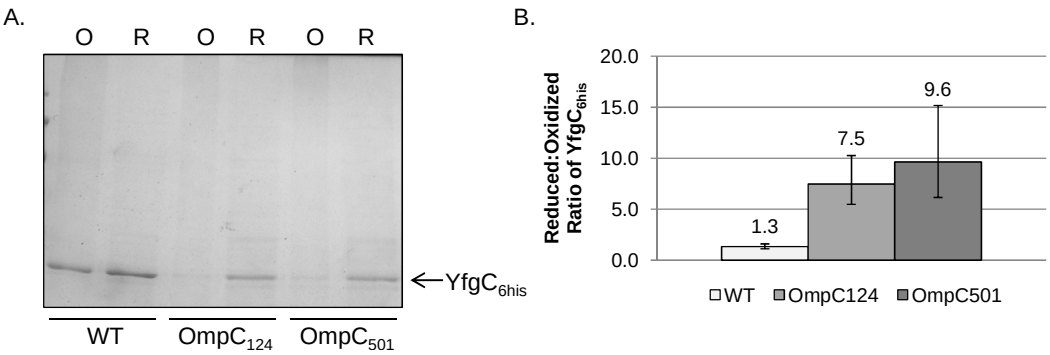
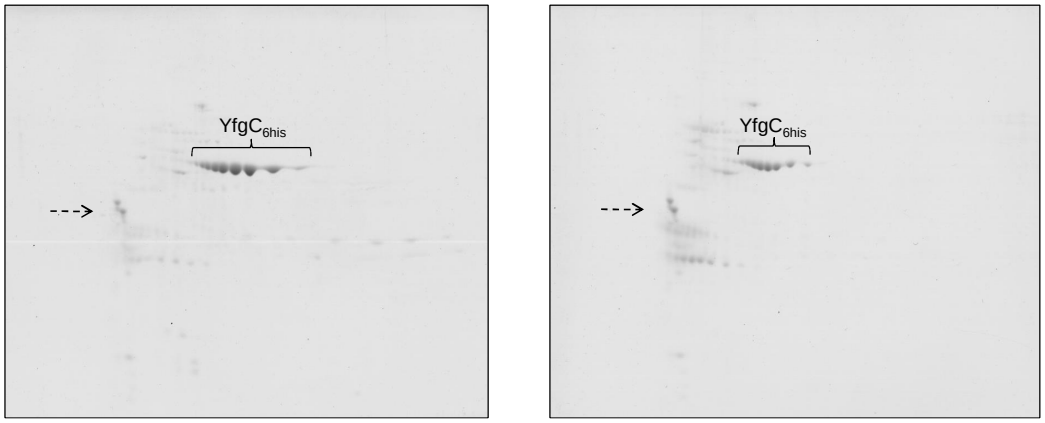


Fig. 53. $YfgC_{6his}$ forms high molecular weight complexes when crosslinked with DSP. A. $YfgC_{6his}$ elution peaks were run on SDS-PAGE gels under oxidizing (O) or reducing (R) conditions and stained with coomassie blue. B. Ratio of reduced to oxidized forms of $YfgC_{6his}$ was calculated by comparing the proteins' relative abundance.

Identification of crosslinked complex composition

This analysis revealed that YfgC preferentially forms crosslinked complexes *in vivo*. Several things remain unclear at this time: whether the σ^E response triggered by misfolding OmpC in the envelope (or simply the presence of misfolded OmpC in itself) enables $YfgC_{6his}$ complex formation, and the nature of the complexes themselves. It is possible that the crosslinked complexes are comprised of $YfgC_{6his}$ and mutant OmpC, but it is also possible that they are multimers of $YfgC_{6his}$ by itself.

$YfgC_{6his}$ elution peaks from wild-type *ompC* and $ompC_{501}$ backgrounds were concentrated using tricarboxylic acid (TCA) precipitation in order to bring out low-abundance proteins. TCA-concentrated samples were analyzed by 2D-GE, and the sample profiles between the two *ompC* alleles were compared to eliminate spurious Ni-NTA column binders, as well as those proteins bound by $YfgC_{6his}$ in a wild-type *ompC* background. As seen in Fig. 54, OmpC was not detected by this analysis in either genetic background tested. Additionally, there was no discernible difference between the profiles of the two backgrounds. The most obvious explanation for this result is that YfgC is “activated” to form multimers in a misfolding environment, although the mechanism by which this occurs is not known at this time.



WT $OmpC_{501}$

Fig. 54. Analysis of crosslinked $YfgC_{6his}$ by 2D-GE. Purified $YfgC_{6his}$ samples after crosslinking with DSP were concentrated with TCA and analyzed by 2D-GE. Proteins were visualized with coomassie blue. Dashed arrow indicates the normal position of OmpC using this method.

Chapter 5

DISCUSSION

Proper protein folding is an essential process in all cells. Without proper folding, toxic intermediates can accumulate, eventually becoming lethal to the cell. Data presented throughout this study show the effects of the accumulation of non-native proteins in the cell envelope, as well as two mechanisms by which the cell is able to suppress lethal phenotypes.

Envelope stress in Δ*degP* mutants

Previous studies have sought to elucidate the role of *degP* in proper envelope biogenesis (Lipinska *et al,* 1990; Misra *et al,* 2000, among others). Indeed, much is known about DegP's mechanistic aspects, including how it performs its cellular function (Krojer *et al,* 2002; Iwanczyk *et al,* 2007; Krojer *et al*, 2008). However, the cellular effects of temperature stress in Δ*degP* cells have not been thoroughly investigated.

As temperature increases, the growth rate of *E. coli* also increases. This in turn increases the demand for envelope proteins, including for the major OMPs. At all growth temperatures, OMPs have a chance of falling off the assembly pathway, where they are subject to either refolding or degradation by DegP (Sklar *et al*, 2007). At elevated temperatures, both the increased amount of OMP intermediates and the increased propensity for misfolding demand the presence of DegP. Work presented in this study explores the consequences to cells lacking this major periplasmic protease, and provides the first investigation of a cytoplasmic response to

envelope stress.

Growth phenotypes of ΔdegP cells

The data presented here show that Δ*degP* cells exhibit a growth defect when grown at the semi-nonpermissive temperature of 39°C. Cells lacking *degP* grow at a slower growth rate and to a lower cell density than wild-type, and are 5-fold less viable after extended thermal stress (Fig. 5). The reason for this defect could be due to the loss of chaperone activity of DegP, the protease activity, or both (Spiess *et al*, 1999). This was investigated by growing wild-type and *ΔdegP* cells expressing wild-type or proteolytically-inactive *degP* heterologously from a plasmid. As shown in Fig. 12, growth of Δ*degP* cells is only restored to that of wild-type in the presence of plasmid-expressed *degP*. Cells expressing protease-defective $degP_{S210A}$ are able to grow to an intermediate cell density between Δ*degP* cells containing empty vector and wild-type *degP*. These results show that it is the protease activity of DegP, and not only the chaperone activity, that is essential for cell survival at high temperatures.

The precise mechanism by which cells lacking *degP* are killed at elevated temperatures is not known. However, it is speculated that the toxic accumulation of OMP and other envelope protein assembly intermediates titrates assembly factors such as SurA and BamA away from their other needed functions. For example, SurA or BamA may continually attempt to refold or assemble a misfolded protein, when that protein should have been degraded by DegP. Consistent with this idea,

proteolytically inactive $DegP_{S210A}$ is only able to partially rescue the growth defect of cells lacking *degP*, but not to the level of wild-type. This most likely reflects the fact that it can *bind* to misfolded target substrates, as shown by Castillo-Keller *et al* (2003), but not *degrade* them. Eventually, even the partially-active DegP variant becomes saturated under the growth conditions tested here.

Cells lacking *degP* are capable of recovering from stress, provided they are returned to a permissive temperature (Fig. 6). This is likely because, when returned to permissive conditions, they then experience a reduced demand for envelope biogenesis, and thus the demand for functional DegP is reduced. Although not tested directly in this work, it is also likely that assembly intermediates no longer accumulate in Δ*degP* cells at the permissive temperature, and so the preexisting accumulation is diluted out into freshly growing cells. This would lessen the stress in each subsequent generation. Indeed, Δ*degP* cells returned to a permissive temperature did resume growth at a slower rate than wild-type, which suggests that the initial outgrowth upon recovery required dilution of toxic, misfolded intermediates.

Cytoplasmic response to envelope stress

Another consequence of growth at high temperatures in Δ*degP* cells is the association of novel proteins in the envelope. These proteins are retained in the insoluble fraction after membranes are treated with sodium carbonate, suggesting that their association is not simply

superificial or transient (Fig. 7). However, unlike typical OMPs, the fact that they can be solubilized by treatment with sarcosyl suggests that the proteins seen have not actually integrated themselves into the membrane fraction (Fig. 8).

In further support of the notion that the phenotype of Δ*degP* cells is caused by the loss of proteolytic function in the envelope, when protease-defective $degP_{S210A}$ was expressed from a plasmid the association of novel proteins with the membranes was only partially reduced relative to empty vector. Only when wild-type *degP* was expressed from a plasmid was the association of the proteins with the membrane abrogated completely. These results are interpreted to mean that DegP does not degrade the associating proteins *per se*, but rather that in cells with full envelope proteolysis, the proteins are prevented from associating with the membranes at all due to a lack of envelope stress.

Because the identity of the novel proteins associated with the envelope of Δ*degP* cells could shed light on the nature of the experienced stress, the carbonate-insoluble membrane fraction in wild-type and Δ*degP* was subjected to two-dimensional gel electrophoretic analysis. Strikingly, the membranes of Δ*degP* cells contained numerous cytoplasmic proteins, among them several cytoplasmic chaperones (e.g. Tig and HtpG) and even the ribosomal subunit S1 (Fig. 9). It appeared as though the two prominent bands running immediately above OmpC in one-dimensional analysis were TnaA and TufA. It was not clear why tryptophanase would

exhibit such a high affinity for the membrane under the conditions tested. However, recent evidence shows that increased amounts of the alarmone molecule indole have a protective effect on cell populations under stress conditions (Kuczyńzka-Wiśnik *et* al, 2010). It is therefore possible that the cell attempts to protect itself or its neighbors from further harm through the secretion of TnaA for the purposes of indole production (Lee *at al,* 2010).

The lower molecular weight (43 kD) protein spot prominent in 2D-GE was identified as TufA, otherwise known as EF-Tu. TufA has the essential role in the cell of shuttling aminoacyl-tRNA to the ribosome during translation elongation. However, it has also been shown to possess general chaperone activity, and has an affinity for hydrophobic peptides (Caldas *et al,* 1998; Fu *et al*, 2008). It is also overexpressed in winter wheat chloroplasts upon heat shock (Ristic *et al,* 2007). Because of these properties, and the fact that it was present in the cell envelope in such high amounts during stress, TufA was chosen as a model cytoplasmic protein potentially playing a role in envelope stress response.

Cells lacking *degP* were grown at high temperature and separated into subcellular fractions. Cytoplasm, periplasm and envelopes were probed with α-TufA antibodies to ensure that the bands seen in Δ*degP* cells were, in fact, TufA. Indeed, TufA localized to the membrane exclusively in Δ*degP* cells, whereas in wild-type the membranes are completely devoid of the protein (Fig. 14). Curiously, the protein also appeared to localize to the periplasm of both wild-type and Δ*degP* cells.

However, it is believed that this was merely an artifact of the periplasm preparation procedure. During osmotic shock, such as was used in this study to isolate periplasm, cells are exquisitely sensitive to mechanical pressure (such as pipetting), and present this pressure can cause TufA and DnaK to enter the periplasm (Berrier *et al,* 2000). Regardless of the presence of TufA in the periplasm, it nonetheless is only present in the membrane fraction of Δ*degP* cells.

It was asked whether the membrane-associating proteins were present immediately upon the subjecting the cells to temperature stress, or rather if their association occurs as a function of time under stress. The novel protein species were not present in the membrane fraction immediately upon outgrowth at elevated temperature, but rather associated with membranes in a time-dependent manner (Fig. 11). It is shown here that their association is related directly to the persistent stress phenotype if Δ*degP* cells at elevated temperature, and not that they are always present in these cells. Indeed, the presence of these proteins in the cell envelope is a function of three conditions: the cell must lack *degP*, the cell must be grown at high temperature, and the stress conditions must persist over time. Without any of those three conditions, the novel proteins do not associate with the envelope.

This is the first reported instance in which cytoplasmic chaperones have been shown to associate with the envelope under the conditions tested in this study. It is not known at this time whether the association of

cytoplasmic chaperones with the membranes of *degP* mutants is a *bona fide* response to envelope stress, or if it is simply a reflection of the level of chaos in these cells. It is tempting, however, to speculate that TufA's presence is an extension of its known chaperone activity; that is, that TufA may in fact be able to exert an effect on misfolded proteins in the envelope. If TufA does ineed have a role in envelope stress in the absence of *degP*, it may constitute a last-ditch effort on the part of the cell to combat what are otherwise lethal conditions. Unfortunately, due to its essentiality in global cell metabolism in *E. coli*, it is impossible to create deletion mutants in *tufA* to study the effects of such mutants at high temperature.

Outer membrane vesicle production

Cells lacking *degP* are known to produce outer membrane vesicles as a unique stress response (McBroom and Kuehn, 2007). Vesiculation appears to be free of regulation from any of the known stress response regulons, and its exact mechanistic aspects are not known. Several groups have attempted to catalog the contents of OMVs using proteomics approaches (Nally *et al,* 2005; Lee *et al*, 2007). However, to date no group has produced data showing the contents of native vesicles produced under stress in Δ*degP* cells.

Data presented in this study show that, contrary to the idea that vesicles produced under stress are only comprised of outer membrane and periplasmic contents, Δ*degP* cells experiencing prolonged

temperature stress produce OMVs containing cytoplasmic proteins as well. It is therefore possible that the conditions tested here are some of the most stressful a cell can encounter. Unlike membrane preparations from Δ*degP* cells, predominant spots excised from the 2D gels were not abundant in cytoplasmic chaperones, with the exception of GroL (Table 2).

The majority of protein spots identified are involved in periplasmic or cytoplasmic metabolic pathways, which could indicate that vesiculation is a general response without a compartment-specific packaging mechanism. This interpretation must be taken with the caveat that not all the proteins in the OMVs were identified, and it is therefore possible that proteins responsible for a packaging mechanism were missed. It should also be noted that the major OMPs, and other proteins whose identities were already known from prior 2D-GE analysis were excluded from MALDI analysis here.

Curiously, although the analysis presented in this work detected both cytoplasmic and periplsmic proteins in the vesicles, it did not detect inner membrane contents (Fig. 16). Presumably, if cytoplasmic proteins were present in OMVs, inner membrane proteins should also be present. However, it is also conceivable that under the conditions tested Δ*degP* cells experience so much stress that the IM ruptures, releasing cytoplasmic contents into the periplasm. However, that possibility is beyond the scope of this work.

Suppression of Δ*degP* temperature sensitivity by a novel *rpoE'* mutation

Suppressor mutation reduces stress phenotypes of ΔdegP cells

Mutations which suppress the temperature sensitivity of Δ*degP* cells have been described before in the literature. Among others, mutations in *rseA* and *envZ* (Gerken, 2009) or in *cya* (Strozen *et al,* 2005) serve to upregulate the σ^E or the Cpx and σ^E stress responses, respectively. This study aimed to identify another suppressor of Δ*degP* temperature sensitivity, originally isolated by an undergraduate in this laboratory in a Δ*degP* Δ*bamB* background.

Before the suppressor mutation was identified, it was asked to what extent it reduced the phenotypes associated with temperature stress in Δ*degP* cells. The mutation restored growth of Δ*degP* cells in liquid media to a level indistinguishable from wild-type (Fig. 19). On solid media, it restored growth of the same cells to nearly that of wild-type (Fig. 18). Strikingly, it did so without significantly lowering the steady-state level of OMPs (Fig. 20; Fig. 32), as would be the case in a classical σ^E response.

It was asked whether, consistent with an abrogation of growth defect in Δ*degP* cells, the suppressor mutation reduced the association of cytoplasmic proteins, including TufA, with the envelope. When analyzed by both one-dimensional (Fig. 20), and two-dimensional (Fig. 21) gel electrophoresis, the appearance of novel proteins in the envelopes of Δ*degP* cells was strikingly reduced. This is consistent with the

interpretation that the association of cytoplasmic proteins with the cell envelope is a function of cell stress. Further, the data show that the suppressor mutation acts through a general lowering of stress in the cell, and not through specific targeting of one or more problem proteins.

Suppressor mutation identified as rpoE'

In order to conclusively identify the suppressor mutant, several approaches were undertaken. The traditional method of P1 transductional mapping ultimately failed to locate the mutation in the chromosome. However, this was found to be a consequence of an unintended gap in the spacing of known mapping drug resistance markers. A Tn*10* insertion was obatained by screening a random Tn*10* insertion library in Δ*degP* cells harboring the suppressor, and was found to reside within ~1' of the suppressor mutation. Further analysis by arbitrarily primed polymerase chain reaction showed that the Tn*10* is inserted in *yfiF*, which is located at 58.5' on the chromosome. A series of three-factor crosses was utilized, narrowing down the target region of interest to be between *glrK* (57.9') and *yfiF*.

Direct sequencing of the region inclusive of *rpoE* and *nadB* revealed an unusual banding pattern in the suppressor strain when the intergenic region was included (Fig. 25; Fig. 26). Although it was not immediately clear why this banding pattern occurred, when the novel PCR fragments were excised from the gel and sequenced it was revealed that they shared the same sequence as wild-type *rpoE*. This indicated that a

duplication event in *rpoE* had likely occurred.

It was reasoned that, if a duplication of *rpoE* was present in the *rpoE-nadB* intergenic region, novel priming sites might converge in the suppressor strain in order to amplify PCR fragments; conversely, in wild-type these priming sites would remain divergent and no PCR product could be amplified (Fig. 27). Therefore, it was attempted to amplify a PCR fragment using normally divergent primers. This "divergently primed PCR" technique, new to this laboratory, indeed allowed the amplification of clean PCR product (Fig. 28B), which was then subjected to sequencing in order to determine the exact molecular nature of the suppressor mutation.

Ultimately, the suppressor mutation was identified as a duplication-truncation of *rpoE*, which was termed *rpoE'*. RpoE' is truncated at F122, followed by five amino acids with the sequence MVWYA. The focus of the investigation then turned to elucidating the mechanism by which *rpoE'* exerted its protective effects in the cell.

RpoE' subtly increases the σ^E stress response

The most obvious mechanism by which RpoE' protected Δ*degP* cells from temperature sensitivity was through direct action of the protein. When cell lysates were probed with antibodies specific to RpoE, it was found that the total level of steady-state, full-length RpoE increased (Fig. 33). However, RpoE must be released into the soluble fraction to become active. In order to determine whether more RpoE was present in active form in *rpoE'* cells, soluble and insoluble fractions were prepared and

probed with α-RpoE antibodies. It was found that RpoE levels increased in both the soluble and insoluble cell fractions (Fig. 33 B; Fig. 33C), which did not provide an immediate answer to the question at hand. Furthermore, there was no evidence from Western blotting that a truncated RpoE' protein was produced from the chromosome of *rpoE'* cells.

To address the question of whether the presence of RpoE' *per se* exerted a suppressive effect on Δ*degP* temperature sensitivity, plasmid borne *rpoE'*$_{6his}$ along with wild-type *rpoE* was introduced into wild-type and Δ*degP* cells. It was found that expression of either gene failed to rescue Δ*degP* cells from temperature sensitivity (Fig. 34). In the case of wild-type *rpoE*, even leaky expression from the plasmid promoter had a deleterious effect on cells, including those with wild-type *degP*. It was reasoned that this was due to the overabundance of an integral cellular regulator. In the case of *rpoE'*, it was speculated that the protein was either not produced, or that the protein was produced but suppression was not simply due to the presence of the RpoE' protein itself.

To address both of these possibilities, soluble and membrane fractions of strains overexpressing wild-type *rpoE* and *rpoE'*$_{6his}$ were prepared and probed with α-RpoE antibodies (Fig. 35A; Fig. 35B). It was found that, as when *rpoE'* was expressed solely from the chromosome, RpoE localized to both the soluble and insoluble fractions. Interestingly, RpoE' was also detected by the antibodies, showing that the truncated,

chromosomally expressed protein is not stable under normal cellular conditions.

Because it was discovered that RpoE' was not stably produced under normal conditions, it was reasoned that the duplication of *rpoE'* could increase the levels of wild-type RpoE sublty when the entire region was transcribed from the chromosome. To address this, an *rybB*::*lacZ* transcriptional fusion was used. The expression of the small RNA *rybB* is controlled exclusively by σ^E, and subtle changes in its transcription could be detected colorimetrically by β-galactosidase assay. Indeed, using this assay it was discovered that σ^E activity increased by 1.3-fold in the presence of *rpoE'*, which is the same as the increase resulting from the lack of *degP* by itself (Fig. 36). This was interpreted to mean that, since the suppressor mutation arose in direct response to the lack of *degP*, the level of suppression is finely tuned to the stress encountered.

Mechanism of rpoE' suppression of envelope stress

One of the hallmarks of envelope stress response is the ability to degrade misfolded proteins. Since *rpoE'* did not operate by significantly lowering the steady-state levels of OMPs in the envelope, it was asked if proteolysis was restored in Δ*degP* mutants. The mutant *acrA* allele, $acrA_{L222Q}$ was utilized, due to the fact that it is stabilized in the absence of *degP* (Gerken and Misra, 2004). If proteolysis was restored in the envelope of Δ*degP* cells, the levels of $AcrA_{L222Q}$ would be seen to drop accordingly. Indeed, cells lacking *degP* but expressing *rpoE'* experienced

a marked reduction in $AcrA_{L222Q}$ levels, indicating that DegP-independent proteolysis was restored in these mutants (Fig. 37).

Because cells lacking *degP* are thought to be crippled in their ability to degrade envelope proteins, it was reasoned that there must be at least one other protease resident in the envelope responsible for the increased proteolysis. Because it would appear to be under the control of the σ^E regulon, the same $AcrA_{L222Q}$ experiment was repeated in cells lacking either *degP* and *rseA*, or both (Fig. 38). In this case, $AcrA_{L222Q}$ was stabilized in Δ*degP* cells, because the major protease was absent. In $degP^+$ Δ*rseA* cells, the protein was degraded due to the upregulation of *degP*. Strikingly, in Δ*degP* Δ*rseA* cells the steady-state levels of $AcrA_{L222Q}$ were no different than in $degP^+$ Δ*rseA* cells, suggesting that another protease resident in the envelope was responsible for the degradation observed. It is not clear at this time which protein or proteins are responsible for this increased proteolysis.

The data presented in this study suggest a model in which a subtly increased σ^E response rescues Δ*degP* cells from temperature sensitivity through the collective action of its regulon members. It is proposed that this is possible due to a very subtly increased amount of *rpoE* mRNA. In wild-type cells, *rpoE* possesses only one promoter region, providing the required level of *rpoE* expression. However, in an *rpoE'* strain, *rpoE* transcription begins from two sites – one upstream of the wild-type copy

and another upstream of the truncated *rpoE'*. In this way, RpoE can be translated from twice the amount of mRNA than would normally be present in the cell (Fig. 55). It is likely that RpoE' is translated from the longer-length mRNA transcript, but that it is rapidly degraded in the cell. In this way, the cell avoids a complete induction of the σ^E response, which can have negative consequences for the cell in the long-term, yet retains the protective effects of a slightly increased amount of σ^E.

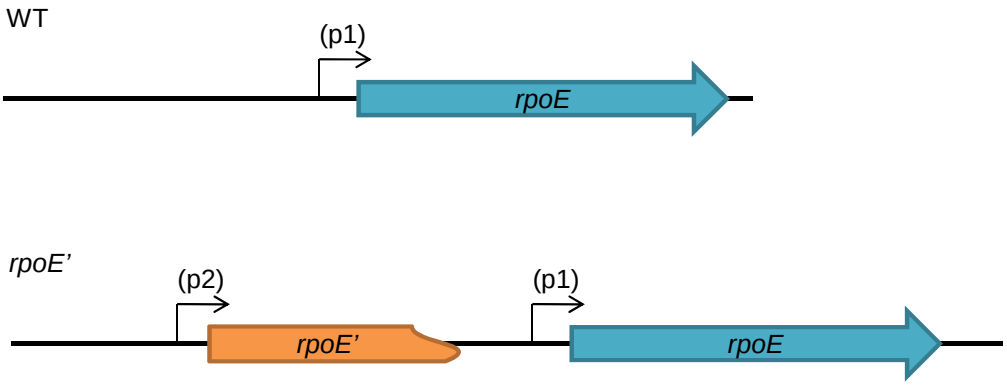


Fig. 55. Schematic representation of *rpoE'* increase in *rpoE* transcription. In wild-type cells, one *rpoE* mRNA transcript is present. In an *rpoE'* strain, the presence of a second transcription start site upstream of *rpoE'* provides an extra mRNA transcript of wild-type *rpoE*, subtly upregulating the steady-state levels of RpoE.

The multicopy suppressor *yfgC*

YfgC is a bona fide member of the σ^E regulon

Rhodius *et al* (2006) showed by microarray expression analysis that *yfgC* was upregulated in cells overexpressing *rpoE*. To confirm this, a *yfgC*::*lacZ* fusion was created and its activity measured by β-

galactosidase assay in the absence of the anti-sigma factor *rseA* (Fig. 41). Its activity was indeed increased 9-fold, definitively placing it in the σ^E regulon. As a confirmation of this placement, cells overexpressing *rseA* had sharply reduced *yfgC*::*lacZ* activity, because σ^E was forced into an inactive state in these cells (Fig. 42).

Since *yfgC* is a member of the σ^E regulon, it stands to reason that its absence would confer a deleterious effect in the cell envelope. One classical way to investigate defects in envelope biogenesis is to challenge cells with antibiotics in a disc assay. If cells possess mutations which confer defects in their envelope permeability barrier, they will be more susceptible to antibiotics than wild-type. In the case presented in this study, Δ*yfgC* cells are slightly more sensitive to antibiotics than wild-type (Fig. 43). Although the increase in sensitivity is moderate, it is nonetheless interpreted to mean that YfgC plays a role in envelope biogenesis.

YfgC acts in a separate, but possibly overlapping pathway as SurA

Another group used pairs of mutants in *degP*, *skp* and *surA* to dissect the assembly pathways in the envelope (Sklar *et al*, 2007). In the same vein, it was asked whether the periplasmic chaperone SurA and YfgC acted in the same or different pathways during envelope assembly. If the two proteins act in the same pathway, then disruption of either gene should have the same effect, due to the fact that the pathway will be

interrupted in either case. However, if they act in separate assembly pathways, *yfgC* and *surA* mutants will show an additive defect. As assayed by antibiotic sensitivity, Δ*yfgC* Δ*surA* cells are more susceptible to antibiotics than either single mutant (Fig. 45), suggesting that they work in separate pathways in the envelope. It has recently been shown that Δ*yfgC* mutants are somewhat more susceptible to antibiotics than wild-type, although this was not explored further by that group (Girgis *et al*, 2009).

A confirmation of this additive defect was obtained when Δ*yfgC* Δ*surA* double mutants were assayed for growth in liquid culture (Fig. 46). Again, if the two proteins act in different pathways, then the simultaneous disruption of both will have a more deleterious effect on the cell than the disruption of a single pathway. It was observed that in cells lacking *surA* both the growth rate and cell density was reduced, reflecting the important nature of SurA to the biogenesis of OMPs. However, Δ*yfgC* mutants grew at a similar rate and to a similar cell density as wild-type. It was therefore somewhat surprising to observe a severe defect in the double mutant, where cells reach exponential phase much more slowly than either single mutant, and failed to reach a cell density even approaching either single mutant by the end of the experiment. These data suggest a minor role for YfgC except when cells lack SurA, perhaps because YfgC is able to compensate somewhat for the lack of *surA* due to partially overlapping

function or because it acts in a partially overlapping pathway.

YfgC exerts an effect on misfolded OmpC

YfgC was isolated as a multicopy suppressor of Δ*degP*-mediated lethality in an $ompC_{2Cys}$ background (Castillo-Keller *et al*, 2006). It was asked if the suppression observed in this background would carry into other *ompC* mutants, ones which misfolded for reasons besides a non-native disulfide bond. $OmpC_{501}$ was one such mutant form of OmpC tested. YfgC was found to exert a protective effect on cells expressing the mutant protein in a Δ*degP* (Fig. 47) or a $degP^{+}$ (Fig. 48) background, indicating that it is not limited to the specific form of OmpC it was isolated against. Rather, YfgC appears to exert a general protective effect against mutant proteins in the envelope.

One possibility for YfgC's mechanism of action is degradation of mutant OmpC. No effect on steady-state levels of wild-type or moderately misfolding OmpC was observed under conditions of *yfgC* overexpression (Fig. 51). However, in an $ompC_{501}$ or $ompC_{2Cys}$ background, $yfgC_{6his}$ overexpression had a profound effect on the relative levels of OmpC and OmpF, suggesting that it acts primarily on aberrantly folded forms of OmpC. It should be noted here that in the misfolding OmpC backgrounds tested, the level of OmpC was reduced at the same time OmpF steady-state levels were increased. This likely reflects a cellular response, mediated by EnvZ/OmpR, to increase OmpF levels as OmpC levels are

reduced by $YfgC_{6his}$.

Purified $YfgC_{6his}$ was able to be crosslinked with unknown substrate(s) in a proportional manner to the misfolding background of the cells tested (Fig. 53), yet no bands belonging to OmpC were detected after affinity purification when $YfgC_{6his}$ elution fractions were reduced with β-mercaptoethanol and analyzed by SDS-PAGE. It is possible that any mutant OmpC proteins present were degraded by $YfgC_{6his}$ itself. When oxidized samples were examined, the protein mixture barely entered the gel, indicating that exceedingly high moelecular weight complexes were formed in these cells. In wild-type *ompC* backgrounds, the formation of high-molecular weight complexes is not nearly as prominent.

Because YfgC possesses a putative substrate binding domain with six lysine residues (its TPR domain), it would be reasonable for it to bind substrate proteins in a crosslinkable form. It is also possible that the high molecular weight complexes are simply crosslinked oligomers of $YfgC_{6his}$. To distinguish between these two possibilities, crosslinked and purified $YfgC_{6his}$ in both wild-type and $OmpC_{501}$ backgrounds was analyzed by 2D-GE. Strikingly, no OmpC was detectable in an *ompC*$_{501}$ background, suggesting that YfgC does not function by binding directly to misfolded OmpC. Furthermore, the protein profile of both strains was not substantially different, confounding efforts to identify YfgC's substrate(s).

The data suggest a function for YfgC primarily during envelope

stress caused by protein misfolding. Under normal conditions, YfgC may only be a minor player in envelope biogenesis. However, its activity appears to be triggered under conditions of accumulation of misfolded OMPs in the envelope, and may in fact form multimers during this process. In this way, YfgC's function is primarily to bring the envelope back to homeostasis.

MATERIALS AND METHODS

Bacterial strains, plasmids and phages

Unless otherwise specified, all bacterial strains used in this study were constructed in an MC4100 background, derived from *E. coli* K-12 (Casadaban, 1976). Strains are listed in Appendix A. Plasmids and phages are listed in Appendix B.

Media and reagents

Unless otherwise specified, all reagents and chemicals were of analytical grade. Luria-Bertani (LB) broth powder was purchased from VWR, and when required agar was added at 1.5% w/v. M63 minimal media were prepared as described previously (Silhavy *et al*, 1984). M63 minimal medium was supplemented with 0.4% glycerol (Fisher) and 0.1% casamino acids (Difco). As required, media were supplemented with 50 μg/ml ampicillin, 25 μg/ml kanamycin, 12.5 μg/ml chloramphenicol, 10 μg/ml tetracycline and 0.2% L-(+)arabinose (Sigma). SuperSignal West Pico Chemiluminescent Substrate was purchased from Thermo Scientific. Mouse α-DnaK antibodies were purchased from Stressgen. Goat anti-rabbit, goat anti-mouse IgG and rabbit anti-goat IgG secondary antibodies were purchased from Sigma. 2-ortho-nitrophenyl-β-D-galactopyranoside (ONPG) was purchased from Acros Organics. All other reagents and chemicals were of analytic grade.

DNA manipulation

Standard bacterial genetic methods were carried out as described

by Silhavy *et al* (1984). PCR, deletion and sequencing primers are listed in Appendix C. Deletion of *yfgC* and *degP* and subsequent removal of the antibiotic resistance cassete was carried out using the λ-red mediated gene deletion method as described previously (Datsenko and Wanner, 2000). Deletions were confirmed using PCR. When required, *lacZ* was recombined into the deletion scar site using the method of Ellermeier *et al* (2002).

Chromosomal fragments containing *yfgC* were amplified by PCR using specific cloning primers. Fragments were digested by *Eco*RI and *Hin*DIII and ligated into appropriately digested pBAD24 (Guzman *et al*, 1995). When cloning $yfgC_{6his}$ the reverse cloning primer incorporated a hexahistidine tag between the last codon of *yfgC* and the stop codon.

PCR products were routinely purified using phenol::chloroform followed by QIAquick PCR Cleanup Kit purchased from Qiagen. Plasmids were routinely purified using the QIAprep Spin Miniprep Kit purchased from Qiagen. Unless otherwise noted, agarose gels were comprised of 0.8% w/v agarose dissolved in TBE buffer.

Cell fractionation

Fresh cultures were grown by subculturing overnight cultures into fresh media with appropriate antibiotics at a starting OD_{600} of 0.025. Cell pellets were resuspended in French Press lysis buffer (Misra *et al*, 1991) consisting of 20 mM Tris-HCl pH 7.5, 2 mM paramethysufonylfloride (PMSF), 10 mM $MgCl_2$ and 40 µg/ml DNase I, followed by two passes of

French Press for maximum lysis. Crude lysates were centrifuged at 100,000 x g for one hour at 4°C to pellet membrane s. To extract periplasm, prior to French Press lysis cell pellets were resuspended in periplasm extraction buffer consisting of 10 mM Tris-HCl pH 7.5, 500 mM sucrose, 10 mM EDTA and 0.2 mg/ml lysozyme, and periplasm extracted using the gentle osmotic shock method (Arié *et al*, 2001). Spheroplast pellets were then subjected to French Press lysis as described above. Membranes were routinely resuspended according to cell density in 10 mM Tris-HCl pH 7.5.

Protein methods

For whole-cell lysate analysis by Western blot, cultures were normalized to cell density and 100 µl pelleted in a microcentrifuge. Pellets were resuspended in SDS-PAGE buffer consisting of 63 mM Tris-HCl pH 6.8, 10% v/v glycerol, 100 µg/ml bromophenol blue, 5% v/v β-mercaptoethanol and 2% w/v sodium dodecyl sulfate (SDS). Samples were boiled and analyzed by SDS-polyacrylamide gel electrophoresis (SDS-PAGE). Where indicated, SDS-PAGE buffer was prepared without β-mercaptoethanol to preserve disulfide bonds in samples. Also where indicated, 4 M urea was added in order to resolve OmpC and OmpF. Membrane, and solube periplasmic and cytoplasmic fractions were prepared in the same way as whole-cell samples. After electrophoresis proteins were transferred to Immobilon-P polyvinylidene difluoride (PVDF) membrane purchased from Millipore using a Bio-Rad mini-transblot.

Membranes were washed briefly in Tris-buffered saline and probed with primary antibodies for 1.5 hours. Rabbit antibody dilutions were LamB 1:10,000, AcrA 1:10,000, TolC 1:10,000, AcrB 1:5,000, MalE 1:10,000 and RpoE 1:5,000. Goat TufA antibody dilution was 1:5,000. Mouse DnaK antibody dilution was 1:25,000.

For analysis of major OMPs, samples were analyzed by urea SDS-PAGE as above and stained with coomassie brilliant blue R-250.

Extraction of membranes with sodium carbonate was carried out using the method of Molloy *et al* (2000). Membrane samples were diluted 1:10 into cold 100 mM sodium carbonate and rocked at 4° C for one hour. Samples were then centrifuged at 100,000 x g for one hour at 4°C to separate carbonate soluble and insoluble fractions. After centrifugation, soluble fraction was drawn off, and insoluble pellets washed in cold 10 mM Tris-HCl pH 7.5. Samples were again centrifuged at 100,000 x g for thirty minutes at 4°C. Insoluble pellets were resuspe nded in 10 mM Tris-HCl pH 7.5.

Extraction of membranes with sarcosyl was carried out using the method of Collin *et al* (2007). Membrane samples were diluted five-fold into 2% w/v final concentration sarcosyl in 10 mM Tris-HCl pH 7.5 and incubated at room temperature for thirty minutes. Samples were centrifuged at 100,000 x g for thirty minutes at 4°C . After centrifugation, soluble fraction was drawn off, and insoluble fraction resuspended in 10 mM Tris-HCl pH 7.5.

Two-dimensional gel electrophoresis was carried out by Kendrick Labs (Madison, WI) per their standard protocol. Isoelectric focusing was carried out in glass tubes of inner diameter 2.0 mm using 1% pH 4-6 ampholines and 1% pH 5-8 ampholines purchased from GE Healthcare for 9600 volt-hours. One µg of an IEF internal standard, tropomyosin, was added to each sample, and is indicated by a wedge in the 2D gels. After equilibration for 10 minutes in Buffer O consisting of 10% v/v glycerol, fo mM dithiothreitol, 2.3% w/v SDS and 0.0625 M Tris pH 6.8 the tube gels were sealed to the top of stacking gels on top of 10% acrylamide slab gels (0.75 mm thick) and SDS slab gel electrophoresis carried out for about 4 hours at 12.5 mA/gel. The comassie brilliant blue R-250-stained gels were then dried between sheets of cellophane. For identification of protein bands from 2D-GE, protein spots were excised and subjected to nano LC-MS/MS analysis at the Proteomics Consortium at the University of Arizona (Tucson, AZ). Returned peptides were analyzed using Scaffold 2 Proteome Software.

RNA isolation and quantitative real-time PCR

For use in qRT-PCR, total RNA was extracted from 5 ml of overnight culture grown at 37°C using TRIzol Max Bact erial RNA Isolation Kit purchased from Invitrogen. Total RNA was purified with the RNeasy kit purchased from Qiagen, and RNA quality was analyzed using an Agilent Technologies Agilent 2100 Bioanlayzer. Total RNA was then converted to single-stranded cDNA for analysis by qRT-PCR.

Single-stranded cDNA was synthesized from 10 µg of RNA using 100 pM random hexamer primers purchased from Integrated DNA Technologies and M-MuLV Reverse Transcriptase purchased from New England Biolabs. After reverse transcription, cDNA was treated with 5 Units of RNaseH purchased from New England Biolabs for 20 min at 37° C followed by purification with the QIAquick PCR purification kit purchased from Qiagen. qRT-PCR Primers were designed according to the manufacturer's instructions using software provided. 300 nM of each primer and 20 ng of prepared cDNA per 20 µl reaction were added to SYBR Green PCR Master Mix purchased from Applied Biosystems. Critical threshold (*ct*) values were determined using an Applied Biosystems ABI Prism 7900HT Sequence Detection System. Quantification of gene expression was calculated according to the ΔΔ*ct* method using *purC* as an endogenous control gene. Each qRT-PCR reaction was performed in triplicate and fold-changes in expression levels, as well as standard deviations, were calculated from at least two independent cultures.

Antibiotic sensitivity assays and growth curves

Overnight cultures were diluted 1:50 into molten top agar (LB supplemented with 0.75% w/v agar) and spread onto LBA plates. After solidifying, paper discs containing 15 µg erythromycin and 5 µg per disc (purchased from VWR) were placed on the growing lawn. Cells were grown overnight, and zones of inhibition measured by the diameter of

clear zone surrounding each disc.

Growth curves were performed by diluting overnight cultures into fresh media supplemented with appropriate antibiotics. Cultures were vigorously shaken in baffle-bottom flasks in a water bath, with cell density checked every thirty minutes.

β-galactosidase assay

Cells were subcultured and grown to late log phase (OD_{600} = 0.7-0.8), at which point growth was stopped by plunging cultures into an ice-water slurry. Cells were permeabilized using 0.04% v/v chloroform and 2 mM SDS. After allowing the chloroform to settle, samples were placed in 96-well plates and mixed 1:1 with Z-buffer (60 mM $Na_2HPO_4 \cdot 7H_2O$, 40 mM NaH_2PO_4, 10 mM KCl, 1 mM $MgSO_4$, 50 mM β-mercaptoethanol, 1.32 mg/ml ONPG, pH 7.0) in a Molecular Dynamics Versamax microtiter plate reader. Activity was calculated as the rate of ONPG cleavage normalized to cell density in each well. All assays were performed in at least quadruplicate using this method.

Purification of $YfgC_{6his}$

Overnight cultures were diluted 1:100 into fresh LB supplemented with appropriate antibiotic and grown to mid-log phase (OD_{600} = 0.3-0.4). Expression of *$yfgC_{6his}$* was induced with 0.2% arabinose, and growth continued for another two hours. Crosslinking with dithiobis[succimidyl propionate] (DSP, purchased from Pierce) was carried out per the manufacturer's instructions. After crosslinking, cells were pelleted and

lysed by French Press as described above. Soluble fraction after centrifugation was filtered through a 0.45 µM low-protein binding syringe filter precharged with binding buffer (20 mM sodium phosphate buffer pH 7.2, 250 mM $NaCl_2$, 20 mM imidazole) prior to affinity purification on a 1 ml HisTrap HP column purchased from GE Healthcare using a Bio-Rad BioLogic LP purification system.

Before purification, the column was washed with six column volumes of binding buffer (as listed above). Samples were loaded on the column and followed by six more column volumes of binding buffer. The column was washed with twelve column volumes of wash buffer (20 mM sodium phosphate buffer pH 7.2, 250 mM $NaCl_2$, 70 mM imidazole), and proteins eluted with 24 column volumes of elution buffer (20 mM sodium phosphate buffer pH 7.2, 250 mM $NaCl_2$, 250 mM imidazole). Elution fractions were analyzed by SDS-PAGE and stained with coomasse blue as described above to find elution peaks.

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APPENDIX A

BACTERIAL STRAINS USED IN THIS STUDY

Strain	Relevant Genotype	Source
MC4100	F- *araD139* Δ(argF-lac)U139 *rspL150 relA1 fibB5301 ptsF25 deoC1 thi*-1 *rbsR*	Casadaban, 1976
JM109	e14–(McrA–) *recA1 endA1 gyrA96 thi-1 hsdR17* ($r_K^- m_K^+$) *supE44 relA1* ∆(*lac-proAB*) [F´ *traD36 proAB lacI*q*Z*∆*M15*]	Promega
RAM1292	MC4100 Δ*ara*714	Werner and Misra, 2005
B431078	MC4100 Δ*ara*714 *yadDpanC*::scar *yfiF*::Tn*10*	This study
B431070	MC4100 Δ*ara*714 Δ*degP*::scar *yfiF*::Tn*10*	This study
B431334	B431078 Δ*rseA*::kan^r	This study
B431336	B431070 Δ*rseA*::kan^r	This study
B431079	MC4100 Δ*ara*714 *yadDpanC*::scar *rpoE'-yfiF*::Tn*10*	This study
B431071	MC4100 Δ*ara*714 Δ*degP*::scar *rpoE'-yfiF*::Tn*10*	This study
B431412	B431078 pACYC184	This study
B431413	B431078 pACYC184-*degP*	This study
B431414	B431078 pACYC184-$degP_{S210A}$	This study
B431418	B431070 pACYC184	This study
B431419	B431070 pACYC184-*degP*	This study
B431420	B431070 pACYC184-$degP_{S210A}$	This study
B431470	RAM1292 Δ*rseC*::kan^r	This study
B431473	B431470 *degP*::Tn*10*	This study
B43960	MC4100 Δ*ara*714 *yfgDE*::scar Δ*degP*::scar *rpoE'*	This study
B431031	MC4100 Δ*ara*714 *yfgDE*::scar Δ*degP*::scar *rpoE'-yfiF*::Tn*10*	This study
B431032	MC4100 Δ*ara*714 *yfgDE*::scar Δ*degP*::scar *yfiF*::Tn*10*	This study
B431356	RAM1292 Δ*csiE*::kan^r	This study

Strain	Relevant Genotype	Source
B431360	RAM1292 Δ*yfhR*::kan^r	This study
B431361	RAM1292 Δ*sseA*::kan^r	This study
B431372	RAM1292 Δ*glrK*::kan^r	This study
B431362	B431070 Δ*csiE*::kan^r	This study
B431363	B431070 Δ*csiE*::kan^r	This study
B431364	MC4100 Δ*ara*714Δ*degP*::scar *rpoE'* Δ*csiE*::kan^r	This study
B431365	MC4100 Δ*ara*714 Δ*degP*::scar Δ*csiE*::kan^r	This study
B431366	B431071 Δ*sseA*::kan^r	This study
B431367	B431070 Δ*sseA*::kan^r	This study
B431368	MC4100 Δ*ara*714 Δ*degP*::scar Δ*sseA*::kan^r	This study
B431369	B431071 Δ*yfhR*::kan^r	This study
B431370	B431070 Δ*yfgR*::kan^r	This study
B431370	MC4100 Δ*ara*714 Δ*degP*::scar Δ*yfhR*::kan^r	This study
B431373	B431071 Δ*glrK*::kan^r	This study
B431374	B431070 Δ*glrK*::kan^r	This study
B431375	MC4100 Δ*ara*714 Δ*degP*::scar Δ*glrK*::kan^r	This study
B431434	B431071 *nadB*::Tn*10*kan	This study
B431435	MC4100 Δ*ara*714 Δ*degP*::scar *rpoE' nadB*::Tn*10*kan	This study
B431436	B431070 *nadB*::Tn*10*kan	This study
B431437	MC4100 Δ*ara*714 Δ*degP*::scar *nadB*::Tn*10*kan	This study
B431451	B431071 Δ*rseC*::kan^r	This study
B431452	B431070 Δ*rseC*::kan^r	This study
B431453	MC4100 Δ*ara*714 Δ*degP*::scar *rpoE'* Δ*rseC*::kan^r	This study
B431454	MC4100 Δ*ara*714 Δ*degP*::scar Δ*rseC*::kan^r	This study
B431621	RAM1292 pBAD24	This study
B431622	RAM1292 pBAD24-*rpoE*	This study

Strain	Relevant Genotype	Source
B431635	RAM1292 pBAD24-*rpoE'*$_{6his}$	This study
B431624	MC4100 Δ*ara*714 *degP*::Tn*10* pBAD24	This study
B431625	MC4100 Δ*ara*714 *degP*::Tn*10* pBAD24-*rpoE*	This study
B431636	MC4100 Δ*ara*714 *degP*::Tn*10* pBAD24-*rpoE'*$_{6his}$	This study
B431651	MC4100 Δ*ara*714 Δ*rybB*::*lacZ yfiF*::Tn*10*	This study
B431653	MC4100 Δ*ara*714 Δ*rybB*::*lacZ* Δ*degP*::cm^r *yfiF*::Tn*10*	This study
B431652	MC4100 Δ*ara*714 Δ*rybB*::*lacZ rpoE'-yfiF*::Tn*10*	This study
B431654	MC4100 Δ*ara*714 Δ*rybB*::*lacZ* Δ*degP*::cm^r *rpoE'-yfiF*::Tn*10*	This study
B43983	MC4100 Δ*ara*714 *yfgDE*::scar *yadDpanC*::scar Δ*tolC*::cm^r *acrA*$_{L222Q}$-*zba*::Tn*10*	This study
B43984	MC4100 Δ*ara*714 *yfgDE*::scar *yadDpanC*::scar Δ*tolC*::cm^r *acrA*$_{L222Q}$-*zba*::Tn*10 rpoE'*	This study
B43985	MC4100 Δ*ara*714 *yfgDE*::scar *yadDpanC*::scar Δ*degP*::scar Δ*tolC*::cm^r *acrA*$_{L222Q}$-*zba*::Tn*10*	This study
B43986	MC4100 Δ*ara*714 *yfgDE*::scar *yadDpanC*::scar Δ*degP*::scar Δ*tolC*::cm^r *acrA*$_{L222Q}$-*zba*::Tn*10 rpoE'*	This study
B431072	MC4100 Δ*ara*714 *yadDpanC*::scar Δ*tolC*::cm^r *acrA*$_{L222Q}$-*zba*::Tn*10*	This study
B431498	B431072 Δ*rseA*::kan^r	This study
B431073	MC4100 Δ*ara*714 Δ*degP*::scar Δ*tolC*::cm^r *acrA*$_{L222Q}$-*zba*::Tn*10*	This study
B431499	B431073 Δ*rseA*::kan^r	This study
B43431	RAM1292 Δ*yfgC*::*lacZ*	This study
B43443	MC4100 Δ*ara*714 Δ*rseA*::scar Δ*yfgC*::*lacZ*	This study
B43452	B43443 pBAD24	This study
B43453	B43443 pBAD24-*rseA*$_{6his}$	This study
B43268	RAM1292 Δ*yfgC*::scar	This study

Strain	Relevant Genotype	Source
B43382	RAM1292 Δ*surA*::kan^r	This study
B43383	B43268 Δ*surA*::kan^r	This study
B43379	B43268 pBAD24	This study
B43380	B43268 pBAD24-*yfgC*	This study
B43119	RAM1292 pBAD24	This study
B43024	RAM1292 pBAD24-$yfgC_{6his}$	This study
B43146	PLB3261 Δ*ara*::cm^r *zei*::Tn*10* pBAD24	This study
B43147	PLB3261 Δ*ara*::cm^r $ompC_{124}$-*zei*::Tn*10* pBAD24	This study
B43148	PLB3261 Δ*ara*::cm^r $ompC_{501}$-zei::Tn*10* pBAD24	This study
B43149	PLB3261 Δ*ara*::cm^r $ompC_{509}$-*zei*::Tn*10* pBAD24	This study
B43150	PLB3261 Δ*ara*::cm^r *zei*::Tn*10* pBAD24-yfgC	This study
B43153	PLB3261 Δ*ara*::cm^r $ompC_{124}$-*zei*::Tn*10* pBAD24-*yfgC*	This study
B43152	PLB3261 Δ*ara*::cm^r $ompC_{501}$-*zei*::Tn*10* pBAD24-*yfgC*	This study
B43151	PLB3261 Δ*ara*::cm^r $ompC_{509}$-*zei*::Tn*10*	This study
B43191	B43146 Δ*degP*::kan^r	This study
B43192	B43147 Δ*degP*::kan^r	This study
B43193	B43148 Δ*degP*::kan^r	This study
B43194	B43149 Δ*degP*::kan^r	This study
B43195	B43150 Δ*degP*::kan^r	This study
B43196	B43151 Δ*degP*::kan^r	This study
B43197	B43152 Δ*degP*::kan^r	This study
B43198	B43153 Δ*degP*::kan^r	This study
B43471	B43431 *zei*::Tn*10*	This study
B43472	B43431 $ompC_{124}$-*zei*::Tn*10*	This study
B43473	B43431 $ompC_{501}$-*zei*::Tn*10*	This study
B43491	B43431 [Δ*ompC*::cm^r]-*zei*::Tn*10*	This study

Strain	Relevant Genotype	Source
B43734	PLB3261 Δ*ara*::scar *zei*::Tn*10* Δ*yfgC*::cm^r pBAD24	This study
B43741	PLB3261 Δ*ara*::scar *ompC*$_{124}$-*zei*::Tn*10* Δ*yfgC*::cm^r pBAD24	This study
B43736	PLB3261 Δ*ara*::scar *ompC*$_{501}$-*zei*::Tn*10* ΔyfgC::cm^r pBAD24	This study
B43738	PLB3261 Δ*ara*::scar *zei*::Tn*10* Δ*yfgC*::cm^r pBAD24-*yfgC*$_{6his}$	This study
B43742	PLB3261 Δ*ara*::scar *ompC*$_{124}$-*zei*::Tn*10* Δ*yfgC*::cm^r pBAD24-*yfgC*$_{6his}$	This study
B43745	PLB3261 Δ*ara*::scar *ompC*$_{501}$-*zei*::Tn*10* ΔyfgC::cm^r pBAD24-*yfgC*$_{6his}$	This study
B43578	MC4100 Δ*ara*714 φ(*ompC'-lacZ*)Hyb10-25 pBAD24	This study
B43579	MC4100 Δ*ara*714 φ(*ompC'-lacZ*)Hyb10-25 pBAD24-*yfgC*	This study

APPENDIX B

PLASMIDS AND PHAGES USED IN THIS STUDY

Name	Plasmid Characteristics	Source
pBAD24	Amp^r, expression vector	Guzman, 1995
pBAD24-*yfgC*	Amp^r, encodes *yfgC*	This study
pBAD24-$yfgC_{6his}$	Amp^r, encodes $yfgC_{6his}$	This study
pACYC184	Tet^r Cm^r, cloning vector	Chan and Cohen, 1978
pACYC184-*degP*	Tet^r Cm^r, encodes *degP*	Gerken, 2009
pACYC184-$degP_{S210A}$	Tet^r Cm^r , encodes $degP_{S210A}$	Spiess *et al*, 1999
pKD3	Cm^r cassette	Datsenko and Wanner, 2000
pKD4	Kan^r cassette	Datsenko and Wanner, 2000
pKD46	Amp^r, harbors λ Red recombinase	Datsenko and Wanner, 2000
pCP20	Amp^r Cm^r, harbors FLP recombinase	Datsenko and Wanner, 2000
pKG136	Kan^r, harbors *lacZ*	Ellermeier *et al*, 2002
P1	Transducing phage	Lab stock

APPENDIX C

PRIMERS USED IN THIS STUDY

Primer Name	Sequence 5'-3'	Source
yadD-panC delF	GTTACCTGTGTTATGACAGATGACGTG TAGGCTGGAGCTGCTTCG	This study
yadD-panC delR	CTTAACTGGCGCTACGGCTGATGCATA TGAATATCCTCCTTAG	This study
yadD-panC seq F	CAGGGAAGACAGGAAGTAAGTCAGGA ATTCGC	This study
yadD-panC seq R	CGATGATATTCAGATTCGCGATGCCG	This study
degP del F	CGTTATCTGTTAATCGAGACTGAAATA CATGAAAAAAACCTGTAGGCTGGAGCT GCTTCG	This study
degP del R	CCCGTTTTCAGGAAGGGGTTGAGGGA GATTACTGCATTAACATATGAATATCCT CCTTAG	This study
degP F	GTGGGATGAATTCCGACGTCTG	This study
degP R	CATTTTGGGATCCAGATGCCAG	This study
ARB1	GGCCACGCGTCGACTAGTTACNNNNN NNNNNGATAT	Fontaine *et al*, 2008
ARB6	GGCCACGCGTCGACTAGTACNNNNNN NNNNACGCC	Fontaine *et al*, 2008
ARB2	GGCCACGCGTCGACTTAGTTAC	Fontaine *et al*, 2008
Tn10 Left	CGGGGCAGAATTGGTAAAGAGAGTCG	This study
Tn10 Right	GCCAACGTTAAGCTGTTGAGTCG	This study

Primer Name	Sequence 5’-3’	Source
Tn10 Int F	GGTAATCGTCACCTCCACAAAGAGC	This study
Tn10 Int R	CGGAAGGTTTCTTCAATCTGCATTCGC	This study
YfiF SeqR	GCCTGATTAACCAGCATCGCGAAGGCG	This study
sseA del F	GGAGATGCCTATGTCCACGACATGGTTTGTATGTAGGCTGGAGCTGCTTCG	This study
sseA del R	GCCAAATTCGGCGGTTTTTTATTGCCTGTAAAATACCATATGAATATCCTCCTTAG	This study
sseA F	CAATCCGAATTTTAGTGAGAATTGTGGCTC	This study
sseA R	CAAGTCAACCTGCTGGAAAGGCCGCG	This study
yfhR del F	ATGGCACTGCCAGTGAACAAACGCGTTCCCAAATGTAGGCTGGAGCTGCTTCG	This study
yfhR del R	CGCGCTAAGGATAAAGTCCACCATCTGTTCCATATGAATATCCTCCTTAG	This study
yfhR PCR F	CCGGTGGTCATAACTACATGC	This study
yfhR PCR R	GCTCAAGGAAATGGCGAAGGGAATGC	This study
csiE del F	GCTATTCTTATCAATTATGCTTATGGGAGATCTACAGTGTAGGCTGGAGCTGCTTCG	This study
csiE del R	CAATGTTTTTGCGCCCGAAAGTTGTTCCCCATATGAATATCCTCCTTAG	This study
csiE F	GCTTCGCTTGCCAACATTTCTGATGATTAGC	This study
csiE R	GGCAGTATCGCTATCATGACCGTTTTCGC	This study

Primer Name	Sequence 5’-3’	Source
qseE F	CCGCCTTATTCCATAACAAAGCCGGG	This study
qseE R	GGACATGGACAGCGCGGAGAAACCGG	This study
glmY F	CGAACGGTATTACGGCAGTCACG	This study
glmY R	CAGGGGCAGCAGAATCAGC	This study
purL F	CGGTTGTCGGCTTTACCAAACC	This study
purL R	GACACGGCAAACCACCCATTGTCG	This study
purL Seq F1	CAGGGCCGTCAGGCGCTGATCG	This study
purL Seq F2	CGCGCCGATCACGTACAAAAAGGCG	This study
purL Seq F3	GCCCGTCTGGCGGTCGGTGAAGCG	This study
purL Seq F4	GGCCTGAATGTAAAACTGTCGTTCG	This study
mltF F	GTGCCAGCAGTTTGTTGATTCGG	This study
yfhB F	GCGCGCCGTATCAGGTTGTAGC	This study
yfhH PCR F	GCGGTTGGCGACGTAGTAAATAGC	This study
yfhL PCR R	CCGGTGAACCAGTCGGACGCACC	This study
shoB-ohsC PCR F	GCCCGATCCCCAATACTATTGTGAAAGATCC	This study
shoB-ohsC PCR R	CGCCGCGTATTAAGCGATAACGAATGGG	This study

Primer Name	Sequence 5’-3’	Source
acpS R	CGCTGTTGTTGAGGGTGCATGCTGC	This study
pdxJ Seq F1	GCGCGATGCCTGCAAACGTCTGG	This study
recO Seq F1	CTTGCACTGCATTCAGTCTCTTGC	This study
era F	CCTCGACAGTGATATTCAAACCGTCG	This study
era Seq F1	GCAATCGTGCGTAAGCATCTACC	This study
rnc F	GTCGCATTGGCGGCATCC	This study
rnc R	CCAGCAGTTTGTTCAACAATGTGG	This study
lepB F	GGTAAGAAACGCATGAAGCAGATCGG	This study
lepB PCR R	CGTGTGCCGTGGCAACCTGGAGG	This study
lepA seq F1	GCGATCCGGAAGGCCCGTTGCAGG	This study
lepA seq F2	GCGCTTCCAGGCGTCCGACATGG	This study
lepA PCR F2	GGCGGGTTCCTGATTGCGCG	This study
lepA R	CGCACCATAAAATGCCCGTCACC	This study
rseC F	GTCCGCTACCGACGATGG	This study
rseC R	GCGAGGAAATGTTCATACCGTATGG	This study
RseB Fwd1	CGTTATTACAGCAGATGAACC	Gerken, 2009

Primer Name	Sequence 5’-3’	Source
RseB Rev1	GCAGAGAATAACAGGCTACC	Gerken, 2009
yfiE-eamB F	GACTCCGGAACAGCAGCGCGACG	This study
yfiE-eamB R	GCGTTCACGCCGCATCCGGC	This study
yfiE seq F	CCGTGGTGGAAGAGGGTTCC	This study
yfiD F	CCTGCGAATCTTCAATCCGCC	This study
yfiD R	GCTGGTCTATTGCGCGGTACGC	This study
ung PCR F	CGATGCAACGCGCTTCGCC	This study
ung PCR R	CCGGATGCCGCACGCGATC	This study
eamB PCR R	CTCCGGAACAGCAGCGCG	This study
yfiC F	GCAGGTGCTGCAACGCTGGCAG	This study
yfiC R	GTTCGCTGTGCAATGATGCG	This study
rpoE-nadB IGR F BspH1	GCTCATGACTGCTCGCTCATCCG	This study
nadB Seq R1	GGCGCGGGCGACAATATCG	This study
nadB Seq R2	GAACCTTCCGTTACCGGGCC	This study
rpoE PCR R	GCAGCTCACTATCCAGCGTTTCGCC	This study
RpoE Fwd1	GATGCGTTACGGAACTTTAC	Gerken, 2009

Primer Name	Sequence 5’-3’	Source
nadB Seq F1	CACCACCTAATCCTCCACCAGCCAG	This study
mscL F	TCGAAAGACTCTTCCAGCCAAGC	This study
mscL R	GATCCCTTATTCCGACAGCGCG	This study
rpoE' F EcoR1	GCGAGAATTCACCATGAGCGAGCAGTTAACG	This study
rpoE' 6his 3R	CGTCTAGACTAATGATGATGATGATGA	This
XbaI 2	TGTGCTGACCAAACCATG	study
rpoE R Xba1	CGTCTAGATCAACGCCTGATAAGCGGTTGAAC	This study
AcrA RT Fwd	CGCGGATCAATCTGGCTTA	This study
AcrA RT Rev	TTCCGTCACGTTCGACTTACC	This study
purC RTPCR FWD	TCGAATTCCGCAATGATACG	Gerken, 2009
purC RTPCR REV	GCGATCAAACTGCTCAATGC	Gerken, 2009
dyfgCfwd	CTCCAGAAATACAGGATAGAGGTTATGTTCAGGAGGCTGGAGCTGCTTCG	This study
dyfgCrev	CATTGTTCTTCCTTTAATGCGAATTACATCTTGGTCATATGAATATCCTCCTTAG	This study
yfgC OF	CGAGCATAAAGACCTTCCCTG	This study
yfgC OR	CTTTCTGGCGCATCAATTCACG	This study
yfgC Seq2	TACACTGGCGGGAACGCGTCAGG	This study
yfgC Seq3	GCTTATTTGCAAGGCGGTCA	This study

Primer Name	Sequence 5’-3’	Source
b2494A F	GATAGAGGTCATGATCAGGC	Castillo-Keller, 2004
b2494 his R	GCTCTAGAAGCTTAGTGATGGTGATGGTGATGCATCTTGGTATAAGGC	Castillo-Keller, 2004
b2494 SDM F	GGCTTCAGTTATGGCGTTCGGAATCTCCCACGTCACC	Castillo-Keller, 2004
b2494 SDM R	GGTGACGTGGGAGATTCCGAACGCCATAACTGAAGCC	Castillo-Keller, 2004
yfgC E201A F	GATCAGTTTCACCCAGCAAAATGCACAGGAAGCGGACCGCATTGG	This study
yfgC E201A R	CCAATGCGGTCCGCTTCCTGTGCATTTTGCTGGGTGAAACTGATC	This study
yfgC dTPR4 F	CTAAATAACCGCGATCAGAGCCTGCAACAAGCGCGTTACG	This study
yfgC dTPR4 R	CGTAACGCGCTTGTTGCAGGCTCTGATCGCGGTTATTTAG	This study

APPENDIX D

INSERTIONS USED TO MAP RPOE' BY P1 TRANSDUCTION

Minute	Gene	*tr* Colonies	Kan^r/Cml^r Colonies
0.0	*thrA*	162	162
1.8	*leuO*	73	73
3.1	*yadD-panC* IGR	406	406
4.9	*yafC*	113	113
6.8	*insF*	62	62
9.2	*yajC*	422	422
11.9	*purK*	150	150
14.1	*pagP*	208	208
16.8	*nadA*	58	58
19.1	*rybB*	306	306
22.0	*ompA*	194	194
24.9	*holB*	80	80
26.6	*fadR*	171	171
29.5	*yjcM*	62	62
33.1	*yddE*	105	105
35.6	*rspAB*	258	258
38.3	*ydiP*	114	114
41.0	*manY*	240	240
43.9	*yedW*	68	68
44.9	*yeeE*	14	14
47.0	*gatZ*	93	93
49.8	*micF*	286	286
52.8	*yfcS*	274	274
60.6	*emrAB*	214	214
63.2	*fucP*	89	89
64.1	*omrAB*	106	106
66.5	*speA*	59	59
69.3	*aer*	122	122
71.8	*sfsB*	94	94
73.9	*smg*	288	288
75.2	*ppiA*	32	32
76.5	*malT*	106	106

Minute	Gene	*tr* Colonies	Kan^r/Cml^r Colonies
78.9	*mdtF*	43	43
84.7	*rbsD*	51	51
87.1	*dsbA*	400	400
88.4	*cpxR*	202	202
90.8	*metA*	82	82
92.8	*alsK*	18	18
95.8	*yftN*	42	42
98.3	*yjiK*	49	49