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The Cpx pathway causes changes in the peptidoglycan structure, turnover, and recycling

by

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Dedicated to my wonderful parents, Juan Carlos and Marcela, for their unconditional support and love, and to my brother, Juan Sebastián.

Abstract

Bacteria need to adapt to the ever-changing conditions in the environment and *Escherichia coli* employs the Cpx two-component system to protect the envelope, which consists of the inner membrane, periplasm, peptidoglycan, and outer membrane. Cpx is known to sense and respond to protein misfolding in the periplasm and changes to the inner membrane. In this study, we investigate the effect of the activation of the Cpx pathway on the peptidoglycan (PG) structure. We confirm the CpxR-mediated regulation of *amiA, amiC, dacC, slt, mltB, ygaU,* and *ycbB* using luminescent reporters. In addition, we confirm that these genes are activated in response to Cpx-inducing conditions. HPLC analysis shows that activation of four genes – *ycbB, ygaU, slt70* and *mltB* - leads to changes in peptidoglycan cross-linking and turn over. Furthermore, absence of *ycbB* and *ygaU* caused activation of the Cpx pathway, suggesting that changes in PG can induce this stress response.

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CHAPTER 1

Introduction

SECTION 1. GENERAL OVERVIEW

The bacterial envelope of enteric bacteria is a complex structure with unique composition and organization. The envelope consists of three different layers; an inner membrane that encloses the cytoplasm, the periplasmic space that contains the peptidoglycan layer, and a second, outer membrane that is located externally. The cytoplasmic membrane and the peptidoglycan layer are similar to that of Gram-positive microorganisms while the external layer is unique to Gram-negative organisms (Glauert and Thornley, 1968).

1.1 The inner Membrane

The inner membrane (IM) or cytoplasmic membrane is a phospholipid bilayer, usually containing proteins that span the membrane using hydrophobic α-helices (Costerton *et al.*, 1974a). This membrane is hydrophobic and fluid in nature, and it is also involved in prevention of cytoplasmic leakage (Weiner and Rothery, 2007). Some of the proteins found on this membrane are involved in signaling, electron transfer activities, cytochromes, permease functions and biosynthetic enzymes (Costerton *et al.*, 1974a). In addition, the inner membrane has membrane-associated stress-responses that are important for adaptation (Raivio *et al.*, 2013). Protein insertion into this membrane is generally mediated by Sec translocases, which open a protein-conducting channel, necessary for protein translocation and insertion into the inner membrane. This layer also contains enzymes that participate in the synthesis of phospholipids, LPS and peptidoglycan (Costerton *et al.*, 1974b).

1.1.2. Phospholipids

The cytoplasmic membrane of bacteria has unique properties that enable it to form a lipid bilayer and maintain its conformation under numerous environmental conditions (DiRusso and Nyström, 1998). One of the main characteristics of the cytoplasmic membrane is its permeability. Phospholipids are selectively permeable to water and other compounds, as they provide the appropriate environment for the function of transporters, integral membrane enzymes, protein export (Gensure *et al.*, 2006). Phospholipids constitute about 40% of the inner membrane dry weight. The three most common phospholipids in *E. coli* are phosphatidyl ethanolamine, phosphatidylglycerol, and cardiolipin that account for 70-90% of all phospholipids found in *E. coli* (DiRusso and Nyström, 1998) .Some other phospholipids such as phosphatidic acid, phosphatidylserine and lysophospholipids can also be found in small quantities in the cytoplasmic membrane as they are turn over products (Weiner and Rothery, 2007). Furthermore, the saturation and the length of the fatty esters will cause variations in the phospholipid content and fluidity of the cytoplasmic membrane (DiRusso and Nyström, 1998).

1.1.3 Respiration and Transport

The phospholipids in the inner membrane must also allow the insertion of proteins that are fundamental for the metabolism of the cell. Proteins integral to the cell membrane are composed of hydrophobic amino acids arranged in α-helical secondary structures and β-sheets (Weiner and Rothery, 2007), maximizing hydrogen bonds, and minimizing the exposure of polar groups to the hydrophobic environment.

One of the functions of the inner membrane consists on respiration. For this process, several dehydrogenases, cytochromes, and reductases are necessary. These proteins contain various prosthethic groups that include iron, haems, flavins, and copper (Unden and Bongaerts, 1997). This diversity of prothethic groups enables the membrane to acquire a wide variety of potentials. *E. coli* respiratory chain proteins can be further divided into two groups: dehydrogenases that oxidize a substrate by generating reduced quinone in the membrane, and reductases that diminish respiratory oxidants by using electrons of reduced quinone (Weiner and Rothery, 2007) that accomplish anaerobic respiration by generating a membrane potential. The membrane potential is then used by the ATPase F_OF_1 to generate ATP (Weiner and Rothery, 2007)

The cytoplasmic membrane is important for influx of various compounds such as water, glycerol, or compounds that are larger than 600 Da as these types of compounds need to be transported in an energy-dependent manner against a concentration gradient (Driessen and Nouwen, 2008). Several proteins participate in the essential process that selectively allows the intake of hydrophilic compounds across the membrane by allowing the transportation of one molecule against its concentration gradient (Weiner and Rothery, 2007). In addition, the plasma membrane is also important for efflux of toxic compounds such as antibiotics (Pedersen *et al.*, 2006).

Furthermore, bacteria lack an endoplasmic reticulum or a Golgi complex. The bacterial periplasm is the equivalent of the lumen of the endoplasmic reticulum in bacteria as some proteins have its final destination in the periplasm, the outer membrane or the external environment. These proteins need to cross the periplasm membrane to reach their final destination (Weiner and Rothery, 2007). The major route for protein translocation across

the cytoplasmic membrane is the SecYEG translocation complex. This complex translocates proteins in an extended conformation often bound by SecB or other chaperones to prevent folding before the protein reaches the periplasm. The Sec translocase is a highly-conserved structure that forms a channel in the cytoplasmic membrane together with the ATP-hydrolyzing SecA protein (Driessen and Nouwen, 2008). Another route that is used by *E. coli* to translocate proteins is the Tat (twin-arginine translocation) translocation system. This system is able to transport folded proteins of different dimensions across the cytoplasmic membrane. Proteins that are translocated by this system have a signal peptide that consists of consecutive arginine residues (Berks *et al.*, 2000).

1.1.4 Signal Tranduction

Bacteria are to adapt to the ever-changing conditions in the environment by using transmembrane phosphotransfer proteins with a receptor facing the periplasm and a phosphorylation domain facing the cytoplasm. The transmembrane phosphotransfer proteins is also known as a histidine kinase (HK). The histidine kinase then transfers the phosphate to an aspartate residue of a response regulator (RR), generating conformational changes in the regulatory domain of the response regulator that result in the transcription or repression of specific genes. (Stock *et al.*, 2000). This signal transduction mechanism is commonly known as a two-component regulatory system. Extracellular stimuli are sensed and serve to modulate the activity of the HK (Weiner and Rothery, 2007).

1.2 The periplasm

The periplasmic layer has a very important role in the development of antibiotic resistance as it contains many enzymes and proteins that reduce the effect of antimicrobials. The periplasm is a compartment bounded by the cytoplasmic and the outer membranes. It contains a variety of molecules that reflect the metabolic state of the cell (Hobot *et al.*, 1984).

This layer is filled with periplasm, which is constituted of a gel matrix made up of peptidoglycan, which is strongly cross-liked to the outer membrane and less so towards the cytoplasmic membrane (Hobot *et al.*, 1984; Costerton *et al.*, 1974b). This periplasm is an oxidizing environment where proteins can be stabilized by disulfide bonds (Merdanovic *et al.*, 2011).

The periplasm is more fluid near the cytoplasm, allowing the proteins that are embedded in this layer to be diffused, making this place a dynamic area with an ever-changing variety of molecules, varying according to the metabolic state of the cell and the environmental conditions (Beveridge and Graham, 1991). The periplasm has a wide variety of macromolecules such as, periplasmic binding proteins involved in nutrient acquisition, trafficking proteins, secreted materials, recently synthesized components of the outer membrane and the peptidoglycan layer, environmental signals, and quorum sensing molecules that are necessary for the adaptation and survival of bacteria. It also contains autolysins and penicillin-binding proteins that participate in the synthesis of peptidoglycan as the cell divides and grows (Beveridge, 1999).

Periplasmic proteins must be transported across the inner membrane and folded correctly upon arrival in the periplasm. The proteins that are found in the periplasm are either

translocated by the general secretion system (sec), which transports unfolded proteins, or by the twin-arginine translocation system (TAT) that is in charge of translocating folded proteins. The integrity of these proteins has to be carefully monitored by different chaperones because the permeability of the outer membrane makes the periplasm more exposed to external factors than the cytoplasmic membrane. These factors can affect the structure of the periplasmic proteins by allowing the passage of chemical stresses, detergents, antibiotics, etc. (Merdanovic *et al.*, 2011).

Molecular chaperones can repair some proteins whose structure has been compromised. Chaperones bind polypeptides in their nonnative conformation and restore its native structure. Some of the main chaperones of *E. coli* include Spy, Skp, and HdeA. Protein refolding by these chaperones involves fast and slow steps. Fast steps consist of the formation of secondary structures and non-covalent bond (Merdanovic *et al.*, 2011). Slow steps include disulfide bond formation and peptidyl-prolyl isomerization (Merdanovic *et al.*, 2011). Disulfide bond formation is catalyzed by DsbA, a disulfide oxidoreductase (Kadokura *et al.*, 2003), while the *cis-trans* isomerization of proline is synthesized by Peptidyl-Prolyl isomerases that include SurA, PpiD, and PpiA (Kadokura *et al.*, 2003).

Proteolysis occurs when peptides cannot be properly folded or assembled. In addition, an increase in proteolytic activity leads to an increase in the activity of stress responses due to modification of inducing cues (see stress responses of *E. coli*). If proteolytic activity is not possible cell death will occur due to protein aggregation. Some proteases such as DegS, DegP, and RseP are well studied, but detailed information on other periplasmic proteases is limited compared to cytoplasmic proteases (Merdanovic *et al.*, 2011;

Gottesman, 1989; Miot and Betton, 2004) . Cytoplasmic proteases defer from periplasmic proteases in various ways. First of all, cytoplasmic proteases are ATP dependent. *E. coli* has at least five ATP dependent proteases: Lon, ClpAP, ClpXP, HslVU and HflB. These enzymes have different substrates. ClpAP and ClpXP are two-component proteases that share the same proteolytic subunit ClpP, but the ATPase regulatory activity is different for both of these proteases (Gottesman, 1989). Lon has one protease and four ATP-binding sites. The activity of this protease depends on its ATPase activity and the rate at which ATP is depleted (Chin *et al.*, 1988; Menon and Goldberg, 1987) . Furthermore, the HslV protease and the HslU ATPase form a proteasome-like complex that is expressed in the presence of cell stress (Gille *et al.*, 2003). The HsIV protease only degrades misfolded proteins in the presence of HslU when it is in it ATP-bound state (Gille *et al.*, 2003)

1.2.1 Periplasmic proteins

-Periplasmic Chaperones

The main chaperones found in *E. coli* are Skp (seventeen kilodalton protein), Spy (spheroplast protein y), and the Acid Chaperone HdeA. Skp is a small periplasmic protein that binds to OmpA, OmpC, OmpF, and LamB and folds these proteins in the presence of phospholipids with a negative charge. Spy is over produced by the induction of the Cpx pathway, Bae, and Rcs stress responses (Merdanovic *et al.*, 2011). Spy is in charge of protein folding and it also prevents aggregation of proteins. HdeA is in charge of protein verification in the presence of acid, suppressing the aggregation of misfolded proteins at low pH. It also releases the misfolded substrates when the pH is normalized (Merdanovic

et al., 2011).

-Periplasmic proteases

The most studied proteases in *E. coli* are DegP, Prc (processing defective), and PtrA (protease III). DegP is in charge or alleviating protein-folding stress. Over production of misfolded, mislocalized or hybrid proteins, leads to DegP protease activity. In contrast, in the presence of misfolded periplasmic amylases such as MalS, DegP has chaperone activity and contributes to proper folding of these proteins. Prc has a role in protein quality control, and it has preference substrates with hydrophobic C-termini. PtrA also participates in quality control. However, more studies are necessary to determine its function (Danese *et al.*, 1995; Merdanovic *et al.*, 2011).

1.2.2 Folding Catalysts

Disulfide bond formation is a very important step in protein synthesis. Disulfide bonds help to stabilize the structure of the protein by forming a covalent linkage between the different subunits. Sometimes proteins can form a disulfide bond with the wrong cysteine, affecting the final structure of the protein (Bardwell, 1994). Thus, it is necessary to carefully verify disulfide bond formation. The periplasmic redox system (Dsb) is in charge for the formation of disulphides in compartments external to the inner membrane, this system has various proteins in charge of the different steps of disulfide bond formation. DsbA has a main role in formation of these bonds, $DsbA^-$ mutants are severly defective in disulphide bond formation (Bardwell, 1994).

Isomerization of peptidyl-propyl bonds is another limiting factor. Peptide bond

formation with proline can either have a *cis* or a *trans* conformation. Peptidyl-isomerases interconvert the different isomers of peptide bonds that have proline. This is usually a slow process, but PPIases catalyze this process. PpiA was the first PPIase discovered in *E. coli.* It is highly up-regulated by the Cpx stress response, and it participates in the assembly of the P-pili (Rowley *et al.*, 2006). PpiD is one type of PPIase. It is also located in the periplasm and it participates in the folding of polypeptides that emerge from the SecYEG translocon (Merdanovic *et al.*, 2011). SurA is another type of PPIase that recognizes substrates with aromatic substrates. Usually these substrates are found on the outer membrane, suggesting that this PPIase participates in outer membrane biogenesis (Merdanovic *et al.*, 2011).

1.3 The cell wall

The cell wall consists of peptidoglycan, a polymer of glycan chains that are cross-linked to form an elastic structure that is strong enough to protect the cell from lysis due to changes in the osmotic pressure and it also confers the cell shape (Costerton *et al.*, 1974a). As mentioned above, it also functions as an anchor for the outer membrane via interactions with phospholipids that hold this membrane in place (Bos and Tommassen, 2004). It is one to three layers thick in Gram-negative bacteria whereas, in Gram-positive organisms, it is at least 10 to 20 layers thick (Scheffers and Pinho, 2005).

Synthesis of the peptidoglycan precursors takes places in three different stages: 1. Assembly of particles takes places in different organelles in the cytoplasm. 2.

Some intermediate products are loaded onto lipid carrier molecules, 3. The glycan strands are incorporated in the sacculus (Tomasz, 1971).

1.3.1 Step I: The cytoplasm

Various enzymes participate in the cytoplasmic steps of peptidoglycan synthesis. These enzymes are: MurA-F, D-Alanine racemases and D-Alanine ligases (Projan, 2002).

Cell wall synthesis initiates with MurA (or UDP-N-acetylglucosamine enolpyruvyl transferase). This enzyme transfers a phosphoenol to 3'-hydroxyl-UDP-N-acetylglucosamine (NAG). Next, an enol ether from NADPH and a proton are reduced to a lactyl ether by MurB reductase to form UDP-N-acetyl muramic acid (NAM). The following steps consist of various ATP-depentend aminoacid ligases (MurC, MurD, MurE and MurF) that catalyze the steps of the synthesis of the pentapeptide side-chain of the N-acetylglucsamine (NAG) (Projan, 2002). Each of these enzymes adds one amino acid with the exception of MurF, which is in charge of catalyzing the addition of D-Ala-D-Ala. In Gram negative bacteria MurE catalyzes the addition of meso-2,6-diaminopimelic acid (Projan, 2002) (Figure 1).

1.3.2 Step 2: Translocation to the inner membrane

For the translocation of the peptidoglycan precursor from the cytoplasm to the plasma membrane two enzymes are necessary, MraY and MurG. Both these enzymes are similar as they are attached to the cytoplasmic membrane, and they

also process lipid intermediates. First, MraY transfers phosphor-MurNAc-peptapeptide onto the lipid carrier undecaprenyl phosphate, forming Lipid I (MurNAc-pentapeptide-pyrophosphoryl undecaprenol). Next, MurG attaches the GlcNAc to form NAG-NAM-pentapeptide-pyrophosphoryl undecaprenol (lipid II) (Bouhss *et al.*, 1999) (Figure 1).

1.3.3 Step 3: Translocation to periplasm and synthesis of the cell wall

The third step of peptidoglycan synthesis consists of the polymerization of the monomer unit on the periplasm and binding the new material to the pre-existing cell wall. Lipid II is translocated across the membrane to the periplasm by MurJ, a lipid flippase (Ruiz and Silhavy, 2005). All murein synthases are anchored to the cytoplasmic membrane by a transmembrane domain close to their N-terminus. The transglycosylase (TG) and transpeptidase (TP) domains are located in the periplasm (Vollmer and Bertsche, 2008). Two major types of enzymes participate in this process; glycosyltransferases and penicillin-binding proteins.

Glycosyltransferases are in charge of catalyzing the formation of linear glycan chains and in transferring the newly made material to the existing peptidoglycan (Vollmer and Bertsche, 2008). Murein synthases are in charge of cross-link formation. They have transpeptidase activity and are also known as penicillin-binding proteins (PBPs), as they are capable of binding β-lactams that resemble the structure of the D-Ala-D-Ala ends of the peptapeptide chains (Vollmer and Bertsche, 2008). In growing cells, these polymerization reactions are accompanied by following peptidoglycan modifications. The covalent structure of

the murein layer is a dynamic structure that must adjust to the requirements of stressful conditions, surface growth and cell division. Peptidoglycan hydrolases are in charge of these changes and they are specific to each microorganism (van Heijenoort, 2011).

Cross-linking of the cell wall takes place by transpeptidation enzymes that link the D-Ala aminoacid at position 4 of the donor peptide and the diaminopimelic-acid residue at position 3 of the acceptor peptide. The energy necessary for the cross-link formation is obtained from the cleavage of the D-Ala-D-Ala bond of the pentapeptide of the donor peptide (Terrak *et al.*, 1999). The final step of peptidoglycan synthesis consists of the recycling of the undecaprenol-phosphate by UppP, a specific lipid pyrophosphorylase (Figure 1) that accounts for 75% of the total Und-PP pyrophosphatase activity that also participates in the synthesis of Und-P. In addition, *E. coli* has other Und-PP pyrophosphatases that were recently identified (YbjG, YejU, PgpB) (Tatar *et al.,* 2007). These pyrophosphaases have less activity than UppP and they have a typical acid phosphatase motif found in other pyrophosphate-recycling pyrophosphatases. Furthermore, YbhG and YeuJ are localized on the periplasmic side of the inner membrane and directly involved in the recycling of Und-PP (Tatar *et al.*, 2007).

1.3.4 Enzymes involved in the last stages of peptidoglycan synthesis

Penicillin-binding proteins (PBPs) belong to the family of acyl serine transferases (Sauvage *et al.*, 2008). This family includes high-molecular-weight (HMW) PBPs, low-molecular-weight (LMW) PBPs and β-lactamases. HMW-PBPs are

located on the exterior of the plasma membrane, the C-terminal domain is the penicillin-binding domain that is necessary for cross-linking and the N-terminal domain has a non-cleavable signal peptide that anchors these proteins to the cytoplasmatic membrane. Depending on their catalytic activity and catalytic domain, they can be classified into two major groups: A or B (Livermore, 2001) Low-molecular-weight (LMW) PBPs have various functions; they can be classified as D,D-peptidases, or D,D-carboxypeptidases (Scheffers and Pinho, 2005).

D,D-peptidases mediate transglycosylation (Frère, 1995) Their function consists of forming peptide bonds of peptidoglycan. D,D-peptidases displace the terminal D-alanine residue from one of the two peptide chains that form the peptidoglycan cross-links (Sauvage *et al.*, 2008). This process requires the removal of a proton from the serine located in the active site of the D,D-peptidase (Zapun *et al.*, 2009). The cleavage reaction provides necessary energy for transpeptidation, which occurs in the absence of ATP (Zapun *et al.*, 2009) (Figure 1).

Transgycosylation is mediated by D,D-carboxypeptidases. These are membrane-bound enzymes involved in the final steps of peptidoglycan growth, cell division, and cell wall structure (Santos *et al.*, 2002). It involves the formation of glycan strands by reducing the N-acetylmuramic acid of the nascent-linked peptidoglycan strand and its transfer to the lipid-linked peptidoglycan precursor in order to yield the lipid carrier, lipid II. Growing peptidoglycan chains are attached to the cytoplasmic membrane via a long hydrophobic moiety (Figure 1) (Vollmer *et al.*, 2008). *E. coli* has six D,D-carboxy peptidases: PBP5 (DacA) is the most

important. Cells lacking PBP5 have altered morphology (Santos *et al.*, 2002). D,D-carboxypeptidases are also a specific target of penicillins and cephalosporins because the enzyme substrate is the D-alanyl-D-alanine terminus of the donor peptide side chain of the growing peptidoglycan strand (Schneider and Sahl, 2010). Frequently, DD-carboxypeptidases will also have dual activity, allowing them to both cleave and synthesize peptidoglycan cross-links (Schneider and Sahl, 2010; Typas *et al.*, 2012) (Figure 1).

1.3.5 Peptidoglycan cross-linking in *E. coli*

Disaccharide strands have a muropeptide subunit. These strands are cross-linked to the peptides of an adjacent chain to form a mesh-like structue, which results in a two- or three-dimensional network. In most bacteria, the stem peptide terminates in two D-alanine residues. D,D-transpeptidases are in charge of cleaving the terminal D-alanine and synthezising a covalent link between the D-alanine at position 4 to the D-aminoacid of the Diaminopymelic acid (DAP), forming the characteristic 3-4 cross-link which is predominant in the sacculi of bacteria. 3-4 cross-links are disrupted by the presence of β-lactam antibiotics due to the irreversible binding of these compounds to the serine active site of the D,D-transpeptidases (Llarrull *et al.*, 2010; Bush and Jacoby, 2010). In addition, *E. coli* and other bacterial species also have 3-3 cross-links. This type of cross-link is the result of the formation of a covalent bond between the L-carboxyl group of DAP in one muropeptide cross-linked to the D-amino group of an adjacent DAP-

DAP cross-link (Cava *et al.*, 2011; Magnet *et al.*, 2008; Magnet *et al.*, 2007) (Figure 1).

1.3.6 HPLC- The gold standard for the analysis of the cell wall composition of *E. coli*

The bacterial cell wall is a key determinant of cell shape, cell stability, and formation of new daughter cells (Matteï *et al.*, 2010). Detailed composition of the cell wall has been obtained by fractionation of the peptidoglycan with amidases, enzymes that cleave the peptides from the glycans, or with muramidases, enzymes that cleave the glycosidic bonds. The resulting degradation products are then analyzed by HPLC (reversed-phase high pressure liquid chromatography) (Glauner, 1988; Höltje, 1998). This technique is based on separating the different muropeptides according to their solubility in the solid phase (porous beads) and the mobile phase (phosphate/methanol). Eluted compounds produce a chromatoGram that is detected with UV absorbance as a function of time. Each particular glycan species elutes at a different time is that known as 'retention time' depending on the mobile and stationary phase (Desmarais *et al.*, 2013). HPLC is a valuable tool for the identification of murein structure and composition of *E. coli* (Glauner, 1988) as it has revealed more than 80 different kinds of muropeptides that can be classified according to their size and their role in the structure of the cell wall. For example, an increase in disaccharides with either 3-3 or 3-4 cross-links can suggest an increase an increase in rigidity of the cell wall (Desmarais *et al.*, 2013). In addition, the structure of the cell in *E. coli* during

early stationary phase has been well characterized (Prats and de Pedro, 1989). Stationary phase peptidoglycan has a higher percentage of 3,4 and 3,3 cross-links, shorter glycan strands, and an increase in peptidoglycan bound to the outer membrane (Glauner, 1988; Prats and de Pedro, 1989; Desmarais *et al.*, 2013) .

HPLC analysis has also contributed to the identification and characterization of peptidoglycan enzymes. Differences in the concentration of different muropeptide species in the presence or absence of an unknown enzyme can lead to the identification of its substrate and function, such as the case of L,D-transpeptidase YcbB (Magnet *et al.*, 2008; Desmarais *et al.*, 2013; Sanders and Pavelka, 2013) or carboxypeptidase DacC (Pedersen *et al.*, 1998).

1.3.6 The various functions of the cell wall

-Turgor pressure

The various components of the bacterial envelope have different functions as previously described. The cytoplasmic membrane forms a semipermeable barrier that separates the cytoplasm from the periplasm. The cytoplasmic membrane can swell depending on the concentration of the solutes found in the cytoplasm, generating an osmotic pressure called turgor pressure, which pushes the inner membrane against the cell wall. The ridigity of the cell wall will limit the cell size during this process (Deng *et al.*, 2011). Atomic force and fluorescence microscopy have shown that the cell wall stiffens in the presence of stressful conditions. Cell wall stiffening prevents changes in cell shape during changes in osmolarity or pressure by maintaining normal peptidoglycan plasticity during

these conditions (Deng *et al.*, 2011) as polymer networks have a nonlinear relationship between strain and stress due to geometry and monomer composition (Gardel *et al.*, 2004).

-Cell shape and cell stability

Various morphological changes during the course of bacterial growth, division, or adaptation, and also the shape uniformity that is seen for every bacterial species indicates that cell shape is important for these microorganisms (Young, 2006). The cell shape of *E. coli* seems to define its ability to survive in different environments as it changes from rod-shaped to cocci at the entry of stationary phase probably due to slow growth conditions (Young, 2006). In addition, *E. coli* can also display a filamentous phenotype depending of nutrient availability without increasing the surface-to-volume ratio, suggesting that *E. coli* can modify its cell shape according to the conditions. Cell shape modification is under the control of various genetic and physiological mechanisms. For example, penicillin-binding protein 2 (PBP2) is essential for rod morphology in *E. coli* and its absence is lethal (Ogura *et al.*, 1989).

Cell wall elongation is a multi-complex process depends on numerous enzymes that include MreB, FtsZ, outer membrane proteins, periplasmic hydrolases, and various PBPs (Matteï *et al.*, 2010). MreB and FtsZ are localized in the cytoplasmic side of the inner membrane whereas the rest of the peptidoglycan hydrolases and transglycosylases are located in the periplasm (Varma and Young, 2009). MreB and FtsZ interact with the peptidoglycan hydrolases and transglycosylases by inner-membrane proteins to determine the overall cell

morphology (Varma and Young, 2009). MreB is an actin-like cytoskeleton protein that has a similar role to the eukaryotic actin as it regulates the rod shape of bacteria (Figge *et al.*, 2004). MreB is also in charge of the localization of the cell wall synthetizing and modifying machinery, which helps to determine the cell shape (Carballido-López, 2006). Furthermore, FtsZ, a tubulin-like protein, is in charge of forming a ring structure and a multi-complex protein necessary for cell division (Masuda *et al.*, 2012) that also has a minor in cell shape as absence or improper function of FtsZ can affect the integrity of the cell. FtsZ interacts with LMW-PBPs and probably other peptidoglycan hydrolases to determine the gross cell shape to generate uniform and unbranched *E. coli* cells (Varma and Young, 2004).

-Antibiotic Target

The bacterial cell wall was the first pathway to be targeted by antibiotics. Several classes of antibiotics target the bacterial cell wall. The biggest class is the beta-lactams (penicillin derivatives, cephalosporins, monobactams, and carbapenems), as well as vancomycin, and bacitracin (Llarrull *et al.*, 2010). β-lactams target the PBPs, enzymes necessary for the last stages of peptidoglycan synthesis and remodeling. For example, penicillin mimics the structure of the D-alanine-D-alanine terminus of the stem peptide, inhibiting PBPs which falsely recognize penicillin as the active site and they form an acyl-enzyme bond with this antibiotic, impairing the peptidoglycan cross-linking capability (Schneider and Sahl, 2010; Sauvage *et al.*, 2008) . This leads to a build up of peptidoglycan precursors, which activates autolytic hydrolases that digest existing peptidoglycan

without the synthesis of new peptidoglycan, enhancing the bactericidal action of β-lactams and causing the cell wall to break and the cell to lyse (Nordmann *et al.*, 2012). Vancomycin exterts its bacteriocidal activity by interfering with cell wall synthesis mostly in Gram positive microorganisms. It binds to cell wall precursor, D-Alanine-D-Alanine, and acts as a steric impediment that prevents the nascent glycan chain from being further processed, as a result cross-linking cannot occur due to the inability of the transpeptidases to cleave the terminal D-alanine (Merchant and Vithlani, 1986). Inhibition of following cell wall synthesis steps such as transglycosylation and transpeptidation, weakens the peptidoglycan layer and leads to cell lysis (Merchant and Vithlani, 1986). On the other hand, bacitracin inhibits the transfer of peptidoglycan precursors synthesized in the cytoplasm to the bactoprenol pyrophosphate (Hancock, 1997), inhibiting the dephosphorylation of C55-isoprenyl pyrophosphate. As a result, the lipid carrier cannot be recycled, and peptidoglycan synthesis is stopped (Stone and Strominger, 1971).

1.4 The outer membrane

The external bilipid layer is known as the outer membrane. It is an assymmetrical bilayer indistinguishable in dimension from the cytoplasmic membrane. Its proper functioning is very important for the physiology of Gram-negative bacteria, as it makes them resistant to external factors that include lysozyme, leukocyte proteins, bile salts and lysins (Nikaido and Vaara, 1985). At the same time this membrane acts as a barrier to numerous antibiotics that are usually effective against other

microorganisms (e.g., macrolides, tetracyclines, glycopeptides) (Nikaido and Vaara, 1985). The outer membrane also slows down the penetration of antibiotics, which gives enough time to the bacteria to inactivate the small amount that has entered the cell. As a result high levels of resistance are easily acquired in Gram-negative bacteria (Bos and Tommassen, 2004).

1.4.1 LPS

Lipopolysaccharide molecules are of great importance for Gram-negative microorganisms as it can be modified to confer resistance to different antibiotics such as CAMPs through variation and modification of structures (Bos and Tommassen, 2004; Tzeng *et al.*, 2005; Bos *et al.*, 2007). LPS structure consists of three clearly defined chemical regions: the lipid A component (which is in charge of anchoring the molecule to the OM and is the endotoxic moiety of toxic LPS), the core region which is linked to the lipid A and which modulates lipid A toxicity, and, the O-specific polysaccharide that is a highly variable antigenic structure and its convalently linked to the core (, 2009). The different serotypes of *E. coli* and *Salmonella spp.* reflect the variations in the O-antigen structure (Tomasz, 1971) due to variation in the *rfb* cluster (Schnaitman and Klena, 1993). Bacteria that lack LPS are usually non-viable. LPS also confers resistance to detergents, antibiotics and other chemicals to Gram-negative organisms (Tomasz, 1971; Costerton *et al.*, 1974a).

1.4.2 Surface associated polysaccharides

The enterobacterial common antigen is also found on the external part of this membrane (Mäkelä and Mayer, 1976). Its structure and composition can be used for diagnosis and taxonomy. This antigen is a glycophospholipid that consists of aminosugar heteropolymers linked to glycerophosphatidyl residue (Schnaitman and Klena, 1993; Mäkelä and Mayer, 1976). The O-antigen is highly variable as more than 60 monosaccharides and 30 non-carbohydrate components have been identified. In addition, the O-antigen can vary in the number of monomer glycoses, the organization and composition of the O-glycosydic bonds, absence or presence of non-carbohydrate substituents. The O-repeat units can have varying amounts of monosaccharides that can form homopolymers or heteropolymers (Raetz and Whitfield, 2002).

Capsules are also surface associated polysaccharides. They are composed of capsular polysaccharides (CPSs), which are anchored to the OM. CPSs are composed of 90% water, providing the bacteria with a gel-like mesh that protects the bacteria from dehaydration. Also, CPSs aid in bacterial colonization, immune system evasion, and biofilm production (Mäkelä and Mayer, 1976).

1.4.3 Proteins

Nearly half of the mass of the outer membrane is composed of proteins. Few proteins cross the outer membrane and the cytoplasmic membrane Most of the outer membrane proteins extend either outside the cell or into the periplasm. They consist of antiparallel amphipathic β-strands that fold into cylindrical β-barrels

with a hydrophilic motif, making this membrane a point of the entry of different particles, such as DNA and quorum sensing molecules. They can also limit or block antibiotic uptake (Bos *et al.*, 2007). The outer membrane also has receptors for extracellular macromolecules and viruses, and it is necessary for macrophage and sexual recognition (Costerton *et al.*, 1974a). The different receptors present on this membrane are regulated by more than 60 proteins, which include: transport carriers, membrane vesicles that carry out specific transport tasks and intravesicle transport of different molecule substrates (Costerton *et al.*, 1974b). The host range of bacteriophages, quorum sensing molecules and competence factors (agents necessary for conjugation), depends on the presence of sensors on this membrane (Tomasz, 1971).

1.4.4 Lipoproteins

The different surface layers are anchored one to another by the presence of various proteins. Some of these proteins are called anchoring proteins, and they are in charge of forming covalent bridges between the ε-amino group of lysine residues in the peptidoglycan and lipoprotein components of the outer membrane (Tomasz, 1971). One of the most studied proteins that is found here is Braun's protein, a highly abundant membrane lipoprotein, covalently bound to the peptidoglycan layer by its lysine domain and to the outer membrane by its acylated, hydrophobic cysteine head (Braun and Hantke, 1974; Nikaido and Vaara, 1985) .

1.4.5 Porins

These proteins are known as porins because they produce non-specific pores. Different genes such as *ompF*, and *ompC* encode them. These pores allow the passage of small hydrophilic molecules across this membrane, making them highly important for the physiology of the cell since they allow the passage of nutrients, antibiotics, waste, and inhibitors (Batchelor *et al.*, 2005). One such protein, Omp85, is an evolutionary conserved protein in all Gram-negative organisms. When this protein is absent, outer membrane proteins cannot not be inserted in this membrane and are accumulated in a unfolded form inhibitors (Batchelor *et al.*, 2005). Porins are assembled into the OM by the β-barrel assembly machinery (BAM) complex, and by periplasmic chaperones SurA, DegP and Skp (See Periplasm section). First, nascent porins are targeted to the SecYEG translocon. After the export through the SecYEG translocon, the nascent porins are recruited by SurA and Skp-DegP, and are transported through the periplasm to the OM (Knowles *et al.*, 2009). Folding and insertion of nascent porins is in charge of the complex (BamA-BamE). One of the models is the pore-folding model, which suggests that BamA offers a pore for the insertion of the nascent porin, and the polypeptide transport-associated domains help the nascent porin to go through the pore. Another model is the β-barrel; it suggests that BamA offers a template for barrel folding near the BAM complex. After, the BAM complex inserts the protein into the membrane. Once this process has completed, DegP verifies the quality of the porins and removes misfolded OMPs (Knowles *et al.*, 2009). The outer membrane protein A (OmpA) is an example of a porin

assembled by the BAM complex. This porin is important for conjugation and serves as a receptor for phages (Livermore, 2001). Other functions of this latter protein consist of anchoring the outer membrane to the peptidoglycan layer. The C-terminal periplasmic domain of this protein binds non-covalently to the cell wall (Ried *et al.*, 1990).

SECTION II. STRESS RESPONSES OF *E.coli* AND THEIR ROLE IN CELL WALL MODIFICATION.

1.5 Adaptation and stress responses

Bacteria need to adapt to adverse conditions in order to persist in the environment. Depending on the external factor that is affecting them, bacteria have different responses that allow them to actively respond to the source of stress and survive. Recently, a number of these adaptive responses have been linked to antibiotic resistance phenotypes, thus making their study important for understanding and preventing antibiotic resistance, and perhaps for developing new therapeutics that might act in concert with, potentiators. *E. coli* utilizes six different envelope stress responses that continually monitor the status of various components of the bacterial envelope and respond according to the sensed damage. These responses include: the Rcs envelope stress response (Rcs), the bacterial adaptive response (Bae), the sigma E outer membrane stress response (σ^E), vesicle formation, the

Psp (phage-shock-protein) response, and the conjugative pilus expression (Cpx) envelope stress response. A number of these responses have been linked to antibiotic resistance phenotypes (Figure 2).

1.5.1 The Bacterial Adaptive Response (Bae)

Another stress response that has an important role in antibiotic resistance is the Bacterial Adaptive Response (Bae), which is regulated by a two-component system consisting of the sensor histidine kinase (BaeS) and the response regulator (BaeR) (Nagasawa *et al.*, 1993). Like typical two-component regulatory systems, BaeS has a histidine at the site of autophosphorylation and BaeR has an aspartate residue at the site of phosphorylation (Baranova and Nikaido, 2002) (Figure 2). Once this stress response is activated, BaeS autophosporylates, then a phosphate is transferred to BaeR, leading to transcription of the BaeSR regulon which includes: mdtABCD (multidrug transporter ABCD), *acrD* (aminoglycoside efflux system) and *spy* (spheroplast protein Y) (Raffa and Raivio, 2002). The main function of BaeSR consists of detecting damage to the envelope that is caused by small toxic molecules and regulating efflux pumps as its activation upregulates transcription of the transporter gene cluster *mdtABCD* and *acrD* (Hu *et al.*, 2011).

1.5.2 The σ^E stress response

The σ^E response is activated in the presence of stress that interferes with outer membrane protein (OMPs) biogenesis such as overexpression of OMP genes, mutations in chaperones required for proper OMP folding, changes in the

structure of OmpF and OmpC and heat shock (sigmaH regulated heat shock) and also by changes in the structure of LPS (Missiakas and Raina, 1997). This stress response is regulated by *rpoE* (Missiakas *et al.*, 1996).

Unstressed cells have low σ^E activity because σ^E is bound at the inner membrane by RseA. RseA prevents σ^E from binding to the RNA polymerase (Ades *et al.*, 1999). Under stress, when porins are unfolded, DegS and RseP, two different proteases, cleave RseA liberating σ^E. Constant degradation of RseA is necessary for maintenance of the σ^E response .

RpoE belongs to a family of conserved sigma factors with extracytoplasmic function that respond to extracytoplasmic stimuli. RpoE is regulated by RseA and RseB, proteins located in the periplasm that negatively regulate RpoE by acting as antisigma factors (Sohn *et al.*, 2009). RseA is the major negative regulator of the $\underline{\sigma^E}$ envelope response as it senses envelope stress signals that activate the $\underline{\sigma^E}$ response and affect RseA activity since RseA degradation by DegS is directly proportional to the folding state of various outer membrane proteins . (Bos *et al.*, 2007) RseB is a weak negative regulator that modulates the stability of RseA. RseB also has the capacity to sense a broader spectrum of unfolded periplasmic proteins, facilitating the identification of different cues for the activation of $\underline{\sigma^E}$ (Chaba *et al.*, 2011)(Figure 2).

1.5.3 Vesicle response

It has been long known that Gram-negative bacteria produce vesicles during the course of normal growth (McBroom and Kuehn, 2007). It has been recently

discovered that vesicles are an additional mechanisms for stress management. Vesicles production is modulated by the state of the envelope; their production is increased in the presence of stress and reduced in its absence. They act as intercellular transport vehicles in the presence of stress functioning as a complement to other stress-responses. Increased vesiculation enhances bacterial survival in the presence of chemical stressors and toxic compounds as vesicles help in the removal of these harmful molecules from the cell, functioning as a protective mechanism (McBroom and Kuehn, 2007)(Figure 2).

1.5.4 Overview of the Psp response

During infection with phage f1, *E. coli* produces a protein at very high concentrations. This protein was called phage-shock-protein A (PspA). Further experiments helped to elucidate that this protein mediates a response called Psp. The Psp response is induced by numerous environmental stimuli such as heat, ethanol, infection by the bacteriophage f1, and osmotic shock. Psp synthesis does not require the heat shock factor σ, σ^{32}, making Psp a parallel response (Brissette *et al.*, 1990). Additionally, this response is activated in the presence of proton leaks, supporting protein translocation under detrimental conditions (Brissette *et al.*, 1990). (Figure 2). PspA could be either directly involved in protein translocation, increasing the kinetics of translocation, or it can increase the activity of proton generating systems responding to the loss of protons by stress (Kleerebezem *et al.*, 1996). The proposed mechanism by which the Psp response is activated is highly hypothetical. In the lack of stress PspA binds to PspF, which

inhibits transcription of the Psp regulon, since PspF is a transcription factor (Darwin, 2005). Cytoplasmic membrane proteins sense inducing cues (Darwin, 2005). Then, they interact with PspA, liberating PspF to activate transcription of the *pspA* operon (Darwin, 2005). PspA concentration increases dramatically, indicating that it has an important physiological role in alleviating stress (Darwin, 2005).

1.5.5 The Regulator of capsular synthesis Rcs

The Rcs envelope stress response is controlled by a phosphorelay that is a modified version of the highly prevalent two-component system that was first identified in *E. coli (Mizuno, 1998)*. Canonical two-component systems consist of a histidine-kinase and a response regulator (Mizuno, 1998). Environmental parameters are sensed by the sensor kinase, modulating the regulation and activation of the response regulator (Mizuno, 1998). In a phosphorelay there are multiple signal inputs. The sensor kinase transfers its phosphoryl group to a second phosphotransferase domain that serves as the primary phosphoryl donor to the response regulator (Hoch, 2000).

One of the roles of the Rcs response consists of the regulation of genes for capsular polysaccharide. *E. coli* has a capsule of colonic acid composed of various mono and polysaccharides (glucose, galactose, and glucuronic acid) (Majdalani and Gottesman, 2005). Usually, the capsule is slim, but under different conditions such as UV light or mutations of the *lon* gene, colonic acid is over produced

making the capsular polysaccharide thick, and leading to heavily mucoid cells (Majdalani *et al.*, 2005).

The Rcs response has four major components: RscC, RscD, RscB and RcsA. RcsB and RcsC are part of the two-component system: RcsC is an inner membrane histidine-phosphotransfer protein (Hpt) with a large periplasmic domain and it interacts with RcsD via its periplasmic domain. RcsB is the response regulator and is in charge of modulating the expression of the Rcs regulon (Stout and Gottesman, 1990). The third component of the Rcs pathway is RcsA, a co-regulator of *rcs*-mediated gene expression. The activation of a RcsA/RcsB heterodimer is necessary for gene expression of the capsule when RcsA has accumulated or when RcsC is active (Majdalani and Gottesman, 2005). RcsC is a hybrid kinase with transmitter and receiver domains. When RcsC is activated, a histidine in the transmitter domain is autophosphorylated and the phosphoryl group is moved to an aspartate in the receiver domain. Next, the phosphoryl group is transferred to RcsD in the Hpt domain and finally to RcsB (Mizuno, 1997) (Figure 2).

Upon activation of RcsB, transcription of various genes takes place. The first one described was *ftsZ,* a gene that codes for a protein responsible for cell division and septum formation (Laubacher and Ades, 2008). RcsB is also responsible for the activation of *rprA*, a small RNA, that in turn activates translation of (stress response transcription factor) (Laubacher and Ades, 2008). RpoS induces the RpoS-dependent genes, such as *osmC* (Majdalani and Gottesman, 2005) and genes involved in biofilm formation. Biofilm formation requires the cells to attach

to a solid surface. Afterwards, colonies are formed and finally architectural development of the biofilm takes place (Leblanc *et al.*, 2011). Various studies have shown that Rcs participates in the final steps of biofilm formation (Ferrières *et al.*, 2009)

-Exposure to β- antibiotics and inhibition of PBPs activates Rcs (Regulator of capsular snynthesis)

In a study by Laubacher & Ades in 2008, it was found that the Rcs stress response was activated in *E. coli* when the structure of the cell wall was compromised by the inhibition of PBP 1a, 1b and 2 (Laubacher and Ades, 2008). It was also observed that the expression levels of the Rcs regulon increased when cells were in the presence of aztreonam and ampicillin (Laubacher and Ades, 2008).

Activation of the Rcs stress response by antibiotics that target the cell wall is specific. There are high levels of transcription of the Rcs regulon when antibiotics that target the cell wall are present. Other perturbations in the cell wall, such as deletion of PB1b (Laubacher and Ades, 2008) and mislocalization of AmiA and AmiC hydrolases due to disruption of the twin-arginine transportation system led to up-regulation of many genes of the Rcs regulon. These two modifications are not lethal for the cell, proving that the Rcs response is not activated in conditions of impending lysis or cell death (Laubacher and Ades, 2008), but that the Rcs pathway is activated when there is a disturbance in the cell envelope as a result of changes to peptidoglycan structure (Laubacher and Ades, 2008). This suggests that the Rcs phosphorelay can sense modifications in the cell wall by interacting

directly with the peptidoglycan layer or by intermediate mediators. Activation of the Rcs regulon is important for survival of bacteria in the presence of β-lactam antibiotics cefsulodin and amdinocillin as various genes activated by this regulon were upregulated by exposure to these compounds. These genes were *rprA*, *ydhA*, an inhibitor of c-type lysozymes, such as human lysozyme (Callewaert *et al.*, 2009), *osmB*, a lipoprotein of unknown function located on the outer membrane that is activated by various stress responses, such as RpoS; and the hypothetical protein *ymgG* (Laubacher and Ades, 2008). Another study demonstrated that bacterial L-forms, osmosensitive bacteria without a cell wall, require the colanic acid capsule and the genes *rcsA* and *rcsF to* survive in the presence of β-lactam antibiotic cefsulodin (Joseleau-Petit *et al.*, 2007).

1.5.6 The CpxRA envelope response

The Cpx (conjugative pilus expression) response was first identified in 1980 when *cpxA* and *cpxB* mutants were observed to be unable to elaborate F-pili or to act as conjugal donors and were resistant to donor-specific bacteriophages (McEwen and Silverman, 1980). In 1986, it was found that *cpxA* shared homology with the C-terminal region of *ntrB* (nitrogen regulatory protein B), a well-defined histidine kinase, and that Cpx was part of a two component-system (Nixon *et al.*, 1986). Subsequently it was found that the Cpx response is primarily activated in the presence of extra-cytoplasmic protein misfolding (Danese *et al.*, 1995) as a result of various cues. The activating cues of the Cpx pathway include misfolding of the Pap pilus subunits in absence of the PapD chaperone (Jones *et al.*, 1997),

elevated pH (Danese and Silhavy, 1998), osmolarity (Jubelin *et al.*, 2005), indole (Raffa and Raivio, 2002), modifications to the lipid composition of the cell membrane and over-production of periplasmic lipoprotein NlpE (New lipoprotein E). This lipoprotein seems to participate in copper homeostasis (Gupta *et al.*, 1995) and its necessary for adhesion to abiotic hydrophobic surfaces (Otto and Silhavy, 2002). It has been reported that a multi-drug resistant (MDR) *Acinetobacter baumanii* strain isolated during a hospital outbreak in Rouen overproduced NlpE, leading to increased adherence when compared to that of a susceptible strain (Nishino *et al.*, 2010), indicating that the Cpx pathway might be involved in biofilm formation on inanimate surfaces, as well as antibiotic resistance. Biofilms protect bacteria against antimicrobial agents and contribute to the development of antibiotic resistance As a result of delayed penetration of the antimicrobial into the biofilm extracellular matrix and slowing down the reproduction rate of the microorganisms that are found inside the biofilm (Price and Raivio, 2009).

Once misfolded proteins accumulate in the periplasm the Cpx pathway is activated. The *cpx* locus encodes a histidine kinase (CpxA) and a cognate response regulator (CpxR), both of which are involved in signal sensing and activating the Cpx regulon respectively (Weber and Silverman, 1988). Once CpxA detects an inducing cue, it autophosphorylates at a conserved histidine residue. Subsequently, CpxA transfers a phosphate to a conserved aspartate in CpxR, making it a functional response regulator and leading to transcription of the Cpx regulon (Raivio and Silhavy, 1997).

Some of the genes that are regulated by the Cpx pathway are proteases and chaperones including *dsbA,* a periplasmic enzyme necessary for disulfide bond formation in extracytoplasmic protein (Pogliano *et al.*, 1997), *degP,* a housekeeping protease and chaperone (Danese *et al.*, 1995), and *ppiA,* the peptidyl-prolyl cis/trans isomerase (Pogliano *et al.*, 1997).

The Cpx pathway has an auxiliary regulator, CpxP (Danese and Silhavy, 1998). In the presence of high levels of CpxP, the Cpx pathway is repressed. It is hypothesized that this happens when CpxP interacts directly with the periplasmic domain of CpxA, keeping CpxA in an ''off'' state by preventing its dimerization and autophosphorylation (Raivio *et al.*, 2000). Additionally, CpxP has weak chaperone activity *in vitro* (Quan et al., 2011).

According to the model proposed by Isaac and colleagues (Isaac *et al.*, 2005) CpxP may bind to misfolded pilus subunits when they are present in the periplasm and escort them to DegP for degradation, together with CpxP itself, titrating away CpxP from CpxA and leading to activation of the Cpx pathway (Peschel and Sahl, 2006) (Figure 3).

-CpxRA upregulates peptidoglycan amidases upon CAMPs exposure

In a study by Weatherspoon-Griffin, it was discovered that the Cpx pathway up-regulates two amidases, amiA and amiC in the presence of cationic antimicrobial peptides (AMPs) (Weatherspoon-Griffin *et al.*, 2011). CAMPs play an important role in mammalian host defense as they are components of the innate immune system against infections caused by microorganisms (Peschel and Sahl, 2006) CAMPs are highly hydrophobic with broad-spectrum activities against microbes

(Peschel and Sahl, 2006). Initially, they interact with outer or cytoplasmic membranes, disrupting them and opening pores that eventually lead to cell leaking and lysis (Peschel and Sahl, 2006). CAMPs can be an alternative to antibiotics, since their action is non-specific, general and lethal (Peschel and Sahl, 2006). Their presence in the cell leads to activation of the Cpx pathway in *Salmonella* and *E. coli (Weatherspoon-Griffin et al., 2011)*. CpxR binds to CpxR boxes in *amiA* and *amiC* promoters, facilitating resistance to protamine. AmiA and AmiC have N-acetylmuramyl-L-alanine activity, they are capable of breaking the cross-link formed by the pentapeptide and polysaccharide strands and they participate in cell wall remodeling (Bernhardt and De Boer, 2003). Disruption of the function of these enzymes affects the integrity of the *E. coli* cell wall, which in turn leads to an increase in the permeability to hydrophobic antibiotics, possibly because of changes in the association of the cell wall with the outer membrane (Weatherspoon-Griffin *et al.*, 2011). In the presence of a functional Cpx pathway and in the absence of the Tat system AmiA and AmiC cannot be translocated. As a result, these amidases that are up-regulated by the Cpx pathway cannot confer resistance to CAMPs WeatherspoonGriffin 2011}. Furthermore, exposure to CAMPs also sensitizes bacteria to antibiotics when the Cpx pathway and the Twin-arginine transportation system (Tat) are inactivated. Possibly, CpxRA confers resistance to CAMPs by reducing envelope stress and fighting the damage that is caused to the cell envelope by these peptides. Over-expression of NlpE also leads to an increase in protamine resistance, showing that this protein might be involved in antibiotic resistance because it leads to up-regulation of some genes of

the Cpx regulon that confer resistance (Weatherspoon-Griffin *et al.*, 2011). One of these genes is *yqjA*, a protein with unknown function that is required for protamine resistance (Weatherspoon-Griffin *et al.*, 2011).

-CpxR over production increases resistance to kanamycin, novobiocin, oxacillin, cefmetazole and aztreonam probably by mediating changes in the peptidoglycan structure.

E. coli has 32 open reading frames (ORFs) that are believed to be response regulators of two-component systems on the basis of bioinformatics. One of these ORFs is CpxR. CpxR modulates the expression of several drug exporter genes and this drug resistance was observed by using drug exporter gene knockout strains. Over-expression of CpxR leads to an increase in the minimum inhibitory concentration and resistance to kanamycin, novobiocin, oxacillin, cloxacillin, nafcillin, cefamandole, carbenicillin, nafcillin, cefmetazole and aztreonam, deoxycholic acid, and low level β-lactam resistance (Hirakawa *et al.*, 2003). CpxR over-expression results in *acrD* (aminoglycoside efflux pump) over-expression, indicating that AcrD might be important for bacterial adaptation (Baranova and Nikaido, 2002). In addition, CpxR over-expression leads to up-regulation of MdtABC (Nishino *et al.*, 2010). NlpE over-expression also increases resistance to all these β-lactams suggesting that activation of the Cpx pathway enhances multidrug resistance in *E. coli by* mediating changes in the cell wall structure (Nishino *et al.*, 2010), These responses also require the up-regulation of BaeSR to activate AcrD and MdtABC. This process is reminiscent of the BaeSR

copper and zinc resistance mechanism mediated by AcrD and MdtABC in *Salmonella enterica (Nishino et al., 2010)*.

The Cpx pathway also influences pathogenicity and antibiotic resistance in *Klebsiella pneumoniae.* The first step of nosocomial infections caused by this pathogen consists on the colonization in the patient's gastrointestinal (GI) tract. *K. pneumoniae* must sense the hosts' different environments to survive and adapt, in order to be able to persist in the GI tract. *cpxRA* deficient mutants have lower survival capabilities than the wild type, showing that the Cpx pathway is necessary for survival of bacteria in the upper part of the GI (Srinivasan *et al.*, 2012). Additionally, deletion of the *cpxRA* makes resistant strains susceptible to β-lactams such as cefepime, cexotaxime, ceftzadime and chloramphenicol. These antibiotics are the substrate of various efflux pumps regulated by the Cpx regulon and inactivation of this response leads to a decrease in resistance (Srinivasan *et al.*, 2012).

-Simultaneous absence of 4 PBPs up-regulates the Cpx pathway and in a Cpx-dependent manner the Rcs response.

PBPs (penicillin-binding proteins) are enzymes in charge of modifying or synthesizing the cell wall. Absence of some of PBPs can lead to changes be lethal or cause changes in cell shape, division, or motility (Vollmer *et al.*, 2008). In a screening by Evans *et. al* it was found that absence of four PBPs (PBPs 4, 5, 7, AmpH) activates the Rcs stress response and the Cpx pathway in *E. coli (Evans et al., 2013)*. However, induction of Rcs was dependent on the presence of the

Cpx pathway. These results suggest that the Cpx pathway also has a role in sensing changes to the peptidoglycan structure by either directly sensing the cell wall fragments or by an unknown mechanism.

SECTION III. RESEARCH OBJECTIVES

The traditional view of the Cpx pathway suggests that the main function of this stress response is the detection, folding, and degradation of mis-folded proteins in the periplasm. However, a more contemporary view proposes that the Cpx pathway is mostly involved in the homeostasis of the inner membrane. In addition, recent studies have found that activation of the Cpx pathway leads to up-regulation of various cell wall genes, indicating that this stress response might be sensing or leading to changes in this structure (Table 1).

The objective of this thesis was to determine whether activation of the Cpx pathway causes changes in the peptidoglycan structure and composition of *E. coli* using various approaches.

1.6 The Cpx pathway up-regulates genes involved in cell wall metabolism and catabolism

Regulation of the Cpx-pathway consists of a *cpxRA* operon that encodes the response regulator CpxR and the histidine-kinase CpxA. Together with SigmaE, RpoS, and RpoH, the Cpx pathway regulates numerous genes to alleviate envelope stress (Pogliano *et al.*, 1997; Danese and Silhavy, 1997) . The

complexity of the regulation and activation of this stress response led to two microarray studies that have tried to identify and characterize the members of the regulon of the Cpx pathway. In a first microarray study by Bore-Moné *et. al* some novel members of the Cpx regulon were identified by CpxR over-expression (Bury-Moné *et al.*, 2009). However, other well-known targets of CpxR, such as *degP* and *dsbA* were not identified in this study (Pogliano *et al.*, 1997). A second analysis by Raivio *et. al (Raivio et al., 2013)* identified numerous new members of the Cpx regulon that had previously not been identified before by overexpression NlpE a well-known inducing cue of the Cpx pathway (Snyder *et al.*, 1995), suggesting that this pathway may have more functions that previously thought. Among these, several genes involved in peptidoglycan synthesis, modification, and recycling were identified: *ycbB, ygaU, slt, mltB, dacC, amiA, and amiC.*

YcbB is a 67 kDa L,D-transpeptidase in charge of synthesizing the uncommon 3-3 cross-link (Rawlings and Salvesen, 2012) by the removal a D-alanine residue of an acyl donor tetrapeptide stem and the binding of the *meso*-diaminopimelyl (DAP) residue to the *meso*-DAP residue of the second acyl acceptor tetrapeptide (Magnet *et al.*, 2008). YgaU is a 16kDa conserved hypothetical protein with a LysM domain, found in enzymes involved in cell wall degradation (Joris *et al.*, 1992), and a BON motif that has a membrane binding domain that contains a conserved glycine residue and several hydrophobic regions (Yeats and Bateman, 2003). YgaU is also up-regulated by RpoS (Weber *et al.*, 2005) when the *E. coli* is grown in hyperosmolar conditions (Weber *et al.*, 2006). Slt70 is a 70kDa

soluble lytic transglycosylase with muramidase activity (Engel *et al.*, 1991). It catalyses the cleavage of the glycosidic bonds between N-acetyl glucosamine and N-acetyl muramic acid, producing N-acetyl glycosamine and 1,6-anhydro N-acetyl muramic acid (Dijkstra *et al.*, 1999). MltB is a 36kDa membrane-bound lytic transglycosylase that also cleaves glycosydic bonds forming N-acetyl glycosamine and 1,6-anhydro N-acetyl muramic acid. It is preferentially localized at the periplasmic side of the outer membrane (van Asselt *et al.*, 2000). Slt70 and MltB have endolytic and exolytic activity (Artola-Recolons *et al.*, 2011), this is the ability to cleave peptidoglycan in the middle or at the end of a chain producing 1,6-anhydro N-acetyl muramic acid (Höltje *et al.*, 1975). In addition MltB is more active than Slt70 in *E. coli* grown to stationary phase as MltB produces more muropeptide residues. MltB also has a greater preference than Slt70 for cross-linked products (Lee *et al.*, 2013).

AmiA and AmiC are N-acetylmuramyl-L-alanine amidases that have a major role in septum cleavage during cell division (Priyadarshini *et al.*, 2007) by removing the peptide stem from the N-acetyl muramic acid, destroying cross-links. AmiA and AmiC are differentially localized in the cell. AmiA is localized throughout the periplasm in all cells whereas AmiC is present throughout the periplasm only in non-dividing cells. In cells with visible constriction, AmiC is found in a ring at the site of constriction, suggesting that AmiC is a component of the septal ring (Heidrich *et al.*, 2001). Both of these amidases are translocated by the twin-arginine translocation system (Bernhardt and De Boer, 2003). DacC is membrane-bound penicillin-binding protein (PBP6) that is highly toxic when

over-expressed in *E. coli* *(Pedersen et al., 1998)*. DacC is one of the two major D-analyl-alanine carboxypeptidases (D,D-carboxypeptidases) of *E. coli*. Its function consists on splitting of the C-terminal D-alanine from the precursor pentapeptide side chains L-Ala-D-Glu-*ms*A_2pm-D-ala-D-Ala, which can either remain uncross-linked in mature peptidoglycan or need to have their C-terminal end removed in order to be cross-linked, thus converting them in L-Ala-D-Glu-*ms*A_2pm-D-ala-D-Ala (MARTIN *et al.*, 1975). DacC is highly expressed in cells when they enter stationary phase (Santos *et al.*, 2002), indicating that the Cpx pathway and RpoS might be regulators of this gene (De Wulf and Lin, 2000; Costanzo and Ades, 2006). Moreover, it was recently reported that the simultaneous absence of 4 PBPs caused activation of the Cpx pathway and the Rcs response. Activation of the Rcs response was, in this case, dependent in this case of the presence of CpxR, showing that small changes to the peptidoglycan structure are sufficient to activate this two stress responses and suggesting that a specific muropeptide might be the inducing cue (Evans *et al.*, 2013).

The probable regulation of *ycbB, ygaU, slt, mltB, dacC* and the extent of the regulation of *amiA* and *amiC* by the Cpx pathway is exciting to us because even though there are few reports that prove that the Cpx pathway regulates cell wall genes (Price and Raivio, 2009; Evans *et al.*, 2013; Weatherspoon-Griffin *et al.*, 2011), no one has studied before how the peptidoglycan structure is modified during the activation of this response. Prior to the characterization of the role of

ycbB, ygaU, slt, mltB, dacC, amiA, and *amiC* during the activation of the Cpx pathway it was necessary to confirm the regulation of these genes.

The initial steps of this project consisted on cloning the promoters of these genes in the plasmid pJW15, which carries a promoterless luciferase operon. Binding of phosphorylated CpxR at the promoter site of each gene should result in the expression of luciferase, which in turn emits light proportional to the amount of the transcription of each gene, allowing the quantification of the gene transcription biofilm (Price and Raivio, 2009). Expression levels were measured in strains with different genetic backgrounds such as: *cpxA** (*cpxA24*), *ΔcpxR*, NlpE and BfpA over production. This allowed us to assess whether these genes are regulated when the Cpx pathway is induced and to confirm the results of the microarray.

Furthermore, we analyzed whether the Cpx pathway directly regulates *ygaU, ycbB, mltB,* and *slt70,* the most up-regulated cell wall genes in the lux assay. We performed electrophoretic mobility shift assays (EMSAs) to observe whether the response regulator CpxR bound directly to the promoter of each of these genes. We found that CpxR binds strongly to the promoter of these genes, leading to the conclusion that activation of the Cpx pathway leads to changes in the peptidoglycan structure. This finding further supports that the Cpx pathway has more roles than initially thought, and that modification of the cell wall might be important for adaptation in the presence of Cpx inducing cues.

1.7 Determination of changes in cell morphology, cell wall composition and structure during activation of the Cpx pathway

For those genes confirmed to be regulated by the Cpx pathway, we made null mutants in various genetic backgrounds such as MC4100, *cpxA** and *Δcpx* (Price and Raivio, 2009). We used standard MIC determination measurements to quantify the MIC to different β- lactams, which is an indicator of changes in the peptidoglycan structure. We also analyzed the effect Cpx pathway had in the cell morphology, cell wall composition and structure, and induction of the Cpx pathway in each of these strains. These studies indicated that *ygaU* is important for cell morphology during activation of the Cpx pathway as absence of this gene leads to an increase in filamentation. In addition, HPLC analysis showed that 3-3 cross-linking is important during activation of the Cpx pathway, and that these cross-links are synthesized by L,D-transpeptidases. We also observed an increase in 1,6-anhydromuropeptides, indicating that during activation of the Cpx pathway murein chains are shorter and cross-linking becomes more frequent, indicating that activation of the Cpx pathway might help to increase peptidoglycan recycling to alleviate stress in the periplasm.

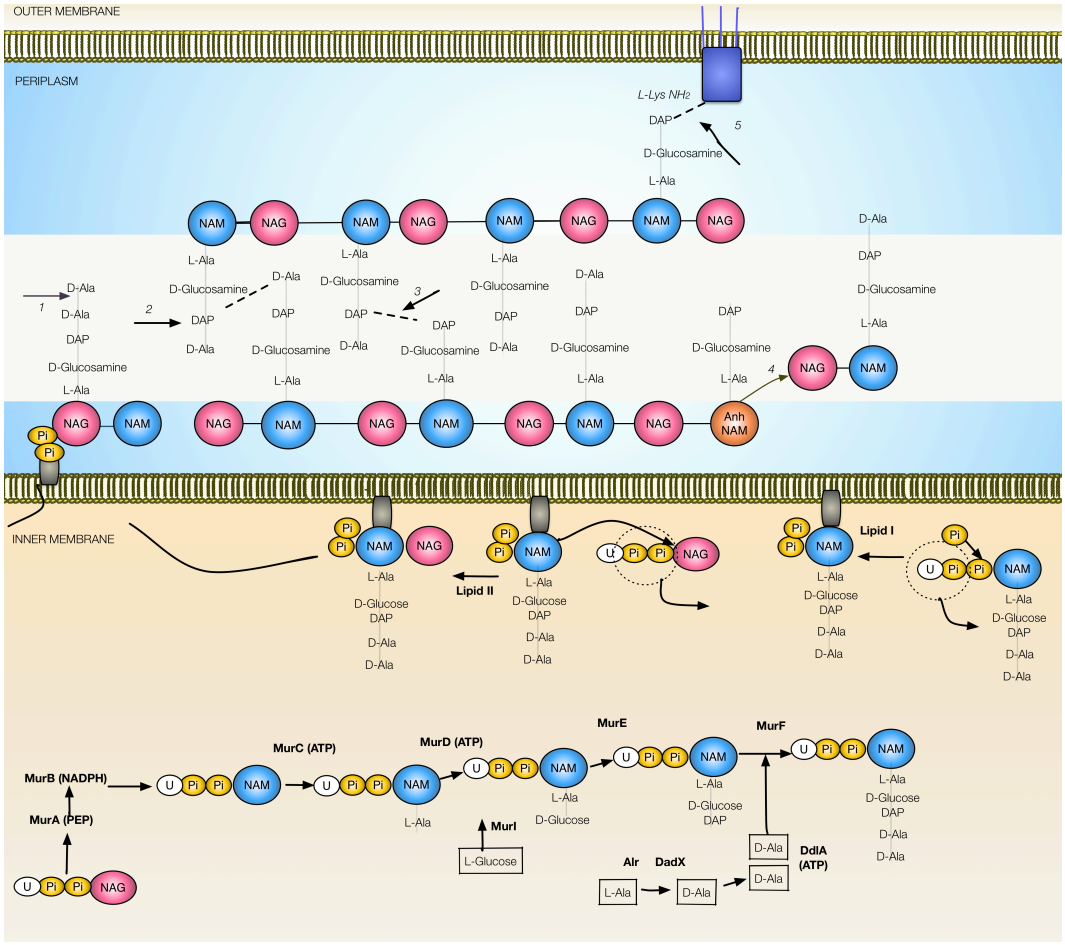


Figure 1: Steps of the synthesis of the cell wall.

Image modified from Typas *et.* al (Typas *et al.*, 2012). The peptidoglycan synthesis is a three-step process that takes places in the cytoplasm, inner membrane, and periplasm. In the first step, Mur enzymes are in charge of synthesizing a UDP-MurNAc (UDP-N-acetylmuramyl) pentapeptide from UDP-N-Acetyglycosamine (GlcNAc) by the addition of pyruvate. In the second step, the UDP-MurNAc pentapeptide is transferred to yield lipid I (undecaprenyl phosphate). Lipid II (undecaprenyl pyrophosphate) is then formed by the transfer of GlcNAc and translocated to the periplasm by FtsZ-RodA or MurJ. Newer peptidoglycan monosaccharides are attached to the cell wall by the activity

transglycosylases that catalyse the polymerization of new peptidoglycan subunits. New peptidoglycan subunits are first trimmed to di, tri, and tetrapeptides by carboxypeptidases, and cross-linked by 3-4 cross-links (DAP-D-Ala) or 3-3 cross-links (DAP-DAP) by the activity of D,D-transpeptidases or L,D-transpeptidases respectively. L,D-transpeptidases are also responsible for the cross-linking between the peptidoglycan and Braun's lipoprotein. Lytic transglycosylases cleave the glycan chains to synthesize the 1,6-anhydro-N-acetyl glucosamine, which is an indicator of the glycan chain length (van Heijenoort, 2010; El Zoeiby *et al.*, 2003).

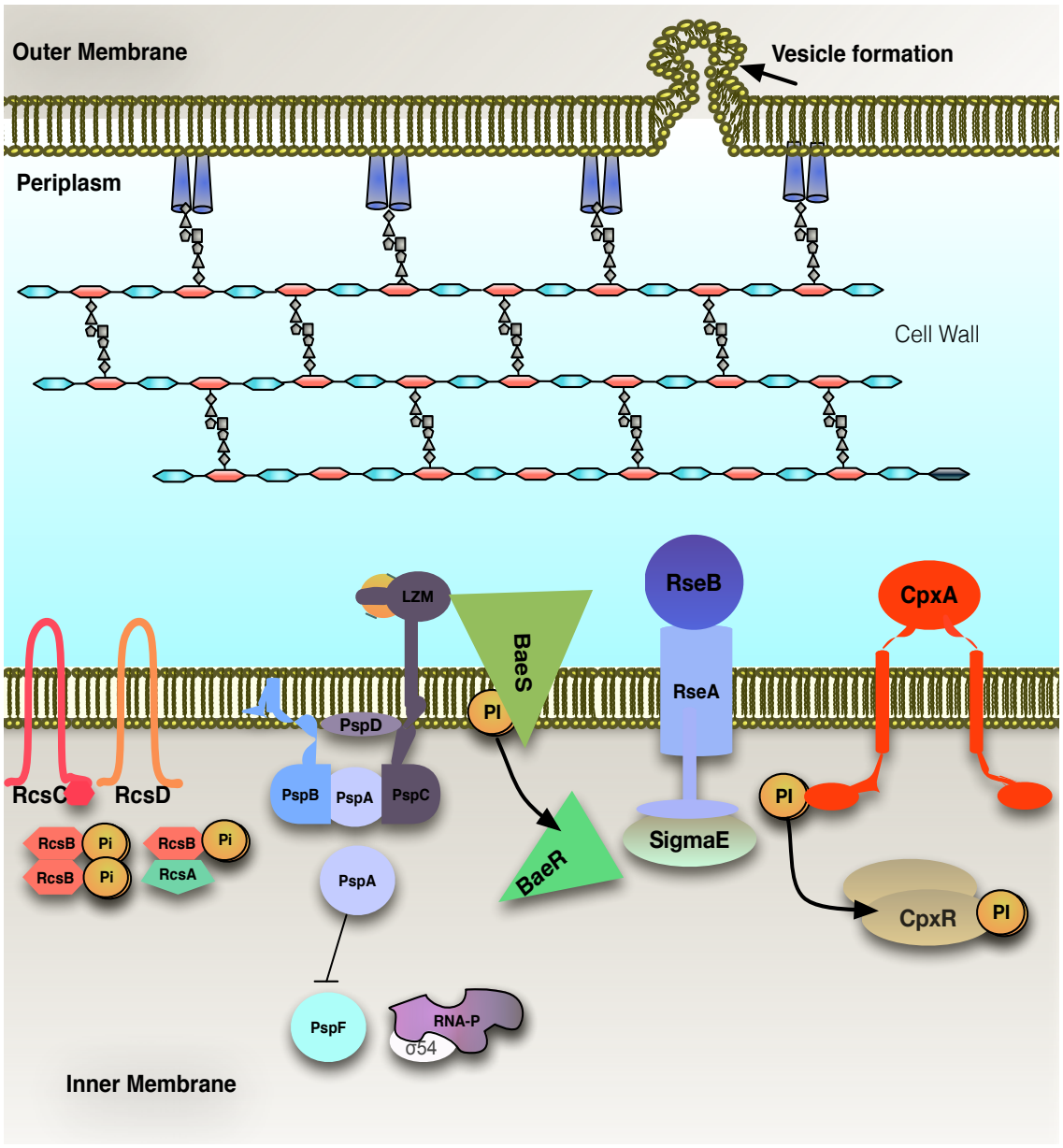


Figure 1-2 Some of the stress responses of *E. coli*.

The Rcs response has four major components: RscC, RscD, RscB and RcsA. RcsB and RcsC are part of the two-component system: RcsC is an inner membra it interacts with RcsD via its periplasmic domain. RcsB is the response regulator and is in charge of modulating the expression of the Rcs regulon (Stout and Gottesman, 1990). The third component of the Rcs pathway is RcsA, a co-regulator of *rcs*-mediated gene expression. 2)The PsP pathway cytoplasmic membrane proteins sense inducing cues (Darwin, 2005). Then, they interact with PspA, liberating PspF to activate transcription of the *pspA* operon (Darwin, 2005). 3,5) The BaeSR stress response and the Cpx pathway are two-component system consisting of the sensor histidine kinase (BaeS, CpxA) and the response regulator (BaeR, CpxR). 4) The SigmaE pathway is activated when porins are unfolded resulting in DegS and RseP, two different proteases, cleaving RseA liberating σ^E (Missiakas *et al.*, 1996). 6) Vesicle production is modulated by the state of the envelope; their production is increased in the presence of stress and reduced in its absence (McBroom and Kuehn, 2007).

References

Ades, S.E., Connolly, L.E., Alba, B.M., and Gross, C.A. (1999) The *Escherichia coli* sigma(E)-dependent extracytoplasmic stress response is controlled by the regulated proteolysis of an anti-sigma factor. *Genes Dev* **13**: 2449-2461.

Artola-Recolons, C., Carrasco-López, C., Llarrull, L.I., Kumarasiri, M., Lastochkin, E., Martínez de Ilarduya, I., *et al.* (2011) High-resolution crystal structure of MltE, an outer membrane-anchored endolytic peptidoglycan lytic transglycosylase from *Escherichia coli*. *Biochemistry* **50**: 2384-2386.

Baranova, N., and Nikaido, H. (2002) The BaeSR Two-Component Regulatory System Activates Transcription of the *yegMNOB* (*mdtABCD*) Transporter Gene Cluster in *Escherichia coli* and Increases Its Resistance to Novobiocin and Deoxycholate. *Journal of Bacteriology* **184**: 4168-4176.

Bardwell, J.C. (1994) Building bridges: disulphide bond formation in the cell. *Molecular microbiology* **14**: 199-205.

Batchelor, E., Walthers, D., Kenney, L.J., and Goulian, M. (2005) The *Escherichia coli* CpxA-CpxR envelope stress response system regulates expression of the porins *ompF* and *ompC*. *J Bacteriol* **187**: 5723-5731.

Berks, B.C., Sargent, F., and Palmer, T. (2000) The Tat protein export pathway. *Molecular microbiology* **35**: 260-274.

Bernhardt, T.G., and De Boer, P.A.J. (2003) The *Escherichia coli* amidase AmiC is a periplasmic septal ring component exported via the twin-arginine transport pathway. *Molecular Microbiology* **48**: 1171-1182.

Beveridge, T.J. (1999) Structures of Gram-negative cell walls and their derived membrane vesicles. *J Bacteriol* **181**: 4725-4733.

Beveridge, T.J., and Graham, L.L. (1991) Surface layers of bacteria. *Microbiological reviews* **55**: 684.

Bos, M.P., and Tommassen, J. (2004) Biogenesis of the Gram-negative bacterial outer membrane. *Curr Opin Microbiol* **7**: 610-616.

Bos, M.P., Robert, V., and Tommassen, J. (2007) Biogenesis of the Gram-negative bacterial outer membrane. *Annu Rev Microbiol* **61**: 191-214.

Bouhss, A., Mengin-Lecreulx, D., Le Beller, D., and Van Heijenoort, J. (1999) Topological analysis of the MraY protein catalysing the first membrane step of peptidoglycan synthesis. *Mol Microbiol* **34**: 576-585.

Braun, V., and Hantke, K. (1974) Biochemistry of bacterial cell envelopes. *Annu Rev Biochem* **43**: 89-121.

Brissette, J.L., Russel, M., Weiner, L., and Model, P. (1990) Phage shock protein, a stress protein of *Escherichia coli*. *Proceedings of the National Academy of Sciences* **87**: 862-866.

Bury-Moné, S., Nomane, Y., Reymond, N., Barbet, R., Jacquet, E., Imbeaud, S., *et al.* (2009) Global analysis of extracytoplasmic stress signaling in *Escherichia coli*. *PLoS Genet* **5**: e1000651.

Bush, K., and Jacoby, G.A. (2010) Updated functional classification of β-lactamases. *Antimicrob Agents Chemother* **54**: 969-976.

Callewaert, L., Vanoirbeek, K.G.A., Lurquin, I., Michiels, C.W., and Aertsen, A. (2009) The Rcs two-component system regulates expression of lysozyme inhibitors and is induced by exposure to lysozyme. *J Bacteriol* **191**: 1979-1981.

Carballido-López, R. (2006) Orchestrating bacterial cell morphogenesis. *Mol Microbiol* **60**: 815-819.

Cava, F., de Pedro, M.A., Lam, H., Davis, B.M., and Waldor, M.K. (2011) Distinct pathways for modification of the bacterial cell wall by non-canonical D-amino acids. *EMBO J* **30**: 3442-3453.

Chaba, R., Alba, B.M., Guo, M.S., Sohn, J., Ahuja, N., Sauer, R.T., and Gross, C.A. (2011) Signal integration by DegS and RseB governs Sigma E mediated envelope stress response in *Escherichia coli*. *Proc Natl Acad Sci U S A* **108**: 2106-2111.

Chin, D.T., Goff, S.A., Webster, T., Smith, T., and Goldberg, A.L. (1988) Sequence of the lon gene in *Escherichia coli*. A heat-shock gene which encodes the ATP-dependent protease. *Journal of Biological Chemistry* **263**: 11718-11728.

Costanzo, A., and Ades, S.E. (2006) Growth phase-dependent regulation of the extracytoplasmic stress factor, sigmaE, by guanosine 3',5'-bispyrophosphate (ppGpp). *J Bacteriol* **188**: 4627-4634.

Costerton, J.W., Damgaard, H.N., and Cheng, K.-J. (1974a) Cell envelope morphology of rumen bacteria. *Journal of bacteriology* **118**: 1132-1143.

Costerton, J.W., InGram, J.M., and Cheng, K.J. (1974b) Structure and function of the cell envelope of Gram-negative bacteria. *Bacteriological reviews* **38**: 87.

Danese, P.N., and Silhavy, T.J. (1997) The sigma(E) and the Cpx signal transduction systems control the synthesis of periplasmic protein-folding enzymes in *Escherichia coli*. *Genes & Development* **11**: 1183-1193.

Danese, P.N., and Silhavy, T.J. (1998) CpxP, a stress-combative member of the Cpx regulon. *Journal of bacteriology* **180**: 831-839.

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

Darwin, A.J. (2005) The phage-shock-protein response. *Mol Microbiol* **57**: 621-628.

Deng, Y., Sun, M., and Shaevitz, J.W. (2011) Direct Measurement of Cell Wall Stress Stiffening and Turgor Pressure in Live Bacterial Cells. *Physical Review Letters* **107**.

Desmarais, S.M., De Pedro, M.A., Cava, F., and Huang, K.C. (2013) Peptidoglycan at its peaks: how chromatographic analyses can reveal bacterial cell wall structure and assembly. *Mol Microbiol* **89**: 1-13.

DiRusso, C.C., and Nyström, T. (1998) The fats of *Escherichia coli* during infancy and old age: regulation by global regulators, alarmones and lipid intermediates. *Molecular microbiology* **27**: 1-8.

Driessen, A.J.M., and Nouwen, N. (2008) Protein translocation across the bacterial cytoplasmic membrane. *Annu Rev Biochem* **77**: 643-667.

El Zoeiby, A., Sanschagrin, F., and Levesque, R.C. (2003) Structure and function of the Mur enzymes: development of novel inhibitors. *Molecular microbiology* **47**: 1-12.

Engel, H., Kazemier, B., and Keck, W. (1991) Murein-metabolizing enzymes from *Escherichia coli*: sequence analysis and controlled overexpression of the *slt* gene, which encodes the soluble lytic transglycosylase. *Journal of bacteriology* **173**: 6773-6782.

Evans, K.L., Kannan, S., Li, G., de Pedro, M., and Young, K.D. (2013) Eliminating a set of four penicillin binding proteins triggers the Rcs phosphorelay and Cpx stress responses in *Escherichia coli*. *J Bacteriol* .

Ferrières, L., Thompson, A., and Clarke, D.J. (2009) Elevated levels of sigma S inhibit biofilm formation in *Escherichia coli*: a role for the Rcs phosphorelay. *Microbiology* **155**: 3544-3553.

Figge, R.M., Divakaruni, A.V., and Gober, J.W. (2004) MreB, the cell shape-determining bacterial actin homologue, co-ordinates cell wall morphogenesis in Caulobacter crescentus. *Mol Microbiol* **51**: 1321-1332.

Frère, J.-M. (1995) Beta-lactamases and bacterial resistance to antibiotics. *Molecular microbiology* **16**: 385-395.

Gangaiah, D., Zhang, X., Fortney, K.R., Baker, B., Liu, Y., Munson, R.S., and Spinola, S.M. (2013) Activation of CpxRA in Haemophilus ducreyi Primarily Inhibits the Expression of Its Targets, Including Major Virulence Determinants. *J Bacteriol* **195**: 3486-3502.

Gardel, M.L., Shin, J.H., MacKintosh, F.C., Mahadevan, L., Matsudaira, P., and Weitz, D.A. (2004) Elastic behavior of cross-linked and bundled actin networks. *Science* **304**: 1301-1305.

Gensure, R., Zeidel, M., and Hill, W. (2006) Lipid raft components cholesterol and sphingomyelin increase H+/OH-permeability of phosphatidylcholine membranes. *Biochem. J* **398**: 485-495.

Gille, C., Goede, A., Schlöetelburg, C., Preißner, R., Kloetzel, P.-M., Göbel, U.B., and Frömmel, C. (2003) A Comprehensive View on Proteasomal Sequences: Implications for the Evolution of the Proteasome. *Journal of Molecular Biology* **326**: 1437-1448.

Glauert, A.M., and Thornley, M.J. (1968) The topography of the bacterial cell wall. *Annual review of microbiology* **23**: 159-198.

Glauner, B. (1988) Separation and quantification of muropeptides with high-performance liquid chromatography. *Analytical biochemistry* **172**: 451-464.

Gottesman, S. (1989) Genetics of proteolysis in *Escherichia coli*. *Annual review of genetics* **23**: 163-198.

Gupta, S.D., Lee, B.T., Camakaris, J., and Wu, H.C. (1995) Identification of *cutC* and *cutF* (*nlpE*) genes involved in copper tolerance in *Escherichia coli*. *Journal of bacteriology* **177**: 4207-4215.

Hancock, R.E. (1997) Peptide antibiotics. *Antimicrob Agents Chemother* **349**: 418-422.

Heidrich, C., Templin, M.F., Ursinus, A., Merdanovic, M., Berger, J., Schwarz, H., *et al.* (2001) Involvement of N-acetylmuramyl-l-alanine amidases in cell

separation and antibiotic-induced autolysis of *Escherichia coli*. *Molecular microbiology* **41**: 167-178.

Hirakawa, H., Nishino, K., Hirata, T., and Yamaguchi, A. (2003) Comprehensive Studies of Drug Resistance Mediated by Overexpression of Response Regulators of Two-Component Signal Transduction Systems in *Escherichia coli*. *Journal of Bacteriology* **185**: 1851-1856.

Hobot, J.A., Carlemalm, E., Villiger, W., and Kellenberger, E. (1984) Periplasmic gel: new concept resulting from the reinvestigation of bacterial cell envelope ultrastructure by new methods. *Journal of bacteriology* **160**: 143-152.

Hoch, J.A. (2000) Two-component and phosphorelay signal transduction. *Current opinion in microbiology* **3**: 165-170.

Höltje, J.V. (1998) Growth of the stress-bearing and shape-maintaining murein sacculus of *Escherichia coli*. *Microbiol Mol Biol Rev* **62**: 181-203.

Höltje, J.V., Mirelman, D., Sharon, N., and Schwarz, U. (1975) Novel type of murein transglycosylase in *Escherichia coli*. *Journal of bacteriology* **124**: 1067-1076.

Hu, W.S., Chen, H.-W., Zhang, R.-Y., Huang, C.-Y., and Shen, C.-F. (2011) The expression levels of outer membrane proteins STM1530 and OmpD, which are influenced by the CpxAR and BaeSR two-component systems, play important roles in the ceftriaxone resistance of Salmonella enterica serovar Typhimurium. *Antimicrob Agents Chemother* **55**: 3829-3837.

Isaac, D.D., Pinkner, J.S., Hultgren, S.J., and Silhavy, T.J. (2005) The extracytoplasmic adaptor protein CpxP is degraded with substrate by DegP.

Proceedings of the National Academy of Sciences of the United States of America **102**: 17775-17779.

Jones, C.H., Danese, P.N., Pinkner, J.S., Silhavy, T.J., and Hultgren, S.J. (1997) The chaperone-assisted membrane release and folding pathway is sensed by two signal transduction systems. *The EMBO journal* **16**: 6394-6406.

Joris, B., Englebert, S., Chu, C.P., Kariyama, R., Daneo-Moore, L., Shockman, G.D., and Ghuysen, J.M. (1992) Modular design of the Enterococcus hirae muramidase-2 and Streptococcus faecalis autolysin. *FEMS Microbiol Lett* **70**: 257-264.

Joseleau-Petit, D., Liébart, J.-C., Ayala, J.A., and D'Ari, R. (2007) Unstable *Escherichia coli* L forms revisited: growth requires peptidoglycan synthesis. *J Bacteriol* **189**: 6512-6520.

Jubelin, G., Vianney, A., Beloin, C., Ghigo, J.-M., Lazzaroni, J.-C., Lejeune, P., and Dorel, C. (2005) CpxR/OmpR interplay regulates curli gene expression in response to osmolarity in *Escherichia coli*. *J Bacteriol* **187**: 2038-2049.

Kadokura, H., Katzen, F., and Beckwith, J. (2003) Protein disulfide bond formation in prokaryotes. *Annu Rev Biochem* **72**: 111-135.

Kleerebezem, M., Crielaard, W., and Tommassen, J. (1996) Involvement of stress protein PspA (phage shock protein A) of *Escherichia coli* in maintenance of the protonmotive force under stress conditions. *The EMBO journal* **15**: 162.

Knowles, T.J., Scott-Tucker, A., Overduin, M., and Henderson, I.R. (2009) Membrane protein architects: the role of the BAM complex in outer membrane protein assembly. *Nat Rev Microbiol* **7**: 206-214.

Laubacher, M.E., and Ades, S.E. (2008) The Rcs phosphorelay is a cell envelope stress response activated by peptidoglycan stress and contributes to intrinsic antibiotic resistance. *J Bacteriol* **190**: 2065-2074.

Leblanc, S.K.D., Oates, C.W., and Raivio, T.L. (2011) Characterization of the induction and cellular role of the BaeSR two-component envelope stress response of *Escherichia coli*. *J Bacteriol* **193**: 3367-3375.

Lee, M., Hesek, D., Llarrull, L.I., Lastochkin, E., Pi, H., Boggess, B., and Mobashery, S. (2013) Reactions of all *Escherichia coli* lytic transglycosylases with bacterial cell wall. *J Am Chem Soc* **135**: 3311-3314.

Livermore, D.M. (2001) Of Pseudomonas, porins, pumps and carbapenems. *Journal of Antimicrobial Chemotherapy* **47**: 247-250.

Llarrull, L.I., Testero, S.A., Fisher, J.F., and Mobashery, S. (2010) The future of the β-lactams. *Curr Opin Microbiol* **13**: 551-557.

Magnet, S., Bellais, S., Dubost, L., Fourgeaud, M., Mainardi, J.-L., Petit-Frère, S., *et al.* (2007) Identification of the L,D-transpeptidases responsible for attachment of the Braun lipoprotein to *Escherichia coli* peptidoglycan. *J Bacteriol* **189**: 3927-3931.

Magnet, S., Dubost, L., Marie, A., Arthur, M., and Gutmann, L. (2008) Identification of the L,D-transpeptidases for peptidoglycan cross-linking in *Escherichia coli*. *J Bacteriol* **190**: 4782-4785.

Majdalani, N., and Gottesman, S. (2005) The Rcs phosphorelay: a complex signal transduction system. *Annu Rev Microbiol* **59**: 379-405.

Majdalani, N., Heck, M., Stout, V., and Gottesman, S. (2005) Role of RcsF in signaling to the Rcs phosphorelay pathway in *Escherichia coli*. *J Bacteriol* **187**: 6770-6778.

MARTIN, H.H., MASKOS, C., and BURGER, R. (1975) d-Alanyl-d-Alanine Carboxypeptidase in the Bacterial Form and L-Form of Proteus mirabilis. *European Journal of Biochemistry* **55**: 465-473.

Masuda, H., Tan, Q., Awano, N., Wu, K.-P., and Inouye, M. (2012) YeeU enhances the bundling of cytoskeletal polymers of MreB and FtsZ, antagonizing the CbtA (YeeV) toxicity in *Escherichia coli*. *Mol Microbiol* **84**: 979-989.

Matteï, P.-J., Neves, D., and Dessen, A. (2010) Bridging cell wall biosynthesis and bacterial morphogenesis. *Curr Opin Struct Biol* **20**: 749-755.

Mäkelä, P.H., and Mayer, H. (1976) Enterobacterial common antigen. *Bacteriological reviews* **40**: 591.

McBroom, A.J., and Kuehn, M.J. (2007) Release of outer membrane vesicles by Gram-negative bacteria is a novel envelope stress response. *Mol Microbiol* **63**: 545-558.

McEwen, J., and Silverman, P. (1980) Chromosomal mutations of *Escherichia coli* that alter expression of conjugative plasmid functions. *Proceedings of the National Academy of Sciences* **77**: 513-517.

Menon, A.S., and Goldberg, A.L. (1987) Binding of nucleotides to the ATP-dependent protease from *Escherichia coli*. *Journal of Biological Chemistry* **262**: 14921-14928.

Merchant, S.M., and Vithlani, N.P. (1986) Current antibiotic usage II: Aminoglycosides, tetracyclines, erythromycin, vancomycin and sulphonamides. *Indian J Pediatr* **53**: 199-213.

Merdanovic, M., Clausen, T., Kaiser, M., Huber, R., and Ehrmann, M. (2011) Protein quality control in the bacterial periplasm. *Annu Rev Microbiol* **65**: 149-168.

Microbial Glycobiology: Structures, Relevance and Applications (2009). Elsevier.

Miot, M., and Betton, J.-M. (2004) Protein quality control in the bacterial periplasm. *Microbial cell factories* **3**: 4.

Missiakas, D., and Raina, S. (1997) Protein misfolding in the cell envelope of*Escherichia coli*: new signaling pathways. *Trends in biochemical sciences* **22**: 59-63.

Missiakas, D., Betton, J.-M., and Raina, S. (1996) New components of protein folding in extracytoplasmic compartments of *Escherichia coli* SurA, FkpA and Skp/OmpH. *Molecular microbiology* **21**: 871-884.

Mizuno, T. (1997) Compilation of all genes encoding two-component phosphotransfer signal transducers in the genome of *Escherichia coli*. *DNA Res* **4**: 161-168.

Mizuno, T. (1998) His-Asp phosphotransfer signal transduction. *Journal of biochemistry* **123**: 555-563.

Nagasawa, S., Ishige, K., and Mizuno, T. (1993) Novel members of the two-component signal transduction genes in *Escherichia coli*. *Journal of biochemistry* **114**: 350-357.

Nikaido, H., and Vaara, M. (1985) Molecular basis of bacterial outer membrane permeability. *Microbiological reviews* **49**: 1.

Nishino, K., Yamasaki, S., Hayashi-Nishino, M., and Yamaguchi, A. (2010) Effect of NlpE overproduction on multidrug resistance in *Escherichia coli*. *Antimicrob Agents Chemother* **54**: 2239-2243.

Nixon, B.T., Ronson, C.W., and Ausubel, F.M. (1986) Two-component regulatory systems responsive to environmental stimuli share strongly conserved domains with the nitrogen assimilation regulatory genes *ntrB* and *ntrC*. *Proceedings of the National Academy of Sciences* **83**: 7850-7854.

Nordmann, P., Dortet, L., and Poirel, L. (2012) Carbapenem resistance in *Enterobacteriaceae*: here is the storm!. *Trends Mol Med* **18**: 263-272.

Ogura, T., Bouloc, P., Niki, H., D'ari, R., Hiraga, S., and Jaffe, A. (1989) Penicillin-binding protein 2 is essential in wild-type *Escherichia coli* but not in *lov* or *cya* mutants. *Journal of bacteriology* **171**: 3025-3030.

Otto, K., and Silhavy, T.J. (2002) Surface sensing and adhesion of *Escherichia coli* controlled by the Cpx-signaling pathway. *Proceedings of the National Academy of Sciences* **99**: 2287-2292.

Pedersen, L.B., Murray, T., Popham, D.L., and Setlow, P. (1998) Characterization of *dacC*, which encodes a new low-molecular-weight penicillin-binding protein in *Bacillus subtilis*. *Journal of bacteriology* **180**: 4967-4973.

Pedersen, S.F., Poulsen, K.A., and Lambert, I.H. (2006) Roles of phospholipase A2 isoforms in swelling- and melittin-induced arachidonic acid release and taurine efflux in NIH3T3 fibroblasts. *Am J Physiol Cell Physiol* **291**: C1286-C1296.

Peschel, A., and Sahl, H.-G. (2006) The co-evolution of host cationic antimicrobial peptides and microbial resistance. *Nat Rev Microbiol* **4**: 529-536.

Pogliano, J., Lynch, A.S., Belin, D., Lin, E.C., and Beckwith, J. (1997) Regulation of *Escherichia coli* cell envelope proteins involved in protein folding and degradation by the Cpx two-component system. *Genes & Development* **11**: 1169-1182.

Prats, R., and de Pedro, M.A. (1989) Normal growth and division of *Escherichia coli* with a reduced amount of murein. *J Bacteriol* **171**: 3740-3745.

Price, N.L., and Raivio, T.L. (2009) Characterization of the Cpx regulon in *Escherichia coli* strain MC4100. *J Bacteriol* **191**: 1798-1815.

Priyadarshini, R., de Pedro, M.A., and Young, K.D. (2007) Role of peptidoglycan amidases in the development and morphology of the division septum in *Escherichia coli*. *J Bacteriol* **189**: 5334-5347.

Projan, S.J. (2002) New (and not so new) antibacterial targets--from where and when will the novel drugs come? *Current opinion in pharmacology* **2**: 513-522.

Quan, S., Koldewey, P., Tapley, T., Kirsch, N., Ruane, K.M., Pfizenmaier, J., *et al.* (2011) Genetic selection designed to stabilize proteins uncovers a chaperone called Spy. *Nat Struct Mol Biol* **18**: 262-269.

Raetz, C.R.H., and Whitfield, C. (2002) Lipopolysaccharide endotoxins. *Annu Rev Biochem* **71**: 635-700.

Raffa, R.G., and Raivio, T.L. (2002) A third envelope stress signal transduction pathway in *Escherichia coli*. *Mol Microbiol* **45**: 1599-1611.

Raivio, T.L., and Silhavy, T.J. (1997) Transduction of envelope stress in *Escherichia coli* by the Cpx two-component system. *Journal of bacteriology* **179**: 7724-7733.

Raivio, T.L., Laird, M.W., Joly, J.C., and Silhavy, T.J. (2000) Tethering of CpxP to the inner membrane prevents spheroplast induction of the Cpx envelope stress response. *Mol Microbiol* **37**: 1186-1197.

Raivio, T.L., Leblanc, S.K.D., and Price, N.L. (2013) The *Escherichia coli* Cpx envelope stress response regulates genes of diverse function that impact antibiotic resistance and membrane integrity. *J Bacteriol* **195**: 2755-2767.

Rawlings, N.D., and Salvesen, G. (2012) Handbook of Proteolytic Enzymes, Volume 1. Access Online via Elsevier.

Ried, G., Hindennach, I., and Henning, U. (1990) Role of lipopolysaccharide in assembly of *Escherichia coli* outer membrane proteins OmpA, OmpC, and OmpF. *Journal of bacteriology* **172**: 6048-6053.

Rowley, G., Spector, M., Kormanec, J., and Roberts, M. (2006) Pushing the envelope: extracytoplasmic stress responses in bacterial pathogens. *Nat Rev Microbiol* **4**: 383-394.

Ruiz, N., and Silhavy, T.J. (2005) Sensing external stress: watchdogs of the *Escherichia coli* cell envelope. *Curr Opin Microbiol* **8**: 122-126.

Sanders, A.N., and Pavelka, M.S. (2013) Phenotypic Analysis of E. coli Mutants Lacking L,D-transpeptidases. *Microbiology* .

Santos, J.M., Lobo, M., Matos, A., De Pedro, M.A., and Arraiano, C.M. (2002) The gene *bolA* regulates *dacA* (PBP5), *dacC* (PBP6) and *ampC* (AmpC),

promoting normal morphology in *Escherichia coli*. *Molecular microbiology* **45**: 1729-1740.

Sauvage, E., Kerff, F., Terrak, M., Ayala, J.A., and Charlier, P. (2008) The penicillin-binding proteins: structure and role in peptidoglycan biosynthesis. *FEMS microbiology reviews* **32**: 234-258.

Scheffers, D.-J., and Pinho, M.G. (2005) Bacterial cell wall synthesis: new insights from localization studies. *Microbiol Mol Biol Rev* **69**: 585-607.

Schnaitman, C.A., and Klena, J.D. (1993) Genetics of lipopolysaccharide biosynthesis in enteric bacteria. *Microbiological reviews* **57**: 655-682.

Schneider, T., and Sahl, H.-G. (2010) An oldie but a goodie - cell wall biosynthesis as antibiotic target pathway. *Int J Med Microbiol* **300**: 161-169.

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

Sohn, J., Grant, R.A., and Sauer, R.T. (2009) OMP peptides activate the DegS stress-sensor protease by a relief of inhibition mechanism. *Structure* **17**: 1411-1421.

Srinivasan, V.B., Vaidyanathan, V., Mondal, A., and Rajamohan, G. (2012) Role of the two component signal transduction system CpxAR in conferring cefepime and chloramphenicol resistance in *Klebsiella pneumoniae* NTUH-K2044. *PLoS One* **7**: e33777.

Stock, A.M., Robinson, V.L., and Goudreau, P.N. (2000) Two-component signal transduction. *Annual review of biochemistry* **69**: 183-215.

Stone, K.J., and Strominger, J.L. (1971) Mechanism of action of bacitracin: complexation with metal ion and C55-isoprenyl pyrophosphate. *Proceedings of the National Academy of Sciences* **68**: 3223-3227.

Stout, V., and Gottesman, S. (1990) RcsB and RcsC: a two-component regulator of capsule synthesis in *Escherichia coli*. *Journal of bacteriology* **172**: 659-669.

Tatar, L.D., Marolda, C.L., Polischuk, A.N., van Leeuwen, D., and Valvano, M.A. (2007) An *Escherichia coli* undecaprenyl-pyrophosphate phosphatase implicated in undecaprenyl phosphate recycling. *Microbiology* **153**: 2518-2529.

Terrak, M., Ghosh, T.K., van Heijenoort, J., Van Beeumen, J., Lampilas, M., Aszodi, J., *et al.* (1999) The catalytic, glycosyl transferase and acyl transferase modules of the cell wall peptidoglycan-polymerizing penicillin-binding protein 1b of *Escherichia coli*. *Mol Microbiol* **34**: 350-364.

Tomasz, A. (1971) The bacterial cell surface. *Nature* **234**: 389 - 392.

Typas, A., Banzhaf, M., Gross, C.A., and Vollmer, W. (2012) From the regulation of peptidoglycan synthesis to bacterial growth and morphology. *Nat Rev Microbiol* **10**: 123-136.

Tzeng, Y.-L., Ambrose, K.D., Zughaier, S., Zhou, X., Miller, Y.K., Shafer, W.M., and Stephens, D.S. (2005) Cationic antimicrobial peptide resistance in *Neisseria meningitidis*. *J Bacteriol* **187**: 5387-5396.

Unden, G., and Bongaerts, J. (1997) Alternative respiratory pathways of *Escherichia coli* energetics and transcriptional regulation in response to electron acceptors. *Biochimica et Biophysica Acta (BBA)-Bioenergetics* **1320**: 217-234.

van Asselt, E.J., Kalk, K.H., and Dijkstra, B.W. (2000) Crystallographic studies of the interactions of *Escherichia coli* lytic transglycosylase Slt35 with peptidoglycan. *Biochemistry* **39**: 1924-1934.

van Heijenoort, J. (2010) Lipid Intermediates in Bacterial Peptidoglycan Biosynthesis, In Handbook of Hydrocarbon and Lipid Microbiology. Berlin, Heidelberg: Springer Berlin Heidelberg, pp. 435-444.

van Heijenoort, J. (2011) Peptidoglycan hydrolases of *Escherichia coli*. *Microbiol Mol Biol Rev* **75**: 636-663.

Varma, A., and Young, K.D. (2004) FtsZ collaborates with penicillin binding proteins to generate bacterial cell shape in *Escherichia coli*. *J Bacteriol* **186**: 6768-6774.

Varma, A., and Young, K.D. (2009) In *Escherichia coli*, MreB and FtsZ direct the synthesis of lateral cell wall via independent pathways that require PBP 2. *J Bacteriol* **191**: 3526-3533.

Vollmer, W., and Bertsche, U. (2008) Murein (peptidoglycan) structure, architecture and biosynthesis in *Escherichia coli*. *Biochim Biophys Acta* **1778**: 1714-1734.

Vollmer, W., Blanot, D., and de Pedro, M.A. (2008) Peptidoglycan structure and architecture. *FEMS Microbiol Rev* **32**: 149-167.

Weatherspoon-Griffin, N., Zhao, G., Kong, W., Kong, Y., Morigen, Andrews-Polymenis, H., *et al.* (2011) The CpxR/CpxA two-component system up-regulates two Tat-dependent peptidoglycan amidases to confer bacterial resistance to antimicrobial peptide. *J Biol Chem* **286**: 5529-5539.

Weber, A., Kögl, S.A., and Jung, K. (2006) Time-dependent proteome alterations under osmotic stress during aerobic and anaerobic growth in *Escherichia coli*. *J Bacteriol* **188**: 7165-7175.

Weber, H., Polen, T., Heuveling, J., Wendisch, V.F., and Hengge, R. (2005) Genome-wide analysis of the general stress response network in *Escherichia coli*: sigmaS-dependent genes, promoters, and sigma factor selectivity. *J Bacteriol* **187**: 1591-1603.

Weber, R.F., and Silverman, P.M. (1988) The Cpx proteins of *Escherichia coli*K12: Structure of the CpxA polypeptide as an inner membrane component. *Journal of molecular biology* **203**: 467-478.

Weiner, J.H., and Rothery, R.A. (2007) Bacterial Cytoplasmic Membrane, In Encyclopedia of Life Sciences. Chichester, UK: John Wiley & Sons, Ltd, pp. 1-10.

De Wulf, P., and Lin, E.C.C. (2000) Cpx Two-Component Signal Transduction in*Escherichia coli*: Excessive CpxR-P Levels Underlie CpxA* Phenotypes. *Journal of bacteriology* **182**: 1423-1426.

Yeats, C., and Bateman, A. (2003) The BON domain: a putative membrane-binding domain. *Trends Biochem Sci* **28**: 352-355.

Young, K.D. (2006) The selective value of bacterial shape. *Microbiol Mol Biol Rev* **70**: 660-703.

Zapun, A., Macheboeuf, P., and Vernet, T. (2009) Penicillin-Binding Proteins and β-Lactam Resistance, In Antimicrobial Drug Resistance. Totowa, NJ: Humana Press, pp. 145-170.

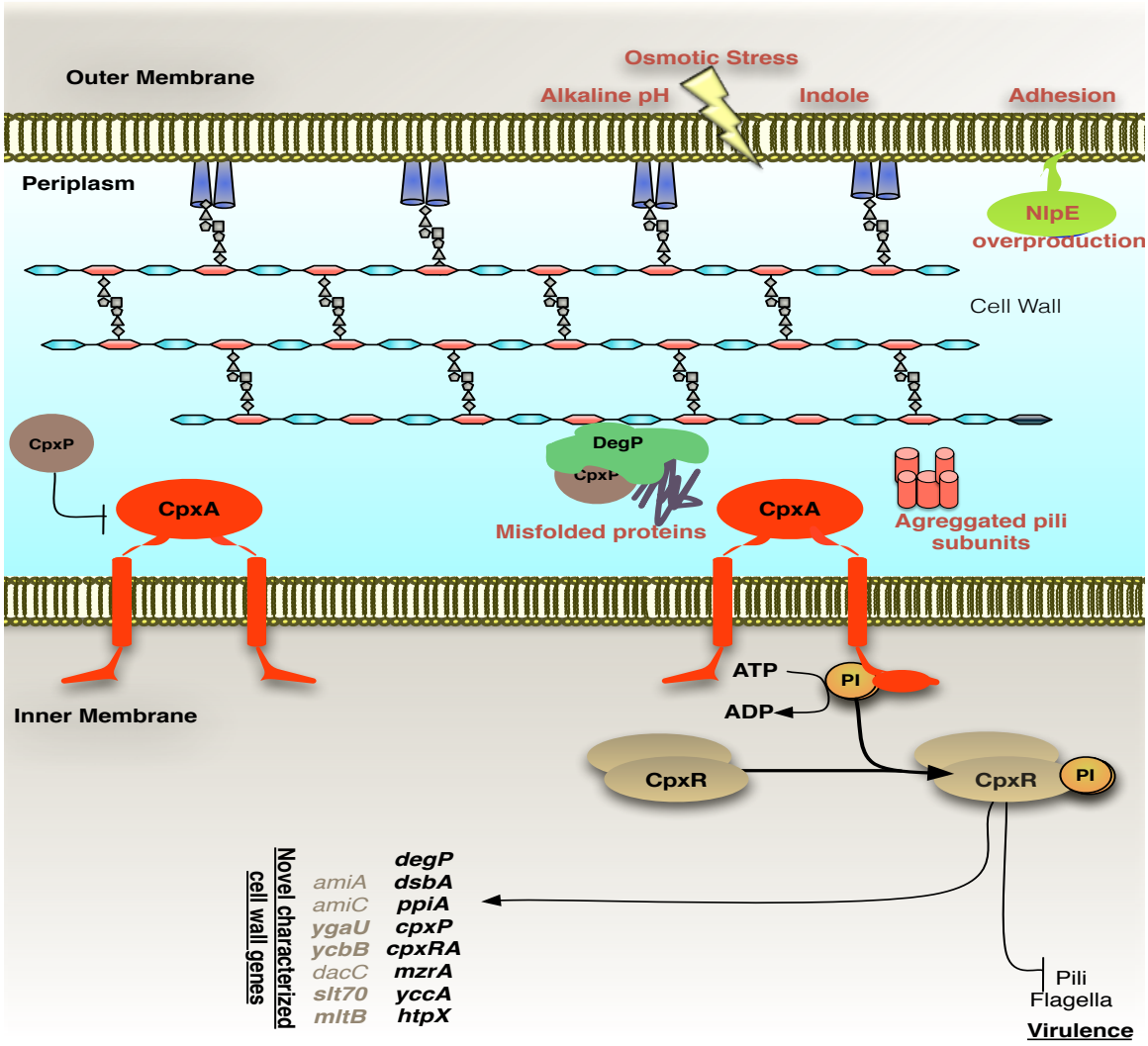


Figure 1-3 The Cpx pathway

The Cpx pathway is an envelope stress response that senses changes in the pH, indole, and in misfolded and mislocalized proteins in the periplasm.

Gene	Function	First Reported	Organism	Methodology
dacC	D,D-carboxypeptidase	(Raivio *et al.*, 2013; Weatherspoon-Griffin *et al.*, 2011)	*E. coli*	Microarray
ycbB	L,D-transpeptidase	(Raivio *et al.*, 2013)	*E. coli*	Microarray
slt70	Lytic transglycosylase (soluble)	(Raivio *et al.*, 2013) (Gangaiah *et al.*, 2013)	-*E. coli* -*Haemophilus ducreyi*	-Microarray -RNA-seq
ygaU	Unknown function	(Raivio *et al.*, 2013)	*E. coli*	Microarray

amiA	Amidase	(Weatherspoon-Griffin *et al.*, 2011)	Salmonella	Screening
amiC	Amidase	(Weatherspoon-Griffin *et al.*, 2011)	Salmonella	Screening
dacA	D,D-carboxy peptidase	(Gangaiah *et al.*, 2013)	-*Haemophilus ducreyi* -*Vibrio cholareae*	-Microarray -Unpublished data
mltB- **Slt35**	Lytic transglycosylase	-----	---	Similar activity to Slt70

Table 1-1: Cell wall genes and their correlation with the Cpx pathway

Microarray analysis and RNA-seq suggests that the Cpx pathway up-regulates various genes involved in peptidoglycan synthesis and recycling.

CHAPTER 2

Materials and Methods

2.1 Growth conditions

Overnight cultures were grown in LB broth or minimal media M63/2 (13.6g KH_2PO (100mM), 2g $(NH_4)_2SO$ (15mM)$_4$ 0.5g $FeSO_4 \cdot 7H_2O$ (1.7 μM)) (Tao *et al.*, 1998) supplemented with 0.2% glucose at 30ºC with shaking at 250 rpm unless otherwise noted. For growth on solid media, strains were grown on LB supplemented with 1.5% agar at 30ºC or 37ºC.

To prevent plasmid curing and to maintain some strains, different media was supplemented with the following antibiotics: amikacin (3μg/ml) ampicillin (100μg/ml), chloramphenicol (25μg/ml), knamycin (50μg/ml), tetracycline (10μg/ml). All antibiotics were purchased from Sigma-Aldrich.

2.2 Strains used in this study

Strains used in this study are listed on Table 2-1 and 2-2.

2.3 Plasmids used in this study

The plasmids used in this study are listed in Table 2-3.

2.4 Primers used in this study

Different sets of primers were designed for the construction of *lux* reporters, synthesis of DNA promoter fragments for EMSAs, and for verification of the insertion or excision of the kn^R cassette in the mutants obtained using the KEIO

library and the FLP2 recombinase (Baba *et al.*, 2006; Hoang *et al.*, 1998) (Sigma-Aldrich). Primers used in this study are listed in Table 2-4.

2.5 Chemical transformations

Transformations were performed as described in (Silhavy *et al.*, 1984). Briefly, a single colony of the strain to be transformed was inoculated in 5ml of LB with the appropriate antibiotic at 30°C or 37ºC depending on the strain used. After 10 hours, the bacterial culture was diluted (1:40) into 5 ml of LB with the appropriate antibiotic and grown for 2 hours to an OD_{600} of approximately 0.2. The bacterial culture was then centrifuged at 14, 000 rpm (Eppendorf 5810R) for 10 minutes at room temperature and the supernatant was discarded, The pellet was resuspended in Magic Formula (10ml 1M $CaCl_2$, 80ml dH_20, 10ml 1M MOPS pH 6.5) and the suspension was incubated on ice 30 minutes. The culture was centrifuged again and resuspended by gentle pipetting in 0.2ml of Magic Formula. 3µl of DNA were added and the cell-plasmid mixture was incubated on ice for 10 minutes, heat shocked at 42ºC for 30 seconds if the cells were not temperature sensitive, and 1ml of LB was added to allow phenotypic expression. The transformants were incubated at 30° or 37ºC and then plated onto LB with the appropriate antibiotic (Silhavy *et al.*, 1984).

2.6 Transductions

Single gene knockouts (Table 2-2) were constructed by P1 transduction of alleles from the KEIO library as previously described (Baba *et al.*, 2006; Silhavy *et al.*, 1984). For the construction of double mutants and triple knockout mutants, the knamycin cassette was first excised by chemical transformation with the pFLP2 plasmid (Section 2.5). Once the knamycin resistance cassette was excised, the pFLP2 plasmid was cured by growing knamycin sensitive strains for 2-3 hours in 5ml of LB at 30ºC and plating 1ml on LB plates supplemented with 5% sucrose without NaCl. Plates were grown at room temperature for two days, and the resulting colonies were screened for ampicillin sensitivity as previously described (Hoang *et al.*, 1998). Ampicillin sensitive colonies were screened by PCR (See section 2.8) for the verification of unmarked deletions using the primers on Table 2-4 before sequentially transducing another gene.

2.7 Techniques in molecular biology

Agarose gel electrophoresis and cloning were performed following the procedures described in (Sambrook *et al.*, 1989). The GenElute Plasmid Miniprep Kit from Sigma-Aldrich was used for the isolation of plasmid DNA from *E. coli* following the recommendations of the manufacturer. PCR products used for cloning or sequencing were purified using the QIAGene PCR purification kit, according to the manufacturer's instructions. Restriction enzymes were purchased from New England Biolabs and Invitrogen.

2.8 PCR

For the preparation of PCR reactions 26 µl of milliQ water, 10µl of betaine (5M, Sigma Aldrich), 5µl of 10X PCR buffer without $MgCl_2$ (Invitrogen), 3 µl of 50 mM $MgCl_2$, 2µl of 10mM dNTPs (Invitrogen), 1 µl of a 100 µM stock of each primer (Sigma Aldrich), DNA (1 colony picked from agar or 0.5µl of plasmid DNA), and 1U of recombinant *Taq* (Invitrogen) were combined for a final volume of 50 µl. The PCR reactions were kept at 4ºC during preparation, and then placed in a Mastercycler thermal-cycler (Eppendorf) and subjected to an initial denaturation cycle of 5 minutes, followed by 30 cycles of denaturation (95ºC for 1 minute), annealing (50-60ºC for 45 seconds), and extension (72ºC for 1 minute/kb). PCR products were visualized by electrophoresis on 1.0% agarose gels, followed by staining with ethidium bromide.

2.9 Constrution of luminescent reporter plasmids.

To create *lux* reporters for the promoters of *ygau, ycbB, slt, mltB, dacC, amiA,* and *amiC,* we constructred primers that annealed +500pb and -100bp from the translational start site to ensure that the CpxR binding site was present in the amplicon (Table 2-4). In addition, we integrated the XhoI recognition site to the forward primer and the EcoRI recognition site to the reverse primer to insert and clone the respective promoter into the pJW15 lux reporter. PCR products were purified using the QiaQuick PCR purification kit (Qiagene), and the pJW15 plasmid was purified using a the Miniprep GenElute Plasmid Kit (Sigma–Aldrich). The pJW15 vector and the amplicon for each promoter were cleaved

with XhoI and EcoRI (New England Biolabs) for 5 hours at 37ºC, purified using the GeneJet Gel Extraction Kit (Fermentas) before overnight ligation at 16ºC with ligase T4 (invitrogen). Ligation products were transformed into TOP10 competent cells (Invitrogen). Verification of each *lux* reporter was performed by colony PCR and subsequent sequencing (View section 2.10).

2.10 DNA sequencing

PCR products were purified using the QiaQuick PCR purification Kit according to the manufacturer's instructions (QiaGen). Sequencing reactions were set up using 100 ng of PCR product and 10 pmol of a primer according to the manufacturer's instructions terminator protocol with BigDye. The samples were analysed on an ABI Prism 310 Automated Sequencer (Applied Biosystems) using the premix provided by the University of Alberta Molecular Biology Service Unit (MBSU).

2.11 Electrophoretic Mobility Shift Assays (EMSAs)

Randi Guest standarized the EMSA protocol and purified the MBP-CpxR fusion protein.

2.11.1 Protein purification

The MBP-CpxR fusion protein was purifed from JM109(pMCR) as previously described with some variations (Raivio and Silhavy, 1997). A single colony of

JM109 (pMCR) strain was grown overnight in 10 ml of LB with with 150 μg of ampicillin, The next morning, all of the overnight culture was used to inoculate 1L of fresh LB with 0.2% glucose. Cells were grown until they reached an OD_{600}=0.3, and MBP-CpxR over-production was induced with 0.300 mM IPTG for 4 hours. Next, cells were pelleted by centrifugation at x4000 prm for 20 minutes. Bacterial culture was disrupted by passage through a French press at 20,000 psi. The crude extract was then treated overnight with 10 ml of amylose resin (New-England Biolabs) for batch purification. This preparation was poured into a column, and treated as previously described to purify the MBP-CpxR fusion protein (Raivio and Silhavy, 1997).

2.11.2 Mobility shift assay

Gene promoters were amplified with the primer son Table 2-4 and purified with the QiaQuick PCR purification kit (QiaGen). Binding buffer (10 mM $MgSO_4$ 20 mM potassium glutamate, 1 mM DTT, 50 μg/ml BSA, 1 mM EDTA, 50 mM KCl, 10 mM Tris pH 7.4) with or without acetyl phosphate (20 mM) was prepared, and stored at 4ºC. 15 μl of binding or non-binding buffer were incubated in the presence of 75 pmol CpxR at 37ºC for 30 minutes. Next, 1.5pmol of purified PCR product of the promoter of interest were added to the mixture without adjusting the volumen. Next, the mixture was incubated at 37ºC for 30 minutes. 6X DNA loading dye (0.25% xylene cyanol FF, 30% glycerol in water, 0.25% bromophenol blue) was added and the mixture was loaded on a 5% non-denaturing TBE polyacrylamide gel (Bio-Rad), electrophoresed in 1X TBE

running buffer (89 mM Tris, 89 mM boric acid, 2 mM EDTA) and stained with ethidium bromide for visualization. Images were obtained using a Molecular Imager Gel Doc (Bio-Rad)

2.12 Assays

2.12.1 *lux* assay

Single colonies of bacterial strains were inoculated in 2ml of LB with the appropriate antibiotics and grown overnight at 30ºC at 225 rpm. The next morning, each strain was subcultured (1:40) in fresh LB with the appropriate antibiotic. After 2 hours, 200 µl of each strain were transferred to a black bottom 96-well plate (Corning® 96 Well Black Flat Bottom Polystyrene). The optical density at 600 nm (OD_{600}) and the luminescence (CPS) were measured every 2 hours until the culture reached stationary phase, using a Wallac Victor 2 multilabel plate reader (Perkin Elmer Life Sciences). Each strain was assayed in triplicate or quintuplicate.

2.12.2 ß-Galactosidase assay

Single colonies of bacterial strains were inoculated in quintuplicate in 2ml of LB with the appropriate antibiotic and grown overnight at 30ºC with shaking. The next morning, each strain was sub-cultured (1:40) in 5ml of fresh LB and grown until late log phase (A_{600}=0.6). Cultures were centrifuged at 4000 rpm for 10 minutes and re-suspended in the same volume of Z-buffer. ß-Galactosidase activity was measured using a previously described protocol (Slauch *et al.*, 1991)

and a Wallac Victor 2 multilabel plate reader (Perkin Elmer Life Sciences) in a 96-well plate (Corning® 96 Well Clear Flat Bottom Polystyrene). The maximum slope (Km/Vmax) was obtained using WorkOut 2.0. Miller Units were calculated by multiplying the slope by a factor of 600000 and dividing the it by the OD_{600} GraphPad Prism 6.0

2.13 Minimum Inhibitory Concentrations (MICs) determination

Minimum inhibitory concentrations of antibiotics required for killing were determined using 96-well plates with clear bottoms. Overnight cultures were grown in 5ml Mueller-Hinton Browth at 30ºC with shaking at 250rpm and diluted in 25ml of Mueller-Hinton until the A_{600} was 0.08-0.130 (Wiegand *et al.*, 2008). 50 µl of cells were inoculated into 50ul of serial dilutions of the antibiotic to be tested in a 96 well plate. Starting concentrations for the antibiotics used were as follows: Aztreonam 256 µg/ml, Ampicillin 256 µg/ml, Piperacillin 256 µg/ml, Cloxacillin 256 µg/ml, Carbanicillin 256 µg/ml, Tobramycin 256 µg/ml. Antibiotics were purchased from Sigma-Aldrich and Santa Cruz Biotechnology. Plates were then incubated at 30ºC with shaking at 250 rpm for 24 hours. The A_{600} was read using a Perkin Elmer Victor 1420 plate reader. The MIC was defined as the lowest concentration of an antibiotic that inhibited the visible growth of *E. coli.*

2.14 HPLC analysis

The preparation, separation, and quantification of muropeptides was performed by Dr. Juan Alfonso Ayala from the Ayala Laboratory at the Centro de Biología Molecular "Severo Ochoa", Agencia Estatal Consejo Superior de Investigaciones Científicas (CSIC-UAM), Campus de Cantoblanco, 28049 Madrid, Spain.

-Culture preparation

Sample preparation for HPLC analysis was done as previously described with some changes (Tayler *et al.*, 2010). Cells were grown in 100ml of LB with the appropriate antibiotics for several hours until the OD_{600}=1.0. The cells were then incubated on ice until the temperature of the culture was 4ºC. Samples were centrifuged at 4.000 rpm for 10 minutes at 4ºC (Eppendorf 5810R), resuspended in 3ml of dH_20. The cell suspension was added to 3ml of boiling SDS drop by drop, and mixed until a homogeneous mixture was seen. The cells were boiled for 5 minutes, then transferred to 10ml glass tubes and heated at 90ºC for 72 hours in a Baxter heat block. Sacculi were concentrated by centrifugation at 65,000 rpm for 15 min at 30°C in an ultracentrifuge (Beckman-Optima TLX, TLA110 rotor). The pellet was washed with water until no SDS was detected by the method of Hayashi (Hayashi, 1975). The last pellet of the washing procedure was suspended in 1 ml of 10 mM Tris-HCl (pH 7.2) and digested first with 100 mg/ml a-amylase (EC 3.2.1.1; Sigma-Aldrich, Saint Louis, MO) for 1 h at 37°C and then with 100 mg/ml preactivated pronase E (EC 3.4.24.4; Merck, Darmstadt, Germany) at 60°C for 90 min. The enzymes were inactivated by boiling for 20 min in 1% (final

concentration) SDS. The cell walls were collected by centrifugation as described above and washed three times with water. The peptidoglycan was stored in water at 4°C.

-Preparation and separation of muropeptides

Peptidoglycan was digested in 50 mM phosphate buffer (pH 4.9) with Cellosyl (Hoechst AG, Frankfurt, Germany) 100 mg/ml final concentration at 37°C overnight. The enzyme reaction was stopped by boiling the sample for 2 min in a water bath and centrifuged (Eppendorf centrifuge at maximum speed for10 min) to remove insoluble debris. The supernatant was mixed with 1/3 volume of 0.5 M sodium borate buffer (pH 9.0) and reduced with excess sodium borohydride (NaBH4) for 30 min at room temperature. The pH was tested with pH indicator strips (Acilit, Merck) and adjusted to 3 with orthophosphoric acid. All samples were filtered (Millex-GV filters; 0.22-mm pore size, 2.5-mm diameter; Millipore, Cork, Ireland) and stored at -20°C.

Separation of the reduced muropeptides was performed essentially by the method of Glauner et al. ((Glauner, 1988; Glauner *et al.*, 1988)). Muropeptides were analyzed using a binary-pump Waters HPLC system (Waters Corporation, Milford, USA) fitted with a reverse phase RP18 Aeris peptide column (250 x 4.6 mm; 3.6 μm particle 500 size) (Phenomenex, USA) and a dual wavelength absorbance detector (Waters UV-1570 spectrophotometer). The eluted muropeptides were monitored by measuring absorbance at 204 nm. When required, the individual peaks were collected, vacuum dried, and stored at _20°C.

-Quantification of muropeptides

Individual muropeptides were quantified from their integrated areas (Waters Breeze) using samples of known concentration as standards. Concentration of the standard muropeptides was determined as described by Work (Work, 1957). Average PG chain length was calculated by dividing the molar amount of anhydro-muropeptide (=chain termini) by total molar amount of muropeptides in muramidase-digested PG, and degree of crosslinking was calculated by calculating the molar ratio of dimers and trimers to total muropeptide, as described by Glauner et al. (Glauner, 1988; Glauner *et al.*, 1988).

2.15 Microscopy

All the microscopy work was performed at the Advanced Microscopy Facility at the Department of Biological Sciences-University of Alberta with the assistance of Arlene Oatway.

2.15.1 Light microscopy

Bacterial samples were grown overnight in 5ml of LB supplemented with the indicated antibiotics overnight at 30ºC with shaking at 250rpm. One drop of culture was added to a glass slide, air dried, heat-fixed, and stained with a drop of Crystal violet (3g Crystal violet, 50ml isopropanol, 50ml ethanol, 900ml of dH_20

for a final volume of 1 liter) for one minute, and rinsed with enough dH_20 until the water running through was completely clear. Cells were visualized at 1000X magnification using a Leica DMRXA Microscope and Nikon DXM1200 digital camera.

2.15.2 Transmission Electron Microscope (TEM)

- Negative stain

Bacterial samples were grown overnight at 30ºC with shaking at 250rpm in 5ml of LB supplemented with the appropriate antibiotics. Cultures were centrifuged at 4000 rpm (Eppendorf 5810R) for 10 minutes and resuspended in 1ml of PBS pH 7.4. One drop of culture was applied to the metallic side of a copper grid copper grid (300 mesh) coated with Formvar/Carbon. Samples sat on the grid for two minutes before staining with one drop of phosphotungstic acid (2% in water; pH 7). Grids were allowed to air dry for five minutes, and visualized with a FEI Morgagni 268 microscope operating at 80 kV.

- Sectioning

Bacterial samples were grown overnight in 5ml of LB supplemented with the appropriate antibiotic. Cultures were centrifuged at 4000 rpm (Eppendorf 5810R) for 10 minutes and resuspended in 1ml of fixative (2.5% glutaraldehyde, 2% paraformaldehyde in 100 mM phosphate buffer at pH 7.2) overnight. Samples were centrifuged for 5 minutes at 14,000 rpm (Eppendorf 5415D), the fixative was removed and samples resuspended in 0.1M PBS, followed by 4 1ml- washes

in PBS over 30 minutes. Buffer was drained off and cells were resuspended and post-fixed overnight in 1ml of 1% osmium tetroxide (OsO_4 in 0.12M phosphate buffer pH 7.2). The next morning, samples were washed three times in 1ml of 100 mM phosphate buffer at pH 7.2. Samples were dehydrated through a graded ethanol concentration (50, 70, 90, 100% ethanol) For this, each sample was resuspended in 1ml of the respective ethanol concentration for 15 minutes (e.g 50%), centrifuged at 12.000 rpm for 4 minutes, and resuspended in the following ethanol concentration for another 15 minutes (e.g 70%) before centrifuging again. Excess 100% ethanol was removed and replaced with 1ml of ethanol::Spurr mixture overnight. The next morning, samples were centrifruged 10 minutes, resuspended in 100% Spurr and transferred to BEEM capsules. Samples were incubated at 70ºC overnight, sectioned (UltracutE Reichert-Jung ultramicrotome), and stained with uranyl acetate and lead citrate stains for TEM observation.

Strain	**Phenotype**	**Antibiotic**	**Reference**
MC4100	MC4100 F^- *araD139*Δ*(argF-lac)* U169 *rpsL*150 (Str^R) *relA*1 *flbB5301 deoC1 ptsF25 rbsR*	N/A	(Casadaban, 1976)
TR10	MC4100 *cpxA24* –strong allele	Amk^R	(Raivio and Silhavy, 1997; Raivio *et al.*, 1999)
TR11	MC4100 *cpxA102* –strong allele	Amk^R	(Price and Raivio, 2009)

TR15	MC4100 *cpxA711* -weak allele	Amk^R	This study
TR50	MC4100 λRS88*[cpxP-lacZ]*	N/A	(Danese and Silhavy, 1998), (Raivio and Silhavy, 1997)
TR51	MC4100 *cpxR*::spc	Spc^R	(Danese *et al.*, 1995; Raivio *et al.*, 1999)
pJW15	MC4100 with pJW15	Kn^R	(Macritchie *et al.*, 2008)
pJW1	MC4100 with pJW1	Kn^R	(Price and Raivio, 2009)

Table 2-1: Strains used in this study.

Strain	Relevant Genotype	Antibiotic	Reference
MMB1	TR15 with pJW25	Kn^R Spc^R	This study
MMB2	MC4100Δ*cpxR*	N/A	This study (Baba *et al.*, 2006; Hoang *et al.*, 1998)
MMB3	MC4100 with pCA-NlpE	Kn^R Cam^R	This study
MMB4	MC4100 with pCA-24N	Kn^R Cam^R	This study
MMB5	MC4100 with pKDS322	KanKn^R Amp^R	This study
MMB6	MC4100 with ptrc99A	KanKn^R Amp^R	This study
MMB7	TOP10 with *ygaU*-pJW15	KanKn^R	This study
MMB8	MC4100 with *ygaU*-pJW15	KanKn^R	This study

MMB9	MC4100 with pCA-NlpE and *ygaU*-pJW15	KanKn^R Cam^R	This study
MMB10	MC4100 with *ygaU*-pJW15 and pCA-24N	KanKn^R Cam^R	This study
MMB11	TR10 with *ygaU*-pJW15	KanKn^R Amk^R	This study
MMB12	TR51 with *ygaU*-pJW15	KanKn^R Spc^R	This study
MMB13	MC4100 with *ygaU*-pJW15 and ptrc99A	Kn^R Amp^R	This study
MMB14	MC4100 with *ygaU*-pJW15 and pKDS302	Kn^R Amp^R	This study
MMB15	TOP10 with *mltB*-pJW15	KanKn^R	This study
MMB16	MC4100 with *mltB*-pJW15	KanKn^R	This study
MMB17	MC4100 *mltB*-pJW15 and pCA-NlpE	KanKn^R Cam^R	This study
MMB18	MC4100 with *mltB*-pJW15 and pCA-24N	KanKn^R Cam^R	This study
MMB19	TR10 with *mltB*-pJW15	KanKn^R Amk^R	This study
MMB20	TR51 with *mltB*-pJW15	KanKn^R Spc^R	This study
MMB21	MC4100 with *mltB*-pJW15 and	KanKn^R	This study

	ptrc99A	Amp^R	
MMB22	MC4100 with *mltB*-pJW15 and pKDS302	Kn^R Amp^R	This study
MMB23	TOP10 with *dacC*-pJW15	Kn^R	This study
MMB24	MC4100 with *dacC*-pJW15	Kn^R	This study
MMB25	MC4100 with *dacC*-pJW15 and pCA-NlpE	Kn^R Cam^R	This study
MMB26	MC4100 with *dacC*-pJW15 and pCA24N	Kn^R Cam^R	This study
MMB27	TR10 with *dacC*-pJW15	Kn^R Amk^R	This study
MMB28	TR51 with *dacC*-pJW15	Kn^R Spc^R	This study
MMB29	MC4100 with *dacC*-pJW15 and ptrc99A	Kn^R Amp^R	This study
MMB30	MC4100 with *dacC*-pJW15 and pKDS302	Kn^R Amp^R	This study
MMB31	MC4100 with *amiC*-pJW15	Kn^R	This study
MMB32	TR10 with *amiC*-pJW15	Kn^R Cam^R	This study
MMB33	TR51with *amiC*-pJW15	Kn^R Cam^R	This study
MMB34	MC4100 with *amiC*-pJW15 and pCA-NlpE	Kn^R Spc^R	This study
MMB35	TR51with *amiC*-pJW15	Kn^R Spc^R	This study
MMB36	TOP10 with *amiA*-pJW15	Kn^R	This study
MMB37	MC4100 with *amiA*-pJW15	Kn^R	This study

MMB38	MC4100 with *amiA*-pJW15 and pCA-NlpE	Kn^R Cam^R	This study
MMB39	MC4100 with *amiA*-pJW15 and pCA-24N	Kn^R Cam^R	This study
MMB40	TR10 with *amiA*-pJW15	Kn^R Amk^R	This study
MMB41	TR51 with *amiA*-pJW15	Kn^R Spc^R	This study
MMB42	TOP10 with *slt70*-pJW15	Kn^R	This study
MMB43	MC4100 with *slt70*-pJW15	Kn^R	This study
MMB44	MC4100 with *slt70*-pJW15 and pCA-NlpE	Kn^R Cam^R	This study
MMB45	MC4100 with *slt70*-pJW15 and pCA-24N	Kn^R Cam^R	This study
MMB46	TR10 with *slt70*-pJW15	Kn^R Amk^R	This study
MMB47	TR51 with *slt70*-pJW15	Kn^R Spc^R	This study
MMB48	TOP10 with *ycbB*-pJW15	Kn^R	This study
MMB49	MC4100 with *ycbB*-pJW15	Kn^R	This study
MMB50	MC4100 with *ycbB*-pJW15 and pCA-NlpE	Kn^R Cam^R	This study
MMB51	MC4100 with *ycbB*-pJW15 and pCA-24N	Kn^R Cam^R	This study
MMB52	TR10 with *ycbB*-pJW15	Kn^R Amk^R	This study
MMB53	TR51 with *ycbB*-pJW15	Kn^R Spc^R	This study
MMB54	MC4100 with *ycbB*-pJW15 and	Kn^R Amp^R	This study

	ptrc99A		
MMB55	MC4100 with *ycbB*-pJW15 and pKDS302	Kn^R Amp^R	This study
MMB56	MC4100 *mltB*::kn	Kn^R	This study (Baba *et al.*, 2006)
MMB57	TR10 *mltB*::kn	Kn^R Amk^R	This study (Baba *et al.*, 2006)
MMB58	TR51 *mltB*::kn	Kn^R Spc^R	This study (Baba *et al.*, 2006)
MMB59	MC4100 ygaU::kn	Kn^R	(Baba *et al.*, 2006) This study (Baba *et al.*, 2006)
MMB60	TR10 *ygaU*::kn	Kn^R Amk^R	This study (Baba *et al.*, 2006)
MMB61	MC4100 *ycbB*::kn	Kn^R	This study This study (Baba *et al.*, 2006)
MMB62	TR10 *ycbB*::kn	Kn^R Amk^R	This study (Baba *et al.*, 2006)
MMB63	MC4100 *slt70*::kn	Kn^R	This study (Baba *et al.*, 2006)
MMB64	TR10 slt70::kn	Kn^R Amk^R	This study (Baba *et al.*, 2006)

MMB65	MC4100 *ycbB*::kn with pCA-*ycbB*	Kn^R Cam^R	This study (Baba *et al.*, 2006)
MMB66	TR10 *ycbB*::kn with pCA-*ycbB*	Kn^R Cam^R Amk^R	This study (Baba *et al.*, 2006)
MMB67	MC4100 *ygaU*::kn with pCA-*ygaU*	Kn^R Cam^R	This study (Baba *et al.*, 2006)
MMB68	TR10 *ygaU*::kn with pCA-*ygaU*	Kn^R Cam^R	This study (Baba *et al.*, 2006)
MMB69	MC4100Δ*cpxR ycbB*::kn	Kn^R	This study (Baba *et al.*, 2006)
MMB70	MC4100Δ*cpxR slt70*::kn	Kn^R	This study
MMB71	MC4100Δ*cpxR* with pCA-*ycbB*	Cam^R	This study
MMB72	MC4100Δ*cpxR* with pCA-*ygaU*	Cam^R	This study (Baba *et al.*, 2006)
MMB73	MC4100Δ*cpxR* with pCA-*slt70*	Kn^R	This study (Baba *et al.*, 2006; Hoang *et al.*, 1998)
MMB74	MC4100Δ*ycbB ygaU*	-N/A	This study (Baba *et al.*, 2006; Hoang *et al.*, 1998)
MMB75	*cpxA** Δ*ycbB ygaU zi*i::Tn10	Tet^R Amk^R	This study Baba 2006} (Hoang *et al.*, 1998)
MMB76	MC4100Δ*ycbB ygaU slt70*::kn	Kn^R	This study, (Baba *et al.*, 2006)
MMB77	cpxA* Δ*ycbB ygaU slt70*::kn	Kn^R Tet^R	This study, (Baba *et al.*,

	zii::Tn10	Amk^R	2006)
MMB78	MC4100Δ*ygaU* with *ycbB*-pJW15	Kn^R	This study, (Baba *et al.*, 2006; Hoang *et al.*, 1998)
MMB79	MC4100Δ*ycbB* with *ygaU*-pJW15	Kn^R	This study, (Baba *et al.*, 2006; Hoang *et al.*, 1998)
MMB80	MC4100Δ*ycbB ygaU* with *ygaU*-pJW15	Kn^R	This study, (Baba *et al.*, 2006; Hoang *et al.*, 1998)
MMB81	cpxA* Δ*ycbB ygaU zii*::Tn10 with pJW25	Kn^R Tet^R Amk^R	This study, (Baba *et al.*, 2006; Hoang *et al.*, 1998)
MMB82	MC4100Δ*ycbB* with *ycbB*-pJW15	Kn^R	This study, (Baba *et al.*, 2006; Hoang *et al.*, 1998)
MMB83	MC4100Δ*ygaU* with *ycbB*-pJW15	Kn^R	This study, (Baba *et al.*, 2006; Hoang *et al.*, 1998)
MMB84	MC4100Δ*ycbB* with *ygaU*-pJW15	Kn^R	This study, (Baba *et al.*, 2006; Hoang *et al.*, 1998)
MMB85	MC4100Δ*ygaU* with *ygaU*-pJW15	Kn^R	This study, (Baba *et al.*, 2006; Hoang *et al.*, 1998)
MMB86	MC4100Δ*ycbB* with *ycbB*-pJW15 with pCA-*ycbB*	Kn^R Cam^R	This study, (Baba *et al.*, 2006; Hoang *et al.*, 1998)
MMB87	MC41000Δ*ygaU* with *ygaU*-pJW15 with pCA-*ygaU*	Kn^R Cam^R	This study, (Baba *et al.*, 2006; Hoang *et al.*, 1998)
MMB88	MC4100Δ*ycbB ygaU* with *ycbB*-pJW15	Kn^R	This study, (Baba *et al.*, 2006; Hoang *et al.*, 1998)

MMB89	MC4100Δ*ycbB ygaU* with *ygaU*-pJW15	Kn^R	This study, (Baba *et al.*, 2006; Hoang *et al.*, 1998)
MMB90	MC4100Δ*ycbB ygaU* with *ycbB*-pJW15 and pCA-*ycbB*	Kn^R Cam^R	This study, (Baba *et al.*, 2006; Hoang *et al.*, 1998)
MMB91	MC4100Δ*ycbB ygaU* with *ycbB*-pJW15 and pCA-*ygaU*	Kn^R Cam^R	This study, (Baba *et al.*, 2006; Hoang *et al.*, 1998)
MMB92	MC4100Δ*ycbB ygaU* with ygaU-pJW15 and pCA-*ygaU*	Kn^R Cam^R	This study, (Baba *et al.*, 2006; Hoang *et al.*, 1998)
MMB93	MC4100Δ*ycbB ygaU* with ygaU-pJW15 and pCA-*ycbB*	Kn^R Cam^R	This study, (Baba *et al.*, 2006; Hoang *et al.*, 1998)

Table 2-2 **:**Strains constructed for this study.

Plasmid	Relevant Genotype	Reference
pJW15	pJW15 with p15 ori	(Macritchie *et al.*, 2008)
pCA-NlpE	pCA-24N that overexpresses *nlpE*	(Kitagawa *et al.*, 2005)
pCA-*ygaU*	pCA-24N that overexpresses *ygaU*	(Kitagawa *et al.*, 2005)
pCA-*ycbB*	pCA-24N that overexpresses *ycbB*	(Kitagawa *et al.*, 2005)
pCA-24N	Vector control for pCA	(Kitagawa *et al.*, 2005)
pB322	Cloning Vector	(Bolivar *et al.*, 1977)
pKDS302	pTR99A with *bfpA* to *bfpL* cloned downstream of the IPTG-inducible trc promoter	(Stone *et al.*, 1996)
ptrc99A	Expression vector with a multiple cloning site following an IPTG-inducible *trc* promoter	Amersham Pharmacia
pJW1	pNLP10 with P*cpxP*::luxCDABE	(Price and Raivio, 2009)
pJW25	pNLP10 with P*cpxP*::luxCDABE	(Price and Raivio, 2009)

pFLP2	FLP recombinase	(Hoang *et al.*, 1998)
pJW15-*ygaU*	pJW15 with *ygaU* promoter	
pJW15-*mltB*	pJW15 with *mltB* promoter	
pJW15-*dacC*	pJW15 with *dacC* promoter	
pJW15-*amiC*	pJW15 with *amiC* promoter	
pJW15-*amiA*	pJW15 with *amiA* promoter	
pJW5-*slt70*	pJW15 with *slt70* promoter	

Table 2-3 : List of plasmids constructed used in this study.

List of primer sets used to construct *lux* reporters

Gene(s) Targeted	Primer name	Primer Sequence
ycbB	*pJW5-ycbB(R)*	AAAGTAATTCTTTGCGTGCGGGGCTTT
ycbB	*pJW5-ycbB(F)*	AAACTCGAGTGGTACAAAGCTGGGAAGAT
ygaU	*pJW5-ygaU(R)*	AAAGAAATTCTACCGGTTTGTTCAGATGC
ygaU	*pJW5-ygaU(F)*	AAACTCGAGGGAACCGCTAAGCATGCACA
dacC	*pJW5-dacC(F)*	AAACTCGAGAGAATGAGATCACTTTCGTC
dacC	*pJW5-dacC(R)*	AAAGAATTCCTCGCGGGATCCAGTTTCTCA
mltB	*pJW5-mltB(F)*	AAACTCGAGTTGTACTTTTCCGGCATAAA
mltB	*pJW5-mltB(R)*	AAAGAATTCCCTGCAACTGCTGACGATGC
slt70	*pJW5-slt70(F)*	AAAGAATTCTCAATGGACTCACGCACGGT
slt70	*pJW5-*	AAAGGATCCTGCTCATCCAGTGAGTCG

	slt70(R)	GC
amiA	*pJW5-amiA(F)*	AAAGAATTCTCAGGTTTTTCTCCAGCCGA
amiA	*pJW5-amiA(R)*	AAAGGATCCGGCGCAAGTGAGTGTTTT
amiC	*pJW5-amiC(F)*	AAAGAATTCGCCATAGACCACCACCAGAC
amiC	*pJW5-amiC(R)*	AAAGGATCCCATTGGCCGGATAGAGGTCC

List of primer sets used to amplify gene promoters for EMSA analysis

Gene(s) Targeted	**Primer name**	**Primer Sequence**
degP	*PdegP-F*	CGCTTATTCCACAAACTCTCG
degP	*PdegP-R*	CGGCTGAGACTTCTTCAGCAACGA
rpoD	*PrpoD-F*	CGAAGAACGCCTGGAGC
rpoD	*PrpoD-R*	TCACCTGAATGCCCATGTCG
ygaU	*PygaU-F*	AAATGCTGTGATGTCGCAGAGG
ygaU	*PygaU-R*	GCGTCCCAGAGTTTTTCTCC
ycbB	*PycbB-F*	AAATGGTACAAAGCTGGGAGAT
ycbB	*PycbB-R*	AAATTTGCGTGCGGGCTTTTTCT
mltB	*PmltB-F*	AAATTGTACTTTTCCGGCATAAA
mltB	*PmltB-R*	AAACCTGCAACTGCTGACGATCG

slt70	*Pslt70-F*	AAATCCTGCGGCAGATAACCAAT
slt70	*Pslt70-R*	AAATCGGTGATCTGGCGGTATTC

	List of primer sets used for KEIO or kn cassette verification	
Gene(s) Targeted	**Primer name**	**Primer Sequence**
ycbB	*KycbB-F*	AAAGTGGCACATTACGGCG
ycbB	*KycbB-R*	AAAGAGAGTGTTGCAAACGCAGG
ygaU	*KygaU-F*	AAATCGGTAAAGGGTTCGGTTGG
ygaU	*KygaU-R*	AAATAAGCTTGAGGCCTGTGACG
mltB	*KmltB-F*	AGCCCGATAAACCACTCTGC
mltB	*KmltB-R*	AGGTGACAAATCCGCGTTCA
slt70	*Kslt70-F*	AAACGAATTGAGCCACGTTGGAC
slt70	*Kslt70-R*	AAATGGCGTCAGCATCAGGTTTA

Table 2-4 :List of primer sets used in this study.

References

Baba, T., Ara, T., Hasegawa, M., Takai, Y., Okumura, Y., Baba, M., *et al.* (2006) Construction of *Escherichia coli* K-12 in-frame, single-gene knockout mutants: the KEIO collection. *Mol Syst Biol* **2**: 2006.0008.

Bolivar, F., Rodriguez, R.L., Betlach, M.C., and Boyer, H.W. (1977) Construction and characterization of new cloning vehicles. Ampicillin-resistant derivatives of the plasmid pMB9. *Gene* **2**: 75-91.

Casadaban, M.J. (1976) Transposition and fusion of the *lac* genes to selected promoters in *Escherichia coli* using bacteriophage lambda and Mu. *J Mol Biol* **104**: 541-555.

Danese, P.N., and Silhavy, T.J. (1998) CpxP, a stress-combative member of the Cpx regulon. *J Bacteriol* **180**: 831-839.

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

Glauner, B. (1988) Separation and quantification of muropeptides with high-performance liquid chromatography. *Analytical biochemistry* **172**: 451-464.

Glauner, B., Höltje, J.V., and Schwarz, U. (1988) The composition of the murein of *Escherichia coli*. *Journal of Biological Chemistry* **263**: 10088-10095.

Hayashi, K. (1975) A rapid determination of sodium dodecyl sulfate with methylene blue. *Analytical biochemistry* **67**: 503-506.

Hoang, T.T., Karkhoff-Schweizer, R.R., Kutchma, A.J., and Schweizer, H.P. (1998) A broad-host-range Flp-FRT recombination system for site-specific excision of chromosomally-located DNA sequences: application for isolation of unmarked *Pseudomonas aeruginosa* mutants. *Gene* **212**: 77-86.

Kitagawa, M., Ara, T., Arifuzzaman, M., Ioka-Nakamichi, T., Inamoto, E., Toyonaga, H., and Mori, H. (2005) Complete set of ORF clones of *Escherichia coli* ASKA library (a complete set of E. coli K-12 ORF archive): unique resources for biological research. *DNA Res* **12**: 291-299.

Macritchie, D.M., Ward, J.D., Nevesinjac, A.Z., and Raivio, T.L. (2008) Activation of the Cpx envelope stress response down-regulates expression of several locus of enterocyte effacement-encoded genes in enteropathogenic *Escherichia coli*. *Infect Immun* **76**: 1465-1475.

Price, N.L., and Raivio, T.L. (2009) Characterization of the Cpx regulon in *Escherichia coli* strain MC4100. *J Bacteriol* **191**: 1798-1815.

Raivio, T.L., and Silhavy, T.J. (1997) Transduction of envelope stress in *Escherichia coli* by the Cpx two-component system. *Journal of bacteriology* **179**: 7724-7733.

Raivio, T.L., Popkin, D.L., and Silhavy, T.J. (1999) The Cpx envelope stress response is controlled by amplification and feedback inhibition. *J Bacteriol* **181**: 5263-5272.

Sambrook, J., Fritsch, E.F., and Maniatis, T. (1989) Molecular cloning. Cold spring harbor laboratory press New York.

Silhavy, T.,., Berman, M.,., and Enquist, L.,. (1984) Experiments with Gene Fusions. *Journal of Basic Microbiology.* **5:**350

Slauch, J.M., Russo, F.D., and Silhavy, T.J. (1991) Suppressor mutations in *rpoA* suggest that OmpR controls transcription by direct interaction with the alpha subunit of RNA polymerase. *Journal of bacteriology* **173**: 7501-7510.

Stone, K.D., Zhang, H.-Z., Carlson, L.K., and Donnenberg, M.S. (1996) A cluster of fourteen genes from enteropathogenic *Escherichia coli* is sufficient for the biogenesis of a type IV pilus. *Molecular microbiology* **20**: 325-337.

Tao, G.-Z., Kobayashi, A., Itoh, H., and Tashima, Y. (1998) Expression of *Escherichia coli* gives a strong tolerance to hypotonic stress. *FEBS letters* **434**: 28-32.

Tayler, A.E., Ayala, J.A., Niumsup, P., Westphal, K., Baker, J.A., Zhang, L., *et al.* (2010) Induction of beta-lactamase production in *Aeromonas hydrophila* is responsive to beta-lactam-mediated changes in peptidoglycan composition. *Microbiology* **156**: 2327-2335.

Wiegand, I., Hilpert, K., and Hancock, R.E. (2008) Agar and broth dilution methods to determine the minimal inhibitory concentration (MIC) of antimicrobial substances. *Nat Protoc* **3**: 163-175.

Work, E. (1957) Reaction of ninhydrin in acid solution with straight-chain amino acids containing two amino groups and its application to the estimation of α - diaminopimelic acid. *Biochemical Journal* **67**: 416.

CHAPTER 3

Identification and verification of Cpx regulated cell wall genes: *ygaU, ycbB, dacC, amiA, amiC, slt70, and mltB*

3.1 Characterization of cell wall genes *ygaU, ycbB, mltB, slt70, dacC, amiA,* and *amiC* as part of the Cpx regulon

At the start of this project, a microarray analysis performed by our laboratory found that cell wall genes *ygaU, ycbB, amiA, dacC* and *slt70* were up-regulated in *E. coli* MC4100 in the presence of NlpE over-expression (Raivio *et al.*, 2013; Snyder *et al.*, 1995). This raised the possibility that changes in the structure of the cell wall could result from the activation of the Cpx pathway. This would suggest that this stress response might have other functions in addition to proper maintenance of protein folding in the periplasm (Vogt and Raivio, 2012). This project was intended to further clarify and characterize the effects of *ygaU, ycbB, amiC,A dacC, slt70,* and other cell wall genes on the peptidoglycan structure, organization, and composition during activation of the Cpx pathway in *E. coli*. We decided to also characterize the role of two more cell wall genes that were not found in the microarray; *amiC* and *mltB*. We selected *amiC* because this gene has been shown to be regulated by the Cpx pathway in Salmonella Weatherspoon-Griffin *et al.*, 2011. In addition, we analyzed activity of mltB because proteolytic degradation of murein hydrolyse lipoprotein MltB results in Slt35. Slt35 is a soluble lytic transglycosylase that has a core domain that closely resembles the fold of the catalytic domain of Slt70 (van Asselt *et al.*, 1999).

To this end, we first performed a preliminary bioinformatic analysis with Jalview and searched for the GTAa-N_5-GTAAa (De Wulf and Lin, 2000) CpxR

binding motif within the promoter of these 7 genes (Figure 3-1A). We found that *ygaU, mltB, slt70,* and *ycbB* had a putative CpxR binding site with close resemblance to the published consensus within their promoter, whereas *dacC, amiA, and amiC* had CpxR binding sites that only weakly matched the consensus. We also found that the promoter of *ycbB* might have more than one CpxR binding site (Figure 3B).

Furthermore, to verify Cpx regulation of these 7 cell wall genes genes we performed luciferase assays with bioluminescent reporter genes (Macritchie *et al.*, 2008). For this, we constructed *lux* reporters for *ygaU, ycbB, amiA, amiC, dacC, slt70*, *mltB*. We first amplified the promoter of each gene by PCR using primers that annealed 500 bp upstream and 100 bp downstream from the translational start site to ensure that the CpxR binding site was present, and we cloned each amplicon into the pJW15 plasmid (Figure 3-2). We sequenced the MCS of each plasmid to verify that there were no mutations present that could affect binding of CpxR. Finally, we transformed each promoter-pJW15 fusion into strains with varying levels of Cpx pathway activity. These included strains carrying the the *cpxA24* allele, which constitutively activates the Cpx response (Cosma *et al.*, 1995; Raivio and Silhavy, 1997), strains over-expressing the inducer NlpE (Snyder *et al.*, 1995), or strains lacking the cognate response regulator *cpxR* where the transcription of Cpx-regulated genes is abolished (Raivio *et al.*, 1999). This enabled us to assess Cpx regulation of *ygaU, ycbB, amiA, amiC, dacC, slt70*, *mltB*, and also assess the relative strength of Cpx regulation of each gene. We expected that positively Cpx-regulated genes would

show an increase in transcription in the presence of the strong allele *cpxA24* (from now on denoted *cpxA**) and NlpE over-expression, and possibly a decrease in transcription in the *cpxR* null background (Price and Raivio, 2009), while weakly or non- Cpx regulated genes would show little or no changes in expression levels in any of these strains.

For those genes whose expression appeared to be Cpx-regulated in the above reporter gene assays, we performed electrophoretic mobility assays (EMSAs) to determine if a MBP-CpxR fusion protein was able to bind to their promoters. For this assay, we also used primers that annealed 500 bp upstream and 100 bp downstream from the translational start site to amplify the transcriptional control region containing the putative CpxR binding sites. The promoter region of *degP was* also assayed for MBP-CpxR binding, as this gene is a well-characterized member of the Cpx regulon, and functioned as an excellent positive control (Danese *et al.*, 1995; Danese *et al.*, 1998) (Figure 3-3a). The promoter region of *rpoD,* a housekeeping sigma factor that is not Cpx-regulated, was used as a negative control (Raivio *et al.*, 2013) (Fig 3-3b). To phosphorylate CpxR, we incubated the MBP-CpxR fusion protein with the small molecular weight phosphodonor acetyl-phosphate (Raivio and Silhavy, 1997). Initially, we performed a gradient EMSA with varying concentrations of MBP-CpxR~P ranging from 0-150 pmol in the presence of the positive control promoter *degP* to determine at which minimum concentration of MBP-CpxR~P we observed complete shifting (Figure 3- 2a). In addition, we also performed a gradient EMSA for the negative control promoter *rpoD* to assess at what minimum concentration

we observed nonspecific binding of MBP-CpxR~P and shifting (Figure 3-3b). With this in mind, we determined that the optimal concentration of MBP-CpxR for binding to the promoter of the known, moderately Cpx-regulated gene *degP* was 50 pmol.. The results of these EMSA gradients are depicted in Figure 3.2. Moreover, these EMSA studies demonstrated the MBP-CpxR protein we utilized for this assay was functional and able to bind DNA at concentrations previously reported (1.25-200 pmol) (Raivio and Silhavy, 1997; Jubelin *et al.*, 2005; Weatherspoon-Griffin *et al.*, 2011) . They also demonstrated that the controls we used were effective, reliable, and specific, as the presence of high concentrations of MBP-CpxR caused minimum shifting of the negative control *rpoD* promoter. These control assays enabled us to establish the conditions for the accurate assessment of CpxR binding to the promoters of our cell wall genes, using the *degP* promoter as competitive DNA and a positive control and the *rpoD* promoter as a non-competitive DNA and a negative control.

3.2 The putative cell wall modification protein ygaU is highly up-regulated by the Cpx pathway.

The *ygaU* gene is predicted to encode a 16kDa un-characterized protein that has a BON domain that is predicted to localize proteins to phospholipid membranes (Yeats and Bateman, 2003). In addition, YgaU has a LysM motif that is associated with binding to various types of peptidoglycan, most likely

recognizing the N-acetylglucosamine moiety, and also with hydrolase activity (Buist *et al.*, 2008).

The microarray analysis previously performed by our laboratory to identify novel members of the Cpx regulon found that *ygaU* was the fourth most highly up-regulated cell wall gene in the presence of NlpE over-expression in *E. coli* K-12 strain MC4100 (Raivio *et al.*, 2013). Expression of *ygaU* is also known to be regulated in response to osmolarity (Weber et al. 2006) and is controlled by the stationary phase sigma factor s^S (Ibanez-Ruiz et al. 2000). We first performed a luciferase assay with the *ygaU*-pJW15 reporter in MC4100 and the *cpxA** and the *cpxR* mutant strain backgrounds over the course of 6 hours to determine at what time point the *ygaU* reporter had the highest activity in the wild-type background to corroborate the fact that *ygaU* is highly up-regulated at the entry of stationary phase(Figure 3A). We determined that the transcriptional activity of *ygaU* was higher at late log-phase in the wild-type background (OD=0.7), consistent with its demonstrated regulation by s^S (Ibanez-Ruiz et al. 2000). We also performed an other *lux* assay to determine if *ygaU* was in fact Cpx-regulated (Figure 3-4B). We measured the transcriptional activity of the *ygaU-lux* reporter at 6 hours. Furthermore, under these conditions we found that transcription levels of *ygaU* were significantly up-regulated by almost four-fold in the *cpxA** background ($P<0.05$, one-way ANOVA) when compared to the wild-type, suggesting that activation of the Cpx pathway leads to up-regulation of this gene. In addition, absence of the cognate regulator CpxR lead to a decrease in the transcription of *ygaU-lux*, indicating that CpxR is necessary for basal expression of this gene. We

also tested whether transcription of *ygaU* was dependent on NlpE over-production (Figure 3-4C). We found that over-expression of NlpE caused elevated expression of *ygaU* compared to the transcriptional activity of *ygaU* in the presence of the vector control pCA-24N. These differences were statistically significant ($P<0.05$, one-way ANOVA). Finally, we also measured the transcriptional activity of *ygaU* in the presence of over-expression of the BfpA-L cluster, another well characterized inducing cue of the Cpx pathway (Figure 3-4D) (Vogt *et al.*, 2010). For this we used the ptrc99A derivative plasmid, IPTG-inducible plasmid PKDS302 that contains the *bfpA-L* cluster. We found that the *ygaU*-pJW15 reporter was significantly ($P<0.05$, one-way ANOVA). up-regulated under these conditions.

Since our reporter gene assays suggested that *ygaU* was Cpx-regulated, we determine whether this might be due to direct regulation by CpxR. For this, we performed two kinds of EMSAs. The first EMSA that we performed was a gradient EMSA to determine the minimum concentration at which MBP-CpxR~P bound. We found that MBP-CpxR~P bound at a concentration of 25pmol and that 50 pmol was sufficient to cause complete shifting of the *ygaU* promoter, confirming that CpxR~P binds to the *ygaU* promoter and is likely directly regulated by the Cpx-pathway (Figure 3-5). However, we needed a higher amount of MBP- CpxR~P to cause shifting of the *ygaU* promoter (25pmol) compared to the promoter of *degP* (10pmol) (Figure 3-5). This may be a result of a single base change away from the consensus CpxR binding site found in the promoter of *ygaU* (A→G) (Figure 3-1A). We performed a second EMSA using 50pmol of

CpxR to observe the behaviour of the *ygaU* promoter in the presence of the *degP* (positive control and competitive inhibitor) and *rpoD* (negative control and non-competitive inhibitor) promoter regions. In the presence of the *degP* promoter region, both the *ygaU* and *degP* DNA fragments were bound by 50 pmol of CpxR, since no unbound DNA fragments were visible (Figure 3-5). This is consistent with the gradient EMSAs done in the absence of competitive DNA (Figure 3-3a and 3-5a), and suggests that CpxR binds with similar affinities to the *degP* and *ygaU* promoter regions. Addition of the *rpoD* promoter region to the *ygaU* EMSA had no effect on binding, supporting the argument that CpxR binds specifically to the *ygaU* promoter.

3.3 *ycbB* is an L,D-transpeptidase regulated by the Cpx pathway

YcbB is one of the five L,D-transpeptidases found in *E. coli.* The function of this protein is to synthesize the rare *meso*-DAP-*meso*-DAP murein cross-link (Magnet *et al.*, 2008). These cross-links are synthesized after D,D-carboxypeptidases cleave the last alanine of the stem peptide creating a tetrapeptide. This tetrapeptide becomes the substrate for the L,D-transpeptidases that recognize and cleave the bond between the L center of the DAP and the D-alanine, attaching the *meso*-DAP to a neighboring chain *meso*-DAP (Sanders and Pavelka, 2013). Although the function of these 3-3 crosslinks is uncertain, they are thought to be important during times of stress (Goffin & Ghuysen, 2002). Furthermore, YcbB was the second most highly up-regulated gene, amongst the

known and predicted cell wall modification enzymes, in the microarray performed by our lab in MC4100 (Raivio *et al.*, 2013).

We first assayed expression of *ycbB* in genetic backgrounds with varying Cpx pathway activity using a *ycbB-lux* transcriptional reporter gene. (Figure 3-6A). Transcriptional activity of the *ycbB*-pJW15 lux reporter was 6-fold higher in the *cpxA** background than in MC4100 ($P<0.05$, one-way ANOVA), which suggests that this gene might be regulated by the Cpx pathway. However, transcriptional activity of the *ycbB* reporter was not significantly significantly different in a *cpxR* background compared to the wild-type. This could mean that CpxR does not play a role in the basal expression of *ycbB* and that there is another regulatory mechanism involved in this process.

When we measured the activity of the *ycbB-lux* reporter in the presence of NlpE over-expression (Figure 3-6B) we observed an 11-fold increase in luminescence compared to the activity of the vector control for NlpE ($P<0.05$, one-way ANOVA). Interestingly, the transcriptional activity of this gene in the presence of NlpE was almost the same as in the *cpxA** mutant (Figure 3-6A,B). Furthermore, we also observed a significant 2-fold increase ($P<0.05$, one-way ANOVA). in the transcription of *ycbB* in the presence of Bfp over-expresssion (Figure 3-6C). Taken together, this data suggest that thus confirming that the Cpx pathway strongly regulates this gene

We performed a gradient and a competitive EMSA in the same way we did for *ygaU* to confirm whether the Cpx pathway directly regulates the transcription of *ycbB*. The gradient EMSA (Figure 3-7A) showed that 25pmol of MBP-CpxR~P

caused complete shifting of this gene. This is the same concentration needed to cause complete shifting of the CpxR regulated promoter of *degP* (Figure 3-3), which shows that CpxR has a strong binding affinity for this gene. This is consistent with the observed strong conservation of the CpxR consensus binding site upstream of the *ycbB* gene (Figure 3-1A). In addition, when we added 200pmol of CpxR to the promoter of *ycbB* we observed a new type of band shifting, this could have occurred due to the fact that *ycbB* has more than one CpxR binding site (Figure 3-1B). Therefore, saturation of the *ycbB* promoter with CpxR can cause CpxR binding at this secondary location, generating another type of CpxR-*ycbB* promoter complex. We also performed a competitive EMSA to analyze whether the presence of the promoters of *degP* or *rpoD* would affect the binding affinity of CpxR to the *ycbB* promoter (Figure 3-7B). In the presence of the *degP* promoter region, both the *ycbB* and *degP* DNA fragments were bound by 25 pMol of CpxR, since no unbound DNA fragments were visible (Figure 3-7B). This is consistent with the gradient EMSAs done in the absence of competitive DNA (Figure 3-3A and 3-7A), and suggests that CpxR binds with similar affinities to the *degP* and *ycbB* promoter regions. Addition of the *rpoD* promoter region to the *ycbB* EMSA had no effect on binding, supporting the argument that CpxR binds specifically to the *ycbB* promoter.

3.4 The soluble lytic transglycosylase Slt70 is regulated by the Cpx pathway

Slt70 is one of the six lytic transglycosylases isolated from *E. coli* (MltA, MltB, MltC, MltD, EmtA). Activity of *slt70* is well described in the literature

(Engel *et al.*, 1991), The function of this enzyme is to convert the glycosidic bond between between N-acetylmuramic acid and N-acetylglucosamine, into a 1→6 anhydro-N-acetylmuramyl bond (Höltje *et al.*, 1975). Furthermore, Slt70 is the only soluble lytic transglycosylase present in *E. coli* as the other five enzymes being membrane bound (Dijkstra *et al.*, 1999), and it also has exo-muramidase activity as it recognizes disaccharides located at one end of the glycan strand (Dijkstra *et al.*, 1999). Interestingly, the activity of Slt70 has been shown to be altered by another stress response, the stringent response (Betzner et al. 1990).

The microarray analysis performed by our laboratory found that *slt70* was the third highest up-regulated cell wall gene in *E. coli* in response to induction of the Cpx pathway by NlpE over-expression (Raivio *et al.*, 2013). RNA-seq analysis has also found that *slt70* is up-regulated in *Haemophilus ducreyi* as a result of Cpx activation (Gangaiah *et al.*, 2013), suggesting that this gene might be a conserved member of the Cpx regulon and that it's activity is important for adaptation to envelope stress and Cpx-inducing conditions.

We performed two luciferase assays with the *slt70*-pJW15 reporter to assess whether the Cpx pathway regulates this gene. When we measured the transcription levels of the *slt70-lux* transcriptional reporter in the *cpxA*, cpxR,* and wild-type backgrounds (Figure 3-8A) we found that the transcription of *slt70* was almost 10-fold higher in the *cpxA** background and 7-fold lower in the *cpxR* null mutant compared to the wild type levels ($P<0.05$, one-way ANOVA). Taken together, this data supports the conclusion that the Cpx pathway regulates expression of this lytic transglycosylase.

We also performed a luciferase assay in the presence of NlpE over-production to determine if induction of the Cpx-pathway caused an increase in the transcription of *slt70 (Snyder et al., 1995)* (Figure 3-8B). Transcription levels of the *slt70*-pJW15 promoter were 3-fold higher than the levels of the pCA-*nlpE* vector control ($P<0.05$, one-way ANOVA), further supporting that *slt70* is another member of the Cpx regulon.

Since activity of *slt70* was not as strongly regulated as *ygaU* or *ycbB*, we only performed a competitive EMSA using a concentration of 50 pmol MPB-CpxR (view section 3.1) to observe in presence of *degP* or *rpoD* could affect specific binding by MPB-CpxR~P (3-9A). To determine whether CpxR regulated *slt70* expression directly, we performed EMSA experiments (Figure 3-9A). We observed complete shifting of the *slt70* promoter using 50 pmol of MPB-CpxR. suggesting that CpxR directly regulates *slt70.* In the presence of equal amounts of *degP* promoter, which binds CpxR at a similar concentration (Figure 3-1 A) both the *slt70* and *degP* promoter fragments were bound, suggesting that CpxR binds to the *slt70* promoter at least as well as it does to that of *degP*. Curiously, equal amounts of the *rpoD* promoter appeared to interfere with CpxR binding to the *slt70* promoter (Figure 3-9 A). At present we have no explanation for this.

3.5 *mltB* is membrane-bound lytic transglycosylase regulated by the Cpx pathway

MltB is a major transglycosylase in *E. coli.* Its function is to cleave the sugar backbone between the N-acetyl muramic acid and the N-acetyl glucosamine

by a process that results in the formation of 1,6-anhydromuropepties. MltB is the only lytic transglycosylase capable of recognizing peptidoglycan fragments that only have two disaccharides; hence this enzyme is responsible for processing short peptidoglycan strands (Suvorov *et al.*, 2008). MltB, and other lytic transglycosylases, play important roles in turning over peptidoglycan during cell growth and division.

Although *mltB* expression was not documented to change when the Cpx regulon was examined by microarray (Raivio et al. 2013), we chose to examine whether it might be Cpx-regulated because of our finding that the expression of the related Slt70 enzyme was part of the Cpx regulon. (van Asselt *et al.*, 2000). Accordingly we constructed and characterized the expression of an *mltB-lux* reporter gene in the presence of a strong *cpxA** allele, *cpxR* null mutation, and upon over-expression of NlpE. We found that the transcription of the *mltB*-pJW15 reporter was significantly up-regulated by 3-fold in the *cpxA** background ($P<0.05$, one-way ANOVA) and significantly down-regulated in the *cpxR* strain ($P<0.05$, one-way ANOVA) suggesting that the Cpx-pathway regulates this gene. (Figure 3-10 A). Remarkably, *mltB* was the second most highly up-regulated gene in the *cpxA** background out of all of *ygaU, ycbB, slt70, dacC, amiA and amiC.* Transcription of *mltB* also increased as a result of NlpE over-expression ($P<0.05$, one-way ANOVA) while it remained unaffected in the presence of the vector control pCA-24N (Figure 3-10B). However, transcription of *mltB* remained un-affected ($P>0.05$, one-way ANOVA). in the presence of Bfp over-expression

(Figure 3-10C), suggesting that transcription of this gene might vary according to the nature of the inducing cue.

We found that the *mltB* promoter had an almost perfect CpxR binding site (Figure 3-1). To assess if CpxR bound to the *mltB* promoter, we performed a competitive EMSA (Figure 3-11). As expected, and observed with other predicted Cpx-regulated cell wall modification genes, CpxR bound to the *mltB* promoter and this binding occurred in the presence of either the Cpx-regulated *degP* promoter or the *rpoD* negative control (Figure 3-11). These results suggest that CpxR binds to the *mltB* promoter at least as strongly as it does to the *degP* promoter.

3.6 The carboxy-peptidase dacC is regulated by the Cpx pathway.

dacC was the most up-regulated cell wall gene in *E. coli* upon NlpE over-expression *(Raivio et al., 2013)*. DacC is also known as PBP6 and it is a membrane-bound D,D-carboxypeptidase (Pedersen *et al.*, 1998) that catalyzes the hydrolysis of the D-alanine-D-alanyl peptide bond of the stem peptide in the murein sacculus resulting in the formation of a tetrapeptide (Baquero *et al.*, 1996). This PBP is one of the limiting factors in the supply of sufficient cross-link precursors for the synthesis of 3-4 cross-links by PBP3 (Santos *et al.*, 2002) and by L,D-transpeptidases YcbB or YnhG (Sanders and Pavelka, 2013).

The *dacC* promoter was found to contain only one half of a potential CpxR binding site (Figure 3-1), suggesting the Cpx pathway might weakly, or indirectly, regulate *dacC*. Surprisingly, expression of a *dacC*-pJW15 *lux* reporter

in wild-type, *cpxA**, and *cpxR* backgrounds suggested that *dacC* transcription is strongly Cpx-regulated (Figure 3-12A). We found that this gene was the most highly up-regulated gene we tested in this study. Transcriptional activity was almost 10-fold higher in the *cpxA** background compared to the wild-type ($P<0.05$, one-way ANOVA), while the transcription levels of the *dacC* reporter in the *cpxR* background were similar to the wild-type, indicating that the Cpx pathway is not necessary for basal activity of this gene. We also determined if activation of the Cpx pathway as a result of NlpE over-production could affect transcription levels of the *dacC*-pJW15 *lux* reporter (Figure 3-12B). We observed a 15-fold increase in the transcription of *dacC* in the presence of NlpE over-expression compared to the transcriptional activity of the NlpE vector control ($P<0.05$, one-way ANOVA). Over-expression of Bfp also showed a significant increase in the transcription of *dacC* ($P<0.05$, one-way ANOVA)., however, this transcription levels were 400-fold level lower than those seen in the presence of NlpE-over expression (Figure 3-12C). Taken together, this transcriptional data showed that *dacC* is another member of the Cpx regulon, although the absence of a consensus CpxR binding site in its upstream region suggests that this regulation may be indirect.

3.7 The *amiA* amidase is weakly regulated by the Cpx pathway

E. coli has three N-acetyl-L-alanine amidases (AmiA, AmiB, and AmiC) that are involved in the removal of cross-links. They accomplish this by cleaving the peptide moiety from the N-acetyl muramic acid (Heidrich *et al.*, 2001). AmiA

is evenly distributed throughout the periplasm, and it is translocated to the periplasm by the twin-arginine protein transport pathway (TAT) (Priyadarshini *et al.*, 2007). *amiA* was the only amidase whose expression was found to be regulated by NlpE over-expression in the microarray performed by our laboratory, and it was the most weakly up-regulated cell wall gene (Raivio *et al.*, 2013). Regulation of *amiA* may vary between *E. coli* and *Salmonella* species, since previous transcriptional and EMSA analyses have shown that CpxR binds directly to the *amiA* promoter, up-regulating this gene during activation of the Cpx pathway in Salmonella (Weatherspoon-Griffin *et al.*, 2011). Our bioinformatic analysis of the *amiA* promoter indicated that no full CpxR consensus binding site exists upstream of this gene in *E. coli* (Figure 3-1).

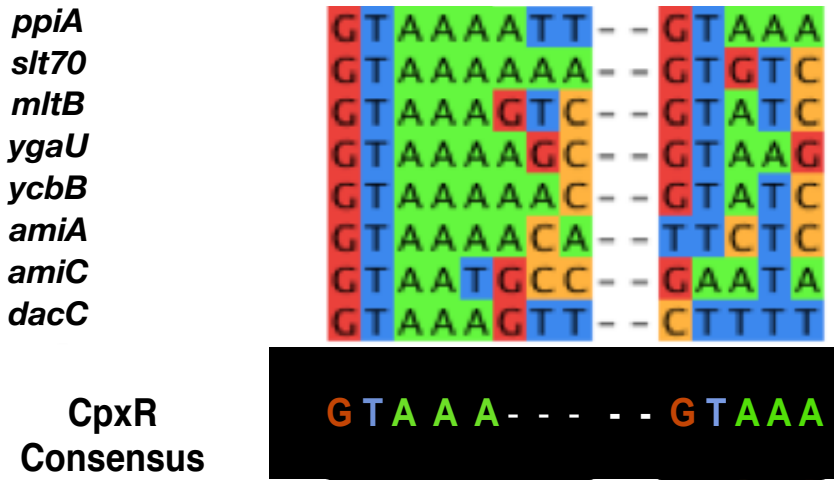
To confirm the suspected difference in *amiA* regulation between *E. coli* and *S. typhimurium,* the transcriptional activity of the *amiA*-pJW15 reporter was measured in wild-type*, cpxA** and *cpxR* backgrounds. The level of luminescence expression from this reporter was very low compared to the other cell wall genes we analyzed (Figure 3-13). Furthermore, the pattern of transcription of *amiA* was complex, exhibiting almost 6-fold higher levels in both the *cpxA** and the *cpxR* backgrounds compared to the wild-type (Figure 3-13) ($P<0.05$, one-way ANOVA). Similarly, we found a 6-fold increase in the activity of the *amiA*-pJW15 reporter as a result of NlpE over-expression compared to the vector control ($P<0.05$, one-way ANOVA). Taken together, these data suggest that the *E. coli* Cpx response impacts *amiA* expression in a complex manner that is likely indirect.

3.8 The *amiC* amidase is regulated by the Cpx pathway

AmiC is an amidase that cleaves the peptide moiety from the N-acetyl muramic acid, removing cross-links present in the cell wall (Heidrich *et al.*, 2001). Although AmiA and AmiC have the same function, AmiC is only found at the division septum (Bernhardt and De Boer, 2003). The microarray analysis of NlpE-regulated genes performed by our laboratory (Raivio *et al.*, 2013) did not detect this gene. However, we wished to determine if activation of the Cpx pathway up-regulated *amiC* in *E. coli* as it has been previously reported that the Cpx pathway directly regulates this gene in Salmonella (Weatherspoon-Griffin *et al.*, 2011). Our bioinformatics analysis showed that *amiC* has a well conserved CpxR binding site in its promoter with only two base changes from the consensus (T-A, T,A). This suggests that this gene might be part of the Cpx regulon of *E. coli.* To test this hypothesis, we measured luminescence produced by a *amiC-lux* reporter gene in the wild-type, *cpxA*,* and *cpxR* backgrounds (Figure 3-14). Surprisingly, we found that activation of the Cpx pathway did not increase transcription of this gene, while the absence of CpxR caused a two-fold increase in the transcription activity of *amiC*-pJW15. Similarly, when we measured the activity of the *amiC*-pJW15 promoter in the presence of NlpE over-expression we did not observe any change in the transcription of this gene (Figure 3-14). These data suggest that, as with *amiA,* the regulation of *amiC* differs in *E. coli* compared to *S. typhimurium*. While *amiA* and *amiC* expression appear to be impacted by changes in the activity of the Cpx response in unexpected ways, the pattern of

gene expression suggests that regulation is complex and likely involves other regulatory factors.

A)



B)

>*ycbB promoter*

GCAGCGCGACTGGATGCTCGTTCTATCGCCACGCTGTTTGAATTGTGTGAGCGTTTGCAAATGCAACTCATCATC
GCAGCGCCGGAAAATATCAGCCCGGAGAAAGGCACCACCTATAAACTGGTGCGTAAAGTCTTCCAGAATACCGAA
CACGTTCATGTCGTCGGCCTGCGAGGATTTGCGCCGCAACTCCCTGAAACGCTTCCAGGAACTGACGAAGCGCCT
TCTCAGGCGAGTTAAAATTAAGGCGGCAGCAATGCCGCCTTTTCTTTTTCCGAAAACTCCGTTTCTGCACTAAAA
AAGTGGCACATTACGGCGCGCTTTTCTTTAAACTTCTTTACATTAGGTTATGTAAAAACGTATCGGCGTTTATAT
ACTGAAGATAAGCCTGATGAGTAACAGGCTTGCTCGTCATACTTTCGTGAGTATTGGCGTTGTACAGGCAAGTCG
TAAAATAACAGCCTGGCTATTCAGAGTATGATAAAAACAGGGGGCAAGGGatg

Figure 3-1 **:** CpxR~P has a putative binding site in various cell wall genes

A) Predicted CpxR~P binding sites upstream of seven cell wall genes. Promoter regions of the seven cell wall genes were analyzed using Jalview for probable putative CpxR~P binding sites (De Wulf and Lin, 2000). Binding sites were based on the CpxR consensus binding sequence 5'-GTAAA-N_5-GTAAA-3'. The *ppiA* promoter is shown as an example of a promoter with a confirmed perfect CpxR~P binding site (Pogliano *et al.*, 1997), the promoters of *amiA* and *amiC* are used as an example of cell wall genes with an

experimentally verified CpxR binding site (Weatherspoon-Griffin *et al.*, 2011). Red color represents the letter G, blue represents the letter T, and green the letter A of the CpxR~P binding site. The generalized consensus of the CpxR~P binding site for *ygaU, mltB, slt70, ycbB, dacC, amiA, and amiC* is shown in the black box at the bottom of the sequences. Only the most conserved putative CpxR~P binding sites are shown in this figure. B) The *ycbB* promoter might have more than one CpxR binding-site. A further analysis indicates that the promoter of *ycbB* might have more than one CpxR binding site.

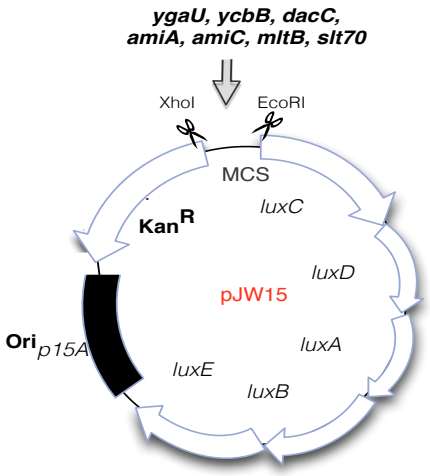


Figure 3-25: Diagram of the pJW15 *lux* reporter and the construction of *lux* reporters for *ygaU, ycbB, dacC, amiA, amiC, mltB, slt70.*

PCR was performed with XhoI and BamHI tagged primers that annealed 500 bp upstream and 100 bp downstream of the translational start site. Clones were verified by sequencing and measuring their ability to confer luminescence.

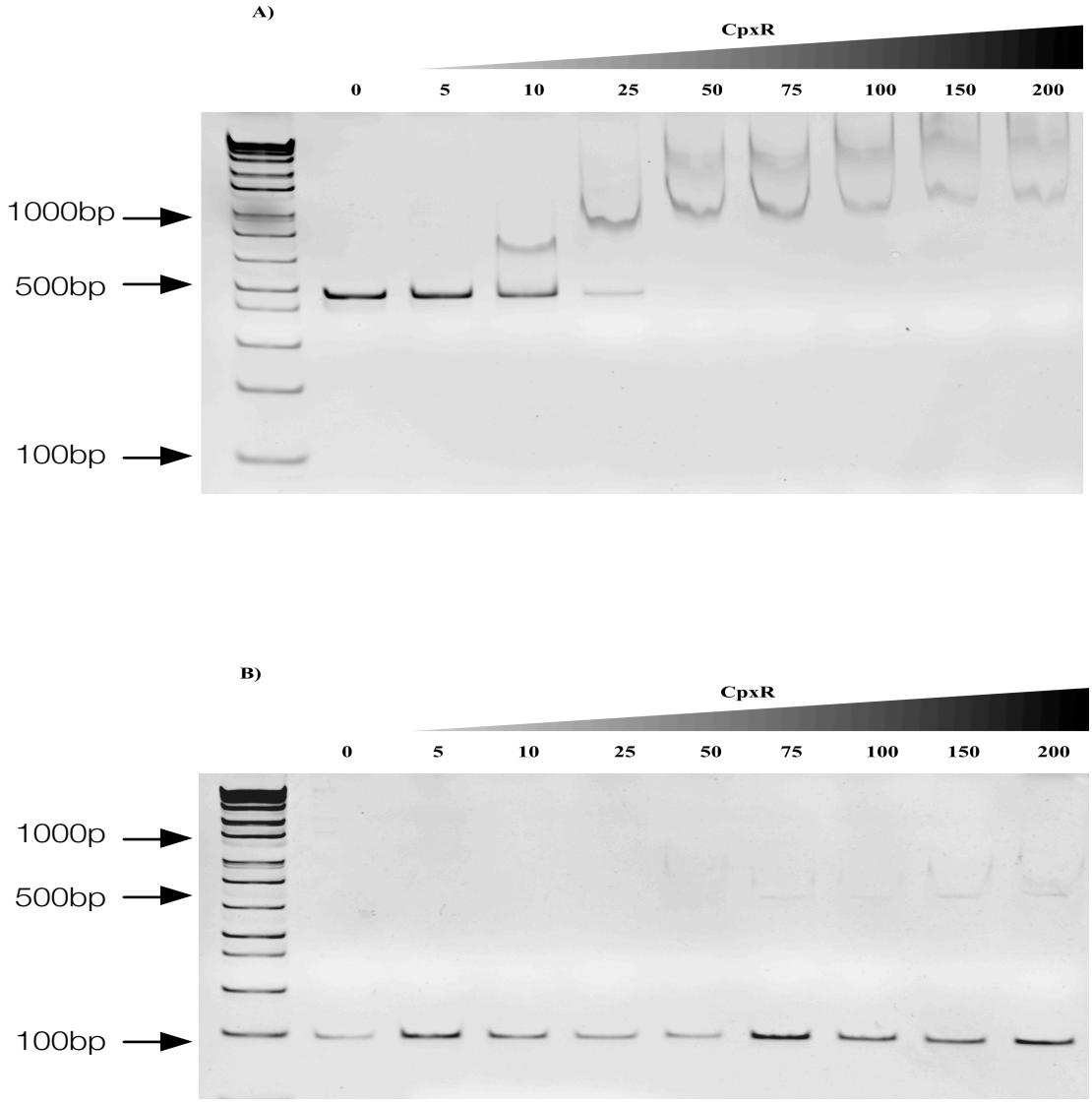
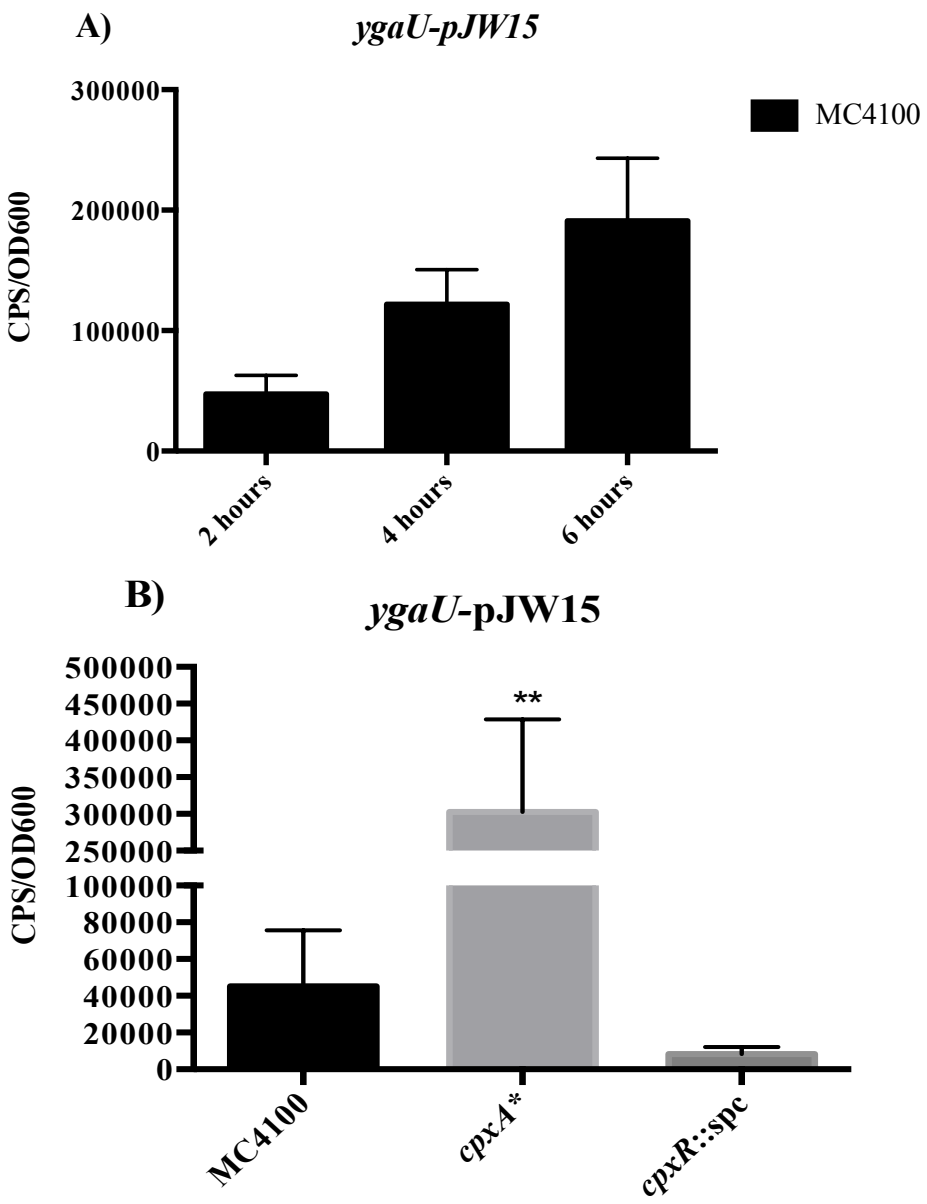
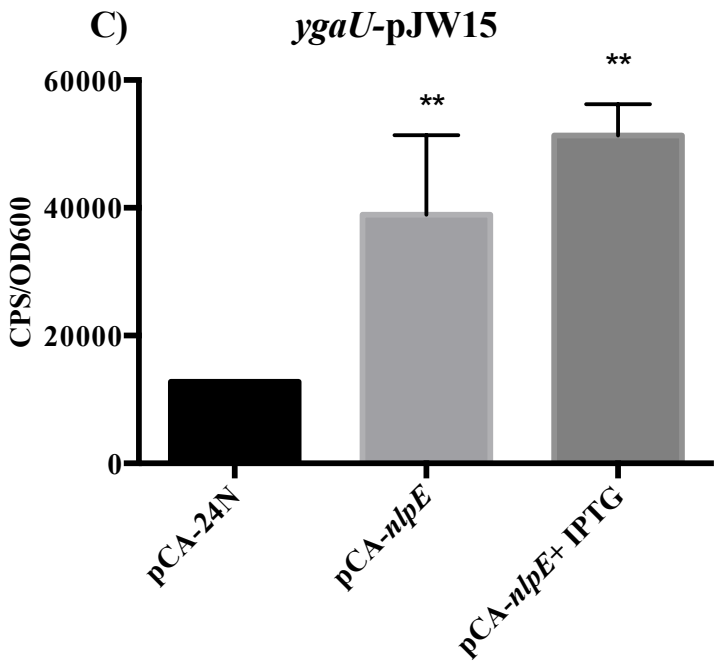


Figure 3-3 **:** CpxR~P binds specifically to the promoter of *degP*.

An electrophoretic mobility assay (EMSA) was performed in the presence of varying concentrations of CpxR~P (0-200pmol) incubated with 1.5pmol of PCR amplified promoters of A) *degP* (positive control) *B) rpoD* (negative control). Samples were loaded to a 5% non-denaturing TBE polyacrylamide gel (Bio-Rad) in 1X TBE running buffer and stained with ethidium bromide for visualization


A)
ygaU-pJW15
MC4100
CPS/OD600
300000
200000
100000
0
2 hours
4 hours
6 hours
B)
ygaU-pJW15
CPS/OD600
500000
450000
400000
350000
300000
250000
100000
80000
60000
40000
20000
0
**
MC4100
cpxA*
cpxR::spc


C)
ygaU-pJW15
60000
40000
20000
0
CPS/OD600
**
**
pCA-24N
pCA-nlpE
pCA-nlpE+ IPTG

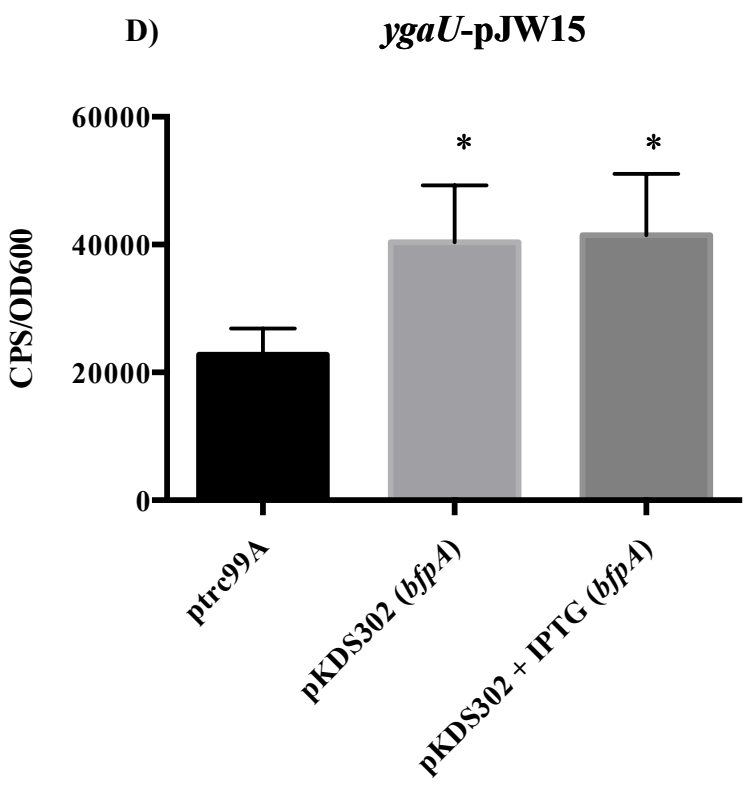

D)
ygaU-pJW15
60000
40000
20000
0
CPS/OD600
*
*
ptrc99A
pKDS302 (bfpA)
pKDS302 + IPTG (bfpA)

Figure 3-4 : *lux* assay*s* demonstrate that the Cpx pathway regulates *ygaU*

A*) lux* assay with *ygaU*-pJW15 vector in the MC4100 over the couse of 6-hours**.** **B***) lux* assay with *ygaU*-pJW15 vector in the MC4100, cpxA*, and *cpxR* backgrounds at 6 hours. **C)** *lux* assay with the *ygaU*-pJW15 vector in the presence the *pCA-nlpE* plasmid and the pCA-24N vector control. D) *lux* assay with the *ygaU*-pJW15 vector in the presence the pKDS302 plasmid and the ptrc99A vector control. A promoter fusion in plasmid pJW15 (Macritchie *et al.*, 2008) was constructed and transformed into wild-type MC4100, and strains carrying a *cpxA** allele, a *cpxR::spc* insertion mutation, the vector control pCA24-N for the NlpE over-expression vector pCA-NlpE,, and the vector control ptrc99A for the *bfpA-L* over-expression vector pKDS302. Strains were grown overnight in triplicate and sub-cultured (1:40) into fresh LB at 30ºC at 225 rpm for 6 hours. NlpE and Bfp over-expression was induced with IPTG (0.1mM) after two hours of sub-culture with IPTG (0.1 mM). Measurements shown were taken after 6 hours. CPS and OD_{600} were measured in a Wallac Victor 2 multilabel plate reader (Perkin Elmer Life Sciences). Asterisks represent significant levels: * $p \leq 0.05$, ** $p \leq 0.01$

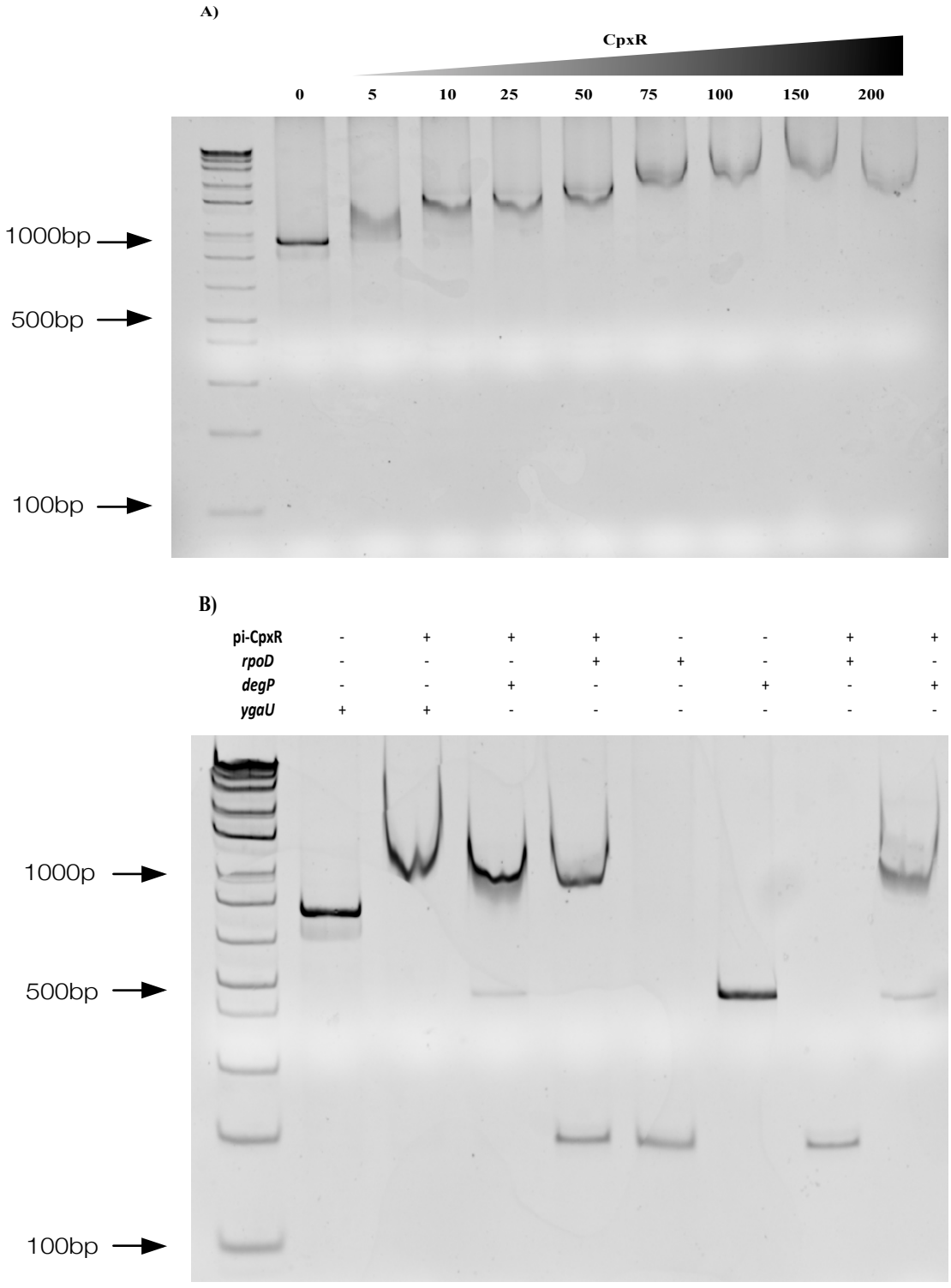

A)
CpxR
0
5
10
25
50
75
100
150
200
1000bp
500bp
100bp
B)
pi-CpxR - + + + - - + +
rpoD - - - + + - + -
degP - - + - - + - +
ygaU + + - - - - - -
1000p
500bp
100bp

Figure 3-5 : CpxR directly binds to the promoter of *ygaU.*

A) Gradient EMSA of *ygaU* **B)** Competitive EMSA of *ygaU.* Electrophoretic mobility shift assays (EMSAs) were performed after PCR products of the promoter region of *ygaU* were purified with the QiaGen purification kit. For the gradient EMSA of *ygaU,* varying concentrations of CpxR-MBP (0-200pMol) where incubated for 30 minutes at 37ªC with 1.5pmol of *ygaU* promoter in phosphorylation buffer. For the competitive *ygaU* EMSA, 50pmol of purified CpxR-MBP were incubated with or without acetyl-phosphate (Ac-Pi).). 1.5 pmol of PCR product two controls to assess binding specificity were present, *degP* (competitive control) and *rpoD* (non-competitive control). Reactions were also incubated for 30 minutes at 37ºC. EMSA reactions were run on a 5% non-denaturing polyacrylamide gel (BioRad) in 1X TBE at 90V. Non-denaturing polyacrylamide gels were stained with ethidium bromide for visualization.

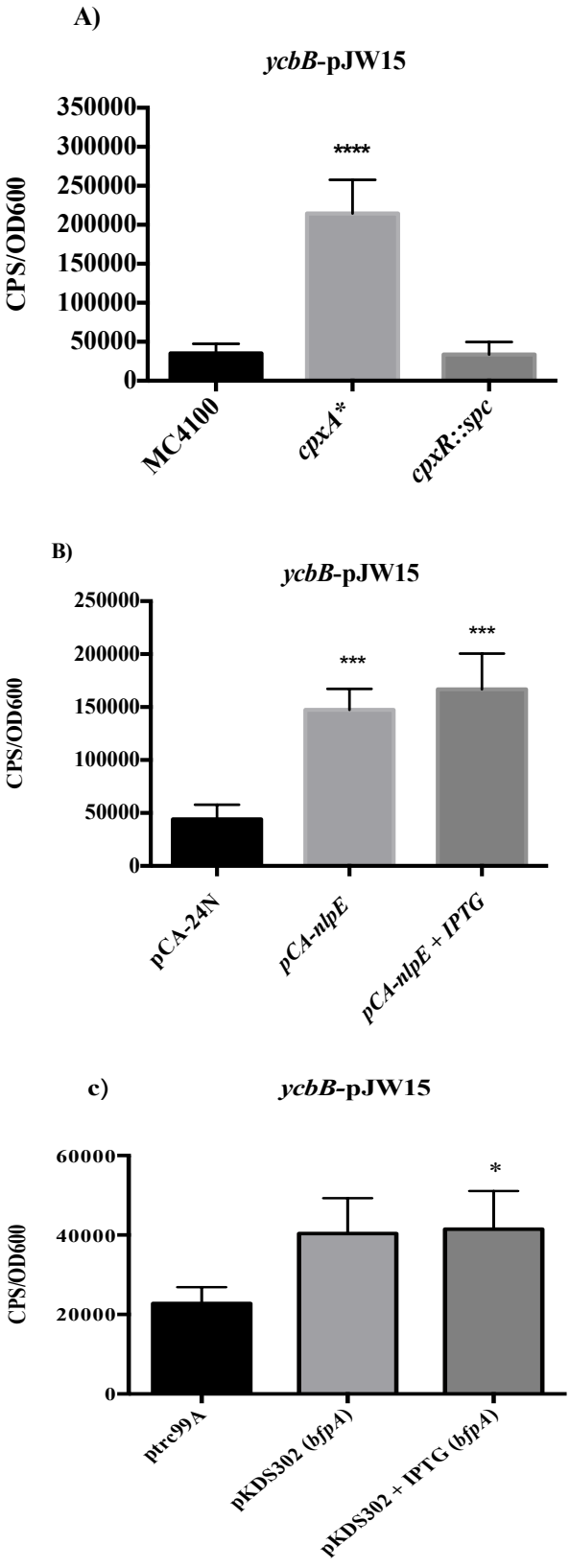
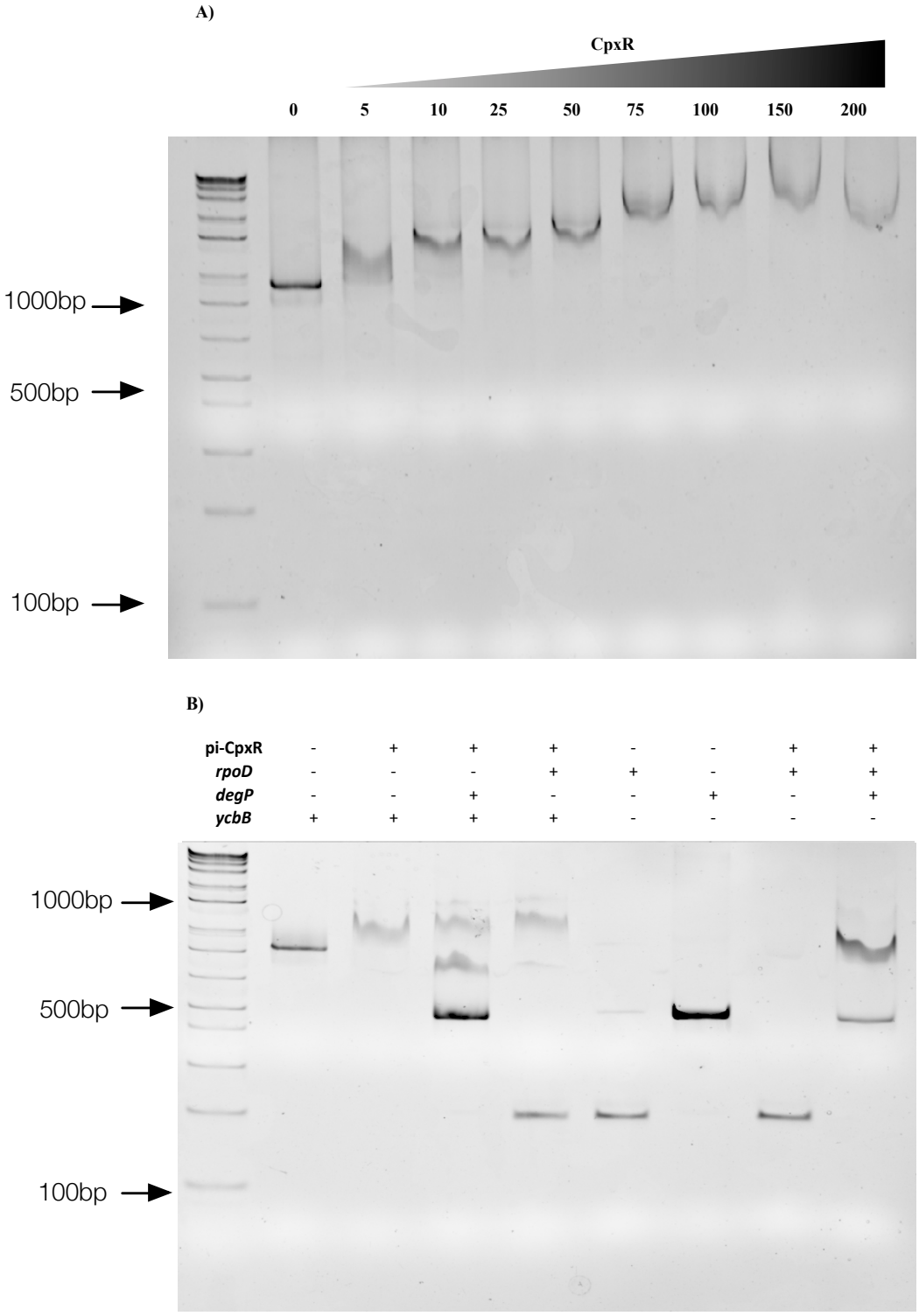

A)
ycbB-pJW15
CPS/OD600
350000
300000
250000
200000
150000
100000
50000
0
****
MC4100
cpxA*
cpxR::spc
B)
ycbB-pJW15
CPS/OD600
250000
200000
150000
100000
50000
0
***
***
pCA-24N
pCA-nlpE
pCA-nlpE + IPTG
c)
ycbB-pJW15
CPS/OD600
60000
40000
20000
0
*
ptrc99A
pKDS302 (bfpA)
pKDS302 + IPTG (bfpA)

Figure 3-6 : The *ycbB-lux* reporter gene is regulated by the Cpx response.

A) *lux* assay with *ycbB*-pJW15 vector in the MC4100, *cpxA**, and *cpxR* backgrounds. **B)** *lux* assay with the *ycbB*-pJW15 vector in the presence the *pCA-nlpE* plasmid and the pCA-24N vector control C) *lux* assay with the *ycbB*-pJW15 vector in the presence the pKDS302 plasmid and the ptrc99A vector control. A promoter fusion in plasmid pJW15 (Macritchie *et al.*, 2008) was constructed and transformed into wild-type MC4100, and strains carrying a *cpxA** allele, a *cpxR::spc* insertion mutation, the vector control pCA24-N for the NlpE over-expression vector pCA-NlpE,, and the vector control ptrc99A for the *bfp* over-expression vector pKDS302. Strains were grown overnight in triplicate and sub-cultured (1:40) into fresh LB at 30ºC at 225 rpm for 6 hours. NlpE and Bfp over-expression was induced with IPTG (0.1mM) after two hours of sub-culturing. Measurements shown were taken after 6 hours. CPS and OD_{600} were measured in a Wallac Victor 2 multilabel plate reader (Perkin Elmer Life Sciences). Asterisks represent significant levels: * $p \leq 0.05$, ** $p \leq 0.01$, *** $P \leq 0.001$, ****$P \leq 0.000$.


A)
CpxR
0
5
10
25
50
75
100
150
200
1000bp
500bp
100bp
B)
pi-CpxR - + + + - - + +
rpoD - - - + + - + +
degP - - + - - + - +
ycbB + + + + - - - -
1000bp
500bp
100bp

B)

Figure 3-7 :CpxR binds directly to the promoter of *ycbB*

Electrophoretic mobility shift assays (EMSAs) were performed after PCR products of the promoter region of *ycbB* were purified with the QiaGen purification kit. **A) Gradient EMSA of *ycbB*.** For the gradient EMSA of *ycbB,* varying concentrations of CpxR-MBP (0-200 pmol) where incubated for 30 minutes at 37ªC with 1.5pmol of *ycbB* promoter in phosphorylation buffer. **B) Competitive EMSA of *ycbB*.** For the competitive *ycbB* EMSA, 50 pmol of purified CpxR-MBP was incubated with or without acetyl-phosphate (Ac-Pi).). 1.5 pmol of PCR product two controls to assess binding specificity were present, *degP* (competitive control) and *rpoD* (non-competitive control). Reactions were also incubated for 30 minutes at 37ªC. The gradient and competitive *ycbB* EMSA reactions were run on a 5% non-denaturing polyacrylamide gel (BioRad) in 1X TBE at 90V. Non-denaturing polyacrylamide gels were stained with ethidium bromide for visualization.

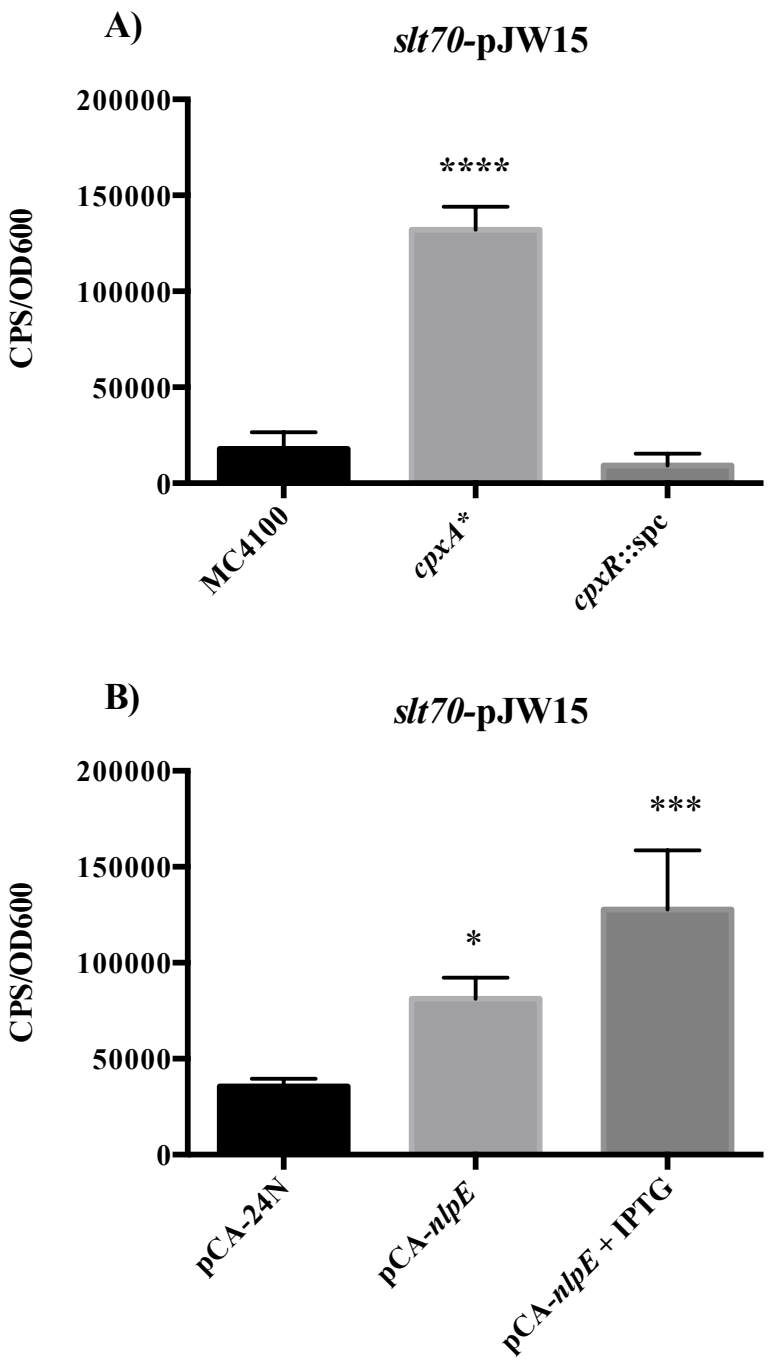

A)
slt70-pJW15
CPS/OD600
200000
150000
100000
50000
0
****
MC4100
cpxA*
cpxR::spc
B)
slt70-pJW15
CPS/OD600
200000
150000
100000
50000
0
*
***
pCA-24N
pCA-nlpE
pCA-nlpE + IPTG

Figure 3-8 *lux* a*ssays* demonstrate that the Cpx pathway regulates *slt70.*

A) *lux* assay with the *slt70*-pJW15 vector in the MC4100, cpxA*, and *cpxR* backgrounds. **B)** *lux* assay with the *slt70*-pJW15 vector in the presence of the *pCA-nlpE* plasmid and the pCA-24N vector control. Strains were grown overnight in triplicate and sub-cultured (1:40) into fresh LB at 30ºC at 225 rpm for 6 hours. NlpE over-expression was induced with IPTG (0.1mM) after two hours sub-culturing with IPTG (0.1 mM). Measurements shown were taken after 6 hours. CPS and OD_{600} were measured in a Wallac Victor 2 multilabel plate reader (Perkin Elmer Life Sciences). Asterisks represent significant levels: * $p \leq 0.05$, ** $p \leq 0.01$, *** $P \leq 0.001$, ****$P \leq 0.000$. Each assay was performed three times

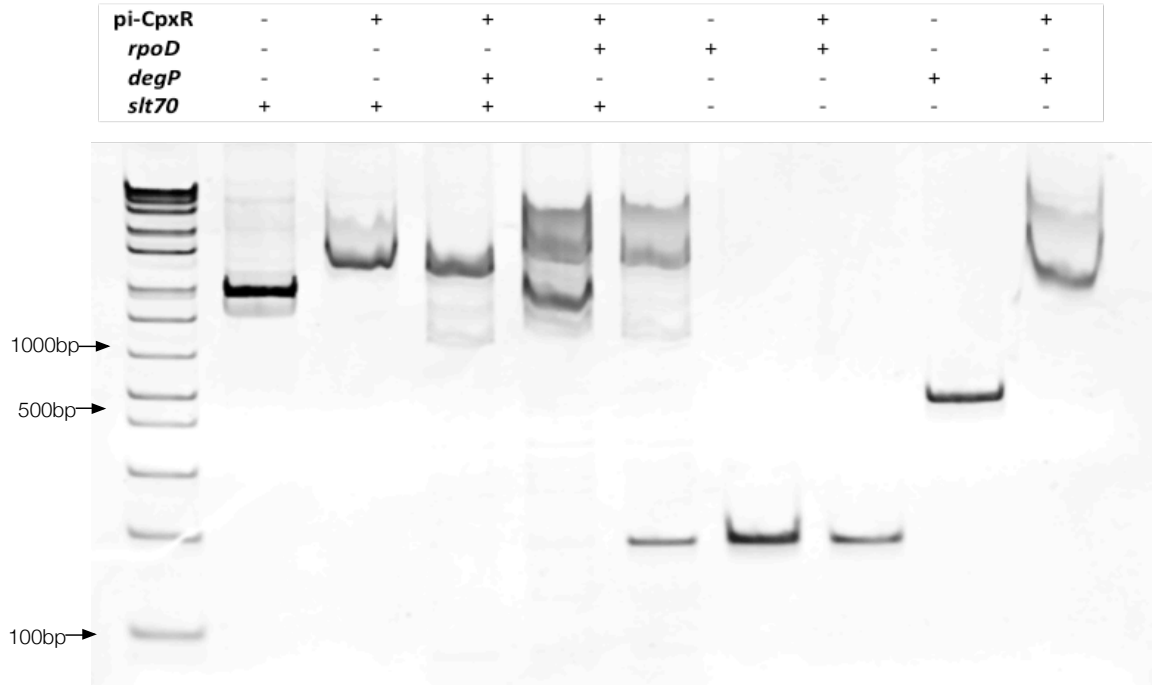


Figure 3-9 **:**CpxR binds directly to the promoter of *slt70*

Competitive EMSA of *slt70.* This Electrophoretic mobility shift assay (EMSAs) was performed after PCR products of the promoter region of *slt70* were purified with the QiaGen purification kit. 50pmol of purified CpxR-MBP was incubated with or without acetyl-phosphate (Ac-Pi).). 1.5 pmol of PCR product g two controls to assess binding specificity were present, *degP* (competitive control) and *rpoD* (non-competitive control). Reactions were incubated for 30 minutes at 37ªC. Reactions were then run on a 5% non-denaturing polyacrylamide gel (BioRad) in 1X TBE at 90V. Non-denaturing polyacrylamide gels were stained with ethidium bromide for visualization

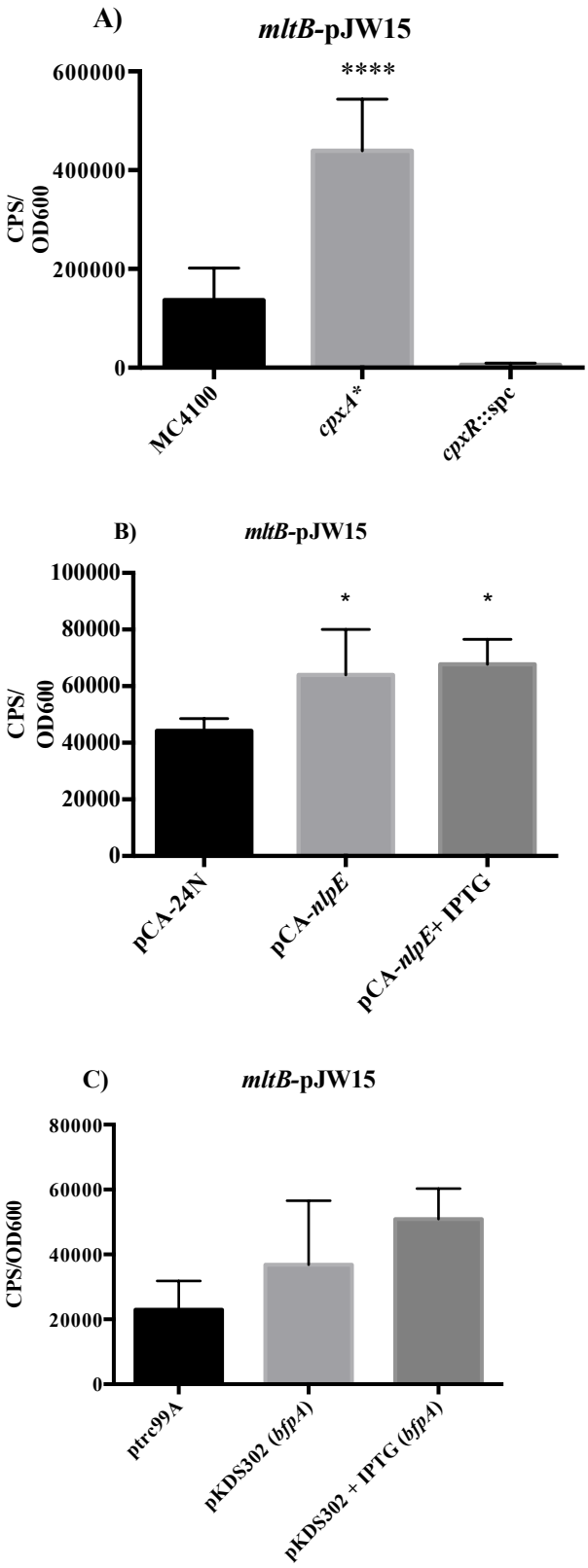


Figure 3-10 **:** *lux* a*ssays* demonstrate that the Cpx pathway regulates *mltB.*

A*) lux* assay with the *mltB*-pJW15 vector in the MC4100, cpxA*, and *cpxR* backgrounds. **B)** *lux* assay with the *mltB*-pJW15 vector in the presence the *pCA-nlpE* plasmid and the pCA-24N vector contro C) *lux* assay with the *mltB*-pJW15 vector in the presence the pKDS302 plasmid and the ptrc99A vector control. A promoter fusion in plasmid pJW15 (Macritchie *et al.*, 2008) was constructed and transformed into wild-type MC4100, and strains carrying a *cpxA** allele, a *cpxR::spc* insertion mutation, the vector control pCA24-N for the NlpE over-expression vector pCA-NlpE,, and the vector control ptrc99A for the *bfp* over-expression vector pKDS302. l. Strains were grown overnight in triplicate and sub-cultured (1:40) into fresh LB at 30ºC at 225 rpm for 6 hours. NlpE and Bfp over-expression was induced with IPTG (0.1mM) after two hours sub-culturing with IPTG (0.1 mM). Measurements shown were taken after 6 hours. CPS and OD_{600} were measured in a Wallac Victor 2 multilabel plate reader (Perkin Elmer Life Sciences). Differences were not statistically significant for C. Asterisks represent significant levels: * $p \leq 0.05$, ** $p \leq 0.01$, *** $P \leq 0.001$, ****$P \leq 0.000$. Each assay was performed three times.

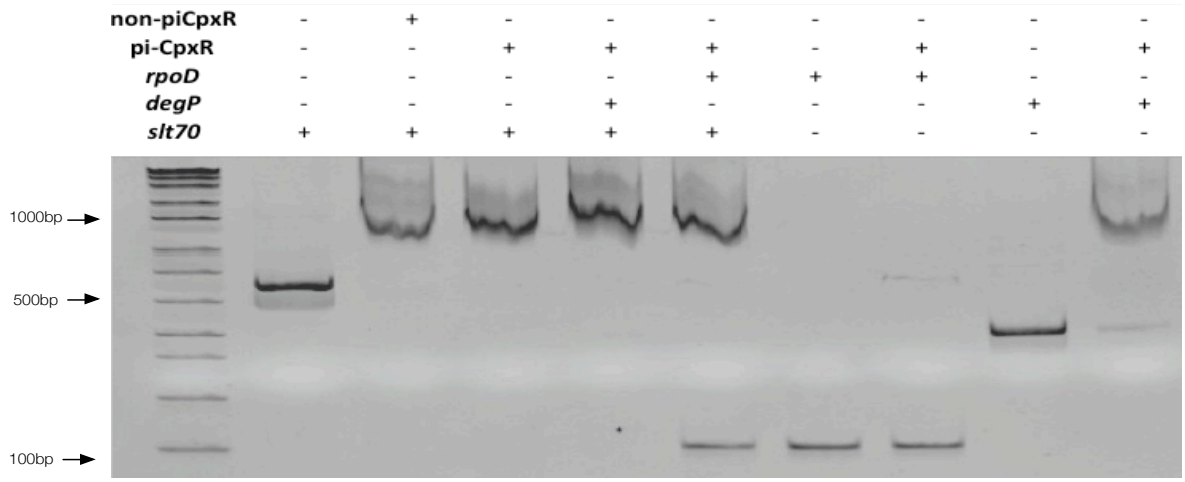


Figure 3-11 **:** CpxR binds directly to the promoter of *mltB*

Competitive EMSA of *mltB*. This Electrophoretic mobility shift assay (EMSAs) was performed after PCR products of the promoter region of *slt70* were purified with the QiaGen purification kit. 50 pmol of purified CpxR-MBP were incubated with or without acetyl-phosphate (Ac-Pi). 1.5 pmol of PCR product of two controls to assess binding specificity were present, *degP* (competitive control) and *rpoD* (non-competitive control). Reactions were also incubated for 30 minutes at 37ªC. Reactions were then run on a 5% non-denaturing polyacrylamide gel (BioRad) in 1X TBE at 90V. Non-denaturing polyacrylamide gels were stained with ethidium bromide for visualization

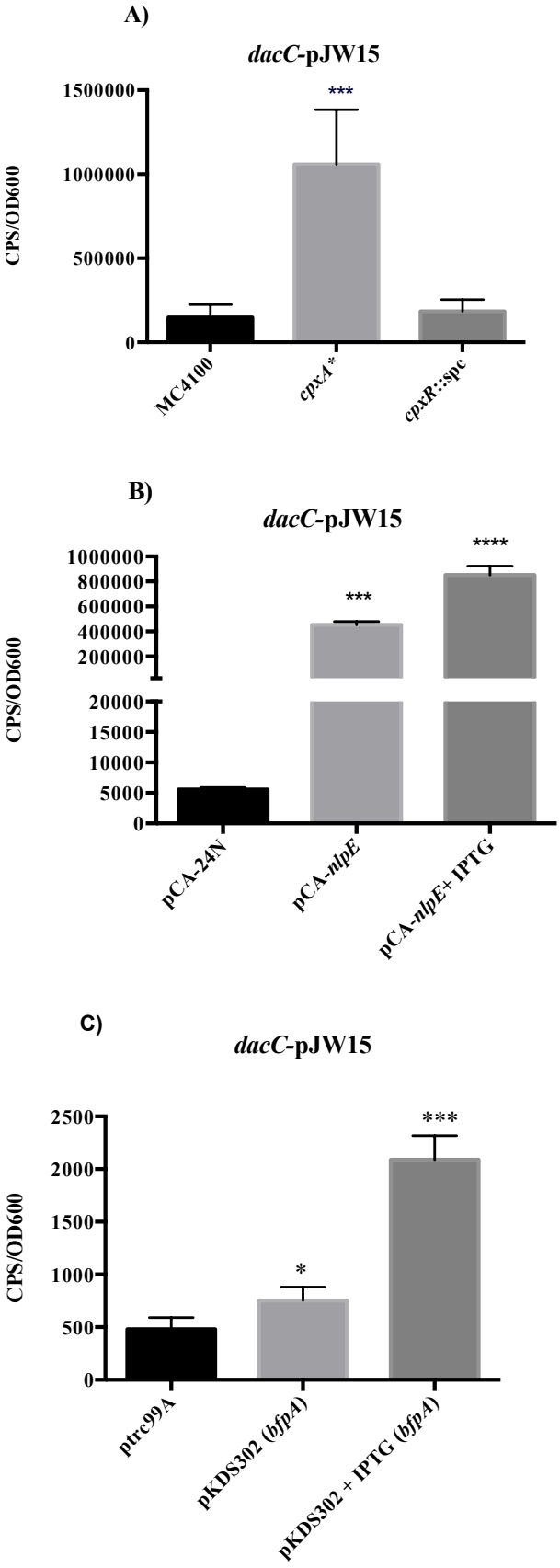

A)
dacC-pJW15
CPS/OD600
1500000
1000000
500000
0
***
MC4100
cpxA*
cpxR::spc
B)
dacC-pJW15
CPS/OD600
1000000
800000
600000
400000
200000
20000
15000
10000
5000
0
***
****
pCA-24N
pCA-nlpE
pCA-nlpE+ IPTG
C)
dacC-pJW15
CPS/OD600
2500
2000
1500
1000
500
0
*
***
ptrc99A
pKDS302 (bfpA)
pKDS302 + IPTG (bfpA)

Figure 3-12 *:lux assays* demonstrate that the Cpx pathway regulates *dacC.*

A*) lux* assay with the *dacC*-pJW15 vector in the MC4100, cpxA*, and *cpxR* backgrounds. **B)** *lux* assay with the *dacC*-pJW15 vector in the presence the *pCA-nlpE* plasmid and the pCA-24N vector control. C) *lux* assay with the *dacC*-pJW15 vector in the presence the pKDS302 plasmid and the ptrc99A vector control. A promoter fusion in plasmid pJW15 (Macritchie *et al.*, 2008) was constructed and transformed into wild-type MC4100, and strains carrying a *cpxA** allele, a *cpxR::spc* insertion mutation, the vector control pCA24-N for the NlpE over-expression vector pCA-NlpE,, and the vector control ptrc99A for the *bfp* over-expression vector pKDS302. Strains were grown overnight in triplicate and sub-cultured (1:40) into fresh LB at 30ºC at 225 rpm for 6 hours. NlpE over-expression was induced with IPTG (0.1mM) after two hours sub-culturing with IPTG (0.1 mM). Measurements shown were taken after 6 hours. CPS and OD_{600} were measured in a Wallac Victor 2 multilabel plate reader (Perkin Elmer Life Sciences). Asterisks represent significant levels: * $p \leq 0.05$, ** $p \leq 0.01$, *** $P \leq 0.001$, ****$P \leq 0.000$. Each assay was performed three times.

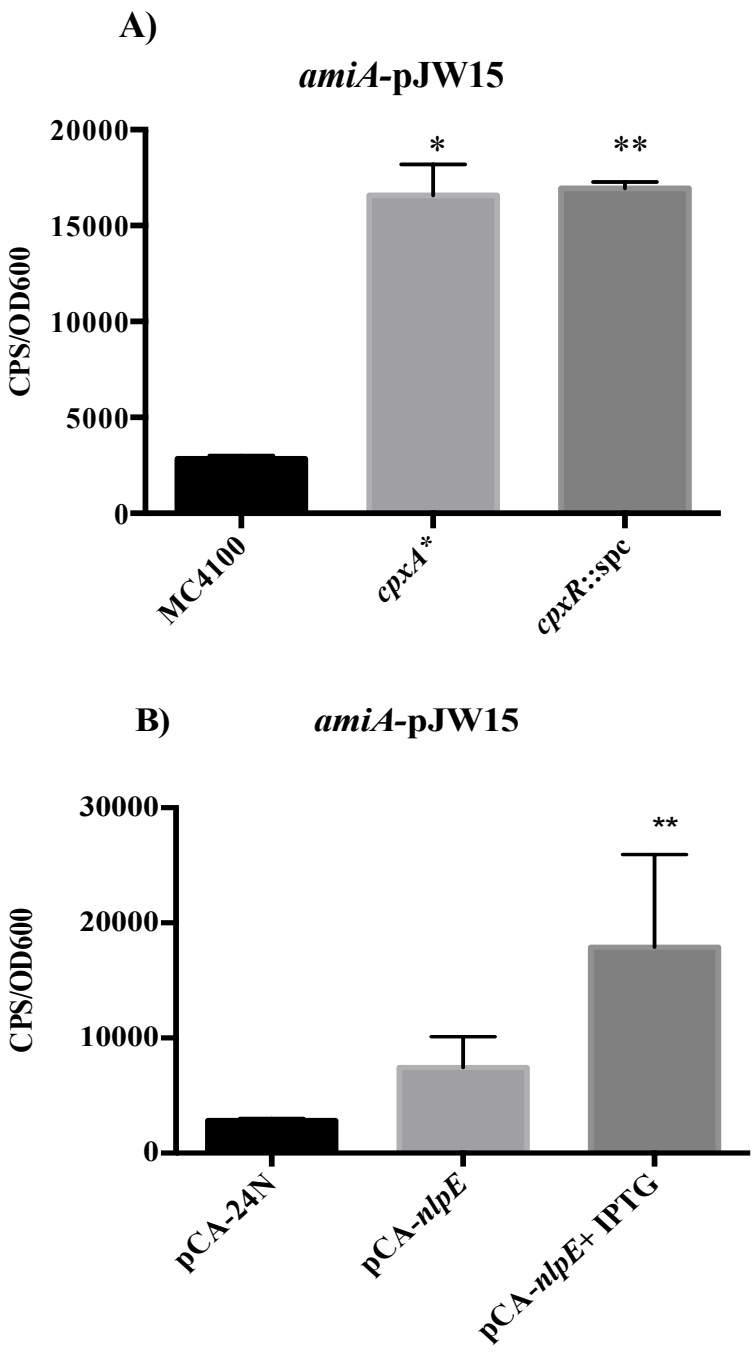


Figure 3-13 *lux* a*ssays* demonstrate that the Cpx regulation of *amiA* expression is complex

A*) lux* assay with the *amiA*-pJW15 vector in the MC4100, *cpxA**, and *cpxR* backgrounds. **B)** *lux* assay with the *amiA* -pJW15 vector in the presence the *pCA-nlpE* plasmid and the pCA-24N vector control. Strains were grown overnight in triplicate and sub-cultured (1:40) into fresh LB at 30ºC at 225 rpm for 6 hours. NlpE over-expression was induced with IPTG (0.1mM) after two hours sub-culturing with IPTG (0.1 mM). Measurements shown were taken after 6 hours. CPS and OD_{600} were measured in a Wallac Victor 2 multilabel plate reader (Perkin Elmer Life Sciences). Asterisks represent significant levels: * $p \leq 0.05$, ** $p \leq 0.01$, *** $P \leq 0.001$, ****$P \leq 0.000$. Each assay was performed three times.

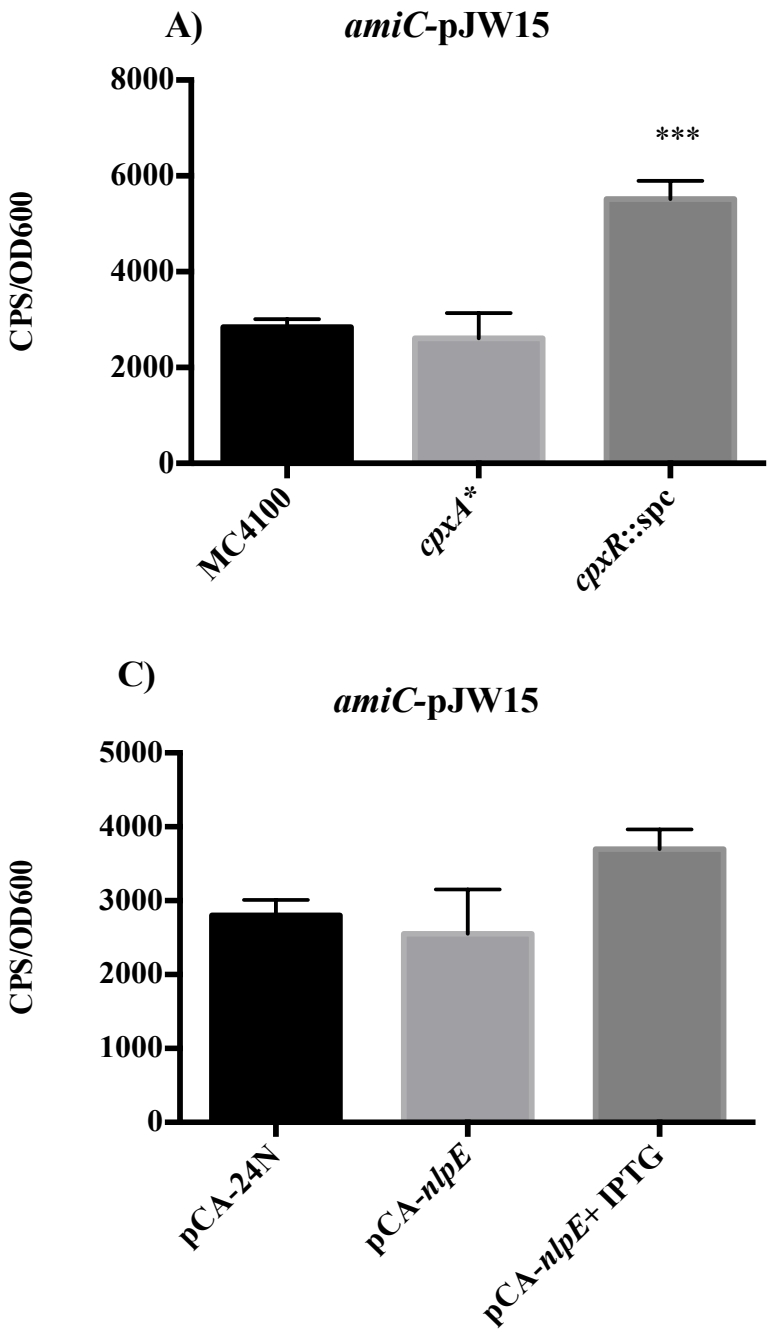


Figure 3-14 **:** *lux* a*ssays* demonstrate that *amiC* expression might not be Cpx-regulated

A*) lux* assay with the *amiC*-pJW15 vector in the MC4100, *cpxA**, and *cpxR* backgrounds. **B)** *lux* assay with the *amiC* -pJW15 vector in the presence the *pCA-nlpE* plasmid and the pCA-24N vector control. Strains were grown overnight in triplicate and sub-cultured (1:40) into fresh LB at 30ºC at 225 rpm for 6 hours. NlpE over-expression was induced with IPTG (0.1mM) after two hours sub-culturing with IPTG (0.1 mM). Measurements shown were taken after 6 hours. CPS and OD_{600} were measured in a Wallac Victor 2 multilabel plate reader (Perkin Elmer Life Sciences).

Asterisks represent significant levels*** $P \leq 0.001$. No significant differences were observed.

References

Baquero, M.R., Bouzon, M., Quintela, J.C., Ayala, J.A., and Moreno, F. (1996) *dacD*, an *Escherichia coli* gene encoding a novel penicillin-binding protein (PBP6b) with DD-carboxypeptidase activity. *J Bacteriol* **178**: 7106-7111.

Bernhardt, T.G., and De Boer, P.A.J. (2003) The *Escherichia coli* amidase AmiC is a periplasmic septal ring component exported via the twin-arginine transport pathway. *Molecular Microbiology* **48**: 1171-1182.

Buist, G., Steen, A., Kok, J., and Kuipers, O.P. (2008) LysM, a widely distributed protein motif for binding to (peptido)glycans. *Mol Microbiol* **68**: 838-847.

Cosma, C.L., Danese, P.N., Carlson, J.H., Silhavy, T.J., and Snyder, W.B. (1995) Mutational activation of the Cpx signal transduction pathway of *Escherichia coli* suppresses the toxicity conferred by certain envelope-associated stresses. *Molecular microbiology* **18**: 491-505.

Danese, P.N., Oliver, G.R., Barr, K., Bowman, G.D., Rick, P.D., and Silhavy, T.J. (1998) Accumulation of the enterobacterial common antigen lipid II biosynthetic intermediate stimulates degP transcription in *Escherichia coli*. *J Bacteriol* **180**: 5875-5884.

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

Dijkstra, B.W., Thunnissen, A.-M.W., and Asselt, E.J.V. (1999) High Resolution Crystal Structures of the *Escherichia coli* Lytic Transglycosylase Slt70 and its Complex with a Peptidoglycan Fragment. *Journal of Molecular Microbiology.* **4:** 877-898

Engel, H., Kazemier, B., and Keck, W. (1991) Murein-metabolizing enzymes from *Escherichia coli*: sequence analysis and controlled overexpression of the *slt* gene, which encodes the soluble lytic transglycosylase. *Journal of bacteriology* **173**: 6773-6782.

Gangaiah, D., Zhang, X., Fortney, K.R., Baker, B., Liu, Y., Munson, R.S., and Spinola, S.M. (2013) Activation of CpxRA in *Haemophilus ducreyi* Primarily Inhibits the Expression of Its Targets, Including Major Virulence Determinants. *J Bacteriol* **195**: 3486-3502.

Heidrich, C., Templin, M.F., Ursinus, A., Merdanovic, M., Berger, J., Schwarz, H., *et al.* (2001) Involvement of N-acetylmuramyl-l-alanine amidases in cell separation and antibiotic-induced autolysis of *Escherichia coli*. *Molecular microbiology* **41**: 167-178.

Höltje, J.V., Mirelman, D., Sharon, N., and Schwarz, U. (1975) Novel type of murein transglycosylase in *Escherichia coli*. *Journal of bacteriology* **124**: 1067-1076.

Jubelin, G., Vianney, A., Beloin, C., Ghigo, J.-M., Lazzaroni, J.-C., Lejeune, P., and Dorel, C. (2005) CpxR/OmpR interplay regulates curli gene expression in response to osmolarity in *Escherichia coli*. *J Bacteriol* **187**: 2038-2049.

Macritchie, D.M., Ward, J.D., Nevesinjac, A.Z., and Raivio, T.L. (2008) Activation of the Cpx envelope stress response down-regulates expression of several locus of enterocyte effacement-encoded genes in enteropathogenic *Escherichia coli*. *Infect Immun* **76**: 1465-1475.

Magnet, S., Dubost, L., Marie, A., Arthur, M., and Gutmann, L. (2008) Identification of the L,D-transpeptidases for peptidoglycan cross-linking in *Escherichia coli*. *J Bacteriol* **190**: 4782-4785.

Pedersen, L.B., Murray, T., Popham, D.L., and Setlow, P. (1998) Characterization of *dacC*, which encodes a new low-molecular-weight penicillin-binding protein in Bacillus subtilis. *J Bacteriol* **180**: 4967-4973.

Pogliano, J., Lynch, A.S., Belin, D., Lin, E.C., and Beckwith, J. (1997) Regulation of *Escherichia coli* cell envelope proteins involved in protein folding and degradation by the Cpx two-component system. *Genes & Development* **11**: 1169-1182.

Price, N.L., and Raivio, T.L. (2009) Characterization of the Cpx regulon in *Escherichia coli* strain MC4100. *J Bacteriol* **191**: 1798-1815.

Priyadarshini, R., de Pedro, M.A., and Young, K.D. (2007) Role of peptidoglycan amidases in the development and morphology of the division septum in *Escherichia coli*. *J Bacteriol* **189**: 5334-5347.

Raivio, T.L., and Silhavy, T.J. (1997) Transduction of envelope stress in *Escherichia coli* by the Cpx two-component system. *Journal of bacteriology* **179**: 7724-7733.

Raivio, T.L., Leblanc, S.K.D., and Price, N.L. (2013) The *Escherichia coli* Cpx envelope stress response regulates genes of diverse function that impact antibiotic resistance and membrane integrity. *J Bacteriol* **195**: 2755-2767.

Raivio, T.L., Popkin, D.L., and Silhavy, T.J. (1999) The Cpx envelope stress response is controlled by amplification and feedback inhibition. *Journal of bacteriology* **181**: 5263-5272.

Sanders, A.N., and Pavelka, M.S. (2013) Phenotypic Analysis of E. coli Mutants Lacking L,D-transpeptidases. *Microbiology* .

Santos, J.M., Lobo, M., Matos, A., De Pedro, M.A., and Arraiano, C.M. (2002) The gene bolA regulates *dacA* (PBP5), *dacC* (PBP6) and *ampC* (AmpC), promoting normal morphology in *Escherichia coli*. *Molecular microbiology* **45**: 1729-1740.

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

Suvorov, M., Lee, M., Hesek, D., Boggess, B., and Mobashery, S. (2008) Lytic transglycosylase MltB of *Escherichia coli* and its role in recycling of peptidoglycan strands of bacterial cell wall. *J Am Chem Soc* **130**: 11878-11879.

van Asselt, E.J., Dijkstra, A.J., Kalk, K.H., Takacs, B., Keck, W., and Dijkstra, B.W. (1999) Crystal structure of *Escherichia coli* lytic transglycosylase Slt35 reveals a lysozyme-like catalytic domain with an EF-hand. *Structure* **7**: 1167-1180.

van Asselt, E.J., Kalk, K.H., and Dijkstra, B.W. (2000) Crystallographic studies of the interactions of *Escherichia coli* lytic transglycosylase Slt35 with peptidoglycan. *Biochemistry* **39**: 1924-1934.

Vogt, S.L., and Raivio, T.L. (2012) Just scratching the surface: an expanding view of the Cpx envelope stress response. *FEMS Microbiol Lett* **326**: 2-11.

Vogt, S.L., Nevesinjac, A.Z., Humphries, R.M., Donnenberg, M.S., Armstrong, G.D., and Raivio, T.L. (2010) The Cpx envelope stress response both facilitates and inhibits elaboration of the enteropathogenic *Escherichia coli* bundle-forming pilus. *Mol Microbiol* **76**: 1095-1110.

Weatherspoon-Griffin, N., Zhao, G., Kong, W., Kong, Y., Morigen, Andrews-Polymenis, H., *et al.* (2011) The CpxR/CpxA two-component system up-regulates two Tat-dependent peptidoglycan amidases to confer bacterial resistance to antimicrobial peptide. *J Biol Chem* **286**: 5529-5539.

De Wulf, P., and Lin, E.C.C. (2000) Cpx Two-Component Signal Transduction in*Escherichia coli*: Excessive CpxR-P Levels Underlie CpxA* Phenotypes. *Journal of bacteriology* **182**: 1423-1426.

Yeats, C., and Bateman, A. (2003) The BON domain: a putative membrane-binding domain. Trends Biochem Sci 28: 352-355.

CHAPTER 4

Activation of the Cpx pathway leads to changes in cell morphology, antibiotic resistance, and peptidoglycan composition of *Escherichia coli* MC4100.

4.1 Overview

As mentioned in section 3, a recent microarray analysis by our group revealed that activation of the Cpx pathway up-regulates seven cell wall genes: *ygaU, slt70, ycbB, dacC* and *amiA* (Raivio *et al.*, 2013). In addition, we investigated if *amiC* is also regulated in *E. coli* as it has been shown that this gene is part of the Cpx regulon in Salmonella (Weatherspoon-Griffin *et al.*, 2011). We also determined if *mltB* is part of the Cpx regulon as the soluble form of MltB, Slt35, is similar in function to Slt70 (van Asselt *et al.*, 2000). After we verified the regulation of these genes by *lux* assays we observed that *ygaU, ycbB, slt70,* and *mltB* were highly up-regulated by the Cpx pathway. Furthermore, electrophoresis mobility assays (EMSAs) revealed that *ygaU, ycbB, slt70,* and *mltB* are directly regulated by CpxR. Interestingly, the fact that the Cpx pathway regulates genes related to peptidoglycan synthesis and remodelling could mean that it is important to modify this structure to withstand the stressful conditions sensed by the Cpx pathway. Modification at the right time to ensure adaptation is crucial as the peptidoglycan layer has to be rigid to protect the integrity of the cell wall, but it must also be flexible to allow growth and adaptation to the environment (Young, 2011), also this structure has to be porous to allow compounds to enter and leave the cells. In

addition, activation of the Cpx pathway down-regulates curli expression (Dorel *et al.*, 1999; Jubelin *et al.*, 2005). Participation of peptidoglycan hydrolases is important for this process as these enzymes need to remove curli subunits without causing breakages to the cell wall that could lead to cell death (Young, 2011). Curli production is also down-regulated in the presence of N-acetylglucosamine 6-phosphate, a resulting product of peptidoglycan recycling (Toba *et al.*, 2011).

With this in mind we decided to further study and characterize the role of *ygaU, slt70, mltB* and *ycbB* in the peptidoglycan structure and composition of *E. coli* during activation of the Cpx pathway. For this, we first assessed the bacterial morphology of various *ygaU, ycbB, mltB,* and *slt70* mutants in wild-type and *cpxA24* backgrounds by transmission electron microscopy (TEM) as bacterial shape can be correlated to changes in the cell wall organization or composition (Cava *et al.*, 2013). Furthermore, we hypothesized that changes in peptidoglycan turn over or recycling could occur due to the absence of specific cell wall genes regulated by the Cpx pathway. We determined the minimum inhibitory concentration (MICs) to different β-lactam antibiotics for the various *ygaU, ycbB, mltB,* and *slt70* mutants in wild-type and *cpxA24* backgrounds and we observed numerous changes in the MICs when these genes were absent in the *cpxA24* background compared to the wild-type. Finally, we analyzed the peptidoglycan composition of MC4100, as well as strains containing combinations of various alleles of *cpxA, cpxR::spc,* and the *ygaU, ycbB, mltB,* and *slt70* mutations. We found that activation of the Cpx pathway causes an increase in peptidoglycan turnover, recycling, and DAP-DAP cross-links.

4.2 Activation or absence of the Cpx pathway leads to changes in cell morphology.

Microorganisms generally vary their cell shape in response to environmental stresses. MC4100, *cpxA24* and *cpxR::spc* strains were observed using transmission electron microscopy (TEM). The *cpxA24* mutant grows slower than the wild-type and the *cpxR::spc* mutant (data not shown). In stationary phase *E. coli* has a high percentage of cocci, while rods and filaments are present in a lesser proportion according to microscopy analysis (Kolter *et al.*, 1993), while the *cpxA24* and the *cpxR::spc* mutants are characterized by an increase in filaments (Pogliano *et al.*, 1998; Herbert *et al.*, 2007) . As expected, these three strains exhibited different significant differences in cell morphology (Figure 4-1). In order to quantify morphological changes, we decided to further classify the bacteria visualized into two categories: bacteria measuring between 1-5 μm, and bacteria measuring more than 5μm (filaments) (Zobell and Cobet, 1964). The *cpxA24* and the *cpxR*::spc mutants exhibited a similar increase in the percentage of filamentation compared to the wild-type (Figure 4-2). Increased filamentation in *cpxA24* mutants has previously been reported in *Escherichia coli (Pogliano et al., 1998)* due to a mislocalization of the FtsZ ring. Furthermore, increased filamentation has also been reported for *Xenorhabdus nematophila* Δ*cpxR* mutants suggesting that CpxR is involved in maintaining normal cell division and shape (Herbert *et al.*, 2007). We also decided to observe transverse sections to visualize further morphological changes for these mutants using TEM (Figure 4-

3). The *cpxA24* mutants exhibited a distinctive enlargement of the periplasmic space and chromatin condensation that may be indicative of changes in the cell wall or the membrane composition (Pawlowski *et al.*, 2011).

4.3 Absence of *ygaU* and *ycbB* cause an increase in filamentation

We also assessed whether the Cpx-regulated genes *ygaU, ycbB, slt,* and *mltB* were important for the increased filamentation observed in the *cpxA24* mutant. We did this by measuring the lengths of MC4100 and *cpxA24* strains carrying mutations in *ygaU, ycbB, slt,* and *mltB*. We only saw changes in the amount of filamentation for the *cpxA24 ygaU::kan* mutant, which displayed a significant increase of the filamentous phenotype compared to the *cpxA24* mutant ($P<0.01$, two-way ANOVA) (Figure 4-4, Figure 4-5). YcbB is an L,D-transpeptidase that catalyzes formation of 3-3 cross-links in peptidoglycan that might be expected to impede cell division, as is observed in the *cpxA24* strain (Figure 4-1). To determine if YcbB might contribute to the increased filamentation already seen for *ygaU* mutant (Figures 4-3, 4-4), we decided to also analyze the *ycbB ygaU::kan* double mutants in the MC4100 and in the *cpxA24* backgrounds (Figure 4-4, Figure 4-5). We observed a significant increase in the percentage of filamentation in both backgrounds. However, the increase in the filamentation percentage was slightly higher in the *cpxA24* than in MC4100 (Figure 4-4, Figure 4-5). Interestingly, various cell wall proteins and PBPs help orient the FtsZ ring.

Absence of these proteins causes changes in cell morphology and filamentation (Varma and Young, 2009). This suggests that *ycbB* and *ygaU* might be some other proteins besides PBPs that participate in morphogenesis of *E. coli* during activation of the Cpx pathway.

4.4 Activation of the Cpx pathway increases resistance to various β-lactams

Resistance to β-lactam antibiotics can be due to a decrease in peptidoglycan turnover and autolysis (Sieradzki and Tomasz, 2003). We decided to determine whether the changes caused to the cell wall during the activation of the Cpx pathway lead to resistance. We tested the minimum-inhibitory concentrations (MICs) of MC4100, *cpxA24, cpxR::spc,* and various single and double mutants in the MC4100 and *cpxA24* backgrounds to Ampicillin, Piperacillin,, Cloxacillin, Carbenicillin, and Tobramycin (an aminoglycoside, which *cpxA24* mutants are known to be resistant to) (Table 4-1). The *cpxA24* strain exhibited just a 2-fold increase in the resistance to most β-lactam antibiotics tested when compared to MC4100, except for carbenicillin to which there was a 16-fold increase. In contrast, the *cpxR::spc* strain exhibited an increase in the sensitivity to most of the β-lactams tested compared to the wild-type, with the exception of carbenicillin, to which there was a 4-fold increase. These results suggest that activation of the Cpx pathway might play a role in resistance to the β-lactam antibiotics tested here.

As a positive control, we also decided to assess the MICs to the aminoglycoside tobramycin, as mutations that activate the Cpx response are known to facilitate resistance to aminoglycosides (Mahoney and Silhavy, 2013). As expected, we

observed that the *cpxA24* mutant exhibited an almost 10-fold increase in the resistance to this antibiotic when compared to the wild-type, while all of the cell wall mutants in the *cpxA24* background abrogated this resistance. This could be an indirect effect due to changes in the structure of the cell wall in the absence of *ycbB, ygaU* or *slt70* as the absence of these *ycbB* (Triboulet *et al.*, 2013) has been linked to weakening of the cell wall and an increased permeability (Monteiro *et al.*, 2011). These two scenarios have been found to increase the efficiency of aminoglycoside antibiotics like tobramycin (Vakulenko and Mobashery, 2003)

4.4.1 Slt70 and MltB participate in the resistance to carbenicillin, piperacillin, and cloxacillin

Mutations in the soluble lytic transglycosylase *slt70* caused a 2-fold decrease in the resistance to carbenicillin in the *cpxA24* background, and a 2 to 3-fold decreased susceptibility to piperacillin and cloxacillin in the MC4100 background. In addition, the *cpxA24 mltB::*kan mutant exhibited no changes in the resistance to carbenicillin compared to its parent strain, while the MC4100 *mltB::*kan strain showed a 2-fold decrease and a 3-fold increase in the resistance to piperacillin and cloxacillin compared to MC4100 (Table 4-1). MltB and Slt70 have been shown to interact with PBP1b, PBP1c, PBP2, and PBP3 (Mitsuyama *et al.*, 1987; Sainsbury *et al.*, 2011). These PBPs bind to carbenicillin and piperacillin, which could explain way absence of *slt70* and *mltB* cause changes in the resistance to carbeniclin and piperacillin.

4.4.3 Mutations in *ygaU* cause an increase to β-lactam resistance during activation of the Cpx pathway

In general, the absence of *ygaU* caused changes in carbenicillin resistance (Table 4-1). The *cpxA24 ygaU:: kan* mutant displayed a 2-fold decrease in the resistance to carbenicillin compared to the *cpxA24* mutant, while the MC4100 *ygaU::kan* mutant displayed a 3-fold increase in the resistance to this β-lactam. In addition, the MC4100 *ygaU::kan* displayed a 3-fold decrease in the resistance to piperacillin These data suggest that this gene confers changes to the peptidoglycan structure probably through changes in the expression of β-lactamases and PBPs PBP1a, 1b, and 5 (Black and Girdwood, 1969; Bell *et al.*, 1985) .

4.4.4 Absence of *ycbB* led to a decrease in resistance to carbenicillin and cloxacillin

The *cpxA24 ycbB* mutant exhibited diminished resistance to all β-lactams, compared to the *cpxA24* parent strain TR10 (Table 4-1). This correlates well with the known L,D-transpeptidase activity of YcbB, which catalyzes 3-3 DAP-DAP cross-links, that cannot be disrupted by β-lactams (Magnet *et al.*, 2008). This data further suggests that during activation of the Cpx response, as in the *cpxA24* strain, YcbB plays an important role in the elevated resistance to β-lactam antibiotics. YcbB may also be active, although less so, in a wild-type strain background, since the MC4100 *ycbB::kan* mutant exhibited a 2-fold decrease in resistance to ampicillin (Table 4-1). This mutant was also curiously more resistant to carbenicillin, although we do not currently understand why (Table 4-1).

4.4.5 Simultaneous absence of *ygaU* and *ycbB* restores or results in slightly increased resistance to β-lactam antibiotics

Interestingly, simultaneous deletion of *ycbB* and *ygaU* in the *cpxA24* mutant background restored β-lactam resistance to that of the parent TR10 strain (Table 4-1). In the wild-type MC4100 background, simultaneous deletion of these genes caused a 3-fold decrease in the resistance to ampicillin and cloxacillin (Table 4-1). Since deletion of *ycbB* caused a decrease in b-lactam resistance in the *cpxA24* background, (Table 4-1), these data suggest that the effects of these two gene products may be additive for this phenotype when the Cpx response is induced as when the inhibitory effect of YgaU on β-lactam resistance is removed, YcbB is no longer required for resistance. Furthermore, these results suggest that YgaU and YcbB might interact with PBP2a which is necessary for resistance to cloxacillin (Parker and Hewitt, 1970), and PBP3, which is necessary for resistance to piperacillin (Mitsuyama *et al.*, 1987).

4.5 HPLC analysis reveals that the Cpx pathway causes changes in the peptidoglycan structure

Observation of the cell morphology and analysis of the MICs to ampicillin, carbenicillin, cloxacillin, and piperacillin for MC4100, *cpxA24,* and *cpxR::spc* strains suggested that the cell wall is modified when the Cpx pathway is activated. To determine what kinds of changes the cell wall undergoes when the Cpx pathway is activate, we isolated the sacculi from wild-type, *cpxA24,* and *cpxR*

strains and performed HPLC analysis with the collaboration of Dr. Juan Alfonso Ayala from the Centre of Molecular Biology Severo Ochoa (Madrid, Spain) (Table 4-2). The general muropeptide profile (Table 4-3) indicated that the *cpxA24* mutant contained high levels of trimers, 3-3 cross-links (DAP-DAP), and a slight increase in the levels of anhydro-disaccharide muropeptides (Anhydro), and cross-linking. Furthermore, the *cpxR::spc* mutant displayed a similar muropeptide profile to MC4100. However, this strain also displayed a decrease in the amount of muropeptides cross-linked with a lipoprotein (Lipo), and anhydro-disaccharides (Anhydro). We also observed a significant increase in the levels of disaccharide-tripeptide-disaccharide-tripeptide with a DAP-DAP acid crosslink (D33D), disaccharide-tripeptide-disaccharide-tripeptide bound to a lipoprotein with a DAP-DAP acid crosslink (D33DL), disaccharide-tripeptide-disaccharide-tetrapeptide with a 3-4 cross-link and a glycan reducing end (D43N) in the *cpxA24* strain. In addition, the *cpxA24* also displayed a decrease in the levels of disaccharide-tetrapeptide with a glycine end (M4G), disaccharide-tripeptide with a glycan-reducing end (M4N) and monomer tripeptides bound to a lipoprotein (M3L), and an increase in monomer tripeptides (M3).

We also analyzed the composition of the cell wall for two additional alleles of *cpxA: cpxA101*, a strongly activating allele, and *cpxA711,* a weakly activating allele (Table 4-4). We observed that only the strong alleles, *cpxA24* and *cpxA101,* exhibited a significant increase in DAP-DAP cross-links and total cross-linking, while the weak *cpxA711* allele displayed only a 7% increase in the DAP/cross-

link ratio, indicating that strong activation of the Cpx pathway might be required to see significant changes in the composition of the cell wall. We also observed that only the *cpxA24* allele showed a significant increase in the amount of trimers (T443D), while the amount of and monomers (M4, M4N, M3L) decreased.

After observing that the percentage of DAP-DAP cross-links varies according to the strength of the Cpx-inducing cue we compared the percentage of DAP-DAP cross-links for the different alleles of *cpxA* (*cpxA24, cpxA101, cpxA711)* and for the *cpxR::*spc mutant (Figure 4-6). The *cpxA24* and the *cpxA101* strong alleles (Raivio *et al.*, 1999) showed more than a 40% increase in the amount of DAP-DAP cross-links relative to wild-type, compared to very small or no increases for the *cpxA711* weak allele or the *cpxR::spc* mutant.

Taken together, these results indicate that activation of the Cpx pathway modifies the composition of the cell wall by mediating an increase in overall cross-linking, mostly through DAP-DAP cross-links (D33D and D33DL in the *cpxA101* and the *cpx711* strains) and 3-4 cross-links (T433D, T43DN only in the *cpxA24* strain). In addition, the overall amount of 1,6-anhydromuropeptides increased slightly in the *cpxA24* mutant while D43N increased significantly *in cpxA24, cpxA101*, and *cpxA711*. Also, monomer containing tetrapeptides (M4, M4N) and monomer tripeptides (M3L) decreased in all of the *cpxA* mutants. These results suggests that the Cpx pathway might cause an increase in peptidoglycan recycling and a decrease in specific muropeptide species (Uehara *et al.*, 2005).

4.6 Lytic transglycosylases Slt70 and MltB are necessary for the production of 1,6-anhydromuramic acid during activation of the Cpx pathway

Since the amount of anhydromuramic acid appeared to increase upon strong activation of the Cpx response (Table 4-5), we decided to characterize the involvement of Slt70 (Soluble lytic transglycosylase B) and MltB (membrane-bound lytic transglycosylase B) in this change in muropeptide composition during induction of the Cpx response. We analyzed the cell wall composition of single mutants of both of these genes in the MC4100 and in the *cpxA24* backgrounds by HPLC. The morphological alterations observed in the absence of *slt70* and *mltB* were different in the *cpxA24* and MC4100 backgrounds.

First, the MC100 *mltB::kan* strain showed an increase in the amount of DAP-DAP cross-links, muropeptides bound to a lipoprotein (Lipo), and anhydro muropeptides (Anhydro), and a reduction in the length of the disaccharide chains (Table 4-5). The *cpxA24 mltB::kan* mutant had a higher percentage of DAP-DAP cross-links than its counterpart in the MC4100 background, and a reduction in the amount of muropeptides bound to a lipoprotein (Lipo). Second, the disaccharide profile showed an increase for both mutants in disaccharide-tripeptide-disaccharide-tripeptide with an L-DAP-DAP crosslink (D33D), as well as disaccharide-tripeptide-disaccharide-tripeptide with an L-DAP-DAP crosslink bound to a lipoprotein (D33DL). In addition, only the *cpxA24 mltB::kan* mutant showed a decrease in monomers (M2, M3L), while both the *cpx24 mltB::kan* and MC4100 *mltB::kan* displayed a decrease in (M4N), and disaccharides (D43N), and it was the only strain that displayed an increase in anhydro trimers (T443D).

These results suggest that MltB actively cleaves short peptidoglycan strands during activation of the Cpx pathway (Suvorov *et al.*, 2008) to synthesize a 1,6-anhydro N-acetyl muramic acid residue that represents the end of the peptidoglycan chain (Höltje, 1998). In addition, MltB plays an important process in peptidoglycan recycling during activation of the Cpx pathway (Suvorov *et al.*, 2008) as it synthesizes the unusual intramolecular glycosyltransferase reaction that results in the nonreducing muropeptide 1,6-anhydro N-acetyl muramic acid that liberates N-acetyl glucosamine (Kraft *et al.*, 1998; van Asselt *et al.*, 2000) that is then transported to the cytoplasm to return to the biosynthetic pathway for cell wall synthesis (Uehara *et al.*, 2005).

In the MC4100 *slt70::kan* and in the *cpxA24 slt70::kan* strains we mainly expected 1,6-anhydro N-acetyl muramic acid to decrease since it has been reported that *slt70* mutants display a significant reduction in the amount of 1,6-anhydro N-acetyl muramic acid (Dijkstra *et al.*, 1999). We observed a decrease in the amount of 1,6-anhydro N-acetyl muramic acid and in muropeptides bound to lipoproteins (M3L) in both strains (Table 4-5), but this reduction was more significant for the *cpxA24 slt70::kan* mutant. In addition, we observed an increase in the length of the peptide chains and in DAP-DAP cross-links just for the *cpxA24 slt70::kan* mutant (Figure 4-7). Absence of Slt70 in the *cpxA24* background also led to an increase in the amount of disaccharides such as (D33D, D33DL), and various types of monosaccharides (M2, M4G), and a decrease in the anhydro monosaccharide (M4N). Taken together, these results suggest that Slt70 also plays an important role in peptidoglycan turnover and recycling during

activation of the Cpx pathway as it is involved in both processes (Höltje *et al.*, 1975), and that absence of MltB and Slt70 have different effects on the peptidoglycan structure during activation of the Cpx pathway.

4.7 The L,D-transpeptidase YcbB increases DAP-DAP cross-links during the activation of the Cpx pathway

Absence of the L,D-transpeptidase YcbB that synthesizes the rare DAP-DAP cross-links (Magnet *et al.*, 2008) caused numerous changes in the sacculi structure that were more severe in the *cpxA24* strain than in the MC4100 background. The percentage of DAP-DAP cross-links for the *cpxA24 ycbB::*kan mutant was more than 60% lower than its parent strain (Figure 4-7). In addition, the MC4100 *ycbB::kan* and *cpxA24 ycbB::kan* strains showed a reduction in the percentage DAP-DAP/overall cross-linking (Table 4-6) that was more significant in the *cpxA24* background. The *cpxA24 ycbB::kan* strain also exhibited a substantial reduction in the percentage of disaccharides (D33D, D43D, D43N), and an increase in M4 and M4N, indicating that this L,D-transpeptidase is highly functional during activation of the Cpx pathway. Interestingly, we also saw an unexpected increase in the amount of disaccharide-tripeptide-disaccharide-tripeptide with a DAP-DAP crosslink and bound to a lipoprotein (D33DL) for this strain, suggesting there might be another L,D-transpeptidase that is highly up-regulated in the absence of YcbB.

The high percentage of DAP-DAP cross-links that we observed during activation of the Cpx pathway suggests that DAP-DAP cross-links are important for

adaptation during Cpx inducing conditions and the data here implicate YcbB in this activity (Sanders and Pavelka, 2013).

4.8 Activity of YgaU causes changes in the peptidoglycan composition during activation of the Cpx pathway

ygaU was the most up-regulated cell wall modification gene that we studied in the *cpxA24* strain and also after induction of the Cpx pathway by NlpE and BfpA over production (See Chapter 3). YgaU is a predicted lipoprotein with unknown function that has a BON motif that participates in membrane-binding and a LysM motif that is found in different enzymes that participate in cell wall degradation (Weber *et al.*, 2006). We decided to further analyze the role of *ygaU* during activation of the Cpx pathway. HPLC analysis showed the *cpxA24 ygaU::kan* mutant had an increase in the percentage of DAP-DAP cross-links (Table 4-7), disaccharide (D33D, D33DL, D43D, T443D), and monosaccharide M5. In addition, the the *cpxA24 ygaU::kan* strain showed a reduction in the amount of disaccharides (D43N) and monomers (M3, M3L, M4N). Comparison of the percentage of DAP-DAP cross-links in the MC4100 *ygaU::kan* and the *cpxA24 ygaU::kan* mutants to the other strains we tested (Figure 4-7) showed that the absence of *ygaU* caused more than a 10% increase of DAP-DAP cross-links in the wild-type background and more than a 60% increase in the *cpxA24* background, suggesting that *ygaU* might play an important role in the regulation of the percentage of DAP-DAP cross-links. Similarly, the MC4100 *ygaU::*kan mutant displayed some differences in the cell wall composition compared to its

counterpart in the *cpxA24* background. These changes were characterized by a decrease in disaccharides (D43D) and trimers (T443D). These results suggest that the activity of YcbB increases in the absence of *ygaU*. This further reinforces the importance of this type of cross-links during activation of the Cpx pathway.

4.9 YgaU is a putative enhancer of L,D-transpeptidase activity

We decided to further characterize the function of YgaU by inducing the overexpression of this protein with the pCA-*ygaU* plasmid in the MC4100 *ygaU::kan* and *cpxA24 ygaU::kan* strains. We found numerous changes in the sacculi of both mutants, however, these changes were more significant in the wild-type background. We did not see significant changes between the induction and non-induction of the pCA-*ygaU* plasmid, suggesting that the pCA plasmid has leaky genetic expression (Table 4-8). The amount of trimers, muropeptides bound to a lipoprotein (Lipo) was reduced in both mutants in almost half of disaccharide chains of the wild-type. Also, the amount of DAP-DAP cross-links and total cross-linking increased by more than 100% compared to MC4100 (Figure 4-8). We also observed a significant increase in DAP-DAP cross-links in disaccharide (D33D). Overexpression of YgaU led to an increase in monosaccharides with a tetrapeptide with a glycine end (M4G) and monomer tripeptides (M3), and an overall decrease in monomers (M4). This suggests that YgaU might have L,D-transpeptidase activity due to the increase in DAP-DAP cross-links.

To determine if this could be true, we performed multiple protein alignments using Clustal Omega and Genious version 7.0 to determine if YgaU shares any homology with any of the L,D-transpeptidases of *E. coli (Magnet et al., 2007)* (Data not shown). Alignments of the protein sequences indicated that YgaU shares less than 11% percent homology with YcbB, YnhG, YbiS, ErfK, YcfS. YgaU, is smaller (149 amino acids) than YnhG, YbiS, ErfK, YcfS (~300 amino acids) and YcbB (615 amino acids), and it lacks the active-site cysteine present in the L,D-transpeptidases (Sanders and Pavelka, 2013) (Figure 4-9). We did not find a putative L,D-transpeptidase domain in YgaU. Furthermore, alignment of YgaU with the Pfam Protein family database showed that YgaU only has a BON domain and a LysM motif, while the remaining 20 aminoacids share no homology with anyother catalytic domains that could explain its function(Figure 4-9). Thus, we hypothesize that another L,D-transpeptidase was induced upon YgaU over-expression, producing the resulting increased 3-3 cross-links. Also, overexpression of YgaU has a differential effect on the global structure of PG of MC4100 *ygaU::kan* and *cpxA24 ygaU::kan*, in spite of the high level of DAP-DAP derived muropeptides and their effect on the global structure. Basal level expression from the pCA plasmid in the MC4100 background was enough to induce changes equivalent to those observed in the *cpxA24* strain under IPTG induction.

4.10 *ycbB* and *ygaU* double mutants in the *cpxA24* and MC4100 strain backgrounds have altered peptidoglycan structure.

Peptidoglycan obtained from the MC4100 Δ *ycbB ygaU* and *cpxA24* Δ *ycbB ygaU* mutants exhibited a number of changes from the wild-type, most significantly a striking reduction in the percentage of DAP-DAP cross-links (Table 4-8), and monomers (M3, M4N). The MC4100 Δ *ycbB ygaU* and *cpxA24* Δ *ycbB ygaU* mutants also exhibited an increase in disaccharide (D33DL), and in monomers with a peptide (M5), and a decrease in trimers (T443D), and monomers (M3L). The *cpxA24* Δ *ycbB ygaU* displayed a decrease in the length of disaccharide chains and an increase in anhydro muropeptides. Our data suggest that YcbB and YgaU participate in the synthesis and regulation of DAP-DAP cross-linking during activation of the Cpx pathway.

4.11 Absence of *ycbB* and *ygaU* activates the Cpx pathway.

We performed a β-galactosidase assay with the chromosomal promoter fusion *cpxP-lacZ* to determine whether absence of the L,D-transpeptidase YcbB and putative L,D-transpeptidase enhancer YgaU induced the Cpx pathway (Figure 4-10). We observed differences in *cpxP* activity in all the mutants tested grown to late log-phase (OD600 ~0.7 in LB). The Δ*cpxR* mutant had no activity compared to the wild-type. The MC4100Δ*ygaU* and MC4100Δy*cbB* mutants showed an increase in Cpx activity (~300 Miller Units) when compared to the wild-type (~180 Miller Units). However, when we tested the MC4100 Δ*ycbB ygaU* double

mutant we did not see a further increase in Cpx activity, suggesting that absence of YgaU and YcbB does not cause an additive effect in this assay.

We also decided to analyze the activity of various *ygaU* mutants to determine if absence of this putative L,D-transpeptidase regulator led to up-regulation of the lux plasmid *ygaU-pJW15* compared to the wild-type. We observed an increase in the *ygaU*-pJW15 in the MC4100 *ycbB ygaU* mutant. Complementation of the *ygaU* deletion using the pCA-*ycbB* or the pCA-*ygaU* plasmid decreased the activity of the *ygaU*-pJW15 plasmid. Induction of the pCA-*ycbB* and pCA-*ygaU* plasmids with IPTG led to a further reduction in the activity of *ygaU. An attractive explanation for* these results is that over-expression of *ygaU* and *ycbB* decreases stress in the periplasm, thereby decreasing Cpx pathway activity and decreasing the CpxR-mediated transcription of these genes.

4.12 *ycbB* can restore the transcriptional activity of *ygaU* back to wild-type levels

To explore transcriptional and regulatory consequences of the loss of *ygaU,* we performed a *lux* assay using the *ygaU*-pJW15 reporter in the MC4100, MC4100 Δ*ygaU*, and MC4100 Δ*ygaU* Δy*cbB* strains. We assessed whether complementation with the pCA-*ygaU* and pCA-*ycbB* plasmids could restore the levels of *ygaU* activity back to wild-type levels. We observed that the transcription of *ygaU* was higher in MC4100 Δy*cbB* Δ*ygaU* and MC4100 Δ*ygaU* than in MC4100 (Figure 4-11). This suggests that *ygaU* is not necessary for its own expression and that it is likely up-regulated by a response regulator like

cpxR. Activity of the *ygaU*-pJW15 reporter was higher when *ycbB* was absent, indicating that the stress levels in the cell are higher and that up-regulation of *ygaU* should restore envelope homeostasis. Complementation with *pCA-ygaU* in both the single and double mutants restored the activity to wild-type levels. We observed a similar result when the single and double mutants were complemented with *ycbB*. Reduction in the activity of *ygaU* was conferred by complementation with either *ycbB* or *ygaU,* implying that both YcbB and YgaU can remove the stress that is causing an increase in the activity of *ygaU*.

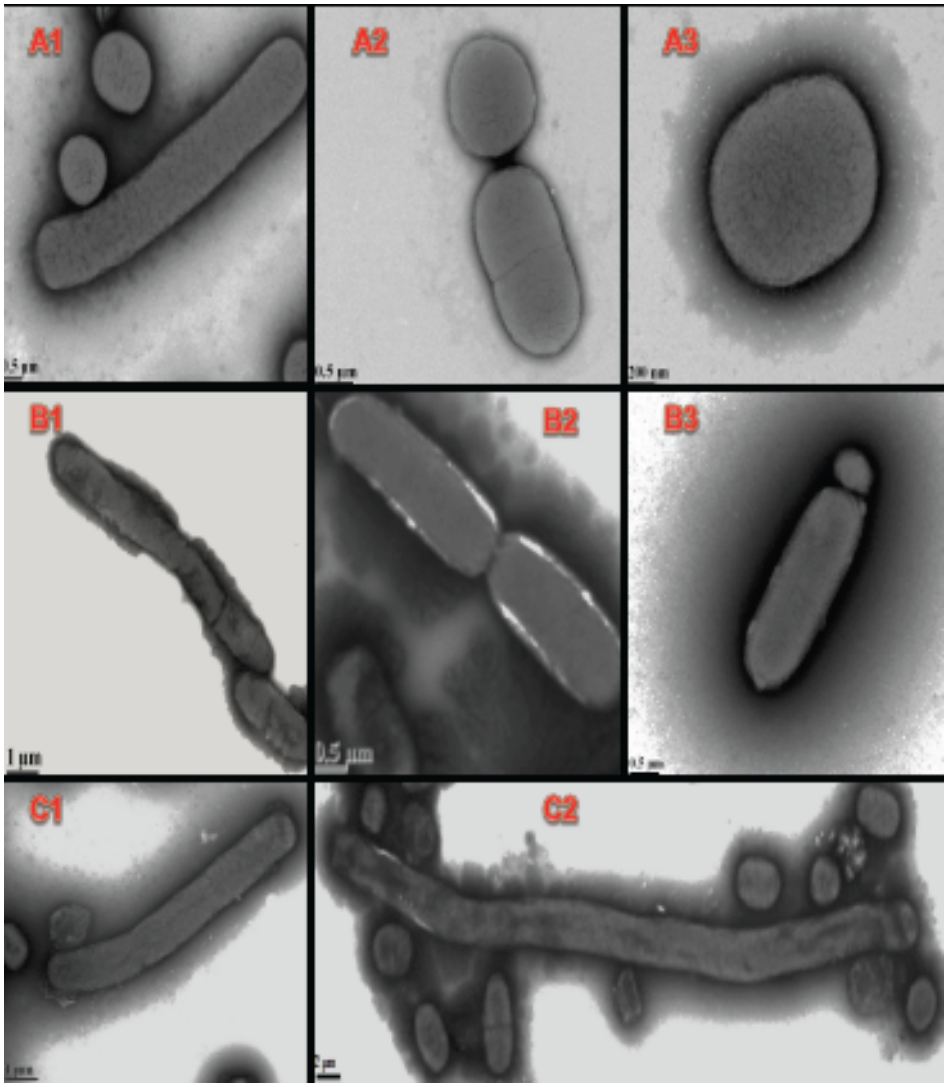


Figure 4-1 The Cpx pathway is important for cell morphology in *E. coli.*

The most common cell morphology observed via TEM is shown for overnight cultures of MC4100, *cpxA24,* and *cpxR::spc* strains **(A1-A3)** MC4100**, (B1-B3)** *cpxA24* , **(C-1-C2)** *cpxR::spc*

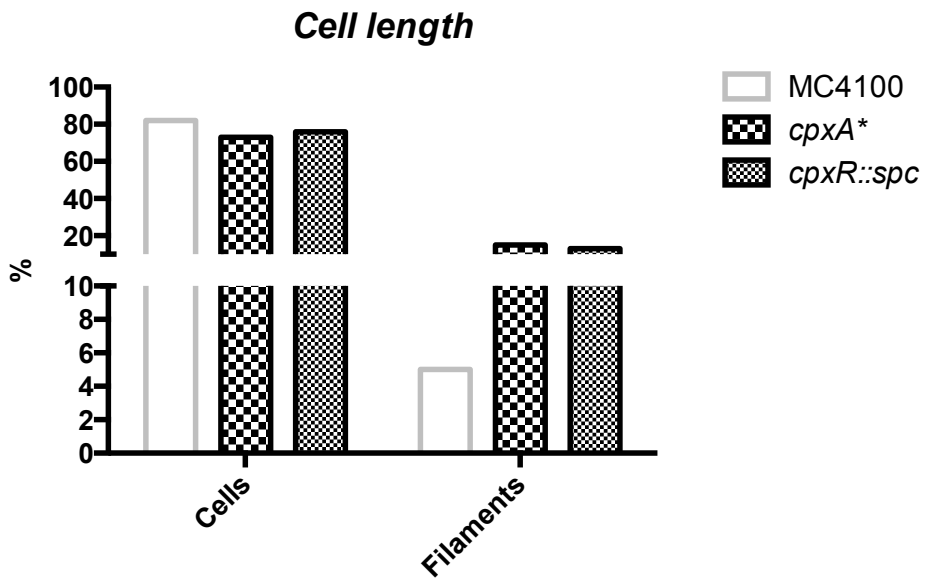


Figure 4-2 :The Cpx pathway is important for normal cell morphology.

The percentage of cells with different cell lengths in overnight cultures was calculated after counting 90 cells for MC4100, *cpxA24,* and *cpxR::spc* using TEM **(1)** Cells: length < 5μm, **(2)** Filaments: length ≥ 5μm (Zobell and Cobet, 1964).

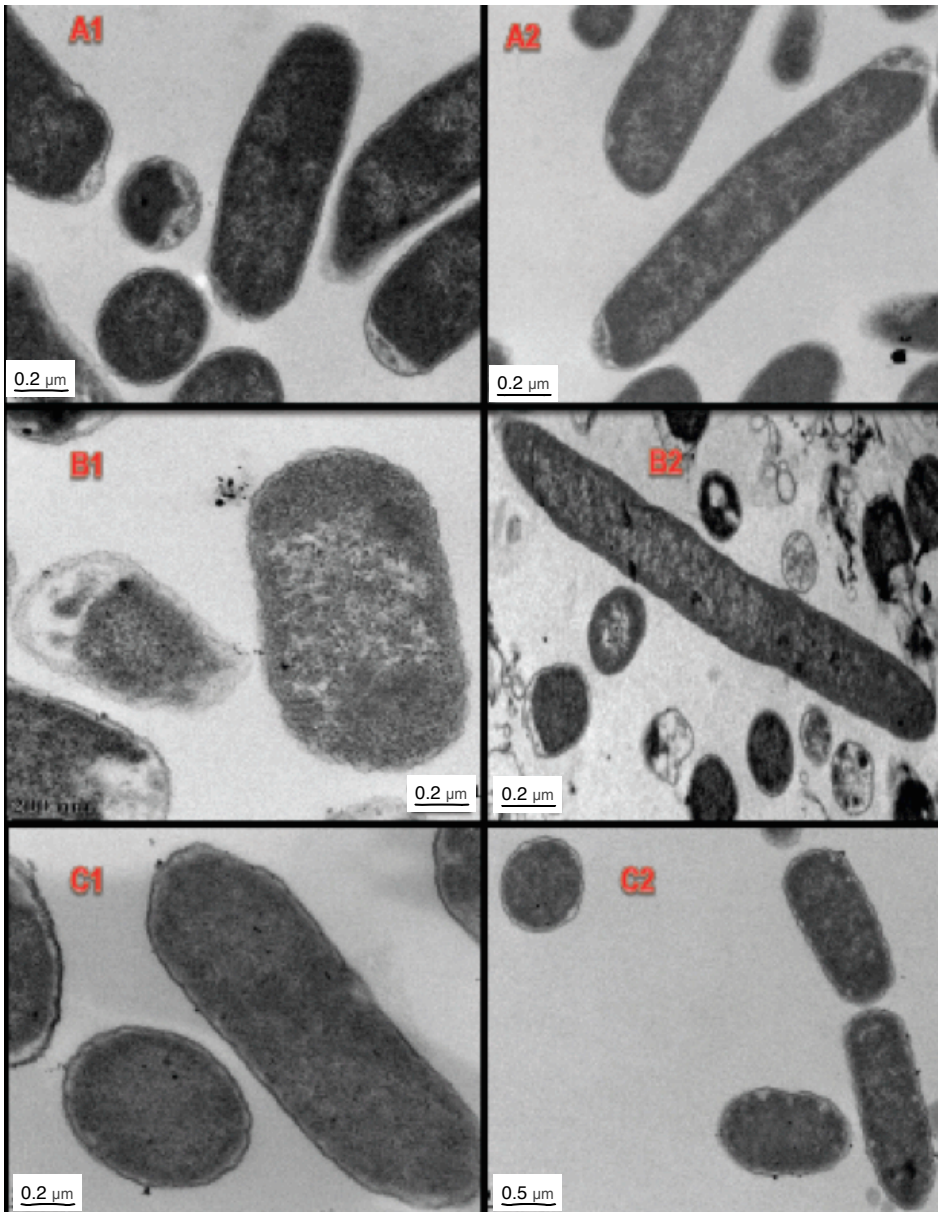


Figure 4-3 : The Cpx pathway is important for cell morphology in *E. coli.*

The most common cell morphology obtained from TEM visualization of transverse section micrographs is shown for overnight cultures of MC4100, *cpxA24*, and *cpxR::spc* strains **(A1-A2)** MC4100**, (B1-B2)** *cpxA24,* **(C-1-C2)** *cpxR::spc*

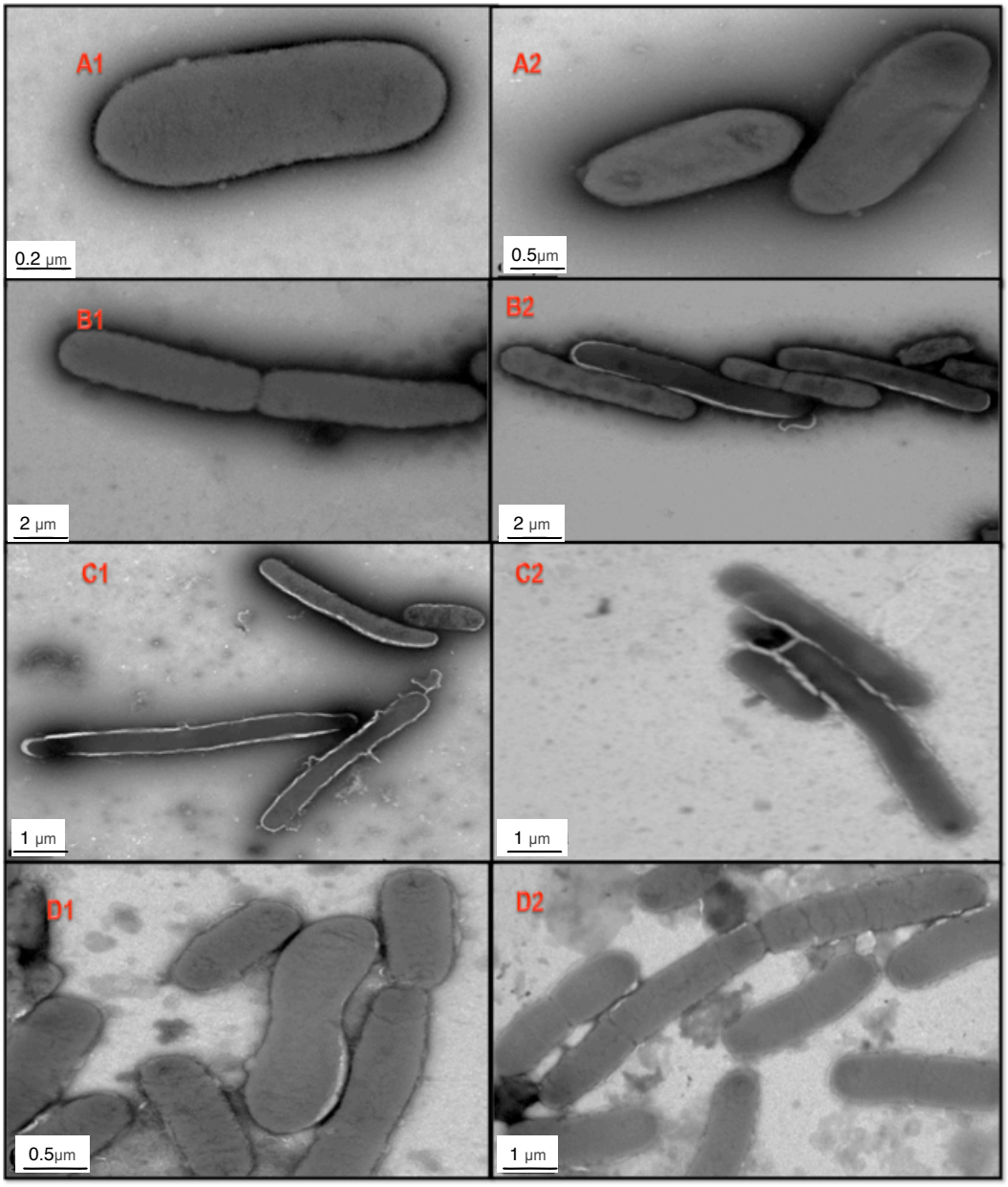


Figure 4-4 **:** *ycbB* and *ygaU* affect cell morphology in *E. coli.*

The most common cell morphology visualized via TEM of overnight cultures of MC4100 *ygaU:: kan*, *cpxA24 ygaU::kan*, *cpxA24 ycbB ygaU:: kan*, and MC4100 *ycbB ygaU:: kan* **(A1-A2)** MC4100 *ygaU:: kan* **(B1-B2)** *cpxA24 ygaU::kan* , **(C1-C2)** *cpxA24 ycbB ygaU:: kan* **(D1-D2)** MC4100 *ycbB ygaU:: kan.*

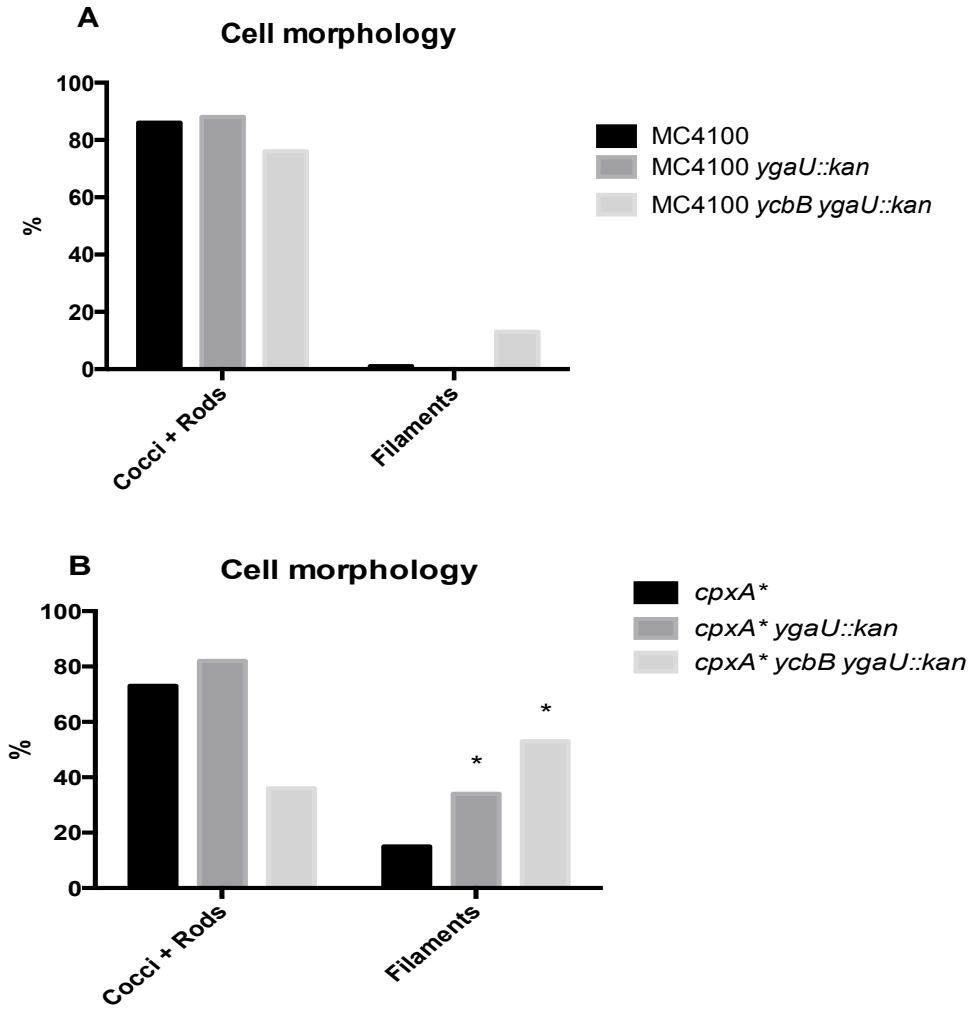


Figure 4-5 :*ygaU* is important for normal cell morphology.

The percentage of cells that can be classified as cocci and rods or filaments was calculated for overnight cultures of **A)** MC4100, MC4100 *ygaU::kan,* MC4100 *ycbB ygaU::kan***, B)** *cpxA24, cpxA24 ygaU::kan, cpxA24 ycbB ygaU::kan. Cocci + rods:* length < 5µm, **(2)** Filaments: length ≥ 5µm (Zobell and Cobet, 1964). * P<0.01, two-way ANOVA. Differences were not statistically significant for A.

STRAIN	AMP	PIPER	CLOX	CARB	TOBR
MC4100	2	8	64	2	0.125
cpxA24	4	16	128	32	1.0
cpxR	0.5	0.25	0.5	8	0.125
*cpxA24 ycbB::ka*n	1	8	4	8	0.25
cpxA24 ygaU::kan	2	16	128	8	0.25
cpxA24 slt70::kan	2	4	128	16	0.25
cpxA24 mltB::kan	2	16	128	64	0.25
cpxA24 ycbB ygaU	4	16	128	32	0.125
MC4100 *ycbB*::kan	0.5	8	64	32	0.125
MC4100 *ygaU::kan*	1	1	64	16	0.125
MC4100 *slt70::kan*	1	2	8	2	0.125
MC4100 *mltB::kan*	2	2	64	8	0.125
MC4100Δ*ycbBygaU*	0.25	4	8	4	01.25

Table 4-1: Minimum inhibitory concentrations (MICs) of cultures (O.D 0.08-1.30) in Mueller-Hinton

Minimum inhibitory concentrations (MICs) of cultures (O.D 0.08-1.30) in Mueller-Hinton to: **Amp**-Ampicillin (highest concentration 256 μg/ml), **Piper**-Piperacillin (highest concentration 256 μg/ml), **Clox**-Cloxacillin (highest concentration 256 μg/ml), Carb-Carbenicillin (highest concentration 256 μg/ml), and Tobr-Tobramycin (highest concentration 256 μg/ml) were calculated for strains: MC4100, TR10, TR51, *cpxA24 ycbB::kan, cpxA24 ygaU::kan, cpxA24 slt70::kan, cpxA24 mltB::kan, cpxA24* Δ *ycbB ygaU zii:*:Tn10. MC4100 *ycbB*::*kan*, MC4100 *ygaU::kan,* MC4100 *slt70::kan,* MC4100 *mltB::kan,* and MC4100Δ*ycbB ygaU.* Dark gray denotes a 2-fold or higher decrease in sensitivity compared to the parents strain while light gray indicates a 2-fold or higher increase in sensitivity.

Units	Title	Composition
M: Monomer	M3	Disaccharide-tripeptide
	M4G	Disaccharide-tetrapeptide with a glycine
	M4	Disaccharide-tetrapeptide
	M2	Disaccharide-dipeptide
	M5	Disaccharide-tetrapeptide
	M3N	Disaccharide-tripeptide with an glycan-reducing end
	M4N	Disaccharide-tripeptide with an glycan-reducing end
D: Dimer	D33D	Disaccharide-tripeptide-disaccharide-tripeptide with an L-Dap-Dap cross-link
	D43D	Disaccharide-tetrapeptide-disaccharide-tripeptide with a L-Dap-Dap cross-link
	D33DL	Disaccharide-tripeptide-disaccharide-tripeptide with an L-Dap-Dap cross-link and bound to a lipoprotein
	D43DL	Disaccharide-tetrapeptide-disaccharide-tripeptide with a L-Dap-Dap cross-link and bound to a lipoprotein
	D43N	Disaccharide-tetrapeptide-disaccharide-tripeptide with a L-Dap-Dap cross-link with an glycan-reducing end
T: Trimer	T443D	Disaccharide-tetrapeptide-disaccharide-tetrapeptide-disaccharide-tripeptide with a L-Dap-Dap cross-link
	Trimers	Total number of crosslinked trimeric muropeptides (NAcGlc-NAcMur-peptide-NAcGlc-NAcMur-peptide-NAcGlc-NAcMur-peptide)
Various	DAP-DAP	Total number of 3-3 type crosslinks between D-aminopimelic acid residues (Activity of LD-transpeptidases)
	Lipo	Total number of muropeptides crosslinked with lipoprotein.
	Anhydro	Total number of anhydro-disaccharide muropeptides (End of chains)
	Penta	Total number of muropeptides with a pentapeptide residue. (Lost by the action of DD-carboxypeptidase)
	Cross-linking	Percentage of muropeptides with a peptide crosslink.
	D-D/total	Ratio of muropeptides with a 3-3 link related to total crosslinking
	Length	Mean length of the disaccharide chains.

Table 4-2 : Summary of muropeptides analyzed by HPLC.

	cpxR::spc	*cpxA24*
Monomers	**98**	**94**
Dimers	**105**	**110**
Trimers	**100**	**136**
DAP-DAP	**97**	**138**
Lipo	**86**	**95**
Anhydro	**90**	**107**
Crosslinking	**104**	**114**
D-D/total	**94**	**119**
Length	**105**	**92**
D33D	**95**	**183**
D43D	**104**	**115**
D33DL	**117**	**100**
D43N	**91**	**142**
T443D	**119**	**210**
M3	**76**	**97**
M4G	**89**	**143**
M4	**108**	**81**
M5	**106**	**76**
M4N	**82**	**67**

Table 4-3 Heat map of the murein ratio of cell wall components (% strain strains/wild-type) of the strains *cpxA24, cpxR::spc.*

Heat map of the murein ratio of cell wall components (% strain strains/wild-type) of the strains *cpxA24, cpxR::spc*,. Different colors indicate an increase (green) or a decrease (red) in the percentage of different kinds of muropeptides within each column

% (Strain/MC4100)	*cpxA24*	*cpxA102 – strong allele*	*cpxA711- weak allele*
Trimers	136.20	96.38	100.36
DAP-DAP	138.10	147.02	107.51
Anhydro	106.68	91.04	94.60
Lipo	94.80	73.17	75.19
Penta	126.10	97.83	84.78
Cross-linking	114.30	101.91	99.66
D-D/total	119.40	143.92	107.56
D33D	183.08	207.69	206.15
D33DL	100.00	198.28	179.31
D43N	142.22	95.56	104.44
T443D	209.52	138.10	138.10
M4	80.65	100.94	99.06
M3L	85.27	58.35	57.17
M4N	66.67	82.76	90.80

Table 4-4 **:** Heat map of the murein ratio of cell wall components (% strain strains/wild-type) of the strains ***cpxA24,*** *cpxA101 (*strong allele), *cpxA711* (weak allele).

Different colors indicate an increase (green) or a decrease (red) in the percentage of different kinds of muropeptides within each column.

% (Strain/parent)	MC4100 *mltB::kan*	cpxA* *mltB*::kan	MC4100 *slt70::kan*	*cpxA** *slt70::kan*
DAP-DAP	135	166	95	61
Lipo	142	77	95	113
Anhydro	133	95	79	75
D-D/total	139	175	97	126
Length	72	101	119	159
D33D	252	197	128	150
D43D	104	187	80	115
D33DL	359	186	110	178
D43DN	98	50	147	106
T443D	0	150	0	120
M3	90	114	110	88
M4G	85	93	91	115
M2	176	59	168	131
M3L	113	76	88	74
M4N	25	91	21	0

Table 4-5 :Heat map of the murein ratio of cell wall components (% strain strains/parent) of the strains MC4100 *slt70::kan,* MC4100 *mltB::kan,* ***cpxA24*** *mltB::kan,* and ***cpxA24*** *slt70::kan*

Different colors indicate an increase (green) or a decrease (red) in the percentage of different kinds of muropeptides within each column.

% (Strain/parent)	MC4100 *ycbB::kan*	*cpxA24* *ycbB::kan*
Trimers	98	84
DAP-DAP	107	39
Anhidro	75	102
Crosslinking	102	93
D-D/total	86	42
Length	126	95
D33D	66	34
D43D	44	20
D33DL	352	236
D43N	124	19
T443D	0	34
M3	81	84
M4	104	129
M3L	77	84
M4N	0	152

Table 4-6 **:**Heat map of the murein ratio of cell wall components (% strain strains/parent strain) of the strains MC4100 *ycbB::kan,* ***cpxA24*** *ycbB::kan.*

Different colors indicate an increase (green) or a decrease (red) in the percentage of different kinds of muropeptides within each column.

% (Strain/parent)	MC4100 *ygaU::kan*	*cpxA24 ygaU::kan*
Trimers	92	90
DAP-DAP	107	150
Lipo	98	80
Cross-linking	109	101
D-D/total	94	145
D33D	123	139
D43D	63	167
D33DL	203	200
D43N	169	22
T443D	62	164
M3	87	54
M4G	116	71
M5	129	369
M2	192	93
M3L	62	79
M4N	30	34

Table 4-7 : Heat map of the murein ratio for monomers (% strain strains/parent strain) of the strains MC4100 *ygaU::kan* and *cpxA24 ygaU::kan.*

Different colors indicate an increase (green) or a decrease (red) in the percentage of different kinds of muropeptides within each column.

% (Strain/ parent strain)	MC4100 *Δ ycbB ygaU::kan*	*cpxA24 Δ ycbB ygaU::kan*
Trimers	**91**	**74**
DAP-DAP	**47**	**72**
Lipo	**119**	**109**
Anhydro	**103**	**113**
Cross-linking	**100**	**95**
D-D/total	**47**	**76**
Length	**92**	**85**
D43D	**32**	**37**
D33DL	**103**	**49**
D43N	**78**	**341**
T443D	**0**	**83**
M3	**91**	**0**
M4G	**68**	**54**
M4	**76**	**130**
M2	**174**	**93**
M5	**129**	**369**
M3L	**90**	**79**
M4N	**23**	**34**

Table 4-8 **:**Heat map of the murein ratio of cell wall components (% strain strains/wild-type) of the strains MC4100 *ycbB ygaU::*, *cpxA24 ycbB ygaU*.

Different colors indicate an increase (green) or a decrease (red) in the percentage of different kinds of muropeptides within each column.

% (Strain/parent)	MC4100 *ygaU*::kan + pCA-*ygaU* +	MC4100 *ygaU*::kan + pCA-*ygaU* +IPTG	*cpxA24* *ygaU*::kan + pCA-*ygaU*	*cpxA24* ygaU::kan+ pCA-*ygaU*+IPTG
Trimers	55	69	52	44
DAP-DAP	239	412	215	248
Lipo	92	85	84	60
Anhidro	96	161	175	133
Penta	120	517	200	147
Cross-linking	109	132	107	107
D-D/total	220	312	202	234
Length	99	58	55	72
D33D	220	909	419	479
D43D	225	148	68	76
D33DL	284	336	269	153
D43N	118	100	75	66
T443D	0	314	214	180
M3	93	134	155	151
M4G	160	266	162	299
M4	93	28	40	32
M2	77	95	72	65
M5	224	447	169	108
M3N	72	94	0	0
M4N	0	0	164	136

Table 4-9 Heat map of the murein ratio of cell wall components (% strain strains/parent) of the strains MC4100 *ygaU::kan + pCA-ygaU* + IPTG, MC4100 *ygaU::kan + pCA-ygaU,* TR10 *ygaU::kan + pCA-ygaU,* TR10 *ygaU::kan+ pCA-ygaU+IPTG*.

Different colors indicate an increase (green) or a decrease (red) in the percentage of different kinds of muropeptides within each column.

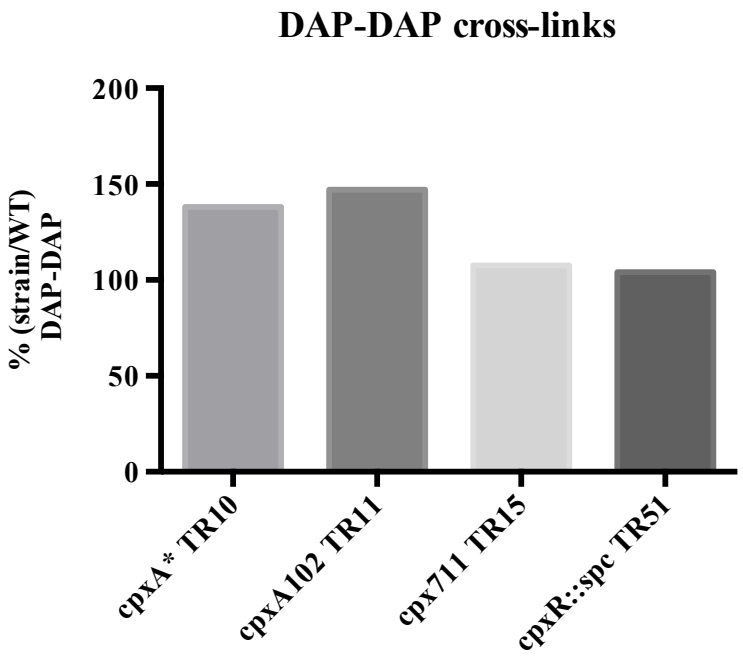


Figure 4-6 : Graphic representation of the percentage of DAP-DAP cross-links for the different alleles of ***cpxA24*** and the *cpxR::spc* mutant.

The intermediate strength allele *cpxA101* has the highest percentage of DAP-DAP cross-links followed by the *cpxA24* mutant.

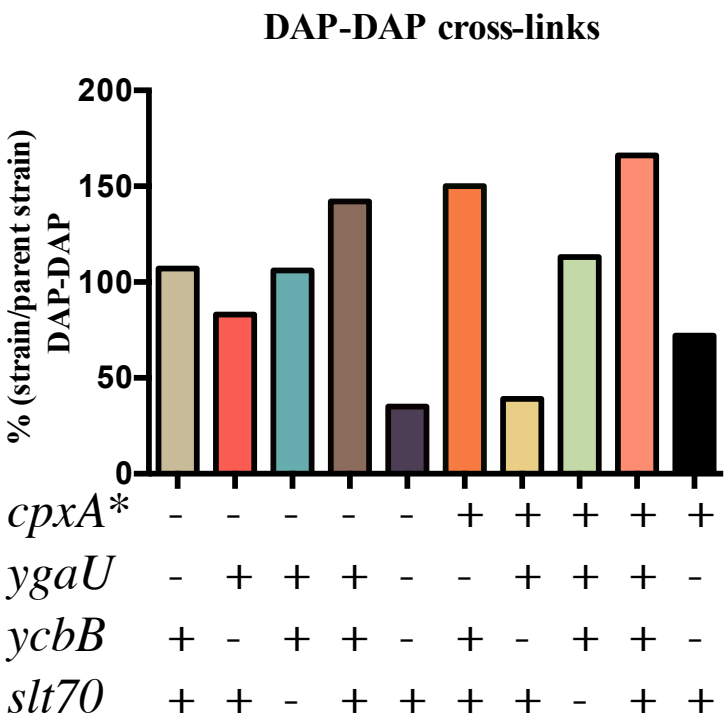


Figure 4-7 **:** Graphic representation of the percentage of DAP-DAP cross-links for the *cpxA24* and the *cpxR::spc* derived mutants tested by HPLC analysis.

The *cpxA24* mutants show a more significant increase/decrease in the amount of DAP-DAP cross-links.

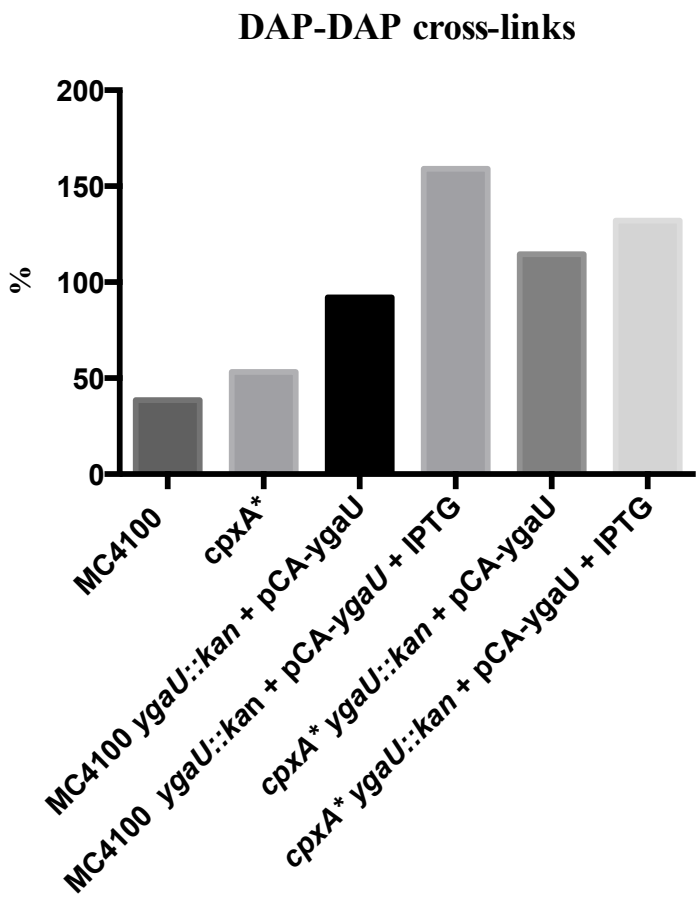


Figure 4-8 : Visual representation of the percentage of DAP-DAP cross-links for the complementation with the pCA-*ygaU* plasmid of the *ygaU::kan* mutation derived mutants tested by HPLC analysis. *ygaU* over-expression causes a significant increase in DAP-DAP cross-links.

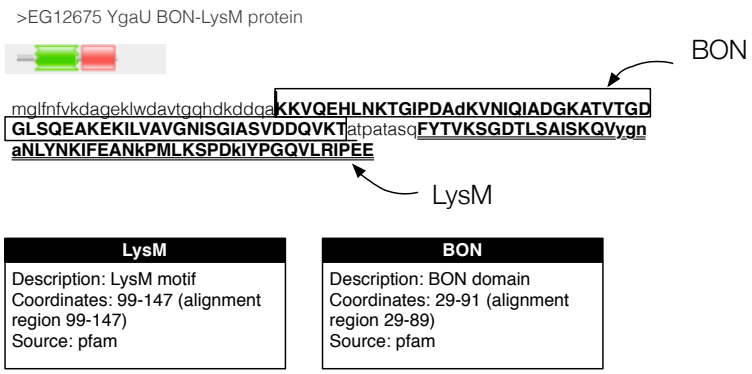


Figure 4-9 **:** Domains and motifs present in YgaU**.**

The consensus sequence of YgaU from the Pfam Protein Family Database indicates that YgaU has a BON domain and a LysM motif. Aminoacids in lower cases are not conserved.

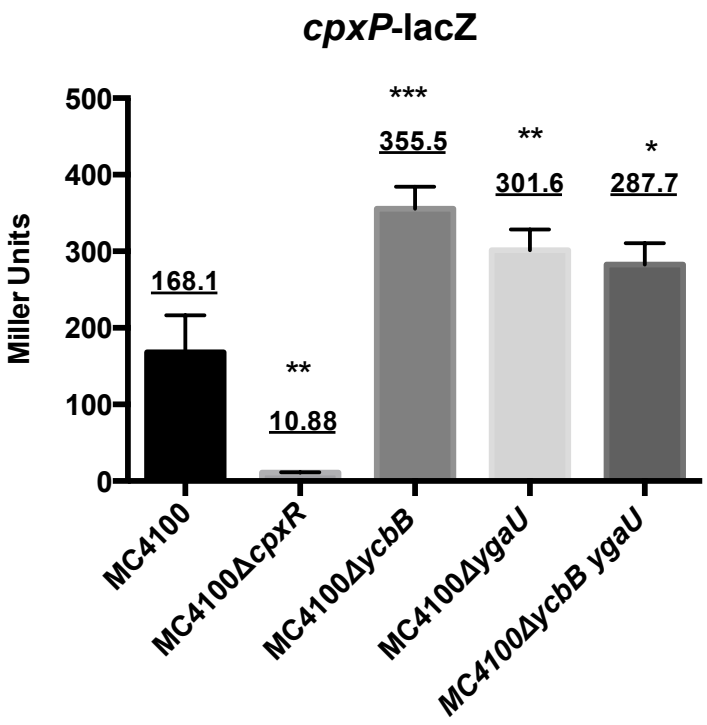


Figure 4-10 : Absence of *ycbB* and *ygaU* activates the Cpx pathway. We performed a β-galactosidase assay using a *cpxP-lacZ* reporter in strains MC4100, MC4100Δ*cpxR*, MC4100Δ*ygaU,* MC4100Δ*ycbB,* MC4100Δ*ycbB ygaU,* MC4100Δ*mltB* indicates that the Cpx pathway was induced in late log-phase. (final $O.D_{600}$ = ~ 0.7). Error bars represent the standard deviation. Asterisks represent significant levels: * $p \leq 0.05$, ** $p \leq 0.01$, *** $P \leq 0.001$, ****$P \leq 0.000$. This assay was repeated at least two times.

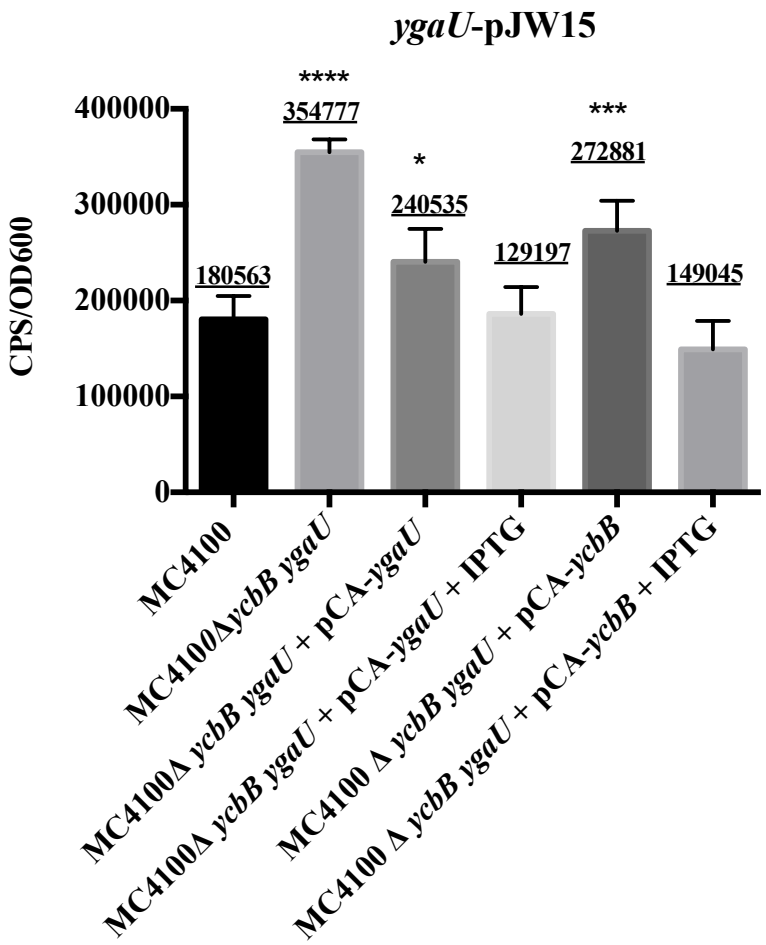


Figure 4-11 **:**Absence of *ygaU* increases transcription of the ygaU-pJW15 reporter and overexpression of *ycbB* and *ygaU* restore its activity back to basal level.

Luciferase assay with the *ygaU*- pJW15 lux reporter in MC4100, MC4100Δ*ycbB ygaU,* MC4100Δ*ygaU,* MC4100Δ*ycbB* in the presence of *pCA-ycbB or pCA-ygaU* with or without induction shows that over expression of YcbB or YgaU reduces the activity of the Cpx pathway (final $O.D_{600}$ = ~ 0.7). * $p \leq 0.05$, ** $p \leq 0.01$, *** $P \leq 0.001$, ****$P \leq 0.000$. This assay was repeated at least two times**.**

References

Bell, S.M., Pham, J.N., and Lanzarone, J.Y.M. (1985) Mutation of *Pseudomonas aeruginosa t*o piperadillin resistance mediated by β-lactamase production. *Journal of Antimicrobial Chemotherapy* **15**: 665-670.

Black, W.A., and Girdwood, R.W. (1969) Carbenicillin resistance in *Pseudomonas aeruginosa. British medical journal* **4**: 234.

Cava, F., Kuru, E., Brun, Y.V., and de Pedro, M.A. (2013) Modes of cell wall growth differentiation in rod-shaped bacteria. *Curr Opin Microbiol* .

Dijkstra, B.W., Thunnissen, A.-M.W., and Asselt, E.J.V. (1999) High Resolution Crystal Structures of the *Escherichia coli* Lytic Transglycosylase Slt70 and its Complex with a Peptidoglycan Fragment. *Journal of Molecular Microbiology.* **4:** 877-898

Dorel, C., Vidal, O., Prigent-Combaret, C., Vallet, I., and Lejeune, P. (1999) Involvement of the Cpx signal transduction pathway of *E. coli* in biofilm formation. *FEMS Microbiol Lett* **178**: 169-175.

Herbert, E.E., Cowles, K.N., and Goodrich-Blair, H. (2007) CpxRA regulates mutualism and pathogenesis in *Xenorhabdus nematophila*. *Appl Environ Microbiol* **73**: 7826-7836.

Höltje, J.-V. (1998) Growth of the stress-bearing and shape-maintaining murein sacculus of *Escherichia coli*. *Microbiology and Molecular Biology Reviews* **62**: 181-203.

Höltje, J.V., Mirelman, D., Sharon, N., and Schwarz, U. (1975) Novel type of murein transglycosylase in *Escherichia coli*. *Journal of bacteriology* **124**: 1067-1076.

Jubelin, G., Vianney, A., Beloin, C., Ghigo, J.-M., Lazzaroni, J.-C., Lejeune, P., and Dorel, C. (2005) CpxR/OmpR interplay regulates curli gene expression in response to osmolarity in *Escherichia coli*. *J Bacteriol* **187**: 2038-2049.

Kolter, R., Siegele, D.A., and Tormo, A. (1993) The stationary phase of the bacterial life cycle. *Annual Reviews in Microbiology* **47**: 855-874.

Kraft, A.R., Templin, M.F., and Höltje, J.V. (1998) Membrane-bound lytic endotransglycosylase in *Escherichia coli*. *J Bacteriol* **180**: 3441-3447.

Magnet, S., Bellais, S., Dubost, L., Fourgeaud, M., Mainardi, J.-L., Petit-Frère, S., *et al.* (2007) Identification of the L,D-transpeptidases responsible for attachment of the Braun lipoprotein to *Escherichia coli* peptidoglycan. *J Bacteriol* **189**: 3927-3931.

Magnet, S., Dubost, L., Marie, A., Arthur, M., and Gutmann, L. (2008) Identification of the L,D-transpeptidases for peptidoglycan cross-linking in *Escherichia coli*. *J Bacteriol* **190**: 4782-4785.

Mahoney, T.F., and Silhavy, T.J. (2013) The Cpx stress response confers resistance to some, but not all, bactericidal antibiotics. *J Bacteriol* **195**: 1869-1874.

Mitsuyama, J., Takahata, M., Yasuda, T., and Saikawa, I. (1987) The mechanism of action of piperacillin-analogues in vitro; effect of the carbon number at the N-4 position of 2, 3-dioxopiperazine on the outer membrane permeability, stability to

beta-lactamase and binding affinity to penicillin-binding proteins. *The Journal of antibiotics* **40**: 868.

Monteiro, C., Fang, X., Ahmad, I., Gomelsky, M., and Römling, U. (2011) Regulation of biofilm components in Salmonella enterica serovar Typhimurium by lytic transglycosylases involved in cell wall turnover. *J Bacteriol* **193**: 6443-6451.

Parker, M.T., and Hewitt, J.H. (1970) Methicillin resistance in Staphylococcus aureus. *The Lancet* **295**: 800-804.

Pawlowski, D.R., Metzger, D.J., Raslawsky, A., Howlett, A., Siebert, G., Karalus, R.J., *et al.* (2011) Entry of *Yersinia pestis* into the viable but nonculturable state in a low-temperature tap water microcosm. *PLoS One* **6**: e17585.

Pogliano, J., Dong, J.M., De Wulf, P., Furlong, D., Boyd, D., Losick, R., *et al.* (1998) Aberrant cell division and random FtsZ ring positioning in *Escherichia coli* cpxA* mutants. *Journal of bacteriology* **180**: 3486-3490.

Raivio, T.L., Leblanc, S.K.D., and Price, N.L. (2013) The *Escherichia coli* Cpx envelope stress response regulates genes of diverse function that impact antibiotic resistance and membrane integrity. *J Bacteriol* **195**: 2755-2767.

Raivio, T.L., Popkin, D.L., and Silhavy, T.J. (1999) The Cpx envelope stress response is controlled by amplification and feedback inhibition. *Journal of bacteriology* **181**: 5263-5272.

Sainsbury, S., Bird, L., Rao, V., Shepherd, S.M., Stuart, D.I., Hunter, W.N., *et al.* (2011) Crystal structures of penicillin-binding protein 3 from *Pseudomonas*

aeruginosa: comparison of native and antibiotic-bound forms. *J Mol Biol* **405**: 173-184.

Sanders, A.N., and Pavelka, M.S. (2013) Phenotypic Analysis of *E. coli* Mutants Lacking L,D-transpeptidases. *Microbiology* .

Sieradzki, K., and Tomasz, A. (2003) Alterations of Cell Wall Structure and Metabolism Accompany Reduced Susceptibility to Vancomycin in an Isogenic Series of Clinical Isolates of *Staphylococcus aureus*. *Journal of Bacteriology* **185**: 7103-7110.

Suvorov, M., Lee, M., Hesek, D., Boggess, B., and Mobashery, S. (2008) Lytic transglycosylase MltB of *Escherichia coli* and its role in recycling of peptidoglycan strands of bacterial cell wall. *J Am Chem Soc* **130**: 11878-11879.

Toba, F.A., Thompson, M.G., Campbell, B.R., Junker, L.M., Rueggeberg, K.-G., and Hay, A.G. (2011) Role of DLP12 lysis genes in *Escherichia coli* biofilm formation. *Microbiology* **157**: 1640-1650.

Triboulet, S., Dubée, V., Lecoq, L., Bougault, C., Mainardi, J.L., Rice, L.B., *et al.* (2013) Kinetic features of L,D-transpeptidase inactivation critical for β-lactam antibacterial activity. *PLoS One* **8**: e67831.

Uehara, T., Suefuji, K., Valbuena, N., Meehan, B., Donegan, M., and Park, J.T. (2005) Recycling of the anhydro-N-acetylmuramic acid derived from cell wall murein involves a two-step conversion to N-acetylglucosamine-phosphate. *J Bacteriol* **187**: 3643-3649.

Vakulenko, .B., and Mobashery, S. (2003) Versatility of Aminoglycosides and Prospects for Their Future. *Clinical Microbiology Reviews* **16**: 430-450.

van Asselt, E.J., Kalk, K.H., and Dijkstra, B.W. (2000) Crystallographic studies of the interactions of *Escherichia coli* lytic transglycosylase Slt35 with peptidoglycan. *Biochemistry* **39**: 1924-1934.

Varma, A., and Young, K.D. (2009) In *Escherichia coli*, MreB and FtsZ direct the synthesis of lateral cell wall via independent pathways that require PBP 2. *J Bacteriol* **191**: 3526-3533.

Weatherspoon-Griffin, N., Zhao, G., Kong, W., Kong, Y., Morigen, Andrews-Polymenis, H., *et al.* (2011) The CpxR/CpxA two-component system up-regulates two Tat-dependent peptidoglycan amidases to confer bacterial resistance to antimicrobial peptide. *J Biol Chem* **286**: 5529-5539.

Weber, A., Kögl, S.A., and Jung, K. (2006) Time-dependent proteome alterations under osmotic stress during aerobic and anaerobic growth in *Escherichia coli*. *J Bacteriol* **188**: 7165-7175.

Young, K.D. (2011) Peptidoglycan, In eLS. Chichester, UK: John Wiley & Sons, Ltd.

Zobell, C.E., and Cobet, A.B. (1964) Filament formation by *Escherichia coli* at increased hydrostatic pressures. *Journal of bacteriology* **87**: 710-719.

CHAPTER 5

Changes in the peptidoglycan structure during activation of the Cpx pathway add a new dimension to the complexity of this stress response.

5. Discussion

The classical view of the Cpx pathway suggests that activation of this stress response alleviates the toxicity caused by misfolded proteins in the periplasm by the up-regulation of chaperones and proteases (Cosma, Danese, Carlson, Silhavy, & Snyder, 1995; Danese, Snyder, Cosma, Davis, & Silhavy, 1995; Danese et al., 1998; Pogliano, Lynch, Belin, Lin, & Beckwith, 1997) . However, recent transcriptome analyses and gene expression quantification in *Escherichia coli* and *Haemophilus ducreyi* have revealed that the Cpx response is a more complex, highly dynamic, and intricate stress response than previously thought (Bury-Moné et al., 2009; Gangaiah et al., 2013; Labandeira-Rey, Brautigam, & Hansen, 2010; Raivio, Leblanc, & Price, 2013). Microarray analysis performed by Raivio *et. al (2013)* showed that some of the novel members of the Cpx regulon were predicted to be involved in the synthesis, recycling, or cross-linking of peptidoglycan in *E.coli,* with activation of the Cpx pathway leading to the up-regulation of *amiA, ygaU, ycbB, slt70,* and *dacC*. Furthermore, a RNA-seq analysis of *Haemophilus ducreyi* also showed that *slt70* is up-regulated in response to Cpx activation

(Gangaiah et al., 2013), suggesting that changes to the structure of the cell wall might be an important function of the Cpx pathway.

In Chapter 3 of this study, we confirmed and characterized the regulation by the Cpx pathway of 7 cell wall genes; *ygaU, ycbB, slt70, mltB, amiA, amiC* and *dacC* in *E. coli,* and in Chapter 4 we identified putative physiological functions of YgaU, YcbB, Slt70, and MltB during the activation of the Cpx pathway.

Peptidoglycan remodelling, recycling, and degradation are fundamental processes for bacteria to survive (Vollmer & Bertsche, 2008). The main functions of the cell wall are the prevention of lysis from changes in osmotic conditions and maintenance of the bacterial cell shape (van Heijenoort 2011). The fact that the Cpx pathway causes changes to the cell wall adds a new dimension to this stress response, indicating that the Cpx stress response has more functions than initially assessed and that this pathway uses a wide variety of mechanisms to ensure that the bacterial envelope is rendered stable so that bacteria can survive in the presence of harmful conditions.

5.1 Activation of the Cpx pathway causes an increase in DAP-DAP cross-links by mediating the up-regulation of *ycbB* and *ygaU*

As mentioned previously, YcbB is one of the 5 L,D-transpeptidases of *E. coli* that synthesizes DAP-DAP cross-links (Magnet, Dubost, Marie, Arthur, & Gutmann, 2008) and YgaU is a protein with unknown function that has a BON domain which suggests that the localization of YgaU might be restricted to a phospholipid

membrane (Yeats & Bateman, 2003). Also, the presence of the widely distributed peptidoglycan binding motif LysM in YgaU suggests that this protein is associated with peptidoglycan (Buist, Steen, Kok, & Kuipers, 2008).

This study presented evidence that the expression of the *ygaU* and *ycbB* genes was up regulated by NlpE over-expression, BfpA over-expression, and the presence of the strong allele *cpxA** (Figures 3-4 A, B, C, Figure 3-6 A and B) (Raivio et al., 2013). These findings support our EMSA analyses as CpxR~P bound with strong affinity to the promoters of *ygaU* and *ycbB* (Figure 3-5, Figure 3-7), demonstrating that this regulation is directly dependant on CpxR, which further supports the findings previously presented in a microarray analysis performed by Raivio *et al. (2013)*

HPLC analysis indicated that the absence of *ycbB* led to a 14% decrease in DAP-DAP cross-links compared to the wild-type background and more than a 50% decrease in the *cpxA** background, suggesting that *ycbB* has an active role in the synthesis of DAP-DAP cross-links (Table 4-6). In addition, absence of *ycbB* also caused a decrease in 3-4 cross-links (D34D, T433D, T443D), this is further supported by the fact that the *cpxA* ycbB* mutant showed an increase in the sensitivity to some β-lactams (ampicillin, cloxacillin) (Islam, Alam, Choudhury, Kobayashi, & Ahmed, 2008) and an increase in the resistance to carbenicillin (Table 4-1). Up-regulation of *ycbB* might suggest changes in the activity of the Penicillin-binding proteins (PBPs) that catalyze these β-lactam sensitive linkages (Sauvage, Kerff, Terrak, Ayala, & Charlier, 2008). Thus, this suggests that the

L,D-transpeptidase YcbB has an active role in altering the peptidoglycan composition of the cell wall during the activation of the Cpx pathway.

Absence of *ygaU* also caused changes in the type of peptidoglycan cross-linking as the *cpxA* ygaU* mutant showed more than a 100% increase in the amount of DAP-DAP cross-links. As a result YgaU might participate in the regulation of DAP-DAP cross-links (Table 4-7). With this in mind, we also analyzed the peptidoglycan structure of MC4100 *ygaU ycbB::kan* and *cpxA* ycbB ygaU* mutants to determine if absence of these genes caused a decrease in the percentage of DAP-DAP cross-links of these mutants (Table 4-8). The MC4100 *ygaU ycbB::kan* strain displayed more than a 50% decrease in this type of cross-link while the *cpxA* ycbB ygaU* showed a 17% decrease in DAP-DAP cross-links. This might be an indication of other L,D-transpeptidases that are up-regulated in the absence of both of these genes and also strengthens the notion that *ygaU* might regulate the synthesis of DAP-DAP cross-links . To further characterize the role of YgaU during activation of the Cpx pathway, we over-expressed *ygaU* from the pCA inducible plasmid in *ygaU* mutants in both the wild-type and *cpxA** backgrounds (Table 4-9). HPLC results of the cell wall from strains in which YgaU was over-expressed showed a number of changes typical of the activity of an L,D-transpeptidase such as an increase in DAP-DAP cross-links, M3 monomers, and a decrease in M4 monomers. However, sequence alignment of the amino acid sequences of YgaU with the 5 L,D-transpeptidases of *E. coli* (YnhG, YcfS, YbiS, Ycbb, ErfK) showed that YgaU had an amino acid sequence shorter than any of the 5 L,D-transpeptidases of *E. coli(Magnet et al., 2008)*.

Furthermore, YgaU shared no more than 10% homology with YnhG, YcfS, YbiS, Ycbb, ErfK and it lacked the well-characterized YkuD catalytic domain that contains a conserved cysteine (Bielnicki et al., 2006) (Figure 4-9). The only homology that was detected was the presence of the LysM motif (Bateman & Bycroft, 2000), which is also shared with YnhG and YcfS. This could be further proof that YgaU has a role in regulating peptidoglycan remodelling. In addition, HPLC analysis has shown that the only L,D-transpeptidases capable of synthesizing DAP-DAP cross-links in *E .coli* are YcbB and YnhG (Magnet et al., 2008), which indicates that YgaU cannot synthesize DAP-DAP cross-links, but that it could be an enhancer or regulator of the activity of YcbB and/or YnhG. YgaU also has an important role in the cell wall shape as absence of this protein caused changes in the cell morphology of *E. coli* (Fgure 4-4).

We observed an increase in the transcription of *ygaU* in the MC4100 Δ *ygaU ycbB* background compared to MC4100 (Figure 4-11). We were able to restore basal activity of the *ygaU* reporter by over expressing either *ycbB* or *ygaU,* suggesting that both of these genes might have similar activity on the bacterial envelope, which in turn down-regulates this gene. In addition, absence of *ygaU* and *ycbB* caused an increase in the activity of the Cpx pathway (Figure 4-10), demonstrating that the composition and organization of the cell wall have an important role in the regulation of the Cpx pathway. These data supports findings by Evans *et al*. (Evans, Kannan, Li, de Pedro, & Young, 2013) that show that the Cpx pathway is activated in the absence of PBPs 4, 6, 7. As hypothesized by Evans, this could signify that: 1) YcbB or YgaU modify or alter a specific

muropeptide that inhibits the Cpx pathway, and in absence of these enzymes they inhibit or can no longer be synthesized; and 2) YcbB and YgaU do not interact directly with CpxA. Cpx inducing cues such as alkaline pH (Danese & Silhavy, 1998a), high osmolarity (Jubelin et al., 2005), and SDS (Cosma et al., 1995) can affect the peptidoglycan structure (Desmarais, De Pedro, Cava, & Huang, 2013) while NlpE over-expression can function as a sensing mechanism of potential threats to the envelope and peptidoglycan stability (Yeats & Bateman, 2003). Thus, YgaU and YcbB may help stabilize the bacterial envelope in the presence of Cpx-inducing conditions, and they might also have an additional role in the protection of the inner membrane (Raivio et al., 2013). The inner membrane is one of the main targets of the Cpx stress response as induction of this stress response causes changes in the transcription of genes for transporters and respiration (2013). Localization of the DAP-DAP cross-links could be directed towards the proximity of the inner membrane due to the presence of the putative BON phospholipid-binding motif present in YgaU, if this protein is acting as an enhancer of YcbB (Yeats & Bateman, 2003).

Taken together, these results suggest that elevated DAP-DAP cross-links, controlled by YcbB and YgaU during activation of the Cpx pathway, cause changes in the structure, metabolism, and reinforcement of the cell wall.

5.3 Increased peptidoglycan recycling and turnover due to an increase in the transcription of various hydrolases and amidases is another main feature of the Cpx pathway.

5.3.1 Lytic transglycosylases Slt70 and MltB increase the percentage of 1,6-anhydromuropeptides during the activation of the Cpx pathway

The main function of Slt70 consists of catalyzing the cleavage of the glycosidic bonds between the N-acetylglucosamine and the N-acetylmuramic acid residues in the peptidoglycan. This results in the formation of 1,6-anhydro bonds within the N-acetylmuramic acid (Dijkstra, Thunnissen, & Asselt, 1999). Synthesis of 1,6-anhydromuropeptides is a critical process for peptidoglycan recycling and turnover (van Heijenoort 2011). MltB is another lytic transglycosylase with activity similar to that of Slt70 (Suvorov, Lee, Hesek, Boggess, & Mobashery, 2008). However, MltB is localized on the outer membrane (Ehlert, Höitje, & Templin, 1995) and it is the only lytic transglycosylase to process short chain fragments (Suvorov et al., 2008). MltB also has a soluble form, Slt35, which is the result of proteolytic cleavage and which shares a high homology with Slt70 (van Asselt et al., 1999). MltB and Slt70 are highly expressed during peptidoglycan turnover, as large amounts of peptidoglycan are released during this process (Kraft, Prabhu, Ursinus, & Höltje, 1999b). This requires bacteria to hydrolyze the peptidoglycan fragments that are released, and recycle them for the synthesis of a new peptidoglycan layer. Peptidoglycan recycling occurs during cell division (Park & Uehara, 2008), but it

has also been demonstrated that peptidoglycan turnover has a regulatory role by sensing the status of the cell wall and responding to the need to repair the cell wall (Park 1995; Park & Uehara, 2008) .

Luciferase assays using the *slt70*-pJW15 and the *mltB*-pJW15 reporters showed that the transcription levels of these two genes was up-regulated in the presence of Cpx-inducing cues, such as the presence of the strong allele *cpxA24*, and the over-expression of NlpE (Figure 3-8 and Figure 3-10), suggesting that these two genes are part of the Cpx regulon. Interestingly, the over-expression of *bfp,* a well-characterized Cpx inducing cue (Nevesinjac & Raivio, 2005), did not cause any changes in the transcription of *mltB* (data not shown). A clue as to why this might be derives from the fact that over-expression of *bfp* caused only a mild increase in the transcription of other strongly Cpx-regulated cell wall modification genes (Figure 3-6B and C, Figure 3-8B and C) such as *ygaU* and *ycbB.* These data suggest that this is only a mild inducing cue (Danese & Silhavy, 1998b; Nevesinjac & Raivio, 2005). Since the transcription levels of *mltB*-pJW15 only showed a slight increase in the presence of *bfp* over-expression, this might indicate that *mltB* is not induced by *bfp* over-expression.

Furthermore, EMSA analysis indicated that CpxR has a strong affinity for the *slt70* and *mltB* promoters (Figure 3-9, Figure 3-11) as the presence of the competitive control *degP* did not affect binding of CpxR~P to any of these promoters. These findings support the transcriptomic data that shows that *slt70* is part of the Cpx regulon of both *E. coli (Raivio et al., 2013),* and *Haemophilus*

ducreyi (Gangaiah et al., 2013). In addition, these results also strongly suggest that *mltB* is a putative novel-member of the Cpx regulon.

The *cpxA* slt70::kan* mutant displayed an increase in the resistance to carbenicllin, while the MC4100 *slt70::kan* mutant showed a decrease in the resistance to cloxacillin (Table 4-1). These changes in the sensitivity to cloxacillin can be a result of changes of the expression of the β-lactamase AmpC, as it has been noted that absence of *slt70* represses the induction of AmpC (Kraft, Prabhu, Ursinus, & Höltje, 1999a). However, we did not see a significant increase in the resistance to cloxacillin. This can be due to an indirect effect due on a negative regulator of AmpC, such as a sRNA or an increase in the expression of *ampD* while the changes in the resistance to carbenicillin can be a result of the up-regulation of lytic transglycosylases such as MltB in the presence of Cpx-induction as over-expression of MltB induces β-lactamase induction (Kraft, Prabhu, Ursinus, & Höltje, 1999a). This is supported by the fact that the MC4100 *mltB::kan* mutant showed a decrease in the resistance to piperacillin. In addition, the *cpxA* mltB* mutant exhibited a 4-fold increase in the resistance to carbenicillin, which in this case can be due to an indirect increase in the levels of Slt70 as this lytic transglycosylase can interteract with different PBPs, forming multi-complex enzymes which can affect the peptidoglycan structure (Sauvage et al., 2008).

Additionally, HPLC indicated that the absence of *slt70* in the wild-type caused a significant decrease in anhydro-muropeptides. The *cpxA* slt70* mutant showed a more significant decrease in anhydro-muropeptides and muropeptides

bound to a lipoprotein, while the percentage of DAP-DAP cross-links increased significantly (Table 4-5). This indicates that Slt70 has an active role during the activation of the Cpx pathway in the synthesis of 1,6-anhydromuropeptides and absence of this lytic transglycosylase causes an increase in the length of the muropeptide chains, which also leads to an increase in the amount of DAP-DAP cross-links since these increase proportionally to the murein chain length (Magnet et al., 2008). The reduction in the percentage of muropeptides bound to a lipoprotein can result from an increase in the activity of the Cpx pathway, as no Lpp-transglycosylases have been characterized as part of the Cpx regulon (Magnet et al., 2007). Therefore, the increase of L,D-transpeptidases YcbB and its putative enhancer YgaU results in an increase of DAP-DAP cross-links and a decrease in peptidoglycan bound to Braun's lipoprotein.

The muropeptide profile of the MC4100 *mltB::kan* (Table 4-5) mutant showed a 34.5% increase in the proportion of DAP/DAP cross-links. However, we observed a 30% increase in the percentage of anhydro-muropeptides and a more than a 40% increase in the amount of muropeptides bound to a lipoprotein. This can be due to the increase in the muropeptide chain length which can enhance the susbtrate availability for other membrane-bound lytic translgycosylases (MltA, MltC, MltD, MltE) (van Heijenoort 2011; Vollmer, Joris, Charlier, & Foster, 2008) and Lpp-transpeptidases. In addition, in the absence of Cpx-inducing cues other Lpp-transpeptidases can be up-regulated (Magnet et al., 2007) causing a higher percentage in this type of muropeptide. Similarly, the *cpxA* mltB* mutant had a lower percentage of anhydro-

muropeptides, a higher proportion of DAP-DAP cross-links, and longer muropeptide chains than its wild-type counterpart. The *cpxA* mltB,* just like the *cpxA* slt70* mutant, also displayed a decrease in the proportion of lipoprotein-bound muropeptides. This is probably due to the combined action of longer muropeptide chains and high activity of YcbB and YgaU and the absence of the only Cpx-regulated lytic transglycosylase.

5.3.2 The Cpx pathway up-regulates to a minor extent amidases *amiA* and *amiC* which are involved in peptidoglycan turn over and recycling

In bacterial peptidoglycan, the peptide stem is bound to the N-acetylmuramic acid by an amide bond between the N-terminal L-alanine residue and the D-lactoyl moiety of the N-acetyl muramic unit (van Heijenoort 2011; Vollmer, Blanot, & de Pedro, 2008). *E. coli* has 6 amidases that have N-acetylmuramoyl-L-activity (AmiA, AmiB, AmiC, AmpD, AmiD, and a 39kDa amidase) that are highly up-regulated during peptidoglycan turnover (Kraft et al., 1999b). In this study we proceeded to analyze the effect of the Cpx pathway on the regulation of AmiA and AmiC.

A previous microarray analysis by Raivio *et. Al (Raivio et al., 2013)* found that *amiA* is a putative member of the Cpx regulon. In this study, we confirmed *amiA* as part of the Cpx regulon of *E. coli.* Lux assays with the *amiA*-pJW15 reporter (Figure 3-13) showed that this gene is activated by the Cpx pathway in the presence of the *cpxA** allele and NlpE over-production. Furthermore, it has been reported that *amiA* is also regulated by the Cpx pathway in *Salmonella* as a

result of NlpE over-expression (Weatherspoon-Griffin et al., 2011a). This suggests that *amiA* might be a conserved member of the Cpx regulon.

Interestingly, the activity of the *amiA* reporter was low compared to the activity of the *ygaU, ycbB, slt70,* or *mltB lux* reporters, indicating that *amiA* is mildly up-regulated during the activation of the Cpx pathway. Mild regulation of *amiA* has also been observed in *Salmonella* (Weatherspoon-Griffin et al., 2011b) as over-expression of NlpE caused a slight increase in the transcription of the reporter *amiA-lacZ*. In this same study by Weatherspoon-Griffin (2011a) *amiC* also was found to be up-regulated by the presence of NlpE over-expression. With this in mind, we sought to determine if *amiC* could be a potential member of the Cpx regulon since the microarray performed by Raivio *et al.* failed to detect the expression of this gene (Figure 3-14) (Raivio et al., 2013). Unexpectedly, we observed that the transcription of the *amiC* reporter increased significantly in the absence of CpxA. Also, induction of the Cpx pathway in the presence of the *cpxA** or via NlpE did not increase the transcription of this gene. This suggests that *amiC* has a different regulation mode in *E. coli* than in *Salmonella*, which is expected due to differences in the Cpx pathway (Humphreys et al., 2004; Kato et al., 2012). Activity of *amiA* and *amiC* has been shown to precede the activity of lytic transglycosylases (Park & Uehara, 2008), which in light of this study would signify that precedes the activity of AmiA the activity of Slt70 and MltB. Furthermore, AmiA is localized throughout the periplasm and has no preferred substrate, while AmiC is restricted to the division septum during active cell division and its substrate preference is tripeptides (Sikdar & Doerrler, 2010;

Vollmer et al., 2008). As such, *amiA* has more generic activity than *amiC (Priyadarshini, Popham, & Young, 2006)*. Related to this, the decrease in activity that was observed for the *amiC* reporter in the presence of the *cpxA** allele compared to the *cpxR* background could signify that cell division is decreased during activation of the Cpx pathway as *amiC* is the last protein to be recruited to the divisome (Arends & Weiss, 2004; Olrichs et al., 2011). Furthermore, it has been noted that mutants that express the *cpxA** allele show defects in cell division due to a mislocalized FtsZ (Pogliano et al., 1998) and possibly due to changes in the expression of divisome proteins such as AmiC (den Blaauwen, de Pedro, Nguyen-Distèche, & Ayala, 2008).

5.3.4 The Cpx pathway up-regulates the peptidoglycan peptidase DacC which might be necessary for the formation of DAP-DAP cross-links

DacC is a D-ananyl-D-alanine carboxypeptidase that cleaves the peptide bond between the D-alanine and the stem peptide (Pedersen, Murray, Popham, & Setlow, 1998). Luciferase assays with the *dacC*-pJW15 reporter showed that this gene is highly up-regulated in the presence of the *cpxA** allele, and as a result of Bfp and NlpE over-production (Figure 3-12), corroborating that *dacC* is a member of the Cpx regulon (Raivio et al., 2013).

DacC has been implicated in the maturation of the cell wall and it is also necessary for the maintenance of normal cell morphology (Santos, Lobo, Matos, De Pedro, & Arraiano, 2002). This suggests that the Cpx pathway might up-

regulate this gene to preserve the cell shape during the presence of Cpx-inducing conditions. Interestingly, in the microarray analysis performed by Raivio *et al*. this was the only PBP to be up-regulated, which could signify that DacC has a very important role in the survival of *E. coli* in the presence of stressful conditions. The activity of DacC is also necessary for L,D-transpeptidases to synthesize DAP-DAP cross-links as these enzymes can recognize tetrapeptide stems but not pentapeptides (Chen et al., 2009; Magnet et al., 2008; Santos et al., 2002). Therefore, DacC might also play an important role in the synthesis of DAP-DAP cross-links by YcbB. Further studies are necessary to confirm this hypothesis.

5.4 Future directions

The results presented in this project open a wide variety of directions for novel projects. We identified *ygaU, ycbB, mltB,* and *slt70* as being directly regulated during the activation of the Cpx pathway. However, we did not perform the necessary analysis to identify if *amiA, amiC,* and *dacC* are directly regulated by the Cpx pathway. In order to further clarify the role of these amidases and hydrolases, I suggest performing an EMSA assay and HPLC analysis of single mutants of these genes in the MC4100 and the *cpxA** backgrounds.

The *cpxP-lacZ* reporter showed an increase in activity in the absence of *ygaU* and *ycbB,* and no changes in the absence of *mltB,* indicating that changes in the peptidoglycan structure can activate the Cpx pathway as it also has been

observed by Evans *et. Al (Evans et al., 2013)*. It would be interesting to examine if absence of *slt70, dacC, amiA*, *and amiC* can also cause induction of the Cpx pathway which would provide further insight in the nature of the inducing cues that affect the cell wall and induce the Cpx pathway.

In addition, previous research has demonstrated that the Rcs pathway is induced by the Cpx pathway (2013) in response to human serum (Miajlovic, Cooke, Moran, Rogers, & Smith, 2013). However, it would be important to assess if the nature of the activation of the Rcs response is due to the changes in the structure of the cell wall mediated by the up-regulation of genes such as *ycbB* and *ygaU* by the Cpx pathway. For this, activation of the Rcs stress response could be measured using an *rcsC lux* reporter (Evans et al., 2013) in *ycbB* and *ygaU* single and double mutants in a *cpxA** background to determine if the changes that the cell wall undergoes are indeed the inducing cue of the Rcs response.

Evans *et al.* have suggested that activation of the Cpx pathway in the absence of certain PBPs, or as we observed in the absence of *ycbB* and *ygaU,* is due to fragmentation of the cell wall. However, we hypothesize that this is due to changes in the peptidoglycan that render the inner membrane more susceptible to Cpx-inducing cues, which in turns activates this response. Therefore it is possible that if we analyzed the induction of the Cpx pathway in the presence of peptidoglycan fragmentation using the *cpxP-lacZ* reporter we could determine if peptidoglycan cleavage is an inducing cue of this stress response. Fragmentation can be obtained by over-expression of a lytic transglycosylase that is not induced

by the Cpx pathway such as MltE, or by growing the cells in the presence of high concentrations of penicillin. (Amoroso et al., 2012).

It has been suggested that the cleavage of the stem peptide by AmiA precedes the activity of Slt70 and MltB, it is possible that if we examined the expression of *slt70* and *mltB* we could see a decrease in the activity of these genes, indicating that *amiA, mtlB,* and *slt70* have a specific role in peptidoglycan turnover during the activation of the Cpx pathway.

Analysis of the role of *dacC* during the activation of the Cpx pathway might also provide further insight in the regulation of the synthesis of DAP-DAP cross-links as the terminal D-alanine of the stem peptide has to be removed in order for YcbB to be able to synthesize 3-3 cross-liks (Desmarais et al., 2013). *dacC* was the only carboxypeptidase to be up-regulated in the microarray performed by Raivio *et. Al* (Raivio et al., 2013), which suggests that the activity of DacC might be necessary for YcbB. Analysis of the activity of *ycbB* in the absence of *dacC* can be measured using the *ycbB*-pJW15 reporter in a *cpxA* dacC::kan* mutant or the peptidoglycan structure of a *dacC* mutant can be determined by HPLC to determine the impact of DacC on DAP-DAP cross-links synthesized YcbB.

Further characterization of the function and activity of YgaU will also provide further insight in how the Cpx pathway modifies the cell wall as *ygaU* was highly expressed during the activation of this response. I would suggest performing an *in vitro* peptidoglycan restriction assay using purified YgaU and a YgaU mutant without its characteristic LysM motif (Born, Breukink, & Vollmer,

2006), followed by HPLC analysis. This will provide a link between the cell wall composition and the function of YgaU (Desmarais et al., 2013). Also, localization studies of YgaU using flourescence protease protection (FPP) will provide more information about the cellular function of this protein (Lorenz, Hailey, Wunder, & Lippincott-Schwartz, 2006). This assay consists on testing the proteolytic digestibility of GFP-tagged proteins with transmembrane domains to determine whether they are facing the periplasm or the cytoplasm.

Finally, recent studies have demonstrated that the Cpx regulon is enriched for inner membrane regulated genes (Raivio et al., 2013). The fact that the Cpx pathway regulates cell wall genes might imply that the changes that this structure undergoes might have a role in the protection of the inner membrane. This could be confirmed by analyzing changes in the membrane potential in the absence of cell wall genes that are members of the Cpx regulon (Zilberstein, Agmon, Schuldiner, & Padan, 1984).

5.5 Concluding Remarks

In this study, we found that the Cpx pathway up-regulates seven cell wall genes *(ycbB, ygaU, slt70, mltB, amiA, amiC, dacC)* involved in peptidoglycan synthesis and recycling. *ycbB, ygaU, slt70, mltB, amiA, dacC* were up-regulated in the presence of the *cpxA** allele and NlpE-over-expression while *amiC* was down-regulated as a result of Cpx activation. In addition, we showed that *ygaU, mltB, slt,* and *ycbB* are directly up-regulated by the Cpx pathway as CpxR~P binds with high affinity to their promoter regions. Analysis of the structure and

composition of the cell wall showed that activation of the Cpx pathway leads to an increase in DAP-DAP cross-links due to the activity of L,D-transpeptidase YcbB and the putative regulator YgaU, and it also causes an increase in peptidoglycan recycling and turnover due to the activities of Slt70 and MltB that cause decrease in chain length and an increase in anhydro muropeptides, which can help remove damaged peptides. We conclude that the Cpx pathway actively modifies the structure of the cell wall in response to Cpx-inducing conditions.

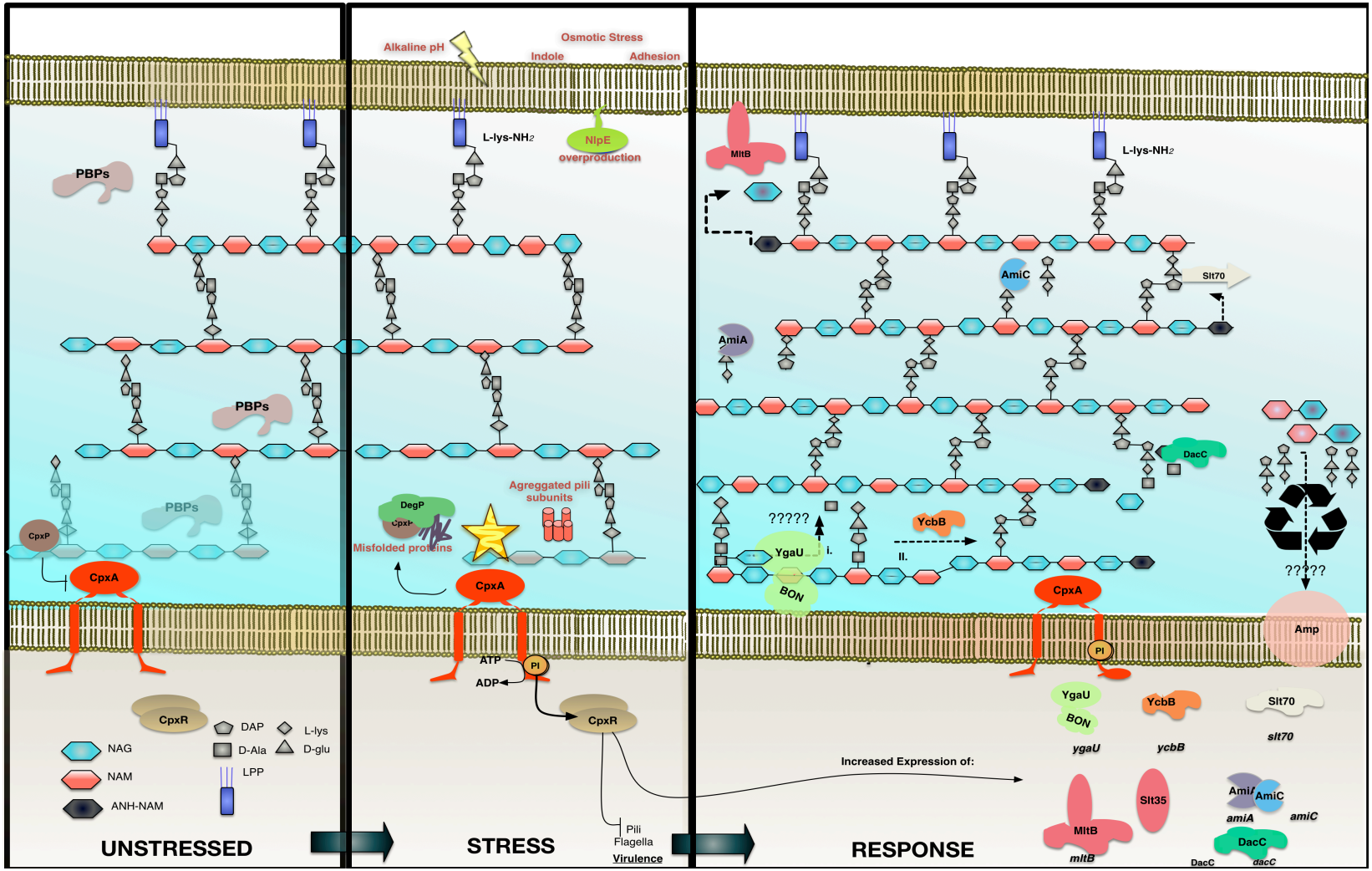

Alkaline pH
Osmotic Stress
Indole
Adhesion
NlpE
overproduction
L-lys-NH2
PBPs
PBPs
PBPs
CpxP
CpxA
CpxR
DegP
Misfolded proteins
Agreggated pili subunits
ATP
ADP
PI
Pili
Flagella
Virulence
NAG
NAM
ANH-NAM
DAP
D-Ala
L-lys
D-glu
LPP
UNSTRESSED
STRESS
RESPONSE
MltB
AmiC
AmiA
Slt70
DacC
?????
YgaU
YcbB
BON
i.
II.
Amp
Increased Expression of:
Slt35
ygaU
ycbB
slt70
amiA
amiC
mltB
dacC

Figure 5-1 : A comprehensive view of the changes that the cell wall undergoes as a result of Cpx activation.

In the absence of stress, it is hypothesized that CpxP interacts with CpxA inhibiting the activity of the Cpx pathway (Raivio, Popkin, & Silhavy, 1999) while the cell wall is characterized by the presence of PBPs that synthesize 3-4 cross-links (Young 2011). In the presence of stress, CpxP is titrated away from CpxA and it is degraded by DegP (Isaac, Pinkner, Hultgren, & Silhavy, 2005). CpxA autophosphorylates and then transfers a phosphate group to the cognate response regulator CpxR, which as we demonstrated in this study directly up-regulates *ygaU, ycbB, slt70, mltB,* and hypothetically *dacC* and *amiA* while down-regulating *amiC.* This causes numerous changes to the cell wall: DacC cleaves the peptide bond between the two terminal D-alanines of the stem peptide (Chen et al., 2009). YgaU acts a hypothethic enhancer of the activity of YcbB, hydrolyzing the different muropeptide subunits and DacC removes so YcbB can synthesize DAP-DAP cross-links (Magnet et al., 2008). Simultaneously, AmiA cleaves the stem peptide from the N-acetyl glucosamine while the membrane-bound lytic transglycosylase (Priyadarshini et al., 2006). MltB and the soluble lytic transglycosilase Slt70 cleave the N-glycosydic bond between the N-acetyl glucosamine and the N-acetyl muramic acid, resulting in the formation of 1,6-anhydro-N-acetyl muramic acid (Dijkstra et al., 1999; Suvorov et al., 2008) . Cleaved peptidoglycan subunits are then transported to the cytoplasm to be recycled by the permease AmpG (Lindquist et al., 1993)

References

Amoroso, A., Boudet, J., Berzigotti, S., Duval, V., Teller, N., Mengin-Lecreulx, D., . . . Joris, B. (2012). A peptidoglycan fragment triggers β-lactam resistance in *Bacillus licheniformis*. *PLoS Pathogens*, *8*(3), e1002571. doi:10.1371/journal.ppat.1002571

Arends, S. J. R., & Weiss, . S. (2004). Inhibiting cell division in *Escherichia coli* has little if any effect on gene expression. *Journal of Bacteriology*, *186*(3), 880-884. doi:10.1128/JB.186.3.880-884.2004

Bateman, A., & Bycroft, M. (2000). The structure of a lysm domain from *E. coli* membrane-bound lytic murein transglycosylase D (*mltD*). *Journal of Molecular Biology*, *299*(4), 1113-9. doi:10.1006/jmbi.2000.3778

Bielnicki, J., Devedjiev, Y., Derewenda, U., Dauter, Z., Joachimiak, A., & Derewenda, Z. S. (2006). *B. Subtilis* Ykud protein at 2.0 A resolution: Insights into the structure and function of a novel, ubiquitous family of bacterial enzymes. *Proteins*, *62*(1), 144-51. doi:10.1002/prot.20702

Born, P., Breukink, E., & Vollmer, W. (2006). In vitro synthesis of cross-linked murein and its attachment to sacculi by PBP1A from *Escherichia coli*. *The Journal of Biological Chemistry*, *281*(37), 26985-93. doi:10.1074/jbc.M604083200

Buist, G., Steen, A., Kok, J., & Kuipers, O. P. (2008). LysM, a widely distributed protein motif for binding to (peptido)glycans. *Molecular Microbiology*, *68*(4), 838-47. doi:10.1111/j.1365-2958.2008.06211.x

Bury-Moné, S., Nomane, Y., Reymond, N., Barbet, R., Jacquet, E., Imbeaud, S., . . . Bouloc, P. (2009). Global analysis of extracytoplasmic stress signaling in *Escherichia coli. PLoS Genetics*, *5*(9), e1000651. doi:10.1371/journal.pgen.1000651

Chen, Y., Zhang, W., Shi, Q., Hesek, D., Lee, M., Mobashery, S., & Shoichet, B. K. (2009). Crystal structures of penicillin-binding protein 6 from *Escherichia coli*. *Journal of the American Chemical Society*, *131*(40), 14345-54. doi:10.1021/ja903773f

Cosma, C. L., Danese, P. N., Carlson, J. H., Silhavy, T. J., & Snyder, W. B. (1995). Mutational activation of the cpx signal transduction pathway of *Escherichia coli.* suppresses the toxicity conferred by certain envelope-associated stresses. *Molecular Microbiology*, *18*(3), 491-505. Retrieved from Google Scholar.

Danese, . N., Snyder, . B., Cosma, . L., Davis, . J., & Silhavy, . J. (1995). The cpx two-component signal transduction pathway of *Escherichia coli* regulates transcription of the gene specifying the stress-inducible periplasmic protease, *degP*. *Genes & Development*, *9*(4), 387-398. doi:10.1101/gad.9.4.387

Danese, P. N., & Silhavy, T. J. (1998a). CpxP, a stress-combative member of the Cpx regulon. *Journal of Bacteriology*, *180*(4), 831-9.

Danese, P. N., Oliver, G. R., Barr, K., Bowman, G. D., Rick, P. D., & Silhavy, T. J. (1998). Accumulation of the enterobacterial common antigen lipid II biosynthetic intermediate stimulates *degp* transcription in *Escherichia coli*. *Journal of Bacteriology*, *180*(22), 5875-84.

den Blaauwen, T., de Pedro, M. A., Nguyen-Distèche, M., & Ayala, J. A. (2008). Morphogenesis of rod-shaped sacculi. *FEMS Microbiology Reviews*, *32*(2), 321-44.

Desmarais, S. M., De Pedro, M. A., Cava, F., & Huang, K. C. (2013). Peptidoglycan at its peaks: How chromatographic analyses can reveal bacterial cell wall structure and assembly. *Molecular Microbiology*, *89*(1), 1-13.

Dijkstra, B.W., Thunnissen, A.-M.W., and Asselt, E.J.V. (1999) High Resolution Crystal Structures of the *Escherichia coli* Lytic Transglycosylase Slt70 and its Complex with a Peptidoglycan Fragment. *Journal of Molecular Microbiology.* **4:** 877-898

Ehlert, K., Höitje, J. -V., & Templin, M. F. (1995). Cloning and expression of a murein hydrolase lipoprotein from escherichia coli. *Molecular Microbiology*, *16*(4), 761-768.

Evans, K. L., Kannan, S., Li, G., de Pedro, M., & Young, K. D. (2013). Eliminating a set of four penicillin binding proteins triggers the Rcs phosphorelay and Cpx stress responses in escherichia coli. *Journal of Bacteriology.* doi:10.1128/JB.00596-13

Gangaiah, D., Zhang, X., Fortney, K. R., Baker, B., Liu, Y., Munson, R. S., & Spinola, S. M. (2013). Activation of CpxRA in *Haemophilus ducreyi* primarily inhibits the expression of its targets, including major virulence determinants. *Journal of Bacteriology*, *195*(15), 3486-502. doi:10.1128/JB.00372-13

Humphreys, S., Rowley, G., Stevenson, A., Anjum, M. F., Woodward, M. J., Gilbert, S., Roberts, M. (2004). Role of the two-component regulator cpxar in the virulence of Salmonella Enterica serotype Typhimurium. *Infection and Immunity*, *72*(8)

Isaac, D. D., Pinkner, J. S., Hultgren, S. J., & Silhavy, T. J. (2005). The extracytoplasmic adaptor protein CpxP is degraded with substrate by DegP. *Proceedings of the National Academy of Sciences of the United States of America*, *102*(49), 17775-17779.

Islam, M. A., Alam, M. M., Choudhury, M. E., Kobayashi, N., & Ahmed, M. U. (2008). Determination of minimum inhibitory concentration (MIC) of cloxacillin for selected

isolates of methicillin-resistant staphylococcus aureus (MRSA) with their antibiocs. *Bangladesh Journal of Veterinary Medicine*, *6*(1), 121-126. Retrieved from Google Scholar.

Jubelin, G., Vianney, A., Beloin, C., Ghigo, J. M., Lazzaroni, J. C., Lejeune, P., & Dorel, C. (2005). CpxR/ompr interplay regulates curli gene expression in response to osmolarity in *Escherichia coli. Journal of Bacteriology*, *187*(6), 2038-49. doi:10.1128/JB.187.6.2038-2049.2005

Kato, A., Hayashi, H., Nomura, W., Emori, H., Hagihara, K., & Utsumi, R. (2012). A connecter-like factor, caca, links rssb/rpos and the cpxr/cpxa two-component system in salmonella. *BMC Microbiology*, *12*, 224. doi:10.1186/1471-2180-12-224

Kraft, A. R., Prabhu, J., Ursinus, A., & Höltje, J. V. (1999a). Interference with murein turnover has no effect on growth but reduces beta-lactamase induction in *Escherichia coli*. *Journal of Bacteriology*, *181*(23), 7192-8.

Kraft, A. R., Prabhu, J., Ursinus, A., & Höltje, J. -V. (1999b). Interference with murein turnover has no effect on growth but reduces β-lactamase induction in *Escherichia coli*. *Journal of Bacteriology*, *181*(23), 7192-7198. Retrieved from Google Scholar.

Labandeira-Rey, M., Brautigam, C. A., & Hansen, E. J. (2010). Characterization of the CpxRA regulon in *Haemophilus ducreyi. Infection and Immunity*, *78*(11), 4779-91. doi:10.1128/IAI.00678-10

Lindquist, S., Weston-Hafer, K., Schmidt, H., Pul, C., Korfmann, G., Erickson, J., . . . Normark, S. (1993). AmpG, a signal transducer in chromosomal β-lactamase induction. *Molecular Microbiology*, *9*(4), 703-715.

Lorenz, H., Hailey, D. W., Wunder, C., & Lippincott-Schwartz, J. (2006). The fluorescence protease protection (FPP) assay to determine protein localization and membrane topology. *Nature Protocols*, *1*(1),

Magnet, S., Bellais, S., Dubost, L., Fourgeaud, M., Mainardi, J. L., Petit-Frère, S., . . . Gutmann, L. (2007). Identification of the L,D-transpeptidases responsible for attachment of the Braun lipoprotein to *Escherichia coli* peptidoglycan. *Journal of Bacteriology*, *189*(10), 3927-31. doi:10.1128/JB.00084-07

Magnet, S., Dubost, L., Marie, A., Arthur, M., & Gutmann, L. (2008). Identification of the L,d-transpeptidases for peptidoglycan cross-linking in *Escherichia coli*. *Journal of Bacteriology*, *190*(13), 4782-5.

Miajlovic, H., Cooke, N. M., Moran, G. P., Rogers, T. R., & Smith, S. G. (2013). The response of extra-intestinal pathogenic *Escherichia coli* to human serum reveals a protective role for rcs-regulated exopolysaccharide colanic acid. *Infection and Immunity*.

Nevesinjac, A. Z., & Raivio, T. L. (2005). The Cpx envelope stress response affects expression of the type IV bundle-forming pili of enteropathogenic *Escherichia coli*. *Journal of Bacteriology*, *187*(2), 672-86.

Olrichs, N. K., Aarsman, M. E., Verheul, J., Arnusch, C. J., Martin, N. I., Hervé, M., . . . den Blaauwen, T. (2011). A novel in vivo cell-wall labeling approach sheds new light on peptidoglycan synthesis in *Escherichia coli*. *Chembiochem : A European Journal of Chemical Biology*, *12*(7), 1124-33.

Park, J. T. (1995). Why does *Escherichia coli* recycle its cell wall peptides? *Molecular Microbiology*, *17*(3), 421-426.

Park, J. T., & Uehara, T. (2008). How bacteria consume their own exoskeletons (turnover and recycling of cell wall peptidoglycan). *Microbiology and Molecular Biology Reviews 72*(2), 211-2.

Pedersen, L. B., Murray, T., Popham, D. L., & Setlow, P. (1998). Characterization of dacc, which encodes a new low-molecular-weight penicillin-binding protein in *Bacillus subtilis*. *Journal of Bacteriology*, *180*(18), 4967-73.

Pogliano, J., Dong, J. M., De Wulf, P., Furlong, D., Boyd, D., Losick, R., . . . Lin, E. C. C. (1998). Aberrant cell division and random ftsz ring positioning in *Escherichia coli* cpxa* mutants. *Journal of Bacteriology*, *180*(13), 3486-3490.

Pogliano, J., Lynch, . S., Belin, D., Lin, . C., & Beckwith, J. (1997). Regulation of *Escherichia coli* cell envelope proteins involved in protein folding and degradation by the Cpx two-component system. *Genes & Development*, *11*(9), 1169-1182.

Priyadarshini, R., Popham, D. L., & Young, K. D. (2006). Daughter cell separation by penicillin-binding proteins and peptidoglycan amidases in *Escherichia coli*. *Journal of Bacteriology*, *188*(15), 5345-55. doi:10.1128/JB.00476-06

Raivio, T. L., Leblanc, S. K., & Price, N. L. (2013). The *Escherichia coli* cpx envelope stress response regulates genes of diverse function that impact antibiotic resistance and membrane integrity. *Journal of Bacteriology*, *195*(12), 2755-67.

Raivio, T. L., Popkin, D. L., & Silhavy, T. J. (1999). The cpx envelope stress response is controlled by amplification and feedback inhibition. *Journal of Bacteriology*, *181*(17), 5263-5272.

Santos, J. M., Lobo, M., Matos, A., De Pedro, M. A., & Arraiano, C. M. (2002). The gene bola regulates *daca* (PBP5), *dacc* (PBP6) and *ampC* (AmpC), promoting normal morphology in *Escherichia coli*. *Molecular Microbiology*, *45*(6), 1729-1740.

Sauvage, E., Kerff, F., Terrak, M., Ayala, J. A., & Charlier, P. (2008). The penicillin-binding proteins: Structure and role in peptidoglycan biosynthesis. *FEMS Microbiology Reviews*, *32*(2), 234-58.

Sikdar, R., & Doerrler, W. T. (2010). Inefficient Tat-dependent export of periplasmic amidases in an *Escherichia coli* strain with mutations in two deda family genes. *Journal of Bacteriology*, *192*(3), 807-18.

Suvorov, M., Lee, M., Hesek, D., Boggess, B., & Mobashery, S. (2008). Lytic transglycosylase mltb of *Escherichia coli* and its role in recycling of peptidoglycan strands of bacterial cell wall. *Journal of the American Chemical Society*, *130*(36),

van Asselt, E. J., Dijkstra, A. J., Kalk, K. H., Takacs, B., Keck, W., & Dijkstra, B. W. (1999). Crystal structure of *Escherichia coli* lytic transglycosylase slt35 reveals a lysozyme-like catalytic domain with an ef-hand. *Structure 7*(10), 1167-1180.

van Heijenoort, J. (2011). Peptidoglycan hydrolases of *Escherichia coli*. *Microbiology and Molecular Biology Reviews : 75*(4), 636-63

Vollmer, W., & Bertsche, U. (2008). Murein (peptidoglycan) structure, architecture and biosynthesis in *Escherichia coli*. *Biochim Biophys Acta*, *1778*(9), 1714-34.

Vollmer, W., Blanot, D., & de Pedro, M. A. (2008). Peptidoglycan structure and architecture. *FEMS Microbiology Reviews*, *32*(2), 149-67.

Vollmer, W., Joris, B., Charlier, P., & Foster, S. (2008). Bacterial peptidoglycan (murein) hydrolases. *FEMS Microbiology Reviews*, *32*(2), 259-86.

Weatherspoon-Griffin, N., Zhao, G., Kong, W., Kong, Y., Morigen, Andrews-Polymenis, H., Shi, Y. (2011a). The CpxR/CpxA two-component system up-regulates two tat-dependent peptidoglycan amidases to confer bacterial resistance to antimicrobial peptide. *The Journal of Biological Chemistry*, *286*(7), 5529-39

Yeats, C., & Bateman, A. (2003). The BON domain: A putative membrane-binding domain. *Trends Biochem Sci*, *28*(7), 352-5.

Young, K. D. (2011). Peptidoglycan. In *ELS.* Chichester, UK: John Wiley & Sons, Ltd.

Zilberstein, D., Agmon, V., Schuldiner, S., & Padan, E. (1984). *Escherichia coli* intracellular pH, membrane potential, and cell growth. *Journal of Bacteriology*, *158*(1), 246-252.