The Cpx envelope stress response and *trkA* mutation

by

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Abstract

The fitness and survival of bacteria depends on their ability to adapt and survive in different ecological niches during its life cycle. Bacteria must therefore be able to sense the different changes in the environment during the transition between different ecological niches to enhance survival. The Cpx two component signal transduction system (TCS) is one of the widely known signal transduction systems employed by bacteria to sense and respond to envelope stress to maintain the integrity of the envelope. The Cpx system consists of an inner membrane sensor kinase, CpxA and its periplasmic inhibitor CpxP as well as a cytoplasmic response regulator, CpxR. In the presence of envelope stress, CpxA is relieved of CpxP inhibition, autophosphorylates and subsequently phosphorylates CpxR. Phosphorylated CpxR acts as a transcription factor and regulates the expression of several genes to mediate envelope stress caused by mis-folded proteins in the periplasm. The Cpx response is also up regulated by mutations that affect transport, particularly by antibiotic efflux pumps. Further, part of the Cpx-mediated response involves alterations of the regulation of a variety of transporter genes. However, the connections between transport and the Cpx response are not fully understood. In this study, we investigated the connection between the Cpx response and the Trk system (A transport system that imports potassium into cell). We confirm that induction of the Cpx pathway is specific to mutation of *trkA* and CpxR dependent but did not require CpxA. The Cpx response is known to be inhibited at acidic pH. We show that when *trkA* is mutated, this inhibition no longer occurs. In addition, excess potassium does not play a role in the activation of the Cpx system. Furthermore, we demonstrate that mutation of *trkA* does not elevate Cpx activity by inhibiting CpxA

phosphatase activity or by altering the levels of acetyl phosphate. Also, mutation of *trkA* alters the levels and/or ratio of NAD^+/NADH in the cell. Finally, we show that induction of the Cpx system diminishes respiration in the *trkA* mutant. Our work demonstrates that the Cpx pathway uses novel mechanism to sense and respond to changing cellular physiology associated with transport and metabolism.

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LIST OF SYMBOLS AND ABBREVIATIONS

A_{420}: absorbance at 420 nm

Amp: ampicillin

ATP: adenosine triphosphate

Cam: chloramphenicol

Cpx: conjugative pilus expression

ECF: extracytoplasmic function

HK: Histidine kianse

IM: inner membrane

IPGT: isopropyl-β-D-thiogalactopyranoside

Kan: kanamycin

LB: Luria-Bertani

OD_{600}: optical density at 600 nm

OM: outer membrane

OMP: outer membrane protein

PCR: polymerase chain reaction

PMF: proton-motive force

Rpm: rotations per minute

RR: response regulator

TCS: two component signal transduction system

WT: wild-type

CHAPTER 1: GENERAL INTRODUCTION

1.1 Overview of envelope stress responses

Enteric bacteria experience drastic changes in their habitat during their life cycle. Several of these, including temperature, pH, osmolarity and nutrient availability, affect bacteria both in the human body and the external environment. The cell envelope of bacteria may experience perturbations, and in some cases damage, as the bacteria undergo these changes. Bacteria therefore employ several envelope stress responses to sense and adapt to drastic changes in their environment. These signal transduction systems function to maintain the integrity of the envelope and to enhance the survival of the bacteria. Change within the environment of the bacteria is detected by these signalling systems which in turn transfer the signal to the interior of the cell to alter gene expression to counteract any damage caused by adverse environmental conditions (Galperin, 2004; McAlpine, 2007)

The most commonly known signal transduction systems in bacteria are the extracytoplasmic function (ECF) sigma factors, for example σ^E, and the two component systems (TCSs). The σ^E stress response mediates adaptation to stresses that affect the outer membrane as a result of mis-folded proteins. In the absence of inducing signal (mis-folded OMPs) the membrane bound anti-sigma factor RseA sequesters σ^E and inhibits its activity (Missiakas et al., 1997). The periplasmic regulatory protein, RseB increases the affinity of RseA for σ^E and protects it from cleavage.(Collinet et al., 2000; Grigorova et al., 2004). In the presence of mis-folded OMPs which accumulate in the periplasm, the σ^E system is activated by the degradation of RseA by DegS. Subsequently, σ^E is released into the cytoplasm to initiate the transcription of genes to

alleviate the outer membrane stress (Ades et al., 1999; Alba et al., 2002; Kanehara et al., 2002).

TCSs typically consists of two components or signal transducers, a membrane localized sensor histidine kinase (HK) and a cytoplasmically localized response regulator (RR), which exchange a phosphoryl group. Signal transduction between a histidine kinase (HK) and a response regulator (RR) is initiated in the presence of an environmental stimulus. HKs sense specific aspects of the environment via one or more amino-terminal sensory domains, dimerize and autophosphorylate a conserved histidine residue using ATP as a phosphoryl donor. The HK subsequently transfers this phosphate to a conserved aspartate residue on the receiver domain of the cognate RR. This alters the conformation of the RR and results in DNA binding and transcriptional regulation by the RR to initiate an adaptive response (Fabret et al., 1999; West and Stock, 2001).

Other envelope stress responses found in bacteria include the phage shock protein (Psp) response, which mediates inner membrane stress from phage infection, heat shock, ethanol, and osmotic shock (Brissette et al., 1990; Flores-Kim and Darwin, 2015); the BaeSR two component envelope stress response system, which controls adaptation to toxic chemicals and metabolites through the regulation of efflux pumps (Raffa and Raivio, 2002), and the Rcs phosphorelay pathway that mediates adaptation to stresses associated with peptidoglycan damage and the outer membrane (Laubacher and Ades, 2008; Majdalani and Gottesman, 2005).

The Cpx TCS been identified to mediate adaptation to misfolded proteins in the periplasm during envelope perturbation by altering the transcription of several protein

folding and degrading factors. The Cpx TCS is activated by several inducing signals, which include alkaline pH, copper, chloride, overexpression of misfolded periplasmic proteins and adherence to abiotic surfaces. Further, recent studies have shown that the CpxAR two component system is also involved in the regulation of transporter genes that impact the bacterial cell envelope in diverse ways (Otto and Silhavy, 2002; Raivio et al., 2013; Vogt et al., 2010).

1.2 The Cpx two component system

1.2.1 History

The Cpx signal transduction system was discovered during a genetic screen of strains incapable of F-plasmid conjugation. The locus, *cpxA* (conjugative pilus expression) is found on the chromosome of *Escherichia coli* and in some other gamma proteobacteria. Strains with mutations in the *cpxA* locus displayed several phenotypes including; temperature sensitivity, altered transport of some carbon substrates, and isoleucine and valine auxotrophy. In addition, it was demonstrated that the mutant strains also showed resistance to aminoglycoside antibiotics and colicins. Because most of these phenotypes were associated with the bacterial envelope in one way or another, the *cpxA* locus was linked to this cellular compartment (McEwen and Silverman, 1980a, b; Newman et al., 1981; Plate and Suit, 1981; Plate et al., 1986). Further studies of the genes encoded by the *cpx* locus demonstrated that it encoded a sensory histidine kinase CpxA, with its cognate response regulator CpxR. As a result of these discoveries, the Cpx pathway was proposed to be a two-component signal transduction system associated with altering envelope activity (Albin et al., 1986; Jianming et al., 1993). Moreover, the Cpx TCS was shown to up-regulate the

expression of the periplasmic chaperone/protease DegP that mediates envelope stress adaptation in the presence of misfolded and/or toxic proteins (Cosma et al., 1995; Danese et al., 1995). These findings supported the notion that the Cpx TCS is involved in maintaining the integrity of the bacterial envelope.

1.3 Mechanism of the Cpx TCS

The Cpx signal sensing pathway involves four proteins in different compartments of the cell: the outer membrane (OM) lipoprotein NlpE, periplasmic protein CpxP, inner membrane (IM) localised HK CpxA, and the cytoplasmic RR CpxR (Vogt and Raivio, 2012). The OM lipoprotein NlpE signals the Cpx TCS in response to adhesion to hydrophobic surfaces while the periplasmic CpxP protein is hypothesized to be involved in a protein-protein interaction with CpxA to keep the Cpx pathway inactive (Vogt and Raivio, 2012). The Cpx pathway is activated by diverse envelope stresses, all of which are predicted to cause protein misfolding. These include physical (osmolarity), chemical (alkaline pH, indole), and biological (altered lipid composition) signals, metal ions (copper, zinc), and adherence to abiotic surfaces (Otto and Silhavy, 2002; Raivio and Silhavy, 2001).

The activation of the pathway is initiated by the relief of inhibition of the sensor histidine kinase, CpxA, through the proteolysis of the periplasmic protein CpxP (Isaac et al., 2005). CpxA is then autophosphorylated at the conserved histidine residue and subsequently transfers the phosphate ion to the conserved aspartate residue on the cognate response regulator, CpxR. Phosphorylated CpxR then functions as a transcription factor that regulates the transcription of target genes to alleviate envelope stress. Target genes transcribed include periplasmic protein folding and degrading

factors (*degP, dsbA, ppiA*), peptidoglycan modification enzymes (*amiA, amiC, ycfS*), inner membrane proteins (*htpX, yccA*, transporters) and envelope localized complexes (pilli, flagella). In noninducing conditions, CpxA remains unphosphorylated and in the inactive state through interaction with the periplasmic protein CpxP, which acts as a negative feedback regulator of the Cpx pathway. Under these conditions, CpxA also acts as a phosphatase of CpxR-P (Buelow and Raivio, 2005; DiGiuseppe and Silhavy, 2003; Raffa and Raivio, 2002; Raivio et al., 2000) (Figure 1-1)

1.4 Inducing cues of the Cpx TCS

The Cpx pathway is primarily induced by envelope stressors like alkaline pH, over-expression of specific membrane associated proteins, alteration in membrane structure, adhesion to abiotic surfaces and several other cues. The majority of the inducing cues of the Cpx pathway are predicted to result in protein misfolding, which subsequently leads to envelope stress (Otto and Silhavy, 2002; Raivio and Silhavy, 2001). Elevated pH is known to activate the Cpx pathway. Cpx mutant strains of *E. coli* showed a hypersensitivity to alkaline pH while strains with the wild-type *cpxA* locus demonstrated an up-regulation of Cpx regulated genes at elevated pH (Danese and Silhavy, 1998). In *Shigella* spp., it was observed that mutation of *cpxA* altered the expression of the virulence regulator *virF* with variation in pH (Nakayama and Watanabe, 1995). The exact mechanism and inducing cue generated from alkaline pH is still unknown. However it has been suggested that alkaline pH is likely to cause denatured proteins which subsequently activate the Cpx response (Nevesinjac and Raivio, 2005).

Induction of the Cpx pathway can also be caused by alterations in the cell membrane. It has been demonstrated that a lack of phosphatidylethanolamine in the cell membrane resulted in the up-regulation of the gene encoding the periplasmic protease *degP* by the Cpx pathway (Mileykovskaya and Dowhan, 1997). In another study, the Cpx TCS was activated by the accumulation of an intermediate of enterobacterial common antigen synthesis in the inner membrane (Danese et al., 1998). All these cases resulted in the alteration of the cell membrane and subsequently activated the Cpx TCS. Although the exact mechanism involved in this activation is not known, it is possible that disruptions in protein folding in the envelope may be the underlying reason for the activation of the Cpx TCS.

Adhesion to abiotic surfaces is another inducer of the Cpx pathway. While sensing of misfolded proteins likely occurs primarily through the periplasmic portion of CpxA, sensing of attachment additionally requires the OM lipoprotein NlpE. It is thought that initial adhesion to a hydrophobic surface induces conformational changes in the OM lipoprotein NlpE which subsequently stimulates CpxA activation to combat any potential envelope stress (Raivio and Silhavy, 2001; Vogt and Raivio, 2012).

1.5 The Cpx TCS regulon

1.5.1 Protein folding and degrading factors

Several studies done on the Cpx regulon indicate that it includes multiple periplasmic chaperones and proteases. This is not surprising as the Cpx pathway is primarily responsible for alleviating envelope stress. These include the molecular chaperone Spy (protein folding), the protease DegP, the disulfide bond oxidoreductase DsbA, and the peptidyl-prolyl isomerase PpiA, among several others (Danese and

Silhavy, 1997; Pogliano et al., 1997; Raivio et al., 2000). These chaperones and proteases alleviate envelope stress by degrading or re-folding misfolded proteins that are detrimental to the survival of the cell. The Cpx pathway therefore mediates adaptation to the envelope stress associated with misfolded proteins by facilitating increased transcription of these genes to enhance the survival of the bacteria

1.5.2 Envelope localized structures

Most of the envelope localized structures that are associated with Cpx induction are down-regulated. These include the type III secretion system, pili and flagella (Dorel et al., 1999; Jubelin et al., 2005; Spinola et al., 2010; Vogt et al., 2010). In most cases, the regulation of these genes is through the direct effect of CpxR on transcription or indirectly through other Cpx regulated genes that are involved in pathogenesis (Vogt and Raivio, 2012). Since the Cpx system alleviates envelope stress mainly by re-folding or degrading mis-folded proteins, it has been suggested that the regulation of these envelope complexes may help conserve energy for other cellular activities and to regulate movement of proteins across the cell membrane (De Wulf et al., 1999; MacRitchie et al., 2008).

1.5.3 Inner membrane transporters

The initial studies done on the Cpx pathway revealed that *E. coli* strains carrying a *cpxA** mutation that constitutively activates the Cpx response showed a reduced ability for the uptake of proline and lactose (Plate and Suit, 1981; Plate, 1976; Plate et al., 1986). Recent studies on the Cpx regulon show that transporter genes for several substrates (succinate, fatty acids, amino acids, and ions) were among the genes down regulated by the Cpx pathway upon overexpression of the lipoprotein NlpE (Raivio et

al., 2013). Similar results were also demonstrated in *Haemophilus ducreyi* (Gangaiah et al., 2013). Furthermore, it has been noted that Cpx pathway activation may result in diminished respiration and proton-motive-force (PMF) through the down regulation of genes encoding succinate and NADH dehydrogenases (Raivio et al., 2013). Altogether, these findings confirm the regulation of inner membrane proteins and/or transporters by the Cpx TCS.

1.6 Cpx Envelope Stress Response in Cellular and Metabolic Activities

1.6.1 Antibiotic resistance

The bacterial cell envelope is the target for many antibiotics due to the crucial role it plays in the cellular and metabolic activities in the cell. Antibiotics such as the beta-lactams inhibit cell wall assembly, while aminoglycosides and fluoroquinolones act by targeting protein synthesis and DNA replication respectively (Manoil, 2013; Silver, 2006). The Cpx system has been known to affect the resistance to aminoglycoside antibiotics since it was discovered (Bryan and Van Den Elzen, 1977; Plate, 1976; Thorbjarnardóttir et al., 1978). Adaptation to mistranslated and misfolded proteins resulting from exposure to aminoglycoside antibiotics is mediated by Cpx-induced up-regulation of expression of the gene *yccA*, which prevents the blockage of the SecYEG secretion channel by preventing its degradation by the FtsH protease (van Stelten et al., 2009). Further, other studies have also linked the Cpx response to resistance to aminoglycosides, to novobiocin, and to beta lactam antibiotics in *E. coli* (Bernal-Cabas et al., 2015; Hirakawa et al., 2003a; Hirakawa et al., 2003b). The expression of Cpx regulated gene *degP* is involved in *E. coli* tolerance of antimicrobial peptides (AMPs) but the exact mechanism is unknown (Audrain et al., 2013). Moreover, the Cpx pathway

increases resistance to fosfomysin by the down-regulation the transporter proteins UhpT and GlpT (Kurabayashi et al., 2014).

Despite these findings, it has been shown that mutational activation of the Cpx pathway in *H. ducreyi* results in sensitivity to antimicrobial peptides (Rinker et al., 2011), while in *Klebsiella pneumonia* the deletion of the Cpx pathway leads to increased sensitivity to beta lactams and chloramphenicol (Srinivasan et al., 2012). While it is clear that the Cpx TCS plays a role in antibiotic resistance, there is no clear mechanism designating the exact role of the Cpx pathway in antibiotic resistance.

1.6.2 Regulation of transport

Membrane transport regulation has been associated with the Cpx pathway since it was discovered many years ago (Plate, 1976). A recent study performed in *E. coli* on genes regulated by the Cpx pathway showed that transporters were the second most enriched group of genes after membrane proteins. Induction of the Cpx pathway down-regulated many transporter genes (Raivio et al., 2013). Similar results were also observed in *H. ducreyi* and *Vibrio cholera* (Acosta et al., 2015; Gangaiah et al., 2013). What is intriguing about these findings is the absence of a CpxR binding site in the promoters of some of these transporters. Hence there are no clear mechanisms known to date about the regulation of these transporters by the Cpx pathway.

Induction of the Cpx pathway in *E. coli* is also suspected to cause a decrease in respiration and PMF (Raivio et al., 2013). The genes encoding a succinate dehydrogenase, NADH dehydrogenase, and cytochrome bo_3 are all down regulated by the Cpx system when it is induced. Other transporters down regulated by the Cpx

system include: NhaB (sodium ions), MglB (galactose periplasmic binding protein), TppB (dipeptides), and several others (Raivio et al., 2013)

Carbon substrate transporter genes are also impacted by Cpx induction. The Cpx TCS in enterohemorrhagic *E. coli* represses transcription of the transporter genes *glpT* and *uhpT.* Both *glpT* and *uhpT* genes encode transporters for glycerol and glucose respectively and also serve as the transporter of fosfomycin into cell. Thus, as previously mentioned, repression of these transporters also confers an increase in fosfomycin resistance in *E. coli* (Kurabayashi et al., 2014).

1.7 Transporters

Transporters are membrane proteins used by living organisms in the movement of substances across cell membranes. Membrane transport systems allow the movement of essential nutrients, mediate the uptake and efflux of ionic species and regulate metabolite concentration by the excretion of metabolic end products. The importance of transporters cannot be overstated as genes encoding for transporter proteins make up approximately 10 to 20% of the genome in living organisms (Conde et al., 2010; Lorca et al., 2007; van der Does and Tampé, 2004). In bacteria, transport systems can be categorized into two major groups based on their energy sources. Primary transporters utilize energy released mainly from ATP hydrolysis, or redox reactions, while secondary transporters utilize energy stored in electrochemical gradients to drive the transport of substrates across the membrane. (Driessen et al., 1997; Enkavi et al., 2013; Lolkema et al., 1998).

Membrane transporters function by a phenomenon known as the "alternating access mechanism" and conformational changes (Enkavi et al., 2013). This mechanism

allows the substrate access from only one side of the membrane at a given time, while changes in conformation allow the manoeuvring of protein structures to transport the substrate across the membrane effectively. Membrane transporter systems consist of proteins made up of either alpha helices or beta barrels that span the inner or outer membranes, respectively, and a substrate-binding protein (SBP) which binds substrates and delivers them from the OM uptake apparatus to the IM for transport. The SBP is a free floating periplasmic protein in Gram negative bacteria. In contrast, it is anchored to the cytoplasmic membrane in Gram positive bacteria (Driessen et al., 1997; Mulligan et al., 2011).

1.8 Potassium transport in bacteria

Potassium (K) is the most abundant intracellular monovalent cation in bacteria. K ions are required for several cellular activities including; homeostasis of cytoplasmic pH, cell turgor pressure, adaptation of cells to osmotic conditions, and activation of cytoplasmic enzymes (Bossemeyer et al., 1989a). The concentration of intracellular K in bacteria is controlled by multiple membrane transport systems and can accumulate to about 1M (Epstein and Schultz, 1965; Martirosov and Trchounian, 1986; Richey et al., 1987). Potassium requirement by the cell is only supplied by accumulation from the external medium and the majority of the intracellular K is used to maintain cell turgor (Epstein, 1986). The cell turgor is the pressure exerted by the cytoplasmic membrane on the cell wall due to the difference in osmolarity between the external environment and the interior of the cell. The cell turgor pressure can amount to values in the range of 1 - 4 Atm and its maintenance is essential for growth as well as the shape of the bacteria (Koch and Pinette, 1987; Reed and Walsby, 1985).

Extensive research on K transport and its role in cellular activities is centered on the two closely related enteric gram negative organisms, *E. coli* and *Salmonella enterica* (Epstein and Davies, 1970; Epstein and Kim, 1971; Parra-Lopez et al., 1993; Parra-Lopez et al., 1994). These two enteric organisms are important human pathogens that regulate K transport via two primary transport systems: the constitutive *trk* gene system and the *kdp* system. The *trk* gene system is also known as *sap* in *S. enterica*. Both organisms maintain intracellular concentrations of K in the range of 0.1 to 0.5 M (Kakinuma, 1998; Silver and Walderhaug, 1992).The intracellular concentration of K of these enteric organisms and several other bacteria is largely determined by the osmolarity of the medium and it is independent of the external K concentration (Christian, 1955; Epstein and Schultz, 1965; Schultz and Solomon, 1961).

High external osmolarity has been shown to increase the accumulation of intracellular K in exponentially growing *E. coli* and *S. enterica* as a result of the decrease in turgor pressure or a reduction in cytoplasmic volume. The loss of turgor or reduction in the cytoplasmic volume has been suggested to be the major signal leading to increased potassium uptake by the cells. Conversely, low external osmolarity stimulated the efflux of K at a rate directly proportional to the decrease in the external osmolarity. Although the mechanism of this regulation is unknown, it is suggested to involve the direct effect of changes in the turgor pressure associated with the changes in the external osmolarity (Epstein and Schultz, 1965; Meury et al., 1985; Stumpe et al., 1996). To better understand K transport and it role in bacteria, the major transport systems and genes will be reviewed.

1.8.1 Influx systems

1.8.1.1 Trk system

The Trk system is a multi-component complex expressed constitutively and found in a large number of bacterial species. The *trk* gene system in *E. coli* consists of at least three genes that are scattered on the chromosome (*trkA, trkE,* and *trkH*) and transport K with low affinity at a very high rate (Figure 1-2). In *S. enterica*, the corresponding genes are designated as *sapG, sapD,* and *sapJ,* respectively (Dosch et al., 1991; Epstein and Kim, 1971; Parra-Lopez et al., 1994; Rhoads et al., 1976). In some strains of *E. coli* K-12 *trkG* is present in addition to *trkH*. Both genes play similar roles in the transport of K. *TrkG* is only found in strains that carry a defective prophage called rac (Schlösser et al., 1991). K is not the only substrate transported by the Trk system though it is the preferred monovalent cation. Rubidium is another substrate but with a 10-fold lower rate of transport. The affinity of the Trk system for rubidium increases to about 10 fold with an increase in external pH but there is no corresponding significant effect on the transport of K (Bakker, 1983; Bossemeyer et al., 1989b; Rhoads and Epstein, 1977). The Trk system utilizes energy from the proton motive force (PMF) and ATP during the transport of K. ATP is suggested to play a regulatory role by activation of the process while the PMF drives the transport process (Rhoads and Epstein, 1977).

The *trk* gene product TrkA is a 458 amino acid peripheral membrane protein bound to the inner membrane in the cytoplasm and it consists of two similar halves. Studies of the nucleotide sequence of the *trkA* gene show that it is well conserved in prokaryotes. TrkA is predicted to facilitate the net uptake of potassium at a neutral or

alkaline pH under aerobic conditions utilizing dual energy requirements derived from PMF and ATP. The TrkA protein contains similar regions to the dinucleotide fold of dehydrogenases and binds to NADH and NAD^+ with a higher affinity for NADH (Bossemeyer et al., 1989a; Schlosser et al., 1993).

The *E. coli trkE* gene, also known as *sapD* in *S. enterica*, was first identified in *S. enterica* as one of the ATP binding components of the ABC transporter encoded by the *sap* operon. The *sap* operon was initially proposed as a transporter of toxic peptides based on sequence comparisons. However, subsequent studies indicated that increased uptake of intracellular K by SapD/TrkE is essential for the cell to resist the effects of toxic peptides. (Parra-Lopez et al., 1993; Stumpe and Bakker, 1997).

The trans-membrane component for K transport by the Trk system is formed by the products of either the *trkG* or *trkH* genes. TrkG (485 amino acid residues) and TrkH (483 amino acid residues) share 41% identity at the amino acid level but *trkG* has low GC content (37%). They are predicted to span the membrane 10 times with their C- and N- termini located in the cytoplasm The uptake of K by the Trk system can be achieved through either TrkG or TrkH depending on the membrane spanning component involved (Schlösser et al., 1991; Schlösser et al., 1995). Although TrkG and TrkH are similar, the *trkE* gene product functions as an absolute requirement for TrkH potassium transport activity while TrkG shows residual activity in the absence of a functional TrkE (Harms et al., 2001).

1.8.1.2 Structure of TrkA complex and mechanism of K transport

TrkA is a peripheral membrane protein that is loosely bound to the cytoplasmic side of the cell membrane. It is made up of two similar halves and each half contains a

complete dinucleotide binding domain. The N-terminal part of the protein is similar to the complete NAD+ binding domain of NAD+ dependent dehydrogenases while the C - terminal is similar to the first 100 residues of the catalytic domain of glyceraldehyde-3-phosphate dehydrogenase (Schlosser et al., 1993).

TrkA is an absolute requirement for Trk transporter activity and facilitates the net uptake of K in symport with protons. TrkA binds to either the transmembrane protein TrkH or TrkG, which are the actual K translocating subunits. The TrkAH complex requires TrkE, an ATP binding protein which is thought to activate the transport process but the TrkAG complex shows residual activity in the absence of a functional TrkE. The movement of potassium from the external environment into the periplasm occurs via diffusion through the outer membrane porins. The periplasmic K is subsequently transported into the interior of the cell by the Trk complex (Bossemeyer et al., 1989a; Harms et al., 2001; Kraegeloh et al., 2005).

Efflux of K by TrkA has been observed in *E. coli.* The efflux of K by TrkA depends on the intracellular concentration of K and the metabolic state of the cell. In non-metabolizing cells (cells without a carbon source), efflux of K occurs at a cellular concentration of 0.1 M while the efflux of K in actively metabolizing cells occurs at a cellular concentration of 0.2 M (Meury and Kepes, 1981).

1.8.1.3 Kdp system

The Kdp system is a P-type ATPase consisting of four proteins encoded by a single operon, *kdpFABC*. It is widely distributed in bacteria and functions as an inducible K scavenging system with high affinity and specificity for K. KdpF is a small hydrophobic peptide with an N-terminus located in the periplasm and a C-terminus located in the

cytoplasm. It is suggested to be involved in stability and assembly of the Kdp complex. KdpA is the main subunit that binds and transports K across the membrane. It is a 557 amino acid residue protein that spans the membrane 10 times. KdpB is made up of 682 amino acid residues and contains a conserved phosphorylation site. It has six putative trans membrane segments with both N- and C-termini located in the cytoplasm. In addition, it shares homology with several other ATPase subunits. The Kdp complex is assembled by KdpC, a 190 residue protein with one predicted membrane span (N - terminal) and an hydrophilic C-terminal domain (Buurman et al., 1995; Gabel et al., 1998; Hesse et al., 1984).

The Kdp system is induced under low concentrations of K (less than 2nM) and upon osmotic upshock. Expression of the operon is under the control of a two-component system encoded by the *kdpDE* operon. KdpD functions as the sensor histidine kinase that undergoes autophosphorylation and subsequently transfers the phosphoryl group to the response regulator KdpE. The response regulator KdpE then binds to the promoter region of the *kdpFABC* operon and facilitates the initiation of transcription (Jung et al., 2000). Unlike the Trk system, transport of K by the *kdp* operon products is independent of PMF. (Fendler et al., 1996; Rhoads and Epstein, 1977). The sensor kinase KdpD which is located in the cell membrane is composed of three domains: a cytoplasmic hydrophilic N-terminus made up of 400 amino acid residues, four putative transmembrane hydrophobic domains made up of 100 residues and an extended hydrophilic cytoplasmic C-terminal domain made up of 400 amino acid residues (Altendorf et al., 1994; Walderhaug et al., 1992). The nature of the signal sensed by the histidine kinase KdpD is not very clear. However, it has been suggested

that a decline in extracellular concentration of K and the physico chemical state of the cytoplasmic membrane due to changes in turgor pressure are the two parameters that serve as the signal recognition for KdpD (Sugiura et al., 1994).

1.8.1.4 Kup system

The *kup* gene, formerly *trkD*, is a constitutive, low affinity K transport system with a low rate of K uptake but functions at maximum under low pH (5.5). The Kup transporter is predicted to contain two domains: an N - terminal hydrophobic domain made up of 440 residues spanning the membrane 12 times and a C-terminal hydrophilic domain. In addition to K, the Kup system is also known to facilitate the transport of Cesium and predicted to be a K^+/H^+ symporter (Bossemeyer et al., 1989b; Schleyer and Bakker, 1993; Trchounian and Kobayashi, 1999).

1.8.1.5 Ktr system

The Ktr K transport system was initially discovered in *Vibrio alginolyticus*, a marine bacterium, and it is found in many other bacteria. The transport system is made up of two gene products, KtrA and KtrB and it is sodium (Na) dependent. KtrA is a cytoplasmic regulatory protein that is distantly related to TrkA and about half the size of TrkA, while KtrB is the peripheral membrane protein that is distantly related to TrkH, with eight trans-membrane helices. KtrA, like TrkA, contains an NAD-binding domain similar to that of some dehydrogenases. However, KtrA has a stronger binding preference for ATP than NAD. This suggests a lack of sensitivity for the redox state of the cell by the KtrA protein (Nakamura et al., 1998; Tholema et al., 1999; Vieira-Pires et al., 2013).

1.8.1.6 Illicit transporters

In some bacteria, the transport of K can occur in the absence of all known K transport systems. This is known to occur via illicit transport, where a transporter whose primary substrate is not K is hijacked to perform the role of a K transporter. In *E. coli*, the transport of K in the absence of all three saturable systems (mutation of TrkA, Kdp, and Kup) occurs through TrkF, an illicit transporter. ATP is not a requirement for this illicit transport activity but PMF is required. The growth of a triple mutant (*trkA, kdp* and *kup*) was severely impaired and required a K concentration of 25 mM. Mutants failed to grow in concentrations of 10 mM or less (Rhoads and Epstein, 1977; Rhoads et al., 1976).

Another group of illicit transporters analogous to TrkF are encoded by the *tetL* gene in *Bacillus subtilis* and the *tetK* gene in *Staphylococcus aureus*. These genes encode tetracycline antiporters and can exchange various monovalent and divalent cations. These genes can facilitate the uptake of K, when expressed in mutants of *E. coli* lacking all saturable K uptake systems (Guffanti et al., 1998; Guffanti et al., 2002).

1.8.2 Efflux systems

1.8.2.1 KefB and KefC systems

Most bacteria can grow and survive in a media of elevated K concentration without accumulating excess K. Bacteria undergo K efflux in order to maintain the normal physiologic concentrations of K needed for cellular and metabolic activities. Also, high turgor pressure due to osmotic down shock is reduced by K efflux to enhance the survival of the bacteria. In *E. coli*, the KefB and KefC systems is one of the K efflux systems that have been well characterized. Similar K efflux systems identical to KefB

and KefC have been found in many gram negative bacteria but none has been identified in gram positive bacteria yet. The two K efflux systems facilitate K efflux by potassium-proton antiport (Bakker and Mangerich, 1982; Ferguson, 1999).

Each of the KefB and KefC proteins consists of a 12 membrane span N-terminal and a cytoplasmic C- terminal with a nucleotide binding site. Both systems are activated by N-ethyl-malemide (K^+ depleting reagent). The efflux of K by the system is accompanied by the influx of protons into the cell. This reduces the intracellular pH and protects the cell by enhancing the rate of repair of damage or slowing down the rate of chemical reactions to minimize any potential damage in the interior of the cell. The activity of the KefB and KefC efflux systems is enhanced by two small ancillary proteins, YheR and YabF, that are encoded by genes adjacent to *kefB* and *kefC* respectively (Booth, 1985; Miller et al., 2000; Munro et al., 1991).

1.9 Role of potassium in cellular and metabolic activities

1.9.1 Regulation of enzyme activity

Potassium acts as an intracellular signaling molecule cation, activating or regulating enzymes that catalyze a host of cellular and metabolic activities. It is involved in the activation of pyruvate kinase during glycolysis. Also, it is necessary in peptidyl transferase activity as it catalyzes the formation of peptide bonds by the 50s ribosomal subunit in *E. coli*. The activity of the enzyme was most effective when activated by K (Boyer et al., 1942; Maden et al., 1968; Monro, 1967). Further, K is required during the uptake of phosphate by the cell. Phosphate is an essential compound in the synthesis of membrane lipids or nucleic acids as well as signal transduction processes (Weiden et al., 1967).

1.9.2 Control of gene expression

The role of potassium in regulating gene expression in response to osmotic stress has been well documented. (Gralla and Vargas, 2006; Lee and Gralla, 2004; Rosenthal et al., 2006). This was first observed with the transporter ProU in *S. enterica* at elevated osmolarity. Increase in cytoplasmic K uptake led to an increase in the expression of ProU (Sutherland et al., 1986). However, the exact mechanism involved in the regulation of these genes remains unknown. One of the proposed mechanistic models asserts that an increase in cytoplasmic K acts directly on target genes to stimulate expression. Another model hypothesizes that changes in cytoplasmic ionic strength may cause an increase in gene expression. The last model proposed that increased cytoplasmic K reacts with a protein or other cell component which subsequently up-regulates gene expression (Epstein, 2003; Stumpe et al., 1996)

1.9.3 Regulation of pH

The movement of intracellular protons across the cell membrane is the primary way of regulating pH in the bacterial envelope. Regulation of intracellular pH depends on the type of K transporter activated. Kdp activity occurs at a slightly higher pH (8.0) than the Trk system (pH 7.7) (Ferguson, 1999; Ferguson et al., 1996). Displacement of protons from the cytoplasm leaves excess charged anionic species. In order to maintain or balance electrical neutrality and pH in the cytoplasm, there is an exchange of the monovalent ion K+ for H+ (Booth, 2007; Epstein, 2003). Conversely, intracellular pH in alkaline conditions is regulated mostly by the KefB and KefC anti-porters. However, efflux of K depends on the presence of toxins to activate the anti-porters (Ferguson, 1999; Ferguson et al., 1996).

1.10 The Trk system and K^+ transport in pathogenesis

The activity of the Trk system has been implicated in the pathogenesis of *S. enterica* and *E. coli*. Mutation of two loci within the *sap* operon, *sapF* (*trkE*) and *sapG* (*trkA*) rendered *S. enterica* avirulent and sensitive to the anti-microbial peptide, protamine. The capacity of the SapG system to transport potassium confers resistance of *S. enterica* to the anti-microbial peptide, protamine (Parra-Lopez et al., 1993; Parra-Lopez et al., 1994). Further, rapid accumulation of K by *E. coli* confers resistance to protamine. Accumulation of K triggers the induction of extracytoplasmic proteases which in turn degrade protamine leading to cell survival (Stumpe and Bakker, 1997).

1.11 Induction of the Cpx pathway by mutation of *trkA*

A recent study conducted in the Raivio lab by Dr. Julia Wong showed that mutation of the gene encoding TrkA, a protein involved in potassium transport, strongly induced the Cpx pathway, in fact five fold more compared to the induction conferred by NlpE over-expression. This is interesting because NlpE over-expression was previously known to be one of the strongest, Cpx-specific activation signals (Snyder et al., 1995). The results was intriguing considering the fact that the Cpx system has been associated with membrane transport regulation since its discovery many years ago (Plate, 1976). Also, in a recent microarray study of the Cpx system, it was revealed that genes encoding inner membrane transport proteins were the most down-regulated genes upon over-expression of the lipoprotein NlpE (Raivio et al., 2013). Furthermore, the mutation of *trkA* homologue, *sapG,* confers a virulence defect in *S. enterica* (Parra-Lopez et al., 1994; Su et al., 2009), as does activation of the Cpx response in numerous organisms.

Although the impact of the Cpx system on the regulation of inner membrane transport proteins has been well established, there is no clear understanding of how the Cpx system and transporters are connected. We are interested in finding the connection between the Cpx system and TrkA.

1.12 Thesis Objectives

This research focuses on understanding why the mutation of *trkA*, which encodes a protein previously associated with potassium transport, results in a strong induction of the Cpx pathway, and to gain more insight into the mechanisms involved in the induction of the Cpx pathway. We hypothesized that some aspect of TrkA function leads to disruption in cellular physiology/function that generates a Cpx inducing signal. The specific objectives of this MSc thesis are:

1. Investigate the effect of *trk* mutants and potassium transport on the Cpx system.

Here we will confirm induction of the Cpx pathway by mutation of *trkA* and determine if mutation of any of the remaining *trk* genes induces the Cpx system (*trkE, trkG*, and *trkH*). Also, we will examine whether change in potassium concentration is involved in the activation of Cpx system.

2. Elucidate potential mechanisms involved in the induction of the Cpx pathway by *trkA* mutation.

Here we will determine the cellular location of the signal involved in the activation of the Cpx system and determine if *trkA* mutation alters the levels of NAD^+ and NADH in the cell.

1.13 Figures

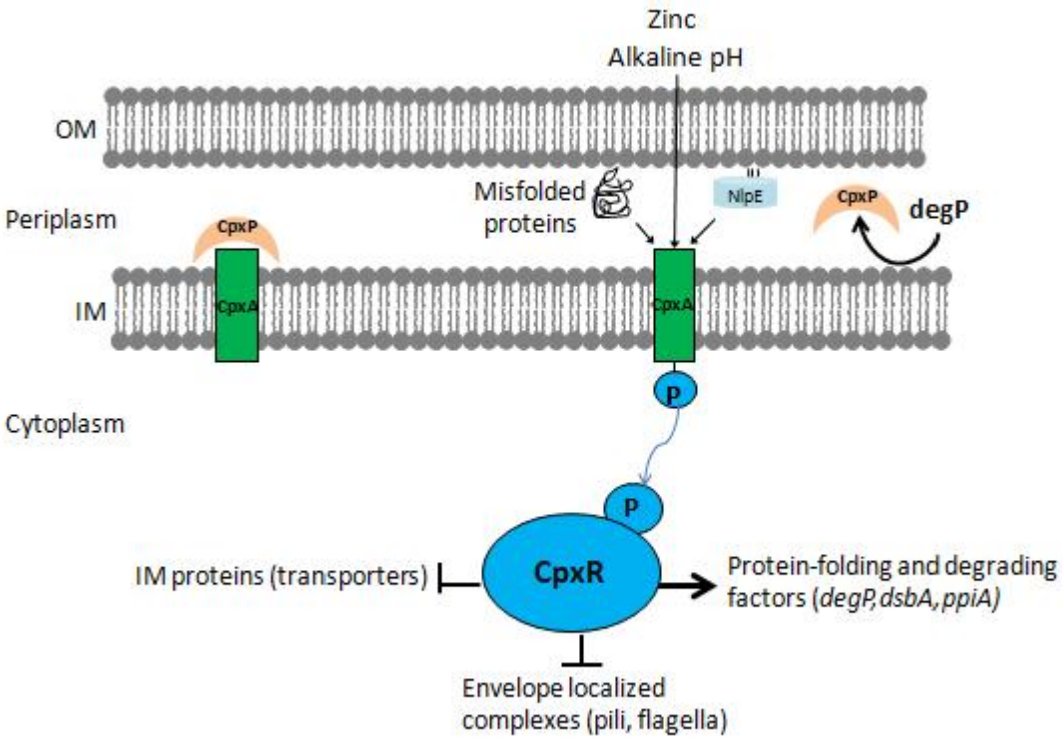

Figure 1-1: Overview of the Cpx system in *E. coli.*

The response regulator CpxR is kept inactive by CpxA phosphatase activity under non inducing conditions (Left side of model). In the presence of inducing cues (misfolded periplasmic proteins, zinc, alkaline pH, over-expression of nlpE), CpxA autophosphorylates and phosphorylates CpxR. Phosphorylated CpxR then regulates the transcription of adaptive response genes such as envelope localized complexes (pili and flagella), IM proteins (transporters), protein folding and degrading factors (*degP, dsbA, ppiA)*. OM denotes outer membrane, IM denotes inner membrane.

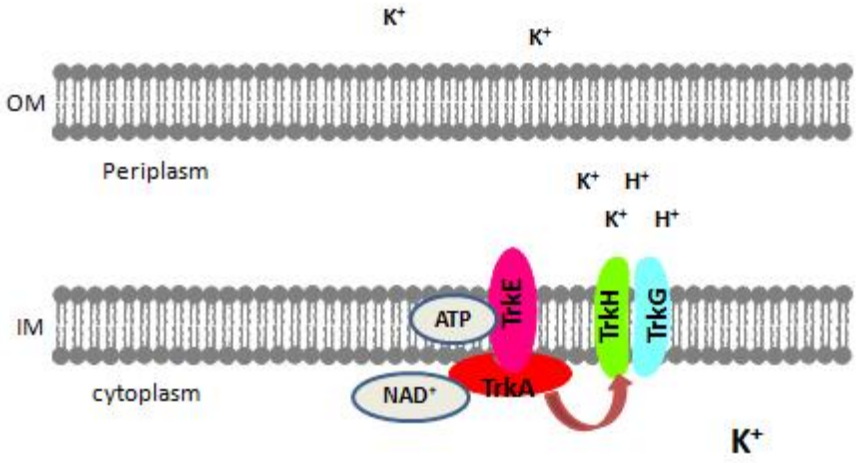

Figure 1-2: Overview of the Trk system.

TrkA binds to either of the potassium translocating subunits, TrkH or TrkG in addition to TrkE to facilitate the uptake of potassium. OM denotes outer membrane, IM denotes inner membrane.

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CHAPTER 2: MATERIALS AND METHODS

2.1 Media and growth conditions

All bacterial strains were grown in Luria-Bertani (LB) broth or agar containing the following ingredients: 10 g tryptone, 5 g bacto yeast extract, 5 g NaCl and 15 g agar. SOB media contained the following ingredients: 20 g tryptone, 5 g yeast extract, 0.5 g NaCl, 2.4 g $MgSO_4$, and 0.2 g KCl. SOC media was made by adding 20% 1M glucose solution to SOB media (Hanahan, 1983). Minimal media consisted of the following: 12.8 g $Na_2HPO_4.7H_2O$, 3 g KH_2PO_4, 0.5 g NaCl, 1 g NH_4Cl, 2 mL 1 M $MgSO_4.7H_2O$, 0.1 mL 1 M $CaCl_2$ and 20 mL 20% glucose. Excess potassium media (115 mM) consisted of the following: 46 mM K_2HPO_4, 23 mM KH_2PO_4, 8 mM $(NH_4)_2SO_4$, 0.4 mM $MgSO_4$, 6 µM $FeSO_4$, 1 mM sodium citrate, 1 mg/L thiamine hydrochloride, and 10 mL 20% glucose (Epstein and Davies, 1970; Epstein and Kim, 1971). For potassium limiting medium, potassium phosphates were replaced by an equal amount of sodium phosphate (Epstein and Davies, 1970; Epstein and Kim, 1971).

Plated cultures were incubated aerobically at 37°C or 30°C for temperature sensitive strains in a standing incubator. Broth cultures were grown aerobically at 37°C or 30°C for temperature sensitive strains with shaking at 225 rpm. The following antibiotics were applied as needed: 30 µg/mL and 50 µg/mL of kanamycin, 25 µg/mL chloramphenicol, 1 µg/mL amikacin, and 50 µg/mL streptomycin.

2.2 Reporter genes

Beta-galactosidase reporter genes were in single copy on the chromosome, integrated at the λ attachment site as part of a modified λRS88 phage (Raivio and Silhavy, 1997a).

2.3 Polymerase chain reaction (PCR)

All PCRs were done using a master mix containing the following: 22 µL MilliQ H_2O, 10µL betaine, 8 µL 5mM dNTPs, 5 µL10X PCR buffer (Invitrogen), 2 µL 50mM $MgSO_4$,1 µL forward primer, 1 µL reverse primer, and 1 µL hot start fusion polymerase (Invitrogen). PCR cycles consisted of 35 cycles of a 98^oC dissociation step for 2 minutes, annealing temperature of 56^oC for 30 seconds and a 72^oC extension for 1.5 minutes, over 35 cycles.

2.4 Preparation of P1 lysates

Single colonies of the needed bacterial strains were inoculated in 5 mL LB broth containing the required antibiotics and incubated overnight at 37^oC with shaking at 225 rpm. The next day, bacteria were subcultured 1:50 into 5 mL of fresh LB broth containing 5 mM $CaCl_2$ and 0.2% glucose and incubated for 30 minutes at 37^oC with shaking at 225 rpm. 100 µL of P1 lysate harvested from *E. coli* MC4100 was added to the culture and incubated at 37^oC until cells were completely lysed. Chloroform (100 µL) was then added to the tubes and vortexed vigorously for 10 seconds. The solution was centrifuged at 4000 rpm for 10 minutes to pellet cell debris. About 4 mL of supernatant was transferred into a sterile glass tube and 100 µL of chloroform was added. The lysate was then stored in a 4^oC refrigerator.

2.5 P1 lysate mediated transductions

Single colonies of the *E. coli* strains were inoculated in 5 mL LB broth containing the required antibiotics and incubated overnight at 37^oC with shaking at 225 rpm. The overnight cultures were centrifuged at 4000 rpm for 10 minutes and re-suspended in 2.5 mL of fresh LB containing 10 mM $MgSO_4$, and 5 mM $CaCl_2$. A 100 µL cell solution was

mixed with 100 µL of P1 lysate in a microfuge tube and the solution was incubated at 30°C for 30 minutes without shaking. LB medium (1 mL) containing 10 mM citrate was added to the cell solution and incubated at 37°C for 30 minutes. The solution was then centrifuged at 4000 rpm for 10 minutes and re-suspended in 100 µL of 1 M citrate. 100 µL of cells was plated on selective media and incubated at 37°C overnight. Single colonies were isolated for pure cultures and incubated at 37°C overnight.

2.6 Calcium Chloride mediated transformation

A single colony of the desired bacterial strain was inoculated in 5 mL of LB broth containing the appropriate antibiotics and incubated at 37°C with shaking at 225 rpm overnight. The bacterial cells were subcultured 1:50 into 5mL LB the next day, incubated at 37°C with shaking at 225 rpm and grown to an OD_{600} of 0.5 (about 2.5 hours). The cells were centrifuged at 4000 rpm for 10 minutes and re-suspended in 1 mL magic formula (0.1 M $CaCl_2$, 0.1 M MOPS in dH_2O). The re-suspended cells were incubated on ice for 30 minutes, centrifuged at 4000 rpm for 10 minutes and resuspended in 200 µL magic formula. The cells were transferred into a microfuge tube and 50-100 ng of DNA was added. The mixture was then incubated on ice for 10 minutes and heat shocked at 42°C for 30 seconds in a water bath. Fresh LB broth (1 mL) was added and the cells were incubated at 37°C with shaking for one hour. The cells were then plated on the appropriate selective media and incubated at 37°C overnight.

2.7 Beta galactosidase assay

Overnight cultures in triplicates were made by inoculating a single isolated colony of each bacterial strain in 2 mL LB broth with appropriate antibiotics, and growing at 30°C

with shaking at 225 rpm. The next day, the cultures were diluted 1:50 into fresh LB broth (2mL) and grown at 30^{o}C with shaking at 225 rpm for four hours. IPTG (0.1 mM final concentration) was added to bacteria with an IPTG inducible expression plasmid after three hours of growth. Beta galactosidase was measured in a microtitre plate assay as described previously (Slaugh and Silhavy, 1991).

2.8 Growth curves

Single isolated colonies of bacterial strains were inoculated in 2 mL LB broth containing the appropriate antibiotics and grown overnight at 37^{o}C or 30^{o}C with shaking at 225 rpm. The cells were subcultured 1:50 the next day into fresh LB broth (2mL) in a 24 well plate. The cells were grown for 10 hours at 37^{o}C or 30^{o}C with shaking at 225 rpm and the OD_{600} was recorded every hour.

2.9 Oxygen consumption assay

Single colonies of each bacterial strain were inoculated in 5 mL of LB broth containing the appropriate antibiotics and incubated at 37^{o}C with shaking at 225 rpm overnight. The following day, the cells were pelleted, washed with PBS and resuspended in fresh LB to an OD_{600}nm of approximately 0.1 and the rate of oxygen consumption was subsequently measured using the Micro Optode Meter oxygen sensor from Unisense as previously described (Liu and Imlay, 2013).

2.10 Plasmid isolation

All plasmid isolations were done using the Sigma-Aldrich GenElute Plasmid Miniprep kit and the accompanying protocol. Single isolated colonies of bacteria containing the plasmid of interest were inoculated in 5 mL of LB broth containing the appropriate antibiotics and grown overnight at 37^{o}C (30^{o}C for temperature sensitive strains) with

shaking at 225 rpm. The next day, the cells were centrifuged and plasmids were isolated according to the manufacturer's instructions (Sigma). Plasmid DNA was eluted into 50 uL milliQ H_2O and stored at -20^{o}C.

2.11 NAD^+/NADH-Glo Assay (Promega)

Overnight cultures were made by inoculating single isolated colonies of each bacterial strain in 2 mL LB broth with appropriate antibiotics, and growing at 30^{o}C with shaking at 225 rpm. The next day, the cultures were diluted 1:50 into fresh LB broth (2mL) and grown at 30^{o}C with shaking at 225 rpm for four hours. The cells were then washed with 2mL PBS (3 times) and resuspended in 2 mL PBS. The cells (50 µL) were transferred into 0.2 mL microfuge tubes and 50 µL of pretreatment solution (Promega) was added to each microfuge tube. The solution was vortexed vigorously to ensure cell lysis and 50 µL of each sample was pipetted into separate microfuge tubes containing 25 µL of 0.4M HCl solution. These served as the acid treated samples while the original samples served as the base treated samples. The samples were incubated at 60^{o}C for 15 minutes and equilibrated at room temperature 10 minutes. The samples were then neutralized using 25 µL 0.5 M Tris base for acid samples and 50 µL 0.5 M Tris HCl for base samples. The samples were transferred into a black 96-well microplate and 100 µL of NAD^+/NADH glo detection reagent (Promega) was added to each well. The microplate was incubated in the dark for 60 minutes and the luminescence was recorded. The ratio of the luminescence to the total protein concentration for each sample was determine to obtain the amount of NAD^+ and NADH for each sample.

2.12 List of bacteria strains, plasmids, and primers

Table 2.1 List of bacteria strains and plasmids

Bacteria strains/plasmids	Description	Reference
MC4100	F^- *araD*139 Δ(*argF-lac*) U169 *rpsL*150(str^R) *relA1fthD5301* deoC1 *ptsF*25	(Casadaban, 1976)
TR10	MC4100 *cpxA24*	(Raivio et al., 1999)
TR50	MC4100 λRS88[*cpxP-lacZ*$^+$]	(Raivio and Silhavy, 1997a)
TR51	MC4100 *cpxR::spec*	(Casadaban, 1976)
pCA24N	Vector control from ASKA library; cam^R	(Kitagawa et al., 2005)
pTrkA	Over expression of trkA from ASKA library; cam^R	(Kitagawa et al., 2005)
LB101	TR50 *trkA::kan*	This study
LB102	TR50 *cpxA::kan*	This study
LB103	TR50 *cpxR::kan*	This study
LB104	TR50 *cpxP::kan*	This study
LB105	TR50 pCA24N	This study
LB106	TR50 *trkD::kan*	This study
LB107	TR50 *trkE::kan*	This study
LB108	TR50 *trkG::kan*	This study
LB109	TR50 *trkH::kan*	This study
LB110	TR50 pTrkA	This study

LB111	TR50 *kdpE::kan*	This study
LB201	LB101 pTrkA	This study
LB202	LB101 Δ*cpxA*	This study
LB203	LB101 Δ*cpxR*	This study
LB204	LB101 Δ*kdpE*	This study
LB206	LB101 pCA24N	This study
LB207	TR50 *ΔtrkGH*	This study
E2348/69	Prototypical EPEC strain	(Levine et al., 1978)

Table 2.2 Lists of primers

trkA-fw	GAGGGCGACGGCTTCTTTTA	Forward primer to confirm *trkA* deletion
trkA- rv	TGTTAAGCCATTTTCCTGCCA	Reverse primer to confirm *trkA* deletion

2.14 References

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CHAPTER 3: RESULTS

3.1 Objective 1: Investigate the effect of *trk* mutants and potassium transport on the Cpx pathway

3.1.1 Mutation of *trkA* activates the Cpx pathway

A study conducted in the Raivio lab by Dr. Julia Wong showed that mutation of the *trkA* gene strongly induced the Cpx pathway, by five fold more compared to the induction on NlpE over-expresssion. This is interesting because NlpE is known to be a specific Cpx activation signal and is used frequently in studies of the Cpx pathway (Snyder et al., 1995). Previously, it was also shown that transporters were among the most down-regulated genes by the Cpx system upon induction by over-expression of the lipoprotein NlpE (Raivio et al., 2013). We sought to confirm that mutation of *trkA* indeed activates the Cpx system. We used a wild type (TR50) *E. coli* K-12 bearing a chromosomal *PcpxP::lacZ* transcriptional reporter to monitor Cpx pathway activity. The expression of *cpxP* is solely dependent on the response regulator CpxR (Danese and Silhavy, 1998), consequently, the expression of *cpxP* is normally used as a measure of Cpx signaling.

We created single *trkA* and *cpxA* mutant strains from TR50 using P1 transductions as described in chapter two. The expression of a Cpx regulated *cpxP-lacZ* reporter gene was monitored by measuring beta galactosidase activity. As previously observed (J.L. Wong. unpublished), there was a strong induction of the Cpx system in the *trkA* mutant. The activation of the Cpx pathway in the *trkA* mutant was elevated over 20 and 11 fold compared to the wild type (TR50) and the *cpxA* mutant respectively. However, there was no significant difference between the wild type and the *cpxA* mutant

(Figure 3-1). This indeed confirmed that the mutation of *trkA* activates the Cpx pathway.

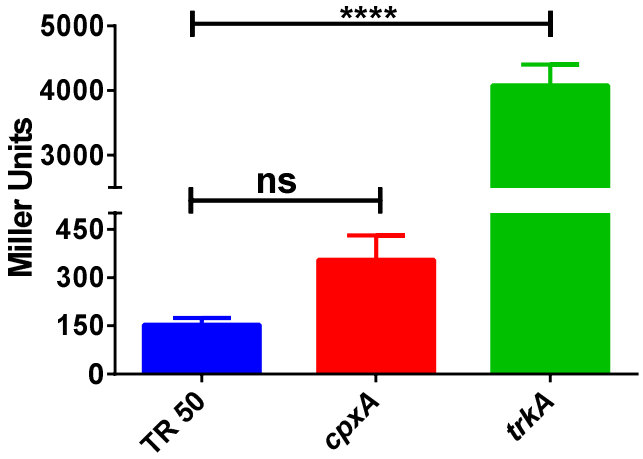


Figure 3-1: Deletion of *trkA* induces the Cpx pathway.

Wild type strain TR50, together with isogenic mutants carrying a mutation in *cpxA* or *trkA* were grown overnight in LB broth and the appropriate antibiotics and then subcultured 1:50 the next day into 2 mL LB broth and grown to mid log phase (A_{600} =0.4-0.6). Cells were pelleted, resuspended in 2 mL 1X Z-buffer and lysed. Beta galactosidase assays were then performed. Bars represent average values between three replicates and error bars represent standard deviation between replicates. Asterisks denote a significant difference compared to wild type and ns denotes no significance difference compared to wild type ($p<0.05$, one way ANOVA test).

3.1.2 Induction of the Cpx pathway is specific to mutation of *trkA*

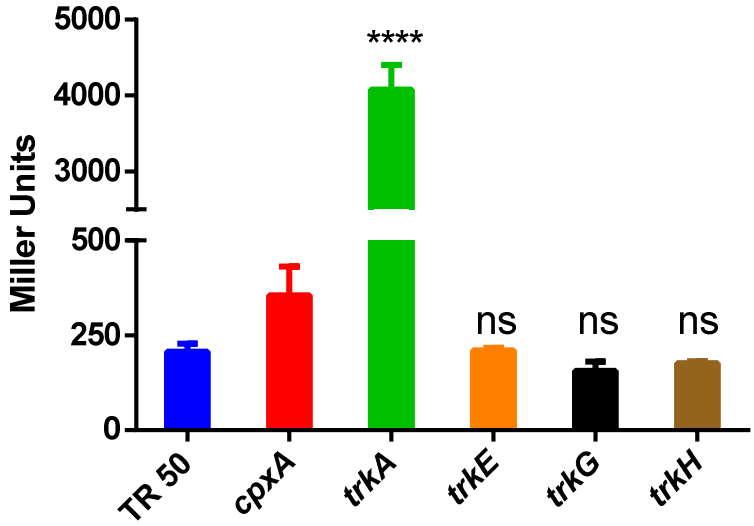
Based on our previous results, which confirmed that the mutation of *trkA* induces the Cpx pathway (Figure 3-1), we pondered the effect of mutation of the remaining *trk* genes (*trkE, trkG*, and *trkH*) on the Cpx pathway. TrkA works by binding with either of the potassium translocating subunits TrkH or TrkG in addition to TrkE during the constitutive uptake of potassium (Dosch et al., 1991; Schlosser et al., 1993). Consequently we were interested in knowing how the mutation of these genes might impact the Cpx pathway.

To test whether mutations in other *trk* genes were capable of inducing the Cpx pathway, single mutants lacking *trkA, trkE, trkG*, and *trkH* were created in an *E. coli* K12 wild type strain (TR50) expression of the Cpx regulated *cpxP-lacZ* reporter gene was monitored by measuring beta galactosidase activity. As previously observed (J.L. Wong. unpublished, Fig. 3-1), Cpx activity in the *trkA* mutant was elevated over 15 fold as compared to the wild type (TR50). However, there was no significant difference in Cpx activity between the remaining *trk* mutant strains and the wild type (Figure 3-2A). These data indicate that, amongst the *trk* genes, induction of the Cpx response is specific to mutation of *trkA.*

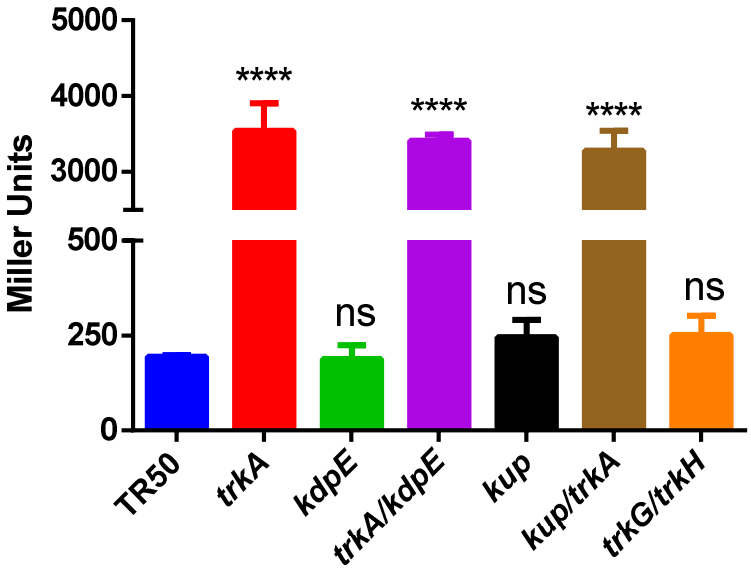
We wondered whether mutations in genes encoding other potassium transport systems might exacerbate potassium transport defects that could be responsible for Cpx pathway induction in the *trkA* mutant. To test this, we created mutants carrying single mutations in *kup* or*, kdpE* and double mutations in *trkA* and *kdpE* or *kup*. The strains were subcultured 1:50 from overnight cultures grown with appropriate antibiotics into fresh LB broth (2 mL) and grown for 4 hours to mid log phase (A_{600} = 0.4-0.6) and

the expression of a Cpx regulated *cpxP-lacZ* reporter gene was monitored by measuring beta galactosidase activity. The *trkA* mutant and double mutants (*trkA/kdpE* and *trkA/kup*) showed elevated Cpx activity compared to the wild type (TR50). However, there was no significant difference in the activity of the Cpx regulated *cpxP-lacZ* reporter gene for *cpxA, kup,* and *kdpE* compared to the wild type. Also, the *trkG/trkH* mutant did not show any significant difference in Cpx pathway activity compared to the wild type (Figure 3-2). The data shows clearly that none of the potassium transport systems contributes to the induction of the Cpx pathway in the *trkA* mutant. Overall this data suggests that the activation of the Cpx pathway is indeed specific to *trkA* mutation.

A.


5000
4000
3000
500
250
0
Miller Units
****
ns
ns
ns
TR 50
cpxA
trkA
trkE
trkG
trkH

B.


5000
4000
3000
500
250
0
Miller Units
****
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ns
ns
ns
TR50
trkA
kdpE
trkA/kdpE
kup
kup/trkA
trkG/trkH

Figure 3-2: Activation of the Cpx pathway is specific to mutation of *trkA*.

A. Wild type strain TR50 was compared to isogenic mutants carrying mutation in *cpxA, trkA, trkE, trkG* and *trkH*. B. Wild type strain TR50 was compared to strains bearing mutations in different potassium transporters. Strains were grown overnight in LB broth and the appropriate antibiotics and then subcultured 1:50 the next day into 2 mL LB broth and grown to mid log phase (A_{600} =0.4-0.6). Cells were pelleted, resuspended in 2 mL 1X Z-buffer and lysed. Beta galactosidase assays were then performed. Bars represent average values between three replicates and error bars represent standard deviation between replicates. Asterisks denote a significant difference compared to wild type and ns denotes no significance difference compared to wild type ($p<0.05$, one way ANOVA test)

3.1.3 Over-expression of *trkA* restores Cpx pathway activity in a *trkA* mutant

To confirm that the effect of *trkA* mutation on the Cpx response was due to the activity of TrkA, we sought to complement the *trkA* mutant with a plasmid that over-expresses the *trkA* gene from an IPTG inducible promoter which was obtained from the ASKA library (Kitagawa et al., 2005). We hypothesized that the over-expression of *trkA* would restore wild type Cpx pathway activity to the *trkA* mutant. We transformed the *ptrkA* plasmid, together with the pCA24N vector control into the TR50 strain carrying the Cpx-regulated *cpxP-lacZ* reporter gene and monitored beta-galactosidase activity to assess Cpx pathway activity after growth to mid-log phase in the presence of the IPTG inducer (Fig. 3-3). As expected, the *cpxP-lacZ* reporter gene activity in the presence of over-expressed TrkA was comparable to that of the wild type strain carrying the pCA24N vector control (Figure 3-3). This supported our theory that over-expression of *trkA* will restore wild type Cpx pathway activity to the *trkA* mutant and confirmed our earlier assertion that the induction of the Cpx system is a consequence of *trkA* mutation.

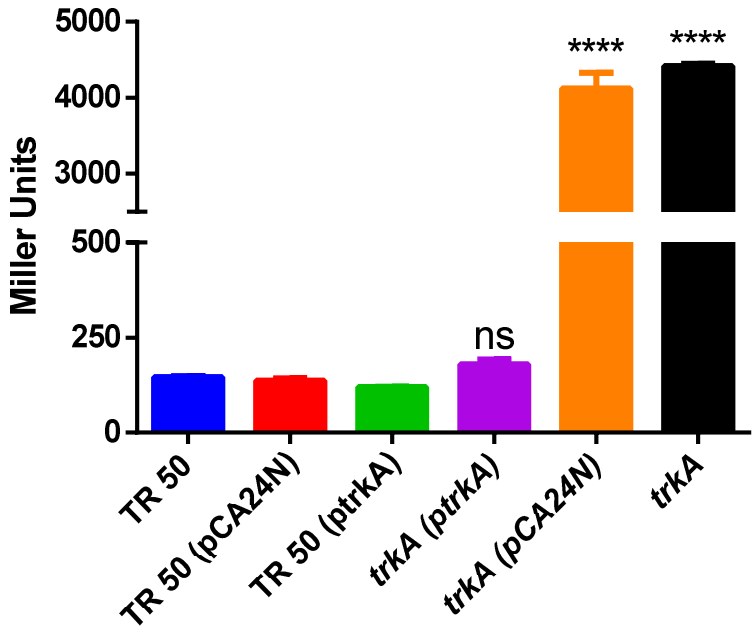


Figure 3-3: Over-expression of trkA restores wild-type Cpx pathway activity to a trkA mutant. Wild type (TR50) and *trkA* mutant strains carrying either the t*rkA* over-expression plasmid (p*trkA)* or the vector control (pCA24N) were grown overnight in LB broth with appropriate antibiotics and then subcultured 1:50 the next day into 2 mL LB broth and grown to mid log phase (A_{600} =0.4-0.6) in the presence of the IPTG inducer. Cells were pelleted, resuspended in 2 mL 1X Z-buffer and lysed. The expression of a Cpx regulated *cpxP-lacZ* reporter gene was then monitored by measuring beta galactosidase activity. Bars represent average values between three replicates and error bars represent standard deviation between replicates. Asterisks denote a significant difference compared to wild type and ns denotes no significance difference compared to wild type ($p<0.05$, one way ANOVA test)

3.1.4 Excess potassium does not play a role in the activation of the Cpx pathway

Our results showed that mutation of individual or multiple genes encoding proteins involved in potassium transport had no impact on Cpx response induction. To further investigate whether potassium transport might be involved in the *trkA* mediated activation of the Cpx response we grew up wild-type and *trkA* mutant strains in the absence and presence of excess potassium in minimal medium. We tested whether potassium concentrations are linked to the activation of the Cpx pathway by monitoring *cpxP-lacZ* reporter gene activity in wild-type and *trkA* mutants grown in the absence or presence of excess potassium. The strains were grown overnight in LB broth with appropriate antibiotics and then subcultured 1:50 the next day into 2 mL minimal media supplemented with 0.4% glucose and either 115 mM potassium salt or no potassium salt and grown to mid log phase (A_{600} =0.4-0.6). Cells were pelleted, resuspended in 2 mL 1X Z-buffer and lysed, and beta galactosidase assay was performed. We found that the level of *cpxP-lacZ* reporter gene expression was unchanged in any of the wild type, *trkA* mutant, as well as the *cpxA* mutant strains, regardless of the amount of potassium salt in the media (Figure 3-4). Our data thus suggest that there is no link between excess potassium and the activation of the Cpx pathway (Figure 3-4).

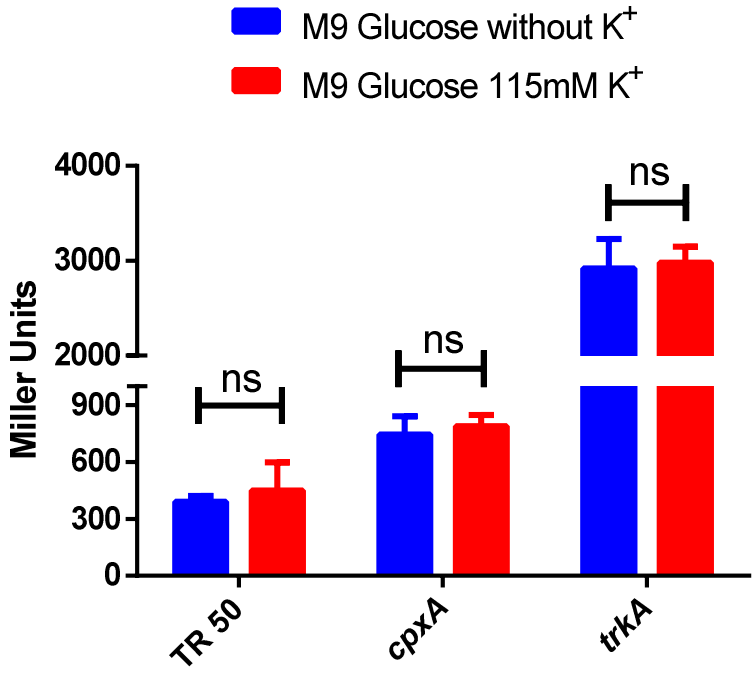


Figure 3-4: Addition of excess potassium has no effect on Cpx pathway pathway activity. Wild type TR50, and isogenic mutants carrying mutations in *cpxA* and *trkA* were grown overnight in LB broth and the appropriate antibiotics and then subcultured 1:50 the next day into 2 mL minimal media supplemented with 0.4% glucose with 115 mM potassium and without potassium. Cells were grown to mid log phase (A_{600} =0.4-0.6), pelleted, resuspended in 2 mL 1X Z-buffer and lysed. Beta galactosidase assays were then performed. Bars represent average values between three replicates and error bars represent standard deviation between replicates. "ns" denotes no significant difference between strains grown in minimal media supplemented with 115 mM potassium as compared to the same medium carrying no potassium. ($p<0.05$, one way ANOVA test).

3.2 Objective 2: Elucidate potential mechanisms involved in the induction of the Cpx pathway by *trkA* mutation

The Cpx pathway responds to a wide variety of signals originating from both the external and the internal environment of the cell (Raivio, 2014). Most of the signals are integrated through the sensor kinase CpxA in a direct or indirect fashion with the exception of a cell growth signal which is sensed independently of CpxA by the response regulator, CpxR (DiGiuseppe and Silhavy, 2003; Raivio, 2014). However, knowledge gaps exist in the mechanisms by which these signals are sensed. Based on our previous results we wondered where in the cell the signaling for the induction of the Cpx pathway that occurs upon mutation of *trkA* is located – in the envelope or the cytoplasm? We sought to further clarify the mechanism of Cpx response induction by the *trkA* mutation by investigating the mechanism by which this signal is transmitted.

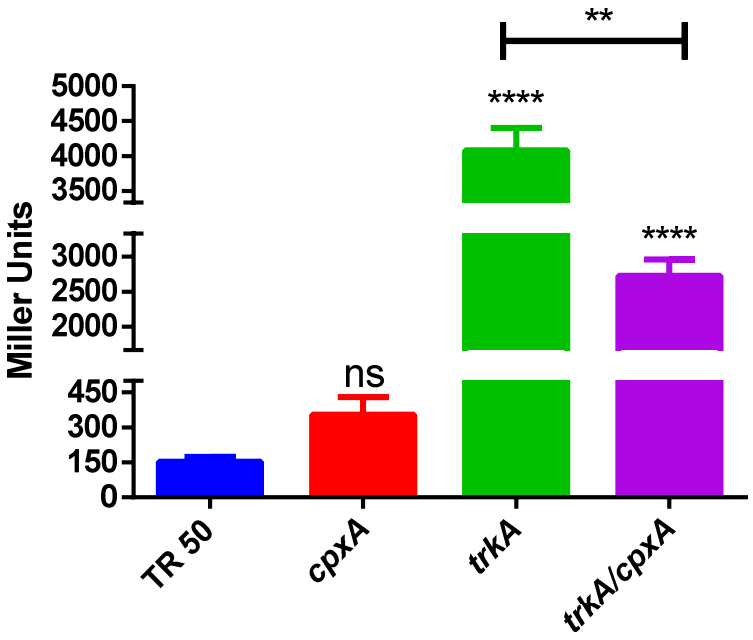
3.2.1 Activation of the Cpx pathway by mutation of *trkA* is independent of CpxA but dependent on CpxR

Our first thought was to determine if the signal for the activation of the Cpx pathway is located in the envelope, and therefore sensed through the sensor kinase CpxA. To test whether the sensor kinase CpxA is involved in signaling the activation of the Cpx pathway in a *trkA* mutant, we created mutant strains in which *trkA* and/or *cpxA* were deleted and monitored the activity of the reporter gene *cpxP-lacZ* using the beta galactosidase assay. As previously observed, mutation of *trkA* alone lead to elevated *cpxP-lacZ* expression (Figure 3-5A). Mutation of *cpxA* also resulted in increased Cpx pathway activity (Figure 3-5A). This is known to be due to elimination of the phosphatase activity of CpxA, which prevents CpxR auto-phosphorylation through the

small molecular weight phosphodonor acetyl-phosphate (Danese and Silhavy, 1998). Although we observed a significant decrease in *cpxP-lacZ* activity between the *trkA* mutant and the *trkA/cpxA* double mutant according to the one-way Anova statistical test (Figure 3-5A), there was still an increase in the induction of *cpxP-lacZ* expression in the *trkA/cpxA* mutant (15 fold), compared to the wild type (Figure 3-5A). These data indicate that, while *trkA* mutation may partially activate the Cpx response via an envelope signal that is transmitted through CpxA, CpxA is not responsible for the majority of the activation of *cpxP-lacZ* expression seen in this mutant.

We then wondered whether the response regulator CpxR was involved in signaling and created *cpxR,* and a *trkA/cpxR* double mutant to test this idea. As previously observed, *cpxP-lacZ* expression was dramatically diminished in the *cpxR* mutant (Figure 3-5B) (Danese and Silhavy, 1998). A double *cpxR trkA* mutant exhibited similar low levels of Cpx pathway activation (Figure 3-5B). These results indicate that the induction of the Cpx pathway by mutation of *trkA* is dependent on the response regulator CpxR (Figure 3-5B). Further, this fact may suggest that the Cpx inducing signal generated by *trkA* mutation originates in the cytoplasm, as opposed to the envelope.

A.


**
****
****
ns
Miller Units
5000
4500
4000
3500
3000
2500
2000
450
300
150
0
TR 50
cpxA
trkA
trkA/cpxA

B.

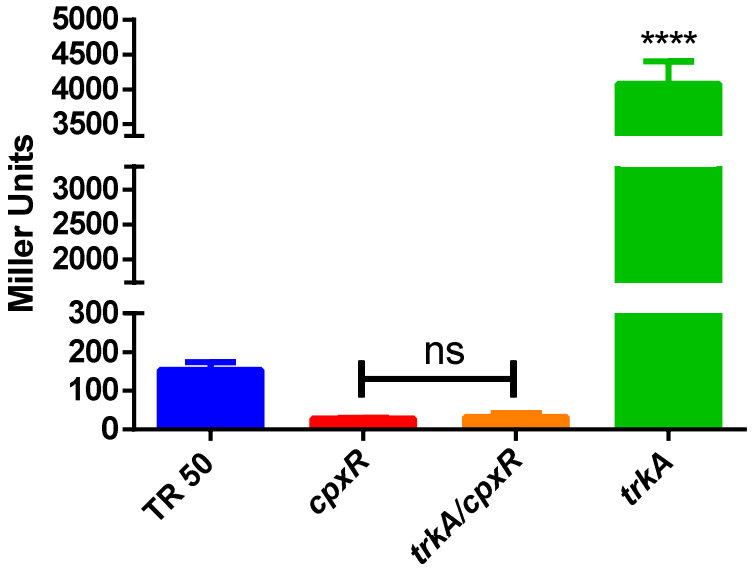

****
ns
Miller Units
5000
4500
4000
3500
3000
2500
2000
300
200
100
0
TR 50
cpxR
trkA/cpxR
trkA

Figure 3-5: Induction of the Cpx pathway by *trkA* mutation is dependent on CpxR but independent of CpxA.

A. Levels of *cpxP-lacZ* expression were compared in wild type strain TR50 and isogenic mutants carrying mutations in *trkA* and/or *cpxA.* B. Levels of *cpxP-lacZ* expression were compared in wild type strain TR50 and isogenic mutants carrying mutations in *trkA* and/or *cpxR.* Strains were grown overnight in LB broth and the appropriate antibiotics and then subcultured 1:50 the next day into 2 mL LB broth and grown to mid log phase (A_{600} =0.4-0.6). Cells were pelleted, resuspended in 2 mL 1X Z-buffer and lysed. Beta galactosidase assays were then performed. Bars represent average values between three replicates and error bars represent standard deviation between replicates. Asterisks denote a significant difference compared to wild type and ns denotes no significant difference compared to wild type ($p<0.05$, one way ANOVA test)

3.2.2: Mutation of *trkA* does not elevate Cpx pathway activity by inhibiting CpxA phosphatase activity

The role of TrkA in pH homeostasis is well documented. TrkA is known to regulate the pH in *E. coli* grown anaerobically at alkaline pH as high as 8.6. Conversely low cytoplasmic pH inhibits TrkA activity (Bakker, 1983; Trchounian et al., 1998). Also, the Cpx pathway is known to be affected by extracellular pH which is dependent on CpxA. Under acidic pH (<6.0), the induction of the pathway is inhibited, and this requires the phosphatase activity of CpxA (R. Malpica and T.L. Raivio, unpublished). On the other hand, the Cpx pathway is induced under alkaline pH and this induction requires the kinase activity of CpxA (Danese and Silhavy, 1998). Based on these findings, and the fact that the effect of *trkA* mutation on the Cpx response seems to be partly dependent on CpxA (Figure 3-5A), it seemed possible that TrkA might impact the enzymatic activity of CpxA. To test this idea, mutant strains carrying mutations in *trkA* and/or *cpxA* were grown in different pH media (5.8, 7.0, and 8.0) and Cpx pathway induction was measured using the *cpxP-lacZ* reporter gene (Figure 3-6). As previously observed, CpxA phosphatase activity was found to be required for inhibition of the Cpx response at acidic pH, as evidenced by elevated *cpxP-lacZ* activity at low pH in the absence of *cpxA* (Figure 3-6). Surprisingly, in the *trkA* mutant, the Cpx response was expressed at elevated levels at all pHs (Figure 3-6). We hypothesized that mutation of *trkA* might prevent CpxA-mediated inhibition of the Cpx response. This in turn, could explain why mutation of *trkA* leads to activation of the Cpx response (ie. it inhibits CpxA phosphatase activity). To test this hypothesis, we created double *cpxA trkA* mutants and measured expression of the *cpxP-lacZ* reporter gene at acidic, neutral, and alkaline pHs

(Figure 3-6). Interestingly, the double *cpxA trkA* mutant also expressed elevated levels of *cpxP-lacZ* at acidic pH, although at somewhat lower levels than that of the single *trkA* mutant. Since mutation of *trkA* can further activate the Cpx response in a strain lacking CpxA (Figure 3-6), these data indicate that *trkA* does not activate the Cpx response by inhibiting the phosphatase activity of CpxA. The diminished level of Cpx pathway activity in the *trkA cpxA* mutant relative to the *trkA* mutant alone might suggest that part of the inducing effect of the *trkA* mutation is due to effects on CpxA

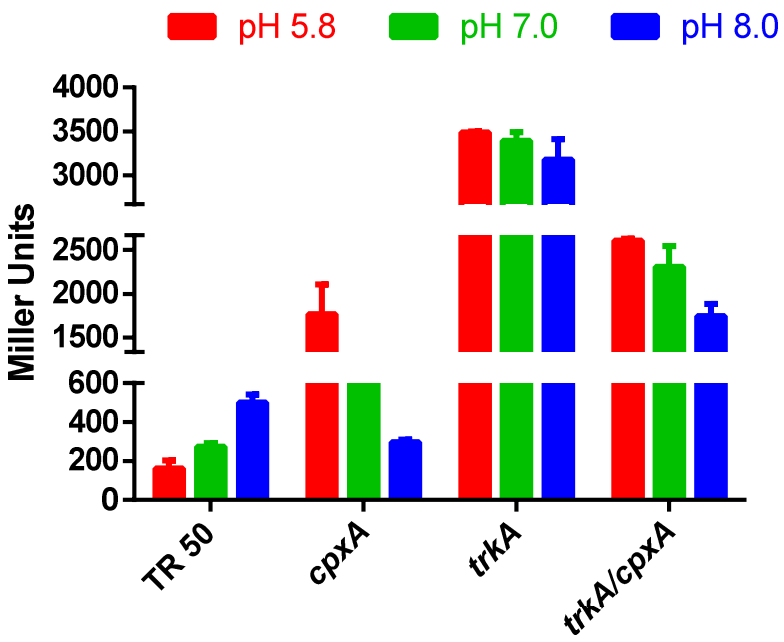


Figure 3-6: Mutation of trkA does not elevate Cpx pathway activity by inhibiting CpxA phosphatase activity. The wild type and strains carrying mutations in *trkA* and/or *cpxA* were grown overnight in LB broth and the appropriate antibiotics and then subcultured 1:50 the next day into 2 mL LB broth with different pHs (5.8, 7.0, and 8.0), and grown to mid log phase (A_{600} =0.4-0.6). Cells were pelleted, resuspended in 2 mL 1X Z-buffer and lysed. Beta galactosidase assays were then performed. Bars represent average values between three replicates and error bars represent standard deviation between replicates.

3.2.3 Mutation of *trkA* strongly induces the Cpx pathway in minimal media

It has previously been demonstrated that CpxR can become phosphorylated independently of CpxA by small molecular weight phosphodonors such as acetyl phosphate (Danese and Silhavy, 1998). We have also shown that activation of the Cpx response by *trkA* mutation is mostly independent of CpxA (Figure 3-5A). Accordingly, we wondered whether the effect of *trkA* might also be dependent on a similar phenomenon, ie the phosphorylation of CpxR by acetyl-phosphate. To test this, we grew wild-type, *cpxA, trkA,* and *cpxA trkA* double mutants under conditions where acetyl phosphate levels are elevated (LB, minimal media plus glucose), and also conditions where they are not (minimal media plus glycerol or succinate), (McCleary et al., 1993; McCleary and Stock, 1994) and measured Cpx response activation using the *cpxP-lacZ* reporter. The wild type and the strains carrying the *cpxA, trkA,* and *cpxA trkA* double mutants were subcultured 1:50 from overnight cultures grown with appropriate antibiotics into fresh 2 mL LB broth or minimal media supplemented with 0.4% carbon source (glucose, glycerol and succinate), and grown for 4 hours to mid log phase (A_{600} = 0.4-0.6). Cells were pelleted, resuspended in 2 mL 1X Z-buffer and lysed. Beta galactosidase assays were then performed.

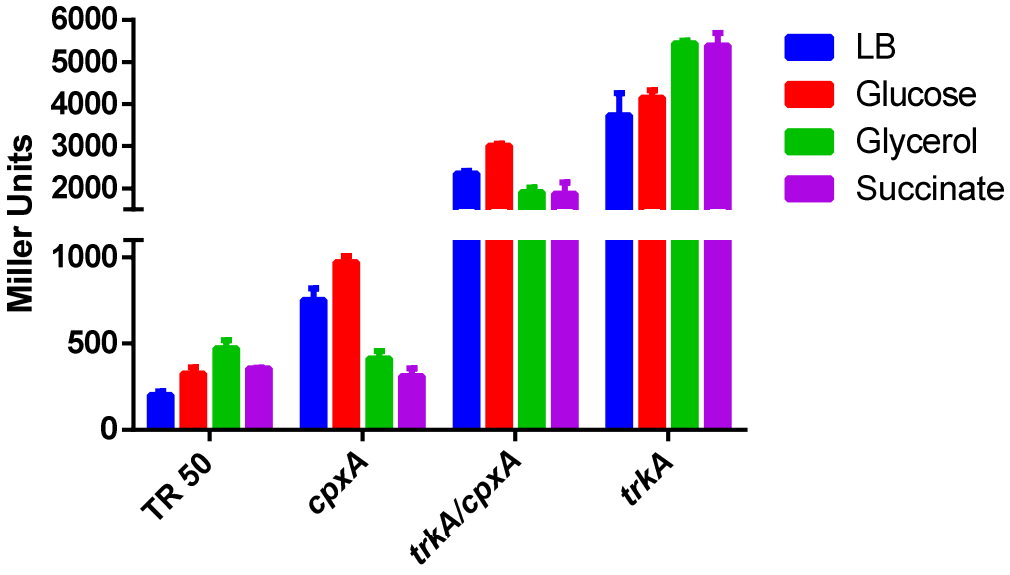
In the wild type strain, the Cpx response was slightly induced by growth in minimal media (Figure 3-7A). In minimal media, *E. coli* relies more heavily on aerobic respiration to generate PMF and ATP, and so these results are consistent with the recent finding that the Cpx response is sensitive to the biogenesis and/or function of membrane bound aerobic respiratory complexes (Guest et al., 2017). In a *cpxA* mutant, *cpxP-lacZ* expression was elevated in both LB and minimal media containing glucose

(Figure 3-7B). These results are also consistent with the previous finding that CpxR can auto-phosphorylate with acetyl-phosphate (Danese and Silhavy, 1998), since high levels of acetyl-phosphate are produced in LB and in the presence of glucose (Danese and Silhavy, 1998; Danese et al., 1995), and there is no CpxA present in these strains to remove this phosphate from CpxR. On the other hand, *cpxP-lacZ* expression was not elevated in the *CpxA* mutant grown in minimal media supplemented with glycerol or succinate relative to LB (Figure 3-7C-D). This is expected due to the low levels of acetyl-phosphophate in glycerol and succinate (McCleary et al., 1993; McCleary and Stock, 1994). In the *trkA* mutant, levels of *cpxP-lacZ* activity were elevated under all conditions, although, as in the wild-type strain, they were higher when the bacteria were grown in minimal media containing either glycerol or succinate (Figure 3-7A). Also, cpx activity was elevated in the *trkA* mutant grown in glycerol and succinate relative to LB (Figure 3-7C-D) but there was no significant difference in cpx activity of the trkA mutant grown in LB and glucose (Figure 3-7B). As previously observed, mutation of *cpxA* in the *trkA* mutant lowered the high levels of Cpx pathway activity partially (Figure 3-7A) in all conditions. In contrast to what was seen with the *cpxA* single mutant, *cpxP-lacZ* expression was not elevated in the *trkA cpxA* mutant grown in glycerol or succinate relative to LB and glucose (Figure 3-7A). These data suggest that, when *trkA* is mutated, elevated Cpx pathway activity is not due phosphorylation of CpxR by acetyl-phosphate.

Overall, these data demonstrate that the Cpx response is slightly activated in minimal media, and that the *trkA* mutation causes a dramatic elevation in Cpx response induction in both rich and minimal media, but to a greater extent under conditions where

aerobic respiration is the predominant means of growth (Figure 3-7A). Altogether, the activation of the Cpx response by *trkA* mutation does not seem to be dependent on the levels of acetyl-phosphate.

A.


6000
5000
4000
3000
2000
1000
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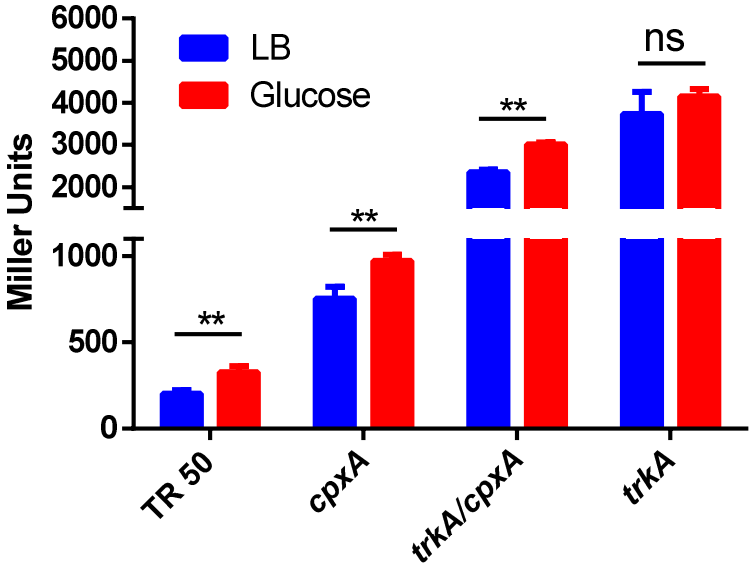
Miller Units
LB
Glucose
Glycerol
Succinate
TR 50
cpxA
trkA/cpxA
trkA

B.


6000
5000
4000
3000
2000
1000
500
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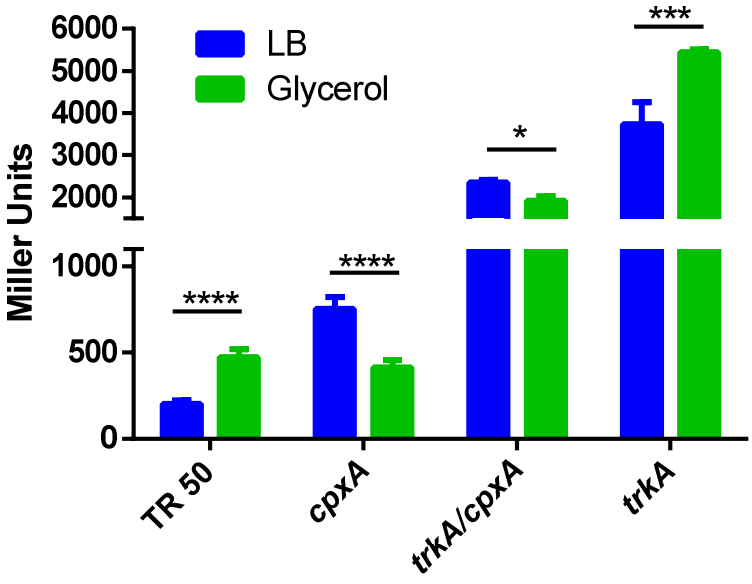
LB
Glucose
**
**
**
ns
TR 50
cpxA
trkA/cpxA
trkA

C.


LB
Glycerol
Miller Units
6000
5000
4000
3000
2000
1000
500
0
****
****
*
***
TR 50
cpxA
trkA/cpxA
trkA

D.

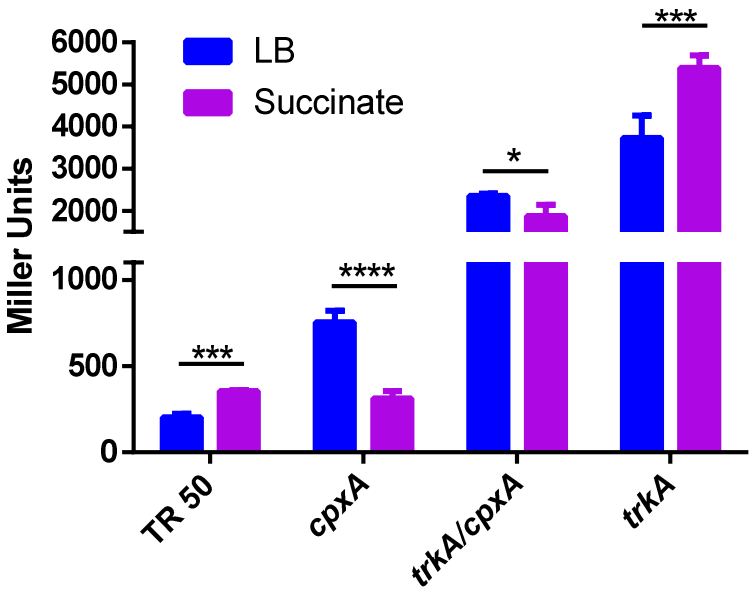

LB
Succinate
Miller Units
6000
5000
4000
3000
2000
1000
500
0
***
****
*
***
TR 50
cpxA
trkA/cpxA
trkA

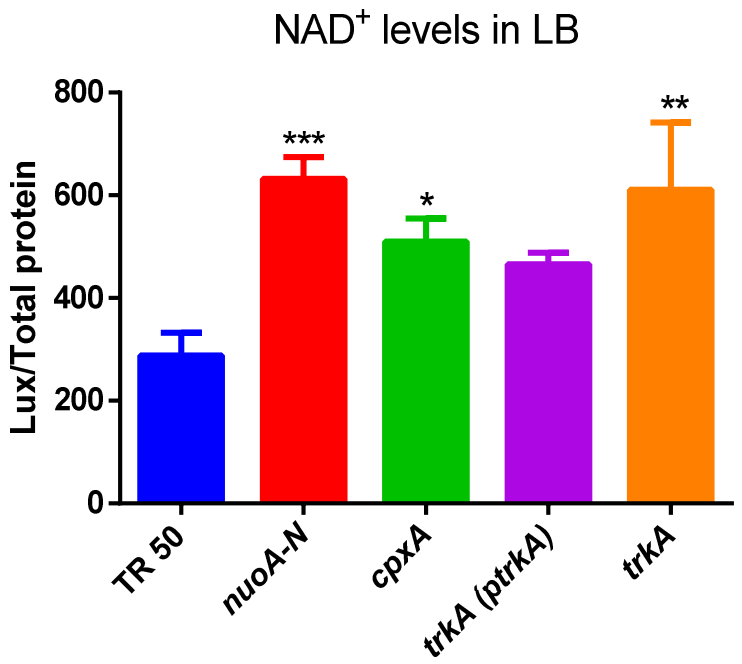
Figure 3-7: Mutation of *trkA* strongly induces the Cpx pathway in minimal media. Wild type TR50 and mutant strains bearing mutations in *cpxA, trkA,* and *cpxA trkA* were subcultured 1:50 from overnight cultures grown with appropriate antibiotics into fresh 2 mL LB broth or minimal media supplemented with 0.4% carbon source (glucose, glycerol and succinate), and grown for 4 hours to mid log phase (A_{600} = 0.4-0.6). Cells were pelleted, resuspended in 2 mL 1X Z-buffer and lysed. Beta galactosidase assays were then performed. Bars represent average values between three replicates and error bars represent standard deviation between replicates. Asterisks denotes a significant difference and ns denotes no significant difference between strains grown under different media conditions ($p<0.05$, one way ANOVA test).

3.2.4 Mutation of *trkA* causes accumulation of NAD^+ in the cell

TrkA has been shown to bind to NAD^+ and NADH (Schlosser et al., 1993). We therefore wondered if, in the absence of *trkA,* the levels of either of these co-factors might be altered, and have some impact on Cpx pathway activity. To test whether the absence of TrkA leads to alterations of NAD^+ and NADH, we tested NAD^+ and NADH levels in the cell by using the NAD^+/NADH glo assay kit from Promega. Wild-type, *trkA, cpxA,* and the *trkA* mutant carrying the t*rkA* over-expression plasmid (p*trkA)* were grown in LB broth and minimal media supplemented with 0.4% glucose or succinate and the levels of NAD^+ and NADH were measured according to the manufacturer's instructions. As a control, we measured NAD^+ and NADH levels in a mutant lacking the *nuoA-N* operon, which encodes the NADH dehydrogenase I complex. The NADH dehydrogenase I complex catalyzes the oxidation of NADH to NAD^+. In this strain, NADH levels were significantly elevated under all growth conditions (LB broth and minimal media supplemented with 0.4% glucose or succinate) due the absence of the NADH dehydrogenase I complex (Figure 3-8D-F). Also, we observed only small, insignificant changes in the NAD^+ levels in the *nuoA-N* mutant compared to the wild type grown under minimal media conditions (Figure 3-8B-C). This is expected due to the absence of NADH dehydrogenase I complex. However, we observed elevated levels of NAD^+ in the *nuoA-N* mutant compared to the wild type in LB (Figure 3-8A). This might be due to an increase in NAD^+ biosynthesis in rich media. Conversely, the *trkA* mutant strain showed an NAD^+ increase of 1.5 to 2 fold compared to the wild type (TR50) under all conditions (Figure 3-8A-C), and this level was reduced partly (Figure 3-8C) or fully (Figure 3-8A, B) to that of wild-type when the *trkA* mutant was complemented with a

plasmid over-expressing *trkA* from an IPTG-inducible promoter. The only other differences observed were an increase in NAD^+ levels in the *cpxA* mutant when it was grown in LB (Figure 3-8A). Lastly, we observed only small insignificant changes in the NADH levels in the *trkA* mutant compared to the wild type under all conditions (Figure 3-8D-F). Altogether, these data support the hypothesis that mutation of *trkA* alters the accumulation or levels of NAD^+ in the cell

A.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Lux/Total protein
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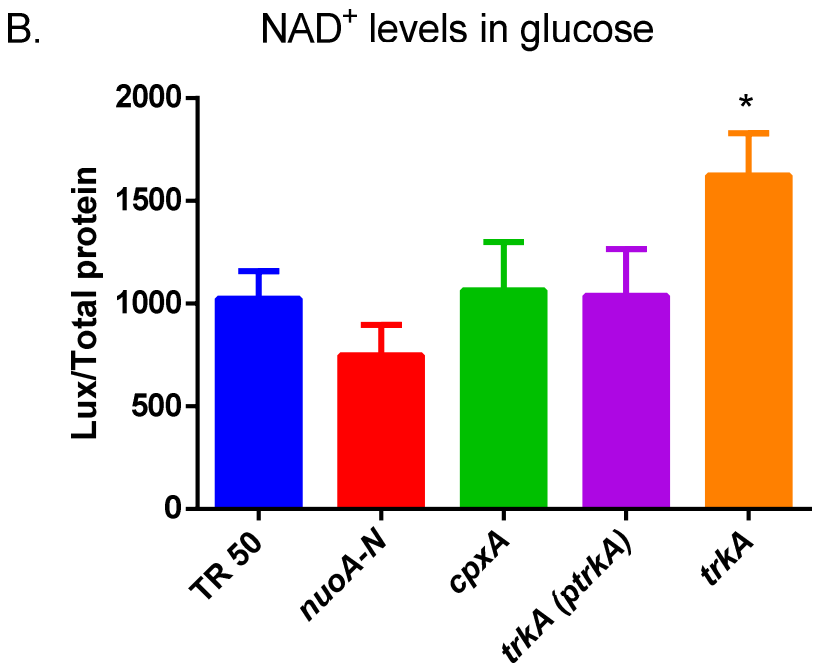
600
400
200
0
***
*
**
TR 50
nuoA-N
cpxA
trkA (ptrkA)
trkA

B.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Lux/Total protein
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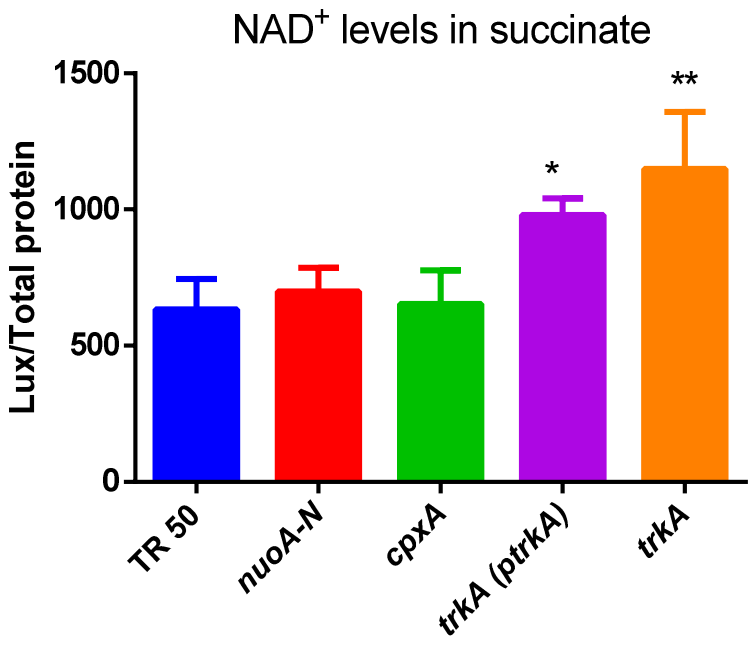
1500
1000
500
0
*
TR 50
nuoA-N
cpxA
trkA (ptrkA)
trkA

C.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Lux/Total protein
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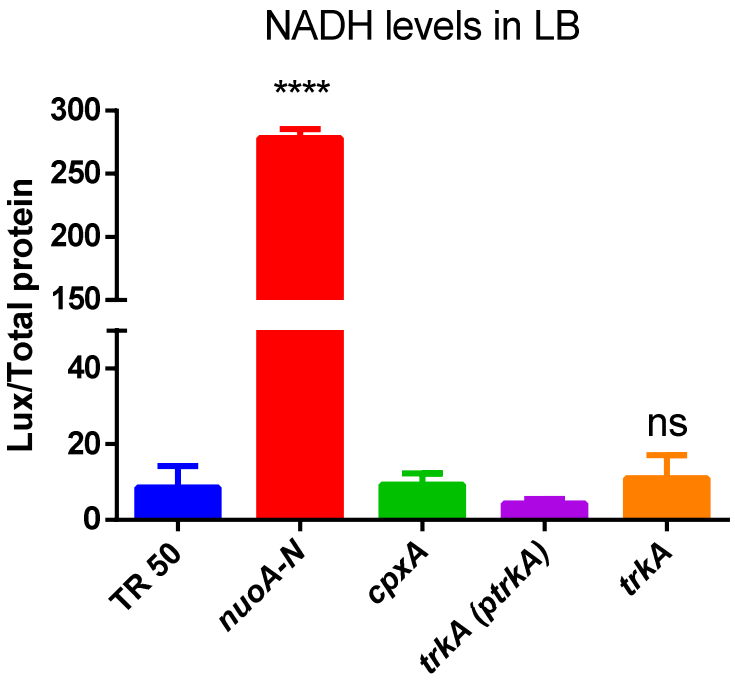
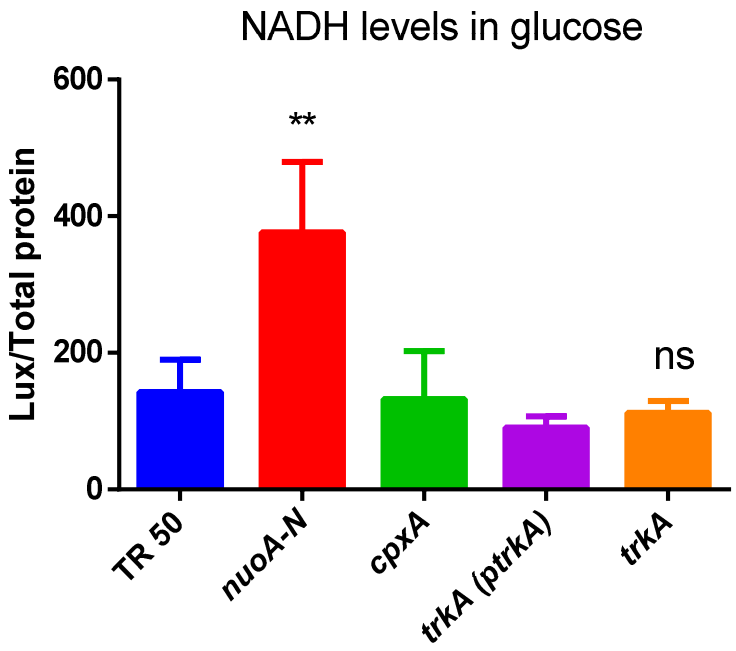
1000
500
0
*
**
TR 50
nuoA-N
cpxA
trkA (ptrkA)
trkA

D.


NADH levels in LB
Lux/Total protein
300
250
200
150
40
20
0
****
ns
TR 50
nuoA-N
cpxA
trkA (ptrkA)
trkA

E.


NADH levels in glucose
Lux/Total protein
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400
200
0
**
ns
TR 50
nuoA-N
cpxA
trkA (ptrkA)
trkA

F.

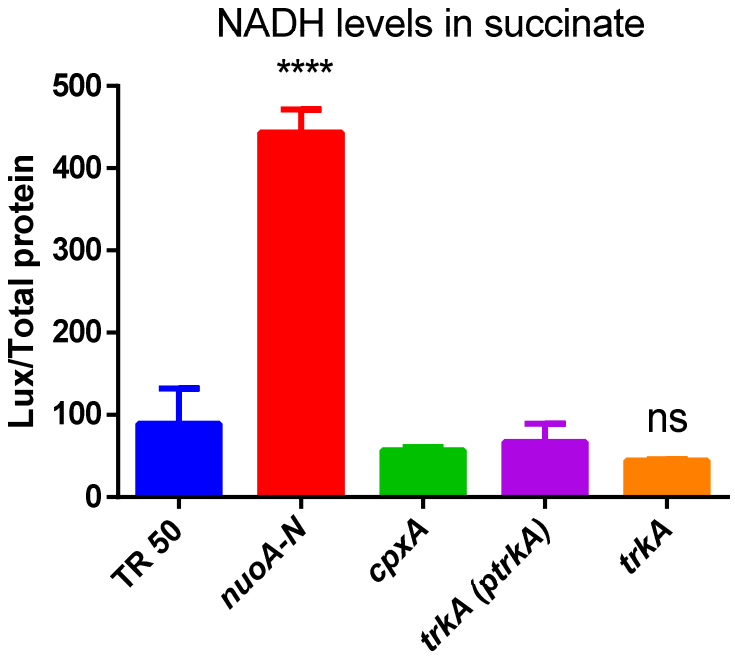
NADH levels in succinate
Lux/Total protein
500
400
300
200
100
0
****
ns
TR 50
nuoA-N
cpxA
trkA (ptrkA)
trkA

Figure 3-8: Mutation of trkA causes accumulation of NAD+ in the cell. Wild type (TR50) together with isogenic mutants bearing mutations in *nuoA-N, cpxA, trkA,*and the *trkA* mutant carrying the t*rkA* over-expression plasmid (p*trkA)* were grown overnight in LB broth with appropriate antibiotics and then subcultured 1:50 the next day into 2 mL LB broth and minimal media supplemented with 0.4% glucose or succinate and grown to mid log phase (A_{600} =0.4-0.6) in the presence of the IPTG inducer. Cells were pelleted and resuspended in 2 mL 1X PBS. The Promega NAD^+/NADH glo assay kit was used to measure levels of NAD^+ (A-LB, B-M9 glucose, and C-M9 succinate) and NADH (D-LB, E-M9 glucose, and F-M9 succinate) according to the manufacturer's instructions. Bars represent average values between three replicates and error bars represent standard deviation between replicates. Asterisks denote a significant difference compared to wild type and ns denotes no significant difference compared to wild type ($p<0.05$, one way ANOVA test).

3.2.5 Activation of the Cpx system by *trkA* mutation causes a decrease in respiration.

Based on the fact that the *trkA* mutant showed elevated levels of NAD^+ in our previous experiment we wondered if the alteration in the levels of NAD^+ and NADH might affect respiration in the *trkA* mutant. The cellular redox state and metabolism are linked to the amounts of NAD^+ and NADH in the cell (Dhamdhere and Zgurskaya, 2010) and changes in the ratio of these compounds can affect the respiratory chain (Calhoun et al., 1993). Also, it has been shown recently that activation of the Cpx pathway can affect the electron transport chain (Guest et al., 2017). It was within reason to posit that accumulation of NAD^+ and the activation of the Cpx response may diminish respiration in the *trkA* mutant. We measured the rate of oxygen consumption in the wild type (TR50) strain as well as isogenic mutants carrying mutations in *trkA, cpxR,* and *cpxA,* as well as in double mutants lacking *trkA* and *cpxA* or *trkA* and *cpxR,* and in the *trkA* mutant carrying the t*rkA* over-expression plasmid (p*trkA)* (Figure 3-9) using the Micro Optode Meter oxygen sensor from Unisense. Strains were grown overnight in 2 mL of LB broth with appropriate antibiotics. Cells were pelleted, washed with 2 mL 1X PBS and resuspended in fresh LB broth to an OD_{600}nm of approximately 0.1 and the rate of oxygen consumption was measured.

As hypothesized, we observed a significant decrease in the rate of oxygen consumption in the *trkA* mutant compared to the wild type (Figure 3-9). These results are also consistent with previous finding that induction of the Cpx pathway decreases respiration, since the Cpx response is activated in the *trkA* mutant (Guest et al., 2017). The *trkA cpxA* double mutant also showed a significant decrease in oxygen

consumption compared to the wild type (Figure 3-9). The *trkA cpxR* double mutant showed an increase in respiration compared to the *trkA* mutant. This was expected due to the absence of *cpxR* and hence a lack of activation of the Cpx response and a failure to repress expression of the aerobic components of the electron transport chain (Figure 3-9). However, we observed a decrease in respiration in the *trkA cpxR* double mutant compared to the wild type (Figure 3-9). We also observed small, insignificant differences in the rate of oxygen consumption in the strains carrying mutations in *cpxA, cpxR*, and the *trkA* mutant bearing the t*rkA* over-expression plasmid (p*trkA)* compared to the wild type (Figure 3-9). All together, our data demonstrate that respiration is diminished in the *trkA* mutant, and this defect is only partly due to activation of the Cpx response.

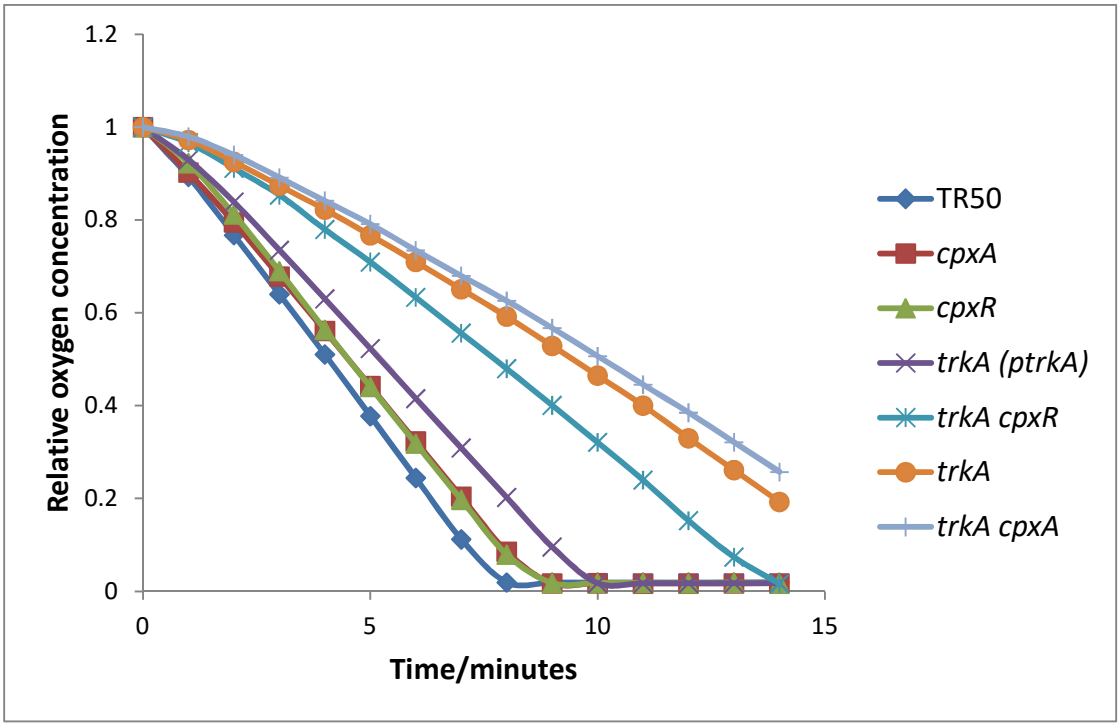

Figure 3-9: Respiration is diminished in the trkA mutant. The wild type (TR50) strain, together with isogenic mutants carrying mutations in *trkA, cpxR,* or *cpxA,* as well as in double mutants lacking *trkA* and *cpxA* or *trkA* and *cpxR,* and a *trkA* mutant carrying the t*rkA* over-expression plasmid (p*trkA)* were grown overnight in 2 mL of LB broth with appropriate antibiotics. Cells were pelleted, washed with 2 mL 1X PBS and resuspended in fresh LB broth to an OD_{600}nm of approximately 0.1 and the rate of oxygen consumption was measured using the Micro Optode Meter oxygen sensor from Unisense.

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CHAPTER 4: GENERAL DISCUSSION

4.1 Induction of the Cpx pathway by mutation of *trkA*

The Cpx envelope stress response system has long been thought to maintain the integrity of the envelope by regulating the transcription of genes associated with protein folding and degrading factors. These genes alleviate envelope stress caused by mis-folded periplasmic proteins (Danese and Silhavy, 1997; Danese et al., 1995; Pogliano et al., 1997). However, a recent microarray study of the Cpx system revealed that genes encoding inner membrane transport proteins were the most down-regulated genes upon over-expression of the lipoprotein NlpE (Raivio et al., 2013). Although the impact of the Cpx system on the regulation of inner membrane transport proteins has been established, there is no clear understanding of how the Cpx system and transporters are connected. Here we have demonstrated that mutation of *trkA*, a potassium transporter, activates the Cpx system by five-fold more compared to the induction by NlpE over-expression.

In this study, we sought to confirm that the mutation of *trkA* indeed activates the Cpx system. We have shown that mutation of *trkA* activated the Cpx pathway by over 15 fold in all cases compared to the wild type (TR50) (Figure 3-1). We observed no significant difference between the *cpxA* mutant and the wild type (Figure 3-1). The results are in agreement with previous observations on the effect of mutation of *trkA* on the Cpx system (J.L. Wong. unpublished). This was not surprising considering the fact that the Cpx pathway has been associated with membrane transport regulation since its discovery many years ago (Plate, 1976). Recently, it was shown by microarray induction of the Cpx pathway regulates many transporter genes (Raivio et al., 2013).

Since the Trk system is made up of three other genes (*trkE, trkG*, and *trkH)* in addition to *trkA* (Bossemeyer et al., 1989a; Dosch et al., 1991), We pondered if mutation of any of the remaining *trk* genes (*trkE, trkG,* and *trkH*) induces the Cpx system. As previously observed (J.L. Wong. unpublished), the *trkA* mutant showed over 15 fold increase in the induction of the Cpx system compared to the wild type (TR50) but there was no significant difference in the induction of the Cpx system between the remaining *trk* genes (*trkE, trkG,* and *trkH*) and the wild type (Figure 3-2A). Thus, the phenotype was specific to only the *trkA gene*. This prompted the hypothesis that perhaps genes encoding other potassium transport systems are exacerbating the potassium transport defects and could be responsible for the induction of the Cpx system in the *trkA* mutant. The two other potassium transport systems are the Kdp system and the Kup system. The Kdp system is a high affinity K^+ transporter under low concentrations (less than 2mM) of K^+ in the cell. While the Kup system is the low affinity potassium transporter that is induced under acidic conditions to transport K^+ into the cell (Dosch et al., 1991; Trchounian and Kobayashi, 1999). We tested this hypothesis by creating single mutations in *kup, kdpE*, and double mutations in *trkA kup* or *trkA kdpE* as well as double mutations *trkG trkH* (genes encoding potassium translocating sub units). Here we found no significant difference in Cpx activity between the single mutants *kup* and *kdp* as well as the double mutant *trkG trkH* compared to wild type (Figure 3-2B). However, the double mutants *trkA kup* and *trkA kdpE* showed comparable Cpx activity to that found in a *trkA* mutant background (Figure 3-2B) as observed previously (Figure 3-1). It is clear from our data that none of the potassium transport systems is responsible for the activation of the Cpx pathway in the *trkA*

mutant. This suggests that the induction of the Cpx pathway is specific to mutation of *trkA*.

Based on our previous results (Figure 3-1), we decided to confirm that the effect of *trkA* mutation on the Cpx response was due to the activity of TrkA. We confirmed this by complementing the *trkA* mutant with a plasmid that over-expresses the *trkA* gene. We predicted that if the Cpx induction phenotype is specific to *trkA*, then complementation of the *trkA* mutant with a plasmid that over expresses *trkA* would abolish induction of the Cpx system. Subsequently, the Cpx activity phenotype of the *trkA* mutant will be restored to the wild type phenotype. As expected, the induction of the Cpx pathway was abolished upon complementation of the *trkA* mutant with a plasmid that over-expresses the *trkA* gene (Figure 3-3). This confirmed our previous discovery that induction of the Cpx pathway is a direct consequence of *trkA* mutation and it is not linked to any of the *trk* genes or any other potassium transporter. This is interesting considering the fact that the exact role of TrkA during the net uptake of potassium by the cell has not been demonstrated but it has been suggested that TrkA plays a role in the activation of the Trk system (Bossemeyer et al., 1989a). Though the exact role of TrkA is not known, TrkA is an absolute requirement for potassium transport and functions by binding with either the potassium translocating unit TrkG or TrkH as well as TrkE (Schlösser et al., 1991; Schlösser et al., 1995). But it is clear from our data that the Cpx pathway is activated very strongly only when *trkA* is deleted.

If TrkA is an absolute requirement in activating the Trk system as suggested previously (Bossemeyer et al., 1989a), then it is reasonable to assume that potassium transport might be involved in the *trkA* mediated activation of the Cpx response. Also we

have demonstrated that mutation of individual or multiple genes encoding proteins involved in potassium transport had no impact on the induction of the Cpx system. We reasoned that perhaps changes in potassium concentrations might be interfering with an enzymatic activity or reaction, which is being sensed by the Cpx system. We tested whether potassium concentrations are linked to the activation of the Cpx pathway. To test this theory we used phosphate buffered media as previously described to alter the concentration of potassium (Epstein and Davies, 1970; Epstein and Kim, 1971) in minimal media. To our surprise there was no difference in the activation of the Cpx system in potassium limiting media or in excess potassium (Figure 3-4). Our data therefore suggest that there is no link between altered potassium concentrations and the activation of the Cpx pathway.

Overall, these data confirm that induction of the Cpx pathway is a consequence of *trkA* deletion and it is not linked to any of the *trk* genes or potassium transporters. Furthermore, changes in the concentration of potassium do not play a role in the activation of the Cpx pathway. We were intrigued with these results and sought to investigate the potential mechanisms involved in the activation of the Cpx pathway

4.2 Mechanisms involved in the activation of the Cpx system by *trkA* mutants

The sensor histidine kinase is the classic signal sensing domain of two component systems and can sense a wide variety of signals in the periplasm which is subsequently transmitted into the cytoplasm and phosphorylation of the response regulator. This alters the conformation of the response regulator and results in DNA binding and transcriptional regulation by the response regulator to initiate an adaptive response (Fabret et al., 1999; West and Stock, 2001). In the Cpx pathway, CpxA acts as an inner membrane sensor histidine kinase that senses inducing signals from the envelope and relays the signal to the cytoplasmic response regulator CpxR via phospho-transfer to activate the pathway (DiGiuseppe and Silhavy, 2003; Raivio and Silhavy, 1997b). Based on our previous results we wondered where in the cell the signaling for the induction of the Cpx pathway that occurs upon mutation of *trkA* is located. Our first thought was to verify if the signal for the activation of the Cpx pathway is located in the envelope and would therefore be sensed through the sensor kinase CpxA. Here we have shown that the activation of the Cpx pathway by mutation of *trkA* is partially independent of CpxA (Figure 3-5A). We observed a slight decrease in Cpx activity (1.5 fold) between the *trkA* mutant and the *trkA cpxA* double mutant. However, there was still an increase in the induction of Cpx system in the *trkA cpxA* double mutant (15 fold), compared to the wild type (Figure 3-5A). This suggests that, while *trkA* mutation may partially activate the Cpx response via an envelope signal that is transmitted through CpxA, CpxA is not responsible for the majority of the activation of *cpxP-lacZ* expression seen in this mutant. The *trkA cpxA* double mutant lacks the signal sensed by CpxA due to its deletion and hence the slight decrease in the activation of

the Cpx system. This is not a rare event as the Cpx system can sense multiple signals from different entry points in the cell (DiGiuseppe and Silhavy, 2003; Wolfe et al., 2008). Our data indicate that CpxA is not responsible for the majority of the activation of the Cpx system seen in this mutant. Therefore, majority of the signal responsible for the Cpx system induction is not located in the envelope.

Although most of the signal integration of the Cpx system is through the Sensor kinase CpxA, It well established that growth activation of the Cpx system is independent of CpxA but dependent on CpxR (DiGiuseppe and Silhavy, 2003). We then wondered whether the signaling originates from the cytoplasm and if CpxR is involved in the signaling of the pathway. Interestingly, we discovered that induction of the Cpx pathway is CpxR dependent (Figure 3-5B). We observed that the Cpx activity diminished considerably in both the *cpxR* mutant as previously shown (Danese and Silhavy, 1998) as well as the *trkA cpxA* double mutant. These data suggest that the Cpx inducing signal generated by *trkA* mutation originates mostly in the cytoplasm, as opposed to the envelope. Although we have not shown how this cytoplasmic signal is relayed to CpxR, it is clear from our results that this signal sensing is independent of CpxA, and it is being transmitted by an unknown mechanism to the response regulator CpxR which activates the Cpx system. In the future, it will be interesting to investigate further how this signal is relayed to CpxR. This will broaden our understanding on how cytoplasmic signals are integrated through CpxR.

We have also demonstrated that mutation of *trkA* does not elevate Cpx activity by inhibiting CpxA phosphatase activity (Figure 3-6). This is important because TrkA is known to regulate the pH in *E. coli* grown anaerobically at alkaline pH as high as 8.6.

Conversely low cytoplasmic pH inhibits TrkA activity (Bakker, 1983; Trchounian et al., 1998). On the other hand, the Cpx pathway is induced under alkaline pH and this induction requires the kinase activity of CpxA (Danese and Silhavy, 1998) but it is inhibited under acidic pH (<6.0) and this requires the phosphatase activity of CpxA (R. Malpica and T.L. Raivio, unpublished). Based on these facts and our previous data which showed that induction of the Cpx pathway seems to be partly dependent on CpxA (Figure 3-5A), We thought there is a possibility that TrkA might impact the enzymatic activity of CpxA which subsequently inhibits it phosphatase activity. As previously observed (R. Malpica and T.L. Raivio, unpublished), the *cpxA* mutant showed elevated levels of Cpx activity in acidic pH due to absence of *cpxA* phosphatase activity (Figure 3-6). On the other hand, the *trkA* mutant showed elevated Cpx activity at all pHs. However, the *trkA cpxA* double mutant also showed elevated levels (slightly lower than *trkA*) of Cpx activity at acidic pH (Figure 3-6). The diminished level of Cpx pathway activity in the *trkA cpxA* mutant relative to the *trkA* mutant alone might suggest that part of the inducing effect of the *trkA* mutation is due to effects on CpxA. Since mutation of *trkA* can further activate the Cpx response in a strain lacking CpxA (Figure 3-6), it implies that, *trkA* does not activate the Cpx response by inhibiting the phosphatase activity of CpxA. Altogether, our data indicate that, *trkA* does not activate the Cpx response by inhibiting the phosphatase activity of CpxA. At this juncture we do not know the reason or the mechanism under which the histidine kinase, CpxA, is blind to the acidic pH and does not inhibit induction of the Cpx pathway.

Small molecular weight phosphodonors such as acetyl phosphate has been shown to phosphorylate CpxR in dependent of CpxA (Danese and Silhavy, 1998) and

we have also shown that activation of the Cpx response by *trkA* mutation is mostly independent of CpxA (Figure 3-5A). Based on these facts we hypothesized that the effect of *trkA* might be dependent on the phosphorylation of CPxR by acetyl phosphate. Here we show that mutation of *trkA* strongly activates the Cpx pathway in minimal media.(Figure 3-7A). Because acetyl phosphate levels are elevated in LB broth and minimal media with glucose but not in minimal media with succinate or glycerol (Danese and Silhavy, 1998; Danese et al., 1995), we expected elevated Cpx activity in LB and minimal media with glucose but not in glycerol or succinate. We observe a slight increase in Cpx activity in the wild type grown in minimal media (Figure 3-7A). These data are consistent with recent finding that show that the Cpx response is sensitive to the biogenesis and/or function of membrane bound aerobic respiratory complexes (Guest et al., 2017) As we expected, the Cpx activity was elevated in the *cpxA* mutant and the *trkA cpxA double* mutant grown in LB and minimal media with glucose (Figure 3-7A) due to phosphorylation of CpxR by acetyl phosphate as shown previously (Danese and Silhavy, 1998). However, the *trkA* mutant showed elevated Cpx activity under all conditions and was even higher in minimal media containing glycerol and succinate. This suggests that induction of the Cpx system by mutation of *trkA* is not dependent on acetyl phosphate. This is contrary to our hypothesis that the effect of *trkA* might be dependent on the phosphorylation of CPxR by acetyl phosphate.

Because *trkA* has been shown to bind to NAD^+ and NADH (Schlosser et al., 1993), we hypothesized that the absence of *trkA* might alter the levels of NAD^+ and NADH and might impact Cpx activity. We examined the levels of NAD^+ and NADH to verify this hypothesis. As expected, the *trkA* mutant strain showed an NAD^+ increase of

1.5 to 2 fold compared to the wild type (TR50) under all conditions (Figure 3-8A-C). We observed an unexpected elevated level of NAD^+ in our control mutant *nuoA-N.* This may be possible if the *nuoA-N* mutant has a higher cellular pool of NAD^+ prior to the reduction to NADH. We observed only small, insignificant changes in the NADH levels in the *trkA* mutant compared to the wild type under all conditions (Figure 3-8D-F). NAD^+ and NADH play essential roles in cell metabolism and dramatic changes in the absolute and relative amounts can affect the reaction equilibria of enzymes as well as the redox state and metabolism of the cell.(Dhamdhere and Zgurskaya, 2010; Prüss et al., 1994). It is possible that an alteration in the levels of NAD+ causes a change in the cell's metabolism which is being sensed by the Cpx pathway. Our theory is in agreement with a previous study that showed that the Cpx system could sense metabolism. Mutants lacking the outer membrane efflux pump, TolC, were found to accumulate some metabolites that induced the Cpx system (Rosner and Martin, 2013). At this time, we do not know the specific change in metabolism being sensed by the Cpx system. But our data suggest that mutation of *trkA* alters the accumulation or levels of NAD^+ which is being sensed by the Cpx system and hence the induction of the pathway. In the future, a metabolic profile of the *trkA* mutant may offer more insights into some of the likely metabolites involved here. This will increase our knowledge of how mutation of *trkA* impacts the Cpx system with respect to the cell's metabolism.

Based on the fact that mutation of *trkA* alters the accumulation or levels of NAD^+ (Figure 3-8A-C), we examined oxygen consumption to verify if the rate of respiration decreases upon induction of the Cpx system in *trkA* mutants. As expected, there was a significant reduction in the rate of oxygen consumption in the *trkA* mutant compared to

the wild type (Figure 3-9). Also, in the *trkA cpxR* double mutant, we observe a decrease in respiration compared to the wild type (Figure 3-9). This is due to the absence of *cpxR.* This is consistent with recent finding that there is a respiratory defect in *cpxRA* mutants (Guest et al., 2017) In our previous microarray experiment, we demonstrated that activation of the Cpx pathway leads to down-regulation of genes that encode the electron transport chain (Raivio et al., 2013). In addition we have shown recently that the Cpx response is sensitive to the biogenesis and/or function of membrane bound aerobic respiratory complexes (Guest et al., 2017). It is possible that induction of the Cpx pathway by mutation of *trkA* affects the genes encoding the electron transport chain which diminishes respiration.

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4.4 Final model and concluding remarks

The experiments detailed in this thesis seek to investigate the effect of *trkA* mutants on the Cpx pathway and the possible mechanisms involved in the activation of the Cpx pathway. Although some questions still remain unanswered, we have shown that mutation of *trkA* activates the Cpx pathway. This is specific to *trkA* mutation and does not involve any of the *trk* genes or the other potassium transporters. Also, the activation of the Cpx system by *trkA* mutation is not linked in any way to changes in potassium concentration. Furthermore, our work demonstrated that activation of the Cpx pathway is independent of CpxA but dependent on CpxR. We discovered that the signal for the induction of the Cpx system is located in the cytoplasm and integrated through CpxR. Also, mutation of *trkA* does not elevate Cpx activity by inhibiting CpxA phosphatase activity or dependent on the levels of acetyl phosphate. In addition, we have shown that mutation of *trkA* alters accumulation or levels of NAD^+ in the cell. Finally, we demonstrated that induction of the Cpx pathway diminishes respiration. Overall, our data support our hypothesis some aspect of TrkA function leads to disruption in cellular physiology/function that generates a Cpx inducing signal. (Figure 4-1). We speculate that alterations in the levels of NAD^+ in the cell interferes with a metabolic activity which is sensed by the Cpx system hence the activation of the pathway.

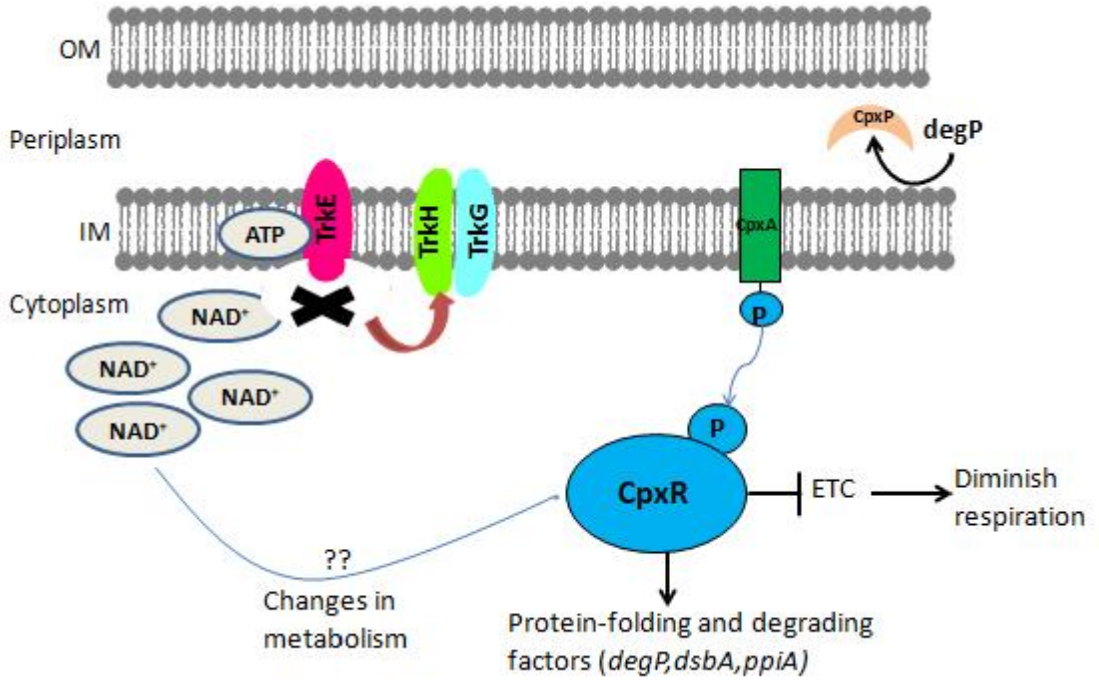

Figure 4-1: Model for activation of the Cpx pathway by mutation of *trkA*.

Mutation of *trkA* causes accumulation of NAD^+ which induces the Cpx response. CpxR then represses the transcription of genes encoding the electron transport chain (ETC) and diminishes respiration. OM denotes outer membrane, IM denotes inner membrane.

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