A proteomics approach to identify the molecular signals for Cpx activation during long-chain fatty acid metabolism in *Escherichia coli*

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Certificate of Examination

This is to certify that the dissertation titled **"A proteomics approach to identify the molecular signals for Cpx activation during long-chain fatty acid metabolism in *Escherichia coli*"** by **Ms. Ardra Nandakumar** (Reg. No. MS16046) for the partial fulfillment of BS-MS dual degree programme of the Institute, has been examined by the thesis committee duly appointed by the Institute. The committee finds the work done by the candidate satisfactory and recommends that the report be accepted.

Prof. Anand Kumar Bachhawat Dr. Rajesh Ramachandran Dr. Rachna Chaba

(Supervisor)

Dated: April 12, 2021

Declaration

The work presented in this dissertation has been carried out by me under the guidance of Dr. Rachna Chaba at the Indian Institute of Science Education and Research, Mohali.

This work has not been submitted in part or in full for a degree, a diploma, or a fellowship to any other university or institute. Whenever contributions of others are involved, every effort is made to indicate this clearly, with due acknowledgement of collaborative research and discussions.

This thesis is a bonafide record of original work done by me, and all sources listed within have been detailed in the bibliography.

Ardra Nandakumar

(Candidate)

Dated: April 12, 2021

In my capacity as the supervisor of the candidate's project work, I certify that the above statements by the candidate are true to the best of my knowledge.

Dr. Rachna Chaba

(Supervisor)

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Abstract

Gram-negative bacteria like *Escherichia coli* are able to thrive in a multitude of environments owing to the presence of a cell-envelope that protects them from various environmental insults. Since the envelope acts as the first line of defense and is crucial to cell survival, its integrity is continuously monitored and maintained by envelope stress response (ESR) pathways. The periplasm, an aqueous component of the bacterial cell-envelope between the inner and outer-membranes, bears a hyper-oxidizing environment that hosts many enzymes for a myriad of vital physiological processes. Amongst the wide range of diverse cellular processes that occur in the periplasm, it facilitates oxidative protein folding in several extracytoplasmic proteins. Previous work from our lab has shown that metabolism of long-chain fatty acids (LCFAs), a rich nutrient source for many bacteria including *E.coli*, shifts the redox environment of the periplasm towards a hypo-oxidising state, thereby hampering disulfide bond formation. Interestingly, an ESR pathway, CpxAR two-component system, gets activated in response to such LCFA-induced envelope stress. However, the molecular signals and mechanistic details for Cpx-activation during LCFA metabolism are still unknown, although a bimodal nature of activation is suggested that may be both redox-dependent and independent. This study focuses on uncovering the players involved in Cpx activation using a proteomics approach. The important players of the Cpx pathway, CpxA and CpxP, were SPA (Sequential Peptide Affinity) tagged to find their interacting partners during LCFA metabolism. Chromosomally tagged CpxA was observed in sufficient amounts that could be used for protein pulldown, however, tagged CpxP had to be cloned onto an inducible plasmid to be produced in detectable amounts. Protein profiles obtained from tagged strains grown with and without LCFAs were very similar and therefore advanced techniques like mass spectroscopy will be required to identify the specific players. The study also briefly discusses the standardization of conjugation, which can be used to screen ordered gene libraries, another strategy by which genes involved in the activation of the Cpx pathway can be uncovered.

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List of Abbreviations

1. βME: 2-mercaptoethanol
2. µl: microlitre
3. APS: Ammonium Persulfate
4. ATP: Adenosine triphosphate
5. $CaCl_2$: Calcium chloride
6. CBP: Calmodulin binding protein
7. CP: Cytoplasm
8. Cpx: CpxAR response
9. DNA: Deoxyribonucleic acid
10. ECF: Extracytoplasmic function
11. EDTA: Ethyelene diamine tetraacetate
12. ESR: Envelope stress response
13. EtBr: Ethidium bromide
14. ETC: Electron Transport Chain
15. $FADH_2$: Flavin adenine dinucleotide
16. His: Histidine
17. H_2O: Water
18. IM: Inner membrane
19. KCl: Potassium chloride
20. LB: Lysogeny broth

21. LCFA: Long-chain fatty acid
22. LPS: Lipopolysachharide
23. MCT: Microcentrifuge tube
24. $MgCl_2$: Magnesium chloride
25. $MgSO_4$: Magnesium sulphate
26. ml: millilitre
27. mM: millimolar
28. Na_2CO_3: Sodium carbonate
29. Na_2HPO_4: Sodium hypophosphate
30. NaCl: Sodium chloride
31. NADH: Nicotinamide adenine dinucleotide
32. NaOH: Sodium hydroxide
33. NEB: New England Biolabs
34. NH_4Cl: Ammonium chloride
35. OD: Optical density
36. OM: Outer membrane
37. OMP: Outermembrane proteins
38. ONPG: 2-nitrophenyl-β-D-galacto-pyranosideside
39. PCR: Polymerase chain reaction
40. pmf: Proton motif force
41. pmol: picomolar

42. PP: Periplasm
43. PPIase: Peptidyl-prolyl-isomerase
44. PSP: Phage shock protein response
45. RNA: Ribonucleic acid
46. ROS: Reactive oxygen species
47. SDS: Sodium dodecyl sulphate
48. SPA: Sequential peptide affinity
49. Taq: *Thermus aquaticus*
50. TCA: Tricarboxylic acid
51. TAE: Tris Acetic Acid EDTA
52. TBK: Tryptone broth K
53. TEMED: Tetramethylethylenediamine
54. TEV: Tobacco etch virus
55. WT: Wild type

Contents

Chapter 1

Introduction

1.1 Bacterial envelope stress responses

Bacteria can thrive in a multitude of environments where they encounter varied biotic and abiotic conditions. Such fluctuating environments can cause stress to the organisms in terms of changes in their cell membrane fluidity/composition, coping with bactericidal products secreted by other organisms, disruptions in membrane transporters due to pH variations, etc. Survival of bacteria in diverse niches is aided by numerous stress response mechanisms that they have evolved which allow them to respond appropriately to their surroundings. These processes are also crucial for pathogenic bacteria since their ability to sense and aptly respond to fluctuating environments within a host helps in their survival and virulence [1].

Gram-negative bacteria like *Escherichia coli* contain an outer membrane (OM) and inner membrane (IM) outside of the cytoplasm (CP). The OM, the IM and the aqueous region between these layers, known as the periplasm (PP), together constitute the cell-envelope (Figure 1). The cell-envelope acts as the first line of defense against external stresses. The stress responses mounted by bacteria in response to various perturbations can be broadly classified into cytoplasmic responses and extracytoplasmic responses [2].Cytoplasmic stresses are mainly caused by the presence of misfolded or unfolded proteins within the cytoplasm, such as when cells undergo heat shock. In *E. coli*, the transcription factor σ^{32}, an alternative σ factor governs response to heat shock by directing the transcription of genes encoding for cytoplasmic chaperones which repair/refold proteins [3].Extracytoplasmic or envelope stress responses (ESRs) are mainly responsible for detecting changes in the external environment and maintaining the integrity of the cell-envelope. Such responses help bacteria modulate their growth and metabolism to combat stress arising either outside of the cell or

that perturb the cell-envelope [4]. In *E. coli*, five dedicated ESRs are known; these include Cpx (conjugative pilus expression), Bae (bacterial adaptive response), Psp (phage shock protein), Rcs (regulator of capsule synthesis) and σ^E (sigma E). Of these, Cpx and σ^E are mainly activated in response to accumulation of misfolded/unfolded secreted proteins [5]. While the σ^E pathway majorly responds to folding and maintenance of the outer membrane proteins (OMPs), the Cpx pathway deals with protein misfolding in the periplasm, although further studies have concluded that the Cpx ESR might also be associated with inner membrane protein misfolding and translocation [5,6].

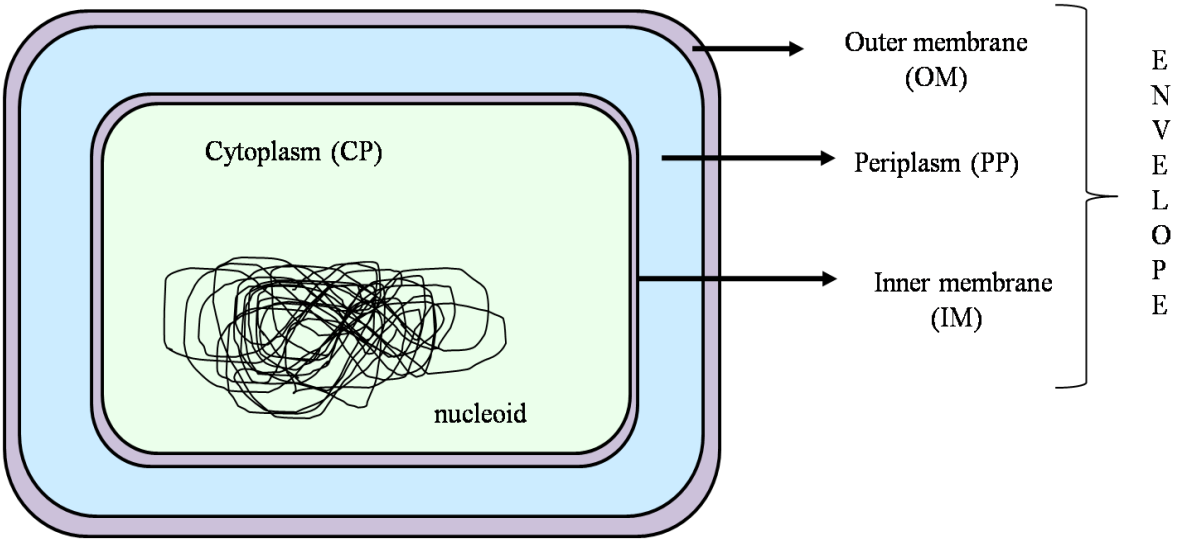


Figure 1: Schematic representation of the compartments in a Gram-negative bacterium

1.2 Two component systems

A two-component signaling system (TCS) consists of a typical histidine sensor kinase in the membrane that can respond to distinct signals through a phosphorelay mechanism along with a cognate response regulator in the cytoplasm. The phosphorylated response regulator can then take part in initiation/inhibition of transcription of effector genes [7]. In this way the TCS can help bacteria to detect physical and chemical changes which can then be relayed to

the genome in the cytoplasm where modulation of gene expression can occur. The TCS systems can modulate numerous processes like motility, nutrient uptake, cell division and even virulence [8]. The ESR pathways Rcs, Bae and Cpx represent some classical examples of a TCS system. The remaining sections will now delve into the Cpx system since this pathway forms the main focus of this study.

1.3 The CpxAR system

1.3.1 History and discovery

The *cpxRA* locus is present on the chromosome of most γ-proteobacteria including *E. coli*. It was discovered between the 1970s and 1980s in association with the absence of varied phenotypes such as the transport of substrates, conjugal plasmid transfer, resistance to antibiotics and even the ability to grow on certain carbon substrates, in cells that carried mutations in the *cpxRA* locus. During its initial discovery, this area of the chromosome was known by various names such as *cpx* (conjugative plasmid expression), *ecfB* (energy coupling factor B), *eup* (energy-uncoupled phenotype) and *ssd* (succinate non-utilizing, high serine deaminase activity). It was hypothesised that the mutations in this locus were affecting some aspect of energy generation at the inner membrane due to the range of phenotypes that the mutations produced. However, various analyses at the molecular and genetic level later revealed that there was no change in the proton motive force (PMF), oxygen consumption or ATP production in these mutants. Instead it was found that the protein composition at both the outer and inner membrane was altered, leading to the conclusion that mutations at this locus cause changes in the envelope or alter the regulatory processes that affect the coupling of the PMF to transport across the IM [6].

To date, the Cpx pathway is known to be involved in many functions including adhesion to abiotic surfaces, biofilm formation, virulence and antibiotic resistance to name a few, although its most important function lies in sensing variations that can cause changes in protein folding in the periplasm and inner membrane [5,9].

1.3.2 Signal transduction

As depicted in Figure 2, the CpxAR system mainly contains CpxA, a sensor histidine kinase located in the inner membrane with a periplasmic and a cytoplasmic domain, and CpxR, a cytoplasmic response regulator. CpxA gets auto-phosphorylated at its conserved histidine residue due to activating signals like changes in the pH, misfolding of proteins, addition of EDTA, etc. This phosphorylated CpxA can then in turn phosphorylate CpxR at its aspartate residue to form phosphorylated CpxR (CpxR~P). CpxR~P then comes in contact with the alpha subunit of RNA polymerase and hence, initiates transcription of downstream genes that are involved in either cleaving misfolded proteins or in assisting their proper folding [2]. These include DegP, a periplasmic protease/chaperone; DsbA, a periplasmic oxido-reductase; PpiA, a peptidyl prolyl isomerase and Spy, a periplasmic protein that may be involved in envelope biogenesis to name a few [10,11].Additionally, CpxR~P autoregulates the *cpxRA* operon. The expression of various periplasmic stress-combative activities under the control of Cpx also overlap with those of σ^E , however some are unique to Cpx [12].

Although Cpx is known as a two-component system, a third element, CpxP is also involved in this pathway. It is a small periplasmic protein known to inhibit CpxA via its periplasmic domain under unstressed conditions. Hence, activation of CpxA requires CpxP to first be titrated away from CpxA. The next section focuses on different modes of Cpx activation that can either be CpxP-dependent or independent [13].

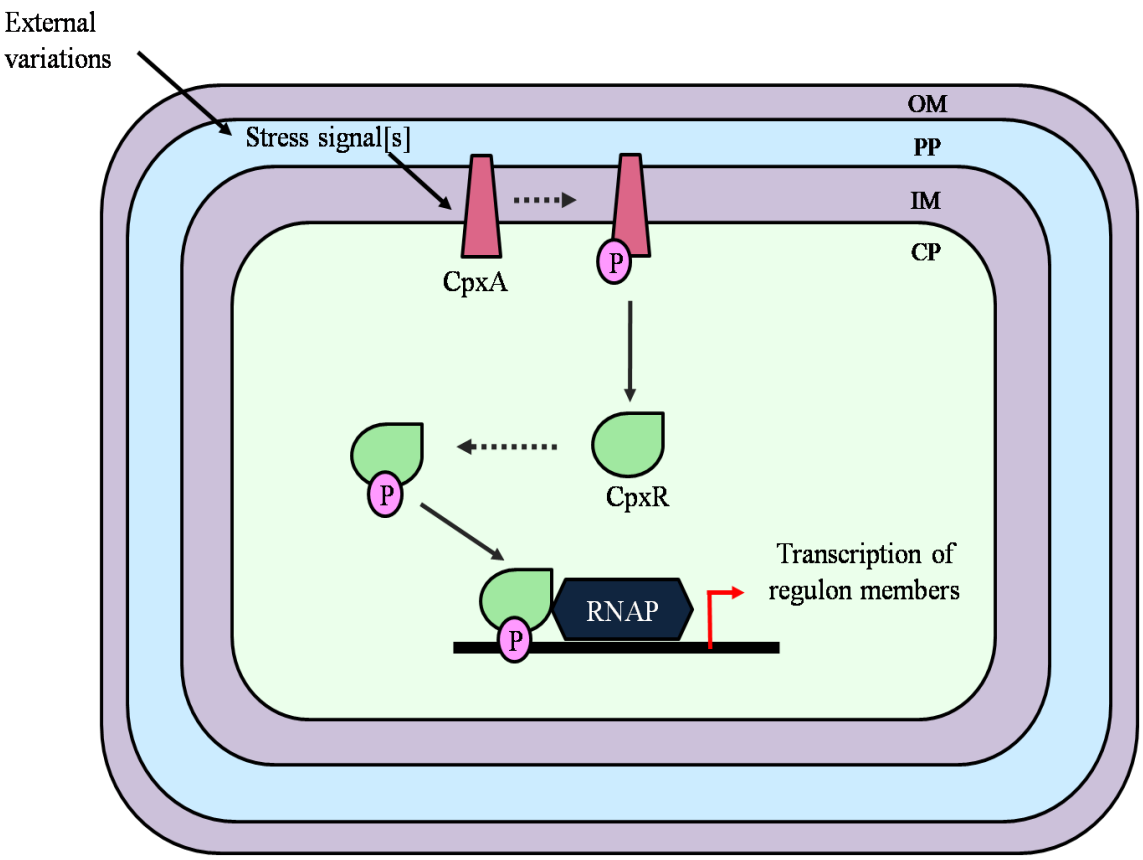


Figure 2: The Cpx signal transduction pathway

1.3.3 Proposed mechanisms of Cpx activation

Some of the conditions that induce Cpx pathway include elevated pH, copper stress, antibiotic stress, alterations in membrane composition, overexpression of outer membrane lipoproteins like NlpE, misfolding of pilus subunits, etc. Amongst all the known cues, the main factor for Cpx activation seems to be the presence of misfolded cell-envelope proteins. Although the molecular mechanisms behind these cues are not well studied, a few models have been proposed for some of them [14,15].

It should also be noted that the Cpx pathway also has a cytoplasmic signal of activation that is independent of the cue from the cell-envelope. It involves CpxR phosphorylation by

acetyl-phosphate, a phosphodonor formed as a product of the phosphotransacetylase (Pta)-acetate kinase (AckA) pathway that is independent of CpxA [16].

1.3.3.1 Elevated pH

In *Shigella sonnei*, it has been shown that the *cpx* locus is involved in the pH regulation of *virF*, a virulence locus. Studies in *E. coli* on this locus speculated that CpxA senses the change in extracellular pH and at elevated pH conditions, this pathway gets activated [17]. Studies have also shown that the transcription of *cpxP* increases almost 50-fold under the condition of elevated pH (pH 5.3 to pH 8.4) [13]. Since *cpxP* is under the regulation of CpxR alone, the transcription of this gene is a good indicator of activation of Cpx pathway [18]. Null mutations of *cpx* were also checked for sensitivity to alkaline pH and it was found that the *cpxR* null mutant confers the highest degree of sensitivity, followed by the *cpxP* null mutant and finally *cpxA* null mutant that was found to be the least sensitive to alkaline pH conditions. Whether CpxA senses these pH changes directly or indirectly is still up for debate. An indirect effect seems more probable since pH fluctuations can cause issues with protein folding and expression.

The proposed mechanisms suggest that alkaline pH might turn on the Cpx pathway either through the direct degradation of CpxP or via the titration of CpxP by proteins that may have become misfolded due to the altered pH. Hence the Cpx pathway is suggested to be regulated by pH in a CpxP-dependent manner as shown in Figure 3 [13]. One of the mechanisms of CpxP degradation in this condition is proposed to be through the periplasmic protease/chaperone DegP, whose expression is controlled by both CpxR and σ^E. Whether DegP directly degrades CpxP to activate the pathway is still not fully understood. It is proposed that CpxP on getting titrated by misfolded proteins may direct the entire complex towards DegP for degradation. [12,19].

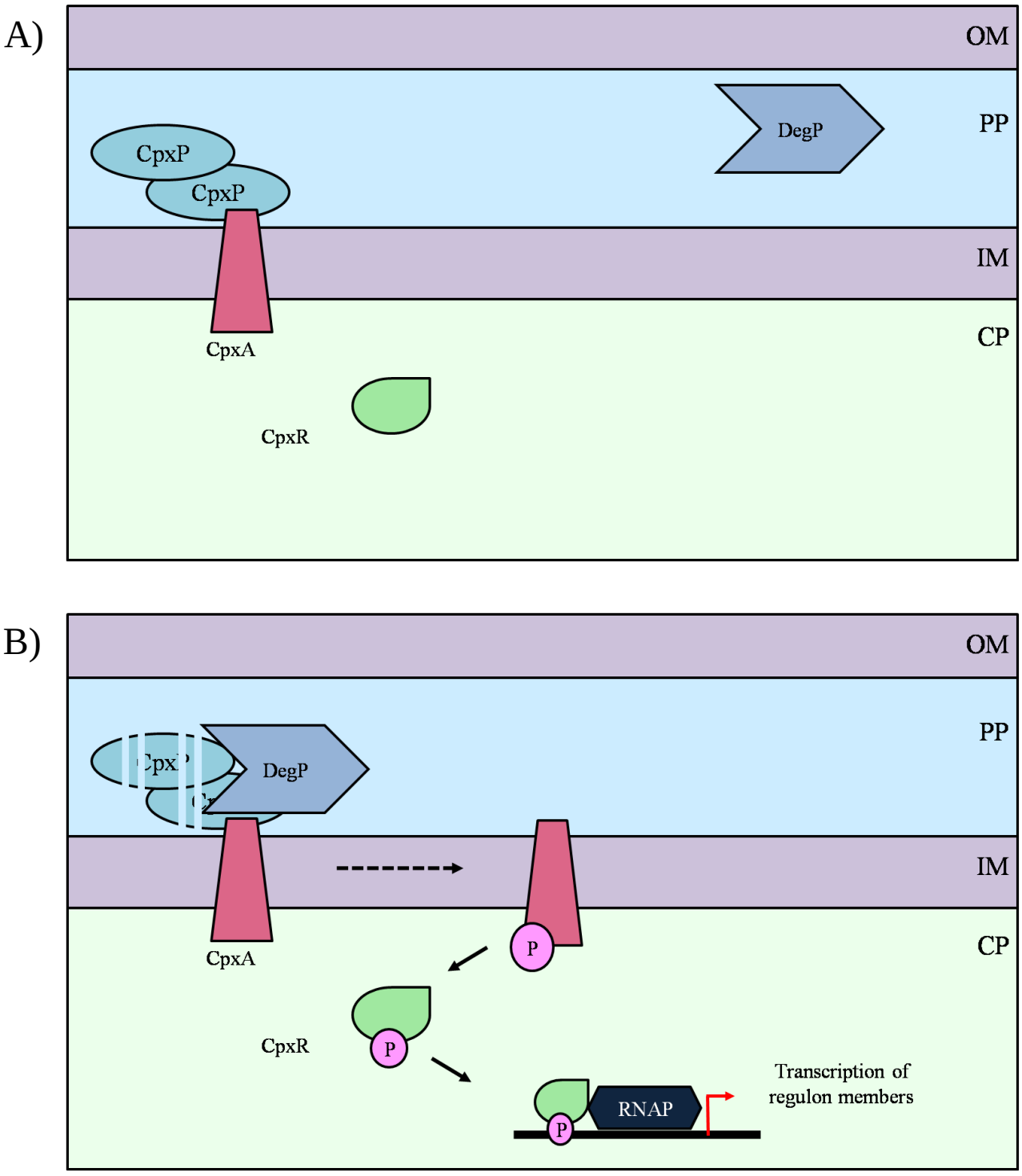
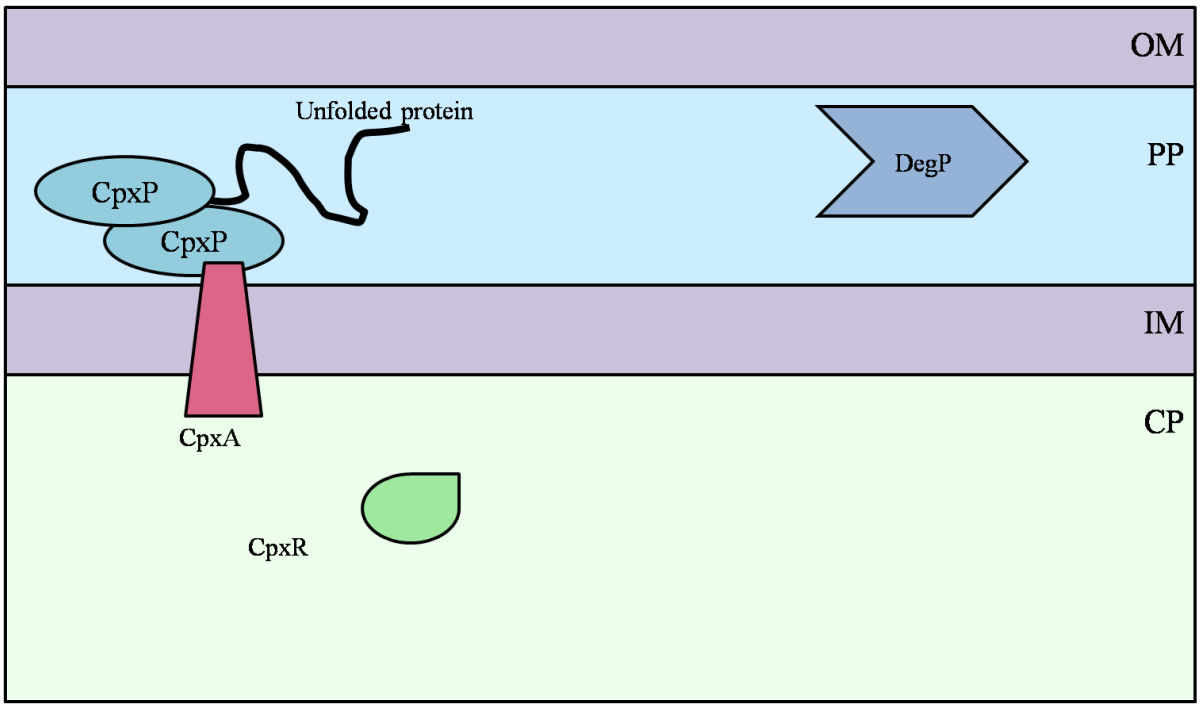


Figure 3: Proposed mechanism by which elevated pH may activate the Cpx pathway. A) Depicts the inactive state (basal growth conditions), and B) depicts the activated state (alkaline pH stress).

1.3.3.2 Misfolding of pilus proteins like PapE and PapG

As displayed in Figure 4, under unstressed conditions, it has been proposed that CpxP inhibits the Cpx pathway by binding to the periplasmic domain of CpxA, hence keeping the pathway in an off state [12].However, when proteins such as the pilin subunits get misfolded (for example in the absence of their periplasmic chaperone, PapD), CpxP acts as an adaptor protein and carries pilins towards DegP protease for degradation, hence activating the Cpx pathway [12]. It has been proposed that the N-terminal extension of the PapE subunit might be important for Cpx activation since in its absence, the pathway fails to get activated [6].Although expression of PapE and PapG in the absence of PapD is able to activate the Cpx pathway, other pilin subunits like PapA, PapF and PapK do not induce Cpx [20].Additionally, it is known that the assembly of P pilin proteins is overseen by the CpxRA system [20].

A)



B)

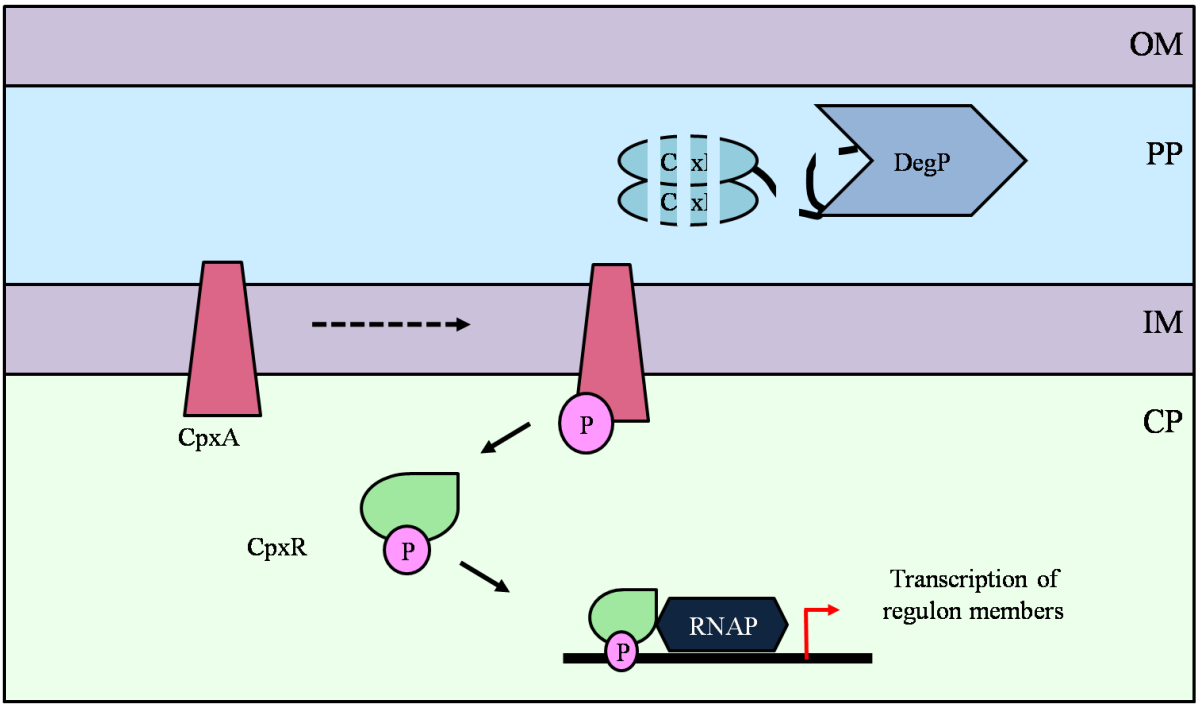


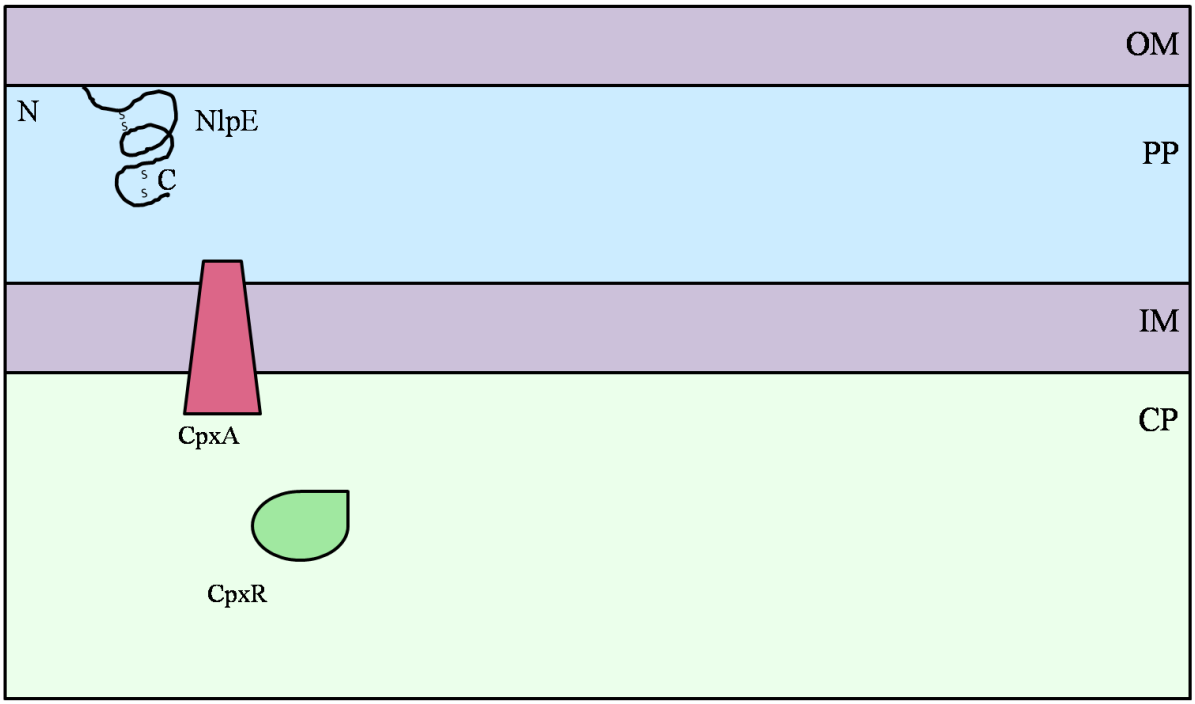
Figure 4: Proposed mechanism by which misfolded pilin proteins activate the Cpx pathway. A) Depicts the pathway in its inactivated state, and B) depicts the mode of activation of the pathway in the presence of misfolded pilin subunits.

1.3.3.3 Overexpression of the outer membrane lipoprotein NlpE

NlpE is an outer membrane lipoprotein with a lipid anchor at its N-terminal cysteine which helps it attach to the outer membrane [21]. It takes on the role of a sensor in multiple processes like surface adhesion, disulfide bond formation and lipoprotein biogenesis [22]. In light of the Cpx response, many studies have overproduced NlpE as a means of activating the Cpx pathway. When expressed at high levels, a portion of NlpE produced fails to assemble or fold correctly which leads to envelope stress and activates the Cpx pathway. However, it was seen that no other lipoprotein was able to activate the Cpx pathway on overexpression and so it was concluded that NlpE might be directly interacting with some component of the Cpx pathway to cause its activation [23]. The Lol machinery is required to transport lipoproteins to the OM via a complex LolCDE, which extracts these proteins from the IM, LolA, a soluble chaperone that transports these proteins across the periplasm and a lipoprotein LolB

which inserts lipoproteins into the OM. Hence a mutant LolA protein will be unable to transport lipoproteins across the periplasm causing their accumulation at the IM. It was observed that the Cpx pathway could be activated even without the overproduction of NlpE ina *lolA*mutant strain, but Cpx activation was not observed in a *lolAnlpE* double mutant strain. The activation of the Cpx pathway by NlpE was observed to be due to the direct interaction of the N-terminal domain of NlpE with the periplasmic domain of CpxA. It was observed that the disruption of disulfide bonds at the C-terminal domain of NlpE(seen in strains devoid of DsbA, a periplasmic thiol disulfide oxido-reductase) activates the Cpx pathway possibly due to the disruption of the export of this protein to the OM. Hence, as shown in Figure 5, this causes NlpE to accumulate at the IM and directly interact with CpxA to turn on the cascade. A *ΔnlpEΔdsbA* strain was also seen to be devoid of Cpx activation hence proving that activation of the Cpx pathway in the absence of DsbA is NlpE dependent. Since the unfolding of NlpE in such a way that its N-terminus reaches out through the periplasm to CpxA at the inner membrane seems unlikely, it was proposed that the accumulation of NlpE at the inner membrane due to defects in lipoprotein trafficking is what allows the interaction of its N-terminus with CpxA. However, it is possible that there are other interacting partners of both NlpE and CpxA that are involved in signal activation [24].

A)



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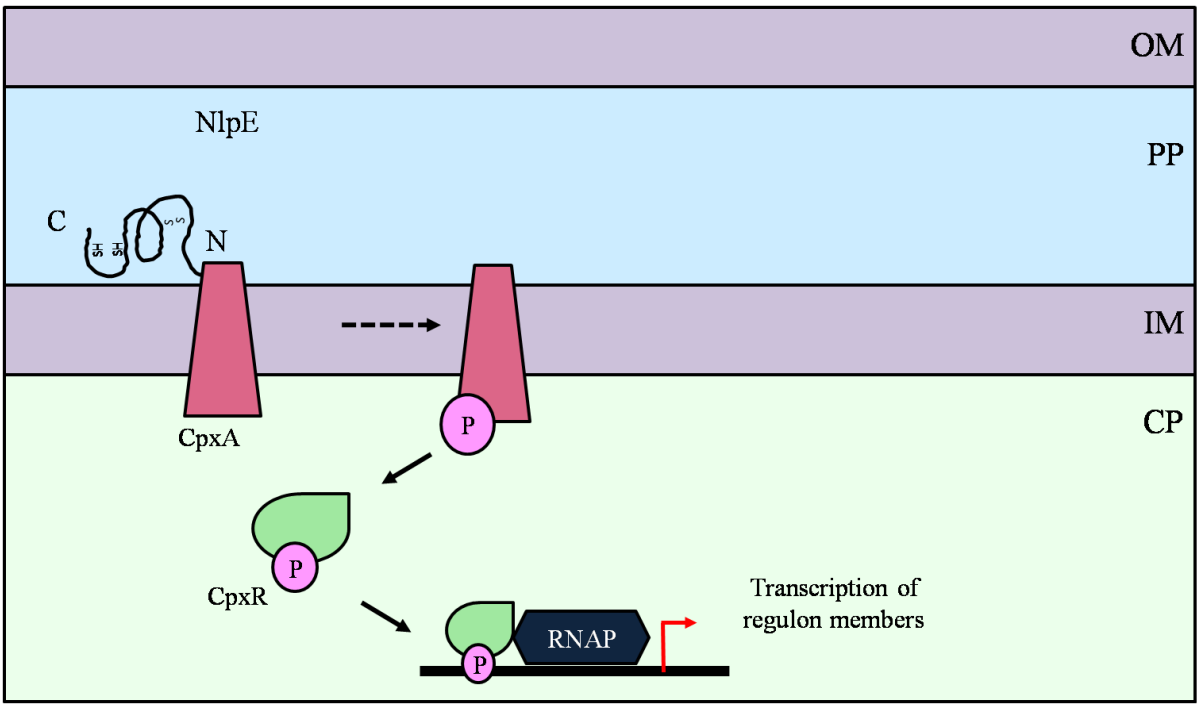

Figure 5: Proposed mechanism by which the outer membrane lipoprotein NlpE activates the Cpx pathway. A) Depicts the pathway in its inactive state with correctly localized NlpE,and B) depicts the pathway in its activated state due to the interaction of CpxA and NlpE. This mode of Cpx activation seems to be independent of CpxP.

1.4 Long-chain fatty acid metabolism and oxidative protein folding

Long-chain fatty acids (LCFAs) consist of aliphatic chains of 12-20 carbon atoms capped with a carboxylic acid group. Many groups of bacteria are able to use LCFAs as a carbon source, of which a good proportion are pathogenic [25]. The LCFA degradation pathway is mediated by *fad* genes, products of which are involved in the metabolism of these compounds through the β-oxidation pathway [26]. Since LCFAs are non-fermentable carbon sources, growth on this media requires optimal functioning of the electron transport chain (ETC) and the presence of a terminal electron acceptor, such as oxygen in case of aerobic metabolism. Published work from our lab has shown that LCFA degradation produces a large number of reduced cofactors which increases electron flow in the ETC [27].Besides its

central role in re-oxidation of the reduced cofactors produced as a consequence of metabolism, the ETC in *E. coli* is also involved in one of the crucial cellular processes, oxidative protein folding, that facilitates disulfide bond formation in several secreted proteins and takes place in the oxidizing environment of the cell-envelope.

Disulfide bond formation forms an important part of protein maturation and functionality, and in *E. coli*, it is mediated by a periplasmic oxido-reductase DsbA which catalyzes disulfide bond formation by taking up electrons from reduced sulfur groups in proteins. Reduced DsbA is then re-oxidised by inner-membrane bound oxido-reductase, DsbB, which finally shuttles electrons to ubiquinone, a lipid-soluble electron carrier in the ETC. Thus metabolism and oxidative protein folding converge at ubiquinone. Therefore, any metabolic condition that produces a large number of reduced cofactors and builds up a load on the ETC might hamper disulfide bond formation thereby altering the envelope redox homeostasis. Published work from our lab has indeed proven that growth in LCFAs represents one such condition where electron flow towards ETC from metabolism is high which compromises disulfide bond formation in the cell-envelope and shifts the periplasmic environment to a hypo-oxidising state. Interestingly, it was also shown that the Cpx pathway is majorly induced in response to this redox imbalance during LCFA metabolism. Moreover, it was seen that although the addition of ubiquinone to LCFA-utilizing cells ameliorated redox stress, as DsbA was found in its oxidized form, the Cpx response was only partially downregulated. This implies that the signal for Cpx activation might be both redox-dependent and redox-independent. Till date, the molecular signals and the underlying mechanism by the Cpx pathway gets activated during LCFA metabolism has not been elucidated. The known activating signals for Cpx activation cannot be the cue during LCFA metabolism due to the following observations: (i) the experiments used to investigate Cpx induction in LCFAs have been performed in buffered media, suggesting elevated pH could not be the inducing signal, (ii) a laboratory strain of *E. coli* (*E. coli* K-12) has been used which is devoid of the *pap* locus that codes for the pilin subunits hence eliminating misfolded pilin subunits as the cue for Cpx activation, and (iii) no significant difference in Cpx induction in the stationary phase was observed between a wild type and a *ΔnlpE* strain grown in LCFAs thereby eliminating NlpE as a prospective signal [28].

Hence, it is necessary to employ an unbiased systems approach (global approach) aimed at uncovering the mechanism of activation and the signals involved in Cpx induction during LCFA metabolism. Oleate is an eighteen-carbon fatty acid used as the representative LCFA throughout our studies. The global approach encompasses techniques like: (i) transposon mutagenesis wherein a strain containing a chromosomal reporter of the Cpx response, P_{cpxP}-*lacZ,* can be mutagenized with a transposome and the colonies obtained can be screened for downregulated Cpx response using the substrate X-gal, (ii) screening ordered gene deletion libraries whereby genes involved in Cpx induction can be comprehensively identified by transferring a P_{cpxP}-*lacZ* reporter fusion to the entire library (via conjugation) and pinning the conjugated library onto a plate containing X-gal, and(iii) a proteomics approach in which known sensors of the Cpx pathway, CpxA and CpxP, can be tagged and used for protein pulldown to identify their interacting partners. This study mainly focuses on the proteomics approach for identifying Cpx-activating signals in LCFA-grown cells although an initial standardization of bacterial conjugation required for screening the gene deletion libraries was also carried out.

Chapter 2

Materials and Methods

2.1 Materials

2.1.1 Chemicals, reagents and their sources

L-arabinose, Bacteriological Agar, Tryptone and Yeast extract were provided by BD Difco^{TM}. Primers were ordered from GCC BIOTECH. 2-mercaptoethanol (β-ME), 2-ONPG, antibiotics,Brij-58, $CaCl_2$, EDTA, glycerol, IPTG, KCl, KH_2PO_4, K_2HPO_4, $MgCl_2$, $MgSO_4.7H_2O$, NaCl, Na_2HPO_4, $NaH_2PO_4.H_2O$, NH_4Cl, sodium citrate, sodium oleate and TEMED were purchased from Sigma. Glucose was purchased from Fisher scientific. Agarose, EtBr, tris base, sodium do-decyl sulfate, ammonium persulfate and Coomassie brilliant blue were purchased from Affymetrix USB. Ethanol, methanol, M2 anti-FLAG primary antibody (raised in mouse) and anti-mouse HRP secondary antibody (raised in goat) were purchased from Merck. Calmodulin resin was purchased from Agilent.

Enzymes (CIP, Taq/Phusion DNA polymerase, restriction enzymes and T4 DNA ligase), Quick load DNA ladder, 100 bp/1Kb DNA ladder, buffers and dNTPs were purchased from New England Biolabs. Plasmid miniprep kit and PCR purification kit were purchased from Promega.

2.1.2 Bacterial strains

Table 1: List of strains used in this study

Strain	Genotype	Source/Reference
WT BW25113	*lacI*^{q} *,rnBT14,ΔlacZWJ16, hsdR514,ΔaraBADAH33, ΔrhaBADLD78*	*E. coli* Genetic Stock Centre
BW25113*ΔcpxR*	BW25113 *cpxR::kan*, Kan^{r}	Keio Collection [29]
DH5α	*F*$^{-}$ *Δ(argF-lac)169, ϕ80dlacZ58(M15) glnX44(AS) λ*$^{-}$ *rfbC1 gyrA96(Nal*^{r}*) recA1 endA1 spoT1 thiE1 hsdR17*	[30]
JA200	*F+, araC14, leuB6, lacY1, glnX44, galK2, galT22, λ*$^{-}$*ΔtrpE63, recA56, xylA5, mtl-1, thiE1*	[31]
SEA4166	MG1655 ΔlacX74λ RS88 (P_{cpxP}-*lacZ*)	Ades Lab
CAG53719	BW25113 *marB-SPA*, Kan^{r}	Gross Lab
RC20002	BW25113*cpxA-SPA*, Kan^{r}	This work
RC20003	P1 (BW25113*cpxA-SPA*) x BW25113	This work
RC20008	BW25113 *cpxP-SPA*, Kan^{r}	This work
RC20004	P1 (BW25113 *cpxP-SPA*) x BW25113	This work
RC20006	P1 (BW25113 *cpxA-SPA*) x SEA4166	This work
RC20007	P1 (BW25113 *cpxP-SPA*) x SEA4166	This work

2.1.3 Plasmids

Table 2: List of plasmids used in this study

Plasmid Name	Description	Reference
pCB112	Mobile plasmid, encoding *lacZ*,Cam^{r}, derived from pACYC184	[32]

pKD46	pSC101*oriaraC repA101*(Ts) P_{araBAD}-λred, Amp^r	[33]
pRC10	pBR322 *ori*, −10 box of P*trc* changed to P*lac* in pTrc99a, ΔNcoIAmp^r	[34]
pAN2	*cpxP-SPA* cloned into pRC10(inEcoRI and BamHI), Amp^r	This work

All plasmids have been transformed into and stored in DH5α.

2.1.4 Primers

Table 3: List of primers used in this study

Primer	Sequence(5'-3')	Purpose
AN01	CCGCTGGGCGGTTTACGGCTGGTGATTTGGTTG CCGCTGTATAAGCGGAGTTCCATGGAAAAGAG AAGATGG	Forward Primer: for SPA tagging *cpxA* at the C-terminus
AN02	CAAATGCCGGATGCGGCGTAAACGCCTTATCC TGCCTGCAAATGCGAAGTCATATGAATATCCTC CTTAG	Reverse Primer: for SPA tagging *cpxA* at the C-terminus
AN03	CTGGCGATTGTTGAAACCG	Forward Primer: for confirmation of *cpxA* SPA tagging at the C-terminus
AN04	CGGAGTGTAGGCCTGATAAGAC	Reverse Primer: for confirmation of *cpxA*-SPA tagging at the C-terminus
AN05	GCAAAAAAGTTCATCGTTGAAGCTATTGAGTA GTAGCAACTCACGTTCCCAGTCCATGGAAAAG AGAAGATGG	Forward Primer: for SPA tagging *cpxP* at the C-terminus
AN06	CATGTGGGGGAAGACAGGGATGGTGTCTATGG CAAGGAAAACAGGGTTTACATATGAATATCCT CCTTAG	Reverse Primer: for SPA tagging *cpxP* at the C-terminus
AN07	GAACTGGAGACAATGCATCGC	Forward Primer: for confirmation of *cpxP*-

		SPA tagging at the C-terminus
AN08	GCAGCAGGCAAATTGAGGAT	Reverse Primer: for confirmation of *cpxP* SPA tagging at the C-terminus
BSC1	AGAAGTTGTCCATATTGGCC	Forward Primer: for confirmation of chloramphenicol cassette
BSC2	CCTACCTGTGACGGAAGATC	Reverse Primer: for confirmation of chloramphenicol cassette
BS25	GCTGTGGTATGGCTGTGCAGG	Forward Primer: for confirmation of cloning in MCS of pRC10
BS26	GCCAGGCAAATTCTGTTTTATCAG	Reverse Primer: for confirmation of cloning in MCS of pRC10
DR015	ACCGGAATTCGAAGGAGATATACATATGCGCATAGTTACCGCTGCC	Forward Primer: for cloning *cpxP-SPA* into pRC10
DR016	CGTGGATCCCTACTTGTCATCGTCATCCTTGTAGTCG	Reverse Primer for cloning *cpxP-SPA* into pRC10
MS23	ATTCAGGCTGCAAACATGCG	Forward Primer: for confirmation of *ΔcpxR*
MS24	CACATTAAATCGTTGGGCGG	Reverse Primer: for confirmation of *ΔcpxR*
SAK1C	GAGGCTATTCGGCTATGACTG	Forward Primer: for confirmation of kanamycin cassette
SAK2C	TTCCATCCGAGTACGTGCTC	Reverse Primer: for confirmation of kanamycin cassette

2.1.5 Antibiotics

Ampicillin and kanamycin stocks as given in table 4 were made in autoclaved MilliQ water whereas chloramphenicol was dissolved in 70% ethanol. These were then filter-sterilized using 0.22 micron filters (Millipore) aseptically. Aliquots of 500µl were made and stored at -20°C.

Table 4: List of antibiotics and their concentrations

Antibiotic	Stock Concentration	Working Concentration
Ampicillin	100 mg/ml	100 µg/ml
Chloramphenicol	20 mg/ml	20 µg/ml
Kanamycin	30 mg/ml	30 µg/ml

2.1.6 LB media composition

2.1.6.1 LB media

The components were dissolved in 1 liter of MilliQ water and autoclaved at 15 lb/inch^2 pressure and 121°C temperature for 15 minutes.

Table 5: Composition of LB media

Composition	Amount (g/l)
Tryptone	10
Yeast extract	5
Sodium chloride (NaCl)	5

2.1.6.2 LB-agar

LB agar was prepared by adding 1.5% (w/v) agar to LB media followed by autoclaving. LB agar plates were prepared by cooling the molten LB agar media to about 50°C (such that one can touch it without scalding) and pouring into sterile petriplates (Tarsons).

Whenever required the desired antibiotics were added into the molten agar media cooled to about 50°C before pouring into petriplates. Plates were stored at 4°C till use. Whenever

required, sodium citrate (1M stock prepared in MilliQ water followed by autoclaving) was added to a final concentration of 10 mM before pouring plates.

2.1.7 Tryptone broth K (TBK) media

The media components were dissolved in MilliQ water and the volume was made up to 1 liter to obtain 2XTBK media such that it could be diluted to 1X when preparing final media. The media was then autoclaved.

Table 6: Composition of TBK media

Composition	Amount (g/l)
Tryptone	20
Potassium chloride (KCl)	10

2.1.8 Super optimal broth (SOB) media

The components were dissolved in MilliQ water and then made up to 1 liter. This solution was then autoclaved.

Table 7: Composition of SOB media

Composition	Amount (g/l)
Tryptone	20
Yeast extract	5
NaCl	0.585
KCl	1.85

To 1 ml SOB, 20 µl of 1 M Glucose and 20 µl of 1 M $MgCl_2$ can be added to make 1 ml SOC.

2.1.9 5% Brij-58

10 g of Brij-58 was added to 140 ml of MilliQ water and was dissolved using a magnetic stirrer at ~40°C. The volume was made up to 200 ml and autoclaved.

2.1.10 10X Phosphate buffer

61.5 ml of K_2HPO_4 (1M made in MilliQ water and autoclaved) and 38.5 ml of KH_2PO_4 (1M made in MilliQ water and autoclaved) were added together to make 100 ml of 10X Phosphate buffer.

2.1.11 Preparation of Sodium oleate (Na-Oleate)

50mM Na-Oleate was prepared by dissolving 760 mg Na-Oleate in 50 ml autoclaved 5% Brij-58. The compound was allowed to dissolve completely at room temperature, filter-sterilized and stored at -20°C.

2.1.12 Composition of TBK media supplemented with different carbon sources

Table 8: Composition of TBK media with different carbon sources

Components	TBK-Brij (15 ml)	TBK-Oleate (15 ml)
Autoclaved MilliQ-Water	4.5 ml	4.5 ml
2XTB media	7.5 ml	7.5 ml
10X Phosphate buffer	1.5 ml	1.5 ml
5% Brij-58	1.5 ml	-
50mM Na-Oleate	-	1.5 ml

TBK-Brij is referred to as -Oleate and TBK-Oleate as +Oleate throughout the text.

2.1.13 Composition of buffers and solutions used in agarose gel electrophoresis

2.1.13.1 50X TAE (stock)

All components were dissolved in 800 ml MilliQ water and pH of the buffer was adjusted to 8.0, (usually already 8.0) and volume was made up to 1 liter with MilliQ water. 50X TAE stock was stored at room temperature and diluted to 1X with MilliQ water before use.

Table 9: Composition of 50X TAE

Composition	Amount (g/l)
Tris base	242
Glacial Acetic Acid	57.1 ml

TetrasodiumEDTA (USB, final concentration 0.5 M)	146

2.1.13.2 6X DNA Loading buffer (stock)

The 6X DNA loading buffer stock was stored in aliquots of 1 ml at 4ºC till use.

Table 10: Composition of 6X DNA loading buffer

Composition	Amount (50 ml)
Bromophenol blue	125 mg
Sucrose	20 g

2.1.14 Composition of buffers and solutions used in SDS-PAGE

Stacking gel

Table 11: Composition of stacking gel

Components	Amount (ml)
Autoclaved MilliQ	1.4
Tris-Cl (pH 6.8)	0.63
30% Bis-acrylamide	0.415
10% SDS	0.025
10% APS	0.03
TEMED	0.004

This composition is used to make one gel of 15%. The components were added to and mixed in a 50 ml falcon tube. The pH of Tris-Cl was adjusted using conc. HCl. Bis-acrylamide was

made in the ratio 29:1 acrylamide: bisacrylamide and components were weighed in the fumehood.

2.1.14.2 Resolving gel

Table 12: Composition of resolving gel of various percentages

Components	10% gel (ml)	15% gel (ml)
Autoclaved MilliQ	1.9	1.1
Tris-Cl (pH 8.8)	1.3	1.3
30% Bis-acrylamide	1.7	2.5
10% SDS	0.05	0.05
10% APS	0.05	0.05
TEMED	0.005	0.005

The components were added to and mixed in a 50 ml falcon tube. The pH of Tris-Cl was adjusted using conc. HCl. Bis-acrylamide was made in the ratio 29:1 acrylamide: bisacrylamide and components were weighed in the fumehood.

2.1.14.3 5X Loading dye

Table 13: Composition of 5X SDS loading dye

Components	Amount (for 10 ml)
1M Tris (pH 6.8)	2.5 ml
SDS	1 g
Glycerol	5 ml
1 M DTT	154.25 mg

Bromophenol blue	10 mg

Bromophenol blue was added once all other components were dissolved in a measuring cylinder on a magnetic stirrer. The volume was made up with MilliQ water. 1ml aliquots of the dye were made and stored at -20˚C.

2.1.14.4 10X SDS running buffer

Table 14: Composition of 10X SDS running buffer

Components	Amount (g/l)
Tris base	30.2
Glycine	144
SDS	10

SDS was weighed inside the fumehood. The components were dissolved in MilliQ water and made up to 1 liter.

2.1.15 Composition of buffers and solutions used for Coomassie staining

Table 15: Composition of staining solution and destaining solution

Components	Staining solution	Destaining solution
Glacial Acetic Acid	10% (v/v)	10% (v/v)
Methanol	40% (v/v)	40% (v/v)
Coomassie Brilliant Blue	0.1% (w/v)	-

MilliQ water	50% (v/v)	50% (v/v)

2.1.16 Composition of buffers and solutions used for Western blot

2.1.16.1 Transfer buffer

14.4 g of Glycine and 5 g of Tris Base were dissolved in 500 ml MilliQ water after which 200 ml Methanol was added. Finally the volume was made up to 1 liter using MilliQ water.

2.1.16.2 10X TBS

Table 16: Composition of 10X TBS

Components	Amount (in grams for 400 ml)
Tris base	9.68
NaCl	32

The pH was adjusted to 7.6 and the volume was made up to 400 ml using MilliQ water.

1X TBS was made from this and 0.1% Tween-20 was added to make 1X TBST.

2.1.16.3 Blocking buffer

0.1% (w/v) of Skim Milk was added to 1X TBST to prepare blocking buffer.

2.1.16.4 Primary antibody preparation

2.5 µl of primary antibody was added to 10 ml of 1X TBST and stored in 4˚C.

2.1.16.5 Secondary antibody preparation

2 µl of secondary antibody was added to 10 ml of 1X TBST and stored in 4˚C.

2.1.17 Composition of buffers and solutions used in β-galactosidase assays

2.1.17.1 Substrate

Ortho-nitrophenyl-β-D-galacto-pyranoside (ONPG) is used as the substrate for this assay. 4 mg of ONPG was dissolved in 1 ml of autoclaved MilliQ water. The substrate is prepared fresh for every assay.

2.1.17.2 Z-buffer

The components were dissolved in 1liter of autoclaved MilliQ water and the pH was then adjusted to 7 with 1 M NaOH.

Table 17: Composition of Z-buffer

Composition	Amount (g/l)
Na_2HPO_4 (anhydrous)	8.52
$NaH_2PO_4.H_2O$	5.5
NaCl/ KCl	0.75
$MgSO_4.7H_2O$	0.246

2.1.17.3 0.1% SDS

0.1 g of sodium do-decyl sulphate (SDS) was dissolved in 100 ml of autoclaved MilliQ water and stored at room temperature.

2.1.17.4 Stop solution

5.3 g of Na_2CO_3 was dissolved in 50 ml of autoclaved MilliQ water to make 1M Na_2CO_3 solution and stored at room temperature.

2.1.18 Composition of buffers and solutions used for protein pulldown

The buffers used for protein pulldown were modified from [35] and [36].

2.1.18.1 Resuspension buffer

Table 18: Composition of resuspension buffer for protein pulldown

Components	Working concentration	Volume (for 50 ml)
1 M Tris (pH 7.5)	20 mM	1 ml
Glycerol (50%)	20%	20 ml
Autoclaved MilliQ water	-	Make up

2.1.18.2 TNG buffer

Table 19: Composition of TNG buffer for protein pulldown

Components	Working concentration	Volume (for 50 ml)
1 M Tris (pH 7.5)	20 mM	1 ml
Glycerol (50%)	10%	10 ml
1 M NaCl	10 mM	5 ml

Autoclaved MilliQ water	-	Make up

2.1.18.3 Lysis buffer

Table 20: Composition of lysis buffer for protein pulldown

Components	Working concentration	Volume (for 50 ml)
1 M Tris (pH 7.5)	20 mM	1 ml
Glycerol (50%)	10%	10 ml
1 M NaCl	10 mM	5 ml
100 mM PMSF	1 mM	0.5 ml
Triton X-100	1%	0.5 ml
Autoclaved MilliQ water	-	Make up

2.1.18.4 Wash buffer

To TNG buffer, add 5 mM β-ME and 1% Triton X-100 to make wash buffer.

2.1.18.5 Elution buffer

To TNG buffer, add 5 mM β-ME, 2.5% SDS and 4 mM EDTA to make elution buffer.

2.2 Methods

2.2.1 Preparation of glycerol stocks

The required strain was inoculated in LB and allowed to grow at the appropriate temperature overnight. Antibiotic was added if necessary. In a labeled, sterile cryotube, 700 µl of this overnight culture was added along with 300µl of autoclaved 50% glycerol. The cryotube was immediately stored at -80°C.

2.2.2 P1 lysate preparation

P1 lysate was prepared according to the Cold Spring Harbor Protocol with minor modifications. The desired strain was inoculated in LB and grown at 37°C overnight. This overnight culture was sub-cultured in 1:100 ratio in a 15 ml falcon tube containing 2 ml LB and 5mM $CaCl_2$ for 1 hour 30 minutes. 20 µl of P1 lysate was added and lysis was allowed to proceed for 3-4 hours. 50 µl chloroform was added and the falcon was vortexed for 30 seconds. Debris was pelleted by centrifuging at 8000 rpm for 10 minutes. 1.5 ml of clear lysate was transferred in a fresh 15 ml falcon containing 50 µl of chloroform. The lysate was stored at 4°C, until further use.

2.2.3 P1 transduction

P1 transduction was carried out according to the procedure mentioned in [37] with slight modifications. 1 ml of overnight culture was pelleted and resuspended in 1 ml of P1 salt solution containing 985 µl of autoclaved MilliQ water, 10 µl of 1 M $CaCl_2$ and 5 µl of 1M $MgSO_4$. 100 µl of re-suspended cells were transferred into micro-centrifuge tubes containing 0 µl (control), 60 µl and 120 µl of P1 lysate and incubated at 37°C for 30 minutes in a water bath. 1 ml LB containing Na citrate (10 mM) was added to the samples and incubated at

37°C for 1 hour in a water bath. After incubation, cells were washed with 1 ml LB two times and then dissolved in 100 µl LB. The cell suspension was spread on LB plates supplemented with Na citrate (10 mM) and an appropriate antibiotic. The plates were incubated at 30°C for 16-18 hours. 60 µl of P1 lysate was also spread on plates as control. Transductant colonies obtained were re-streaked twice on LB agar plates supplemented with appropriate antibiotic and 10 mM Na citrate followed by incubation at 37°C. For every parent strain, two transductants were cultured and preserved as glycerol stocks.

2.2.4 Chromosomal tagging

2.2.4.1 PCR amplification

The tag was amplified from a previously tagged strain. Colonies of the tagged strain were dissolved in 20 µl of NFW and 5 µl was taken from this for a 50µl reaction. 200 µl was set up as 50 µl reactions per tube for amplification.

Table 21: Composition of PCR mix for amplification

Components	Working concentration	Volume for 10 µl reaction
Phusion buffer (5X)	1X	2 µl
$MgCl_2$ (50 mM)	1 mM	0.2 µl
dNTPs (10 mM)	0.2 mM	0.2 µl
Phusion DNA polymerase	2.5 units/µl	0.1 µl
Forward Primer	0.25 µM	0.5 µl
Reverse Primer	0.25 µM	0.5 µl
DNA Template	-	1 µl
Nuclease Free Water (NFW)	-	5.5 µl

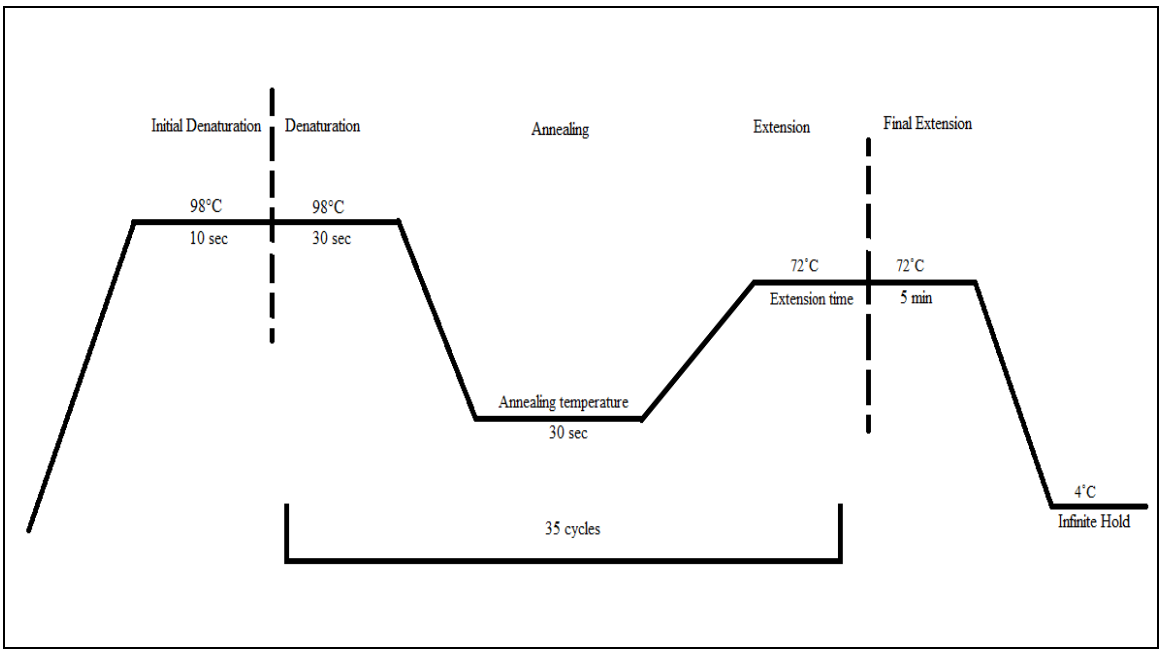

Figure 6: PCR program for the amplification of a gene

Figure 6 depicts the PCR program set in the cycler. The annealing temperature is dependent on the primer pair in use (2-5˚C below their melting temperature) and the extension time is dependent on the size of the PCR product (taking into account that the extension time for Phusion polymerase is 1 kb/30sec).

2.2.4.2 Preparation of electrocompetent *E. coli* containing pKD46 for homologous recombination

The required strain transformed with pKD46 was cultured overnight in LB, with antibiotic if necessary, at 30˚C. A secondary inoculation was set up in 50 ml SOB with 10 mM of $MgCl_2$, 10 mM $MgSO_4$ and 10mM arabinose with initial OD_{600} ~ 0.01 and was grown at 30˚C at 220 rpm till OD_{600} reached 0.5. The culture was chilled on ice for 10-15 minutes and pelleted at 4000 rpm for 10 minutes at 4°C in a 50 ml falcon tube. Supernatant was discarded and the pellet was dissolved in equal volume of pre-chilled 10% glycerol only via tapping, and centrifuged again at 4000 rpm for 10 minutes at 4°C. Similar wash with pre-chilled 10% glycerol was given but with half the volume from the previous step. The next wash too was

given with pre-chilled 10% glycerol using half the volume as compared to the previous step. A final wash was given with the same volume as the previous step with pre-chilled 10% glycerol in a 15 ml falcon. The pellet was re-suspended in 600 µl of 10% glycerol and 60 µl aliquots of the same were made in pre-chilled MCTs. Aliquots were stored at -80°C till use.

2.2.4.3 Homologous recombination using pKD46

The amplified PCR product was PCR purified and then digested using DpnI. This digested product was then run on a preparatory agarose gel and then gel purified. The electro-cuvette was washed thoroughly with distilled water and 100% ethanol such that no salt is left. ~100 ng of the gel purified PCR product was added into 60 µl of pKD46 containing electrocompetent cells and then added into the electro-cuvette of 1 mm width gap. A 1.8 kV pulse was made to pass through the electrocuvette and 1 ml of SOC was added to the cuvette instantly. The culture was transferred to a sterile MCT and incubated at 37ºC for 1 hour in a roller drum. 500 µl of the culture was plated on an LB plate containing the required antibiotic and incubated at 37ºCfor 16-18 hours. The remaining culture was left at room temperature for 24 hours and plated on an LB plate containing the required antibiotic and incubated at 37ºC for 16-18 hours. The colonies were then checked for recombination using colony PCR.

2.2.5 Plasmid isolation

Plasmid isolation was performed using the Promega mini prep kit. Strains containing plasmid of interest were inoculated in 5 ml LB media containing appropriate antibiotic and grown at the required temperature in a roller drum for 14-16 hours. Cells were pelleted by centrifugation at 6000 rpm for 5 minutes. The Miniprep DNA Purification System kit (Promega) was used to isolate plasmid DNA according to manufacturer's instructions.

2.2.6 Gel and PCR purification

PCR products were run on an agarose gel and extracted or purified directly using the Promega Gel and PCR purification kit. In case of gels, the weight of the gel was measured to add equal volume of Membrane binding solution so as to melt the gel at 55°C before proceeding with the rest of the protocol. The product was then purified according to the manufacturer's instructions.

2.2.7 Cloning a gene onto a plasmid

The gene of interest was amplified using primers with restriction sites at the end and was purified using the PCR clean-up system. The purified product and the isolated plasmid (vector) were digested separately using the restriction enzymes at 37˚C for 3 hours. The vector was also treated with 2 µl of CIP (calf-intestincal alkaline phosphatase) and incubated at 37˚C for 2 hours. The digested products were then run on 1% preparatory agarose gel (fresh gel and buffer) and the specific bands were cut out from the gel and purified. The ligation reaction was set up at 25˚C for 2 hours and the ligated product was then transformed into electrocompetent or chemically competent cells of our strain of interest and plated on LB plates with the required antibiotic. The colonies obtained were then screened for the presence of the cloned plasmid.

Table 22: Reaction mix for restriction digestion

Components	For Vector (50 µl)	For Insert (50 µl)
10X NEB buffer 4	5	5
Enzyme 1	1.5	1
Enzyme 2	1.5	1

Template	Volume for 3000 ng	Volume for 1500 ng
Autoclaved MilliQ	Make up	Make up

Table 23: Reaction mix for ligation

Components	For Test Reaction (20μl)	For Self Reaction (10 μl)
10X NEB DNA ligase buffer	2	1
Vector	Volume for 100 ng	1
Insert	Volume for ~50 ng	-
T4 DNA ligase	2	0.5
Autoclaved MilliQ	Make up	Make up

2 μl of each was added to another MCT before adding the enzyme and it was labeled as Pre-Test and Pre-Self.

2.2.8 Preparation of electrocompetent *E. coli*

The required strain was cultured overnight in LB, with antibiotic if necessary, at the appropriate temperature. A secondary inoculation was set up with initial OD_{600} ~ 0.01 and grown at the required temperature at 220 rpm till OD_{600} reached 0.5. The culture was chilled on ice for 10-15 minutes and pelleted at 6000 rpm for 10 minutes at 4°C in a 50 ml falcon tube. Supernatant was discarded and the pellet was dissolved in equal volume of pre-chilled autoclaved MilliQ water and centrifuged again at 6000 rpm for 10 minutes at 4°C. Similar

wash with pre-chilled autoclaved MilliQ water was given but with half the volume from the previous step. The next wash was given (using same volume as previous step) with pre-chilled 10% glycerol. A final wash was given with half the volume from the previous step with pre-chilled 10% glycerol in a 15 ml falcon. The pellet was re-suspended in 600 µl of 10% glycerol and 60µl aliquots of the same were made in pre-chilled MCTs. Aliquots were stored at -80°C till use.

2.2.9 Transformation by electroporation

The electro-cuvette was washed thoroughly with distilled water and 100% ethanol such that no salt is left. 70-100 ng of plasmid was added in 60 µl of electrocompetent cells and then added into the electro-cuvette of 1 mm width gap. A 1.8 kV pulse was made to pass through the electrocuvette and 1 ml of LB was added to the cuvette instantly. The culture was transferred to a sterile MCT and incubated at 37ºC for 60 minutes in a roller drum. The culture was centrifuged at 4000 rpm for 2 min and the pellet was resuspended in 100 µl of LB. It was spread on LB plates containing the appropriate antibiotics. The plates were incubated at 37ºC for 16-18 hours.

2.2.10 Transformation by the heat shock method

Chemically competent cells were taken from -80˚C and kept on ice for 20 mins or till the cells thawed. Required volume of plasmid or ligation product was added to the competent cells (if ligation product was added then equal volume of T3 buffer was also added. T3 buffer contains 100 mM $CaCl_2$ and 50 mM $MgCl_2$ and is used when transforming ligation mixtures directly so as to improve transformation efficiency). This mixture was left on ice for 30 mins. The water bath was set to 42˚C and the MCTs were kept in it for 45 seconds after which they were immediately dunked in ice and kept there for 5 mins. 1 ml LB was added to the mixture

and kept in 37˚C roller drum for 1 hour. The cells were then centrifuged at 6000 rpm for 5 mins and 900 µl of the supernatant was discarded. The pellet was dissolved in the remaining supernatant and plated on an LB plate that contained the required antibiotic. The plate was kept for overnight incubation at the appropriate temperature.

2.2.11 Bacterial conjugation

The desired donor strain containing the mobile plasmid and the recipient strain were inoculated in 3 ml of LB each with the required antibiotic and allowed to grow overnight at 37˚C. The OD_{600} of both cultures were checked with 10X dilution. 100 µl of the donor strain culture was spread as a lawn on an LB plate (without any antibiotic). 1 ml of the recipient culture was made at 0.5 OD_{600} by taking culture volume corresponding to 0.5 OD_{600} and making up the volume to 1 ml using LB. 6 10X serial dilutions of this 0.5 OD_{600} culture were made in MCTs by taking 100 µl from the first culture and adding it to 900µl of LB and using 100 µl of this culture for the next dilution. 10 µl from each of the serial dilutions and the undiluted 0.5 OD_{600} culture were spotted onto the LB plate spread with the donor (liquid to solid). In another marked out section of the same plate, one colony of the recipient was picked up and spotted on scraped onto the donor lawn. This plate was then incubated for 13 hours at 37˚C for mating.

Colonies from these mated plates were picked up and streaked onto sections of an LB-Cam-Kan plate and allowed to grow for 9-12 hours at 37˚C. If single colonies were not formed then the growths were streaked out again onto LB-Cam-Kan plates so as to obtain single colonies which can then be checked for successful conjugation using colony PCR.

2.2.12 Colony PCR to confirm transductants or transformants

Table 24: Composition of PCR mix for confirmation

Components	Working concentration	Volume for 10 µl reaction
Taq buffer (10X)	1X	1 µl
$MgCl_2$ (50 mM)	1.5 mM	0.3 µl
dNTPs (10 mM)	0.2 mM	0.2 µl
Taq DNA polymerase	1 - 2.5 units/µl	0.05 µl
Forward Primer	0.25 µM	0.5 µl
Reverse Primer	0.25 µM	0.5 µl
DNA Template	-	1 µl
Nuclease Free Water (NFW)	-	6.95 µl

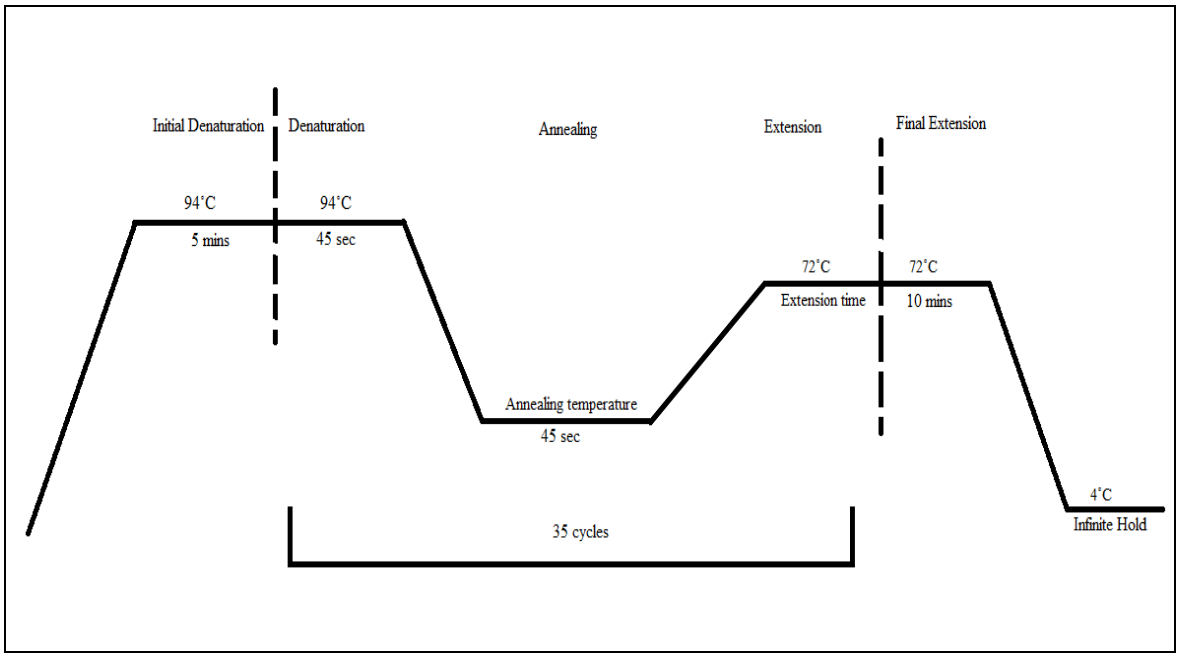

Figure 7: PCR program for the confirmation of transductants and transformants

The colony is dissolved in 20 µl of NFW and 1 µl from this is taken for a 10 µl reaction.

Figure 7 depicts the PCR program set in the cycler. The annealing temperature is dependent on the primer pair in use (2-5˚C below their melting temperature) and the extension time is dependent on the size of the PCR product (taking into account that the extension time for Taq polymerase is 1 kb/90 sec).

2.2.13 Agarose gel electrophoresis

Samples were mixed with 6X Bromophenol blue DNA loading dye (1X final concentration) and were loaded in 1 % (w/v) agarose gel in 1X TAE buffer. The gel was run at 120V till the dye front reaches three quarters of the gel and was observed using UV trans-illuminator or Gel documentation system from BioRad.

2.2.14 β -galactosidaseassay

A single colony of desired culture for which the assay is to be performed was inoculated in 3 ml of LB a day before the assay (primary culture) and incubated at 37ºC for 10-12 hours under shaking. The secondary culture inoculations were done in 125 ml flasks with initial OD_{600} as 0.01 in 15 ml TBK media with desired carbon sources and incubated at 37ºC till the desired phase at which assay was to be performed was reached. OD_{600} of the culture was recorded and the samples were harvested in 1.5 ml MCT at 6,000 rpm for 5 minutes. The cells are then washed 3 times using Z-buffer prepared. The washings were done to get rid of Brij-58 interference. After washing, the culture was normalized to OD_{450} of ~ 0.5 using Z-buffer. The OD_{450} of the normalized culture was again recorded. The following reaction for the assay was set up in test tubes at 30ºC. 2-mercaptoethanol (β-ME) was added to the Z-buffer. The other reagents were added using dropper.

The test tube with the reaction mix was vortexed and then kept at 30ºC for 15 minutes. 200 µl of ONPG was added to each reaction mix and the time of addition was recorded. The stop solution (500 µl) was added when the yellow color develops and this time was recorded as

well. The stopped reaction was then transferred to MCTs and centrifuged twice at maximum speed for 10 minutes. OD_{420} and OD_{550} of the supernatant were recorded and miller units were calculated using the formula:-

$$1 \text{ Miller Unit (M.U.)} = \frac{1000 \times OD_{420} - (1.75 \times OD_{550})}{t \times v \times OD_{450 \text{ normalised}}}$$

Table 25: Reaction composition for the β-galactosidase assay

Composition	Amount
Z- buffer + β-ME (2.7 µl/ml)	500 µl
0.1% SDS	1 drop
Chloroform	2 drops
Sample ($OD_{450 \text{ normalised}}$)	500 µl

2.2.15 Inducing expression of protein from gene cloned on a plasmid

Overnight cultures were inoculated into 16 ml of fresh media with the required antibiotic and grown at 37˚C at 220 rpm. Different final concentrations of IPTG were added (from a 1M stock) 3 hours after inoculation (T1 time point corresponding to the exponential phase, see Results and Discussion) to induce expression of the gene from the plasmid. Volumes corresponding to 0.5 OD_{600} of the cultures were taken at different phases of growth from induced as well as uninduced samples and prepared for SDS-PAGE.

In some experiments, IPTG was added 2-3 hours before the required growth stage at which cultures were harvested.

2.2.16 Protein pulldown protocol

The tagged strains of interest were inoculated in 3 ml of LB (with antibiotic if necessary) and allowed to grow overnight at 37˚C. This overnight culture was then inoculated in a secondary culture of 100 ml with a starting OD_{600} of 0.01. The culture was allowed to grow at 37˚C till the required phase of growth (in this study T5 time point corresponding to the late stationary phase, see results and discussion). The culture was then spun down at 4650 g for 20 mins at 4˚C. 8 ml of resuspension buffer was added and the pellets were dissolved in it. The tubes were spun down at 3700 g for 20 mins at 4˚C. The supernatant was discarded and the pellets were dissolved in 8 ml of lysis buffer. The suspension was sonicated for 10 mins on ice at 12 Amp, 10 seconds On, 30 seconds Off. After 50% of the process was completed, the tubes were kept on ice for 5 mins before continuing. The sonicated samples were then spun down at 10000 rpm, 40 mins at 4˚C. The supernatant was transferred to another tube, to which $CaCl_2$and β-ME were added such that their final concentrations were 2mM and 5mM respectively. 60µl of the calmodulin beads were washed and equilibrated with equal volume of the lysis buffer with 5mM β-ME and 2mM $CaCl_2$. The beads were then resuspended in 60 µl of equilibration buffer so that 120 µl of beads and buffer solution could be added to the supernatant. All steps with the beads were performed with a cut 1 ml tip to avoid damaging the beads. The supernatant was then incubated with the beads overnight on ice at 30 rpm.

The following day, the ice was replaced and the beads were incubated with the supernatant for additional 30 mins at 30 rpm. The beads were then allowed to settle down and most of the flow through was discarded. The beads were washed with 1 ml of wash buffer, transferred to MCTs and allowed to settle for 5 mins. The MCTs were then spun at 1200 rpm for 2 mins and allowed to stand for 3 mins before the supernatant was discarded by pipetting. This was repeated three more times. The beads were then incubated with 150 µl of elution buffer for 15 mins at room temperature and then spun down at 1200 rpm for 5 mins. The supernatant was collected into another MCT. This was repeated thrice.

The samples were prepared for SDS-PAGE and the gel was stained with Coomassie post run to check for bands in the elutions. A Western blot of the samples was also performed.

2.2.17 SDS-PAGE

Optical density of the culture was measured at the required time point and volume of culture corresponding to 0.5 OD_{600} was taken into an MCT and spun down at 6000 rpm for 5 minutes. The supernatant was discarded and 37.5 µl of autoclaved MilliQ water, 20 µl of 5X loading dye and 5 µl of β-ME was added to it. This mixture was then spun down at 10,000 rpm for 10 seconds. The mixture was then kept at 95˚C for 10 mins after which it was spun down once again at 10,000 rpm for 10 seconds. The samples were then directly loaded onto the gel or stored at -20˚C (after which they were thawed and heated again before loading).

To make the gel, initially the notch plate and the glass plate (1 mm plates from Bio-Rad) were clamped and attached to the set up. The leakiness of this set up was tested using ethanol. The resolving gel was made accordingly in a falcon and added in between the glass plates. Ethanol was added to the brim immediately to ensure that the gel forms an even surface. This was allowed to set for 30-40 minutes. The ethanol was discarded after which the stacking gel was prepared in a falcon and poured on top of the resolving gel. A 10-well comb was then placed into the gel and allowed to set for 30 mins.

The glass plate was then removed from the clamps and placed in a cassette (Bio-Rad) along with a buffer dam/ dummy plate. The cassette was then placed in the tank and 1X SDS Running buffer (made from 10X SDS Running buffer) was added between the plates and in the tank (quarter volume). The comb was removed and the samples and 4 µl of the pre-stained ladder/ unstained ladder were added. The electrodes were fixed and the gel was run at 120mV till the dye front reached the bottom of the gel. The gel wasthen processed for Western blot or Coomassie staining.

2.2.18 Coomassie staining

The gel was transferred into a box containing the Coomassie staining solution and was allowed to stain for 45 minutes at 45 rpm on the orbital shaker. The staining solution was removed and MilliQ water was added for 30 mins at 45 rpm. The water was discarded and

destaining solution was added and kept at 45 rpm till the solution accumulated a dark blue hue. The destaining solution was changed till the bands were visible and the gel attained a light blue or white hue.

2.2.19 Western blot

2.2.19.1 Transfer

The gel was transferred to a set up containing a cassette, filter paper and foam base, all of which were soaked in Transfer buffer (Bio-Rad Mini Trans-Blot cell). The gel was placed on the black face. A membrane was placed over the gel to cover it and the second filter paper and foam was placed on top of it. The cassette was closed and placed in a gasket with the black side of the cassette facing the black side of the gasket. The gasket was then placed in a tank with an ice pack behind it. The tank was then filled with chilled Transfer buffer and the set up was placed in an ice bucket and filled with ice. The transfer was set up at 90 mV for 90 mins.

Post transfer the membrane was placed in a box containing 0.1% Ponceau stain and kept at 60 rpm for 5 mins. The stain was removed and MilliQ water was added and kept at 60 rpm till the background stain was removed. Once equal loading was ascertained, 1X TBST was used to remove the Ponceau staining. The blot was then soaked in 15 ml of 0.1% Blocking buffer and kept at 4˚C overnight.

2.2.19.2 Antibody incubation

The membrane with blocking buffer was kept on the orbital shaker at 20 rpm for 30 minutes after which it was given 3 1X TBST washes for 10 mins each at 60 rpm. The primary antibody was added to the membrane and was allowed to incubate for 1 hour 30 minutes at 20 rpm. 4 1X TBST washes were given for 10 mins each at 60 rpm. The blot was then incubated with the secondary antibody for 1 hour 30 minutes at 20 rpm. 4 1X TBST washes were given for 10 mins each at 60 rpm after which the blot was stored in 1X TBS.

2.2.19.3 Developing the blots

The development of the blot takes place in the dark room. The hypercassette was cleaned with MilliQ water and dried. An X ray film was cut in half and bent at a corner to distinguish the loading order post development. Any liquid in the box containing the blot was discarded and the ECL substrate (Pierce) was added to it (500 µl of Reagent 1 + 500 µl of Reagent 2 mixed in an MCT) and re-pipetted till the blot was soaked with the substrate. The blot was then placed in the plastic film, on top of which the second layer was smoothed over. The X-ray films were placed congruently over the blot and the cassette was closed and exposure was allowed from 1-5 minutes. The films were then passed through the developer vertically.

Chapter 3

Results and Discussion

Previous work from our lab has shown that *E. coli* grown in the presence of oleate (a representative LCFA) shows several hallmarks of insufficient disulfide bond formation suggestive of altered envelope redox homeostasis during LCFA metabolism. More importantly, the Cpx and σ^E pathways are activated in response to this LCFA-induced redox imbalance, It was also demonstrated that the Cpx system is the major ESR involved in combating the envelope stress induced during oleate metabolism[29]. Although there are many signals that have been proposed for Cpx activation in response to several known cues,, none of these are applicable to the specific condition during oleate metabolism. For all studies where Cpx activation in response to LCFA metabolism was observed, *E. coli* K-12 (non-pathogenic laboratory) strain was grown in buffered tryptone broth (TBK) media. Buffered media negated the effect of elevated pH as an activator. Moreover, *E. coli* K-12 is devoid of the *pap* locus which encodes for pilin proteins. Importantly, previous work from our lab also showed that the outer membrane lipoprotein NlpE, a well-known activator of the pathway, is also not the signal involved in Cpx activation during oleate metabolism [29]. For these reasons, this study was part of an attempt to discern what the probable activating signals for Cpx activation are during LCFA metabolism using a global approach. One such methodology entails the use of proteomics to elucidate the mechanism of activation. This study majorly focuses on attempting to uncover the signal by identifying the major interacting partners of the proteins CpxA and CpxP by tagging them using a sequential peptide affinity tag or SPA tag. Both of these proteins which are essential players in the Cpx pathway behave as sensors and hence tagging these proteins would allow one to perform a protein pulldown to obtain the proteins interacting with CpxA and CpxP during Cpx

induction and probably help elucidate the mechanisms involved in activating the pathway during LCFA metabolism. Another method by which the players involved in the activation of this pathway can be uncovered is via screening ordered single-gene deletion library of *E. coli* to find the genes involved, although we understand that this method presents a drawback in the form of an inability to detect essential genes involved in Cpx induction. Nevertheless, for this approach, the library will first need a P_{cpxP}-*lacZ* reporter fusion transferred to it, after which the genes involved in Cpx induction can be comprehensively identified by pinning the library containing the construct onto a plate containing X-gal and oleate and screening for white or light blue colonies, that is colonies with downregulated Cpx activity. One method by which the reporter construct can be transferred to the entire library *en* masse is via transfer of a mobile plasmid carrying the reporter construct through the process of bacterial conjugation. Hence this study also minorly focuses on standardizing the conjugation protocol that can be used for this approach.

3.1 SPA-Tagging of CpxA and CpxP

The translational tag used in this study is the sequential peptide affinity Tag (SPA tag) which contains both a calmodulin binding protein domain (CBP) and a 3X FLAG domain. This would assist in two step purification of the tagged protein to reduce the chances of non-specific binding of proteins. These domains are also interspersed by a tobacco etch virus (TEV) cleavage site, in case the 3X FLAG domain has to be removed. Additionally, the small size of the tag (8 kDa) also ensures that any effect on the functionality of the protein caused by the tag is minimized [37].

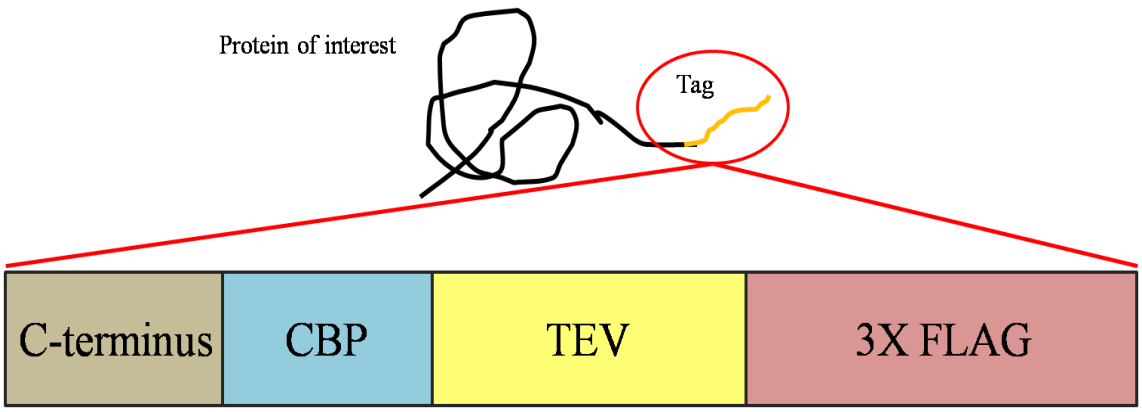

Figure 8: A schematic of the domains of the SPA tag. The SPA tag contains a calmodulin binding protein (CBP) domain, a tobacco etch virus (TEV) cleavage site and a 3X FLAG domain. The protein of interest is usually SPA tagged at the C-terminus.

Two important players in the Cpx pathway, CpxA and CpxP were tagged at their C-termini using the SPA tag. The primers were constructed such that the 5' end of the primer has the regions homologous to the site of insertion whereas the 3' end of the primer was complementary to the regions to the tag itself. The tags were amplified from a previously tagged strain using primers AN01 and AN02 for *cpxA* and AN05 and AN06 for *cpxP*. Post homologous recombination, the colonies were confirmed using colony PCR. The confirmed clones were then freshly transduced into a WT BW25113 background as well as in a chromosomal *lacZ* transcriptional reporter strain of P_{cpxP}-*lacZ* (MG1655 P_{cpxP}-*lacZ*)(strain routinely used to check for *cpxP* promoter activity in our lab which is a direct indicator of Cpx activation). The transductants were re-confirmed using colony PCR. In this reaction, a pair of gene specific primers (AN03 and AN04 in case of *cpxA* and AN07 and AN08 in case of *cpxP*), a pair of tag specific primers (specific to the kanamycin (Kan) cassette in the SPA tag, SAK1C and SAK2C) and a combination of these primers were used to compare the colony PCR profile of the transductants with that of the untagged strain to confirm that the tag was inserted into the required position. The fact that the gene specific primers produced a larger band in the tagged strains as compared to the untagged strain proves that our genes of interest have been successfully tagged. The presence of bands produced by the Kan specific primers in the transductants also confirms the same. The size of the bands corresponding to the junctions, that is where a combination of the gene specific and Kan specific primers were used, shows that the tag has been inserted in the correct location, that is adjacent to the gene such that it replaces the stop codon of the gene. Hence tagged strains were successfully constructed and subsequently confirmed. Henceforth, the strain containing tagged CpxA will be denoted as *cpxA-SPA* and the strain containing tagged CpxP as *cpxP-SPA*.

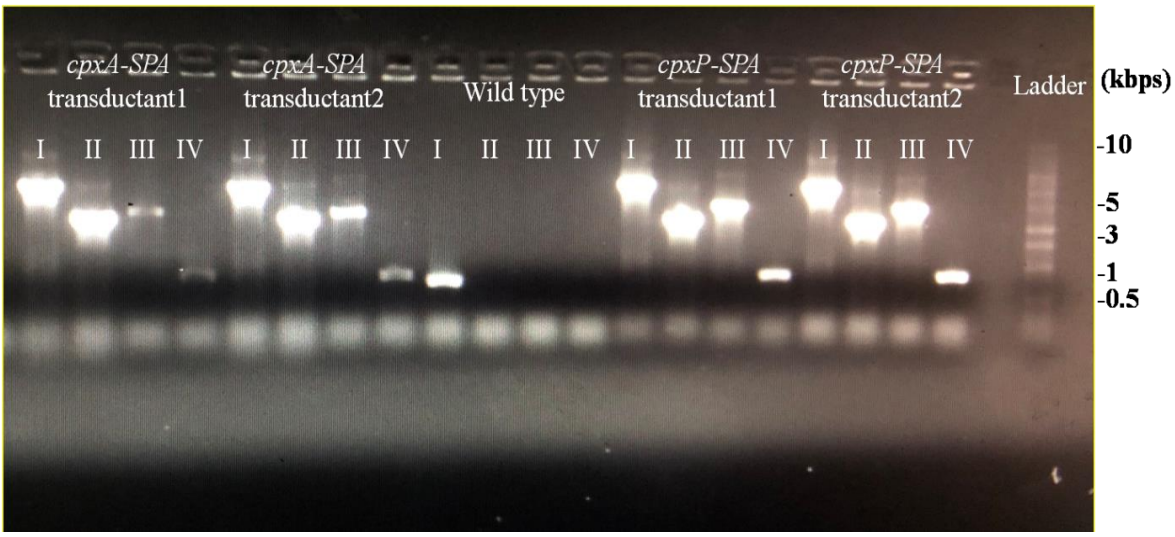

Figure 9: The colony PCR profile of the tagged strains as compared to the untagged strain. PCR was performed with primer sets I (AN03 and AN04 for *cpxA-SPA*, and AN07 and AN08 for *cpxP-SPA*), II (SAK1C and AN04 for *cpxA-SPA*, and SAK1C and AN08 for *cpxP-SPA*), III (AN03 and SAK2C for *cpxA-SPA*, and AN07 and SAK2C for *cpxP-SPA*) and IV (SAK1C and SAK2C). Primer set I with the gene specific primers produced bands in case of the tagged strain as well as the untagged strain. Primer set IV with Kan cassette specific primers produced bands in the tagged strain only. A combination of the gene specific and the Kan cassette specific primers, primer sets II and III, were also used to check for the junction sites which were present in the tagged strains but absent in the untagged strain.

3.2 Tagging of CpxA does not affect the phenotype of the strain

3.2.1 Chromosomal tagging of *cpxA* does not affect the growth of the strain

Although *cpxA* was successfully tagged, it was important to ensure that the incorporation of the tag did not affect the growth of the strain. Previously, it has been shown that WT *E. coli* strain grown in TBK medium supplemented with oleate (TBK-Oleate) as a carbon source attains a higher optical density (OD_{600}) in the stationary phase as compared to when grown in TBK medium supplemented with Brij-58, detergent used for solubilizing oleate (TBK-Brij)[29].To check whether *cpxA-SPA* grows in a similar manner to untagged WT, both the strains were grown either in TBK-Oleate or TBK-Brij, and$OD_{600}$of the samples were measured at regular time intervals. *cpxA-SPA* showed a similar pattern of growth as the WT

untagged strain when grown in TBK-Brij or TBK-Oleate as can be observed in Figure 10. The arrows indicating time points T1 to T5 represents different time points at which samples were harvested for subsequent assays as mentioned later. The SPA tag does not impede or stimulate the growth of the strain.

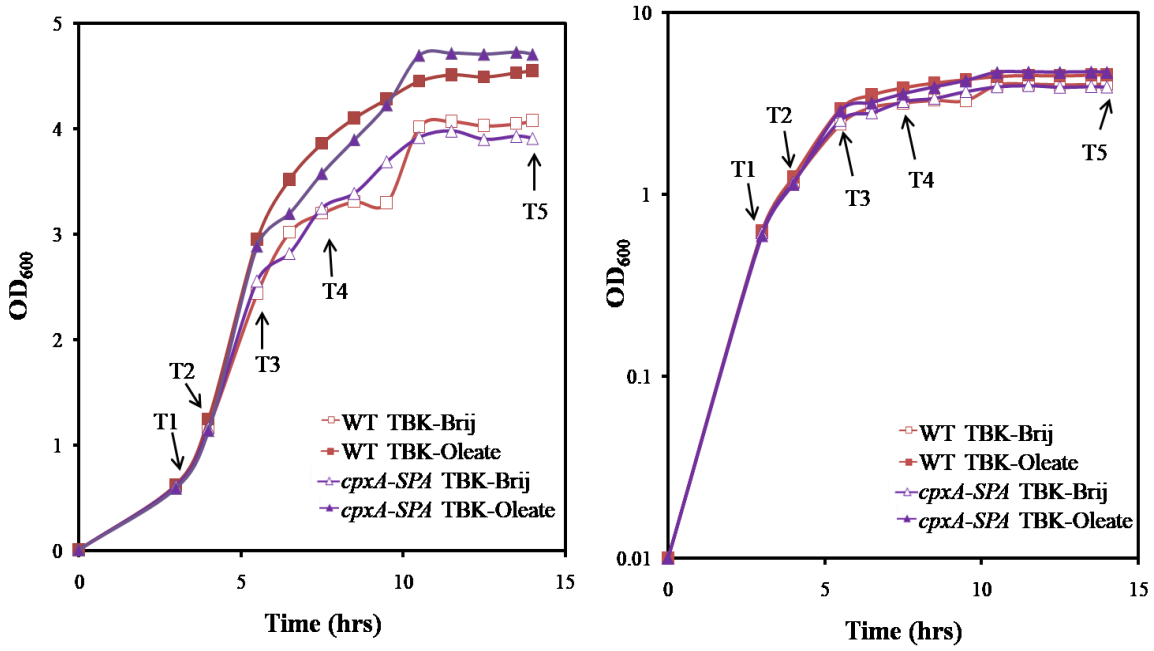

Figure 10: Growth profiles of *cpxA-SPA* as compared to WT BW25113 in TBK-Brij and TBK-Oleate. Strains were cultivated in either TBK-Brij or TBK-Oleate. OD_{600} of the cultures was measured, and growth curves were plotted on a linear scale[left] and semi-log scale[right]. T1, T2, T3, T4, and T5 indicate time points where cultures were harvested for various assays.

3.2.2 Tagging of CpxA does not affect Cpx activation during LCFA metabolism

Although the tagging did not affect the growth of *cpxA-SPA*, it was also necessary to confirm that the functioning of the protein was not affected. This was done by checking Cpx induction in the tagged strain. Previous studies have shown that the Cpx pathway gets activated in stationary phase *E. coli* cells grown in the presence of oleate [28]. The activation

of Cpx was measured using a strain harboring a chromosomal reporter fusion of *lacZ* with the promoter of *cpxP*, and β-galactosidase activity was measured as a read out for Cpx induction. To confirm whether tagging of CpxA did not alter the activation of Cpx pathway during LCFA metabolism, the WT untagged strain and *cpxA-SPA* were grown either in TBK-Brij or TBK-Oleate and samples were collected at different time points, T1-T5, where time point T1 (exponential phase), T2 (late exponential phase), T3 (entry into stationary phase), T4 (mid-stationary phase) and T5 (late stationary phase) represents various phases of growth. These samples were used to perform β-galactosidase assay to check for expression of the *lacZ* gene which in turn is used to ascertain the activity of the *cpxP* promoter. Since the activity of this promoter is only controlled by CpxR, it is used as a direct indicator of the activity of the Cpx pathway. *cpxA-SPA* showed a similar pattern of Cpx induction as the WT untagged strain when grown in oleate, that is, the Cpx pathway showed a ~5-fold induction in the late stationary phase when grown in oleate. Hence, one can conclude that the SPA tag does not affect the Cpx induction and in turn the functionality of the CpxA protein.

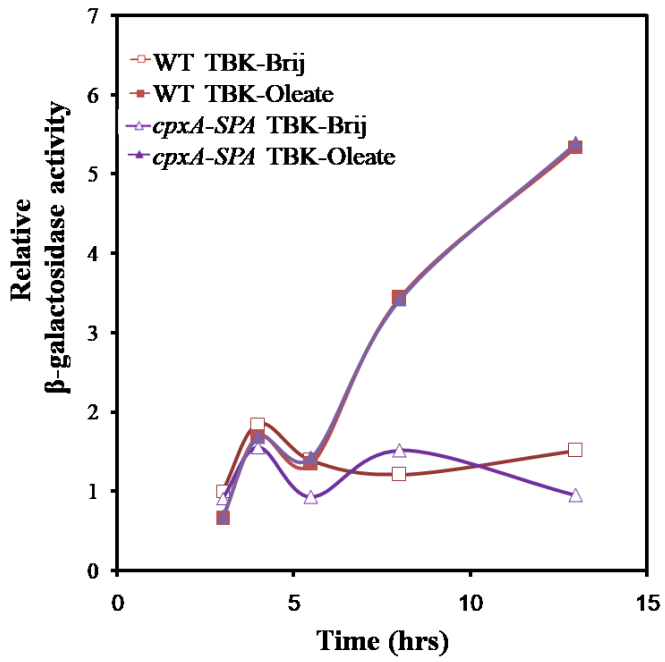

Figure 11: Comparing the Cpx induction between *cpxA-SPA* strains and WT MG1655 carrying chromosomal fusion of *lacZ* with the promoter of a known regulon member of the Cpx response system (P_{cpxP}-*lacZ*) were grown either in TBK-Brij or TBK-Oleate. Cultures were harvested at different phases of growth corresponding to the time points indicated in Fig.10, and β-galactosidase activity was measured. The data was normalized to the β-galactosidase activity of the WT in TBK-Brij at time point T1. The wild type strain grown in oleate

shows a ~5-fold Cpx induction as compared to when grown without oleate. *cpxA-SPA* showed a similar pattern of Cpx induction when grown in oleate.

3.3 Protein pulldown using calmodulin beads showed specific bands in the elution of tagged stains grown in oleate

In a protein pulldown experiment, we hope to obtain the tagged protein and its interacting partners. Although the tagged protein can be identified by Western blotting with an antibody specific to the tag, the identity of the interacting proteins can only be revealed through more advanced and sensitive techniques like mass spectroscopic analysis. Protein mass spectroscopy analyses the mass to charge ratio of peptides which helps in the identification of the proteins in question [38]. However, to eliminate the chances of mislabeling non-specific proteins as hits and to obtain the interacting proteins specific to Cpx induction in oleate, it is necessary to include the elutes obtained from the pulldown of controls for mass spectroscopy. The controls required in this experiment would include the untagged strain grown in TBK-Oleate, to negate any effects due to oleate and the tagged strain grown in TBK-Brij, to identify and negate the proteins common to conditions with and without oleate. Since only calmodulin beads were available at the time of the experiment, the pulldown was carried out only against the CBP domain. However, there is a possibility that native *E. coli* proteins containing a CBP domain may also get eluted in the tagged and untagged strains. For this reason, most protocols suggest performing the two-step purification, first against the 3X FLAG domain and then against the CBP domain for an additional level of purity. However, the single step pulldown will still allow us to see if any major non-specific bands are being eluted in the pulldown of the untagged strain. Furthermore, it is important to note that even if the tagged strain grown without oleate pulls down other proteins with it, just by looking at the profile, one would be unable to determine if the bands are common to both conditions and would require the use of more nuanced techniques such as mass spectroscopy.

The untagged strain and the CpxA tagged strain were cultured in LB overnight at 37˚C. These overnight cultures were used to set up secondary cultures in TBK-Brij and TBK-

Oleate. After 14 hours (T5 time point), volume corresponding to 0.5 OD_{600} was collected from each flask and processed for SDS-PAGE. The remaining cultures were spun down and sonicated in a buffer containing detergent. This was done to extricate CpxA, an inner membrane protein, from the membrane and obtain it in the supernatant. The sonicated sample was spun down to separate the supernatant from the cell pellet fraction. The supernatant and cell pellet fractions were also processed for SDS-PAGE. The whole cell extracts (of both tagged and untagged strains) and post-sonication fractions (of the tagged strain grown in oleate) were run on a 10% gel and the bands on the gel were transferred to a nitrocellulose membrane to perform a Western blot. The membrane was first blocked and then incubated with the M2 anti-FLAG antibody after which it was incubated with the anti-HRP antibody. The blot was then incubated with the ECL substrate (from Pierce) and exposed to X-ray films. Although non-specific bands were seen at around 55 kDa in both the tagged (both whole cell extract and supernatant fraction)and untagged strains (whole cell extract alone), only the tagged strain showed a band at ~60 kDa which corresponds to the size of SPA tagged CpxA (since CpxA alone is ~52 kDa). This implies that the amount of CpxA produced from the chromosome is sufficient enough to be detected. The band intensities of CpxA-SPA in both TBK-Brij and TBK-Oleate in T5 (from the whole cell extracts) were found to be equal and furthermore sufficient amount of CpxA-SPA was obtained in the supernatant; however, the cell pellet did not contain any CpxA-SPA (Figure 12).

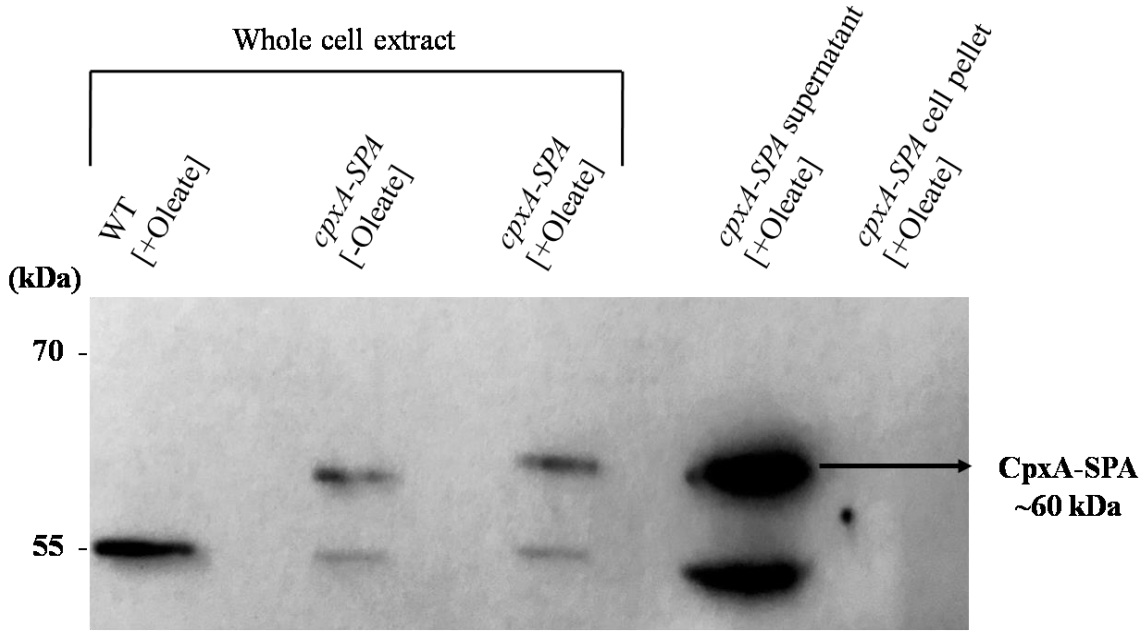

Figure 12: Sufficient amounts of CpxA-SPA could be detected in the supernatant. *cpxA-SPA* and WT BW25113 were grown either in TBK-Brij or TBK-Oleate, and cultures were harvested at time point T5, as in Figure 10. Culture volume corresponding to 0.5 OD_{600} was taken from each set and processed for running on SDS-PAGE gels. The remaining culture of the tagged strains was resuspended in the pulldown buffer, sonicated and separated into supernatant and cell pellet fractions. The supernatant and cell pellet fractions were processed for Western blot to see if CpxA-SPA can be detected. From this figure, it is seen that CpxA-SPA is detected at 60 kDa in the cell extracts of the tagged strain grown in TBK-Brij or TBK-Oleate as well as in the supernatant fraction of the tagged strain grown in TBK-Oleate. No such band was seen in the cell pellet fraction or in the sample of the untagged strain.

The supernatant obtained from both the tagged and the untagged strains were then incubated with calmodulin beads overnight on ice. The following day, the beads were allowed to settle after which the supernatant was discarded. The beads were washed with wash buffer and then eluted thrice with elution buffer. The elutes from both the tagged and untagged strains were run on a 10% SDS-PAGE gel and stained using Coomassie brilliant blue as in Figure13. Although one or two bands were seen in the elute of the untagged strain, which may be attributed to the CBP containing proteins in *E.* coli, there were additional bands that were only present in the tagged strains. However, we were unable to find any visual difference between the band profiles of the tagged strain grown with oleate and when grown without it.

Hence, more sensitive techniques like mass spectroscopy may be required to find the proteins specific to Cpx induction in oleate.

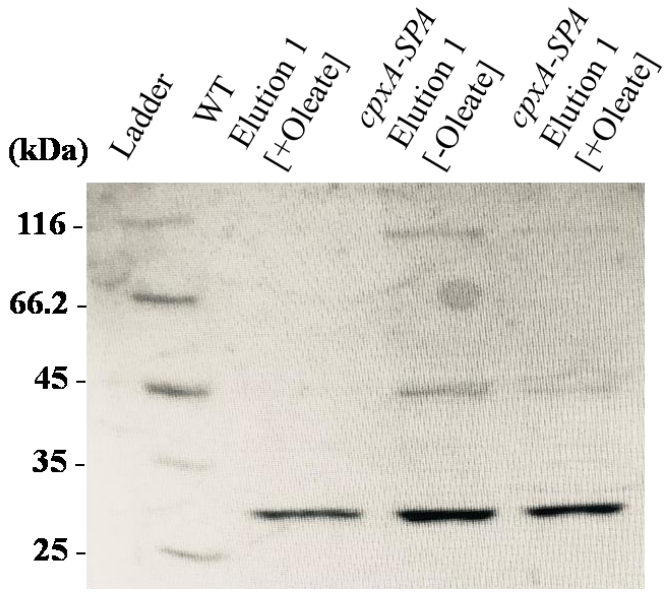

Figure 13: Bands were seen in the elutes of the calmodulin resin pulldown. The supernatants of the tagged strain (*cpxA-SPA*) grown with and without oleate and the untagged strain (WT BW25113) grown in oleate were incubated with calmodulin beads, washed to remove non-specific binding and eluted thrice. The elutes from the tagged and the untagged strains were run on an SDS-PAGE gel. The gel was then Coomassie stained and bands could majorly be seen only in the elutes of the tagged strains, although one or two bands were also seen in the elute of the untagged strain grown in oleate.

The elutes of the tagged and untagged strains grown in oleate were also run on a 15% gel, since that would allow one to see the entire profile of proteins, including the proteins of smaller size. This gel was also subject to Coomassie staining and as seen in Figure 14, a few non-specific bands were seen in the untagged strain elutes as well. This may be attributed to the cellular proteins with CBP domain which implies that the two step purification is necessary with pulldown against the anti-FLAG beads as the first step. Furthermore, the two-step purification would also help eliminate other non-specific bands that may arise during pulldown against the anti-FLAG beads.

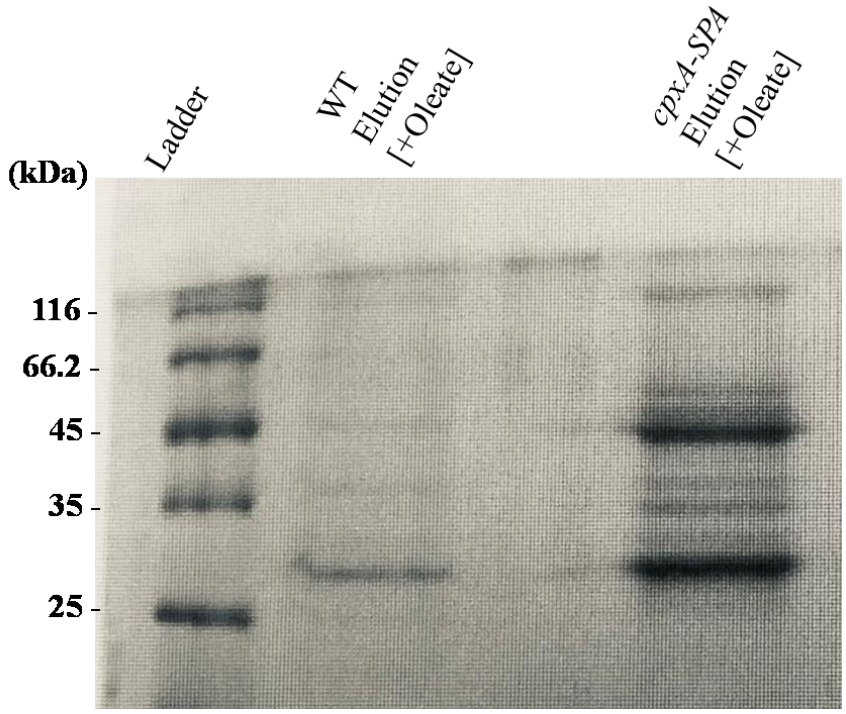

Figure 14: Faint bands were seen in the elutes of the untagged strains on a 15% gel after pulldown using calmodulin resin. The elutes obtained from the tagged and the untagged strains grown in oleate (as described above) were run on a 15% SDS-PAGE gel, to obtain the entire protein profile. The gel was then Coomassie stained. Although bands could majorly be seen in the elutes of the tagged strains, a few faint bands were also observed in the elutes of the untagged strains. This indicates that there may be minor non-specific binding and that incubation with calmodulin beads should be preceded by incubation with anti-FLAG beads.

To confirm that CpxA-SPA was primarily pulled down during the protocol, the elutes of strains grown in oleate were once again subjected to SDS-PAGE on a 15% gel and the proteins were transferred to a membrane. The membrane was probed with an anti-FLAG antibody as the primary antibody and anti-HRP as the secondary antibody. As can be seen in Figure 15, the elute of the tagged strain did contain CpxA-SPA at ~60 kDa. However, it also contained some degradation products. The supernatant of the tagged strain, the elute of the untagged strain and the whole cell extracts of the tagged and the untagged strains were used as controls to confirm that indeed the tagged protein, that is CpxA-SPA, is the major product of the elutes obtained from the pulldown using calmodulin beads. Hence this experiment confirms that the pulldown was successful and has been aptly standardized for CpxA-SPA.

However, it must also be noted that it would be more apt to first perform the two-step purification before sending the samples for mass spectroscopic analysis.

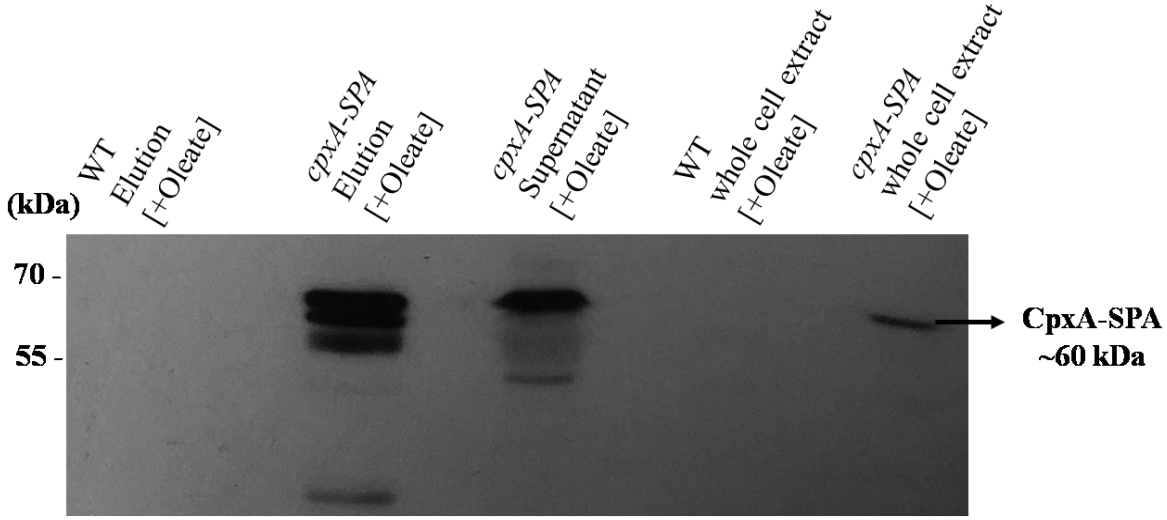

Figure 15: CpxA-SPA could be detected in the elutes of the tagged strains using anti-FLAG antibodies.The elutes of both the untagged strain and the tagged strain grown in oleate were run on an SDS-PAGE gel, followed by Western blot using an anti-FLAG antibody. The supernatant of the tagged strain grown in oleate and the whole cell extracts of the untagged and the tagged strains grown in oleate were used as a control. The band corresponding to CpxA-SPA was seen at ~60 kDa. Some additional bands were also seen in the supernatant and elute fractions of the tagged strain that may be attributed to degradation products.

3.4 Chromosomal tagging of *cpxP* results in alteration in the phenotype of the strain

3.4.1 Chromosomal tagging of *cpxP* slightly affected the growth of the strain in stationary phase

CpxP was successfully tagged on the chromosome with SPA tag. As in the case of CpxA, it was necessary to check that tagging the protein did not affect its functionality. To check whether *cpxP-SPA* grows in a similar manner to untagged WT, both the strains were grown either in TBK-Oleate or TBK-Brij and$OD_{600}$of samples were measured at regular time

intervals. *cpxP-SPA* showed a similar pattern of growth as the WT untagged strain when grown in TBK-Brij or TBK-Oleate as can be observed in Figure 16. The arrows indicating T1 to T5 represent different time points at which samples were harvested for subsequent assays as mentioned later. The result indicates that the SPA tagging of *cpxP* slightly reduces the growth of the strain in stationary phase.

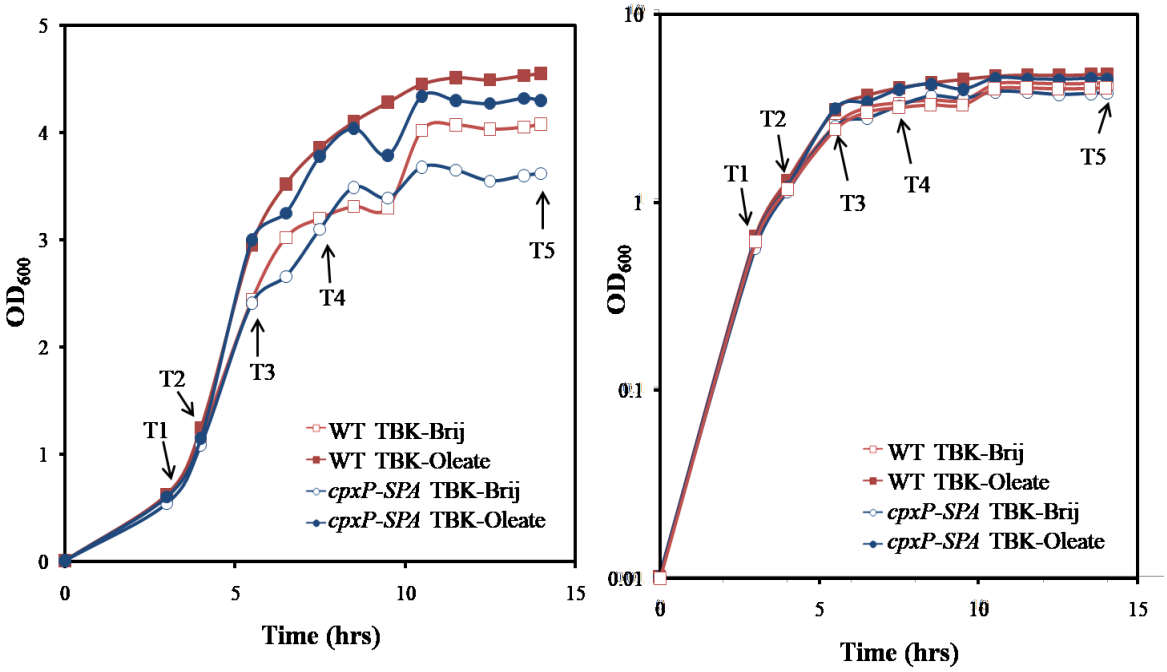

Figure 16: Growth profiles of the *cpxP-SPA* strain as compared to the WT BW25113 in TBK-Brij and TBK-Oleate. Strains were cultured in either TBK-Brij or TBK-Oleate. OD_{600} of the cultures was measured and growth curves were plotted on a linear scale[left] and semi-log scale[right]. T1, T2, T3, T4, and T5 indicate time points where cultures were harvested for various assays.

3.4.2 Chromosomal tagging of *cpxP* resulted in ~2-fold increase in Cpx induction

As in the case of *cpxA-SPA*, it was also necessary to check if strains in which CpxP was SPA tagged had any effect on Cpx induction. As done in case of *cpxA-SPA*, activation of Cpx was measured using a strain harboring a chromosomal reporter fusion of *lacZ* with the promoter

of *cpxP*, and β-galactosidase activity was measured as a read out for Cpx induction. To confirm whether tagging of CpxP did not alter the activation of Cpx pathway during LCFA metabolism, the WT untagged strain and *cpxP-SPA* were grown either in TBK-Brij or TBK-Oleate and samples were collected at different time points, T1-T5, where time points T1, T2, T3, T4 and T5 represents various phases of growth. These samples were used to perform a β-galactosidase assay to check for the expression of the *lacZ* gene which in turn is used to ascertain the activity of the *cpxP* promoter. Unlike the strain in which CpxA was tagged, the CpxP tagged strain did not show a similar pattern of Cpx induction as compared to the untagged WT, as can be seen in Figure 17. Although Cpx induction in the late stationary phase of the tagged strain grown in oleate was ~5-fold more than when it was grown without oleate, as was the case with wild type, the overall induction in this tagged strain was two times more compared to the wild type strain, in both without and with oleate conditions. Since CpxP is an inhibitor, this overall increase in Cpx induction, irrespective of the oleate conditions, might have to do with the stability of the protein. It has been previously reported that SPA tagging to a protein reduces the half-life of the tagged proteins [39]. Therefore, it can be speculated that CpxP-SPA may also be rendered unstable due to SPA tagging. However, another possibility is that the production of a small RNA, CpxQ, which is involved in the degradation of CpxP might be altered due to the tagging. CpxQ is a small RNA produced as a result of the cleavage of the 3' UTR of the *cpxP* transcript and is known to regulate mRNAs of genes involved in inducing stress in the IM. More specifically, CpxQ is known to dampen the synthesis of protein in the IM that are more prone to misfolding [40]. Although CpxP and CpxQ mature from the same mRNA, they work to combat stresses at different parts of the envelope. CpxQ tethers and degrades proteins that help in the insertion of proteins into the IM whereas CpxP alleviates stress caused by protein misfolding in the periplasm. Studies have shown that CpxQ reduces CpxP production without increasing *cpxP* mRNA degradation so CpxQ most likely exerts translational control over CpxP production. So there is reason to believe that the tagging may be altering the *cpxP* transcript in such a way that the synthesis of CpxQ is increased. Another possibility in this same respect is that the promoter for the kanamycin cassette within the SPA tag may work to further increase the production of CpxQ thereby increasing the degradation of CpxP hence increasing Cpx induction regardless of the media condition [41]. Hence, in conclusion, Cpx induction was affected in strains in which CpxP was tagged.

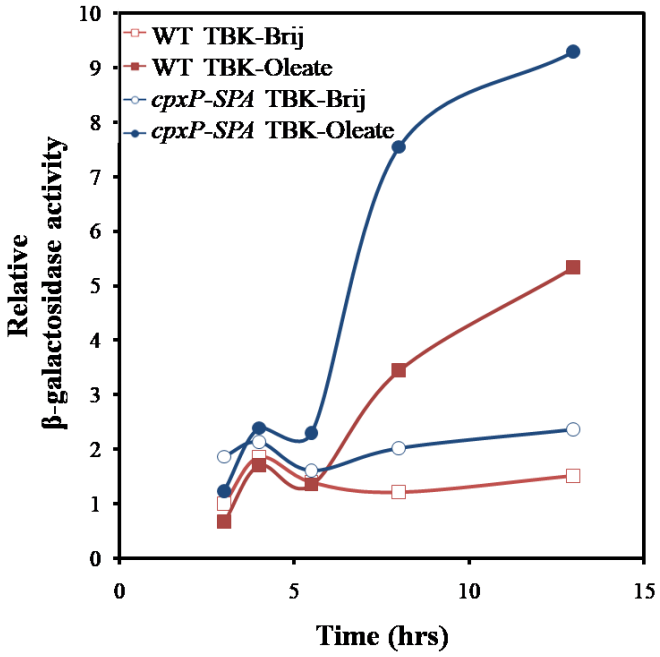

Figure 17: Comparing Cpx induction between *cpxP-SPA* and WT strains. WT MG1655 and its isogenic *cpxP-SPA* carrying chromosomal fusion of *lacZ* with the promoter of a known regulon member of the Cpx response system (P_{cpxP}-*lacZ*) were grown either in TBK-Brij or TBK-Oleate. Cultures were harvested at different phases of growth corresponding to the time points indicated in Fig 16, and β-galactosidase activity was measured. The data was normalized to the β-galactosidase activity of the WT in TBK-Brij at time point T1. WT strain grown in oleate shows a 5-foldCpx induction as compared to when grown without oleate. On the other hand, although *cpxP-SPA* shows a similar trend with and without oleate, there is twice as much Cpx induction in the tagged strain as compared to the untagged strain in both conditions. This may be attributed to the instability of CpxP, the inhibitor of the pathway, due to tagging or due to alterations to the functionality of the small RNA CpxQ.

3.5 Chromosomally tagged CpxP could not be detected using anti-FLAG antibodies

Although overall Cpx induction was affected by tagging CpxP, since the fold difference in Cpx induction remained the same in the tagged strain grown with and without oleate, one could still check for the presence of the protein and proceed with pulldown experiments. Although Cpx induction takes place in the late stationary phase (T5), since according to most models CpxP gets degraded during Cpx induction, samples were collected at early stationary

phase (T3), that is when the Cpx pathway is not induced, and at T5 when the pathway is induced. Both the WT untagged strain and the tagged strains were cultured in TBK-Brij and TBK-Oleate conditions and samples were harvested at time points T3 and T5. The samples were then processed for SDS-PAGE on a 15% gel and the proteins were transferred to a membrane. The membrane was probed with an anti-FLAG antibody as the primary antibody and anti-HRP as the secondary antibody. Since CpxP is a small protein of approximately 18kDa and CpxP-SPA of ~26 kDa, a 15% gel was used to run the samples. However, as seen in Figure 18, there were many non-specific bands in all the samples, including the untagged samples in both T3 and T5. Furthermore, a set of non-specific bands were seen at the position at which CpxP-SPA was expected to be detected.

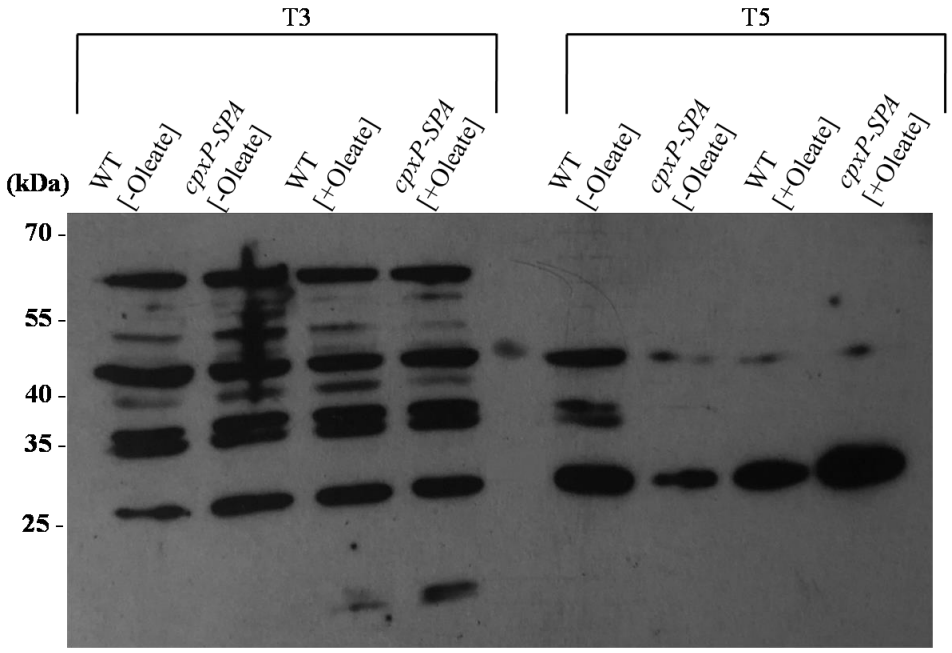

Figure 18: Chromosomally expressed CpxP-SPA could not be identified. *cpxP-SPA* and WT BW25113 were grown either in TBK-Brij or TBK-Oleate, and cultures were harvested at time points T3 and T5, as in Figure 16. Culture volume corresponding to 0.5 OD_{600} was taken from each set and cells were processed. The samples were run on an SDS-PAGE gel, followed by Western blot using an anti-FLAG antibody. Non-specific bands littered the lanes of both the untagged and the tagged strains. Since one set of non-specific bands lay at 25 kDa, it was not possible to identify the presence or absence of CpxP-SPA, a protein of ~26 kDa.

Hence, using only one of the untagged strain samples as the control, along with a sample containing chromosomally expressed CpxA-SPA (as a positive control), T3 and T5 samples of the CpxP tagged strains grown in TBK-Brij and TBK-Oleate conditions were run on a 10% gel and transferred and probed as mentioned before. As seen in Figure 19, although CpxA-SPA could be detected at ~60 kDa and a non-specific band was seen in both the untagged sample and in the *cpxA-SPA* sample, no bands were seen in any of the *cpxP-SPA* samples. Hence we may conclude that chromosomally tagged CpxP cannot be detected, possibly owing to the low amounts of CpxP present in the cell or due to degradation caused by the SPA tag.

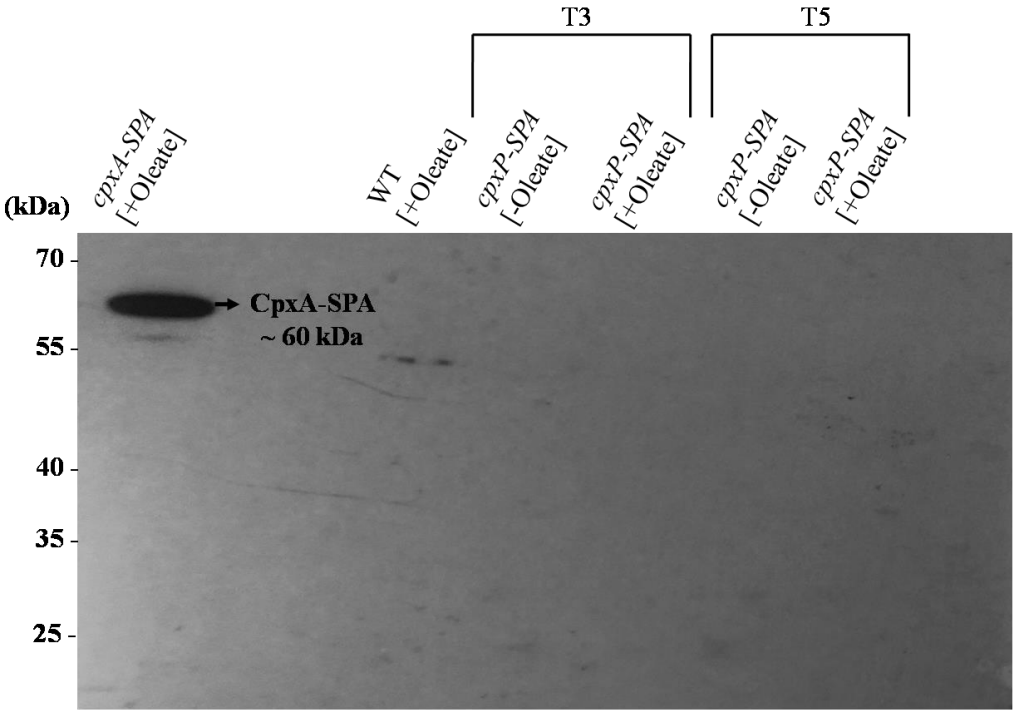

Figure 19: Chromosomally expressed CpxP-SPA could not be detected. *cpxP-SPA* and WT BW25113 were grown either in TBK-Brij or TBK-Oleate, and cultures were harvested at time point T5, as in Figure 16. Culture volume corresponding to 0.5 OD_{600} was taken from each set and cells were processed. The samples were run on an SDS-PAGE gel, followed by Western blot using an anti-FLAG antibody. A sample containing CpxA-SPA was also run as a control. Although the band corresponding to CpxA-SPA could be seen at ~60 kDa, no bands were seen in the other lanes. A faint non-specific band was seen in the untagged sample at ~55 kDa which was also seen in the sample with CpxA-SPA. Hence, CpxP-SPA could not be detected when expressed chromosomally.

3.6 Tagged CpxP expressed from a plasmid could be detected

3.6.1 Cloning of tagged *cpxP* on a plasmid

As CpxP-SPA expressed from the chromosome could not be detected, we decided to clone *cpxP-SPA* on an inducible plasmid so that the protein could be expressed at detectable levels. However, it was desired that the protein should not be expressed at very high quantities so as to alter the physiology of the cell. For this reason, the plasmid pRC10 was chosen as the vector for cloning. pRC10 is an IPTG inducible plasmid that contains a mutated *lac* promoter. The mutation at the *lac* promoter was performed to reduce the activity of the promoter and tighten the regulation and as a result leaky expression from the plasmid is also reduced. Hence the *cpxP* gene, excluding its promoter, along with the nucleotides corresponding to just the SPA tag, was cloned in the MCS adjacent to the mutated *lac* promoter on pRC10 between the *Eco*RI and *Bam*HI sites. The gene was first amplified from the chromosomally tagged *cpxP-SPA* strain using primers that contained the *Eco*RI restriction site, *Bam*HI restriction site and the RBS (DR015 and DR016). pRC10 and the amplicon were then digested using *Eco*RI and *Bam*HI enzymes and the digested products were run on a gel and purified. These purified products were then used to set up a ligation reaction using DNA ligase. The ligation product was transformed into chemically competent DH5α using the heat shock method and the transformation mix was plated on LB plates containing ampicillin. Using primers that are adjacent to the MCS (BS25 and BS26), the cloning was confirmed using colony PCR, with pRC10 as the vector control, as shown in Figure 20.

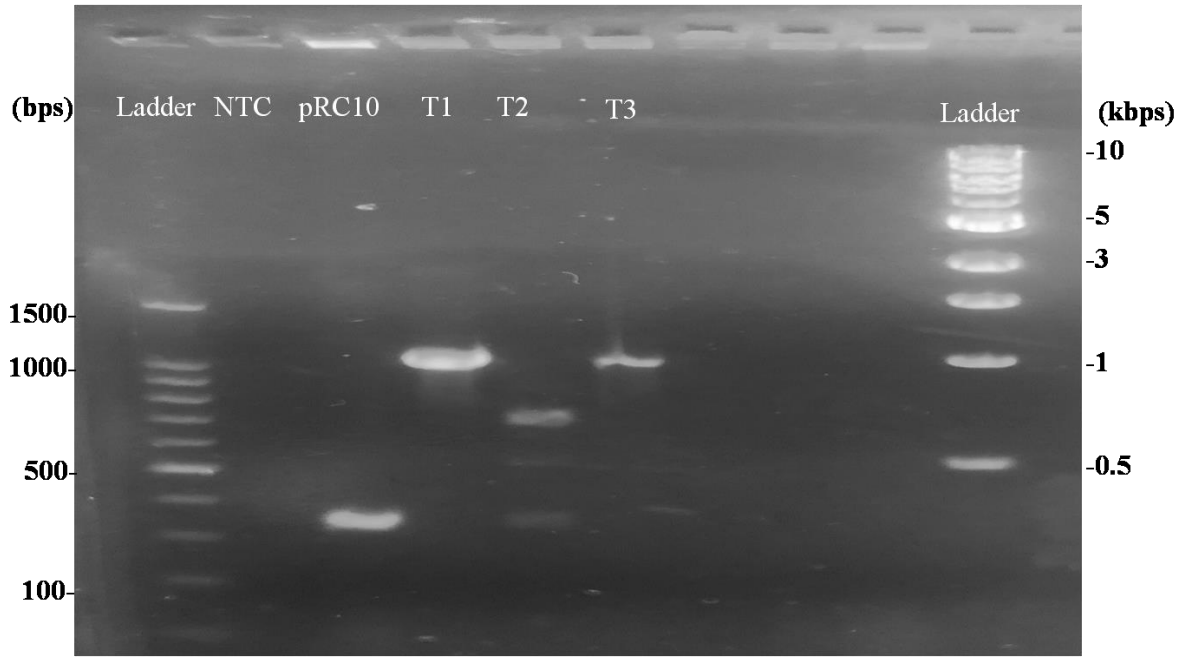

Figure 20: Cloning of *cpxP-SPA* into pRC10 was confirmed by colony PCR. Colonies obtained from chemical transformation were subjected to colony PCR using the primers BS25 and BS26. The vector pRC10 was used as a control along with a no template control (NTC), Bands were obtained at around 1040 bps in the two of the three selected colonies from the transformed plate (T1 and T3). T2 was not a positive clone. The vector control produced a band of a lower size, ~350bps, since it did not contain any cloned gene in the MCS.

3.6.2 Tagged CpxP expressed from the plasmid could be detected using anti-FLAG antibodies

Since *cpxP-SPA* was successfully cloned on a plasmid (pAN2), we next checked if the protein could be detected. The clone pAN2 was transformed into WTBW25113and the vector pRC10 was also transformed to act as a control. The strains containing either the clone or the vector were cultured in TBK-Brij and TBK-Oleate conditions in duplicate. 0.4 mM IPTG was added 3 hours after inoculation (at T1 time point) to one set of cultures and the other set was left uninduced. Samples were harvested at time point T3, i.e., 8 hours post inoculation, from both sets of cultures. The samples were then processed for SDS-PAGE on a 10% gel and transferred and probed as mentioned above. As shown in Figure 21, no bands corresponding to the expected size of CpxP-SPA were seen in uninduced cultures of the

strain containing either the vector or the clone. In case of the samples induced using IPTG, bands corresponding to CpxP-SPA were seen only in the strain containing the clone. Hence, tagged CpxP is produced in detectable amounts when expressed from the inducible plasmid pRC10.

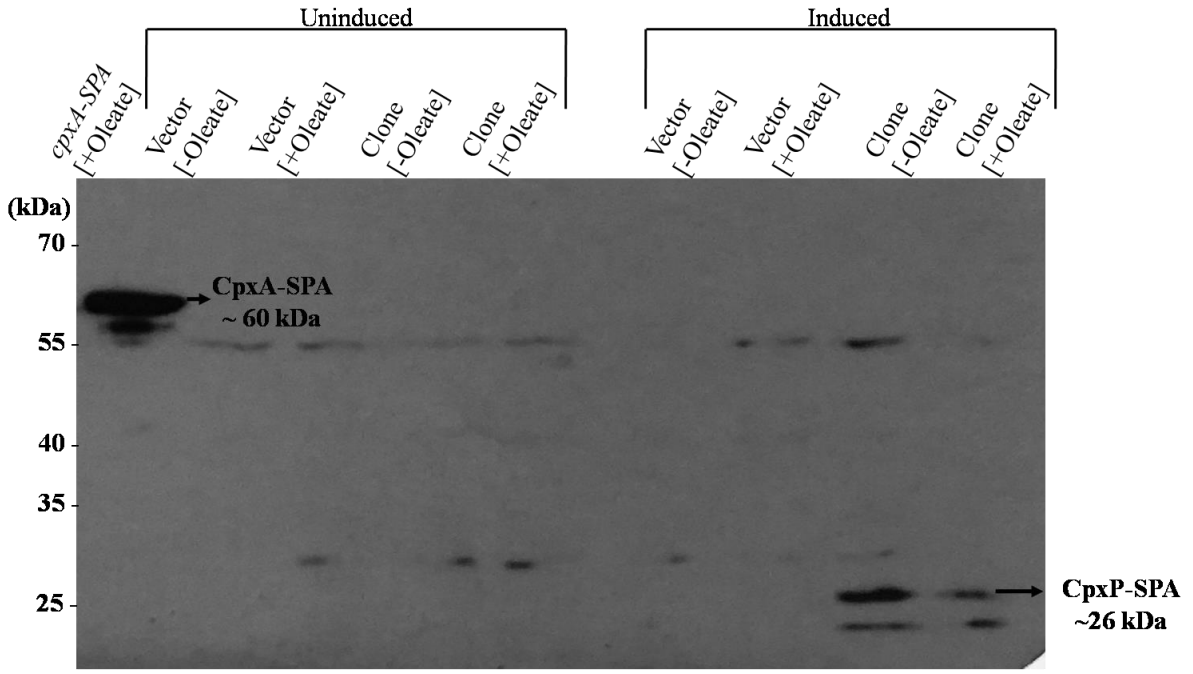

Figure 21: Tagged CpxP could be detected using anti-FLAG antibodies when expressed from a plasmid. WT BW25113 was transformed with the vector, pRC10 (lanes labeled as Vector), and with the plasmid containing *cpxP-SPA*, (lanes labeled as Clone), and these strains were grown either in TBK-Brij or TBK-Oleate. 0.4 mM IPTG was added at time point T1 to one set of cultures and cultures were harvested at time point T3, as in Figure 16, from both the induced and uninduced sets. Culture volume corresponding to 0.5 OD_{600} was taken from each set and cells were processed. CpxP-SPA was identified by running the samples on an SDS-PAGE gel, followed by Western blot using an anti-FLAG antibody. Bands at ~26 kDa were seen only in the lanes with the induced strain containing the clone grown with or without oleate. No bands corresponding to 26 kDa were seen in any of the lanes containing the vector or in case of the uninduced clones. A sample containing chromosomally expressed CpxA-SPA was also run as a control and the band corresponding to it was seen at ~60 kDa.

3.6.3 Tagged CpxP expressed from the plasmid could not be detected in the stationary phase

Since it was confirmed that CpxP-SPA on induction with IPTG at T1 could be detected at T3, when the Cpx pathway is uninduced, it was also necessary to check if CpxP-SPA could be detected when the Cpx pathway is induced, that is at T4 or T5. Hence as previously done, pRC10 and the clone pAN2 were transformed into WT BW25113. The strains were cultured in TBK-Brij and TBK-Oleate conditions. 0.4 mM IPTG was added 3 hours after inoculation (at T1 time point) and samples were harvested at time points T3 and T5, as in Figure 16. The samples were then processed for SDS-PAGE on a 10% gel and transferred and probed as mentioned above. As shown in Figure 22, in case of samples taken at T3, the band corresponding to CpxP-SPA could be detected at ~26 kDa in lanes containing the clone. However in samples taken at T5, bands corresponding to CpxP-SPA could not be detected in any of the samples, including the samples taken from the cultures without oleate, where we don't expect to see Cpx induction [28]. One reason may be the degradation of CpxP-SPA in T4 and T5. Another reason may be that the regulation of the promoter on the plasmid is undergoing some changes during the growth of the strain perhaps due to some sort of stress caused by expression of non-physiological levels of CpxP. One way to test the latter theory would be to change the time of induction to a time closer to T4 or T5.

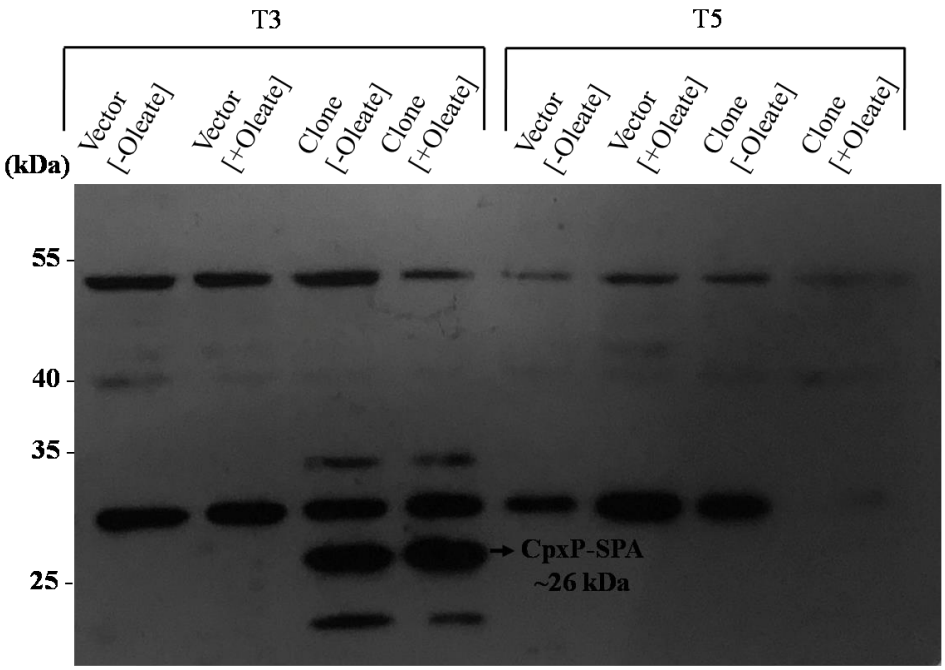

Figure 22: CpxP-SPA expressed from a plasmid at T1 could be detected at T3 but not T5. WT BW25113 was transformed with the vector, pRC10 (lanes labeled as Vector), and with the plasmid containing *cpxP-SPA* (lanes labeled as Clone), and these strains were grown either in TBK-Brij or TBK-Oleate. 0.4 mM IPTG was added at T1, and cultures were harvested at time points T3 and T5, as in Figure 16. Culture volume corresponding to 0.5 OD_{600} was taken from each set and cells were processed. CpxP-SPA was identified by running the samples on an SDS-PAGE gel, followed by Western blot using an anti-FLAG antibody. Band at ~26 kDa was seen only for the strain containing the clone where samples were taken at T3.No band corresponding to 26 kDa was seen either for the strain transformed with the vector or the strain transformed with the clone when the samples were taken at T5.

3.6.4 Tagged CpxP expressed from the plasmid could be detected in the stationary phase on changing the time of induction

Since CpxP-SPA could not be detected at T5 when IPTG was added at T1, we proposed that perhaps delaying the time of addition of IPTG to a later phase may allow us to detect CpxP-SPA. The strains containing the clone or the vector were cultured in TBK-Brij and TBK-Oleate conditions. This time, 0.4 mM of IPTG was added at T3 instead of at T1. Samples were harvested at time point T4, as shown in Figure 16. The samples were then processed for

SDS-PAGE on a 10% gel and transferred and probed as mentioned above. As depicted in Figure 23, bands corresponding to CpxP-SPA, at 26 kDa, were seen in samples of the strain transformed with the clone. Hence it may be concluded that if we are to use tagged CpxP for a pulldown experiment as done in case of CpxA-SPA, induction would have to be performed three hours (or less) prior to sample collection so as to obtain sufficient amount of the protein at detectable quantity.

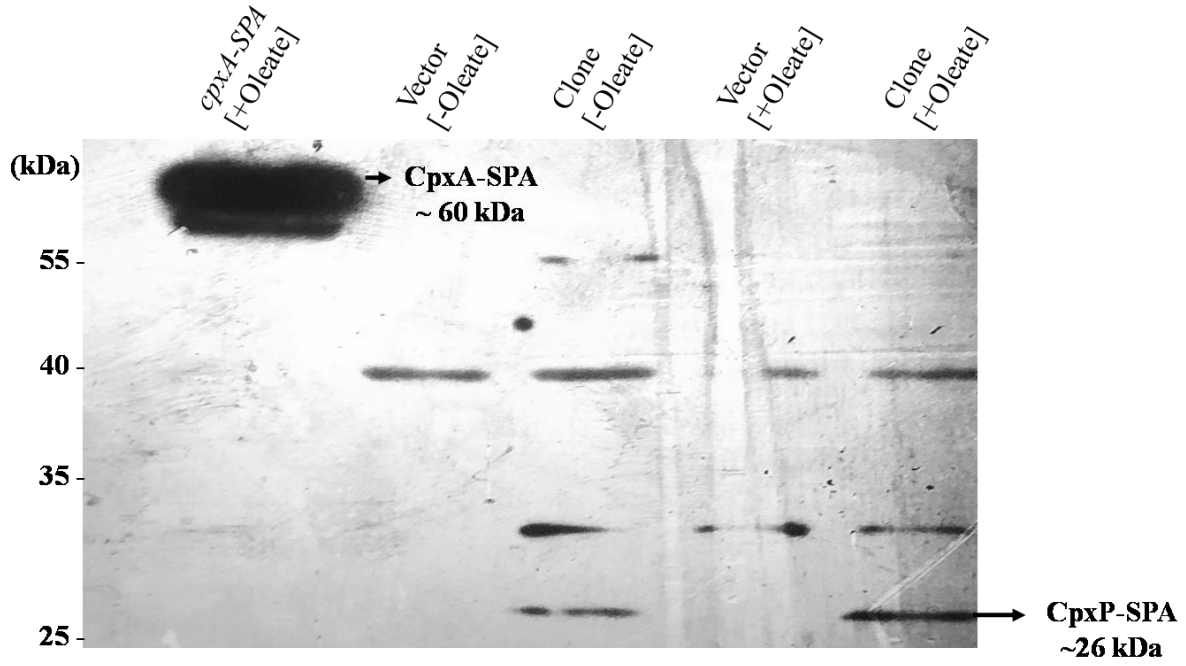

Figure 23: CpxP-SPA induced from the plasmid at T3 could be detected in T4.WT BW25113 was transformed with the vector, pRC10 (lanes labeled as Vector), and with the plasmid containing *cpxP-SPA*, (lanes labeled as Clone), and these strains were grown either in TBK-Brij or TBK-Oleate. 0.4 mM IPTG was added at T3 and cultures were harvested at time point T4, as in Figure 16. Culture volume corresponding to 0.5 OD_{600} was taken from each set and cells were processed. CpxP-SPA was identified by running the samples on an SDS-PAGE gel, followed by Western blot using an anti-FLAG antibody. A band at ~26 kDa was seen only in the strain containing the clone grown with or without oleate. A sample of chromosomally expressed CpxA-SPA was also run as a control and the band corresponding to it was seen at ~60 kDa.

3.7 Bacterial conjugation was successfully standardized

A different approach to elucidating the players involved in Cpx activation is by using a high throughput approach. One method of doing this would involve screening an ordered gene deletion library such as the Keio library and checking for genes that affect Cpx induction. This would involve transferring a *cpxP* reporter construct such as P_{cpxP}-*lacZ* to all the strains of the Keio collection. One method to do this would be via bacterial conjugation. A study had shown that bacterial conjugation may be used to transform an entire library with a plasmid containing the required reporter construct by having it within a donor strain capable of conjugation. In this way, the entire library may be pinned onto the donor strain containing the plasmid and successful conjugants can be selected for by subsequent antibiotic selection [32,42]. The successful conjugants can then be pinned onto a media containing X-gal in case of *lacZ* constructs, to identify the deletions that affect Cpx induction by checking for a difference in the color of the colonies. White and light blue colonies would indicate the downregulation of Cpx activity. One disadvantage of this method is that it cannot give any information regarding the essential genes involved in Cpx induction.

Here an attempt at standardizing this technique in our lab was made wherein an F^+, Hfr^- strain, JA200, was used as the donor strain and *ΔcpxR*(*cpxR::kan*) was used at the recipient. pCB112, a mobile plasmid with a chloramphenicol marker, was used to check for conjugation. As mentioned in the methods section, a lawn of the donor strain was made onto which the recipient was either spotted as varying dilutions (liquid→solid) or pinned from a colony (solid→solid). This was done on a plate without antibiotics to allow unhindered mating and the plate was incubated at 37 ˚C for thirteen hours. After the incubation period required for mating, colonies were taken from the plate and streaked onto plates containing the antibiotics against both the donor (chloramphenicol) and the recipient (kanamycin). Colonies from this double antibiotic plate were then streaked onto another double antibiotic plate to get streaks of a single colony which was then used for colony PCR to assess if conjugation has taken place and the products were run on an agarose gel and the bands were visualized as in Figure 24.

Primers, MS23 and MS24, were used to check for the presence of *cpxR*deletion while SAK1C and SAK2C were used to amplify the kanamycin cassette. Combinations of these

primers were also used to obtain bands corresponding to the amplification of the junctions. While bands obtained from the above primer sets confirm the recipient's genotype, primers BSC1 and BSC2 were used to amplify the chloramphenicol cassette on the plasmid. The parent donor and recipient strains were used as a control along with a no template control. Since bands were obtained from all the primer sets in the colonies from the conjugation plates, it was confirmed that conjugation had successfully taken place and that this method may now be extrapolated to be used for screening the ordered libraries for players involved in Cpx induction. It would be more efficient and less time consuming to use the solid→solid method since making dilutions of the entire Keio collection may be avoided. An additional step may be required in the form of cloning P_{cpxP}-*lacZ* onto pCB112 as well.

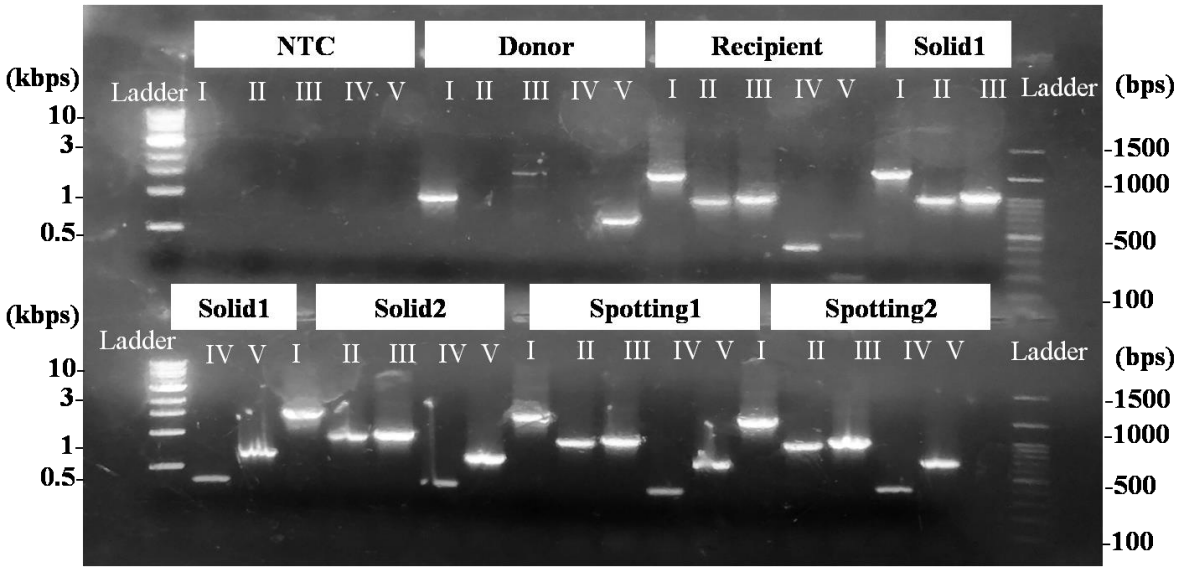

Figure 24: Confirmation of Bacterial Conjugation using colony PCR with primer sets I (MS23 and MS24), II (SAK1C and MS24), III (MS23 and SAK2C), IV (SAK1C and SAK2C) and V (BSC2 and BSC1). Colonies were taken from the double antibiotic plates and their colony PCR profile was obtained using gene specific primers for *ΔcpxR* (MS23 and MS24), kanamycin cassette specific primers (SAK1C and SAK2C) and a combination of these primers to confirm the presence of the recipient background in the conjugants. Chloramphenicol cassette specific primers (BSC2 and BSC1) were used to confirm the presence of the mobile plasmid pCB112 in both the donor control as well as the conjugants. A no template control (NTC) was also used with all the primer sets. The labels Solid1 and Solid2 are used to denote the colonies obtained from solid→solid conjugation and Spotting1 and Spotting2 denote the colonies obtained from liquid→solid conjugation. Since all the primer sets produced bands for the conjugants, it was confirmed that conjugation of the mobile plasmid pCB112 from the donor into the recipient had taken place.

Chapter 4
Summary and Future Directions

The Cpx pathway is a two-component system involved in combating envelope stresses experienced by Gram-negative bacteria such as *Escherichia coli*. Although many mechanisms have been proposed to elucidate the activation of this pathway, to date, no hypotheses have been suggested to understand Cpx activation in the late stationary phase during oleate metabolism. Published work from our lab has shown that the outer membrane lipoprotein, NlpE, a well-known activator of the pathway, is not the signal involved in Cpx activation when the cells are grown in oleate. Further, the experimental conditions used to study oleate metabolism suggest that neither alkaline pH nor P pilus subnits, two other known Cpx inducers, are the signals for Cpx activation during growth in LCFAs. Hence through this study, we hoped to elucidate and identify the players involved in the activation of the Cpx pathway during oleate metabolism. The major approach taken was the proteomics method wherein important sensors in this pathway, CpxA and CpxP, were tagged using the SPA tag and the methods were standardized to be able to detect the proteins and use them for the pulldown protocol.

At first, it was checked whether tagging of the proteins was successful and if so if the tag itself affected the functionality of the protein or the phenotype of the strain. CpxA-SPA could be detected even when produced chromosomally using anti-FLAG antibodies against the 3X FLAG domain of the SPA tag. Since the protein could be detected, the buffer conditions and elution methods were then standardized for the pulldown of this protein. Specific bands were seen when the elutes were run on a gel and Coomassie stained, and the elutes were also seen to contain the tagged protein by Western which is a major indicator that the pulldown was successful.

However when the same elutes were run on a higher percentage of gel, some non specific bands were also seen in the elutes of the untagged strains suggesting that perhaps the pulldown with calmodulin should first be preceded with a pulldown using anti-FLAG beads.

In case of strain in which *cpxP* was chromosomally tagged, the protein could not be detected using anti-FLAG antibodies. This may be attributed to the low amounts of CpxP produced from the chromosome and for this reason, the gene corresponding to the protein and the tag were cloned onto an IPTG inducible plasmid. On addition of IPTG at T1 time point, CpxP-SPA could be detected in T3 but not in T5 time point. This may be attributed to the instability of the protein due to the tag itself or due to some mutations on the plasmid during the growth of the strain after IPTG induction. This was tested by changing the time of addition of IPTG from T1 to T3 and samples were then collected at T4. In this case, CpxP-SPA could be detected at T4 in sufficient amounts. This result suggests that when pulldown experiments are conducted to check for the interacting partners of CpxP during Cpx activation in T5 in oleate, IPTG should be added 2-3 hours prior to T5 to ensure that CpxP can be detected and can be used for pulldown. Hence future experiments would include standardizing the pulldown buffer conditions for CpxP and checking if the elutes contain sufficient amounts of the tagged protein.

Another method by which players involved in the activation can be identified is by using genetic studies. This includes screening ordered gene deletion libraries to check for deletions that affect Cpx induction in oleate. However to use this methods, a reporter construct has to first be transferred into all the strains in the library and hence minor part of this study also focused on standardizing the conditions for bacterial conjugation. The conditions for the same such as time of mating, plate conditions and spotting conditions were optimized and conjugation was confirmed using colony PCR. In the future, this same standardized methodology (solid→solid) will be used in a high throughput framework and the library will also be spotted on a media containing X-gal, to see the effects of the deletion on Cpx induction. It should be noted however that one drawback of this method as compared to the proteomics approach is that it can screen only for the effects of non-essential genes.

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