To investigate the regulation on ubiquinone during long-chain fatty acid metabolism in *Escherichia coli*

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Certificate of Examination

This is to certify that the dissertation titled "**To investigate the regulation on ubiquinone during long-chain fatty acid metabolism in *Escherichia coli***" submitted by **Ms. Liz Maria Luke** (Reg. No. MS16107) for the partial fulfilment 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.

Dr. Anand K Bachhawat Dr. Rajesh Ramachandran Dr. Rachna Chaba

(Supervisor)

Dated: April 30, 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.

Liz Maria Luke
(Candidate)

Dated: April 30, 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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Abbreviations

1. ETC: Electron Transport Chain
2. ATP: Adenosine triphosphate
3. ADP: Adenosine diphosphate
4. LCFA: Long-chain fatty acid
5. NAD^+: Nicotinamide adenine dinucleotide
6. FAD: Flavin adenine dinucleotide
7. TCA: Tricarboxylic acid cycle
8. cAMP: Cyclic adenosine monophosphate
9. CRP: cAMP receptor protein
10. Q_8: Ubiquinone-8
11. MQ_8: Menaquinone-8
12. DMQ_8: Demethylmenaquinone-8
13. 4-HB: 4-Hydroxy benzoate
14. OPP: 2-octa-prenyl phenol
15. OHB: octaprenyl-4-hydroxybenzoate
16. SAM: S-Adenosylmethionine
17. SAH: S-adenosylhomocysteine
18. FNR: Fumarate and nitrate reduction regulatory protein
19. NarL: Nitrate/nitrite response regulator
20. PCR: Polymerase chain reaction
21. H_2O: Water
22. NH_4Cl: Ammonium chloride
23. NaCl: Sodium chloride
24. KCl: Potassium chloride
25. $MgSO_4$: Magnesium sulphate
26. $MgCl_2$: Magnesium chloride
27. Na_2HPO_4: Sodium hypophosphate
28. KH_2PO_4: Potassium dihydrogen phosphate
29. $CaCl_2$: Calcium chloride
30. CIP: Calf Intestinal Alkaline Phosphatase

31. dNTPs: deoxyribonucleotide triphosphate
32. LB: Lysogeny broth
33. TYE: Tryptone yeast extract
34. SOB: Super optimal broth
35. SOC: Super optimal broth with catabolite repression
36. TB: Tryptone broth
37. SDS: Sodium dodecyl sulphate
38. EDTA: Ethylenediaminetetraacetate
39. DNA: Deoxyribonucleic acid
40. Taq: *Thermusaquaticus*
41. MCT: Micro centrifuge tubes
42. TAE: Tris base, acetic acid and EDTA
43. βME: 2-mercaptoethanol
44. ONPG: Ortho-Nitrophenyl-β-galactoside
45. HPLC: High performance liquid chromatography
46. SI: Single integrant
47. MI: Multiple integrant
48. WT: Wild type
49. T1: Transducatnt 1
50. TBK-O: Tryptone broth medium supplemented with oleate
51. TBK-B: Tryptone broth supplemented with Brij-58
52. CRIM: Conditional replication, integration, and modular

Contents

Abstract

All organisms, including bacteria, require energy for performing different cellular activities which they acquire by metabolism. During metabolism, nutrients undergo breakdown via several catabolic reactions producing reduced cofactors and various growth intermediates. The reduced cofactors are oxidized by the electron transport chain (ETC) ultimately resulting in ATP (adenosine triphosphate) production by oxidative phosphorylation. Our lab works on the metabolism of long-chain fatty acid (LCFA), a rich nutrient source for several bacteria including *Escherichia coli*. LCFAs are carboxylic acids containing long unbranched aliphatic chains of 12-20 carbon atoms. Previously our lab showed that LCFA metabolism produces high levels of reduced cofactors, the oxidation of which creates an increased electron flow in the ETC. Ubiquinone, a lipid-soluble electron carrier in the ETC, plays a critical role in metabolism by rapidly transferring electrons from respiratory dehydrogenases to terminal oxidases. Additionally, ubiquinone plays a vital role in the uptake of electrons from the disulfide bond forming machinery, that catalyses disulfide bond formation in several extracytoplasmic proteins in the bacterial cell envelope. Work from our lab showed that as electrons from both carbon metabolism and disulfide bond formation converge at the level of ubiquinone, LCFA metabolism which generates large number of electrons, renders ubiquinone limiting for its electron transfer function, resulting in redox stress in *E. coli*. Moreover, our lab showed that *E. coli* counteracts the LCFA-induced stress by upregulating ubiquinone levels ~1.8 fold. However, the mechanism behind the upregulation of ubiquinone during LCFA metabolism is unknown. My project aims to investigate whether there is a transcriptional regulation of the genes involved in ubiquinone biosynthesis (*ubi* genes) during LCFA metabolism. Previous studies have identified a ubiquinone biosynthesis multi-protein complex comprising of at least 12 proteins in *E. coli*. To check for transcriptional regulation, transcriptional reporter constructs for all 12 *ubi* genes were created and β-galactosidase activity assays were performed. We found that several *ubi* genes are upregulated during stationary phase in the LCFA-utilising cells. We further investigated the role of FadR, a well-known transcriptional regulator during fatty acid metabolism, for the transcriptional regulation on *ubi* genes, however, we did not observe any FadR regulation on the *ubi* genes. Future studies aimed at investigating the role of other transcriptional regulators for induction of *ubi* genes is required to unravel the mechanistic details for ubiquinone upregulation during LCFA metabolism.

Chapter 1

Introduction and Review of literature

1.1Carbon metabolism in bacteria

Metabolism, a central process to microbial life, refers to all the biochemical reactions occurring in a cell for energy generation. Metabolism can be divided into two: the energy generating component called catabolism and the biosynthetic or energy consuming part called anabolism (1). Anabolic reactions are bond-forming processes; therefore, from an energy perspective, these reactions which require energy are termed endergonic reactions (2). Some examples of anabolic reactions are the formation of nucleic acids from nucleotides, proteins from amino acids, and the synthesis of glycogen from glucose (3). Catabolic reactions involving bond breaking and energy release are called exergonic reactions (2). An example of catabolic reaction occurs during the process of respiration when cells break down sugars into carbon dioxide and water, releasing energy (3). Catabolic reactions produce energy and are temporarily stored in the high-energy bonds of ATP, the "energy currency" of the cell. ATP molecules are highly unstable because of the repulsion of the negative charges on the phosphate group; therefore, ATP molecules cannot be stored for a long time inside the cells. As a result, microbial cells produce large organic compounds like lipids and glycogen by anabolic processes for the long-term storage of energy (2). As metabolic reactions either require or release energy, metabolism can be defined as an energy-balancing act (3).

Bacteria thrive in almost every habitat on earth, including harsh environments like hydrothermal vents, glaciers, arctic ice, and hot springs. They exhibit tremendous ability to adapt to these conditions because of their variations in the modes of metabolism and energy generation. They derive energy by diverse reactions depending upon their growth conditions (4). However, they generally use two primary mechanisms for the production of ATP from ADP (adenosine diphosphate).

(i) Substrate-level Phosphorylation: A high-energy phosphate is directly transferred to ADP from a phosphorylated substrate, thereby producing ATP (3).

$$\text{C-C-C}\sim\text{P} + \text{ADP} \longrightarrow \text{C-C-C} + \text{ATP}$$

(ii) Oxidative phosphorylation: Electrons from the organic compound are transferred to a group of electron carriers (like NAD^+ and FAD) that transfers electrons to a series of electron carriers present in the cell membrane of a bacterial cell. The molecular O_2 or other oxidized organic and inorganic molecules act as the final electron acceptor. As electrons are transferred through the electron transport chain (ETC), proton motive force is generated, which is then used for the generation of ATP (3).

1.1.1 Fermentable and non-fermentable carbon sources

Depending upon the mode of ATP generation, carbon sources can be divided into two categories: fermentable carbon sources and non-fermentable carbon sources. When bacteria are grown in fermentable carbon sources, they can generate energy by substrate-level phosphorylation and oxidative phosphorylation. Glucose, maltose, and glucosamine are examples of fermentable carbon sources. Whereas in the case of non-fermentable carbon sources, they generate energy only through oxidative phosphorylation. Thus, the growth of bacteria on non-fermentable carbon sources such as acetate, succinate, and fatty acids requires optimal functioning of ETC for energy production.

Microbial metabolism is classified based on its relationship with oxygen. Microorganisms that have adequate mechanisms to protect them from the oxidizing nature of molecular O_2 are adapted for their growth in the presence of oxygen. They use aerobic respiration as their principal form of energy production and are termed aerobes. The oxidizing nature of O_2 makes it a highly effective terminal electron acceptor during aerobic respiration. Some microorganisms, known as anaerobes, live in habitats where the oxygen is completely excluded as they lack the protective mechanisms against the harmful effects of O_2 (5). They follow anaerobic respiration using NO_3^-, SO_4^{2-}, and CO_2 as the terminal acceptor of electrons (6).

1.2 Aerobic metabolism in *E. coli*

Bacteria can use a wide variety of metabolites for energy production and change their mode of energy generation from one form to another. This is commonly found in facultative microorganisms like *Escherichia coli* that conduct aerobic as well as anaerobic respiration depending upon their growth conditions (7). In *E. coli*, the metabolism of the carbon sources like glucose and fatty acids occurs through a multi-step process involving a specific set of proteins and co-factors. In the initial step, glucose and fatty acids are degraded to acetyl-CoA (a two-carbon compound; 2C) by the process of glycolysis and β-oxidation pathway, respectively. The acetyl-CoA enters the tricarboxylic acid (TCA) cycle or to the glyoxylate cycle and gets metabolized to CO_2, and generates ATP and reduced co-factors (NADH and $FADH_2$). The reduced co-factors produced during the stages of metabolism are oxidized by the transfer of electrons to various electron acceptors in the ETC, resulting in the production of ATP (8, 9). The metabolism of various carbon sources converges into central metabolism at the level of the TCA cycle.

1.2.1 Electron transport chain (ETC) in *E. coli*

ETC comprises a series of electron carriers that are capable of oxidation and reduction reactions. In gram-negative bacteria, the respiratory complexes are present in the inner cell membrane, and most of the ATP during respiration is produced in the ETC. When electrons are passed through the ETC, proton motive force is generated, which is then used for the production of ATP (3). The NADH and $FADH_2$ produced during central metabolic reactions are oxidized by transferring electrons to Complex I (NADH dehydrogenases) and Complex II (succinate dehydrogenase), respectively. Electrons from the upstream respiratory dehydrogenases are then transferred to a lipid-soluble electron carrier, ubiquinone, which is capable of diffusing through the membranes (10). Ubiquinol, the reduced form of ubiquinone, donates electrons to the terminal oxidases, cytochrome *bo*, and cytochrome *bd* (11). The energy released during this series of redox reactions pumps protons across the membrane. The resultant electrochemical gradient causes diffusion of protons back into the cell through an integral membrane ATP synthase protein, driving ATP synthesis.

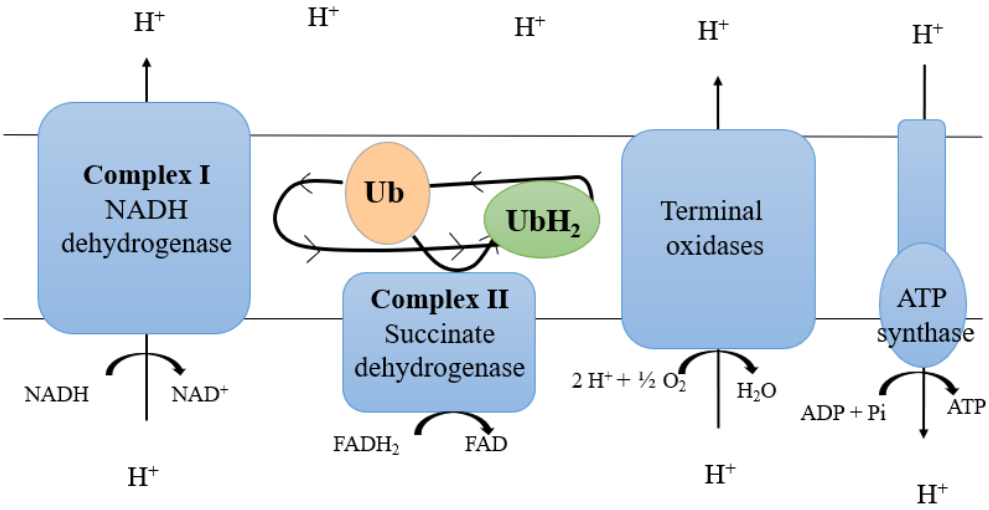

Figure 1.1 Aerobic electron transport chain in *E. coli*

The work presented in my thesis deals with the metabolism of one particular carbon source called long-chain fatty acid (LCFA), which is a rich source of metabolic energy. Several bacteria, including pathogens such as *Mycobacterium tuberculosis*, *Pseudomonas aeruginosa*, *Salmonella typhimurium* and *Vibrio cholerae* utilize host-derived LCFAs for their survival and virulence (12-15).

1.3 Long-chain fatty acid (LCFA) metabolism

Several bacteria use fatty acids as an important energy source and as essential components of the cellular membrane. Fatty acids are carboxylic acids with an un-branched aliphatic chain which are classified based on the number of carbon atoms present. LCFAs contain 12-20 carbon atoms, medium-chain fatty acid contains 5-11 carbon atoms, and short-chain fatty acids contain only 2-4 carbon atoms.

LCFA metabolism is catalysed by the proteins encoded by fatty acid degradation (*fad*) genes. The *fad* genes regulate the transport, activation, and degradation of fatty acids into acetyl-CoA. When LCFA is provided in the growth medium, an outer membrane transporter, FadL transports LCFA across the cell membrane. An inner membrane-associated acyl-CoA synthase converts LCFAs to acyl-CoA (acyl-activation step), which enters the β-oxidation pathway. The first step in the β-oxidation pathway is the FadE-mediated conversion of acyl-CoA to enoyl-CoA. The remaining steps of fatty acid degradation involve hydration, oxidation, and thiolytic cleavage performed by FadA and FadB tetrameric complex. Each cycle of the β-oxidation pathway releases an acetyl-CoA, thereby reducing the acyl-CoA length by two carbon atoms. The acetyl-CoA is further degraded by TCA and glyoxylate cycles, and the shortened acyl-CoA re-enters the β-oxidation pathway. The reduced cofactors NADH and $FADH_2$ produced during the β-oxidation pathway and TCA cycle enter the ETC and generate ATP by oxidative phosphorylation (16, 17).

The LCFA degradation and its biosynthetic pathways has to be regulated by switching on and off in order to maintain lipid homeostasis (18). There are three regulation systems which control *fad* genes at the transcriptional level:

(i) FadR, a transcriptional regulator exerts negative regulation on *fad* genes. Binding of acyl-CoA, a metabolic intermediate of LCFA degradation pathway, makes FadR incapable of binding to DNA, thus relieving its repression (19, 20).

(ii) The cyclic AMP receptor protein (CRP) - cyclic AMP (cAMP) complex, exerts positive regulation on *fad* genes (8). In enteric bacteria like *E. coli*, when glucose is available in the growth medium, the level of phosphorylation of enzyme IIA^{Glc}, an enzyme involved in glucose transport, is reduced. It is proposed that the activation of adenylate cyclase, an enzyme involved in cAMP synthesis, requires the phosphorylated form of enzyme IIA^{Glc}, and thus in the presence of glucose, the levels of cAMP are lowered (21, 22). Glucose also reduces the levels of CRP by exerting autoregulation on the *crp* gene (23-26) .

iii) The anoxic redox control ArcA-ArcB two component system is comprised of a sensor kinase ArcB and a response regulator ArcA. When there is a reduction in oxygen level, the transmitter and receiver domains of ArcB undergo autophosphorylation and transfer the phosphate group to ArcA. The phosphorylated ArcA acts as a negative regulator of many genes including *fadB* and *fadE* (27).

1.4 Role of ubiquinone during LCFA metabolism

Ubiquinone, a lipid-soluble electron carrier in the ETC involves in the shuttling of electrons from respiratory dehydrogenases to the terminal oxidases. Besides its pivotal role in metabolism, ubiquinone is also involved in an essential cellular process, i.e., disulfide bond formation in the secreted proteins which occurs in the cell envelope. In *E. coli*, the oxidising nature of envelope favours the formation of disulfide bonds between the thiol groups of cysteines. The process of disulfide bond formation is aided by the disulfide bond forming machinery which gets reduced upon the uptake of electrons from the thiol group of substrate proteins. The machinery is re-oxidised by the transfer of electrons to ubiquinone in the ETC. As electrons from carbon metabolism and disulfide bond formation converge at the level of ubiquinone, metabolic conditions that increase electron flow in the ETC are likely to render ubiquinone limiting for the uptake of electrons from the disulfide bond forming machinery (28). Previous published work from our lab has shown that LCFA metabolism produces a large number of reduced cofactors (NADH and $FADH_2$). Oxidation of these reduced co-factors by respiratory dehydrogenases causes an increased electron flow in the ETC (29). Therefore, LCFA metabolism represents an instance in which the requirement of ubiquinone is high.

Additionally, the work from our lab has shown that during the growth of *E. coli* in oleate (C18:1 cis-9), a representative LCFA, ubiquinone level increases ~ 2-fold. Previously, it was believed that ubiquinone is present in excess over other ETC components. Thus, under normal conditions, ubiquinone is sufficient for its electron transfer function. However, the increased accumulation of ubiquinone suggests a physiological condition where ubiquinone might be limiting and highlights an important role of ubiquinone during LCFA utilization (29). But the mechanism by which ubiquinone is upregulated during LCFA metabolism is completely unknown and this forms the focus of my thesis project. Thus, below I provide a brief overview of the ubiquinone biosynthesis pathway and the known regulation on ubiquinone.

1.5 Quinones in *E. coli*

Depending on the type of central carbon ring, quinones in *E. coli* are of two types: benzoquinone (a derivative of benzene ring) and naphthoquinones (structurally similar to naphthalene). Ubiquinone-8 (Q_8) is a benzoquinone, whereas demethylmenaquinone-8 (DMQ_8) and menaquinone-8 (MQ_8) are naphthoquinones. MQ_8 and DMQ_8 are predominant during anaerobic electron transport, while under aerobic conditions Q_8 is predominant and MQ_8 is very low (30). In my project, I have focused on aerobic metabolism of LCFAs in *E. coli*, therefore, I will be discussing about ubiquinone, its biosynthesis and regulation aspects.

1.5.1 Ubiquinone biosynthesis in *E. coli*

Ubiquinone biosynthesis is a multistep process involving prenylation, decarboxylation, and three alternating hydroxylation and methylation reactions carried out by 12 *ubi* genes. The quinoid nucleus of the ubiquinone is formed from the shikimate pathway via chorismate (31). The first committed step is the removal of pyruvate from chorismate to produce 4-hydroxybenzoate (4-HB) catalyzed by UbiC (32). The 4-HB is prenylated by the membrane-bound UbiA using the side chain precursor octaprenyl diphosphate, formed by the methylerythritol phosphate pathway in *E. coli* (31, 32). *E. coli* contains Q_8 as the major quinone with minor amounts of Q_1 to Q_7 and Q_9 (In Q_n, 'n' denotes the number of prenyl units present in the side chain). The length of the predominant chain is constant depending on the species (31).

The prenylated form of 4-HB called octaprenyl-4-hydroxybenzoate (OHB) undergoes decarboxylation by the UbiD-UbiX system to form 2-octa-prenyl phenol (OPP). The enzymes, UbiG to UbiK, which catalyze the last six reactions of the pathway, i.e., the alternating hydroxylations and methylations, form a Ubi complex. The Ubi complex also contains UbiK-UbiJ (2:1) heterotrimer that presents the ubiquinone intermediates to Ubi enzymes in the multiprotein complex (32). Hydroxylation reaction occurs at C-5, C-1, and C-6 by the monooxygenases UbiI, UbiH, and UbiF, respectively (31, 32). The three methylation reactions occur by the addition of methyl groups at 6-OH, C-3, and 5-OH (two are on O and one at C). The non-specific enzyme UbiE methylates the ring at the C-3 position and the O-methylations are catalyzed by UbiG at 6-OH and 5-OH. The methyl donor is S-Adenosylmethionine (SAM) which itself gets converted to S-adenosylhomocysteine (SAH) (31). Another important accessory protein involved in Q_8 biosynthesis is UbiB, which is hypothesized to be involved in

making the Q_8 precursors available for the biosynthetic enzymes by extracting them from the membrane, coupling with ATP hydrolysis (32).

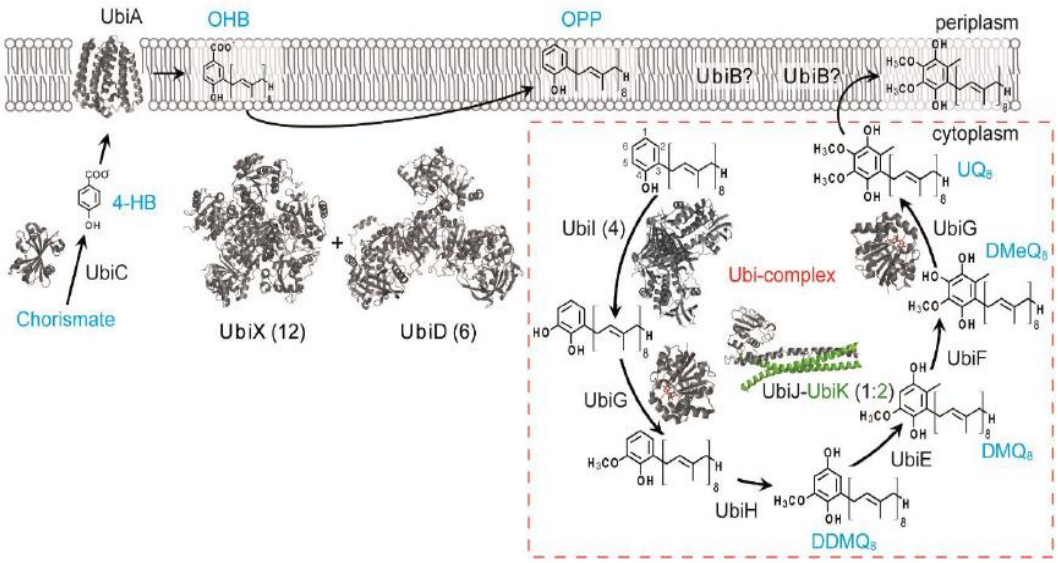

Figure 1.2 Model of ubiquinone biosynthesis in *E. coli* (32)

1.5.2 Regulation on ubiquinone biosynthesis genes

The expression of *ubi* genes in *E. coli* is regulated depending on the growth conditions like shift from aerobic environment to anaerobic environment, variations in carbon-source supplied in media, etc. Till date, the majority of the studies have investigated the regulation on ubiquinone at a transcriptional level. Earlier studies using transcriptional reporter constructs have shown that *ubiCA* genes are regulated as an operon with a promoter upstream of *ubiC* gene, whereas *ubiA* gene lacks its own promoter. An increased expression (~ 2-3-fold) of this promoter was found in oxidizable carbon sources such as succinate, pyruvate, acetate, and lactate compared to fermentable carbon source like glucose (33). Expression of *ubiD* and *ubiX* was also found to be higher in succinate, followed by glycerol, and then glucose in aerobic conditions (34). ArcA and Fnr are global transcription factors, regulating genes depending on oxygen availability. Under anaerobic conditions, *arcA* mutation increased *ubiCA* gene expression (25%-50%), while *fnr* mutation did not affect the expression of *ubiCA* (33). Mutation in *fnr*, *arcA*, or both increased the expression of *ubiD* (2.5- 3-fold) and *ubiX* (1.5-fold). Since no enhancement of inhibition was observed in a mutant carrying deletion of the two regulators, it indicated a common mechanism of regulation. Moreover, the study revealed that in a strain devoid of *hemA*, an enzyme involved in porphyrin biosynthesis, expression of

ubiX increased 2-fold in aerobic and 3.5-fold in anaerobic conditions, whereas the expression of *ubiD* did not change in either of the conditions (34). Another pathway governing the anaerobic respiratory gene expression in *E. coli* in response to nitrate and nitrite is NarX-NarL system, where NarX is a membrane-bound sensor kinase and NarL is the response regulator. Interestingly, the *ubiCA* gene expression has been shown to be increased (50%) in *narXL* mutant (33). Studies have shown that the aerobiosis induced ubiquinone and anaerobiosis induced menaquinone levels are not affected despite the inhibition of transcription by chloramphenicol, suggesting that quinone levels are regulated at a post-transcriptional level (30).

Table 1.1 Regulation on *ubi* genes by various transcriptional regulators.

Regulator	***ubi* genes and the effect**
ArcA-ArcB two-component system	*ubiCA* (-) (33), *ubiD* (-), and *ubiX* (-) (34)
FNR (fumarate and nitrate reduction regulatory protein)	*ubiD* (-), and *ubiX* (-) (34)
CRP (cAMP receptor protein)	*ubiCA* (+) (35), and *ubiG* (+) (35)
NarL (nitrate/nitrite response regulator)	*ubiCA* (-) (33)
HemA (enzyme involved in heme synthesis)	*ubiX* (-) (34)

(+) indicates positive regulation and (-) indicates negative regulation

Chapter 2

Materials and Methods

2.1 Materials

2.1.1 Chemicals, Reagents and their sources

Bacteriological agar, tryptone and yeast extract were purchased from BD Difco™. NH_4Cl, NaCl, KCl, $MgSO_4$, $MgCl_2$, Na_2HPO_4, KH_2PO_4, K_2HPO_4, $CaCl_2$, glycerol, sodium oleate, sodium citrate, ethanol, ethylenediaminetetraacetic acid (EDTA), 2-mercaptoethanol (β-ME), Brij-58 and 2-nitrophenyl-β-D-galactopyranoside (ONPG) were purchased from Sigma. Agarose, Tris-base and EtBr were from Affymetrix USB product. Primers were ordered from GCC Biotech. Plasmid mini prep kit, and SV Gel and PCR Clean-Up System used were from Promega. NEB Buffers, dNTPs, 100bp/1Kb ladder, Enzymes [Taq/Phusion DNA Polymerase, restriction enzymes, T4 DNA ligase and Calf Intestinal Alkaline Phosphatase (CIP)] were purchased from New England Biolabs.

2.1.2 Bacterial strains and plasmids

Table 2.1 List of strains used in this study

Strains	**Genotype**	**Source/ Reference**
BW25113	*lac*I^q, *rnB*T14, Δ*lacZ*WJ16, *hsdR514*, Δ*araBAD*AH33, Δ*rhaBAD*LD78	Genetic Stock Center (36)
BW25141	*lacI^q rrnB3 ΔlacZ4787 hsdR514* Δ(*araBAD*)*567* Δ*(rhaBAD*)*568* Δ*phoBR580 rph-1 galU95* Δ*endA9 uidA*(Δ*MluI*)*::pir*(*wt*) *recA1*	Rao lab (37)
BW25142	*lacI^q rrnB3* Δ*lacZ4787 hsdR514* Δ(*araBAD*)*567* Δ(*rhaBAD*)*568* Δ*phoBR580 rph-1 galU95* Δ*endA9 uidA*(Δ *MluI*)*::pir-116 recA1*	Rao lab (37)
CR700	*attλ*::[Kan *marR*'*-yfp -lacZ oriR6K*], Kan^r (Single integrant control	Rao lab (38)
RC12054	Multiple integrant control	Chaba lab
RC15080	BW25113 *attλ*::pAH125	Chaba lab

RC19006	BW25113 *attλ*::[Kan P_{ubiH} -*lacZ oriR6K*], Kan^r	This work
RC19041	BW25113 *attλ*::[Kan P_{ubiB} -*lacZ oriR6K*], Kan^r	This work
RC19042	BW25113 *attλ*::[Kan P_{ubiE} -*lacZ oriR6K*], Kan^r	This work
RC19043	BW25113 *attλ*::[Kan P_{ubiF} -*lacZ oriR6K*], Kan^r	This work
RC19044	BW25113 *attλ*::[Kan P_{ubiI} -*lacZ oriR6K*], Kan^r	This work
RC19045	BW25113 *attλ*::[Kan P_{ubiJ} -*lacZ oriR6K*], Kan^r	This work
RC19046	BW25113 *attλ*::[Kan P_{ubiK} -*lacZ oriR6K*], Kan^r	This work
RC19048	BW25113 *attλ*::[Kan P_{ubiC} -*lacZ oriR6K*], Kan^r	This work
RC19051	BW25113 *attλ*::[Kan P_{ubiX} -*lacZ oriR6K*], Kan^r	This work
RC19056	BW25113 *attλ*::[Kan P_{ubiG} -*lacZ oriR6K*], Kan^r	This work
RC19055	BW25113 *attλ*::[Kan P_{ubiD} -*lacZ oriR6K*], Kan^r	This work
RC19033	P1 (BW25113 *fadR*::Cam) x RC19006, Kan^r	This work
RC19052	P1 (BW25113 *fadR*::Cam) x RC19042, Kan^r	This work
RC19053	P1 (BW25113 *fadR*::Cam) x RC19043, Kan^r	This work
RC19057	P1 (BW25113 *fadR*::Cam) x RC19046, Kan^r	This work
RC19061	P1 (BW25113 *fadR*::Cam) x RC19048, Kan^r	This work
RC19062	P1 (BW25113 *fadR*::Cam) x RC19055, Kan^r	This work
RC19063	P1 (BW25113 *fadR*::Cam) x RC19051, Kan^r	This work
RC15125	pMS10 in BW25142	Chaba lab
RC19012	pLZ01 in BW25141	This work
RC19013	pLZ02 in BW25141	This work
RC19014	pLZ03 in BW25142	This work
RC19018	pLZ07 in BW25141	This work
RC19019	pLZ06 in BW25142	This work
RC19021	pLZ05 in BW25141	This work

RC19026	pLZ08 in BW25142	This work
RC19029	pLZ10 in BW25141	This work
RC19030	pLZ11 in BW25142	This work
RC19034	pLZ12 in BW25141	This work

Table 2.2 List of plasmids used in this study

Plasmids	**Description**	**Reference**
pAH125	*oriR6K*, MCS-*lacZ t0 attλ* Kan^r	Rao lab (37)
pINT-ts	*oriR6K* int Amp^r	Rao lab (37)
pMS10	*oriR6K*, MCS P$_{ubiH}$-*lacZ t0 attλ* Kan^r	Chaba lab
pLZ01	*oriR6K*, MCS P$_{ubiB}$-*lacZ t0 attλ* Kan^r	This work
pLZ02	*oriR6K*, MCS P$_{ubiE}$-*lacZ t0 attλ* Kan^r	This work
pLZ03	*oriR6K*, MCS P$_{ubiI}$-*lacZ t0 attλ* Kan^r	This work
pLZ05	*oriR6K*, MCS P$_{ubiF}$-*lacZ t0 attλ* Kan^r	This work
pLZ06	*oriR6K*, MCS P$_{ubiJ}$-*lacZ t0 attλ* Kan^r	This work
pLZ07	*oriR6K*, MCS P$_{ubiK}$-*lacZ t0 attλ* Kan^r	This work
pLZ08	*oriR6K*, MCS P$_{ubiC}$-*lacZ t0 attλ* Kan^r	This work
pLZ10	*oriR6K*, MCS P$_{ubiX}$-*lacZ t0 attλ* Kan^r	This work
pLZ11	*oriR6K*, MCS P$_{ubiG}$-*lacZ t0 attλ* Kan^r	This work

pLZ12	*oriR6K*, MCS P_{ubiD}-*lacZ t0 attλ* Kan^r	This work

2.1.3 Primers

Table 2.3 List of primers used in this study

Primers	Sequence (5'-3')	Purpose
MS72	ACCGGGTACC ATCAACTACTTAACGGCCTGG	Forward primer for cloning *ubiH cis*-acting element (1000 bp upstream of *ubiH*) in pAH125
MS73	CGTGAATTC CCGACGATGATTACGCTCAT	Reverse primer for cloning *ubiH cis*-acting element (1000 bp upstream of *ubiH*) in pAH125
LZ1	ACCGGGTACCAGCTTACCGCACTGATTC	Forward primer for cloning *ubiB cis*-acting element (350 bp upstream of *ubiB*) in pAH125
LZ2	CGTGAATTCACTTCACCTGGCGTCAT	Reverse primer for cloning *ubiB cis*-acting element (350 bp upstream of *ubiB*) in pAH125
LZ3	ACCGGGTACCTCAAAGTCTCGACAAAGC	Forward primer for cloning *ubiE cis*-acting element (350 bp upstream of *ubiE*) in pAH125
LZ4	CGTGAATTCTTCTTGTGACTTATCCACCAT	Reverse primer for cloning *ubiE cis*-acting element (350 bp upstream of *ubiE*) in pAH125
LZ5	ACCGGGTACCACGCCGATAATCAGGTCT	Forward primer for cloning *ubiF cis*-acting element (400 bp upstream of *ubiF*) in pAH125

LZ6	CGTGAATTCCGTTGGTTGATTTGTCAT	Reverse primer for cloning *ubiF cis*-acting element (400 bp upstream of *ubiF*) in pAH125
LZ7	ACCGGGTACCAAACTCTGCACCCGATTG	Forward primer for cloning *ubiI cis*-acting element (350 bp upstream of *ubiI*) in pAH125
LZ8	CGTGAATTCGCTACATCAACACTTTGCAT	Reverse primer for cloning *ubiI cis*-acting element (350 bp upstream of *ubiI*) in pAH125
LZ9	ACCGGGTACCTTTGGTCTGCGTAACGTC	Forward primer for cloning *ubiJ cis*-acting element (350 bp upstream of *ubiJ*) in pAH125
LZ10	CGTGAATTCCACTAAAGGTTTAAAAGGCAT	Reverse primer for cloning *ubiJ cis*-acting element (350 bp upstream of *ubiJ*) in pAH125
LZ11	ACCGGGTACCTTACCAGAATCAGGGCAG	Forward primer for cloning *ubiK cis*-acting element (350 bp upstream of *ubiK*) in pAH125
LZ12	CGTGAATTCTTTTTCGGGTCAATCAT	Reverse primer for cloning *ubiK cis*-acting element (350 bp upstream of *ubiK*) in pAH125
LZ13	ACCGGGTACCAAGTTGATGAAATTCGCC	Forward primer for cloning *ubiX cis*-acting element (350 bp upstream of *ubiX*) in pAH125
LZ14	CGTGAATTCCCTACAATGAGTCGTTTCAT	Reverse primer for cloning *ubiX cis*-acting element (350 bp upstream of *ubiX*) in pAH125
LZ15	ACCGGTCGACATGAAGATCTCGATGGTTATATC	Forward primer for cloning *ubiC cis*-acting element (1000 bp upstream of *ubiC*) in pAH125

LZ16	CGTGGTACCAACGCGGGGTGTGACAT	Reverse primer for cloning *ubiC cis*-acting element (1000 bp upstream of *ubiC*) in pAH125
LZ17	ACCGGGTACCAGATTAAGCAATAGCATGG	Forward primer for cloning *ubiD cis*-acting element (600 bp upstream of *ubiD*) in pAH125
LZ18	CGTGAATTCATATTTCATGGCGTCCAT	Reverse primer for cloning *ubiD cis*-acting element (600 bp upstream of *ubiD*) in pAH125
LZ19	ACCGGGTACCTTATAGGCTTTGTTCCAG	Forward primer for cloning *ubiG cis*-acting element (350 bp upstream of *ubiG*) in pAH125
LZ20	CGTGAATTCGATTTTTCGGCATTCAT	Reverse primer for cloning *ubiG cis*-acting element (350 bp upstream of *ubiG*) in pAH125
GA22	GGCATCACGGCAATATAC	Forward primer for confirmation of single copy integration of reporter plasmid, specific for *E. coli* chromosome *attλ* site
GA23	ACTTAACGGCTGACATGG	Forward primer for identifying multiple-copy integration, specific to pAH125 plasmid
GA25	TCTGGTCTGGTAGCAATG	Reverse primer for confirmation of single copy integration of reporter plasmid, specific for *E. coli* chromosome *attλ* site
GA29	TGCGAGGCTTTGTGCTTC	Reverse primer for identifying multiple-copy integration, specific to pAH125 plasmid

Restriction sites are underlined.

2.1.4 Antibiotics

Table 2.4 List of antibiotics and their concentration

Antibiotic	Stock concentration	Working concentration
Kanamycin	30 mg/mL	30 µg/mL
Ampicillin	100 mg/mL	100 µg/mL

The antibiotic stocks mentioned in the above table were made in autoclaved MQ water and filter-sterilized using 0.22-micron filters (Millipore). Aliquoted 500 µL to 1.5 mL MCTs and stored at -20°C.

2.1.5 Media Composition

a) Lysogeny Broth Media

Table 2.5 Composition of LB Media

Composition	Amount (L^{-1})
Tryptone	10 g
Yeast Extract	5 g
NaCl	5 g

Dissolved the above given LB components in 1000 mL of Milli-Q (MQ) water and autoclaved at 121°C for 15 minutes at 15 lb/inch^2 pressure.

b) LB Agar

LB agar was made by adding 1.5% (w/v) agar to the LB Broth composition followed by autoclaving. LB agar was poured into sterile petri plates (Tarsons) after cooling the media to about 50°C (antibiotic added if necessary). Stored the plates at 4°C till use.

c) TYE (Tryptone Yeast Extract) Agar

Table 2.6 Composition of TYE-agar

Composition	Amount (L^{-1})
Tryptone	10 g
Yeast extract	5 g
NaCl	8 g
Agar	15 g

Components were dissolved in 1000 mL MQ water and autoclaved. TYE agar was prepared by supplementing TYE broth with 1.5% agar before autoclaving. TYE agar plates were prepared by cooling the molten TYE agar media to about 50°C (bearable to touch) and pouring into sterile petriplates (Tarsons). Whenever required, kanamycin was added (final concentration of 10 μg/ml) to molten agar media cooled to about 50°C before pouring into petriplates. Plates were stored at 4°C till use.

d) SOB (Super Optimal Broth) Media

Table 2.7 Composition of SOB Media

Composition	Amount (L^{-1})
Tryptone	20 g
Yeast extract	5 g
NaCl	0.585 g
KCl	1.85 g

Dissolved the above given SOB components in 1000 mL of MQ water and autoclaved it.

e) SOC (Super Optimal Broth with Catabolite repression) Media

SOC was made by adding 20 mM glucose and 10 mM $MgCl_2$ to SOB.

f) TBK (Tryptone Broth supplemented with KCl) Media

Table 2.8 Composition of 2X TBK Media

Composition	Amount (L^{-1})
Tryptone	20 g
KCl	10 g

Dissolved the above given TBK components in 1000 mL of MQ water and autoclaved it.

g) 5% Brij-58

Added 5 g of Brij-58 to 70 mL MQ water and dissolved it using magnetic stirrer. The final volume was then made up to 100 mL and autoclaved it.

2.1.6 Preparation of carbon source, buffers and solutions used in β-galactosidase assay

a) Carbon source

Preparation of Na-Oleate
50 mM Na-Oleate was made by dissolving 760 mg of Na-Oleate in 50 mL autoclaved 5% Brij-58 at room temperature. Filter-sterilized the solution and then stored at -20°C.

b) Substrate

Substrate must be prepared fresh for every assay. Dissolved 4 mg of ONPG (Ortho-nitrophenyl-β-D-galactopyranoside) in 1 mL autoclaved MQ water.

c) Z-buffer

Table 2.9 Composition of Z-buffer

Composition	Amount(L^{-1})
Na_2HPO_4 (anhydrous)	8.52 g
$NaH_2PO_4.H_2O$	5.5 g
NaCl/KCl	0.75 g

$MgSO_4.7H_2O$	0.246 g

Dissolved the Z-buffer components in 1L autoclaved MQ water and the pH was adjusted to 7.

d) 0.1% SDS (Sodium dodecyl sulphate)

Dissolved 0.1 g of SDS in 100 mL autoclaved MQ and stored at room temperature.

e) Stop solution

10.6 g of Na_2CO_3 was dissolved in 100 mL autoclaved MQ water and stored at room temperature.

f) TBK Media for β-galactosidase assay

Table 2.10 Composition of TBK-Brij and TBK-Oleate media

Composition	Working concentration	TBK-Brij (15 mL)	TBK-Oleate (15 mL)
2X TBK	1X	7.5 mL	7.5 mL
10X Phosphate	10 mM	1.5 mL	1.5 mL
Brij (5%)	0.5%	1.5 mL	-
50 mM Na-Oleate	5 mM	-	1.5 mL
Autoclaved MQ	-	4.5 mL	4.5 mL

2.1.7 Buffers and solutions used in Agarose Gel Electrophoresis

a) 6X DNA Loading Buffer (stock)

Table 2.11 Composition of 6X DNA loading buffer

Composition	Amount (50 mL)
Bromophenol blue	125 mg (0.25% w/v)
Sucrose	20 g (40.0% w/v)

b) 50X TAE (Tris base, acetic acid and EDTA) buffer (stock)

Table 2.12 Composition of 50X TAE buffer

Composition	Amount (L^{-1})
Tris base	242 g
Glacial acetic acid	57.1 mL
Tetrasodium EDTA (USB, final concentration, 0.5M)	146 g

Dissolved the components in 800 mL MQ water, adjusted the pH to 8.0 and made up the volume to 1L with MQ water. 50X TAE buffer was diluted to 1X with MQ water for use and can be stored at room temperature.

2.2 Methods

2.2.1 Glycerol stock preparation

Overnight inoculation of the required bacterial strain was set-up at 37°C in 3 mL LB containing antibiotic (if necessary). 700 µL of the primary culture was added to a labelled sterile cryotube containing 300 µL of 50% autoclaved glycerol (15% final concentration). The cryotube was stored at -80°C.

2.2.2 Plasmid Isolation

Strains containing the desired plasmid was inoculated in 5 mL LB with appropriate antibiotic (primary culture). The culture was grown overnight (12-16 hours) in a roller drum at 37°C. Plasmid isolation was done using Promega miniprep kit. Cells were pelleted at 8000 rpm for 2 min using MiniSpin centrifuge. Plasmids were isolated using Promega mini prep kit following manufacturer's instruction.

Isopropanol method, another method for plasmid isolation, was performed when a high concentration of plasmid was required for the integration of plasmid to the chromosomal DNA of *E. coli*. Slightly modified version of the plasmid isolation using Promega kit was used for plasmid isolation using isopropanol method. Briefly, the culture was pelleted, resuspended, lysed and neutralised using the buffers provided with the Promega kit and centrifuged at 13,400

rpm for 5 minutes. 750 µL of the supernatant was transferred to a 1.5 ml MCT, followed by precipitation of plasmid DNA using -80°C chilled isopropanol (1:1 ratio of supernatant to isopropanol). The DNA was pelleted at 13,400 rpm for 5 min and the supernatant was discarded. The pellet was washed with 700 µL of 70% ethanol at 13,400 rpm for 5 min. Finally, the dried pellet was resuspended in 50 µL of nuclease free water (NFW).

2.2.3 Colony PCR for amplification of the *cis* acting-element of various *ubi* genes

The PCR reaction mix and the program used are given below:

Table 2.13 Composition of PCR reaction mix

PCR components	Working concentration	Volume for 10 µL reaction
Water	-	7.25 µL
Taq buffer (10X)	1X	1 µL
dNTPs (10 mM)	0.2 mM	0.2 µL
Template (colony suspended in autoclaved water)	-	1 µL
Forward primer (10 µM)	25 U/mL	0.25 µL
Reverse primer (10 µM)	0.25 µM	0.25 µL
Taq polymerase (5000 U/mL)	0.25 µM	0.05 µL

PCR reaction cycle

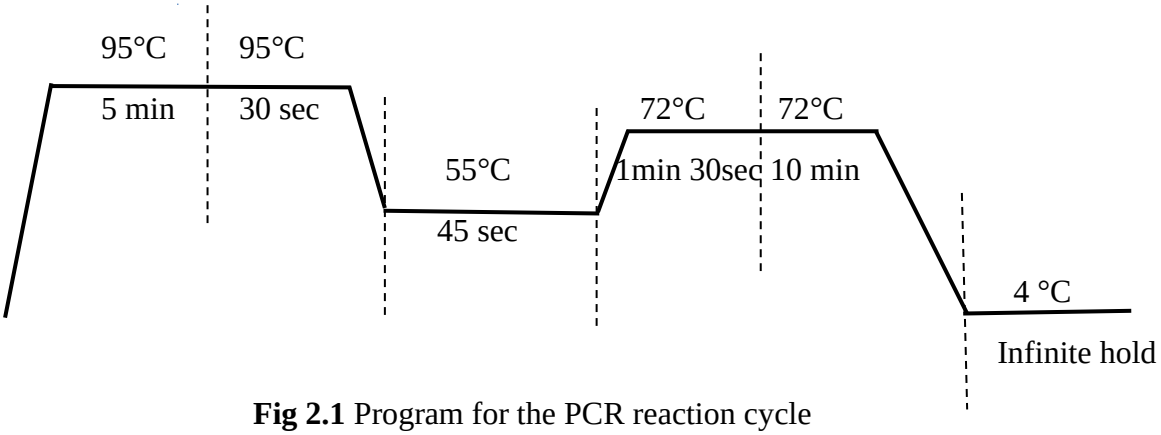

Fig 2.1 Program for the PCR reaction cycle

2.2.4 Restriction digestion of vector and insert DNA

Around 1.5 µg insert (amplified *ubi cis*-acting element) and 2.5 µg vector (pAH125) were digested with *Kpn*I (HF) and *Eco*RI (HF). Samples were incubated for 3 hours at 37°C in water bath.

Table 2.14 Composition of restriction digestion mix

Components	**Insert (50 µL)**	**Vector (50 µL)**
10X NEB Buffer (4)	5 µL	6 µL
*Kpn*I (restriction enzyme)	1 µL	1.5 µL
*Eco*RI (restriction enzyme)	1 µL	1.5 µL
Template	Added accordingly (1.5 µg of DNA)	Added accordingly (2.5 µg of DNA)
Autoclaved MQ	Make up to 50 µL	Make up to 50 µL

Further, 1 µL CIP was added to the digested vector and incubated at 37°C in water bath for 2 hours. Gel purification was done using Promega kit following manufacturer's instruction protocol.

2.2.5 Ligation of insert with vector using T4 DNA ligase

The digested insert and vector were ligated in 1:3 ratio using T4 DNA ligase and incubated at 25°C for 2 hours. 2 µL of pre-ligation mix (before adding ligase enzyme) was taken and run alongside ligation mix sample to ascertain whether ligation has taken place.

Table 2.15 Composition of ligation mix

Components	**Test (20 µL)**	**Self (10 µL)**
H_2O	Make up to 20 µL	Make up to 10 µL
10X Ligation Buffer	2 µL	1 µL
Vector	Added accordingly (100 ng of DNA)	Added accordingly (100 ng of DNA)
Insert	Added accordingly	-
10X T4 Ligase enzyme	2 µL	0.5 µL

2.2.6 Preparation of electrocompetent *E. coli*

The desired *E. coli* strain was cultured overnight at 37°C in 3 mL LB containing antibiotic (if necessary). Secondary culture was set-up with initial $O.D_{600}$ ~0.01 in LB at 37°C shaker at 220 rpm. The culture was taken out when $O.D_{600}$ reached 0.5 and chilled on ice for 15-20 minutes. The culture was transferred to a falcon and pelleted at 4°C for 10 minutes at 4000 rpm. The supernatant was discarded and the pellet was dissolved in equal volume of pre-chilled autoclaved water. The cells were pelleted at 4°C for 10 minutes at 4000 rpm. Similar washing and pelleting were done again with half-the volume of pre-chilled autoclaved water from the previous step. The remaining washings were done with pre-chilled 10% glycerol with half the volume of previous step for 3 times. After the final wash, the pellet was re-suspended in appropriate volume of pre-chilled 10% glycerol. 60 µL cells were aliquoted to pre-chilled MCTs and stored at -80°C.

A slight modification of this protocol was followed for the electrocompetent cell preparation of pINT (encoding for phage integrase enzyme) transformed BW25113 strain, used for the integration of plasmid in the chromosomal DNA of *E. coli*. Secondary cultures with initial $O.D_{600}$ ~0.01 were set-up in SOB containing 10 mM $MgCl_2$, 10 mM $MgSO_4$, and ampicillin (working concentration 100 µL/mL). Cultures were incubated at 30°C until $O.D_{600}$ reached ~0.05. Cultures were incubated on ice for 15 min and then at 42°C for 15 min. Final washings were done like the above written protocol.

2.2.7 Electroporation for the transformation of plasmid

The electro-cuvette was washed with distilled water and 100% ethanol, and dried in a laminar air flow hood to get rid of any remaining droplets. The electrocompetent cells were thawed on ice to which 70-100 ng of plasmid was added and then transferred to 0.1 cm electro-cuvette. 1.8 kV pulse was passed through the electro-cuvette using Biorad Gene Pulser X cell and 1 mL LB was added to the cuvette immediately. The sample was transferred to 1.5 mL MCT and incubated at 37°C for 60 minutes in a roller drum. The sample was centrifuged at 6000 rpm for 5 min. The pellet was resuspended in 100 µL LB and plated on an LB plate containing suitable antibiotic. The plates were incubated at 37°C for 16-18 hours. In case of pINT transformation in BW35113 *E. coli* strain, 1-hour incubation was done at 30°C in a roller drum.

For the integration of plasmid in the chromosomal DNA, the plasmid isolation was done using isopropanol method. The plasmid was then transformed in BW25113 containing pINT. After electroporation, recovery was done using 1mL SOC. The suspension was initially incubated at

37°C for 1 hour followed by incubation at 42°C for 30 minutes (for integrase expression and plasmid clearance). Sample was plated on TYE plate containing suitable antibiotic.

2.2.8 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 110 volts and was observed using UV trans-illuminator or Gel documentation system from BioRad.

2.2.9 P1 Lysate preparation

The primary culture was set up in 3 mL LB at 37°C overnight. The primary culture was sub-cultured in 15 mL LB such that the initial OD_{600} was ~0.01. The secondary culture was incubated at 37°C with shaking at 220 rpm until OD_{600} reached 0.2. $CaCl_2$ (1M stock) was added to the flask at a final concentration of 10 mM. P1 lysate (150 µL) was added to only the test cultures and not to the control. The cultures were incubated at 37°C with shaking at 220 rpm for 4-6 hours until complete lysis occurred. 500 µL chloroform was added. Lysed cultures were centrifuged at 5000 rpm for 10 min. The lysate was stored at 4°C in a falcon containing 500 µL chloroform.

2.2.10 P1 Transduction

P1 transduction was done with slight modifications to the protocol mentioned in (39). 1 mL of overnight culture was pelleted at 6000 rpm for 5 minutes and resuspended in P1 salt buffer containing 985 µL autoclaved MQ, 10 mM $CaCl_2$ and 5 mM $MgSO_4$. 100 µL resuspended cells were transferred to MCTs and P1 lysate was added (0 µL for control sample, and 60 µL for the test sample). The samples were incubated at 37°C in water bath for 30 minutes. Post incubation, 1 mL LB containing 10 mM Na citrate was added to the samples and incubated for 1 hour at 37°C in water bath. The cells were pelleted at 6000 rpm for 3 min and given 2 washes with LB. The cells were resuspended in 100 µL LB and plated on TYE plate supplemented with 10 mM Na citrate and suitable antibiotic. The plates were incubated at 30°C for 16-18 hours. The obtained transductant colonies were re-streaked and plates were incubated at 37°C. Two transductant colonies were selected for each parent strain and then preserved as glycerol stock.

2.2.11 β-galactosidase assay

A single colony of desired strain 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} ~0.01 in 15 mL TBK media with

desired carbon sources and incubated at 37ºC till the desired phase of growth 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 were then washed ~3 times using Z-buffer prepared. The washings are done to get rid of Brij-58 interference. After washing, the culture was normalised to OD_{450} ~ 0.5. The OD_{450} of the normalised culture was again recorded. The following reaction for the assay was set up in test tubes at 30ºC. 2-mercaptoethanol was added to Z-buffer. The other reagents were added using dropper.

Table 2.16 Composition for β-galactosidase assay

Reaction components	**Amount**
Z-buffer + β-ME (2.7 µL/mL)	500 µL
0.1 % SDS	1 drop
Chloroform	2 drops
Sample (culture normalised)	500 µL

The test tube with the reaction mix were vortexed and then kept at 30ºC for 15 minutes. 200 µL of substrate ONPG was added in each reaction mix and the time of addition was recorded. The stop solution (500 µL) was added when the yellow colour develops. 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:

$$1\ Miller\ Unit = \frac{1000 \times O.D_{420} - (1.75 \times O.D_{550})}{t \times v \times O.D_{450Normalised}}$$

Where, v = volume of culture used for assay in mL, t = reaction time in minutes

Chapter 3

Results and discussion

3. Results and Discussion

My project aim was to understand the regulation behind ubiquinone upregulation during LCFA metabolism in *E. coli*. Published work from our lab has shown that the total ubiquinone content increases ~2 fold in oleate-utilizing *E. coli*. The overall increase in ubiquinone levels can be a consequence of regulation at any of the transcriptional, post-transcriptional, or translational level. For this study, my objective was to investigate the transcriptional regulation on genes involved in ubiquinone biosynthesis (*ubi* genes). I first created transcriptional *lacZ* reporter constructs of the *ubi* genes and then performed β-galactosidase assay. I further checked whether these genes are under the transcriptional regulation of FadR, a known repressor of *fad* genes involved in LCFA metabolism.

Given below are the steps followed for the construction of transcriptional reporter strains for all the 12 *ubi* genes using the protocol as mentioned in Haldimann & Wanner (37).

Amongst the 12 genes involved in ubiquinone biosynthesis, some of these *ubi* genes are present as a single unit on the chromosome, whereas many of them are part of the operon. In this study, I have taken the upstream region of each individual *ubi* gene while making transcriptional reporter construct in order to cover the presence of any internal promoter that might be induced during LCFA metabolism.

3.1 Construction of chromosomal *lacZ* reporter fusions of ubiquinone biosynthesis genes

The transcriptional reporter constructs were made by cloning the *cis*-acting element of all *ubi* genes upstream of *lacZ* in a conditional-replication, integration, and modular (CRIM) plasmid pAH125 and then integrating the plasmid in the chromosome of WT BW25113 *E. coli* strain to get a single copy transcriptional reporter fusion. The steps followed for the cloning and integration are given below:

STEP: 1

Cloning of the *cis*-acting element of *ubi* genes upstream of *lacZ* in a conditional replication, integration, and modular (CRIM) plasmid

CRIM plasmid has γ replication origin of R6K and can replicate at high or medium copy number in a host encoding trans-acting Π protein (encoded by *pir* gene) for example, BW52141 (*pir*$^{+}$, medium copy number), and BW25142 (*pir*-116, high copy number mutant). For the

cloning of *cis*-acting element of all the *ubi* genes, I used pAH125 (5739 bp) CRIM plasmid, which has a multiple cloning site upstream of *lacZ* (gene coding for β-galactosidase).

STEP: 2

Integration of cloned plasmid in the bacterial chromosome and the selection of single integrants

The plasmid pAH125 can be integrated at the *attB* attachment site of the bacterial chromosome, by providing phage integrase (Int), followed by the selection of single integrants to eliminate the chance of high-copy number artifact.

STEP: 3

Transduction of the single integrant of transcriptional reporter construct to a fresh WT background

The single integrant once confirmed is then freshly transduced into a BW25113 strain by P1 transduction. The detailed stepwise protocol is discussed in the following sections of the results.

3.1.1 PCR amplification of *cis*-acting elements of all *ubi* genes followed by cloning into pAH125 upstream of *lacZ*

Primers were designed for amplifying the *ubi cis*-acting elements and restriction sites were added at their 5'end to favour restriction digestion and ligation with the pAH125 vector. *Kpn*I and *Eco*RI restriction sites were added in the 5' end of forward and reverse primer, respectively, for all *ubi* genes except *ubiC* gene. For *ubiC* gene, *Sal*I and *Kpn*I were added since the *Eco*RI restriction site was already present in the insert region. The desired upstream region containing the promoter element of all the *ubi* genes was PCR amplified. Figures 3.1 shows the agarose gel images of the amplified product of *ubi* genes (*ubiB*-*ubiK* and *ubiX*)

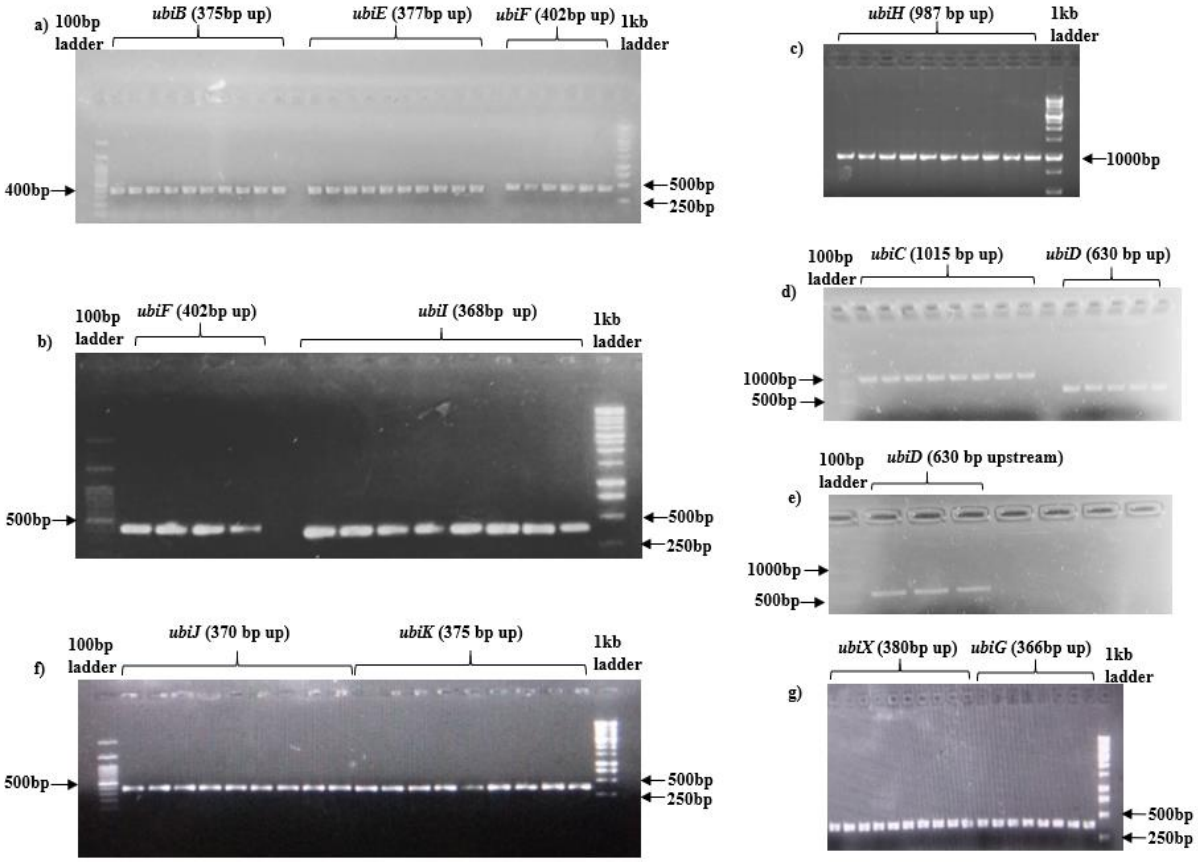

Figure 3.1 PCR amplification of *cis*-acting element of *ubi* genes: **a)** *ubiB* (375 base pair (bp) upstream region (up)), *ubiE* (377 bp up), and *ubiF* (402 bp up), **b)** *ubiF* (402 bp up), and *ubiI* (368 bp up). **c)** *ubiH* (987 bp up), **d)** *ubiC* (1015 bp up), and *ubiD* (630 bp up), **e)** *ubiD* (630 bp up **f)** *ubiJ* (370 bp up), and *ubiK* (375 bp up), **g)** *ubiX* (380 bp up), and *ubiG* (366 bp up). NTC indicates no template control.

3.1.2 PCR confirmation of *ubi cis*-acting elements cloned in pAH125 (CRIM plasmid)

The amplified PCR products were PCR-purified, and restriction digestion was performed using respective restriction enzymes for the insert and the plasmid pAH125. Further, gel extraction of the digested products was carried out followed by ligation and transformation of the ligated product into BW25141/ BW25142 electro-competent cells. Successful transformants were selected on LB-kanamycin plates and PCR was performed to confirm the cloning using the vector-specific primers MS49 (forward primer) and BS106 (reverse primer).

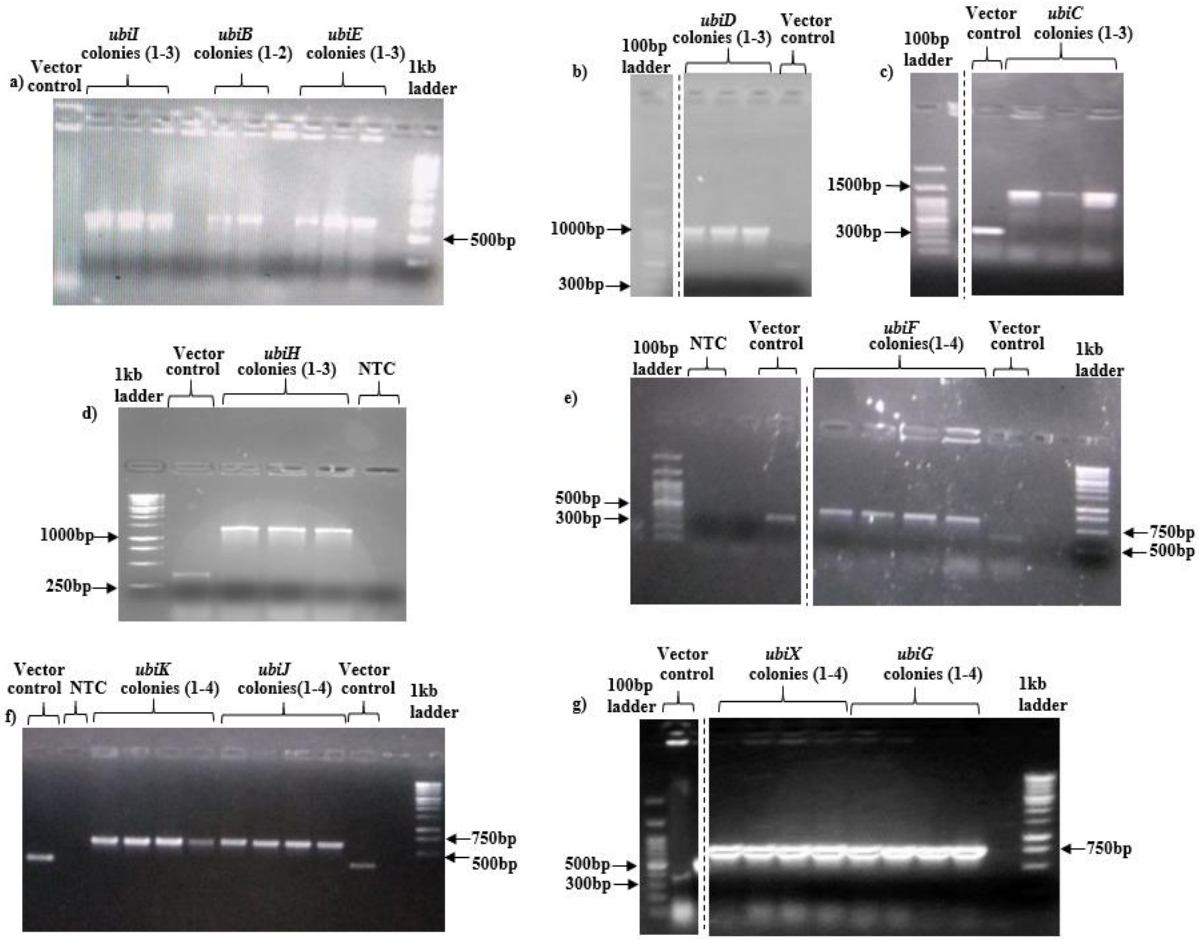

Figure 3.2 PCR confirmation of *ubi cis*-acting element cloned in pAH125 using vector specific primers. Empty vector pAH125 was used as a control (fragment of size 345 bp was amplified) and NTC represent the no template control. **a)** *ubiI*, *ubiB*, and *ubiE cis*-acting element cloned in pAH125, expected length for positive clones, *ubiI* = 719 bp, *ubiB* = 720 bp, and *ubiE* = 722 bp. Colonies (1-3) were positive for *ubiI*, colonies (1-2) were positive for *ubiB*, and colonies (1-3) were positive for *ubiE*, **b)** *ubiD cis*-acting element cloned in pAH125, expected length = 975 bp. Three positive clones for *ubiD cis*-acting element are shown, **c)** *ubiC cis*-acting element cloned in pAH125, expected length = 1372 bp. Three positive clones for *ubiC cis*-acting element are shown, **d)** *ubiH cis*-acting element cloned in pAH125, expected length = 1332 bp. Three positive clones for *ubiH cis*-acting element are shown, **e)** *ubiF cis*-acting element cloned in pAH125, expected length = 759 bp. Four positive colonies for *ubiF cis*-acting element are shown, **f)** *ubiK* and *ubiJ cis*-acting element cloned in pAH125, expected length for *ubiK* = 720 bp, *ubiJ* = 727 bp. Four positive clones for both *ubiK* and *ubiJ cis*-acting element are shown,

and **g)** *ubiX* and *ubiG cis*-acting element cloned in pAH125, expected length = 725 bp for *ubiX*, and 711 bp for *ubiG*. Four positive clones for both *ubiX* and *ubiG cis*-acting element are shown.

The positive clones were sequenced to ensure that there was no mutation in the *cis*-acting elements. The transformants carrying positive clones were also saved as glycerol stocks.

3.1.3 PCR confirmation of single integration of pAH125 carrying *ubi cis*-acting elements in BW25113

The confirmed clones of pAH125 carrying *ubi cis*-acting elements were integrated at the chromosomal "*attB*" attachment site of BW25113. Colonies were screened for single integration by PCR using P2, P3, and P4 primer sets, mentioned below.

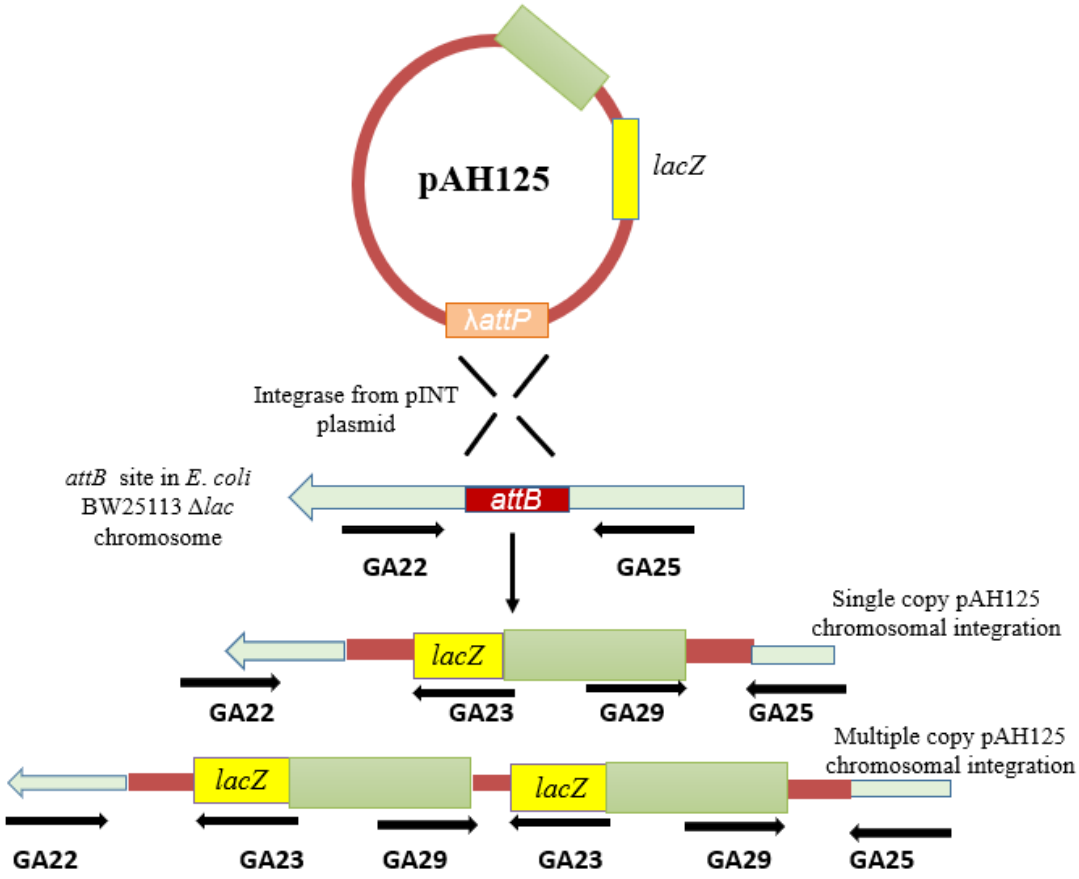

Figure 3.3 Integration of pAH125 at "*attB*" site of chromosomal DNA. The primer sets used for the PCR confirmation of WT, single integration (32), and multiple integration (MI) are also shown.

P1 (GA22+GA25), P2 (GA22+GA23), P3 (GA29+GA25), and P4 (GA29+GA23) primer sets can be used to check whether the strain is WT, single integrant, or multiple integrant (MI).

Table 3.1 The expected size of the amplified product for each of the primer sets in WT, SI, and MI condition are given.

Primer set	Combination used	WT	SI	MI
P1 set	GA22 + GA25 Chromosome specific forward and reverse primer	700bp	~6kb	>6kb
P2 set	GA22+GA23 Chromosome specific forward and pAH125 specific reverse	-	700bp	700bp
P3 set	GA29+GA25 pAH125 specific forward and chromosome specific reverse	-	600bp	600bp
P4 set	GA23+GA29 Both are pAH125 specific. It gets amplified only when multiple integration of plasmid occurs.	-	-	400bp

Because the fragment size for the integrants obtained using P1 set was very large to be amplified using *Taq* polymerase, PCR confirmation for all the *ubi* genes was done using only P2, P3 and P4 primer sets. Here, as an example, I have shown the gel images for the confirmation of integrants carrying pAH125 with *cis*-acting element of *ubiH*.

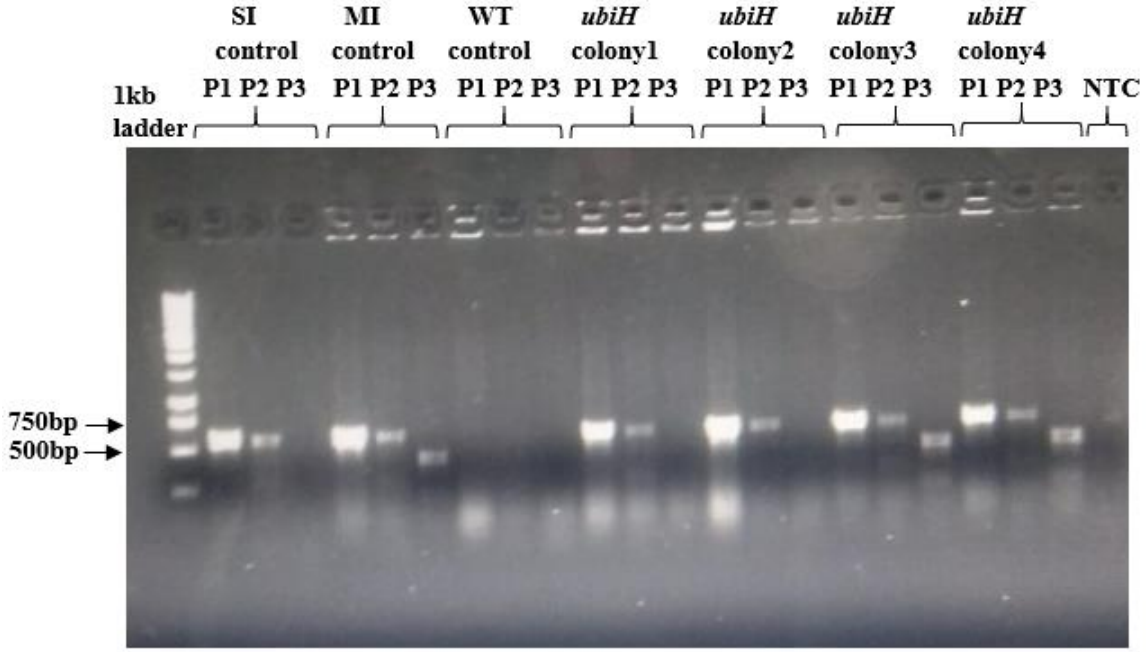

Figure 3.4 PCR confirmation of integration of pAH125 carrying *cis*-acting element of *ubiH* in BW25113. Single integrants were confirmed by using P2, P3, and P4 primer sets (as shown in the gel image). SI represents the single integrant control, MI represent the multiple integrant

control and WT is the negative control where no integration has taken place. Control colonies for SI, MI and WT showed bands as expected. Colony 1 and 2 were positive for single chromosomal integration. Colony 2 and 3 were multiple chromosomal integration.

The confirmed single integrants for all *ubi* genes were stored as glycerol stocks.

3.1.4 PCR confirmation of transductants

The confirmed single integrants were transduced into a fresh WT background using P1 transduction. Transductants carrying single integration of plasmid were confirmed by using the same PCR as discussed above. PCR confirmation for single integrant transductant was done for all the *ubi* genes. Here, as an example, I am showing the gel image for the confirmation of single integrants carrying pAH125 with *cis*-acting element of *ubiD* and *ubiG*.

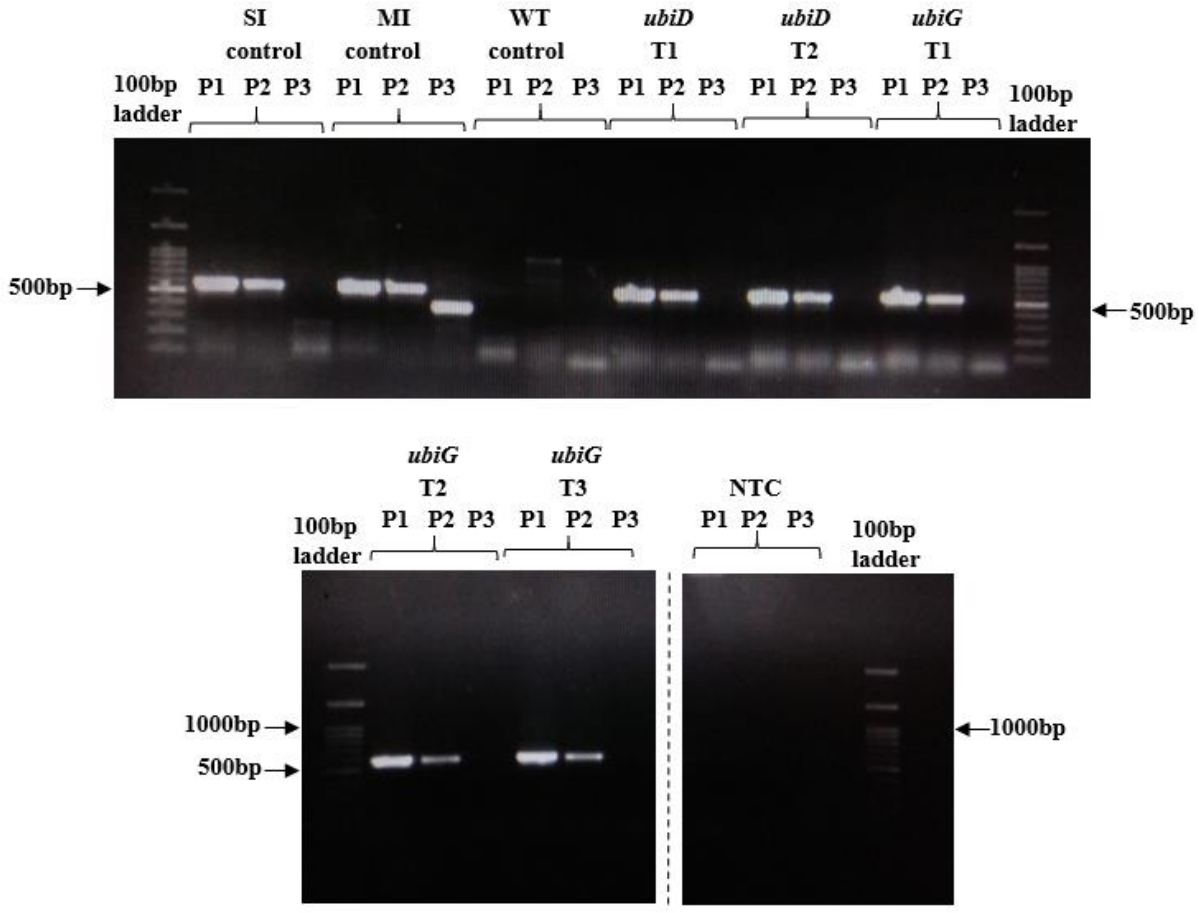

Figure 3.5 PCR confirmation of single integrant transduction to a fresh WT BW25113. Confirmation of transduction of single integrants carrying pAH125 with *cis*-acting element of *ubiD* and *ubiG*. SI represents the single integrant control, MI represents the multiple integrant control and WT is the negative control where no integration has taken place. SI, MI, and WT

control colonies showed expected bands. T1 and T2 were positive for *ubiD* single integrant transduction. T1, T2, and T3 were positive for *ubiG* single integrant transduction. No band was observed in NTC.

The confirmed transductants were stored as glycerol stocks.

Thus, transcriptional reporter construct was made for all 12 *ubi* genes.

3.2 Transcriptional regulation of ubiquinone biosynthesis genes in oleate

To check the upregulation of genes involved in ubiquinone biosynthesis, transcriptional reporter constructs were cultured in buffered tryptone broth medium supplemented with oleate (TBK-O) and tryptone broth supplemented with Brij-58 (TBK-B) (Brij-58 was used for solubilizing oleate). β-galactosidase assay was performed with cultures in both log and stationary phases of growth. Whereas none of the *ubi* genes were upregulated during log phase, we observed that several *ubi* genes were upregulated in oleate during stationary phase (Figures 3.6 and 3.7). This suggests that ubiquinone is upregulated transcriptionally in stationary phase during LCFA metabolism.

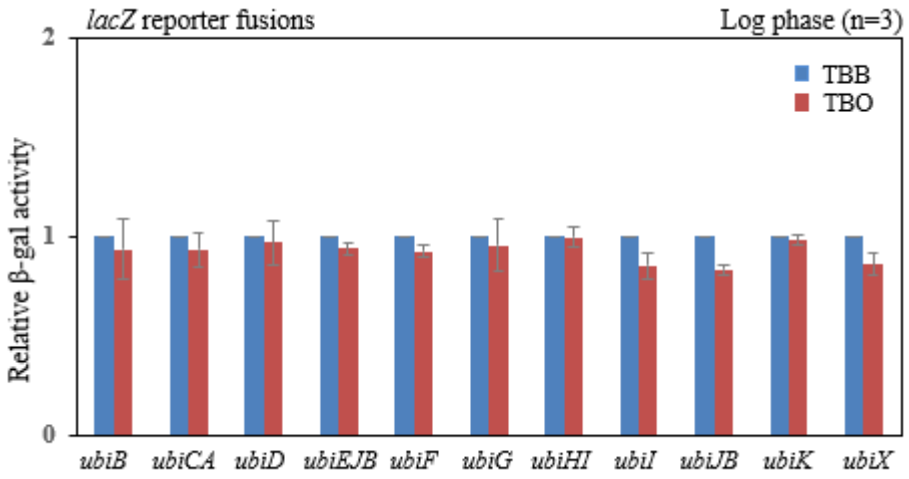

Figure 3.6 No transcriptional induction of *ubi* genes occurs in the log phase of cultures grown in oleate. WT BW25113 strain containing *ubiB-lacZ, ubiCA-lacZ, ubiD-lacZ, ubiEJB-lacZ, ubiF-lacZ, ubiG-lacZ, ubiHI-lacZ, ubiI-lacZ, ubiJB-lacZ, ubiK-lacZ,* and *ubiX-lacZ,* were cultured in TBK-O and TBK-B media, and β-galactosidase assay was performed. The data in TBK-O was normalized to that in the TBK-B condition, and represents average (± SD) of 3 independent experiments. The average β-gal activity of various reporter constructs of *ubi* genes

in TBK-B media were *ubiB-lacZ* (8±1 Miller units), *ubiCA-lacZ* (51±7 Miller units), *ubiD-lacZ* (96±13 Miller units), *ubiEJB-lacZ* (183±13 Miller units), *ubiF-lacZ* (58±5 Miller units), *ubiG-lacZ* (283±81 Miller units), *ubiHI-lacZ* (1112±191 Miller units), *ubiI-lacZ* (11±4 Miller units), *ubiJB-lacZ* (12±4 Miller units), *ubiK-lacZ* (195±28 Miller units), and *ubiX-lacZ* (45±3 Miller units).

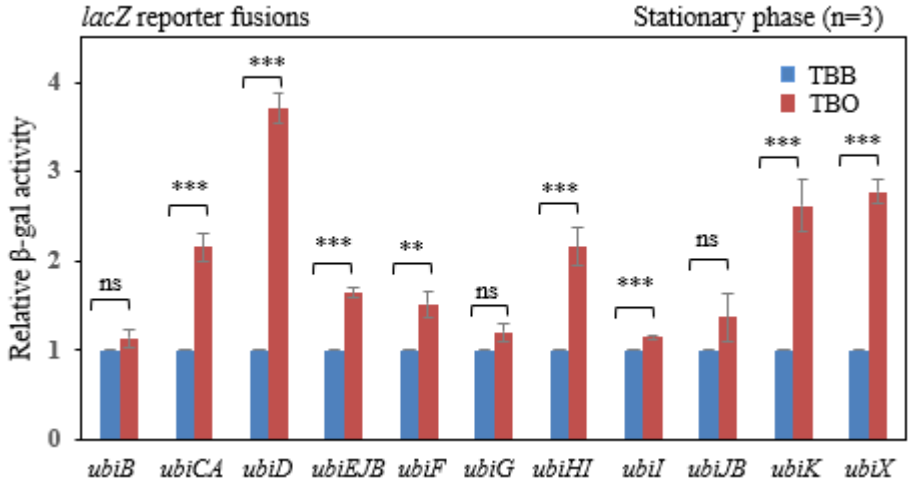

Figure 3.7 Several *ubi* genes are transcriptionally induced in the stationary phase of cultures grown in oleate. The *ubi* genes that were transcriptionally upregulated in TBK-O compared to TBK-B were *ubiCA-lacZ* (2.15±0.16 fold), *ubiD-lacZ* (3.70±0.16 fold), *ubiEJB-lacZ* (1.63±0.05 fold), *ubiF-lacZ* (1.51±0.14 fold), *ubiHI-lacZ* (2.16±0.2 fold), *ubiK-lacZ* (2.61±0.29 fold), and *ubiX-lacZ* (2.76±0.13 fold). Data were normalized to the β-galactosidase assay of TBK-B media and represented average (± SD) of 3 independent experiments.The average β-gal activity of various reporter constructs of *ubi* genes in TBK-B media were *ubiB-lacZ* (19±5 Miller units), *ubiCA-lacZ* (45±1 Miller units), *ubiD-lacZ* (34±5 Miller units), *ubiEJB-lacZ* (155±12 Miller units), *ubiF-lacZ* (73±11 Miller units), *ubiG-lacZ* (126±28 Miller units), *ubiHI-lacZ* (542±91 Miller units), *ubiI-lacZ* (15±1 Miller units), *ubiJB-lacZ* (23±9 Miller units), *ubiK-lacZ* (121±25 Miller units), and *ubiX-lacZ* (19±5 Miller units). The p-values were calculated using the unpaired two-tailed Student's t test (*** $P<0.001$; **$p<0.01$; ns, $P>0.03$).

3.3 FadR does not regulate any of the ubiquinone biosynthesis genes in oleate

Since several *ubi* genes were transcriptionally induced in oleate in the stationary phase of growth, we were interested in identifying the transcriptional regulator involved in this induction. FadR is a transcriptional regulator that acts as a repressor of the *fad* genes involved in LCFA transport and degradation. In the presence of LCFAs, an intermediate of LCFA degradation, acyl-CoA, binds to FadR and relieves its repression on *fad* genes. As our above result shows the upregulation of *ubi* genes during oleate condition and FadR is a known regulator during LCFA metabolism, we initiated our investigation by checking whether FadR is the regulator behind the induction of *ubi* genes in oleate-utilizing cells.

We obtained *fadR* deletion strain from the ASKA gene deletion library, transduced *fadR* deletion in the *ubi-lacZ* strains and confirmed the transductants by PCR. β-galactosidase assay was performed for *ubi-lacZ* and their isogenic *fadR* deletion strain in TBK-O and TBK-B media at stationary phase for checking the regulation on *ubi* genes by FadR. We observed that the relative β-galactosidase activity of *fadR* deletion strains were similar to that of the WT in both TBK-B and TBK-O media, suggesting that FadR does not regulate ubiquinone biosynthesis genes during oleate metabolism.

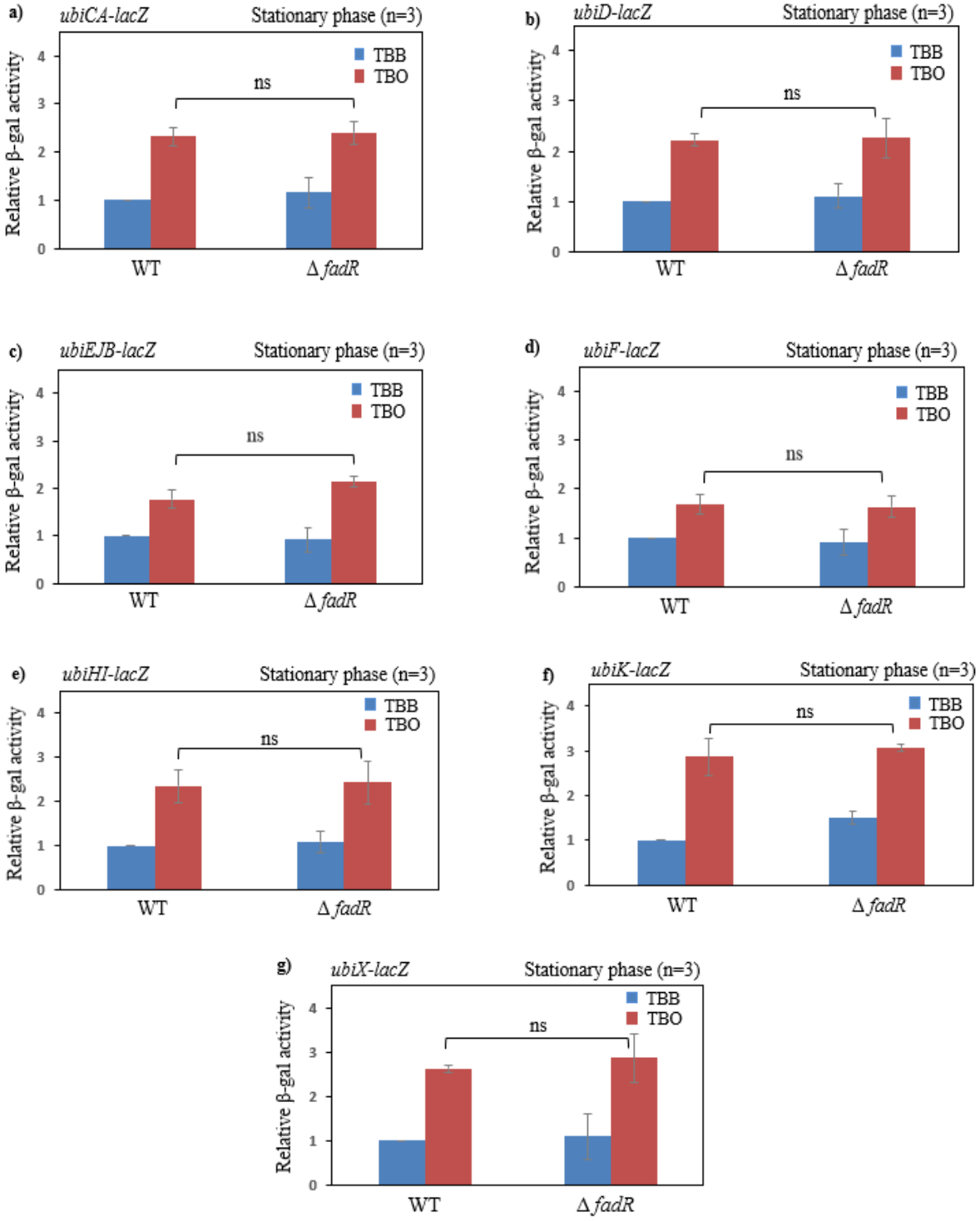

Figure 3.8 FadR does not regulate *ubi* genes: *ubiC* (**a**), *ubiD* (**b**), *ubiEJB* (**c**), *ubiF* (**d**), *ubiHI* (**e**), *ubiK* (**f**), and *ubiX* (**g**). β-galactosidase assay was performed in TBK-O and TBK-B media. The data is normalized to the reporter strain in WT background in TBK-B media. Data represent the average (± SD) of 3 independent experiments. The average β-gal activity of various reporter constructs of *ubi* genes in TBK-B media were WT *ubiC* (42±6 Miller units),

WT *ubiD* (65±12 Miller units), WT *ubiEJB* (149±12 Miller units), WT *ubiF* (64±8 Miller units), WT *ubiHI* (434±46 Miller units), WT *ubiK* (87±34 Miller units), and WT *ubiX* (21±3 Miller units). The p-values were calculated using the unpaired two-tailed Student's t test (*** $P<0.001$; **$p<0.01$; ns, $P>0.03$).

Summary and Future goals

In this project, we investigated the regulation on ubiquinone biosynthesis genes during LCFA metabolism in *E. coli*. Earlier studies showed that ubiquinone, the lipid-soluble mobile electron carrier in ETC, is present in excess over other ETC components. Thus, ubiquinone was considered to be non-limiting for its electron carrier function. However, work from our lab has shown that during LCFA metabolism, insufficiency of ubiquinone for its electron carrier role causes redox stress in *E. coli*. Consequently, as a combat strategy, *E. coli* increases ubiquinone levels ~1.8 fold. But the mechanism for ubiquinone upregulation during LCFA metabolism is unknown. Regulation on the ubiquinone biosynthesis genes can occur at the transcriptional level, post-transcriptional and translational level. I started my project by checking the transcriptional regulation on ubiquinone biosynthesis genes. The first step was creating the chromosomal *lacZ* transcriptional reporter constructs for all 12 *ubi* genes that are committed for ubiquinone biosynthesis. For this, the *ubi cis*-acting element was cloned into a conditional-replication, integration, and modular (CRIM) plasmid pAH125 upstream of *lacZ*. The clones were transformed in BW25141 or BW25142, the pir+ *E. coli* strains which allow replication of CRIM plasmids that have γ replication origin of R6K. With the help of another plasmid pINT that codes for phage integrase enzyme, the cloned *ubi-lacZ* CRIM plasmids were integrated into BW25113 Δ*lac* strain at the *attB* site to create single copy chromosomal *lacZ* fusions. The induction of *ubi* genes during LCFA metabolism was checked using β-galactosidase assay. Whereas none of the 12 *ubi* genes were upregulated in the log phase, several *ubi* genes were upregulated ~1.5 to ~3.5 fold during stationary phase of growth. We hypothesized that FadR could be a potential regulator of *ubi* genes, since FadR is a transcriptional regulator that controls genes involved in LCFA transport and degradation. However, we observed that none of the *ubi* genes that were upregulated in the presence of oleate are under FadR regulation.

In future studies, we aim to check the regulation on *ubi* genes by other transcriptional regulators such as CpxR and σ^E. Published work from our lab has shown that Cpx and σ^E pathways are induced during stationary phase in oleate-utilizing *E. coli*. Importantly, another parallel work done in our lab showed that the increased ubiquinone levels during LCFA metabolism are abolished in a *cpxR* deletion strain suggesting that CpxR might have a role in regulating ubiquinone during oleate metabolism. Thus, further investigations are required to unravel the mechanistic details for ubiquinone upregulation during LCFA metabolism.

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