Identifying new players involved in ubiquinone biosynthesis in *Escherichia coli*

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Certificate of Examination

This is to certify that the dissertation titled "Identifying new players involved in ubiquinone biosynthesis in *Escherichia coli*" submitted by Ms. Himanshi Balecha (Reg. No. MS12097) 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. Kavita Babu Dr. Lolitika Mandal Dr. Rachna Chaba

(Supervisor)

Dated: April 21, 2017

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.

Himanshi Balecha

(Candidate)

Dated: April 21, 2017

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. µl: microliter
2. Amp^R: Ampicillin resistance
3. ATP: Adenosine triphosphate
4. $CaCl_2$: Calcium chloride
5. Cm^R: Chloramphenicol resistance
6. DMK: Demethylmenaquinone
7. DMSO: Dimethyl sulfoxide
8. DNA: Deoxyribonucleic acid
9. dNTP: Deoxynucleotide triphosphate
10. EDTA: Ethyelene diamine tetraacetate
11. EtBr: Ethidium bromide
12. ETC: Electron Transport Chain
13. FLP: Flippase
14. HCl: Hydrochloric acid
15. His: Histidine
16. HPLC: High performance liquid chromatography
17. H_2O: Water
18. Kan^R: Kanamycin resistance
19. KCl,: Potassium chloride
20. kDa: kilo Dalton
21. KH_2PO_4: Monopotassium phosphate
22. kV: kilo Volt
23. LB: Lysogeny broth
24. LCFA: Long Chain Fatty Acid
25. MCT: Microcentrifuge tube
26. $MgSO_4$: Magnesium Sulphate
27. MK: Menaquinone
28. ml: millilitre
29. mM: millimolar
30. Na_2HPO_4: Disodium phosphate

31. NaCl: Sodium chloride
32. NEB: New England Biolabs
33. NH_4Cl: Ammonium chloride
34. OD: Optical density
35. OHB: 3-octaprenyl-4-hydroxylbenzoate
36. Ori: Origin of replication
37. PEG: Polyethylene glycol
38. PCR: Polymerase chain reaction
39. Q: Ubiquione
40. SD: Standard deviation
41. Taq: *Thermus aquaticus*
42. TAE: Tris Acetic Acid EDTA
43. TB: Tryptone broth
44. Tet^R: Tetracycline resistance
45. TSS: Transformation and storage solution
46. UV: Ultra violet
47. WT: Wild type

Contents

Abstract

Quinones are lipid soluble electron carriers in the electron transport chain (ETC) and help in energy generation. They can be broadly divided into benzoquinones (e.g. ubiquinone) involved in aerobic ETC, and naphthoquinones (e.g. menaquinone and demethylmenaquinone) involved in anaerobic respiration. Our lab conducted a high-throughput genetic screen on a long chain fatty acid (LCFA), oleate, in *Escherichia coli* and compared the LCFA dataset with published genome-wide screens on other carbon sources. This comparative analysis revealed highest requirement of ubiquinone for growth in oleate. In particular, a Δ*ubiI* strain (UbiI is a hydroxylase involved in ubiquinone biosynthesis), which produces very low level of ubiquinone exhibited growth defect only in oleate. We thus hypothesized that some of the uncharacterized genes (*y*-genes) in *E. coli*, deletion of which led to significant growth defect only in oleate, might also be involved in ubiquinone biosynthesis. Detailed studies on one such candidate, *yqiC* (*yqiC* encodes a small protein with predicted scaffold function) showed that it is involved in ubiquinone biosynthesis since its deletion resulted in low ubiquinone content. Moreover, the phenotype of Δ*yqiC* in terms of reduced ubiquinone levels and accumulation of an intermediate was similar to Δ*ubiI*. Furthermore, a Δ*yqiC*Δ*ubiI* double knockout showed a synthetic sick phenotype with no detectable ubiquinone suggesting that the two genes act at the same step in ubiquinone biosynthesis. Introduction of multicopy *yqiC* clone in Δ*ubiI* showed slight growth recovery in oleate suggesting that YqiC might promote the function of some other hydroxylase in the absence of UbiI to rescue ubiquinone levels. Since YqiC in *Salmonella typhimurium* is involved in menaquinone biosynthesis, it is also possible that overexpression of *yqiC* in Δ*ubiI* rescues the growth defect of Δ*ubiI* in oleate by regulating the synthesis of naphthoquinones. In addition to understanding the role of YqiC in ubiquinone biosynthesis, we are also investigating additional *y*-genes for their involvement in ubiquinone biosynthesis.

Chapter 1

Introduction and Review of Literature

1.1 Quinones are electron carriers in the Electron Transport Chain

Escherichia coli, a gram-negative bacterium, is a facultative anaerobe which can survive under both aerobic and anaerobic conditions. During metabolism, cells mainly generate energy in the Electron Transport Chain (ETC). In the aerobic ETC, reduced cofactors, NADH and $FADH_2$, produced during metabolism are oxidized by NADH dehydrogenase and succinate dehydrogenase respectively. The electrons released from oxidation of reduced cofactors are transferred to quinone converting it to quinol which in turn donates electrons to the terminal oxidases. Electrons are further transferred to molecular oxygen reducing it to water molecule. During the flow of electrons through ETC, proton motive force is generated which drives ATP synthesis through ATP synthase (Fig 1.1).

Quinones are membrane bound lipids and act as electron carriers in the ETC. They can be broadly divided into benzoquinones (e.g. ubiquinone) and naphthoquinones (e.g. menaquinone and demethylmenaquinone) [1]. Ubiquinone (Q) is involved in aerobic respiration with a higher mid-point potential (E'°= +100 mV) [2,3]. On the other hand, menaquinone (MK) and demethylmenaquinone (DMK) are involved in anaerobic respiration and have a low mid-point potential (E'°= -74 mV and +36 mv respectively) [4].

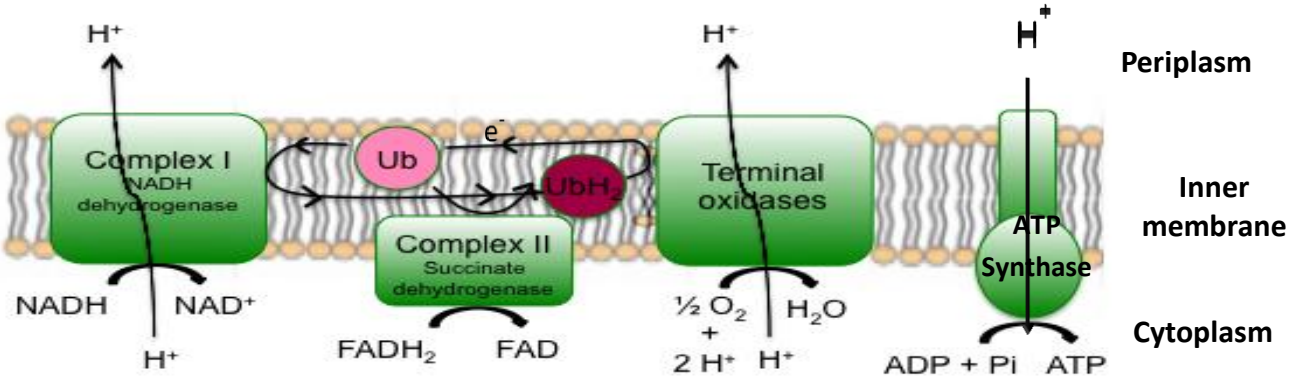
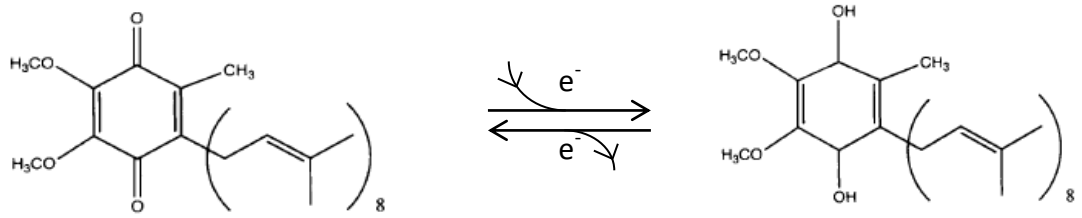


Fig 1.1 Aerobic Electron Transport Chain in *Escherichia coli*

Ubiquinone, a benzoquinone, has a conserved aromatic ring and an isoprenoid tail. The hydrophilic aromatic ring allows it to interact with various membrane proteins while the hydrophobic tail enables it to adopt a lipid-soluble character and perform vital functions in membrane lipid bilayers [5]. The number of isoprenoid unit on the

ubiquinone hydrophobic tail varies from species to species. For example, in humans the number of isoprenoid unit is 10, hence human ubiquinone is represented as Q_{10} while *E. coli* ubiquinone has 8 isoprenoid units and is therefore denoted by Q_8 [1].



Ubiquinone–8 (Q_8) Ubiquinol-8 (Q_8H_2)

Fig 1.2 Two forms of ubiquinone. Ubiquinone (Q_8) gains electron and gets reduced to ubiquinol (Q_8H_2) and ubiquinol loses electron and gets oxidized to ubiquinone.

Menaquinones are thought to be the most ancient types of quinones as they are found in both archaea and bacteria [2,5-9]. MK consists of methylnaphthoquinone as the head group and a polyprenyl side chain (Fig 1.3). The degree of saturation of the side chain varies in different microorganisms. The major MK in *E. coli* is MK-8 [5]. Most of the gram-positive and strictly anaerobic bacteria contain only MK while most of the strictly aerobic bacteria contain only Q. Since the reduced form of MK is very reactive with molecular oxygen in the cell, and is subject to non-catalytic oxidation, it is thought to function inefficiently in oxygen-containing environment [5]. Demethylmenaquinone (DMK) has a similar structure as MK but it lacks a methyl group in the ring (Fig 1.3) [5]. DMK can function under both aerobic and anaerobic environment [10].

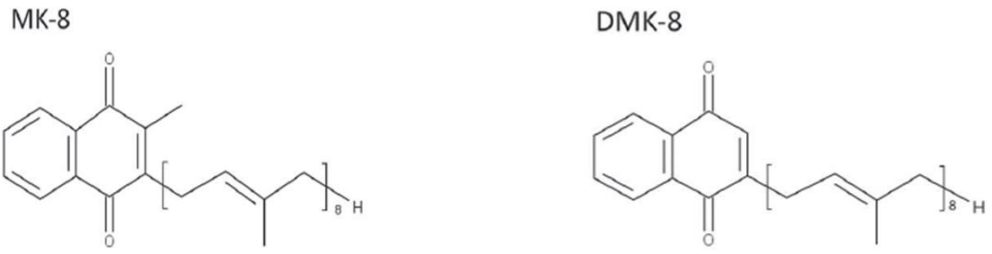


Fig 1.3 Structures of Menaquinone and Demethylmenaquinone

1.2 Ubiquinone and Menaquinone biosynthesis

For both ubiquinone and menaquinone, the head group and the tail are synthesized separately and joined together by an enzyme in the prenyltransferase family. The prenyl chain for both the quinones is derived from prenyl PPi and the head group is derived from the shikimate pathway. The precursor for Q is chorismate, while that for MK is isochorismate which is derived from chorismate. The aromatic head group undergoes many modifications like decarboxylation, hydroxylation and methylation by various enzymes. Despite originating from the same shikimate pathway, MK and Q biosynthesis are different in many ways as evident from their biosynthesis pathway. Biosynthesis of Q_8 has additional requirement of flavoprotein, NADH and oxygen. MK biosynthesis requires 2-ketoglutarate and thiamine PPi, coenzyme A and ATP as cofactors [11].

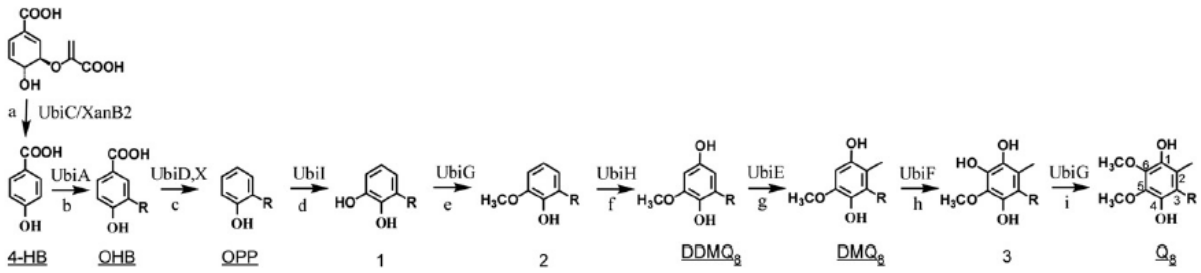


Fig 1.4 Ubiquinone biosynthesis pathway [1]

The ubiquinone biosynthesis pathway consists of around 11 genes. These genes encode for enzymes that modify the aromatic ring through methylation, carboxylation, hydroxylation, etc. Ubiquinone biosynthesis pathway has been extensively studied and major players involved in this pathway have been characterised but still there are many knowledge gaps. For example, both UbiD and UbiX are thought to function together for the decarboxylation of OHB (Fig 1.4) but these proteins have very little structure similarity [12,13] and hence their respective involvement in the decarboxylation step is not well established. Interestingly, on deleting certain genes of the ubiquinone biosynthesis pathway, some amount of residual ubiquinone is still synthesized. Hence, there must be redundant players involved that carry forward the reactions sub-optimally leading to the formation of ubiquinone [1].

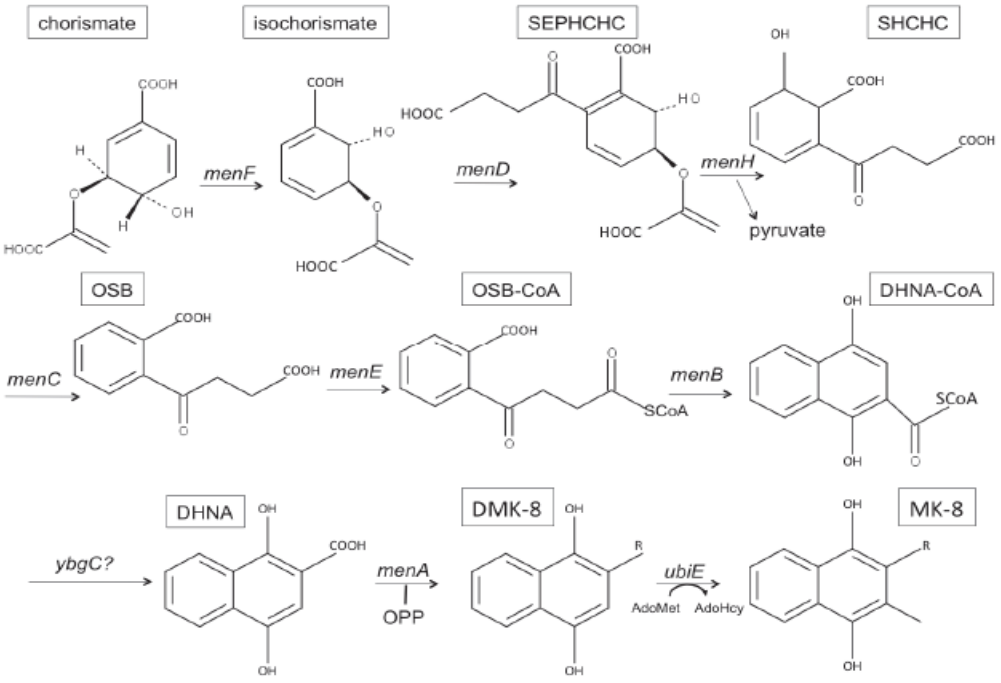


Fig 1.5 Menaquinone biosynthesis pathway [5]

In contrast to fermentable carbon sources which can generate energy through substrate level phosphorylation in addition to energy generation through oxidative phosphorylation in the ETC, non-fermentable carbon sources can generate energy only through ETC. Thus the requirement of ubquinone is higher for growth on non-fermentable carbon sources than on fermentable carbon sources. This rationale has been used to identify genes involved in ubiquinone biosynthesis for the last several decades. The growth of *E. coli* mutants generated by chemical mutagenesis is compared on glucose, a fermentable carbon source, and succinate, a non-fermentable carbon source, and the mutants with severe growth defect only on succinate are studied further for their involvement in ubiquinone biosynthesis.

A high-throughput genetic screen was set up in our lab to identify players involved in long chain fatty acid (LCFA) metabolism. *E. coli* gene deletion library containing 3994 strains were grown on minimal media with either glucose (control) or oleate (a long chain fatty acid with 18 carbon atoms) as sole carbon sources. The colony size was quantified as a measure of growth and the fitness scores were assigned to each strain by comparing the colony size on oleate with respect to glucose control. The data from our high throughput genetic screen on oleate was comapred with previously published screens [14], to facilitate comparison between oleate and other fermentable and non-fermentable carbon sources. This analysis with follow-up detailed studies in our lab

led us to conclude that ubiquinone is required more for growth on oleate as compared to other non-fermentable carbon sources and this increased requirement is to alleviate oxidative stress caused due to LCFA metabolism.

As mentioned earlier, the increased requirement of ubiquinone for growth on succinate as compared to glucose has been the rationale for identifying genes involved in ubiquinone biosynthesis. Despite this, it was surprising that a recent study showed that a Δ*ubiI* strain, where ubiquinone levels are reduced to only ~10% of wild-type level, exhibits normal growth in succinate [15]. Our comparative analysis showed growth defect of *ubiI* deletion strain on oleate, suggesting that our screen can be used as a reliable source for identifying new players involved in ubiquinone biosynthesis.

Another top candidate from the screen (Rank 17), *yqiC* was selected and some of the preliminary experiments were performed by a PhD student (Shashank Agrawal) suggesting its involvement in ubiquinone biosynthesis. Moreover, in a recent high-throughput study where the gene deletion library of *E. coli* was profiled on >100 different stress conditions, the phenotypes of Δ*yqiC* were highly correlated with Δ*ubiI* (correlation coefficient 0.78), and deletion strains of several other ubiquinone biosynthesis players [Table 1.2] [14]. *yqiC* was thus selected to study its role in ubiquinone biosynthesis.

Table 1.1 Correlation coefficient of Δ*yqiC* with other *ubi* deletion strains

Strain 1	**Strain 2**	**Correlation coefficient**
Δ*yqiC*	Δ*ubiI*	0.775682000
Δ*yqiC*	Δ*ubiC*	0.472577000
Δ*yqiC*	Δ*ubiX*	0.321542000
Δ*yqiC*	Δ*ubiH*	0.317641000
Δ*yqiC*	Δ*ubiE*	0.310051000
Δ*yqiC*	Δ*ubiF*	0.278182000

YqiC is a small protein with a molecular weight of 11.3 kDa. Although the function of *yqiC* has not been studied in *E. coli*, certain studies performed in *Salmonella typhimurium* suggest that YqiC is a membrane fusogenic protein, involved in menaquinone biosynthesis [16, 17]. Further, YqiC regulates flagella and fimbriae expression, motility and colonization of *S. typhimurium* [17].

In this work, we found YqiC to have a role in ubiquinone biosynthesis in *E. coli*. Our data suggests that this protein might be functioning at the same step as UbiI. Introduction of of multicopy *yqiC* in Δ*ubiI* strain shows growth recovery on oleate after prolonged incubation suggesting that YqiC, in absence of UbiI might be promoting the activity of some other hydroxylase (UbiF or UbiH) [15]. Alternatively, since *yqiC* is involved in menaquinone biosynthesis in *S. typhimurium*, it is possible that *yqiC* might regulate the synthesis of other quinones (MK or DMK) in *E. coli* and hence its overexpression leads to growth recovery of Δ*ubiI* on oleate.

In order to identify additional players involved in ubiquinone biosynthesis, few *y*-genes were selected from our screen on the basis of their rank and correlation with *ubi* genes. These *y*-gene deletion strains were studied further to confirm the data from our screen and check if they are indeed involved in ubiquinone biosynthesis. One of these genes, *ygeH*, showed growth defect specific to oleate, validating the data from genetic screens. Preliminary experiments show a slight reduction in ubiquinone levels in a Δ*ygeH* strain, suggesting its role in ubiquinone biosynthesis; however, these results have to be verified.

Chapter 2

Materials and Methods

2.1 Materials

2.1.1 Chemicals, Reagents and their Sources

Tryptone, yeast extract and bacteriological agar were purchased from BD Difco™. Primers were ordered from IDT. $MgSO_4$, Na_2HPO_4, KH_2PO_4, NH_4Cl, KCl, bromophenol blue, sodium citrate, methanol, brij-58, sodium-acetate, sodium-succinate sodium-oleate, polyethylene glycol (PEG), dimethyl sulfoxide (DMSO) and glass beads were purchased from Sigma. Glucose was purchased from Fisher scientific. Acetonitrile, n-hexane, 2-propanol, ethanol, HCl were purchased from Merck. NaCl, Agarose, Tris base and EtBr was ordered from USB.

Antibiotics were purchased from Sigma. Taq enzymes, 100bp/1Kb DNA ladder and dNTPs were purchased from New England Biolabs. Plasmid miniprep kit was purchased from Thermo Scientific.

2.1.2 Bacterial strains

Table 2.1 List of *E. coli* strains

Strain	Genotype	Source	Reference
WT BW25113	*lacI*q *rrnB*T14 Δ*lacZ*WJ16 *hsdR514* Δ*araBAD*AH33 Δ*rhaBAD*LD78	Genetic Stock Center	[18]
DH5α	F- *endA1 glnV44 thi-1 recA1relA1 gyrA96 deoR nupG Φ80dlacZΔM15* Δ(*lacZYAargF*) U169, *hsdR17*(rK⁻ mK+), λ–	NEB	[19]
***ubiI*::kan (Δ*ubiI*)**	*ubiI*::kan in BW25113	Keio collection	[20]
***yqiC*::kan (Δ*yqiC*)**	*yqiC*::kan in BW25113	Keio collection	[20]
RC8007	BW25113 *ubiI*::kan, Kan cassette flipped out	Chaba Lab	This work
RC8009	P1 (BW25113 *yqiC*::kan) x	Chaba Lab	This work

	RC8007, Kan^R		
***menA*::kan (Δ*menA*)**	*menA*::kan in BW25113	Keio collection	[20]
***ubiE*::kan (Δ*ubiE*)**	*ubiE*::kan in BW25113	Keio collection	[20]
***yhcB*::kan (Δ*yhcB*)**	*yhcB*::kan in BW25113	Keio collection	[20]
***ygeH*::kan (Δ*ygeH*)**	*ygeH*::kan in BW25113	Keio collection	[20]
***ydeS*::kan (Δ*ydeS*)**	*ydeS*::kan in BW25113	Keio collection	[20]
***ydfU*::kan (Δ*ydfU*)**	*ydfU*::kan in BW25113	Keio collection	[20]
***fadL*::kan (Δ*fadL*)**	*fadL*::kan in BW25113	Keio collection	[20]
RC8011	pACYC184 in BW25113	Chaba Lab	This work
RC8012	pSA4 in BW25113	Chaba Lab	This work
RC8013	pACYC184 in Δ*yqiC*	Chaba Lab	This work
RC8014	pSA4 in Δ*yqiC*	Chaba Lab	This work
RC8019	pBAD24 in BW25113	Chaba Lab	This work
RC8020	pKJ7 in BW25113	Chaba Lab	This work
RC8021	pKJ7 in Δ*yqiC*	Chaba Lab	This work
RC8022	pACYC184 in Δ*ubiI*	Chaba Lab	This work
RC8023	pSA4 in Δ*ubiI*	Chaba Lab	This work
RC8028	pACYC184 in Δ*fadL*	Chaba Lab	This work
RC8030	pBAD24 in Δ*yqiC*	Chaba Lab	This work
RC8031	pBAD24 in Δ*fadL*	Chaba Lab	This work

2.1.3 Plasmids

Table 2.2 List of Plasmids

Plasmid Name	Description	Source	Reference
pACYC184	Vector, p15A ori, Cm^R, Tet^R	NEB	[21], [22]
pBAD24	Vector, f1 ori, pBR322 ori, Amp^R		[23]
pKJ7	*ubiI*-6His in pBAD24, Amp^R	Chaba Lab	Kanchan Jaswal (Unpublished)
pSA4	putative *yqiC* promoter and *yqiC*-6His in pACYC184, Tet^R	Chaba Lab	Shashank Agrawal (Unpublished)
pCP20	Vector, Amp^R, Cm^R, temperature sensitive replication and thermal induction of FLP synthesis		[18]

2.1.4 Primers

Table 2.3 List of primers

Primers	Sequences	Purpose
SA53	5'-CGGCAGCAACGCATAGCTTCAC-3'	Forward Primer: For confirmation of *yqiC* gene disruption with kanamycin cassette
SA54	5'-TGGTTTTCTGAATGGGATTACGCG-3'	Reverse Primer: For confirmation of *yqiC* gene disruption with kanamycin cassette
SA36	5'-GCTGATGACGATGGAATTATTC-3'	Forward Primer: For confirmation of *ubiI* gene disruption with kanamycin cassette
SA37	5'- GAGATGAAAGTGTGATGGGTATC-3'	Reverse Primer: For confirmation of *ubiI* gene disruption with kanamycin cassette
SAK1	5'- GAGGCTATTCGGCTATGACTG-3'	Forward primer for confirming

		kanamycin gene in strains having gene disruption with kanamycin cassette
SAK2	5'- TTCCATCCGAGTACGTGCTC-3'	Reverse primer for confirming kanamycin gene in strains having gene disruption with kanamycin cassette

2.1.5 Antibiotics

Ampicillin and Kanamycin stock as given in the table were made in autoclaved milliQ-H_2O and were filter-sterilized using 0.22 micron filters (Millipore). Tetracycline stocks were made in 70% ethanol and filter sterilized. Antibiotic stock solutions were stored at -20°C.

Table 2.4 List of antibiotics and their concentrations

Name of the antibiotics	**Stock concentration**	**Working Concentration**
Ampicillin	100 mg/ml	100 µg/ml
Kanamycin	30 mg/ml	30 µg/ml
Tetracycline	12.5 mg/ml	12.5 µg/ml

2.1.6 LB media Composition

A) LB (Lysogeny Broth) media

Table 2.5 Composition of LB media

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

The media components were dissolved in 1000 ml of milli-Q H_2O and autoclaved at 15 lb/inch^2 pressure and 121°C temperature for 15 minutes.

B) LB Agar

For preparing LB agar, LB broth was supplemented with 1.5 % agar. For preparing LB agar plates, molten LB agar media was allowed to cool to about 50°C, antibiotics were added wherever required and the media was poured into sterile petriplates (Tarsons).. Plates were stored at 4°C till use.

2.1.7 Tryptone Broth Media

Table 2.6 Composition of 2X TB media

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

The media components were dissolved in milliQ-H_2O and the volume was made up to 1000 ml. The media was then autoclaved.

2.1.8 M9 minimal media

Composition of M9 minimal media is described in Table 2.7. All components were prepared separately as mentioned below and sterilized. Each component was added in required concentration to make 1X M9 minimal media and the volume was made up by milliQ-H_2O.

Table 2.7 Composition of M9 minimal media

Composition	**Working concentration**
10X M9 Salts	1 X
1 M $MgSO_4$	1 mM
250X vitamins	1 X

The compositions of each of the components are as follows:

A) 10X M9 salts

The composition of 10X M9 salt is described in Table 2.8. The components were first dissolved in 600 ml of milli-Q water, the volume was made up to 1000 ml and autoclaved.

Table 2.8 Composition of 10X M9 salts

Composition	Amount (L^{-1})
Na_2HPO_4	53 g
KH_2PO_4	30 g
NaCl	5 g
NH_4Cl	10 g

B) 250 X Vitamin mix

Composition of 250 X vitamin mix is described in Table 2.9. The following components were dissolved in 200 ml of milli-Q H_2O and filter-sterilized.

Table 2.9 Composition of 250X vitamin mix (200 ml)

Composition	Amount
Biotin	100 mg
Nicotinamide	100 mg
Riboflavin	10 mg
Thiamine	100 mg

2.1.9 5% Brij-58

5 gm of Brij-58 was added to 70 ml of milli-Q H_2O, which was dissolved using magnetic stirrer at ~40°C. The volume was made up to 100 ml and autoclaved.

2.1.10 Preparation of different carbon sources

A) Preparation of Na-Oleate

50mM Na-Oleate was prepared in autoclaved 5% Brij-58 and allowed to dissolve completely at room temperature. The solution was then filter-sterilized and stored at -20°C.

B) Preparation of other carbon sources (glucose, Na-acetate and Na-succinate)

50 mM of each carbon source was prepared in milli-Q H_2O and autoclaved.

Working concentration of each of the carbon source was 5 mM, however for the detection of Q_8 in Δ*yqiC*Δ*ubiI* double mutant, strains were grown in LB supplemented with 0.2% glucose.

2.1.11 M9 minimal solid media with different carbon sources

Table 2.10 Composition of M9 minimal solid media containing various carbon sources

Components	**M9+ D-glucose**	**M9+ Na-Acetate**	**M9+ Na-Succinate**	**M9+ Na-Oleate**
10XM9 Salts	8 ml	8 ml	8 ml	8 ml
1M Mg_2SO_4	80 µl	80 µl	80 µl	80 µl
250X Vitamins	320 µl	320 µl	320 µl	320 µl
50mM D-glucose	8 ml	-	-	-
50mM Na-Acetate	-	8 ml	-	-
50mM Na-Succinate	-	-	8 ml	-
50mM Na-Oleate	-	-	-	8 ml
5% Brij-58	8 ml	8 ml	8 ml	-
Autoclaved MiliQ water	Volume made up to 40 ml	Volume made up to 40 ml	Volume made up to 40 ml	Volume made up to 40 ml
2X-Agar	40 ml	40 ml	40 ml	40 ml

For preparing 80 ml M9 minimal agar media supplemented with various carbon sources, 40 ml 2X molten agar was mixed with 40 ml of the remaining premixed components. The media was then poured on plates and allowed to solidify. Antibiotics were added whenever required according to their working concentration. Since Na-oleate is dissolved in 5% Brij-58, 5 mM of Brij-58 was added to other plates as control. Plates were covered and stored at 4^0C for 12-18 hours. Na-oleate plates were stored at room temperature.

2.1.12 M9 minimal liquid media with different carbon sources

Table 2.11 Composition of M9 minimal liquid media containing various carbon sources

Components	M9 + D-glucose	M9 + Na-Acetate	M9 + Na-Succinate	M9 + Na-Oleate
10X M9 Salts	15 ml	15 ml	15 ml	15 ml
1M Mg_2SO_4	150 µl	150 µl	150 µl	150 µl
250X Vitamins	600 µl	600 µl	600 µl	600 µl
50mM D-glucose	15 ml	-	-	-
50mM Na-Acetate	-	15 ml	-	-
50mM Na-Succinate	-	-	15 ml	-
50mM Na-Oleate	-	-	-	15 ml
5% Brij-58	15 ml	15 ml	15 ml	-
Autoclaved Water	Volume was made up to 150 ml	Volume was made up to 150 ml	Volume was made up to 150 ml	Volume was made up to 150 ml

5 mM of Brij-58 was added to all other carbon sources except Na-oleate as control.

2.1.13 Agarose Gel Electrophoresis

50X TAE buffer composition is described in table 2.12

A) 50X TAE

Table 2.12 Composition of 50X TAE

Composition	Amount (L^{-1})
Tris	242 gm
Glacial acetic acid	57.1 ml
0.5M EDTA(pH8.0)	100 ml

The pH of the buffer was adjusted to 8.0 and volume was made up to 1 L with milli-Q water. The 50X stock was stored at room temperature.

B) 6 X DNA Loading Dye (Stock)

Table 2.13 Composition of 6X DNA loading dye

Composition	Amount (for 50 ml)
Bromophenol blue	125 mg (0.25 %)
Sucrose	20 gm (40.0 %)

The 6 X DNA loading dye stock was stored in aliquots of 1ml at 4^0C till use.

2.1.14 Composition of Solutions required for HPLC

A) Mobile Phase

Table 2.14 Composition of Mobile Phase

Composition	Amount (for 200 ml)
Acetonitrile	80 ml
Ethanol	80 ml
1M Lithium Perchlorate	4 ml
2-Propanol	36 ml

1 M Lithium perchlorate was prepared in milli-Q H_2O.

B) Wash Solution

Table 2.15 Composition of Wash Solution

Composition	Amount (for 100 ml)
Acetonitrile	5 ml
Milli-Q H_2O	95 ml

2.1.15 Preparation of Transformation and storage solution (TSS)

Table 2.16 Composition of TSS

Composition	Amount
MilliQ H_2O	100 ml
Tryptone	0.8 g
Yeast extract	0.5 g
NaCl	0.5 g
PEG	20 g
1M $MgSO_4$	10 ml
DMSO	10 ml

To prepare TSS, tryptone, yeast extract, NaCl and PEG were dissolved in 50 ml milli-Q water. 1M $MgSO_4$ and DMSO were added to the solution and the pH was then adjusted to 6.5. The volume was made up to 100 ml by milli-Q H_2O. The solution was filter-sterilized and stored at -20°C.

2.1.16 Confirmation of transductants by colony PCR

The composition and PCR program used is as follows:

Table 2.17 Composition of PCR mix

PCR component	Vol. for 10 µl reaction
Water	7.75 µl
10X Taq buffer	1 µl
10 mM dNTPs	0.2 µl
Template	0.5 µl
25 units/ml Taq Polymerase	0.05 µl
10 µM Forward primer	0.25 µl
10 µM Reverse primer	0.25 µl

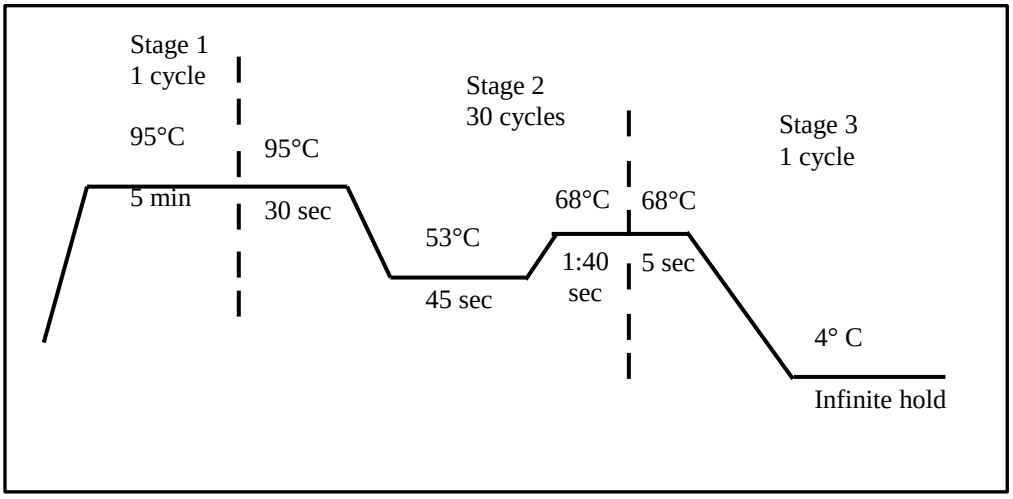


Fig. 2.1 PCR program for confirmation of transductants.

2.2 Methods

2.2.1 Plasmid Isolation

Strains bearing plasmid of interest were inoculated in 3 ml LB media containing appropriate antibiotic and grown at 37°C, in a roller drum for 14-16 hours. Cells were pelleted by centrifugation at 6000 rpm for 2 minutes. Plasmid DNA was isolated using plasmid miniprep kit (Thermo Scientific) according to manufacturer's instructions.

2.2.2 Preparation of Competent Cells

A) Electro competent cells

Single isolated colonies were picked, inoculated in 3 ml LB media and grown at 37°C, in a roller drum for 14-16 hours (primary culture). Cells from primary culture were inoculated in 15 ml LB to an initial OD_{600} of 0.01 and grown at 37°C, 220 rpm till OD_{600} reached 0.5. The secondary culture was centrifuged at 4°C, 5000 rpm for 15 min and the supernatant was discarded. The pellet was resuspended in 15 ml pre-chilled autoclaved water and centrifuged again. The supernatant was discarded and the pellet was resuspended in 7.5 ml pre-chilled autoclaved water and centrifuged again. The supernatant was discarded and the pellet was resuspended in 7.5 ml of pre-chilled 10%

glycerol and centrifuged again. The supernatant was discarded; the pellet was resuspended in 3.75 ml 10% glycerol and centrifuged again. The supernatant was discarded and the pellet was resuspended in 100 µl of 10% glycerol and stored at -80°C in aliquots.

2.2.3 Transformation

A) TSS Transformation

TSS competent cells were prepared following the protocol as described in [24]. Single isolated colonies were picked, inoculated in 3 ml LB media and grown at 37°C, in a roller drum for 14-16 hours (primary culture). Cells from primary culture were inoculated in 25 ml LB with an initial OD_{600} of 0.01 and grown at 37°C, 220 rpm till OD_{600} reached 0.5. The secondary culture was then placed on ice for 10 min. 10 ml of the chilled culture was mixed with 10 ml pre-chilled TSS in a sterile 50 ml centrifuge tube and incubated on ice for 2 hours to make the cells competent. 10-50 ng of plasmid was added to a micro-centrifuge tube containing 1 ml of the competent cells and again kept on ice for 2 hours. Tubes were further incubated at 37ºC for 1 hour in the roller drum. 1ml culture was spread on LB plates containing appropriate antibiotic. Plates were allowed to dry and were then incubated at 37ºC for 16-18 hours.

B) 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. A 1.8 kV pulse was made to pass through the electro-cuvette 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.

2.2.4 Agarose Gel Electrophoresis

1% of agarose was added to 1X TAE and dissolved by heating. Once the agarose was dissolved, EtBr was added and poured into a gel casting tray to prepare a solidified gel. DNA samples were mixed with 6 X Bromophenol blue DNA loading dye (1 X final concentration) and 1X TAE buffer. Samples were loaded in 1 % agarose gel in 1X TAE buffer and run at 110 volts. The gel was observed using UV transilluminator or Gel documentation system from BioRad.

2.2.5 Transduction

P1 transduction was carried out according to the procedure mentioned in [25]. 1 ml of overnight culture was pelleted and resuspended in 1 ml solution containing 100 mM $MgSO_4$ and 10 mM $CaCl_2$. 100 µl of resuspended cells was transferred into the micro-centrifuge tubes containing 0 µl, 1 µl, 10 µl or 100 µl of P1 lysate and incubated at 37°C for 25 minutes in a water bath. The cells were again pelleted, resuspended in 100 µl LB containing Na citrate (10 mM) and incubated at 37°C for 1 hour in water bath. After incubation, cells were 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. 100 µl of P1 lysate was also spread on plates as control. Transductant colonies obtained were re-streaked twice. The successful transduction of various genes interrupted by Kanamycin cassette, was checked by colony PCR (Material 2.1.15) using combinations of four primers in four primer sets, P1: gene-specific forward primer (FP)+gene-specific reverse primer (RP), P2: FP+SAK2, P3: SAK1+RP, P4: SAK1+SAK2. Two transductants were cultured and preserved as glycerol stocks.

2.2.6 Growth curve assays

A) Shake flask

E. coli strains were streaked on LB plates (supplemented with antibiotics as required) and incubated at 37°C for 14-16 hours. For primary culture, single colony of each strain was inoculated in 3 ml LB (appropriate antibiotics were added when required) in autoclaved culture tubes and incubated in roller drum at 37°C for 14-16 hours. For secondary cultures, cells were washed with 1X M9 minimal buffer and re-inoculated in different media in shake flasks such that the initial OD_{450} was ~0.01. Secondary cultures were

incubated at 37°C in a water bath shaker set at a speed of 220 rpm. OD_{450} of various cultures was measured at regular intervals of time. OD_{450} was plotted against time to generate growth curve.

B) Liquid Handling System

For primary culture, single isolated colonies were inoculated in 3 ml LB and incubated overnight at 37°C in roller drum. Overnight cultures grown in LB were pelleted, washed and re-suspended in M9 minimal media. Cells were re-inoculated in 200 µl M9 minimal media containing the desired carbon source and antibiotic to a starting OD_{450} of ~0.03, in 96-well plates, using a robotic liquid handling system (Tecan). Plates were incubated in a shaker at 37°C, and OD_{450} of the cultures was measured at designated time intervals (Tecan Infinite M200 monochromator). The incubator shaker and microplate reader were integrated with the liquid handling system, and the transfer of plates between shaker and reader was automated. OD_{450} was plotted against time to generate growth curve.

2.2.7 Dilution spotting

Primary culture of different strains was set up in 3 ml LB and allowed to grow at 37ºC for 14-16 hours. Cells were pelleted and re-suspended in M9 minimal media. OD_{450} of the strains was normalized. 200 µl of various dilutions (10^{-1}, 10^{-2}, 10^{-3}, 10^{-4}, 10^{-5} and 10^{-6}) of the cultures were prepared in 96-well plates. 5µl of these dilutions (10^{-2}, 10^{-3}, 10^{-4}, 10^{-5} and 10^{-6}) was spotted on plates containing media: LB, M9 minimal media supplemented with one of the carbon sources- D-glucose, Na-acetate, Na-succinate, Na-oleate. Plates were incubated at 37ºC and images were taken at different time intervals using Bio-Rad gel documentation system.

2.2.8 Determination of Quinone content using HPLC

OD_{600} of the secondary overnight culture in 1X TB was measured. Equal number of cells of each culture were pelleted in pre-weighed MCTs. Actual weight of the pellet was obtained. The pellet was resuspended in 100 µl 0.15M KCl, 200 µl glass beads, 600 µl Methanol and 15 µl of 0.75 mg/ml Q_{10} standard. The tubes were vortexed for 15 min, 400 µl of Hexane was added, again vortexed for 3 min and centrifuged at 6000 rpm for 1 min. 100 µl of the supernatant was vacuum dried and resuspended in 100 µl of Mobile Phase. The lipids in the extract were separated through Reverse-Phase liquid chromatography

using C-18 column, and detected using Photodiode array detector. The samples were run in Mobile Phase with 1 ml/min flow rate. The peak for ubiquinone-8 was determined at 275 nm and for ubiquinol-8 at 290 nm. The peak area was normalised by pellet mass and Q_{10}-standard peak area.

Chapter 3:

Results and Discussion

3. Results and Discussion

This project was aimed at identifying new players involved in ubiquinone biosynthesis in *E. coli*. Traditionally, the non-fermentable carbon source, succinate, has been used to identify genes involved in ubiquinone biosynthesis since mutants defective in ubiquinone biosynthesis display significant growth defects in succinate compared to growth on a fermentable carbon source e.g. glucose. Contrary to this, a recent study reported that a Δ*ubiI* strain, where ubiquinone levels are reduced to only ~10% of wild-type level, exhibits normal growth in succinate. Unpublished work from our lab has shown that strains defective in ubiquinone biosynthesis exhibit higher growth defect in oleate, a LCFA, as compared to other non-fermentable carbon sources. Strikingly, Δ*ubiI* also showed significant growth defect in oleate. Follow-up studies revealed that the increased requirement of ubiquinone on oleate is to counteract oxidative stress generated by LCFA metabolism. These results suggest that the dataset from high throughput genetic screen on oleate can be a useful resource for identifying missing players in the ubiquinone biosynthetic pathway.

3.1 High throughput genetic screen revealed new players involved in ubiquinone biosynthesis

In order to identify new players involved in ubiquinone biosynthesis, *y*-genes from top 100 candidates, deletion of which showed significant growth defect in oleate (Table 3.1), were selected and their correlation coefficient with other *ubi* deletion strains was accessed from a published database [13].

Table 3.1 *y*-genes among top 100 candidates, which exhibit growth defect in oleate, and their fitness scores on different carbon sources

Strain	Rank in the oleate screen	N-acetyl glucosamine [-] {0}	acetate [-] {0}	glucosamine [-] {0}	glycerol [-] {0}	maltose [-] {0}	succinate [-] {0}	oleate [-] {5}
Δ*yqiC*	17	0.47171	0.46184	-0.3372	-0.482	0.82565	0.27313	-7.245
Δ*yhcB*	37	1.16831	1.34461	1.09921	1.5389	0.22784	1.77324	-3.164
Δ*yebK*	39	0.09342	-1.2886	0.55508	0.05131	-0.1178	-0.0729	-3.061
Δ*ybhP*	48	-0.8448	0.29211	-0.0316	-0.0627	0.16586	-0.9357	-2.725
Δ*ybgA*	54	0.20854	-1.7197	0.34754	-0.7424	0.03197	-1.3245	-2.633

Δ*yqaA*	55	1.06223	-0.5315	1.14	0.91929	0.52495	1.0629	-2.565
Δ*ydeS*	66	0.1505	-1.0369	0.14699	-0.5754	0.0328	-0.9229	-2.345
Δ*ygeH*	67	1.18481	-0.3755	0.23343	-0.7043	-0.7791	-0.0415	-2.299
Δ*yidK*	68	0.26697	-0.81	0.41293	1.09481	0.0803	-0.6129	-2.281
Δ*ygcR*	70	0.35089	0.20777	-0.0739	0.50224	0.26913	0.21909	-2.275
Δ*yhbT*	72	-0.2931	0.14139	-0.6443	-0.2583	-0.2959	-0.6383	-2.261
Δ*yjjM*	76	0.11629	-0.3209	-0.786	-0.4864	-0.1579	-0.4988	-2.167
Δ*yeaN*	78	1.19923	-0.1181	0.12564	0.84178	0.14875	-0.1702	-2.128
Δ*yqiH*	79	-0.6429	0.40907	-0.6316	-0.3661	-0.194	-0.5082	-2.117
Δ*ydfU*	81	0.07628	-0.2627	0.94159	-0.0615	0.49939	-0.5851	-2.099
Δ*yjcZ*	82	-3.2441	0.07384	-2.5677	-1.0496	-0.5958	-2.5637	-2.085
Δ*yegT*	87	-1.4277	-0.0358	-1.5598	-0.5474	-1.2155	-1.2372	-2.076
Δ*ybaP*	88	-0.8654	-1.3688	-0.6877	-0.7077	-1.4727	-0.4676	-2.062
Δ*ytfL*	93	1.68645	-0.3834	1.41459	0.80868	-0.199	2.01467	-2.004
Δ*yeaD*	98	-0.5971	-0.7004	0.17657	0.29517	0.28823	0.39441	-1.983
Δ*yfcS*	99	-1.5207	-1.6612	-1.776	-1.3658	-0.3878	-1.2585	-1.981
Δ*yfjS*	101	-0.8918	-0.349	-0.1553	-0.7891	-0.3813	-0.2387	-1.964

Among these 22 *y*-genes, 5 were selected for further study that showed more significant growth defect in oleate and had higher correlation coefficients with the *ubi*-deletion strains (Table 3.2). The selected *y*-genes are *yqiC*, *yhcB*, *ydeS*, *ygeH* and *ydfU*.

Table 3.2 Selected y-genes and their correlation coefficient with other *ubi* deletion strains.

Strain	**Rank in the oleate screen**	**Δ*ubiC***	**Δ*ubiX***	**Δ*ubiE***	**Δ*ubiF***	**Δ*ubiH***	**Δ*ubiI***
Δ*yqiC*	17	0.47	0.32	0.31	0.28	0.32	0.77
Δ*yhcB*	37	-0.05	0.07	0.13	0.25	0.25	0.144
Δ*ydeS*	66	0.28	0.12	0.09	0.105	0.086	0.27
Δ*ygeH*	67	0.38	0.26	0.15	0.19	0.04	0.31
Δ*ydfU*	81	-0.248	-0.067	-0.29	-0.178	-0.13	-0.2

3.2 Deletion of *yqiC* shows growth defect specific to oleate

It was shown by a PhD student, Shashank Agrawal, in our lab that *yqiC* deletion strain exhibits growth defect on oleate but not on glucose. On transforming the normal copy of *yqiC* on plasmid in a Δ*yqiC* strain, the phenotype was complemented (upublished data). In the present study the experiment was repeated to reproduce the phenotype and

also to check the growth of this strain on other non-fermentable carbon sources (acetate and succinate). Different dilutions of WT, Δ*fadL*, Δ*yqiC* were spotted on M9 minimal media plates supplemented with glucose, acetate, succinate or oleate as sole carbon source and images were taken after every 6 hrs. Since oleate requires a detergent to solubilize, the detergent was also added to other carbon sources as a control. Δ*yqiC* showed growth defect only on oleate (Fig 3.1).

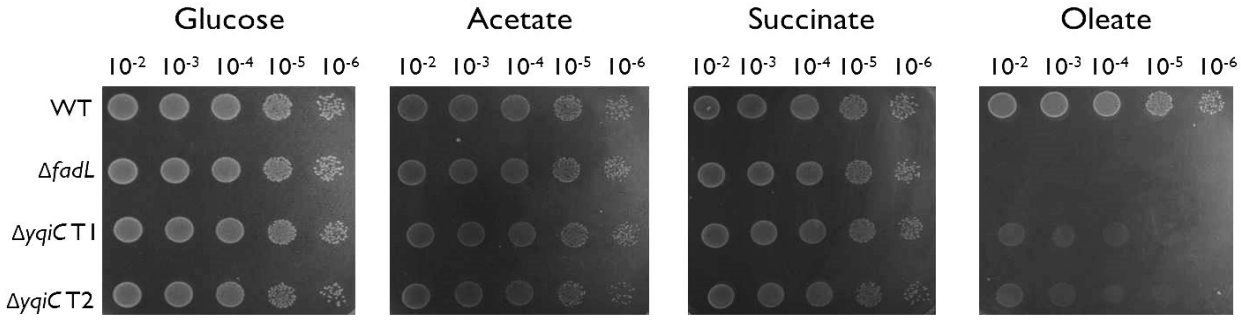


Fig 3.1 Growth phenotype of *yqiC*::kan on glucose, acetate, succinate and oleate. *fadL*::kan was used as a control and two transductants of Δ*yqiC* (T1 and T2) were spotted to check if both have the same phenotype. This experiment was repeated three times and a representative set is shown.

The growth defect of Δ*yqiC* on oleate was rescued when *yqiC* cloned in pACYC184 was transformed into Δ*yqiC* (pSA4: *yqiC* clone was constructed by PhD student, Shashank Agrawal) (Fig 3.2). The growth defect of Δ*yqiC* only on oleate suggested that YqiC might be a player involved in ubiquinone biosynthesis.

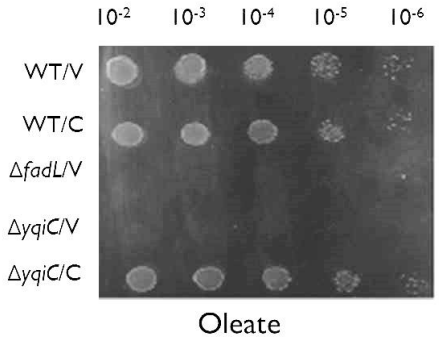
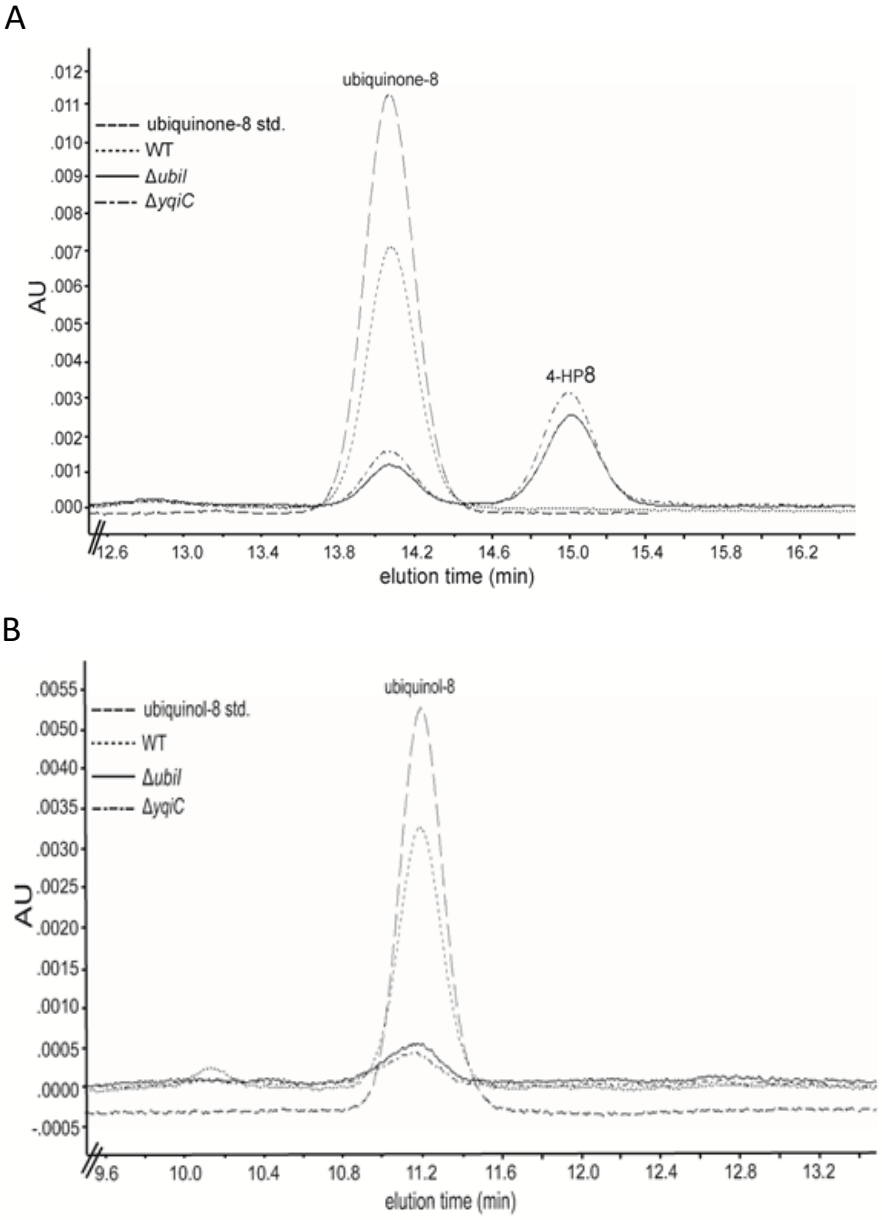


Fig 3.2 *yqiC* cloned in a plasmid rescues the growth defect of *yqiC*::kan on oleate. The plasmid used is pACYC184 with tetracycline selection. V refers to pACYC184 and C refers to *yqiC* cloned in pACYC184 (pSA4). This experiment was done once.

3.3 Δ*yqiC* strain has lower ubiquinone content compared to WT

The specific growth defect of Δ*yqiC* on oleate is similar to the growth phenotype of Δ*ubiI*. Also, the correlation coefficient of Δ*ubiI* and Δ*yqiC* is very high (Table 3.2). These relationships led us to investigate whether *yqiC* is involved in ubiquinone biosynthesis. WT and Δ*yqiC* strains were grown in TB media and ubiquinone levels were measured using HPLC. Δ*yqiC* showed a similar reduction in ubiquinone levels as the Δ*ubiI* strain, i.e., ubiquinone levels were ~20% of ubiquinone levels of WT. Interestingly, both these strains also accumulate the same intermediate, 4-HP_8 (Fig 3.3) [14].



C

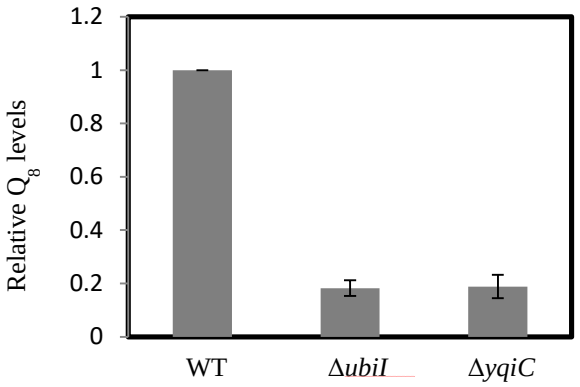
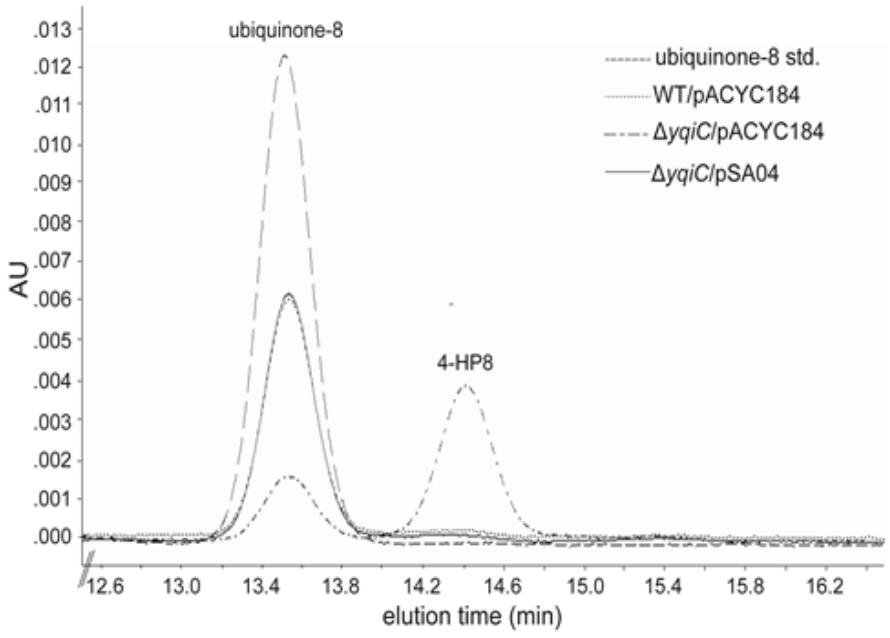


Fig 3.3 Ubiquinone levels are reduced to ~20% in Δ*yqiC*, similar to Δ*ubiI*. A) HPLC chromatogram showing ubiquinone peak in Q_8 standard, WT, Δ*ubiI* and Δ*yqiC*. B) HPLC chromatogram showing ubiquinol peak in Q_8H_2 standard, WT, Δ*ubiI* and Δ*yqiC*. C) Total ubiquinone content of Δ*ubiI* and Δ*yqiC* with respect to WT. Data represent Mean ± SD of three independent experiments.

The low ubiquinone content in Δ*yqiC* strain was restored to WT levels when this strain was transformed with a plasmid containing *yqiC* (pSA4). Moreover, after complementation, there was no accumulation of 4-HP_8 (Fig 3.4).

A



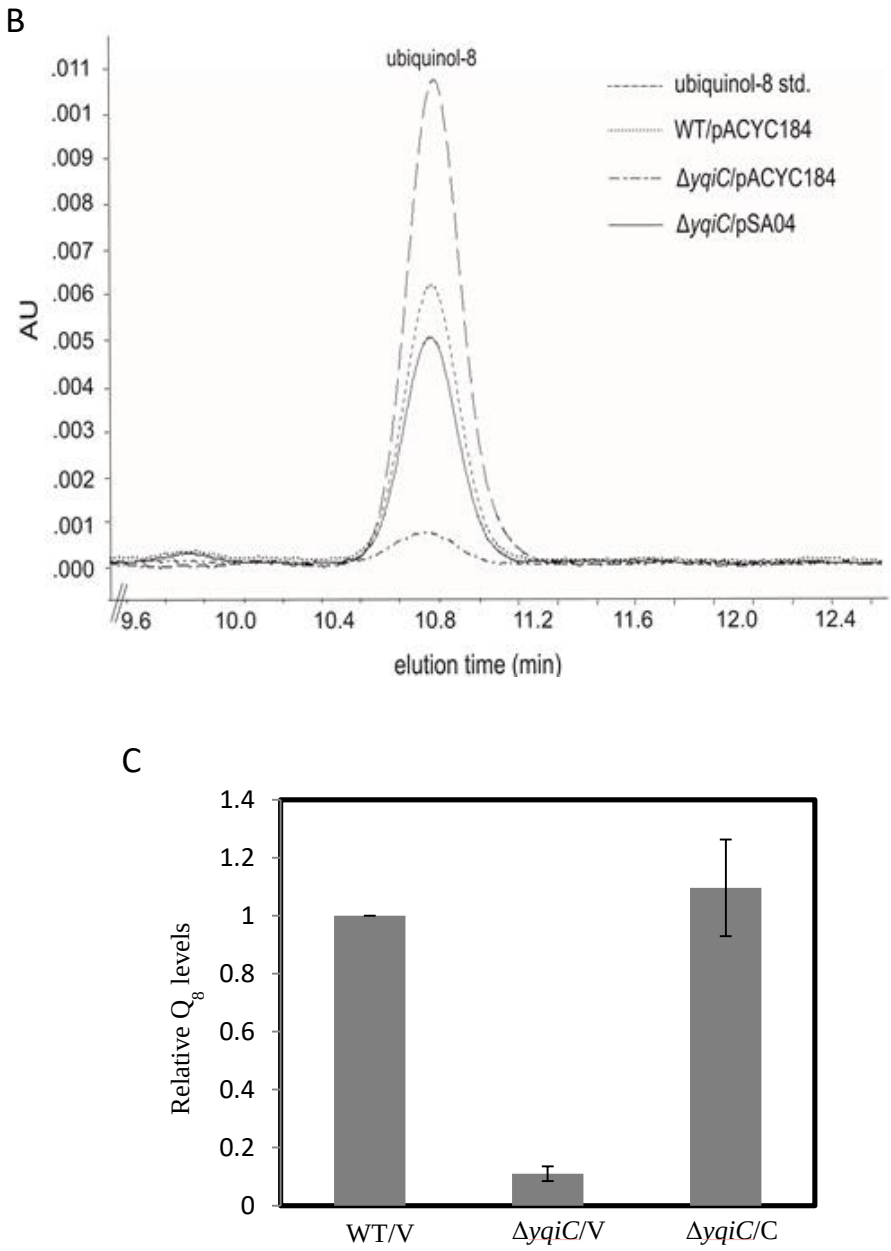


Fig 3.4 Ubiquinone levels are restored in Δ*yqiC* strain transformed with plasmid carrying *yqiC*. V refers to empty plasmid pACYC184 and C refers to *yqiC* cloned in pACYC184 (pSA4). A) HPLC chromatogram showing ubiquinone peak in Q_8 standard, WT transformed with pACYC184 and Δ*yqiC* containing either pACYC184 or pSA4. No accumulation of 4-HP_8 was observed in the *yqiC* complemented strain. B) HPLC chromatogram showing ubiquinol peak in Q_8 standard, WT transformed with pACYC184 and Δ*yqiC* containing either pACYC184 or pSA4. C) Total ubiquinone content in Δ*yqiC* containing either pACYC184 or pSA4 with respect to WT transformed with pACYC184. The cultures were grown in TB media. Data represent Mean ± SD of three independent experiments.

3.4 Δ*yqiC*Δ*ubiI* double knockout shows a synthetic sick phenotype with no detectable ubiquinone

The related phenotypes of Δ*yqiC* and Δ*ubiI* suggested involvement of these players in a common step of ubiquinone biosynthesis and this prompted us to examine the phenotype of Δ*yqiC*Δ*ubiI* double mutant. In order to study this, a Δ*yqiC*Δ*ubiI* double knockout strain was made by flipping out the Kanamycin cassette from *ubiI*::kan using a plasmid, pCP20, which codes for flippase. This strain was then transduced with P1 lysate of *yqiC*::kan and confirmed through colony PCR. In contrast to the normal growth of Δ*yqiC* and Δ*ubiI* in LB media, the double mutant formed tiny colonies on LB reflecting a synthetic sick phenotype of Δ*yqiC*Δ*ubiI* (Fig 3.5).

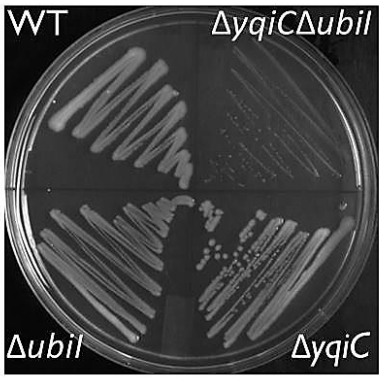


Fig 3.5 Δ*yqiC*Δ*ubiI* shows a synthetic sick phenotype on LB plate. This phenotype was checked two times.

The ubiquinone content in the double knockout strain was determined using HPLC by growing the strain overnight in LB containing 0.2% glucose. The other strains were also grown in the same media condition for comparison. No ubiquinone was detected in the double knockout strain although there was still some accumulation of the intermediate, 4-HP_8 (Fig 3.6).

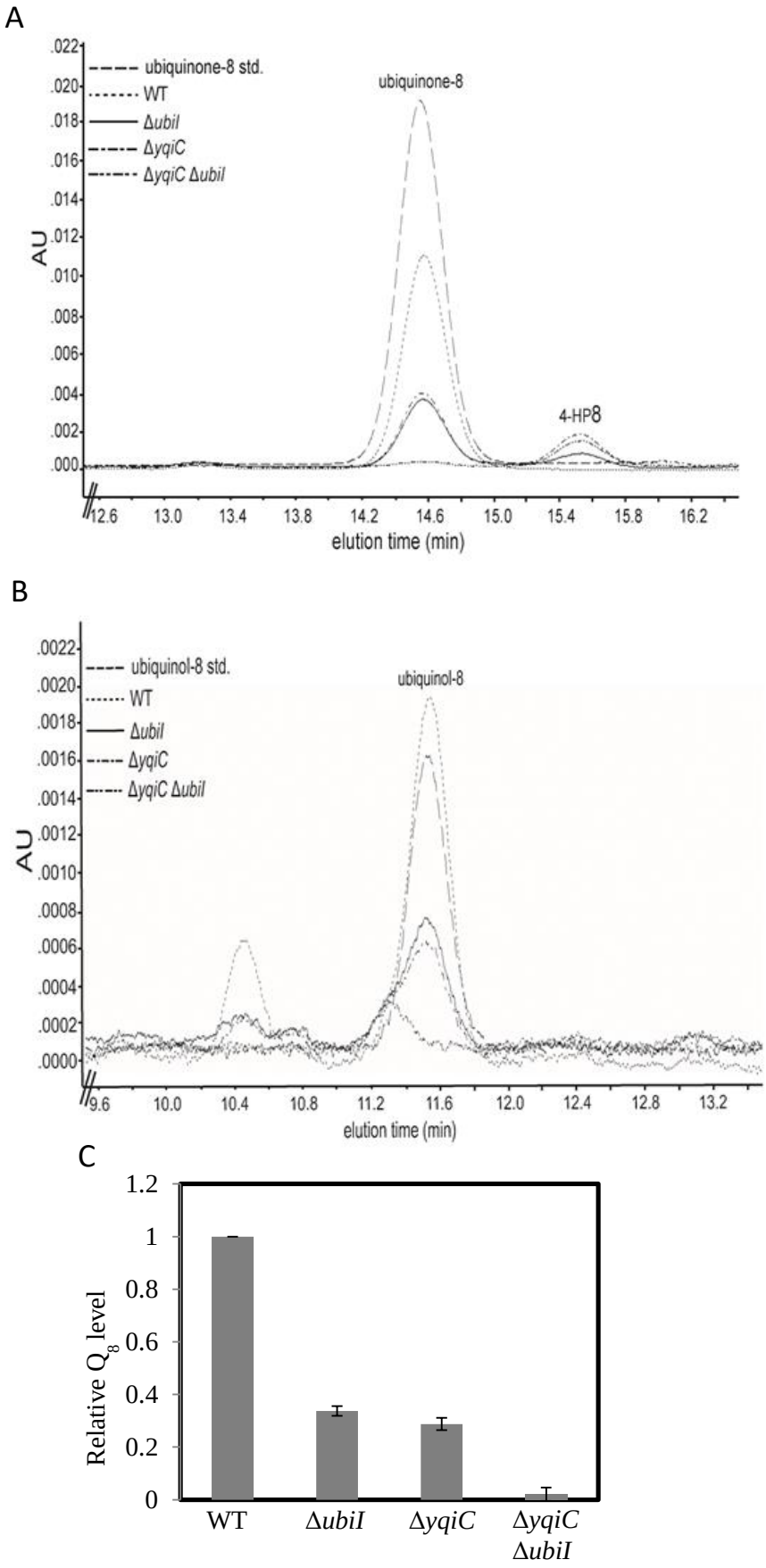


Fig 3.6 Δ*yqiC*Δ*ubiI* has no detectable ubiquinone. A) HPLC chromatogram showing ubiquinone peak in Q_8 standard, WT, Δ*yqiC*, Δ*ubiI* and Δ*yqiC* Δ*ubiI*. B) HPLC

chromatogram showing ubiquinol peak in Q_8H_2 standard, WT, Δ*yqiC*, Δ*ubiI* and Δ*yqiC* Δ*ubiI*. C) Total ubiquinone content in Δ*yqiC*Δ*ubiI* with respect to WT. Data represent Mean ± SD of three independent experiments.

3.5 Δ*yqiC* strain transformed with *ubiI* on multicopy plasmid shows no growth recovery on oleate

Since the Δ*yqiC*Δ*ubiI* strain shows a synthetic sick phenotype, it suggests that the two genes might not be involved in different steps of the same pathway. Moreover, since both Δ*ubiI* and Δ*yqiC* single knockouts show similar reduction in ubiquinone levels and accumulate similar intermediate, 4-HP_8, it is unlikely that the two genes are involved in redundant pathways. We speculate that *ubiI* and *yqiC* might be involved at the same step of ubiquinone biosynthesis: when both the genes are present, they function together to give 100% ubiquinone, and when only one of them is present it works sub-optimally to result in ~20% ubiquinone levels. Hence we determined if the overexpression of one gene in the absence of the other can rescue the phenotype to some extent. We first transformed *ubiI* cloned in a plasmid, pBAD24 (pKJ7: clone was constructed by a PhD student in the lab, Kanchan Jaswal) into WT and Δ*yqiC*. The strains were spotted on M9 minimal media plates containing glucose, acetate, succinate or oleate as sole carbon source. The plates were imaged after every 6 hrs. The Δ*yqiC* strain carrying *ubiI* clone showed growth defect specific to oleate, a phenotype similar to Δ*yqiC* transformed with vector control (Fig 3.7), and also had similar ubiquinone levels as Δ*yqiC* carrying vector (Fig 3.8).

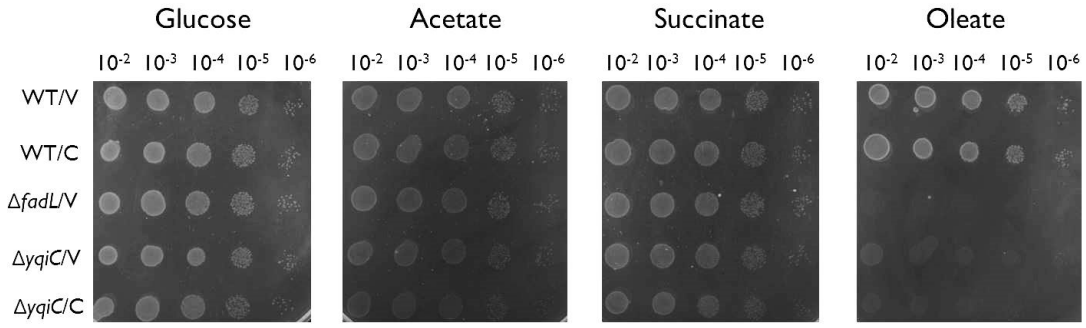


Fig 3.7 Δ*yqiC* transformed with *ubiI* shows no growth recovery in oleate. The growth phenotype is similar to that of Δ*yqiC*. V refers to empty vector pBAD24 and C refers to *ubiI* cloned in pBAD24 (pKJ7). Δ*fadL* containing empty vector pBAD24 was used as a control. This experiment was performed two times and a representative set is shown.

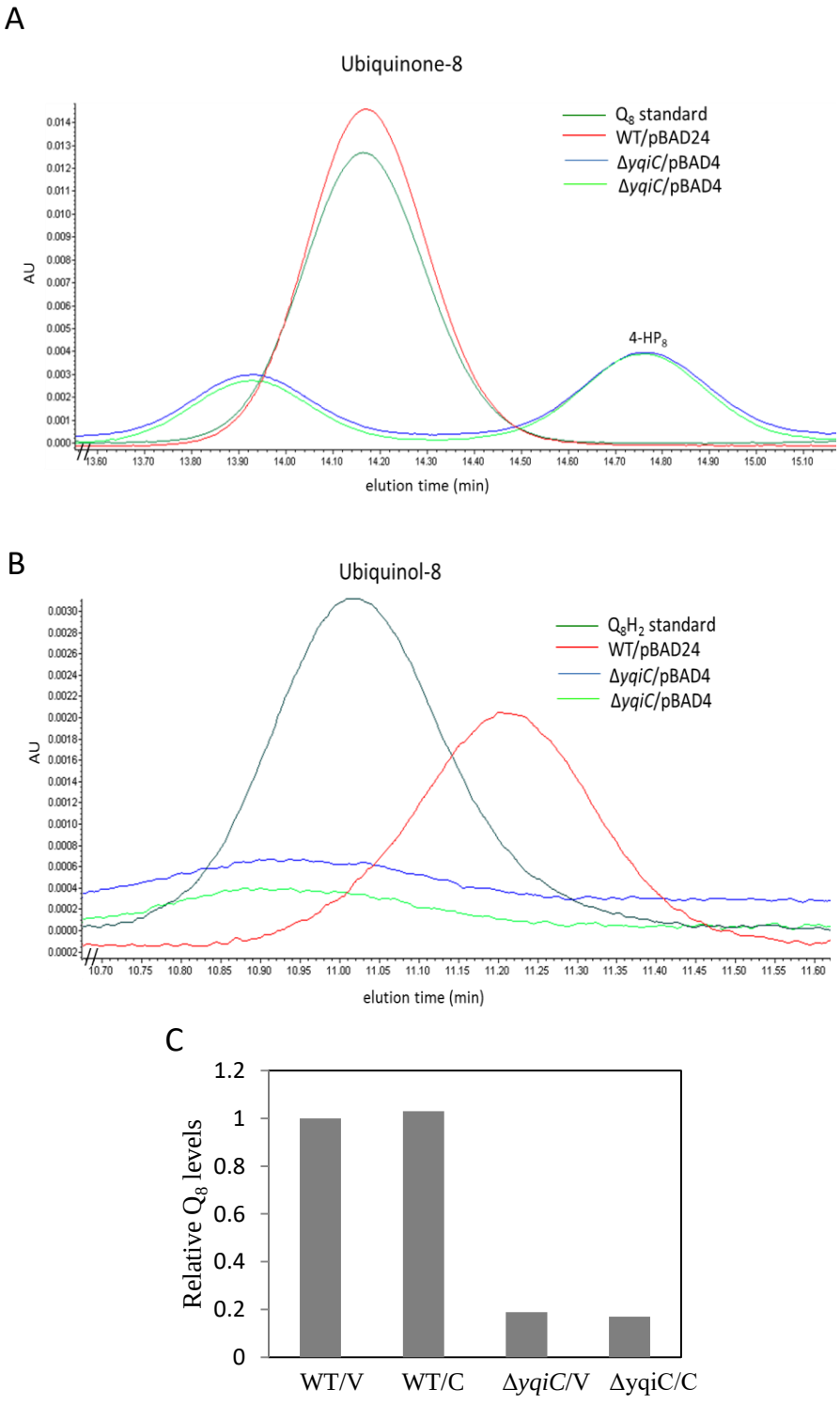


Fig 3.8 Δ*yqiC* transformed with multicopy *ubiI* on plasmid shows similar ubiquinone level as Δ*yqiC*. V refers to pBAD24 and C is *ubiI* cloned in pBAD24 (pKJ7). A) HPLC chromatogram showing ubiquinone-8 peak in Q_8 standard, WT transformed with pBAD24 and Δ*yqiC* containing either pBAD24 or pKJ7. B) HPLC chromatogram showing ubiquinol peak in Q_8H_2 standard, WT transformed with pBAD24 and Δ*yqiC* containing either pBAD24 or pKJ7. C) Total ubiquinone content in WT transformed with

pKJ7 and Δ*yqiC* containing either pBAD24 or pKJ7 with respect to WT transformed with pBAD24. This experiment was done once and the cultures were grown in TB media.

3.6 Δ*ubiI* strain transformed with multicopy *yqiC* on plasmid shows slight growth recovery on oleate after prolonged incubation

Δ*yqiC* strain transformed with multicopy *ubiI* showed similar growth pattern and ubiquinone level as Δ*yqiC*. In order to determine whether multicopy *yqiC* transformed in Δ*ubiI* recovers the growth defect of Δ*ubiI* in oleate, *yqiC* cloned in a plasmid, pACYC184 (pSA4) was transformed into WT and Δ*ubiI* strains. These strains were then spotted on M9 minimal media plates containing glucose, acetate, succinate or oleate as sole carbon source. Images were taken after every 6 hrs.

Intriguingly, multicopy *yqiC* transformed in Δ*ubiI* strain showed a slight growth recovery on oleate after prolonged incubation (~80 hrs). Even WT transformed with *yqiC* showed a slight growth advantage than WT containing empty vector on oleate. On the contrary, both WT and Δ*ubiI* strains transformed with *yqiC* showed growth defect on acetate and succinate (Fig 3.9). This result suggests that the regulation of ubiquinone synthesis might be different depending upon the carbon source. Till date, there is no detailed study on differential regulation of quinone synthesis by different carbon sources but our data hints at this and it would be an interesting area of investigation.

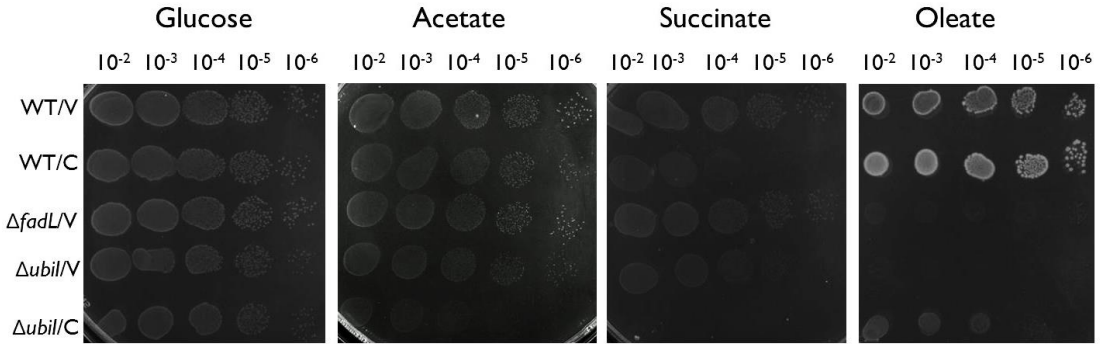
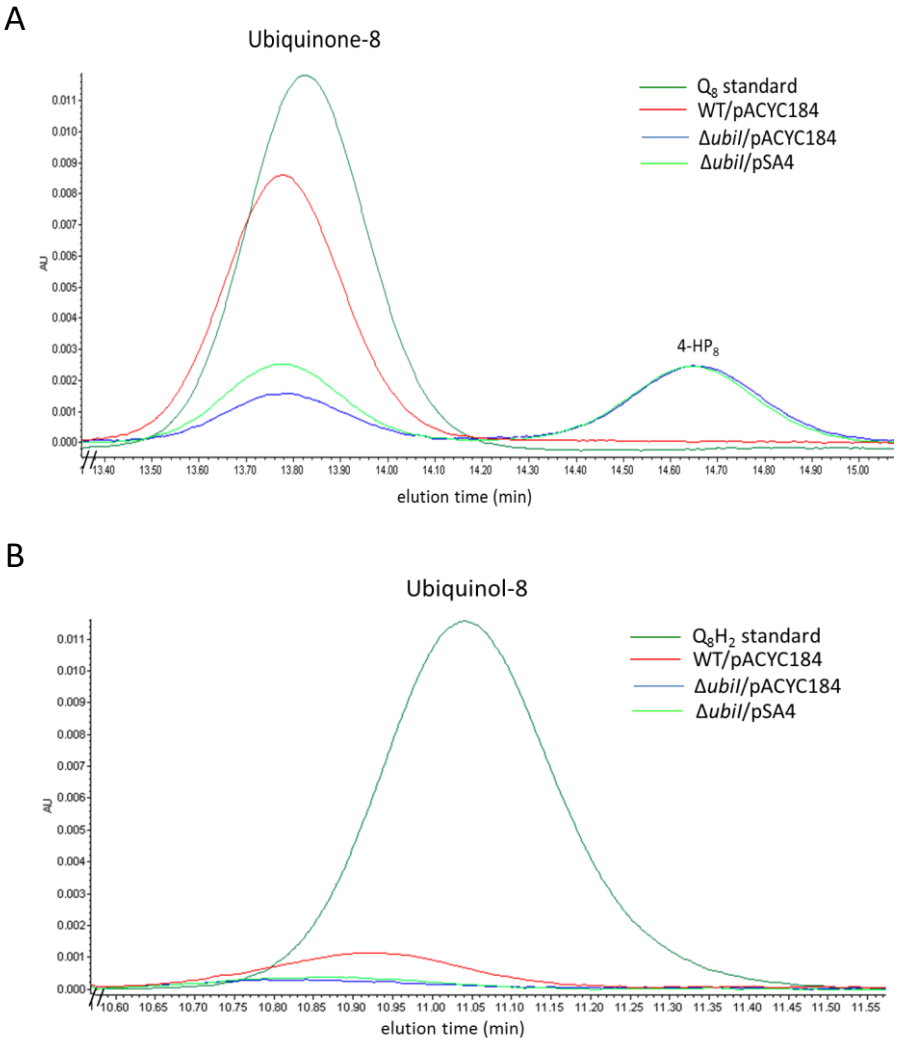


Fig 3.9 Growth phenotype of Δ*ubiI* strain transformed with multicopy *yqiC* on minimal media supplemented with different carbon sources. V refers to empty plasmid pACYC184 and C refers to *yqiC* cloned in pACYC184 (pSA4). WT and Δ*ubiI* strains transformed with *yqiC* show growth defect on acetate and succinate while a slight growth

recovery on oleate. This experiment was repeated three times and a representative set is shown.

We argued that *yqiC* transformed in Δ*ubiI* strain is causing growth recovery on oleate due to increased ubiquinone level. Hence this strain was grown in TB media and the ubiquinone level was determined using HPLC. Contrary to our expectation, the Δ*ubiI* strain transformed with *yqiC* showed similar ubiquinone level as Δ*ubiI* carrying empty plasmid (Fig 3.10). This data suggests that either *yqiC* transformed in Δ*ubiI* strain shows growth recovery on oleate by some mechanism other than rescuing ubiquinone levels or since we speculate that the regulation of quinone synthesis is different in different carbon sources, the increased ubiquinone levels might be occurring only in oleate condition. Thus we would like to determine the ubiquinone level in Δ*ubiI* strain transformed with *yqiC*, cultured in minimal media supplemented with oleate.



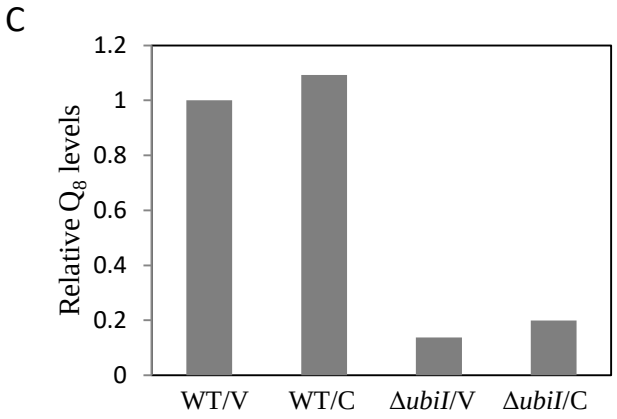


Fig 3.10 Δ*ubiI* strain transformed with multicopy *yqiC* shows similar ubiquinone level as Δ*ubiI*. V refers to pACYC184 and C is *yqiC* cloned in pACYC184 (pSA4). A) HPLC chromatogram showing ubiquinone-8 peak in Q_8 standard, WT transformed with pACYC184 and Δ*ubiI* containing either pACYC184 or pSA4. B) HPLC chromatogram showing ubiquinol-8 peak in Q_8H_2 standard, WT transformed with pACYC184 and Δ*ubiI* containing either pACYC184 or pSA4. C) Total ubiquinone content in WT containing pSA4 and Δ*ubiI* transformed with either pACYC184 or pSA4 with respect to WT containing pACYC184. This experiment has been done once and the cultures were grown in TB media.

Another explanation for the growth recovery of Δ*ubiI* strain transformed with *yqiC* on oleate might be the role of YqiC in menaquionone or demethylmenaquinone biosynthesis. Since YqiC is important for menaquinone biosynthesis in *S. typhimurium*, it might be performing the same function in *E. coli*. From previous studies in our lab, we know that Δ*ubiI* strain when grown on oleate is under excessive oxidative stress and ubiquinone is a key antioxidant during oleate metabolism. *yqiC* transformed in Δ*ubiI* might lead to increased menaquinone biosynthesis which would then act as an antioxidant. Similarly in case of WT transformed with *yqiC,* the increased menaquinone, acting as an antioxidant, would facilitate growth on oleate. In addition to MK, we would also determine the levels of DMK, another quinone that can function under both aerobic and anaerobic conditions.

3.7 Standardizing conditions for determining Menaquinone (MK) and Demethylmenaquinone (DMK) levels

To determine the MK and DMK levels, we first standardized the peaks and wavelengths corresponding to both MK and DMK separately. We used *menA*::kan and *ubiE*::kan strains for this purpose. MenA is an enzyme involved in MK biosynthesis while UbiE methylates and converts DMK into MK. Hence, in case of *menA*::kan, both MK and

DMK peaks would be missing while in *ubiE*::kan, we would expect accumulation of DMK while the MK peak would be missing (Fig 1.5). Keeping this rationale in mind, the MK and DMK peaks were assigned and the area under the peak was scanned at various wavelengths. The wavelength at which maximum intensity peak for a compound was observed was considered as wavelength maxima of that particular compound. It was found that the wavelength maxima for DMK is 245 nm while for MK is 249 nm (Fig 3.11).

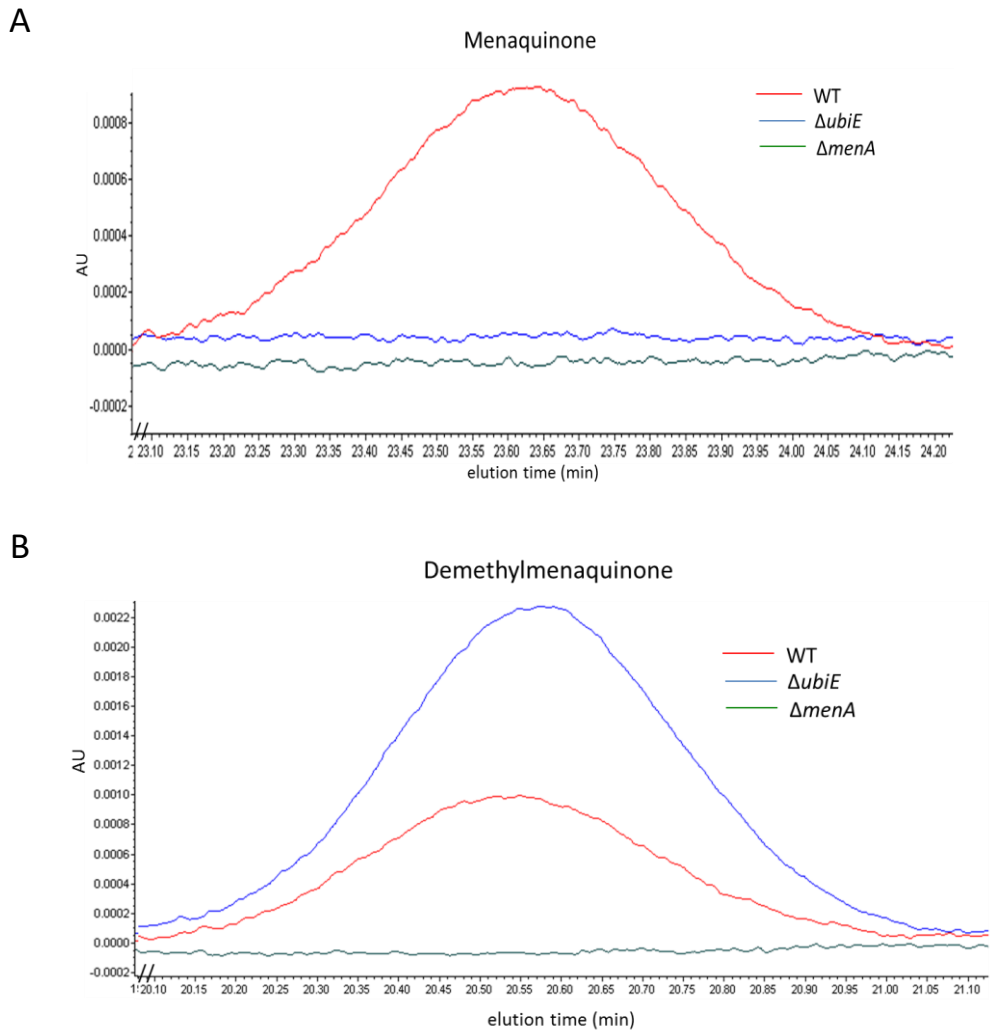
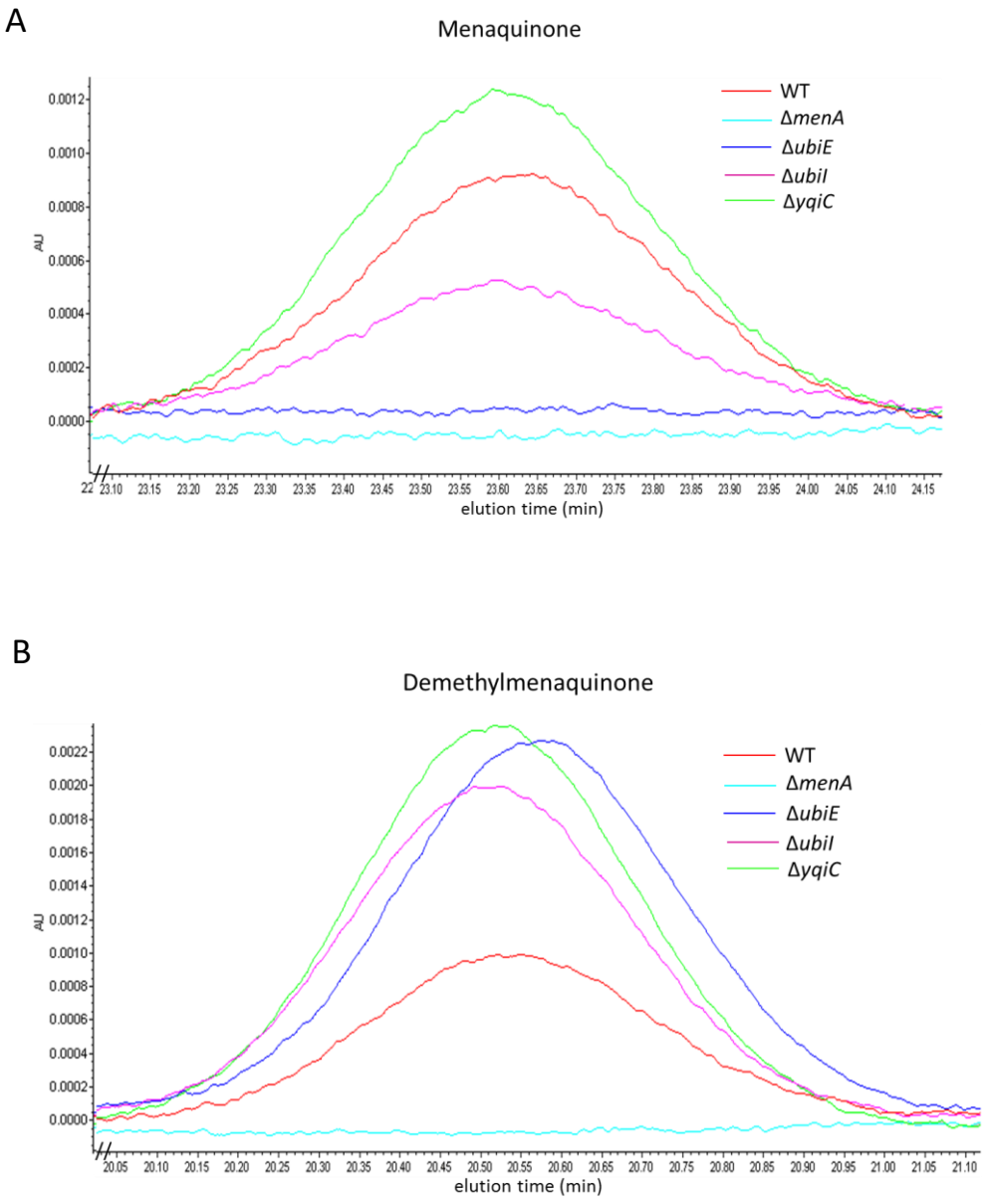


Fig 3.11 Menaquinone (MK) and Demethylmenaquinone (DMK) peaks in HPLC. A) HPLC chromatogram showing MK peak in WT, Δ*menA* and Δ*ubiE* at 249 nm. B) HPLC chromatogram showing DMK peak in WT, Δ*menA* and Δ*ubiE* at 245 nm. This experiment was done four times and a representative chromatogram overlay is shown.

3.8 Δ*yqiC* and Δ*ubiI* exhibit variations in MK and DMK levels

Δ*yqiC* and Δ*ubiI* strains were grown in TB media and the MK and DMK levels were determined using HPLC. Δ*yqiC* strain had MK level similar to WT whereas there was a 2-fold increase in DMK level. On the other hand, in Δ*ubiI* strain, the MK level was almost half of WT whereas the DMK level was 2- fold higher than WT (Fig 3.12).



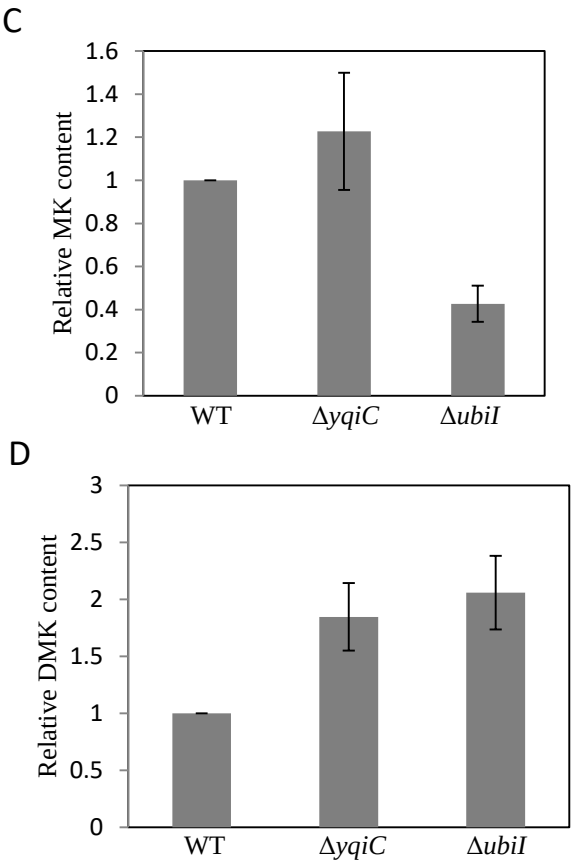
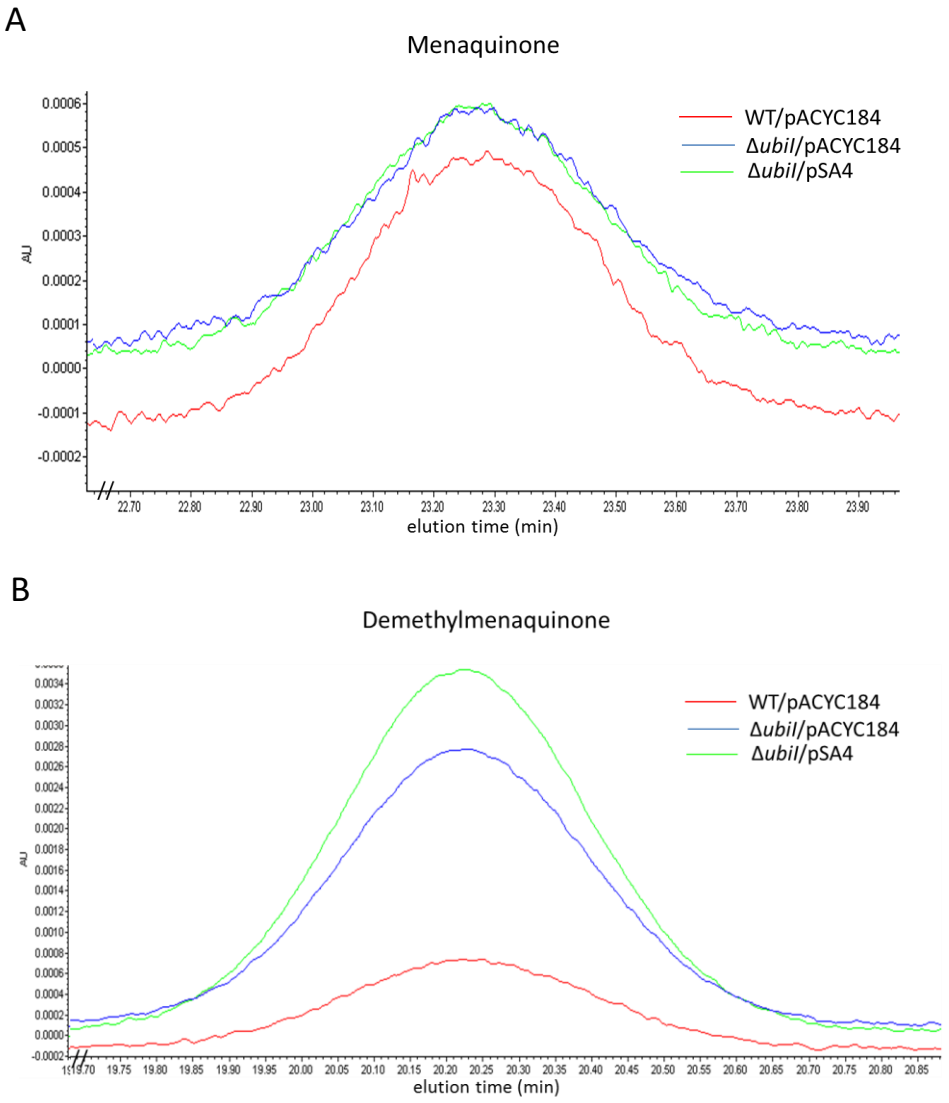


Fig 3.12: Menaquinone (MK) and demethylmenaquinone (DMK) content in Δ*yqiC* and Δ*ubiI*. A) HPLC chromatogram showing MK peak in WT, Δ*yqiC* and Δ*ubiI* at 249 nm. B) HPLC chromatogram showing DMK peak in WT, Δ*yqiC* and Δ*ubiI* at 245 nm. Δ*menA* and Δ*ubiE* strains were used as controls. C) Total MK content in Δ*yqiC* and Δ*ubiI* with respect to WT. D) Total DMK content in Δ*yqiC* and Δ*ubiI* with respect to WT. Data represent Mean ± SD of three independent experiments. A representative chromatogram overlay is shown.

The result obtained for Δ*yqiC* in *E. coli* in this study is contrary to that observed for *S. typhimurium* i.e, there is no decrease in MK levels in Δ*yqiC* in *E. coli* (Fig 3.12) whereas MK levels are significantly reduced in a Δ*yqiC* strain in *S. typhimurium* [17]. There are two possible explanantions for such a difference. First, we know from our experiments that the regulation of quinone biosynthesis might be media dependent. *S. typhimirium* was cultured in LB broth [17] while in our experiments *E. coli* was cultured in TB media. Second, the difference in menaquinone level in Δ*yqiC* in *E.coli* as compared to *S. typhimirium* might be due to the difference between these two strains. The *S. typhimurium* strain used in the previous study is a virulent strain while the strain used in our experiments is a non-pathogenic lab-strain of *E. coli*.

3.9 Δ*ubiI* strain transformed with multicopy *yqiC* does not show any change in MK or DMK level

Since we thought that the growth recovery observed in Δ*ubiI* strain transformed with multicopy *yqiC* on oleate, might be due to the involvement of MK or DMK as major quinones, the levels of these quinones were determined in the relevant strains. All the strains were grown in TB media and the lipids were extracted for HPLC. Δ*ubiI* strain transformed with multicopy *yqiC* showed similar MK and DMK level as Δ*ubiI* carrying empty plasmid (Fig 3.13).



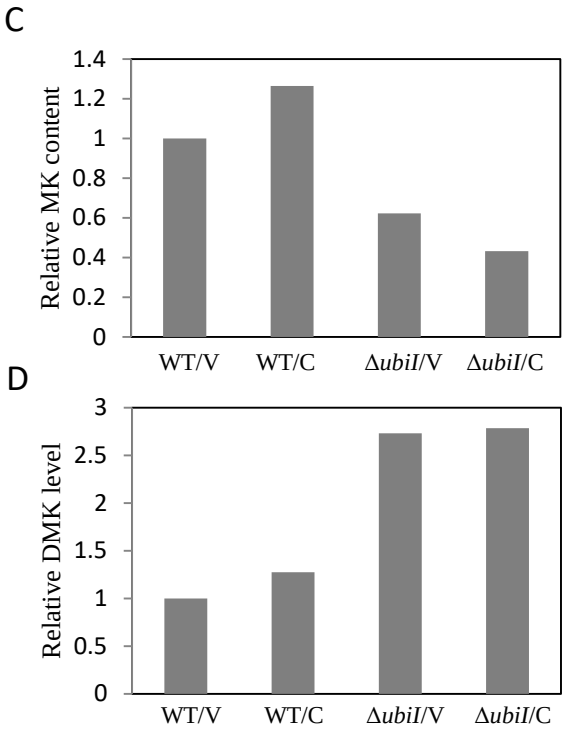


Fig 3.13 MK and DMK levels in Δ*ubiI* strain transformed with *yqiC* in a plasmid. V refers to pACYC184 and C refers to *yqiC* cloned in pACYC184 (pSA4). A) HPLC chromatogram showing MK peak in WT transformed with pACYC184 and Δ*ubiI* containing either pACYC184 or pSA4. B) HPLC chromatogram showing DMK peak in WT transformed with pACYC184 and Δ*ubiI* containing either pACYC184 or pSA4. C) Total MK content in WT containing pSA4 and Δ*ubiI* transformed with either pACYC184 or pSA4 with respect to WT containing pACYC184. D) Total DMK content in WT containing pSA4 and Δ*ubiI* transformed with either pACYC184 or pSA4 with respect to WT containing pACYC184. This experiment was done once and the cultures were grown in TB media.

3.10 Validation of growth phenotype of other *y*-genes on oleate and investigation of their role in ubiquinone biosynthesis

In order to identify new players involved in ubiquinone biosynthesis, four other *y*-gene deletion strains were considered. Their growth phenotype on different carbon sources (glucose, acetate, succinate and oleate) was assessed either by growth curve or dilution spotting and the ubiquinone level was determined using HPLC.

Δ*yhcB* and Δ*ydfU* strains did not show growth defect on any of the carbon sources (Fig 3.14 and 3.15). Further, the ubiquinone level in these strains was similar to WT (Fig 3.16 and 3.17) suggesting that these genes might not have a role in ubiquinone biosynthesis.

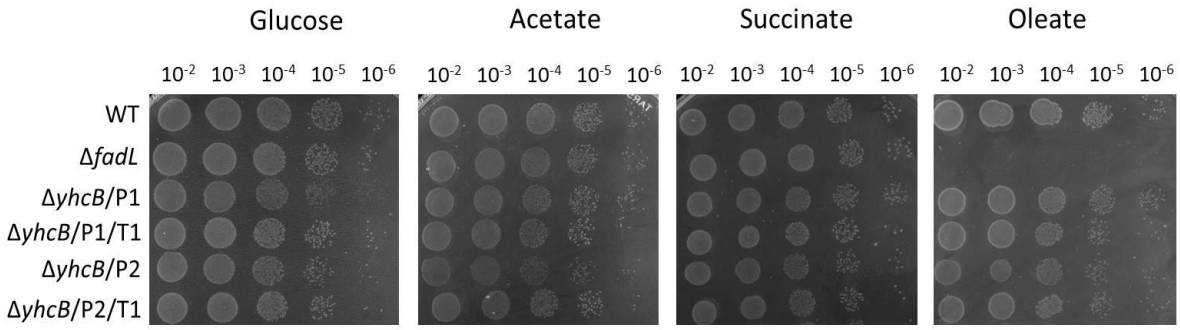


Fig 3.14 Growth phenotype of *yhcB*::kan on glucose, acetate, succinate and oleate. *fadL*::kan was used as a control and two parents (P1 and P2) along with one transductant each (T1) from each parent of Δ*yhcB* were spotted to check if they have the same phenotype. This experiment was done once.

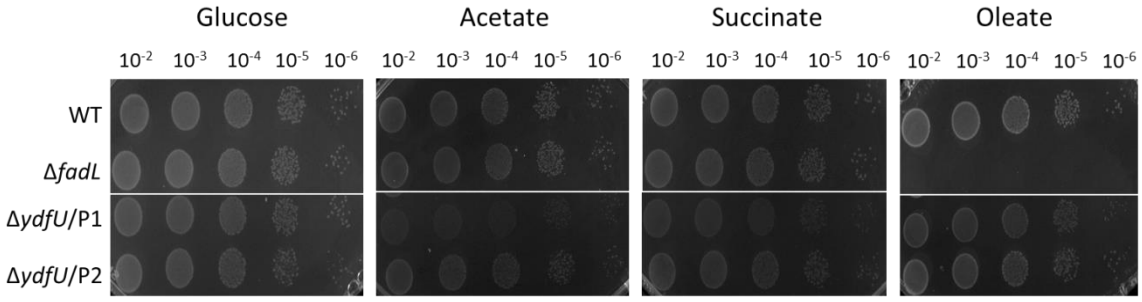


Fig 3.15 Growth phenotype of *ydfU*::kan on glucose, acetate, succinate and oleate. *fadL*::kan was used as a control and two parents (P1 and P2) of Δ*ydfU* were spotted to check if they have the same phenotype. This experiment was done once.

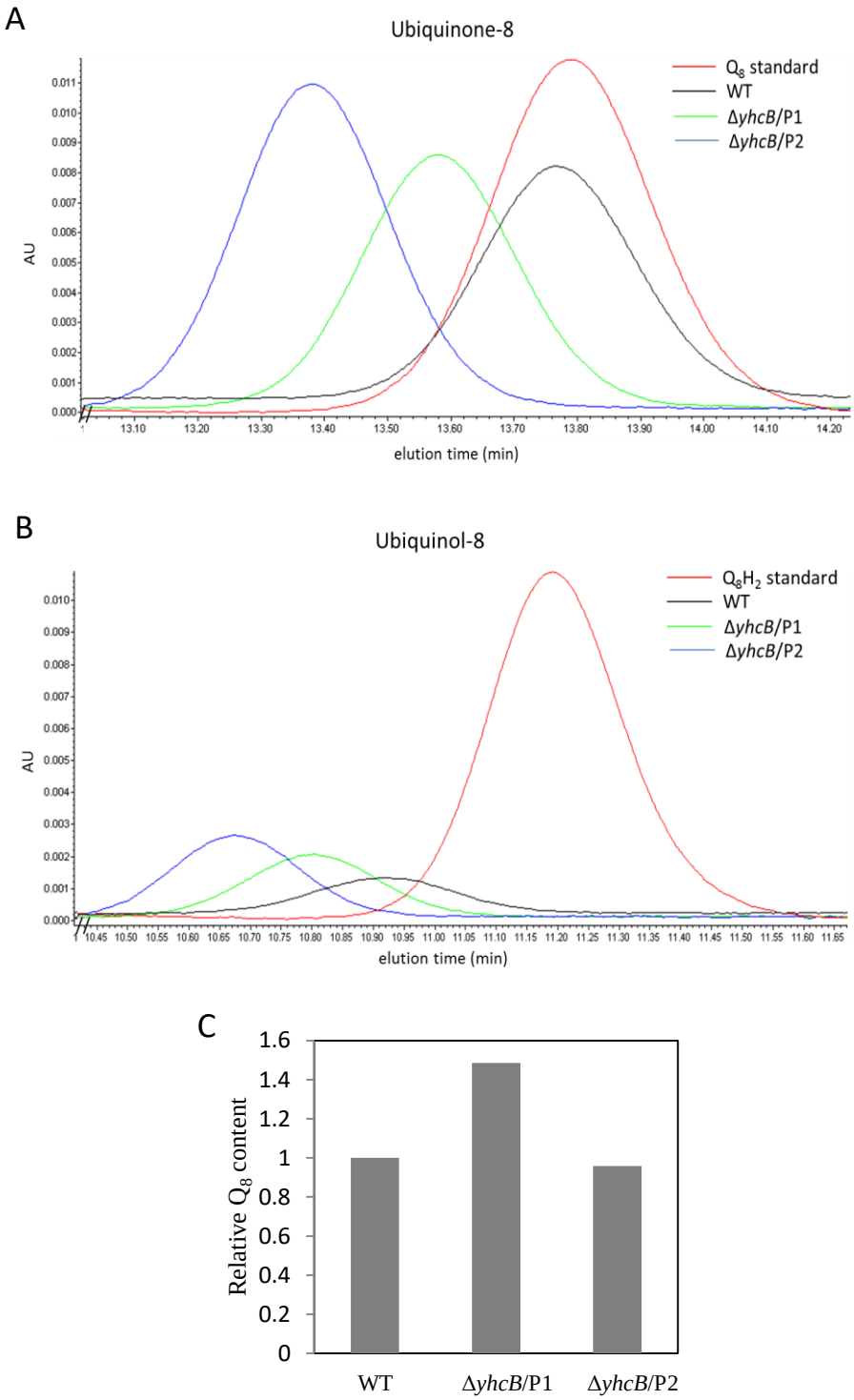


Fig 3.16 Δ*yhcB* has ubiquinone level similar to WT. A) HPLC chromatogram showing ubiquinone peak in Q_8 standard, WT and Δ*yhcB* (Parent 1 and 2) B) HPLC chromatogram showing ubiquinol peak in Q_8H_2 standard, WT and Δ*yhcB* (Parent 1 and 2) C) Total ubiquinone content in Δ*yhcB* (Parent 1 and 2) with respect to WT. This experiment was done once. The strains were cultured in TB media.

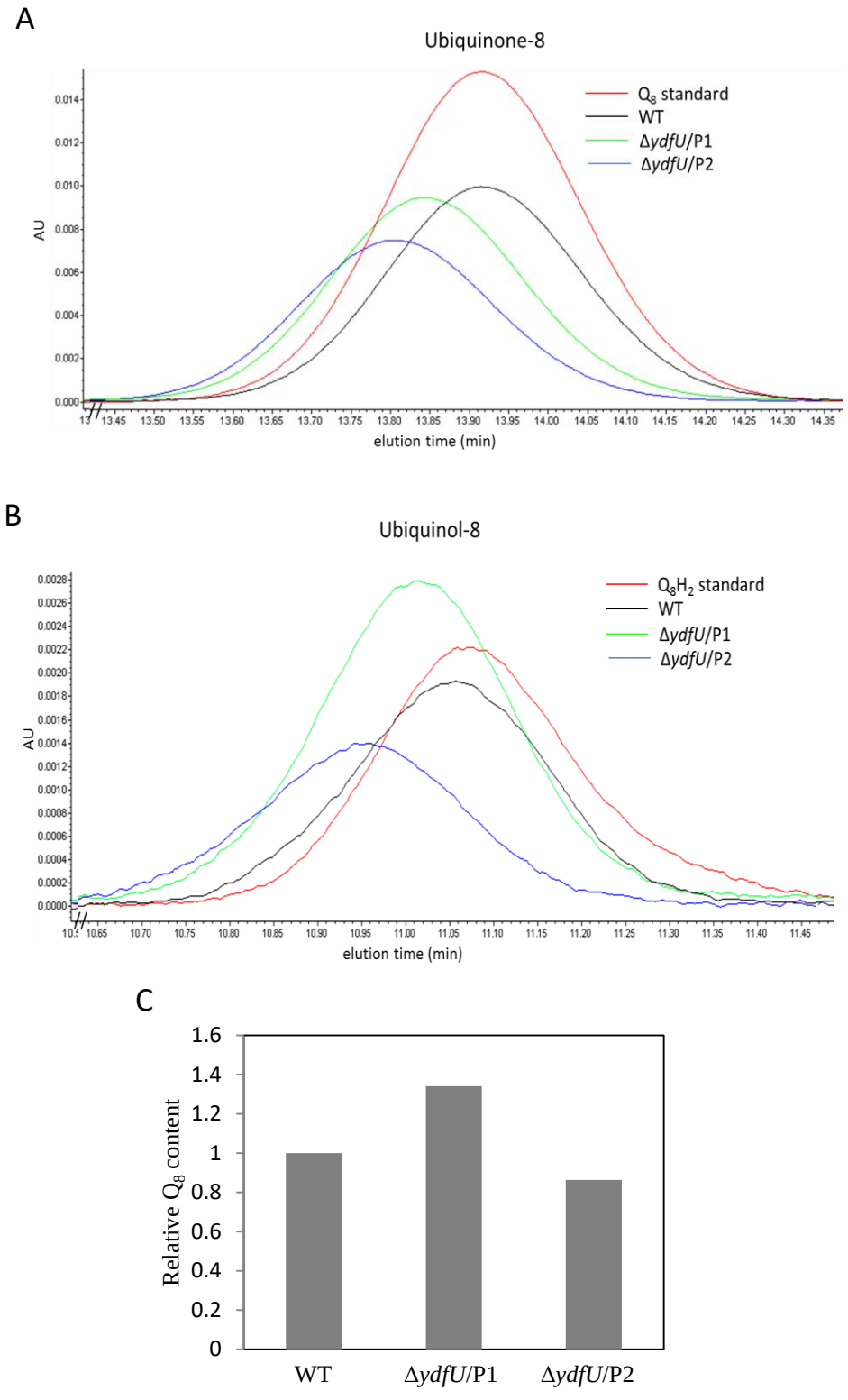


Fig 3.17 Δ*ydfU* has similar ubiquinone level as WT. A) HPLC chromatogram showing ubiquinone peak in Q_8 standard, WT and Δ*ydfU* (Parent 1 and 2) B) HPLC chromatogram showing ubiquinol peak in Q_8H_2 standard, WT and Δ*ydfU* (Parent 1 and 2) C) Total ubiquinone content in Δ*ydfU* (Parent 1 and 2) with respect to WT. This experiment was done once. The strains were cultured in TB media.

Δ*ygeH* showed significant growth defect in oleate (Fig 3.19), whereas Δ*ydeS* showed slight growth defect in succinate and oleate (Fig 3.20). The ubiquinone level in these strains was slightly lower than WT (Fig 3.21 and 3.22) suggesting that these genes might have a role in ubiquinone biosynthesis but these experiments have to be repeated to check the reproducibility of the above results.

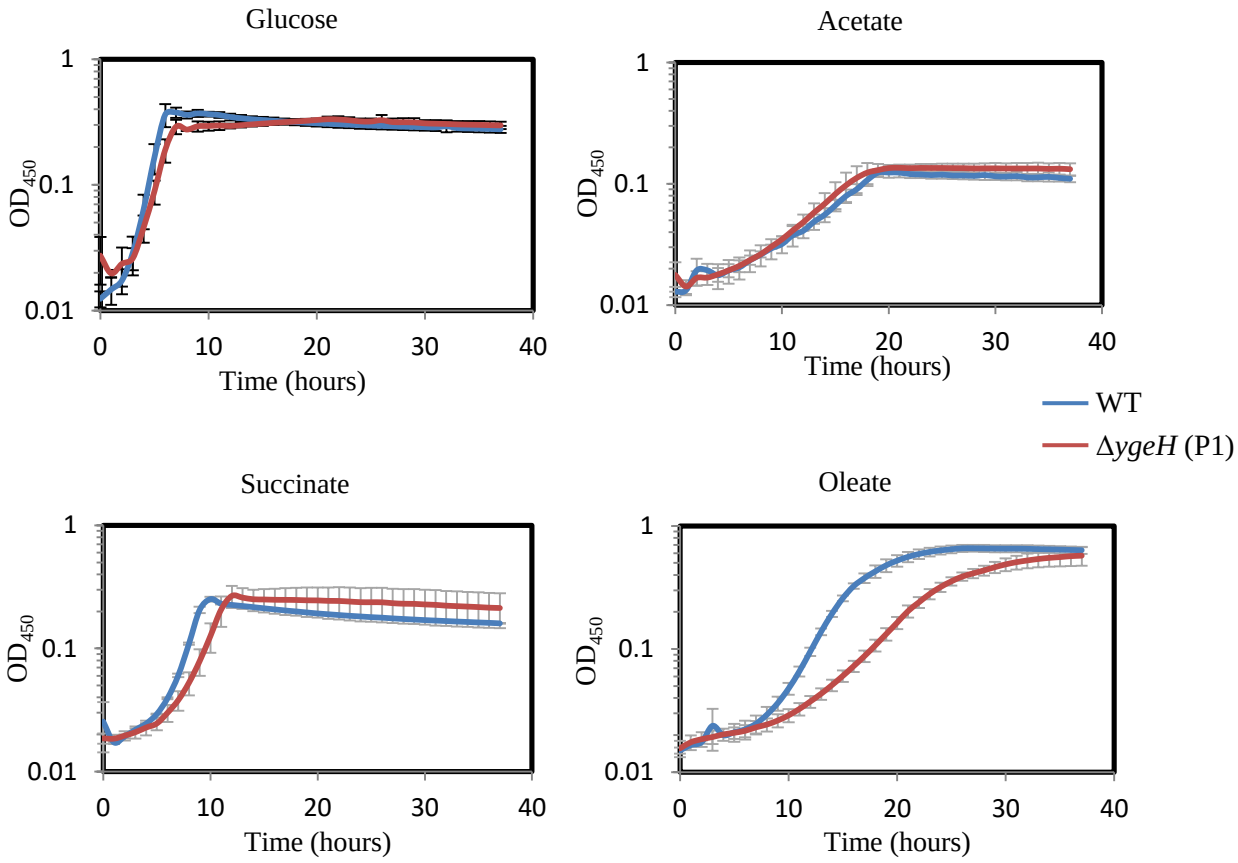
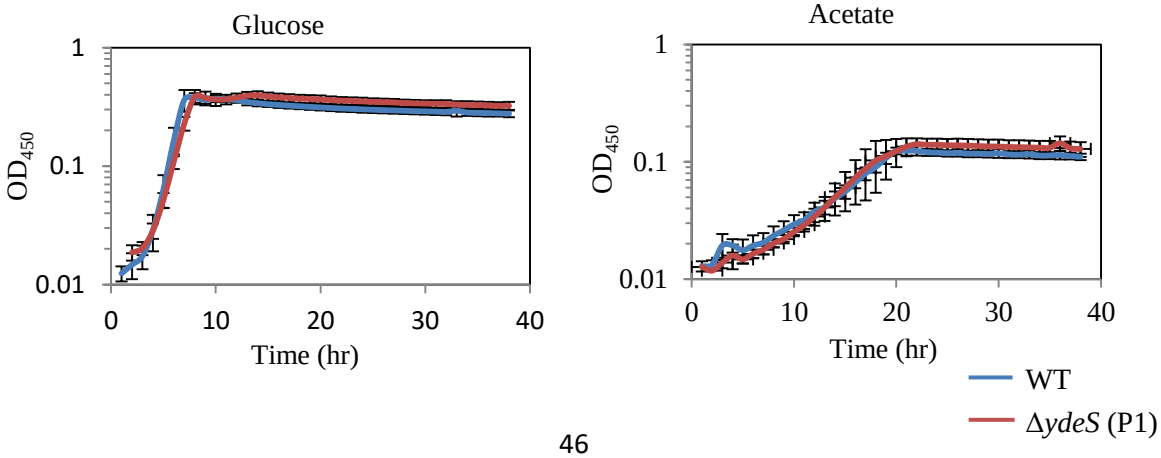


Fig 3.18 Growth curve of Δ*ygeH* strain in minimal media supplemented with different carbon sources. Data represent Mean ± SD of three technical replicates of one biological replicate. Data for one parent is shown; similar results were obtained with second parent from the library.



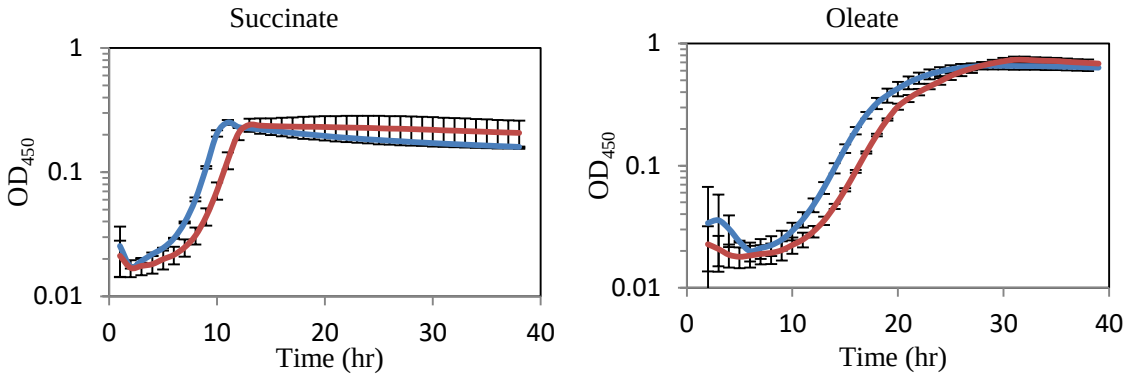
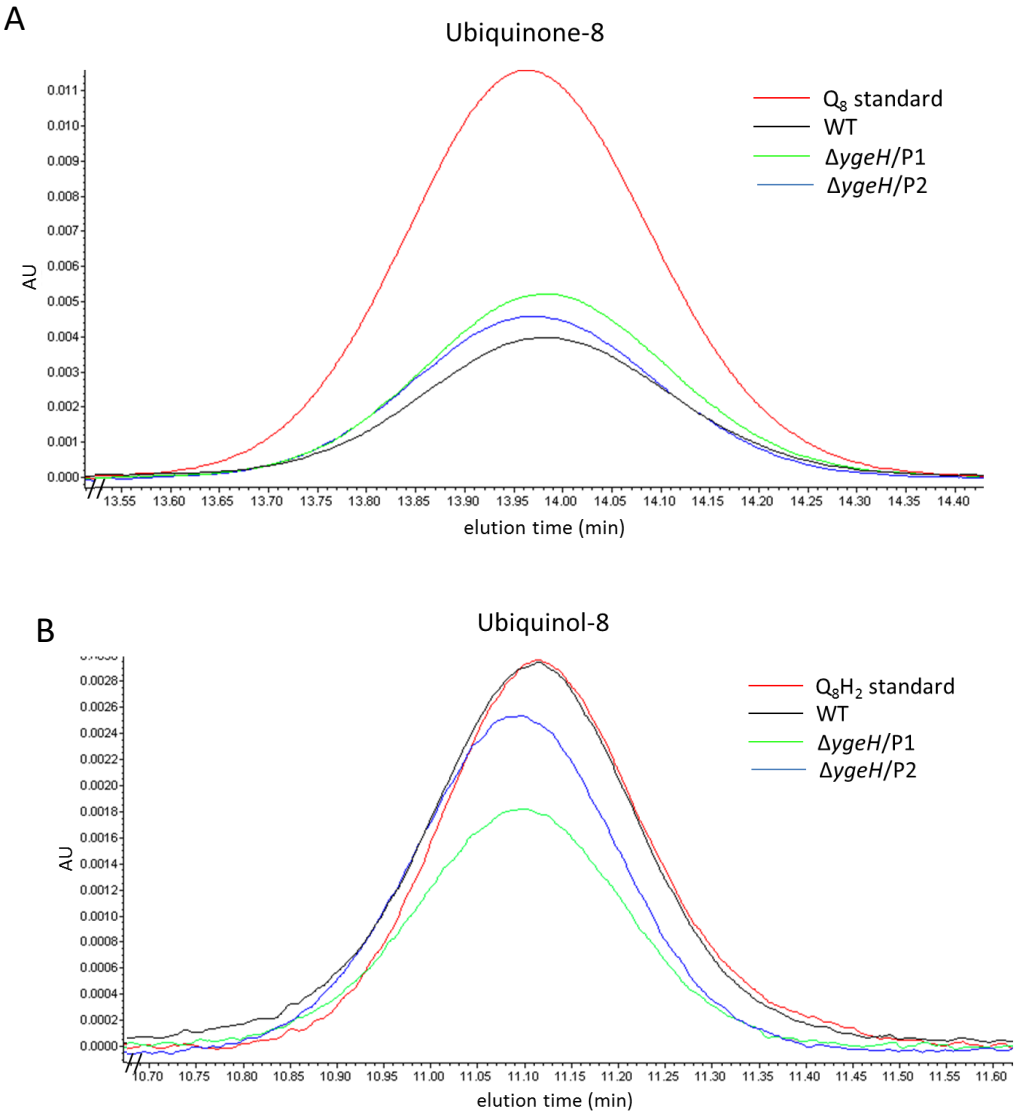


Fig 3.19 Growth curve of Δ*ydeS* strain in minimal media supplemented with different carbon sources. Data represent Mean ± SD of three technical replicates of one biological replicate. Data for one parent is shown; similar results were obtained with second parent from the library.



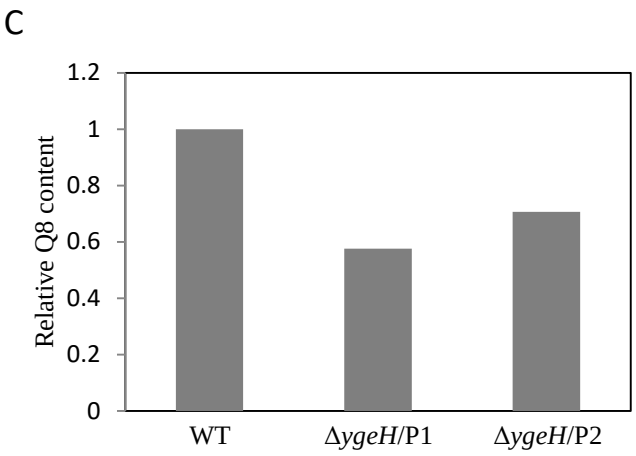
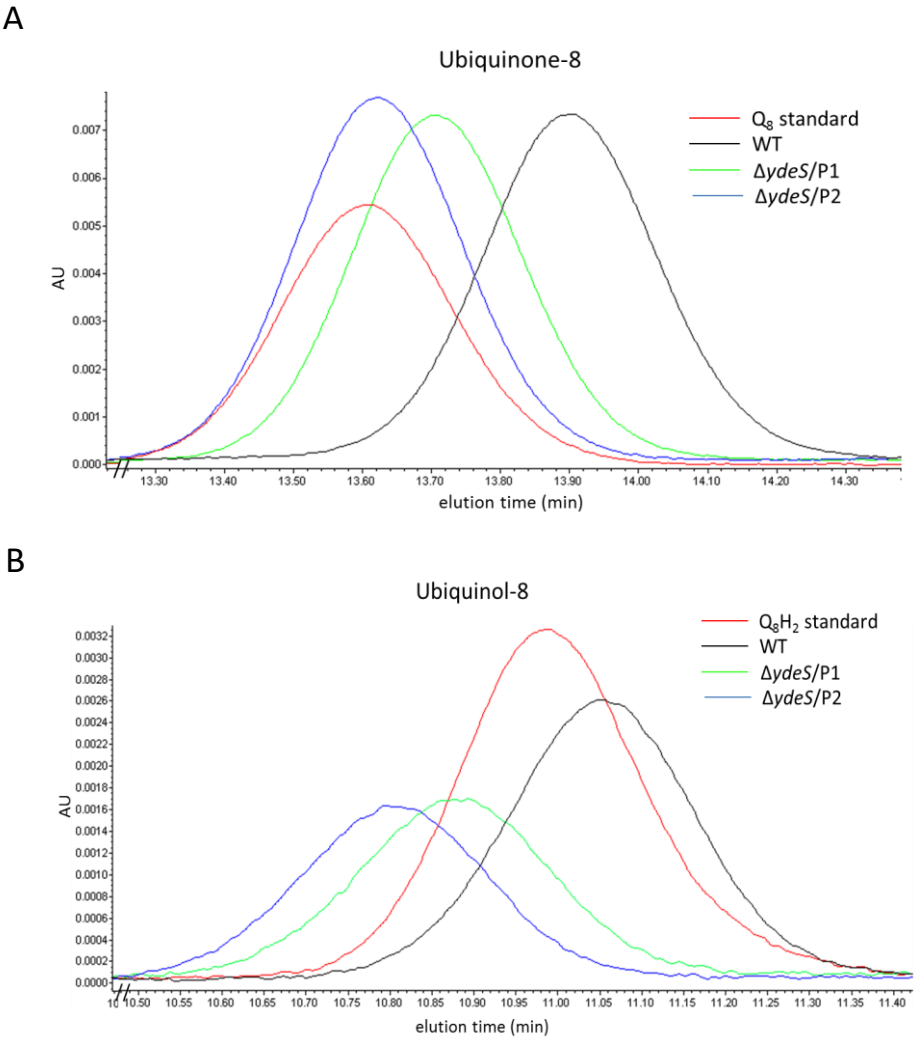


Fig 3.20 Δ*ygeH* has lower ubiquinone level compared to WT. A) HPLC chromatogram showing ubiquinone peak in Q_8 standard, WT and Δ*ygeH* (Parent 1 and 2) B) HPLC chromatogram showing ubiquinol peak in Q_8H_2 standard, WT and Δ*ygeH* (Parent 1 and 2) C) Total ubiquinone content in Δ*ygeH* (Parent 1 and 2) with respect to WT. This experiment was performed once. The strains were cultured in TB media.



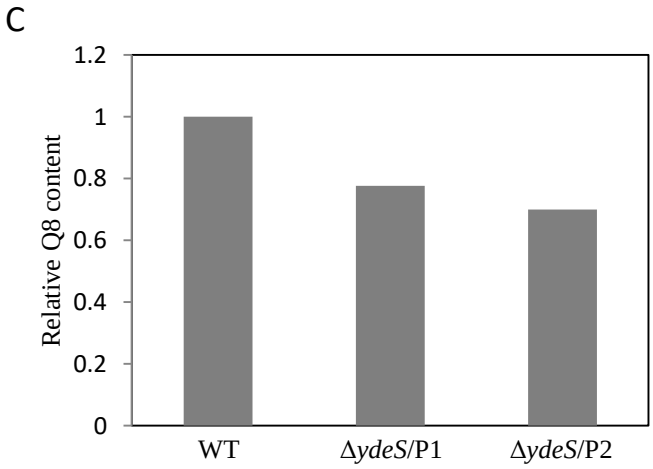


Fig 3.21 Δ*ydeS* has ubiquinone level slightly lower than WT. A) HPLC chromatogram showing ubiquinone peak in Q_8 standard, WT and Δ*ydeS* (Parent 1 and 2) B) HPLC chromatogram showing ubiquinol peak in Q_8H_2 standard, WT and Δ*ydeS* (Parent 1 and 2) C) Total ubiquinone content in Δ*ydeS* (Parent 1 and 2) with respect to WT. This experiment was performed once. The strains were cultured in TB media.

Chapter 4:

Summary and Future Goals

Summary and Future goals

Ubiquinone is a major electron carrier in aerobic respiration. Unpublished work from our lab showed that ubiquinone is maximally required for growth of *E. coli* on oleate when compared to other non-fermentable carbon sources. Follow-up studies revealed that ubiquinone is a key player that combats oxidative stress generated by oleate metabolism. The increased requirement of ubiquinone for growth on oleate can be exploited to identify new players involved in ubiquinone synthesis. In this project, using the above rationale, we identified *yqiC* as a novel ubiquinone biosynthetic player. Δ*yqiC* showed significant growth defect only in oleate in the genetic screens. In the present study, we reproduced the growth phenotype of Δ*yqiC* strain. Further, we showed that Δ*yqiC* has only ~20% ubiquinone level compared to WT. The growth phenotype and the ubiquinone level of Δ*yqiC* strain was similar to Δ*ubiI* strain (UbiI is a hydroxylase involved in ubiquinone biosynthesis). The related phenotypes of Δ*yqiC* and Δ*ubiI* prompted us to examine the phenotype of Δ*yqiC*Δ*ubiI* double mutant. The Δ*yqiC*Δ*ubiI* double knockout strain showed a synthetic sick phenotype. The similar phenotype of Δ*yqiC* and Δ*ubiI* strains, and the synthetic sick phenotype of the double knockout suggested the involvement of these genes at the same step of ubiquinone biosynthesis. Transformation of multicopy *yqiC* in Δ*ubiI* strain showed growth recovery on oleate after prolonged incubation suggesting that during oleate metabolism *yqiC* might promote the activity of some other hydroxylase (UbiF or UbiH) to produce more ubiquinone. Alternatively, considering the role of YqiC in menaquinone biosynthesis in *S. typhimurium*, *yqiC* might regulate menaquinone biosynthesis in *E. coli* and alleviate oxidative stress of Δ*ubiI* strain generated by oleate metabolism. Further studies are required to test these hypotheses. Since our data suggests that the regulation of quinone synthesis is media specific, we would like to determine ubiquinone content after growing the Δ*ubiI* strain transformed with *yqiC* in minimal media supplemented with oleate. Using the method standardized in this work, we would also like to determine the menaquinone and demethylmenaquinone levels in this strain during oleate metabolism.

To investigate other players that might be involved in ubiquinone biosynthesis, we picked four additional *y*-gene deletion strains from our high-throughput genetic screen and tested these strains at a candidate level for their growth phenotype on oleate. Among them, Δ*ygeH* strain showed growth defect specific to oleate and slightly lower ubiquinone

level. The preliminary results would be reproduced using transductants and if the results are verified, this gene would be further investigated for its involvement in ubiquinone biosynthesis.

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