Investigating Role of DgoR in Enterobacteriaceae Family

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A dissertation submitted for the partial fulfilment of BS-MS dual degree in science

Indian Institute of Science Education and Research, Mohali

December,2014

Certificate of Examination

This is to certify that the dissertation titled "**Investigating Role of DgoR in Enterobacteriaceae Family**" submitted by **Mr. Yatendra Singh Arya** (Reg. No. MS09139) for the partial fulfillment of BS-MS dual degree programme of the Institute, has been examined by the thesis committee duly appointed by the Institute. The committee finds the work done by the candidate satisfactory and recommends that the report be accepted.

Dr. Arunika Mukhopadhaya Dr. Mahak Sharma Dr. Rachna Chaba

(supervisor)

Dated:

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.

Yatendra Singh Arya

(Candidate)

Dated:

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)

Acknowledgement

It is generally said that well begun is half done. The maxim holds true in this case as well, chosing Dr. Rachna Chaba's Lab was indeed the perfect begining that I could have given to begin my Final Year Project. It was pleasure working with Dr. Rachna and the wonderful team of young budding scientists which she leads at the Microbiology Research Lab at IISER Mohali. Further, I would like to thank my evaluation committee members, Dr. Mehak Sharma and Dr. Arunika Mukhopadhaya, for their valuable comments and suggestions to eliminate any flaw from the work.

Apart from the academics, I would like to thank my physicians, Dr. Shailendra Srivastava and Dr. Maya Sharma, who cured me of my ailments during the course of my final year project. Without their treatment and counselling it would not have been possible for me to work with full concentration on my research topic.

Few and highly gracious words for my friend, philosopher and guide in the lab Mr. Bhupinder who cherished me in the blues and always kept me up in my spirits to accomplish the task in hand.

Above all, I would like to thank my father and mother for being there and always believing in me for whatever I have done so far. Also, my elder brother who has been a friend for lifetime and a source of inspiration.

Final few words for that anonymous who despite being around me in physical form has been an essence of lifetime.

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Abbreviations

ATP	Adenosine triphosphate
AMP	Adenosine monophosphate
APS	Ammonium Persulfate
Bp	Base pair
CRP	Catabolite repression
dNTP	ddeoxynucleotide triphosphate
DMSO	Dimethyl sulfoxide
DNA	Deoxyribonucleic acid
EDTA	Ethidium bromide
ETC	Electron transport chain
FADH	Flavin adenine dinucleotide
His	Histidine
kDa	Kilo Dalton
KH_2PO_4	Monopotassium phosphate
KCl	Potassium chloride
LB	Luria-Bertani media
LCFA	Long chain fatty acid
µg	Micro gram
µl	Micro litre
mM	Millimolar
$MgSO_4$	Magnesium sulfate
MCFA	Medium chain fatty acid
NaCl	Sodium Chloride

$NaNH_4HPO_4.4H_2O$	Ammonium sodium phosphate dibasic tetrahydrate
NH_4Cl	Ammonium chloride
Na_2HPO_4	Disodium phosphate
NADH	Nicotinamide adenine dinucleotide
PCR	Polymerase Chain Reaction
Rpm	Rotations per minute
SDS-PAGE	Sodium dodecyl sulfate polyacrylamide gel electrophoresis
SS	Salmonella shigella
SOB	Super optimal broth
SOC	SOB with Catobolic repressorGlucose
SCFA	Short chain fatty acid
TBST	Tris buffered saline with triton
TE	Tris EDTA
TAE	Tris-acetate-EDTA
TCA	Tricarboxylic acid cycle
TSS	Transformation storage solution
pH	Power of hydrogen
TEMED	Tetramethylethylenediamine
UV	Ultra violet
V	Volt
VLCFA	Very long chain fatty acid

Abstract

Long chain fatty acids (LCFAs) are used as a carbon source by several bacteria including many important pathogens. The LCFA transport and degradation pathway has been extensively studied in *E. coli*. In a high-throughput genetic screen performed in *E. coli*, a putative transcriptional regulator, DgoR, was identified as a novel component required for the successful growth of the bacterium. In the present study, we determined whether DgoR plays a role in LCFA degradation in other enteric bacteria. We chose to study the requirement of DgoR in LCFA metabolism in *S. typhimurium*, a bacterium very closely related to *E. coli*. In this direction, a *dgoR* deletion strain was constructed in *S. typhimurium* LT2 by homologous recombination. The phenotypic analysis of *dgoR* deletion strain on LCFAs showed that DgoR is required for the successful growth of the organism. The *dgoR* deletion strain could be complemented by *E. coli dgoR* cloned on the plasmid. However, *S. typhimurium dgoR* cloned on the plasmid failed to complement the deletion strain. Importantly, *S. typhimurium dgoR* cloned on the plasmid inhibited the growth of the wildtype strain. We are currently investigating whether a high-level expression of *S. typhimurim* DgoR from the plasmid is the reason for lethality. We are also cloning *S. typhimurium dgoR* on a low copy plasmid. In addition to our studies on *S. typhimurium* DgoR, in the present work we have also devised an important tool for monitoring the expression of *E. coli* DgoR in different carbon sources. We have tagged *dgoR* gene of *E. coli* with 3xFLAG on the chromosome. We find that the chromosomal construct expresses tagged DgoR and shows a growth pattern similar to the wildtype strain on LCFAs. These results thereby confirm that the chromosomally tagged DgoR strain can be used for physiological experiments.

Chapter 1

Introduction and Review of Literature

Fatty acid metabolism in bacteria

Fatty acids are carboxylic acids with an aliphatic hydrocarbon chain. They are categorized into four groups based on the number of carbon atoms in the chain i.e., short chain fatty acids (SCFAs) with fewer than 6 carbon atoms e.g. acetic acid, medium chain fatty acids (MCFAs) with 6-11 number of carbon atoms e.g., decanoic acid, long chain fatty acids (LCFAs) with 12-21 carbon atoms e.g., oleic acid, and very long chain fatty acids (VLCFAs) which have more than 22 carbon atoms e.g., lignoceric acid. Fatty acids are an important requirement for life because they are an important source of metabolic energy and are an essential component of membrane (1). Several bacteria including many important pathogens utilize fatty acids as carbon source and fatty acid degradation pathways have been implicated in their virulence. Thus identification of novel components involved in fatty acid metabolism and understanding their role in the fatty acid pathway is central to designing new drugs in order to combat bacterial infections. Below sections provide an overview of our current knowledge of the LCFA degradation pathway in bacteria, and their role in bacterial pathogenesis.

1.1. Fatty acid Transport and Degradation:

Both gram-negative and gram-positive bacteria use fatty acids as carbon source. Metabolism of LCFAs has been studied extensively in *E. coli* and consequently, a number of components involved in the transport, degradation, and regulation of LCFA metabolism have been identified in this bacterium (2,3,4). As shown in Fig. 1.1, LCFAs are transported inside the cell by an outer membrane transporter, FadL, and an inner membrane associated fatty acyl-CoA synthetase, FadD, which converts fatty acids into fatty acyl-CoA (5). Acyl-CoA are further degraded to two carbon chain length compound acetyl-CoA through the activity of FadB, FadA and FadE enzymes of β-oxidation pathway (6,7,8,9,10,11). Acetyl-CoA is then fed to the TCA cycle where reduced molecules like NADH & $FADH_2$ are produced. These reduced molecules are further channeled to the electron transport chain where ATP is generated.

1.2. Regulation of Fatty acid Transport and Degradation:

There are three well-known transcriptional regulators that control LCFA metabolism (see Fig. 1.2).

(a) Global CRP / cyclic AMP regulator:

CRP/cyclic AMP system keeps *fad* regulon under repression when a preferred carbon source like glucose is available (12).

(b) LCFA-specific transcriptional regulator, FadR:

FadR is an LCFA-specific repressor. It represses regulatory enzymes responsible for degradation of fatty acids under both aerobic and anaerobic conditions. FadR binds to the promoter of the *fad* (fatty acid degradation) genes through its N-terminal DNA binding domain thereby inhibiting the transcription of *fad* genes. When fatty acids are available, they are converted to acyl-CoA by the enzyme FadD. Acyl-CoA binds to the C-terminal regulatory domain of FadR bringing out a conformational change in the protein due to which FadR loses affinity for the promoter sites of *fad* genes. Thus binding of acyl-CoA de-represses FadR to up regulate enzymes involved in LCFA degradation (12, 3,4, 13,14).

(c) Oxygen sensitive ArcA-ArcB two-component system: The transcriptional regulator ArcA represses the transcription of *fad* genes and plays a major role under anaerobic conditions. The repressor activity of ArcA is dependent on its phosphorylation status, which is carried out by the kinase, ArcB (15).

1.3. Role of LCFA metabolism in pathogenesis:

Several gram-negative bacteria including important pathogens like *Pseudomonas aeruginosa*, *Salmonella typhimurium* and *Vibrio vulnificus* use LCFAs as carbon source. There are various studies that have shown the role of LCFA metabolism in pathogenesis, hence underlining the importance of studying this pathway to identify novel drug targets aimed at combating bacterial diseases. *P. aeruginosa* upregulates genes involved in LCFA metabolism to utilize fatty acids released from phospholipids in the lung tissues of patients suffering from cystic fibrosis (16,17). In the pathogen *V. vulnificus* known to cause lethal septicemia, FadR is important for its virulence activity (18). *S. typhimurium*, the causative agent of typhoid, has been shown to evade host immune response system by degrading the host LCFAs released as a part of the pro-inflammatory response (19).

Investigations on the role of a novel transcriptional regulator, DgoR, in LCFA metabolism

The pathway of LCFA transport and degradation has been studied in *E. coli* over the last several decades, mainly by classical genetic techniques, like transposon mutagenesis and chemical mutagenesis. Despite extensive investigations on the LCFA pathway, a high throughput genetic screen using the gene-deletion library of *E. coli* performed by Dr. Rachna Chaba identified several new players to be required for successful growth of *E. coli* on LCFAs {(20) Fig. 1.3, unpublished data}. In this screen, deletion of a putative transcriptional regulator, *dgoR*, resulted in significant growth defect of the bacterium on LCFAs. Growth defect of *E. coli dgoR* deletion strain was further validated in the lab of Dr. Rachna Chaba (Bhupinder Singh, unpublished data). DgoR belongs to the FadR subfamily of GntR family of transcriptional regulators (21,22). Despite low sequence similarity between FadR and DgoR, there is significant structural similarity between the two proteins. These observations motivated us to ask whether DgoR, like FadR, is another regulator of LCFA metabolism. In earlier studies DgoR has been implicated as a transcriptional regulator of genes involved in D-galactonate metabolism (23,24).

One of the goals of the present study was to determine whether DgoR is required for the growth of other enteric bacteria in addition to *E. coli*. We chose *Salmonella typhimurium*, a member of enterobacteriaceae family for our studies. Although there are a lot of similarities in the pathway of LCFA metabolism between *E. coli* and *S. typhimurium*, there are certain differences as well. Under aerobic conditions, *E. coli* can use only LCFAs and few SCFAs like acetate as carbon source but *S. typhimurium* in addition can also use MCFAs (25). Another difference among these two enteric bacteria lies in efficiency of fatty acid metabolizing enzymes, fatty acid metabolizing enzymes in *S. typhimurium* are more efficient than those of *E. coli* (25). These differences in fatty acid metabolism can be due to differences in regulation of fatty acid metabolism amongst the two bacteria. In the present study, we constructed a *dgoR* deletion strain of *S. typhimurium* using homologous recombination technique and assessed its growth phenotype on LCFAs (26). Additionally, we tagged *dgoR* of *E. coli* with FLAG tag on the chromosome, with the motive of using this tagged construct to check the expression of DgoR in various carbon sources.

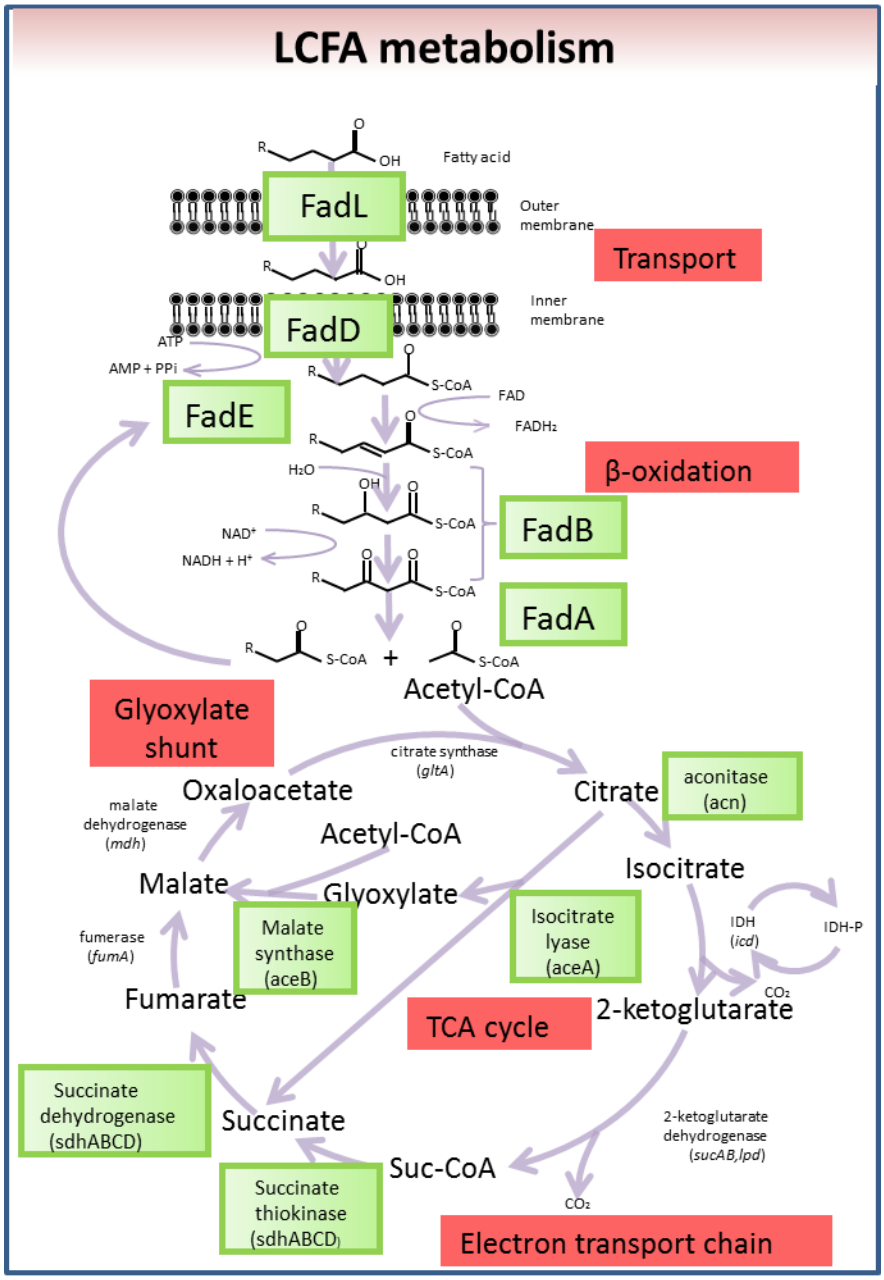
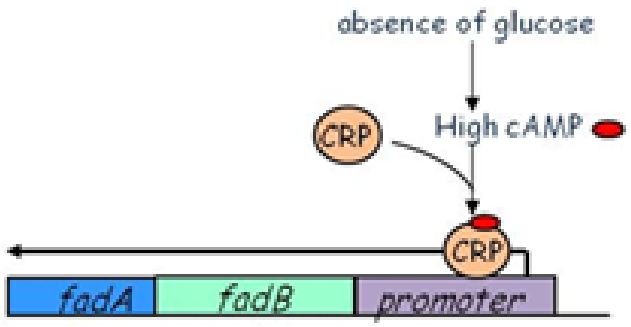
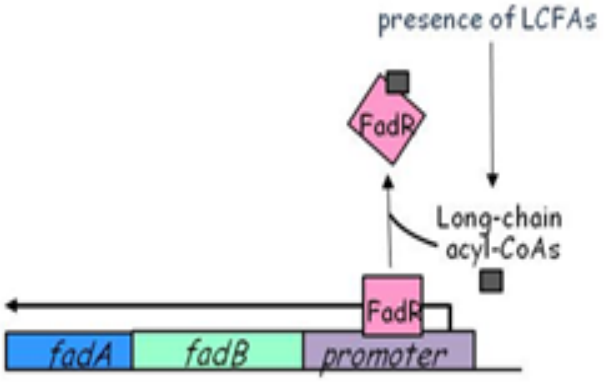


Fig. 1.1. *The aerobic pathway of LCFA transport and degradation in E. coli.* {image adapted from Fujita et al., 2007(31), and modified}.

(a) CRP/ cyclic AMP regulator:



(b) LCFA specific FadR repressor:



(c) Oxygen sensitive ArcA repression:

Fig. 1.2. *Transcriptional regulators which regulate LCFA metabolism.*

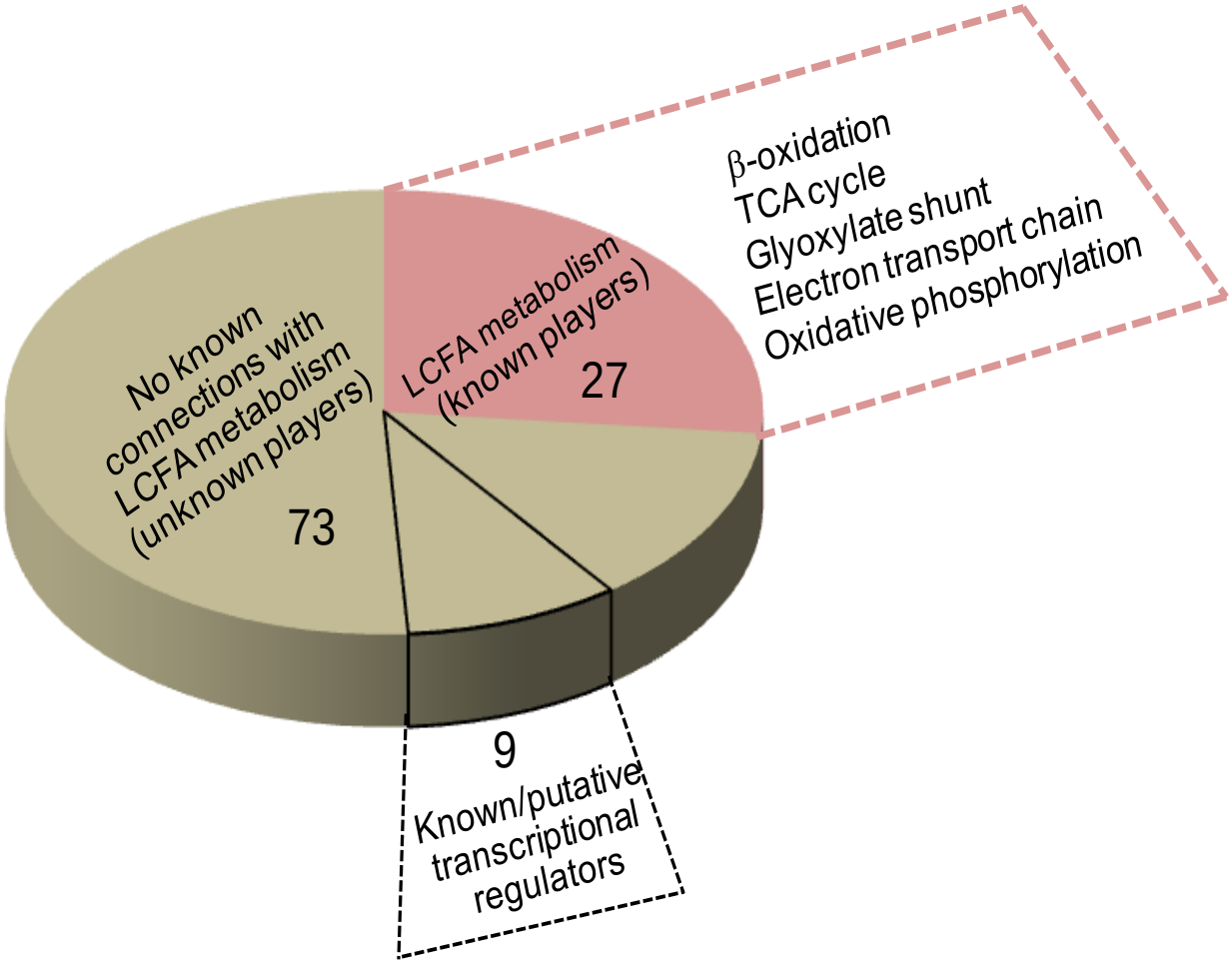


Fig.1.3. *Several known/putative transcriptional regulators show significant growth defect on LCFAs in a high-throughput genetic screen performed using the single gene deletion library of E. coli.* Of the top 100 genes with severe growth defect on LCFAs are 9 known/putative transcriptional regulators. Amongst all transcriptional regulators, DgoR showed the most significant growth defect.

Chapter 2

Materials & Methods

2.1 Materials

2.1.1 Chemicals and Reagents:

Media components for LB were purchased from BD Difco. Antibiotics, media components for M9 minimal media, sodium oleate, detergent (Brij-58), and primary (M2) and secondary (anti-mouse IgG) antibodies were obtained from Sigma. Glucose was purchased from Fisher scientific. SS media was obtained from HiMedia. Enzymes (CIP, Taq/Phusion DNA polymerase, restriction enzymes, T4 DNA ligase), Quick load DNA ladder, 100bp/1Kb DNA ladder and dNTPs were purchased from New England Biolabs. Plasmid miniprep, PCR purification, genomic DNA and gel extraction kits used were from Fermentas. Primers were designed using Gene Runner and Ape softwares, and ordered from IDT and Sigma. ECL chemiluminescent detection kit was purchased from Peirce. Amersham Hyperfilm^{TM} ECL films and nitrocellulose membranes were purchased from GE Healthcare.

2.1.2 Bacterial strains and plasmids: Bacterial strains and plasmids used in the present study are listed below in table 2.1.

Table 2.1: List of bacterial strains and plasmids

Strain name	**Genotype**	**Source**	**Reference**
Escherichia coli BW25113	*lacI*^{q} *rrnB*$_{T14}$ Δ*lacZ*$_{WJ16}$ *hsdR514* Δ*araBAD*$_{AH33}$ Δ*rhaBAD*$_{LD78}$	Carol Gross Lab, UCSF	Wanner, B. L., (1983).{29}
Escherichia coli DH5α	F^{-} endA1 glnV44 thi-1 recA1 relA1 gyrA96 deoR nupG Φ80d*lacZ*ΔM15 Δ(*lacZYA-argF*)U169, hsdR17(r_K^- m_K^+), λ–	Carol Gross Lab, UCSF	FOCUS (1986) 8:2, 9. Meselson M. and Yuan R. (1968) Nature 217:1110 PMID 4868368.

			Hanahan, D. (1985) in DNA Cloning: A Pratical Approach (Glover,DM., ed.), Vol. 1, p. 109, IRL Press, McLean, Virginia. Grant, S.G.N.et al. (1990) Proc. Natl. Acad. Sci. USA 87:4645-4669.
Salmonella typhimurium LT2 MTCC98	----------	MTCC98, IMTECH, Chandigarh, India	R.C.Clowes and W. Hayes,"Microbial Genetics" Blackwell Sci.Publc.1968.

Plasmid name	**Description**	**Source**	**Reference**
pKD13	Template plasmid used for gene disruption. Kanamycin cassette is flanked by FRT sites. Genebank seq. AY048744	Carol Gross Lab	Datsenko & Wanner et. al., 2000(26)
pKD46	λ Red recombinase expression plasmid carrying 2154 bp of λ phage. Genebank seq. AY048746	Carol Gross Lab	Datsenko & Wanner et. al., 2000(26)
pACYC184	A suspension of E. coli strain ER2420 containing the plasmid pACYC184 which	Carol Gross Lab	NEB# E4152S

	has been grown in rich medium and brought to 50% glycerol. ER2420 background: EcoK r- m-		

2.1.3 Primers: Primers used in the present study are listed below in table 2.2.

Table 2.2: List of primers

Primer name	Nucleotide sequence (5`-3`)	Purpose
YA1	CTATCGCGGTAAAGTAAGAGAGTTCACATCG AGCACAAGGACTCTCTATG*ATTCCGGGGATCC GTCGACC*	Amplification of kan cassette from pKD13(Italic region represents priming sites of pKD13), forward primer
YA2	TCCCCAGTCAATTGCGATGTAGCGAGCTGTCA TGTGATTTCCTTTAACCTT*GTAGGCTGGAGCTG CTTCG*	Reverse primer to amplify kan cassete from pKD13.
YA3	CATGCCATGGGCGATCAGGAAAACGACATT GC	Forward primer to ampify *dgoR* with its promoter.(Nco1 sites at 5` end)
YA4	ACCGGAATTCTCAGTGATGATGATGATGATG TGTGATTTCCTTTAACCTTCGTGTC	Reverse primer to amplify *S. typhimurium dgoR* with its putative promoter(6His and EcoR1 added with the primer 5`end)
YA5	CGGCGTAATTCAGCTATCG	Forward primer to check the S. typhimurium dgoR::kan knockout strain.

YA6	ATTTGTCGCCCTGGTAAAGC	Reverse primer to check the S. typhimurium dgoR::kan knockout strain.
BS13	TACAAAGTTGCCGCGTTATG	Forward primer: *E. coli dgoR* gene specific primer.
BS14	AGTGGTCGCCCTGATAAAGC	Reverse primer: *E. coli dgoR* gene specific primer.
BS33	GCCACTCATCGCAGTACTGTTG	Forward primer to cofirm clone.
BS34	TCCCAATGGCATCGTAAAGAAC	Reverse primer to confirm clone.
YA9	CGCCAGCTCGACACGAAGGTTAAAGGAAATC ACA*GACTACAAAGACCATGACG*	Amplification of 3xFLAG with kan cassette.
YA10	GGTCGATCCCCAGTCAATTGCGATGTAGCGA GCTGTCATGTGATTTCCT*CATATGAATATCCTC CTTAG*	Amplification of 3xFLAG with kan cassette
SAK1	GAGGCTATTCGGCTATGACTG	Kanamycin cassette specific primer
SAK2	TTCCATCCGAGTACGTGCTC	Kanamycin cassette specific primer

2.1.4 Buffers and Solutions for Microbiological work:

(a) Antibiotics: Ampicillin and Kanamycin stocks, 100mg/ml and 25mg/ml respectively, were prepared in water. Tetracycline stock (12.5 mg/ml) was prepared in 70% ethanol. After the

dissolution of antibiotics, they were sterilized using 0.22µ filters (Millipore) and stored at -20°C in 1ml aliquots. Ampicillin, Kanamycin and Tetracycline antibiotics were used at a final concentration of 100µg/ml, 30µg/ml and 12.5µg/ml respectively.

(b) Media Composition:

1) LB media: *E. coli* and *S. typhimurium* strains were routinely grown and maintained in LB media.

Preparation of LB broth: Tryptone: 10gm, Yeast Extract: 5gm, and NaCl: 5gm were dissolved in 1L of deionized water. The media was autoclaved at 15lb/inch^2 pressure and 121°C temperature for 15 minutes.

Preparation of LB agar: For preparing LB agar, LB broth was supplemented with 1.5% agar, and autoclaved. When the temperature of LB agar media was ~50°C, the media was poured in plates. Antibiotics were added when required.

2) M9 minimal media: *E. coli* and *S. typhimurium* strains were grown in M9 minimal media supplemented with either glucose or oleate as the sole carbon source in both solid and liquid media. The procedure for preparing stocks of various components of M9 media is given below.

i) 10X M9 salts

Na2HPO4	53gm
KH2PO4	30gm
NaCl	5gm
NH4Cl	10 gm

Above contents were dissolved in 600ml deionized water and final volume was made upto 1 liter. Dissolved components were Autoclaved.

ii) 1 M MgSO4: 24.7gm dissolved in 100ml and sterilize by autoclaving.

iii) 250X Vitamin stock:

Biotin	100mg
Nicotinamide	100mg
Riboflavin	10mg
Thiamine	100mg

Dissolved in 200ml dH2O and filter sterilized.

iv) 5% Brij-58: 10gm dissolved in 200ml dH_2O and autoclaved.

v) 20% Glucose: 8gm dissolved in 40ml dH2O and filter sterilized.

vi) 50mM Sodium oleate: 0.76gm dissolved in 35ml sterile Brij-58 and make up volume till 50ml Brij-58. Solution was filer sterilize by 0.22µM filters.

The final composition of liquid M9 minimal media containing either glucose or oleate as carbon source is given below.

Components	**M9+ oleate**	**M9+G+D**
Water	39.75ml	39.25ml
10×M9 salts	5ml	5ml
1M $MgSO_4$	50µl (1mM)	50µl (1mM)
250X Vitamins	200µl	200 µl
5% Brij-58	-	5ml (0.5%)
20% Glucose	-	500µl (4mM)

50mM Sodium Oleate	5ml	-
Final Volume	50ml	50ml

The final composition of solid M9 minimal media containing either glucose or oleate as carbon source is given below.

Components	**M9+G**	**M9+G+Det**	**M9+oleate**
10X M9 salts	8ml	8ml	8ml
1M $MgSO_4$	80µl (1mM)	80µl (1mM)	80µl (1mM)
250X Vitamins	320µl	320µl	320µl
20% Glucose or 50mM Sodium oleate	800µl (4mM)	800µl (4mM)	8ml (5mM)
5% Brij-58	-	8ml	-
2X molten agar	40ml	40ml	40ml
Final Volume	80ml	80ml	80ml

3) Media used for *S. typhimurium*:

For the identification of *S. typhimurium*, strains were grown in SS media. To detect P22 phage contamination in *S. typhimurium* strains, the strains were streaked on green indicator plates. Composition of these media is given below.

i) SS media: 12.6 grams of SS agar was dissolved in 200ml deionized water and heated gently till complete dissolution of SS media and poured in plates. Caution was taken to avoid overheating of the media. The media was not autoclaved.

ii) Green Indicator Plates: The composition of the media is given below.

Bacto-tryptone	4gm
Yeast-Extract	0.5gm
NaCl	2.5gm
Dextrose	3.8gm
Agar	7.5gm
Methyl-Blue	0.033gm
Alizarin Yellow	0.315gm

The above contents were dissolved in 300ml of water and final volume was made upto 500ml. (Note: both dyes were dissolved prior to add media components).

Top agar: 0.7% agar was used to overlay green indicator plates.

4) SOB and SOC media: SOB and SOC media were used in the process of homologous recombination for preparing electro-competent cells and for recovery of electroporated cells respectively.

i) SOB media:

Tryptone	20gm

Yeast Extract	5gm
NaCl	0.5gm
KCl (3M)	0.84ml

Above components were dissolved in 900ml water and adjusted to pH7.0 using 5N NaOH. Volume was made up to 995ml. Media was autoclaved and 5ml of sterile $MgCl_2$(2M) was added before using the SOB media.

ii) SOC media: 10ml of 1M glucose is added to l L SOB media, prior to use.

5) Media and buffers used for P22 phage preparation:

50X E-salts:

Citric acid monohydrate	10gm
$MgSO_4.7H_2O$	0.962gm
$K_2HPO4.3H_20$	65.5gm
$NaNH_4HPO_4.4H_20$	17.5gm

Above media components were dissolved in 90ml water & dissolution was done in given order. Media volume was made upto 100ml and autoclaved.

P22 phage buffer: 100 ml of LB media was supplemented with 1ml 50X E-salts, 20% dextrose, and 15µl P22 lysate. Buffer aliquots were stored at 4°C.

2.1.5 Buffers and solutions for DNA work:

50X TAE:

Tris	242gm
Glacial acetic acid	57.1ml

0.5M EDTA(pH8.0)	100ml

Added deionized water to bring the volume to 1L.

TE: Used elution buffer from plasmid miniprep kit.

6X DNA gel Loading buffer:

Bromophenol blue	0.25% (125mg 50ml)
sucrose	40.0% (20gm in 50ml)

Buffer was prepared in deionized water.

TSS:

PEG (MW3350)	10%(wt/vol)
DMSO	5%(vol/vol)
$MgCl_2$	50mM

LB broth was supplemented with above-mentioned components and pH was adjusted to 6.5.

2.1.6 Buffers and solutions for protein work:

2.1.6.1. Solutions for SDS-PAGE:

Acrylamide (30%)	Acrylamide 30gm N,N`-methylene bis acrylamide 0.8gm Final volume was adjusted to100ml.

Lower Tris (for Resolving gel) pH 8.8	Tris 18.17gm 10% SDS 4ml pH was adjusted to 8.8 with 6N HCl. Volume was made up to 100ml.
Upper Tris (for Stacking gel) pH 6.8	Tris 6.06gm 10% SDS 4ml pH was adjusted to 6.8 with 6N HCl. Volume was made up to 100ml.
5X sample buffer	Tris-Cl (pH6.8) 0.15M SDS 5% Glycerol 25% β-mercataphenol Blue 12.5% Bromophenol Blue 0.06%
Tris Glycine gel Running Buffer (pH 8.3)	SDS 0.1% Tris Base 25mM Glycine 250mM

Compositions of Stacking and resolving gel:

Components	**Resolving gel**	**Stacking gel**
Water	1.1ml	1.4ml
Tris	1.3ml of pH 8.8 (Lower Tris)	630µl of pH 6.8 (Upper Tris)

30% Acrylamide	2.5ml	415 µl
SDS (10 %)	50 µl	25 µl
10% APS	50 µl	30 µl
TEMED	5 µl	4 µl

2.1.6.2 Solutions for Western Blotting:

Transfer Buffer	Glycine : 39mM Tris : 48mM SDS : 0.037% Methanol : 20%
TBST	Tris Base : 2.42gm NaCl :8gm Dissolved in 800ml water 1ml of 0.1% Tween was added to the above solution. pH was adjusted to 7.6 with conc. HCl and final volume was made to 1L.
Blocking agent	5% skimmed milk in TBST.

2.2 Methods:

2.2.1 Growth and maintenance of bacterial cultures:

E. coli and S. *typhimurium* strains were routinely grown and maintained in LB media. For short term storage of the bacterial cultures, the plates were kept at 4°C. For long term storage stocks of the bacterial cultures were prepared in glycerol (f.c. 15%) and stored at -80°C. *E. coli* and *S. typhimurium* strains were grown in M9 minimal media supplemented with either glucose or oleate as the sole carbon source in experiments involving phenotypic analysis of the strains. Both solid and liquid media cultures were incubated at 37°C. Liquid cultures were aerated in a water bath shaker at a speed of 220rpm. Antibiotics were added to the media at desired concentrations when required.

2.2.2 Genomic DNA Isolation:

E. coli and *S. typhimurium* strains were inoculated in 5ml LB media and incubated at 37°C for 16 hrs. Cells were harvested by centrifugation. Genomic DNA was isolated following the protocol mentioned in the manufacturers instruction manual provided alongwith the Genomic DNA isolation kit.

2.2.3 Plasmid Isolation:

Strains harboring plasmids were inoculated in 3ml LB media containing antibiotics at the desired concentration. Cultures were grown at 37°C for 16hrs. Since pKD46 is a temperature sensitive plasmid, for isolation of pKD46, the cultures were incubated at 30°C. Cells were pelleted down by centrifugation at 8000rpm for 2min. Plasmid DNA was isolated following the protocol mentioned in the manufacturers instruction manual provided alongwith the plasmid miniprep kit.

2.2.4 Agarose Gel Electrophoresis:

DNA was analyzed by agarose gel electrophoresis. Samples prepared in gel loading buffer were loaded on 0.8-1.5% agarose gels chosen depending on the molecular weight of the DNA fragments to be analyzed. Gels were run at 110V in 1X TAE buffer. DNA fragments were visualized using BIO-RAD UV transilluminator.

2.2.5 Disruption of *dgoR* gene of *S. typhimurium* by one step inactivation (26):

(a) Transformation of pKD46 plasmid in *S. typhimurium*:

pKD46, a plasmid which expresses a λ red recombinase, was transformed in freshly prepared electrocompetent cells of *S. typhimurium*. Transformed cells were spread on LB media containing ampicillin. Plates were incubated overnight at 30°C.

(b) Amplification of Kanamycin cassette from pKD13:

Template DNA was amplified from pKD13 using primers YA1 and YA2 (refer table 2.1), and Phusion polymerase. The components of PCR reaction mixture and the protocol used for amplification of template DNA are mentioned below.

Components of PCR reaction:

Components	**reaction mixture (20 µl)**
5xphusion buffer	4µl
10mM dNTP	0.4µl
10µM forward primer	1µl
10µM reverse primer	1µl
Template	2µl
DNA polymerase	0.2µl
Auto MQ water	11.4µl

Table 2.3 PCR protocol for amplification of kanamycin cassette from pKD13.

Steps	Time	Temperature
Initial denaturation	0:30 min	98°C
Denaturation	0:10 min	98°C
Annealing	0:25 min	67°C
Elongation	0:30 min	72°C
Final extension	10min	72°C
Hold	∞	4°C

PCR product was digested with Dpn1 at 37°C for 3hrs. Digested product was run on agarose gel and DNA was purified using gel extraction kit.

(c) Transformation of template DNA in cells carrying λ red recombinase system:

Amplified kanamycin cassette obtained in step b was transformed in *S. typhimurium* cells carrying pKD46 plasmid (obtained in step a). Transformed cells were plated on LB media containing kanamycin. The plates were incubated at 30°C. Colonies obtained after overnight incubation were streaked twice on LB media containing kanamycin.

(d) Verification of the *dgoR* deletion strain by colony PCR: P1 (YA5 and YA6), P2 (YA5 and SAK2) and P3 (YA6 and SAK1) primer sets were used to check if *dgoR* gene was successfully replaced by kanamycin cassette. PCR was carried out using Taq DNA polymerase. Components of the PCR reaction mixture are given below:

Components	Reaction mixture (25µl)

10xtaq buffer	2.5 µl
10mM dNTP	0.5 µl
10µM forward primer	1 µl
10µM reverse primer	1 µl
Template	2.5 µl
DNA polymerase	0.125 µl
Auto MQ water	19.87 µl

The protocol used for colony PCR is given below in table 2.4.

Steps	**Time**	**Temperature**
Initial denaturation	5 min	95°C
Denaturation	0:30 min	95°C
Annealing	0:45min	49°C
Elongation	1:40 min	68°C
Final extension	5 min	68°C
Hold	∞	4°C

(e) P22 transduction and screening out lysogens and pseudolysogens:

P22 phage titer was determined by agar suspension plaque assay (27) and P22 transduction was carried out as described in Experimental techniques in Bacterial genetics(28). The titer of P22 phage received from the lab of Prof. James Slauch (University of Illinois), was determined to be 10^{11} pfu/ml. These phage particles were further used to prepare phage from *S. typhimurium* LT2 (MTCC98) and the titer was determined to be 6.7×10^{11} pfu/ml. P22 phage from WT strain was used to infect *dgoR::kan* parent strains. The lysate thus prepared was used to infect clean WT strain thus transferring *dgoR::kan* into a clean WT background. *S. typhimurium dgoR::kan* transductants were purified from lysogens and pseudolysogen following the protocol mentioned in Experimental techniques in Bacterial genetics(28) .

2.2.6 Cloning of *dgoR* gene of *S. typhimurium* with its native promoter in pACYC184.

dgoR gene was PCR amplified with its native promoter from *S. typhimurium* genomic DNA using primers YA3 and YA4. PCR protocol used for amplification was similar to 2.2.5.b, except that the annealing temperature was set at 61°C. Both the PCR amplified fragment and pACYC184 plasmid were digested with NcoI and EcoRI. The plasmid was further treated with CIP. The digested fragment and plasmid were gel purified, ligated and further transformed in *E. coli* DH5α strain. Clones were verified by colony PCR using the same protocol as mentioned in 2.2.5.d except that annealing temperature: 52°C. BS33 and BS34 primers were used for verification of clones.

2.2.7 Tagging of *dgoR* gene of *E. coli* with FLAG tag:

The protocol used was essentially the same as mentioned in 2.2.5 with differences being the primers, kanamycin template source and the strain used for recombineering. Kanamycin cassette with FLAG tag was amplified from the *E. coli* strain that had tagged kanamycin cassette on the chromosome, using primers YA9 and YA10. The components of the PCR reaction mixture were as follows:

components	Reaction mixture (20µl)

5xphusion buffer	4µl
10mM dNTP	0.4µl
Forward primer	1µl
Reverse primer	1µl
Template	2µl
Phusion enzyme	0.2µl
$MgCl_2$	0.4µl
DMSO	1.6µl
dH_2O	9.3µl

The PCR program was similar to the one given in Table 2.3(Except that the annealing temperature was 54°C).

dgoR tagged with FLAG tag was then transferred from the recombineering strain into WT *E. coli* by P1 transduction according to the protocol mentioned in A short course in bacterial genetics(30). *dgoR* flagged construct was verified by colony PCR using the same protocol as mentioned in 2.2.5.d. P1 (BS13 and BS14), P2 (BS13 and SAK2) and P3 (SAK1 and BS14) primer sets were used for the verification of *dgoR* tagged construct.

2.2.8 Dilution spotting:

Strains were inoculated in 3ml LB media (antibiotics were added when required), and grown at 37°C for 16 hrs. 1ml culture was pelleted at 4000rpm for 2 minutes. Pellet was resuspended and washed with M9 minimal media. OD_{450} of the resuspended cells was measured and normalized to the same OD_{450} by adding required volume of M9 minimal media. Serial dilutions (10^{-1} -10^{-6}) of cultures were prepared in M9 minimal media. 5µl of 10^{-2} to 10^{-6} dilutions was spotted on M9 minimal media plates supplemented with either glucose or oleate as the sole carbon source. Plates were observed at 6 hrs time intervals, and images were taken using Biorad gel documentation system.

2.2.9 Growth curve analysis:

Strains were inoculated in 3ml LB media (antibiotics were added when required), and grown at 37°C for 16 hrs. 1ml culture was pelleted at 4000rpm for 2 minutes. Pellet was resuspended and washed with M9 minimal media. OD_{450} of the resuspended cells was measured. Cultures were reinoculated in 50 ml M9 minimal media containing either glucose or oleate as the sole carbon source (in a 250 ml flask). The number of cells added to 50 ml media was such that the initial OD_{450} of the reinoculated culture was 0.01. Culture flasks were incubated in a water bath shaker at 37°C with a shaking speed set at 220 rpm. For media containing glucose OD_{450} was recorded at an interval of 1 hrs whereas for media containing oleate OD_{450} was recorded at an interval of 3 hrs. OD_{450} was plotted against time period and generation time was calculated for each strain by dividing the natural logarithm of 2 by growth exponent.

2.2.10 SDS-PAGE:

SDS PAGE was carried out according to the protocol given in Laemmli (1970). Briefly, 5X sample buffer was added to the samples such that the final concentration of the sample buffer was 1X. Prior to loading, protein samples were boiled at 95°C (in a dry heat block) for 5min and centrifuged. Protein samples were loaded into the gel along with protein molecular weight marker (gel composition is given in section 2.1.6.1). Gel was electrophoresed in Tris glycine running buffer at 110V.

2.2.11 Western blotting:

Tris glycine SDS buffer was used to transfer proteins from gel to nitrocellulose membrane (0.45µm) in a mini-trans blot apparatus (Bio-Rad). Transfer was carried out at 90V for 1 hr. After completion of transfer, the nitrocellulose membrane was blocked with 5% skimmed milk in TBST for overnight at 4°C. The membrane was washed with TBST four times for 15 minutes each on an orbital shaker. Membrane was incubated with primary antibody (M2 anti-FLAG, dilution 1:1000 in TBST) for 2hrs. Membrane was again washed with TBST four times for 15 min each. Membrane was then incubated with secondary antibody (IgG anti-mouse, dilution: 1: 5000 in TBST) for 1 hr. Membrane was washed four times with TBST for 15min each. Blot was developed using ECL detection kit (based on HRP peroxidase). Blot was exposed to X-ray film for 1 minute and developed using X-ray film processing machine.

Chapter 3

Results and Discussion

3. Results and Discussion:

The first aim of the present study was to determine whether the putative transcriptional regulator, DgoR, is required for the growth of *S. typhimurium* on LCFAs as the carbon source. In this direction, *S. typhimurium dgoR* gene was deleted and growth of the deletion strain was assessed in both solid and liquid media containing LCFAs as the sole carbon source. Complementation tests were performed to determine whether the growth phenotype of the *dgoR::kan* strain was due to the protein function of DgoR. The second aim of the study was to tag the chromosomal copy of *dgoR* in *E. coli* with FLAG tag. This construct was designed with the objective of easy detection of DgoR protein by anti-FLAG antibodies in experiments aimed at monitoring the induction of DgoR in the presence of multiple carbon sources.

3.1 *Phenotypic analysis of the dgoR::kan strain of S. typhimurium LT2 on LCFAs as the carbon source*

a) WT *S. typhimurium* grows on LCFAs as the sole carbon source.

To initiate studies on the role of DgoR in LCFA metabolism in *S. typhimurium* we first standardized conditions for the growth of the bacteria in both solid and liquid media containing LCFAs as the sole carbon source. For determining the growth of *S. typhimurium* on solid media the WT strain was spotted on M9 minimal media containing oleate (a monounsaturated LCFA with 18 carbon atoms) as the carbon source. Growth of the WT strain was observed in ~30 hours of incubation of the solid media plates at 37°C (Fig. 3.1A). The time duration for growth of *S. typhimurium* on oleate plates was similar to that observed for *E. coli* earlier in our lab. The growth of WT strain was also monitored in liquid M9 minimal media containing oleate as the carbon source. As shown in Fig. 3.1B, *S. typhimurium* grows in oleate. The growth parameters of *S. typhimurium* in liquid media (lag phase duration: 10 hrs, and generation time: 133 min), were almost similar to that of *E. coli* (lag phase duration: 9 hrs, and generation time: 130 min) previously determined in our laboratory. However, the cell density in the stationary phase for *S. typhimurium* ($O.D._{450}$: 2.5) was slightly lower than the stationary phase cell density observed for *E. coli* ($O.D._{450}$: 4.0).

b) Disruption of *dgoR* in *S. typhimurium* and strain verification.

i. Deletion of *dgoR* by homologous recombination: The *dgoR* gene in *S. typhimurium* was deleted using one step inactivation method (26). The kanamycin cassette was amplified from the plasmid, pKD13, using primers YA1 and YA2 (see Materials and methods, section 2.2.5.b), and transformed in *S. typhimurium* LT2 harboring plasmid, pKD46, which expresses λ-red recombinase system for homologous recombination. ~60 colonies were observed after overnight incubation. Few of these colonies were streaked and verified for *dgoR* disruption by colony PCR using gene-specific (YA5 and YA6) and kanamycin cassette specific (SAK1 and SAK2) set of primers. As shown in Fig. 3.2A, using gene-specific primers (YA5 and YA6) the fragment size obtained for the four clones (C1-C4) was larger than that obtained for the WT strain. This result was expected if the *dgoR* gene was replaced by kanamycin cassette in the clones. Similarly, as expected, amplification was solely observed in the clones when a gene-specific primer was used in combination with a kanamycin cassette specific primer for amplification. We named these clones (C1-C4) as parent *dgoR::kan*.

ii. P22 transduction for transferring *dgoR* deletion to a clean WT background: To overcome the effect of any background changes that might have happened in the parent *dgoR::kan* strains during the process of homologous recombination, we moved *dgoR* deletion into a clean WT background. The parental clones were infected with P22 phage and the lysates thus prepared were used to infect WT *S. typhimurium* LT2. The transductants were streaked and verified for *dgoR* deletion by colony PCR using a gene-specific primer in combination with a kanamycin cassette specific primer. As expected, amplification was observed only in the parent and transductant clones (Fig. 3.2B).

iii. Screening out the transductants carrying lysogens and pseudo-lysogens: During the process of transduction there is a possibility that some of the transductants may also get infected with P22 phage. Thus it is important to screen and select transductants that do not carry any phage. For this, we first screened out pseudolysogens by streaking the transductants at least four times on green indicator plates. Colony which carries pseudolysogen results in dark green color on the plates, while the colony that is either a non-lysogen or a true-lysogen results in light green color on the indicator plates. The colonies that resulted in light green color were selected for the final screening. The non-lysogens were separated from the true-lysogens by checking the sensitivity of the transductants to P22 infection. True-lysogens are resistant to infection whereas non-

lysogens are sensitive to infection. As shown in Fig. 3.3, all the transductants are sensitive to P22 phage infection and thus are free of any phage. These transductants were used in all the subsequent experiments.

c) *S. typhimurium dgoR::kan* strain shows growth defect on LCFAs.

To determine whether DgoR is required for the growth of *S. typhimurium* on LCFAs, WT, parent *dgoR::kan* and transductant *dgoR::kan* strains were spotted on M9 minimal media containing either glucose or oleate as the carbon source. Since oleate was solubilized in a detergent, for a direct comparison with oleate plates, detergent was included in the minimal media containing glucose. As shown in Fig. 3.4, whereas all the *dgoR::kan* strains showed growth comparable to the WT on glucose, they exhibited significant growth defect on oleate thereby validating that DgoR is required for the growth of *S. typhimurium* on LCFAs. In these experiments *E. coli dgoR::kan* strain, which has previously been shown in our lab to show a growth defect on LCFAs was used as a control (Bhupinder Singh, unpublished data).

We also checked whether *dgoR::kan* strain exhibits growth defect in liquid media. For this, we monitored the growth of WT, parent and transductant *dgoR::kan* strains in M9 minimal liquid media containing either glucose (with detergent) or oleate as the carbon source. All 3 strains showed similar growth characteristics in the glucose media (Fig. 3.5A). On the other hand, in oleate media the final cell density of the *dgoR*::kan strains ($O.D._{450}$: 2.2) was slightly lower than the WT strain ($O.D._{450}$: 2.47) (Fig. 3.5B). Similar results were obtained earlier in our lab for *E. coli*. Overall, the experiments in solid and liquid media suggest that DgoR might play a role in regulating LCFA metabolism in *S. typhimurium*.

There can be multiple reasons for the difference in growth phenotype of *dgoR::kan* strain in solid and liquid media. The growth conditions in solid and liquid media are different as the liquid media is well aerated by shaking while the solid media remain poorly aerated due to no such provision. The second reason for the difference in growth phenotype could be due to local accumulation of toxic metabolites on solid media whereas due to shaking toxic metabolites are dispersed in the liquid media. Another difference is that cells grow in proximity in solid media forming a biofilm as compared to uniform distribution of cells in liquid media.

d) Complementation of the *S. typhimurium* LT2 *dgoR::kan* strain with *E. coli* and *S. typhimurium dgoR* cloned on plasmid.

S. typhimurium LT2 *dgoR* alongwith its putative promoter (~450 bp upstream of the start codon of *dgoR*) was cloned in the plasmid, pACYC184. The clones were confirmed by colony PCR (Fig 3.6) and sequencing, and named as *S. typhimurium* p*dgoR*. To determine whether the growth defect of the *S. typhimurium dgoR::kan* strain is due to the loss of DgoR protein, complementation test was performed. One of the *S. typhimurium* p*dgoR* clones was transformed in both *S. typhimurium* WT and *dgoR::kan* parental strains. The *S. typhimurium* WT and *dgoR::kan* strains were also transformed with *E. coli dgoR* cloned in pACYC184 (*E. coli* p*dgoR*) (Bhupinder Singh, unpublished construct). The growth of all the above transformants and their empty vector controls was assayed on solid media containing either LCFAs or glucose as the sole carbon source. As shown in Fig. 3.7 whereas all the strains grew equally well in glucose media, strains showed differences in growth pattern in oleate media. Whereas the *E. coli* p*dgoR* complemented the growth defect of *S. typhimurium dgoR::kan* strain, the *S. typhimurium* p*dgoR* failed to complement the *S. typhimurium dgoR::kan* strain.

The failure of *S. typhimurium* p*dgoR* to complement *dgoR::kan* strain may be due to lethal level expression of DgoR from the plasmid as *S. typhimurium* p*dgoR* also affects the growth of WT *S. typhimurium*. It would be important to compare the level of expression of DgoR from *S. typhimurium* p*dgoR* and *E. coli* p*dgoR* since *E. coli* p*dgoR* complemented *S. typhimurium dgoR::kan* strain. The difference in DgoR expression from *E. coli* p*dgoR* and *S. typhimurium* p*dgoR* could arise due to differences in their promoter sequences. A follow-up strategy will be to clone the *S. typhimurium dgoR* in a low copy number plasmid and check if DgoR expressed from low copy number plasmid complements *S. typhimurium dgoR::kan* strain.

3.2 Tagging of *E. coli dgoR* on the chromosome

a) *E. coli dgoR* was tagged with 3xFLAG on the chromosome by homologous recombination.

The *dgoR* gene of *E. coli* BW25113 was tagged with 3xFLAG by homologous recombination. The 3xFLAG tag with kanamycin cassette at the 3’ end was amplified from an *E. coli* strain that carried the tag on the chromosome using primers YA9 and YA10 (see Materials and methods, section 2.2.7). The amplified fragment was transformed in *E. coli* BW25113 harboring plasmid,

pKD46. Few of the colonies obtained after overnight incubation were verified by colony PCR. Using gene-specific primers (BS13 and BS14), the size of the fragments obtained for the three clones (C1-C3) was larger than that obtained for the WT strain as expected (Fig. 3.8A). Moreover, amplification was observed only in the clones when a gene-specific primer was used in combination with a kanamycin cassette specific primer. The clones, C1-C3, were named as parent clones. 3xFLAG tagged *dgoR* was then moved into a clean WT *E. coli* strain by P1 transduction, and the transductants were also verified by colony PCR (Fig. 3.8B).

b) *E. coli* expressing 3xFLAG tagged DgoR from the chromosome shows a phenotype similar to the WT strain in media containing LCFAs as the carbon source.

Since the FLAG tagged DgoR construct will be used in physiological experiments, it is important to determine: (i) the expression of tagged DgoR from the chromosome, and (ii) the effect of FLAG tag at the C-terminus of DgoR on the growth phenotype of the strain. The expression of FLAG tagged DgoR was checked by Western blotting using M2 antibody. As shown in Fig 3.9A, DgoR-3xFLAG expresses in both the parent and the transductant. The tagged protein runs at a molecular weight of ~28 kDa which is very close to the molecular weight theoretically calculated based on the amino acid sequence (27.7 kDa). Further, the growth of the tagged construct was compared with the WT strain on minimal media plates containing LCFAs as the carbon source. WT and the tagged constructs (both parent and transductant) exhibited similar growth pattern (Fig. 3.9B). The growth phenotype of the strains was also similar in liquid media containing LCFAs (Shachikanta Nongthombam, unpublished data). These results indicate that the 3xFLAG tagged DgoR construct can be used in experiments aimed at monitoring the induction of DgoR protein in the presence of multiple carbon sources.

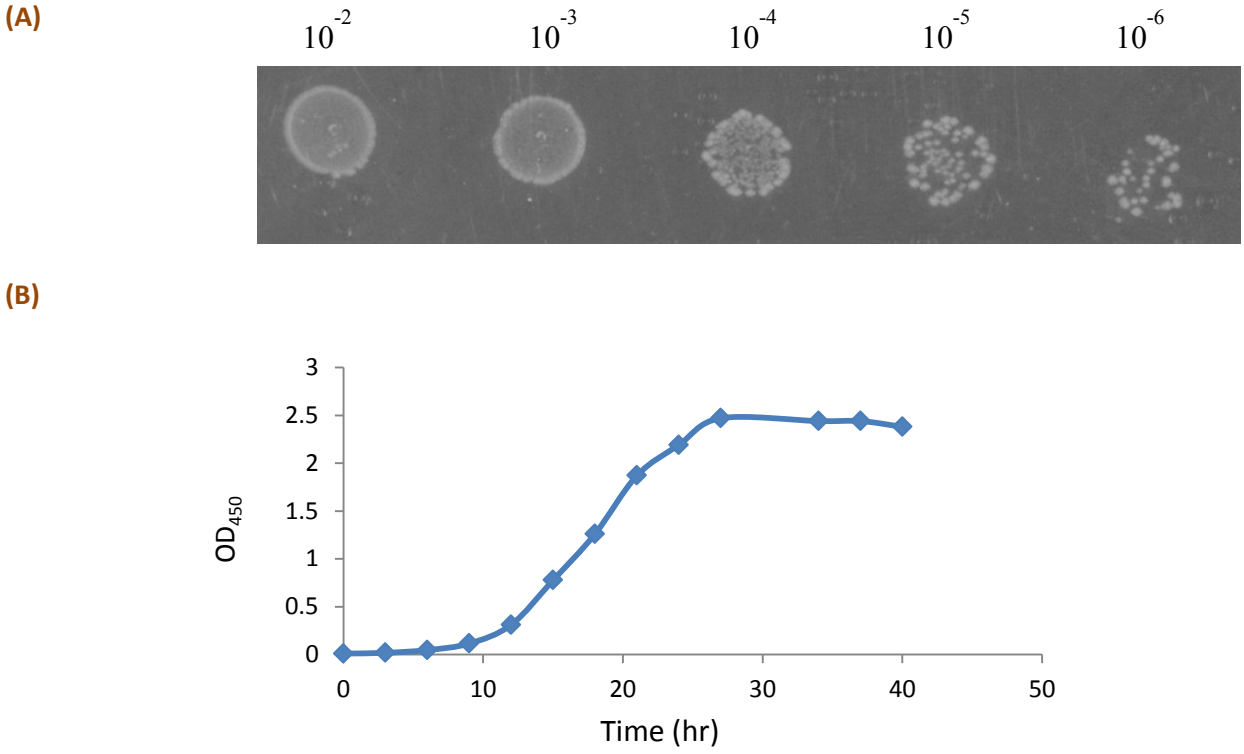
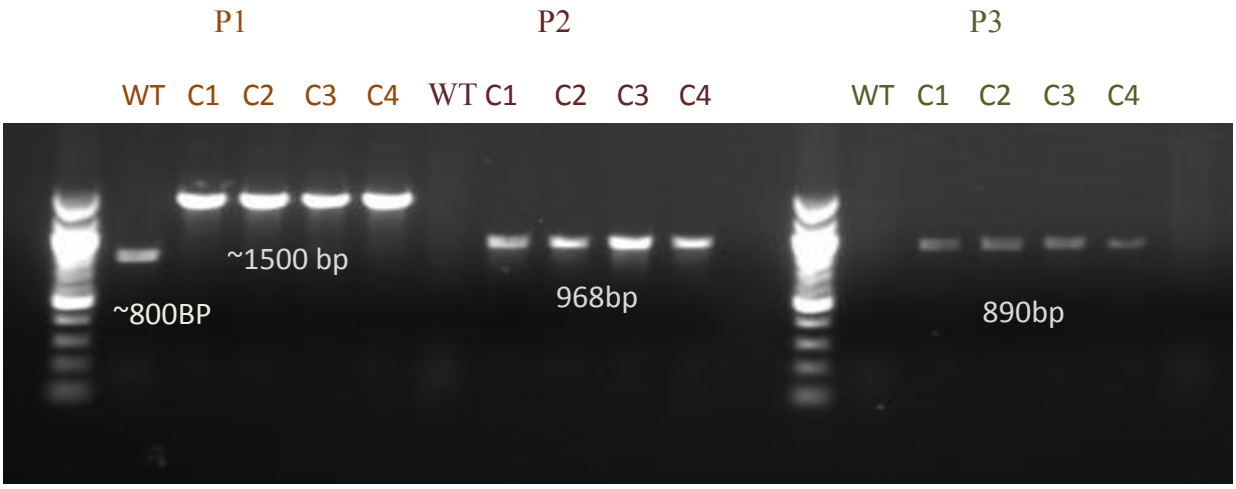


Fig. 3.1 *S. typhimurium grows on oleate as the carbon source in both solid & liquid media.* (A) Growth of *S. typhimurium* on solid media: Various dilutions of the overnight culture of WT strain grown in LB media was spotted on M9 minimal media containing oleate as the sole carbon source, and (B) Growth profile of *S. typhimurium* in liquid media: The overnight culture of WT strain grown in LB media was re-inoculated in M9 minimal media containing oleate as the sole carbon source and growth was monitored at indicated time intervals for a period of 48 hours.

(A)



(B)

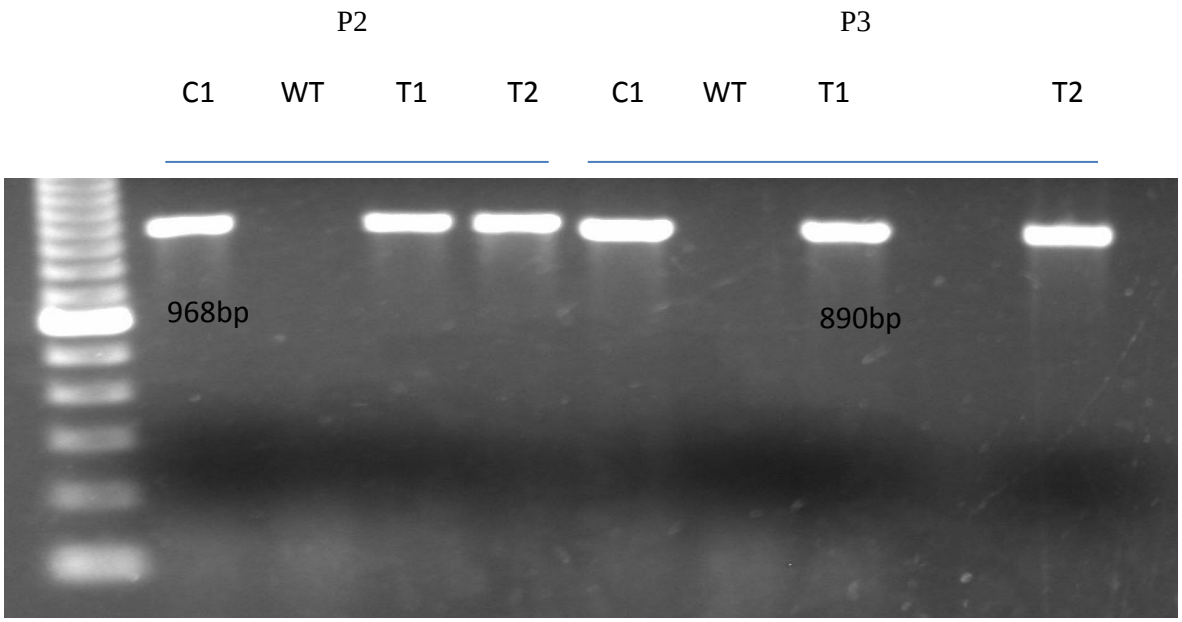


Fig. 3.2 *Verification of parent and transductant dgoR::kan strains by colony PCR.* (A) Four *S. typhimurium dgoR::kan* parent clones were verified by colony PCR using P1 (YA5 and YA6), P2 (YA5 and SAK2) and P3 (YA6 and SAK1) primer sets, where YA5 and YA6 are gene specific primers, and SAK1 and SAK2 are kanamycin cassette specific primers. WT is wildtype *S. typhimurium LT2*, and C1, C2, C3 and C4 are *S. typhimurium dgoR::kan* parent clones. (B) Two *S. typhimurium dgoR::kan* transductant clones were verified by colony PCR using P2 and P3 primer sets. T1 and T2 are *S. typhimurium dgoR::kan* transductant clones.

S. typhimurium WT

S. typhimurium *dgoR*::kan (T1)

S. typhimurium *dgoR*::kan (T2)

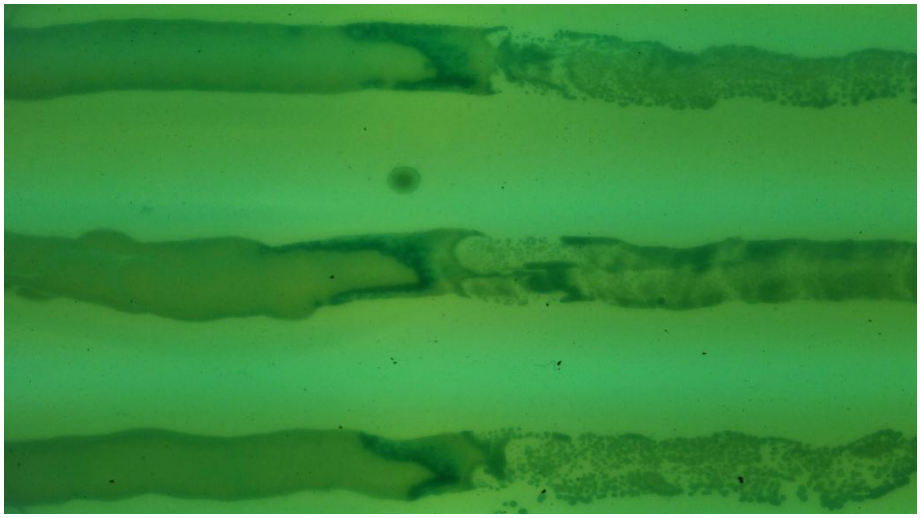


Fig. 3.3 *Screening out lysogens from S. typhimurium dgoR::kan transductants by checking sensitivity to P22 phage.* P22 phage was streaked along the middle of the green indicator plate. The WT and *dgoR::kan* transductants were streaked perpendicular across the phage streak. All the three strains lysed upon touching the phage streak (plates observed after overnight incubation).

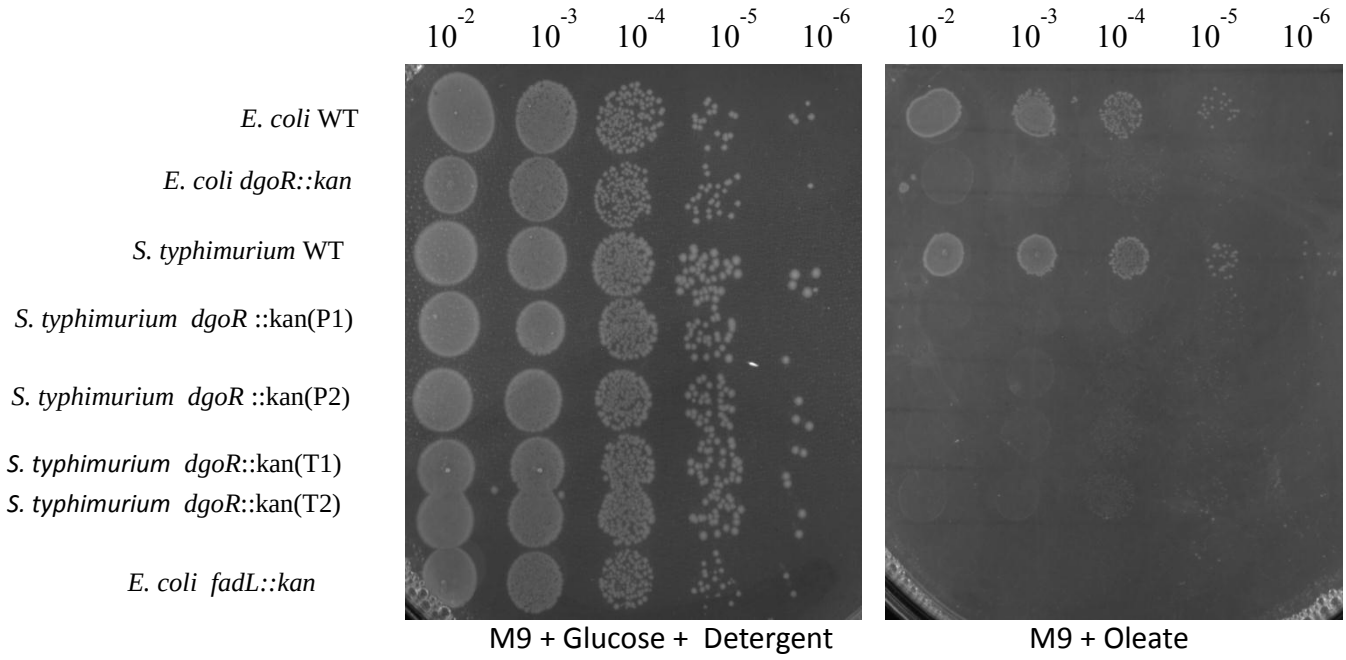
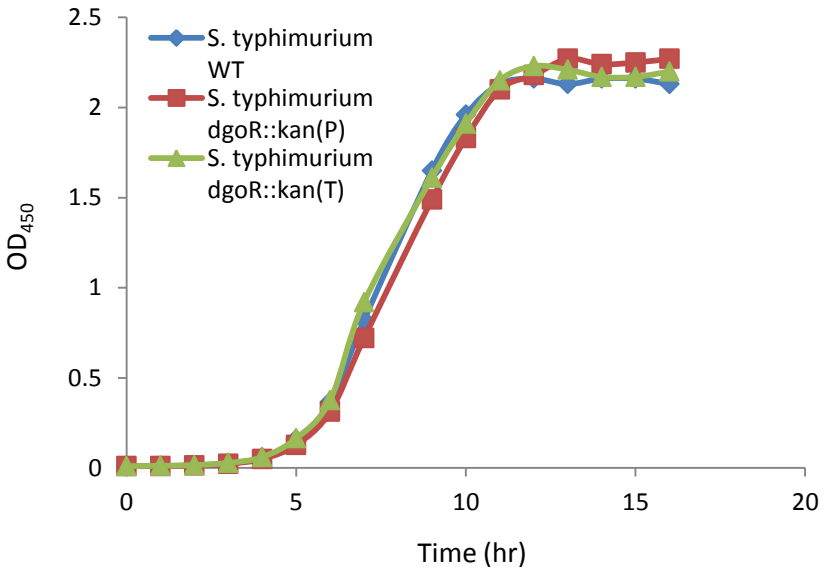


Fig. 3.4. *S. typhimurium dgoR::kan strain shows growth defect in solid media containing oleate as the carbon source.* Dilutions of the cultures of *E. coli* WT, *E. coli dgoR::kan*, *S. typhimurium* WT, *S. typhimurium dgoR::kan* [parents (P1 and P2), and transductants (T1 and T2)], and *E. coli fadL::kan* were spotted on minimal media plates containing either glucose with detergent, or oleate. Since oleate was solubilized in a detergent, glucose with detergent was used as a control for direct comparison with oleate plates. Deletion of the outer membrane LCFA transporter, FadL, is known to result in complete loss of growth when oleate is used as the sole carbon source. Therefore, *fadL::kan* was used as a control in the experiment.

(A)



(B)

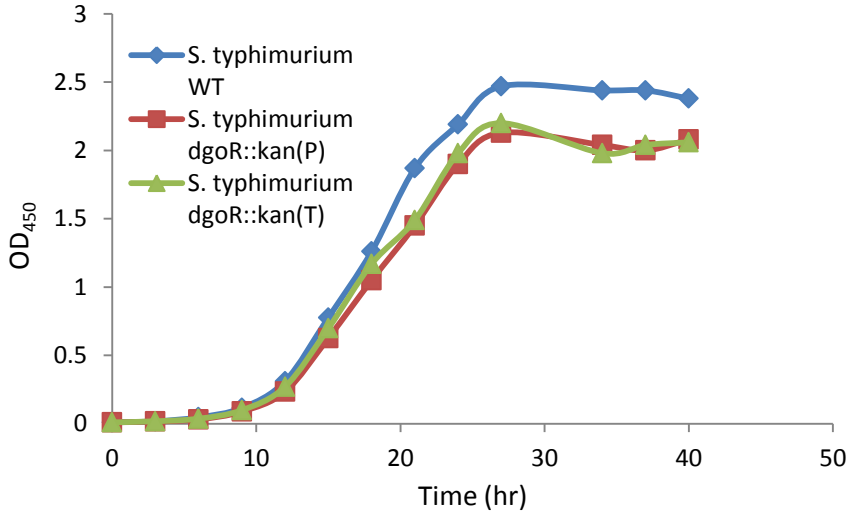


Fig. 3.5. *S. typhimurium dgoR::kan* exhibits slight growth defect in liquid media containing oleate as the carbon source. (A) Growth curve of *S. typhimurium LT2* WT, *dgoR::kan* parent (P), and *dgoR::kan* trasductant (T) in minimal media containing glucose with detergent. (B) Growth curve of *S. typhimurium LT2* WT, *dgoR::kan* parent (P), and *dgoR::kan* trasductant (T) in minimal media containing oleate.

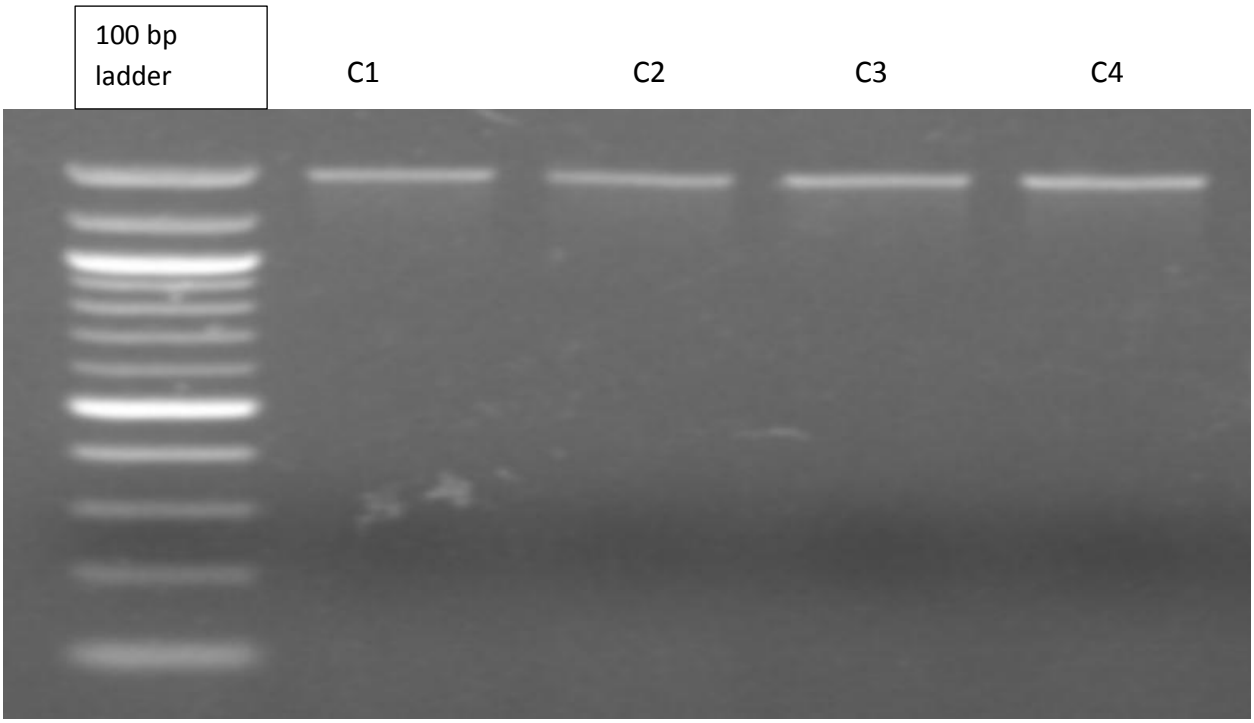


Fig. 3.6. *S. typhimurium dgoR* cloned with its native promoter in plasmid, pACYC184, was verified by colony PCR using primers, BS33 and BS34. Expected band size of 1400bp was observed for all the clones (C1, C2, C3 and C4).

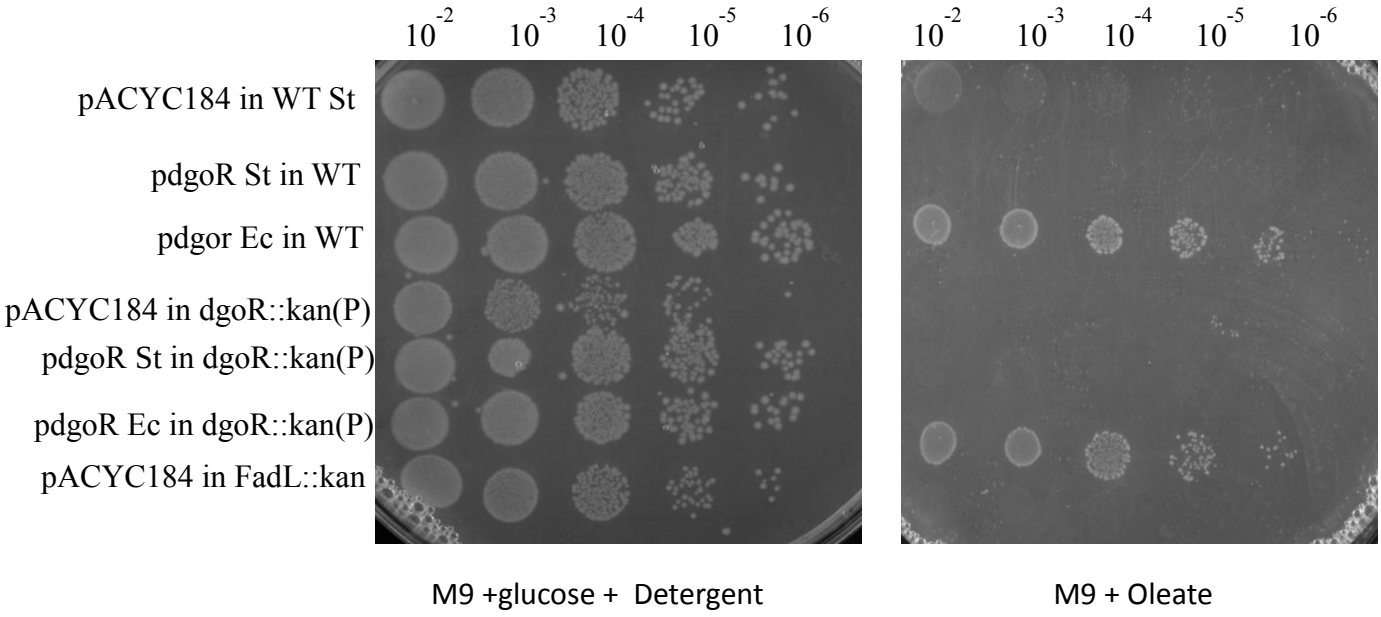


Fig. 3.7. *E. coli DgoR expressed from plasmid rescues the growth defect of S. typhimurium dgoR::kan strain whereas S. typhimurium DgoR expressed from plasmid fails to complement dgoR deletion strain.* Empty plasmid (pACYC184) and plasmid carrying either *E. coli dgoR* (p*dgoR* Ec) or *S. typhimurium dgoR* (p*dgoR* St) were individually transformed in *S. typhimurium* WT (WT St) and *dgoR::kan* parent strains (*dgoR::kan* P). The resulting transformants were spotted on minimal media plates containing either glucose with detergent or oleate. Empty plasmid was transformed in *E. coli fadL::kan*, and used as a control in the experiment.

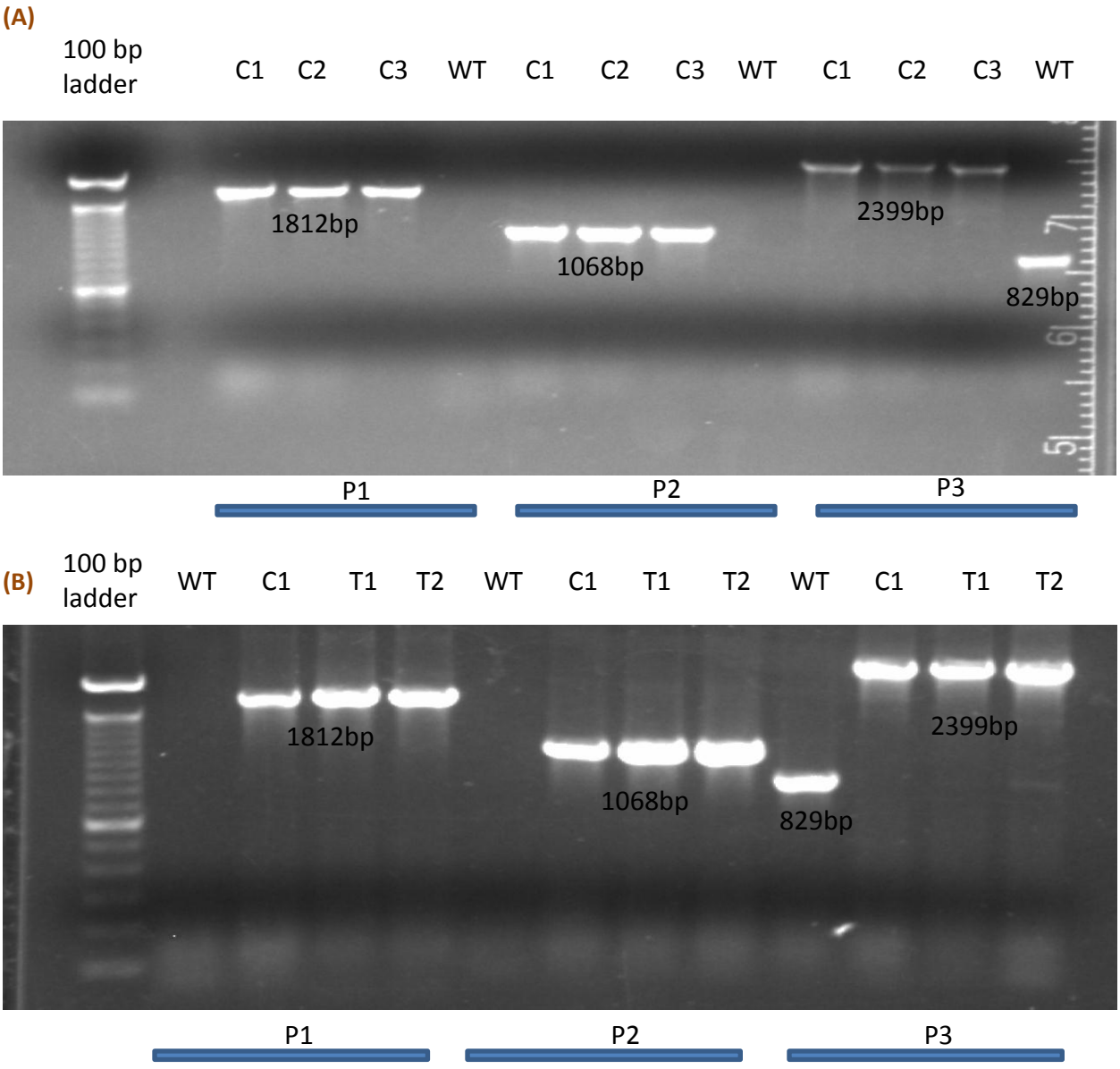
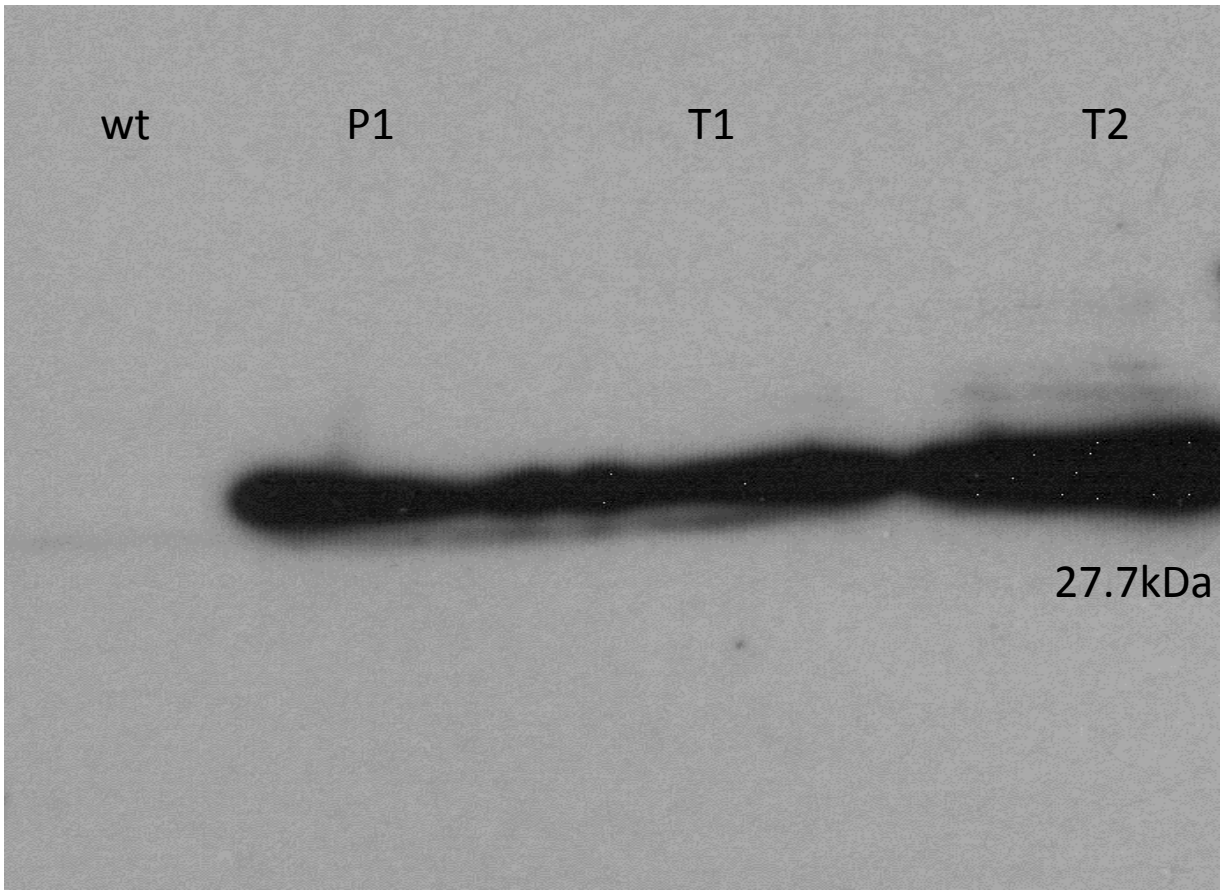


Fig. 3.8. *Tagging of the chromosomal copy of dgoR with 3xFLAG in E. coli was verified by colony PCR.* (A) Three *dgoR-3xFLAG-kan* parent clones were verified by colony PCR using P3 (BS13 and BS14), P1 (BS13 and SAK2) and P2 (SAK1 and BS14) primer sets, where BS13 and BS14 are gene specific primers, and SAK1 and SAK2 are kanamycin cassette specific primers. WT is wildtype *E. coli*, and C1, C2, and C3 are *dgoR*-3xFLAG-*kan* parent clones. (B) Two *dgoR-3*xFLAG-*kan* transductant clones were verified by colony PCR using P1, P2 and P3 primer sets. T1 and T2 are *dgoR*-3xFLAG-*kan* transductant clones

(A)


wt
P1
T1
T2
27.7kDa

(B)

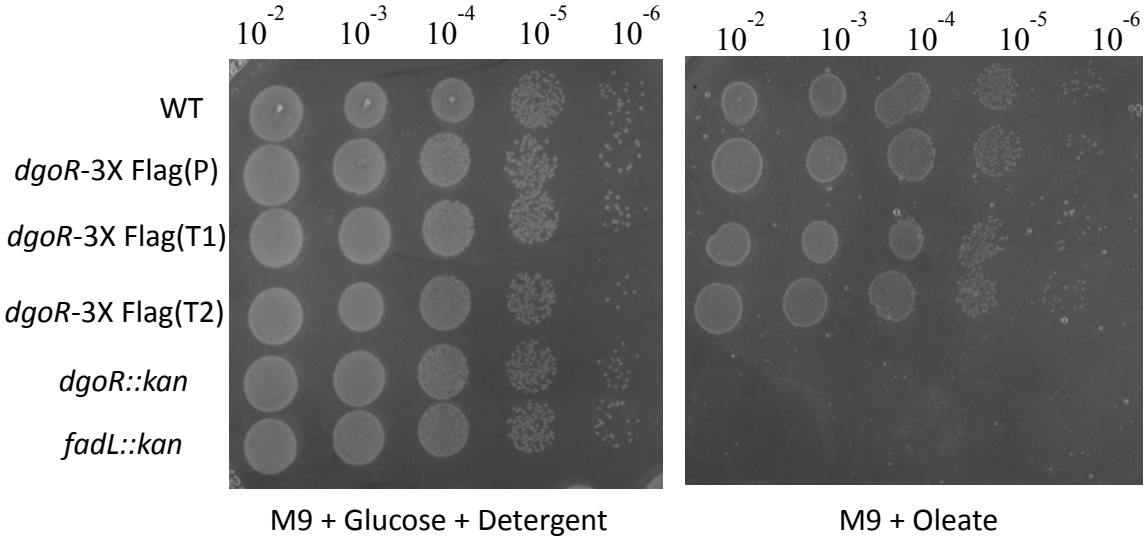

10-2 10-3 10-4 10-5 10-6
10-2 10-3 10-4 10-5 10-6
WT
dgoR-3X Flag(P)
dgoR-3X Flag(T1)
dgoR-3X Flag(T2)
dgoR::kan
fadL::kan
M9 + Glucose + Detergent
M9 + Oleate

Fig. 3.9. *E. coli expressing FLAG tagged DgoR from the chromosome shows a phenotype similar to the WT strain in media containing LCFAs as the carbon source.* (A) Expression of DgoR-FLAG was observed in both parent (P) and the two transductant (T1 and T2) dgoR-3xFLAG-kan strains using anti-FLAG antibody. WT strain expressing untagged DgoR was run as a negative control. The observed molecular weight of ~28kDa of the FLAG tagged DgoR is indicated by an arrow. (B) Various dilutions of the cultures of WT and dgoR-3xFLAG-kan strains (parent, P, and transductants, T1 and T2) were spotted on minimal media plates containing either glucose with detergent or oleate. Both *dgoR::kan* and *fadL::kan* were used as controls.

Chapter 4

Conclusion and Future Outlook

Conclusion and Future Outlook

In a high-throughput genetic screen conducted recently by Dr. Rachna Chaba, a predicted transcriptional regulator in *E. coli*, DgoR, was identified to be required for growth in media containing LCFAs as the sole carbon source (unpublished data). The growth phenotype of the *dgoR* deletion strain of *E. coli* was validated in our lab (Bhupinder Singh, unpublished data). Despite the fact that *E. coli* and the related enteric bacteria, *S. typhimurium*, have similar pathways for fatty acid degradation, there are differences in the types of fatty acids that these bacteria can utilize as carbon source. In the present study, in order to initiate a comparative study of the regulatory role of DgoR in LCFA metabolism in *E. coli* and *S. typhimurium*, *dgoR* was deleted in *S. typhimurium* and its growth phenotype on LCFAs was assessed. Similar to *E. coli*, deletion of *dgoR* in *S. typhimurium* showed significant growth defect on LCFAs. *S. typhimurium dgoR* was cloned with its promoter in pACYC184 to check if it complements *S. typhimurium dgoR* deletion strain. The *S. typhimurium* p*dgoR* clone did not complement *dgoR* deletion strain. Moreover, the clone was lethal when transformed in WT *S. typhimurium*. However, *E. coli dgoR* cloned on a plasmid could complement and rescue the growth of *S. typhimurium dgoR* deletion strain. Currently we are trying to clone *S. typhimurium dgoR* in a low copy number plasmid to check if low-level expression of DgoR complements *S. typhimurium dgoR* deletion strain. Considering the structural similarity of DgoR with FadR, our future experiments will be aimed at checking whether like FadR, DgoR also binds acyl-CoA directly to regulate LCFA metabolism. In addition to the studies performed on *S. typhimurium dgoR*, an important resource was constructed for the lab in the present study. The *E. coli dgoR* gene was tagged at its C-terminus with 3xFLAG on the chromosome. This tagged construct showed the same phenotypic behavior as WT *E. coli* strain in LCFAs. The expression of DgoR-3xFLAG protein was confirmed by Western blotting. We are planning to use this tagged strain in ChIP-seq experiments to identify the genes targeted by DgoR for regulation of LCFA metabolism. This tagged strain will find another use in determining the expression of DgoR in various carbon sources.

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