Investigating the role of *dgo* operon in D-galactonate metabolism and its regulation in *Escherichia coli*

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

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Certificate of Examination

This is to certify that the dissertation titled "Investigating the role of *dgo* operon in D-galactonate metabolism and its regulation in *Escherichia coli*" submitted by Mr Shachikanta Nongthombam (Reg. No. MS10013) 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. Shravan Kumar Mishra Dr. Rachna Chaba

(Supervisor)

Dated: April 24th, 2015

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

Shachikanta Nongthombam (MS10013)

Dated: April 24th, 2015

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. APS: Ammonium persulfate
3. ArcA: Anaerobic response control
4. ATP: Adenosine triphosphate
5. bp: Base pair
6. CIP: Calf Intestinal phosphatase
7. Cm^R: Chloramphenicol resistance
8. *dgo* operon: D-galactonate operon
9. DgoA: D-galactonate operon aldolase
10. DgoD: D-galactonate operon dehydratase
11. DgoK: D-galactonate operon kinase
12. DgoR: D-galactonate operon regulator
13. DgoT: D-galactonate operon transporter
14. DNA: Deoxyribonucleic acid
15. dNTP: Deoxynucleotide triphosphate
16. DTT: Dithiothritol
17. ECL: Enhanced chemiluminescence
18. EDTA: Ethyelene diamine tetraacetate
19. EtBr: Ethidium bromide
20. HCl: Hydrochloric acid
21. His : Histidine
22. HTH: Helix turn helix
23. IgG: Immunoglobulin G
24. IPTG: Isopropyl β-D-1-thiogalactopyranoside (IPTG)
25. KCl,: Potassium chloride
26. kDa: kilo Dalton
27. KDPG: 2-keto-3-deoxy-6-phosphogluconate
28. KH_2PO_4: Monopotassium phosphate
29. LB: Lysogeny broth
30. $MgSO_4$: Magnesium Sulphate
31. ml: millilitre

32. mM: millimolar
33. Na_2HPO_4: Disodium phosphate
34. NaCl: Sodium chloride
35. NEB: New England Biolabs
36. NH_4Cl: Ammonium chloride
37. NMR: Nuclear Magnetic Resonance
38. NTA: Nitrilo tri acetate
39. Ori :origin of replication
40. PAGE: Poly Acrylamide Gel Electrophoresis
41. RBC: Red blood corpuscles
42. SDS: Sodium dodecyl sulphate
43. TAE: Tris Acetic Acid EDTA
44. TBE: Tris borate EDTA
45. TBST: Tris-Buffered saline and Tween
46. TEMED: Tetramethylethylenediamine
47. Tet^R: Tetracyclin resistance
48. UV: Ultra violet
49. w/v: Weight by volume

Abstract

Carbon source is one of the basic requirements for bacterial growth. D-galactonate, an aldonic sugar acid, can be used as a carbon source by *Escherichia coli*, a common gram-negative bacterium. Galactosemic patients who cannot utilize galactose in their diet excrete large amounts of D-galactonate in urine. It has been shown by microarray studies that *E. coli* grown in urine up regulates the expression of enzymes involved in D-galactonate metabolism thereby suggesting that galactosemic patients might be more prone to infections by bacteria that have the ability to utilize D-galactonate. Classical mutagenesis and mapping studies performed in 1970's identified the genes involved in D- galactonate metabolism; however, the phenotypes of strains carrying clean deletion in these *dgo* genes were not studied. The D-galactonate metabolic pathway includes a transporter, a dehydratase, a kinase and an aldolase, and the genes encoding these components are organized in a putative D-galactonate operon (*dgo*). The first gene of the operon, *dgoR*, encodes a putative transcriptional regulator. Till date, the regulation of D-galactonate metabolism has not been studied. In the present work, we have used various *dgo* gene deletion strains to show that *dgo* genes are involved in D-galactonate metabolism. We also provide evidence that DgoR is a negative regulator of *dgo* operon. We observed that deleting *dgoR* shortens the lag phase of bacteria when D-galactonate is used as the carbon source; hence it acts as a repressor of *dgo* genes. Western blotting experiments to detect the expression of 3X-FLAG tagged DgoR from chromosome revealed the induction of DgoR by D-galactonate. During the course of exploring the regulation of *dgo* genes, we identified a second transcriptional regulator of D-galactonate metabolism, ArcA. In future, we will carry out detailed characterization of the regulatory role of DgoR and ArcA in D-galactonate metabolism.

Contents:

Chapter 1

Introduction and Review of Literatures

1.1 Sugar Acids are used as carbon source by *Escherichia coli*

Carbon sources are the basic requirements for growth because they serve as a source of metabolic energy and provide structural framework for synthesis of various cellular macromolecules. *E. coli*, a gram negative bacterium can utilize various carbon sources such as sugars like D-glucose, D-galactose, lactose; sugar acids like D-gluconate, D-galactonate; fatty acids like acetic acid, oleic acid; and amino acids. Sugar acids are monosaccharaides containing a carboxyl group. There are four main classes of sugar acids which include aldonic acids, ulosonic acid, uronic acid and aldaric acid (structure shown in Fig. 1.1). In humans, mucus layer covering the epithelial tissue of intestine provides various sugar acids as carbon source for several gut microbes including *E. coli* [1].

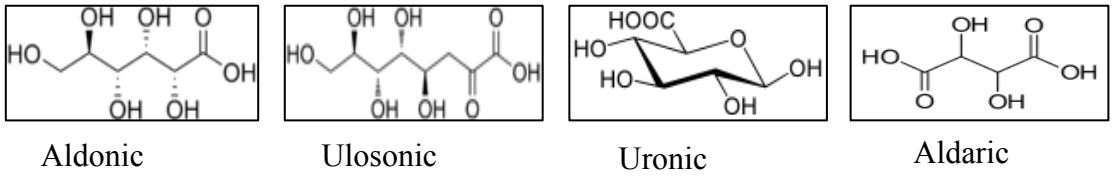

Fig. 1.1. Chemical structure of various classes of sugar acids. [Structure reference: Wikipedia]

1.2 General pathway of sugar acid metabolism and its regulation

The general scheme of sugar acid metabolism involves two independent and interconnected pathways namely Enter-Doudoroff Pathway and Ashwell pathway. In Ashwell pathway, D-glucouronate and D-galactouronate, upon the activity of various enzymes (Fig. 1.2) get converted to 2-keto-3-deoxygluconate which is then metabolized to 2-keto-3-deoxy-6-phosphogluconate (KDPG). KDPG is also produced as an intermediate during catabolism of D-gluconate by Enter-Doudoroff Pathway. KDPG is further metabolised by a reversible reaction to give rise to glyceraldehyde-3-phosphate and pyruvate which are fed to glycolysis and tri-carboxylic acid cycles respectively.

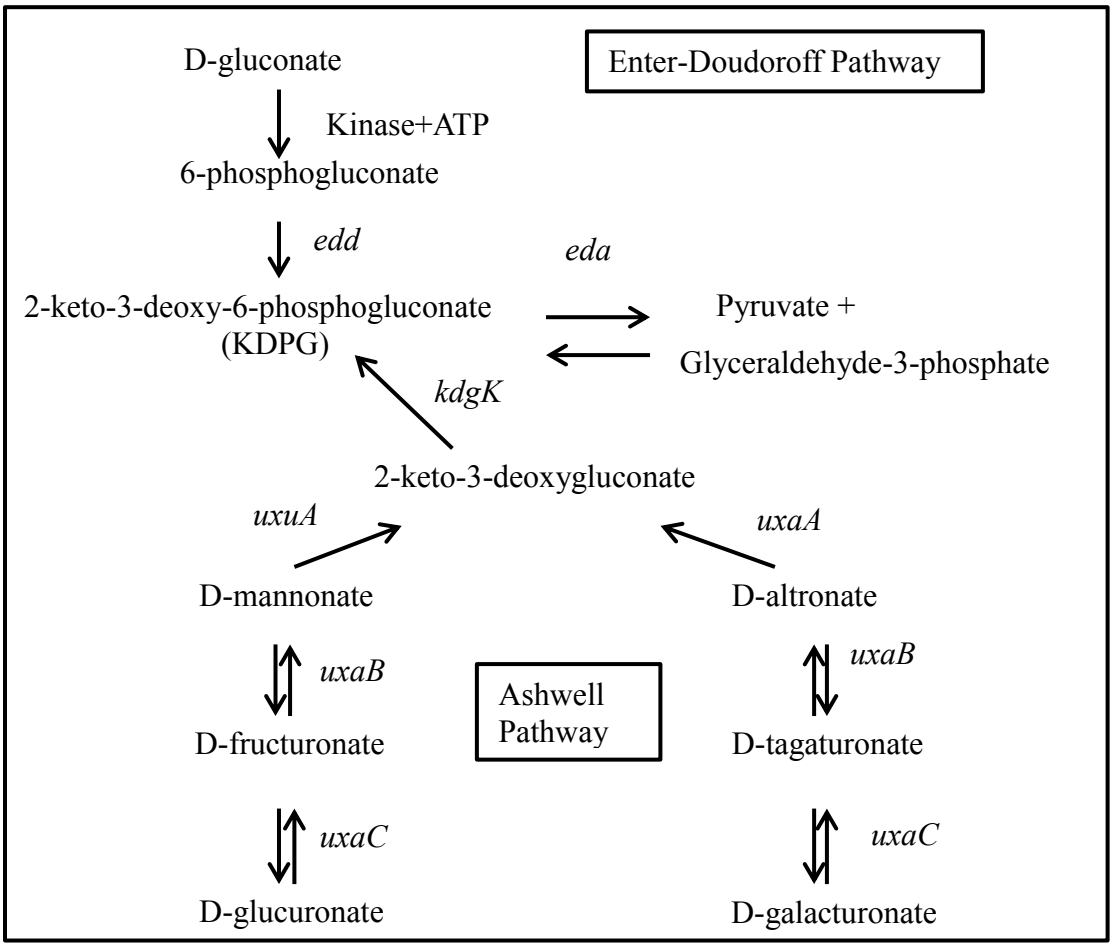

Fig. 1.2. General pathway showing the metabolism of various sugar acids including D-gluconate, D-glucuronate and D-galacturonate. Pathway was modified from [2].

Depending on the availability of carbon source in the environment, bacteria rapidly adjust their metabolic machinery for the transport and degradation of nutrient. Metabolic pathways for the utilization of carbon sources are mainly regulated at a transcriptional level. Transcriptional regulators governing the regulation of sugar acid metabolic pathways mainly belong to GntR family of transcription factors [13]. The transcriptional regulators belonging to this family contain an N-terminal DNA-binding domain which bind DNA through a winged helix-turn-helix (HTH) motif and a C-terminal effector binding/ oligomerization domain (Fig. 1.3) that binds a ligand. Binding of ligand to the C-terminal regulatory domain enables the binding or release of transcription factor from DNA which results in the regulation of gene expression. For example, D-fructuronate metabolism is regulated by a transcriptional regulator UxuR

which belongs to GntR family of transcriptional regulators. UxuR acts as a negative regulator of D-mannonate dehydratase (UxuA), D-mannonate oxidoreductase (UxuB) and D-fructuronate transporter (GntP) required for D-fructuronate metabolism. In the absence of D-fructuronate, UxuR binds to cis-element of *uxuAB* operon and *gntP*, and represses the expression of these genes. When D-fructuronate is available, it is transported inside the cell by the basal level of GntP transporter. D-fructuronate binds to UxuR and dislodges it from the *uxuA-uxuB* and *gntP* cis-element leading to up-regulation of the transporter GntP as well as enzymes UxuA and UxuB for metabolism of D-fructuronate [16].

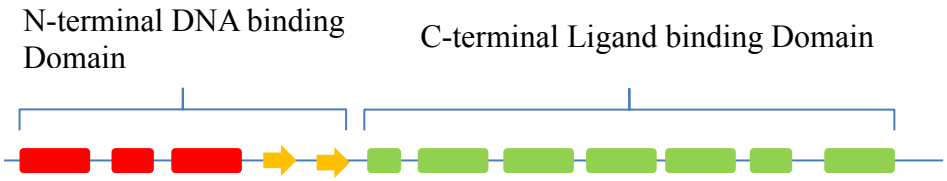

Fig. 1.3. Schematic showing N- and C- terminal domains of transcriptional regulators belonging to GntR family.

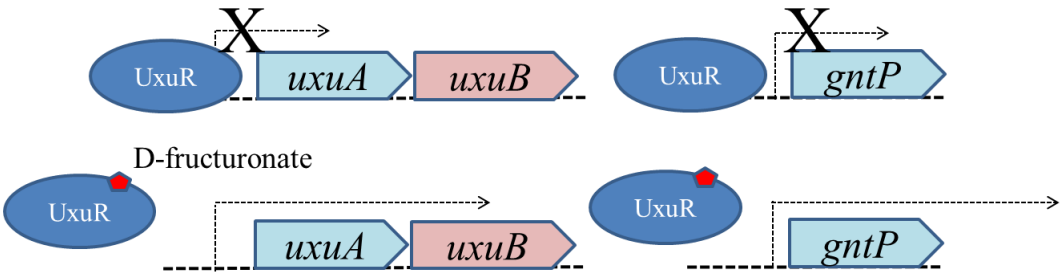

Fig. 1.4 Transcriptional regulation of D-Fructuronate metabolism by UxuR, a transcriptional regulator belonging to GntR family of transcriptional regulators [16].

1.3 D-Galactonate metabolism in *E. coli*

Galactonate is an aldonic sugar acid derived from the oxidation of anomeric carbon of galactose. It exists in two forms, L-galactonate (Fig. 1.5 A) and D-galactonate (Fig. 1.5 B). In humans, D-galactonate is found in RBC [15], gut and excreted in urine [14]. *E. coli* can utilize both L- and D- forms of galactonate [3 and 4], and metabolize these carbon sources to glyceraldehyde-3-phosphate and pyruvate. Metabolism of D-

galactonate has been studied in *E. coli* in 1970's [3 and 5]. In these studies chemical mutagenesis was used to isolate mutants unable to grow on D-galactonate. Crude cell lysate of these mutants were used for enzymatic assays and a pathway for D-galactonate metabolism was proposed (Fig. 1.6). According to the proposed pathway, D-galactonate is transported inside the cell with the help of a transporter, DgoT. Inside the cytoplasm, D-galactonate is dehydrated by a dehydratase, DgoD, phosphorylated by a kinase, DgoK, and cleaved into pyruvate and glyceraldehyde-3-phosphate with the help of an aldolase, DgoA. Glyceraldehyde-3-phosphate and pyruvate are fed into glycolysis and tricarboxylic acid cycles respectively. Various mutations in strains unable to grow on D-galactonate were mapped and were found to be located very close to each other suggesting a putative D-galactonate operon (*dgo*). In these studies, a mutant with constitutive expression of D-galactonate catabolizing enzymes was isolated; therefore, it was proposed that there might be a repressor of *dgo* genes (DgoR). The putative organization of D-galactonate operon is shown in Fig. 1.5 C. Although the pathway for D-galactonate metabolism has been proposed, this metabolic pathway has not been verified using clean deletion strains of various *dgo* genes. In a more recent study DgoR has been predicted to be a member of GntR family of transcriptional regulators [13].

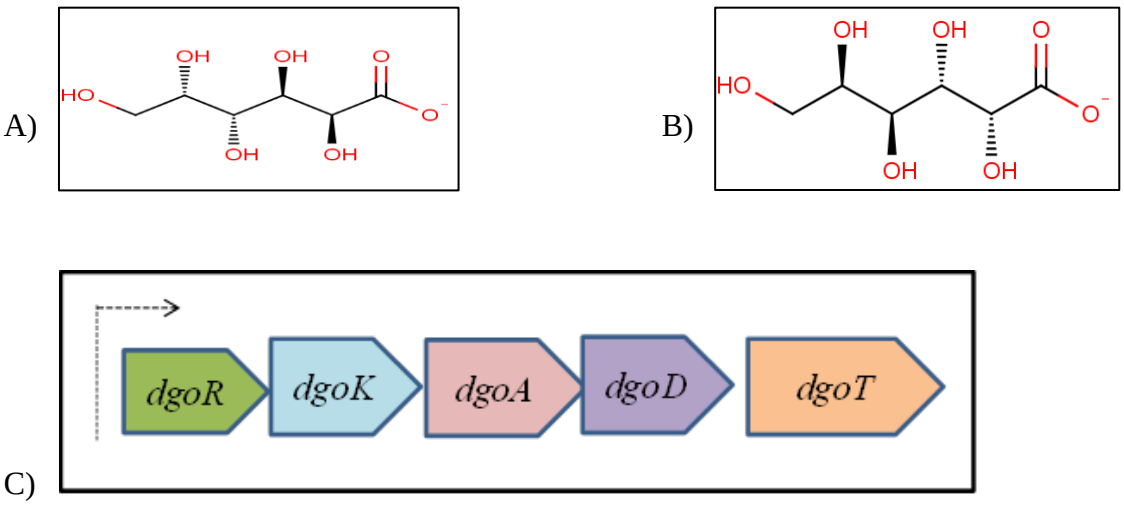

Fig. 1.5 A) Structure of L-galactonate, B) Structure of D-galactonate, and C) Predicted operon (*dgo*) for D-Galactonate metabolism in *E. coli*.

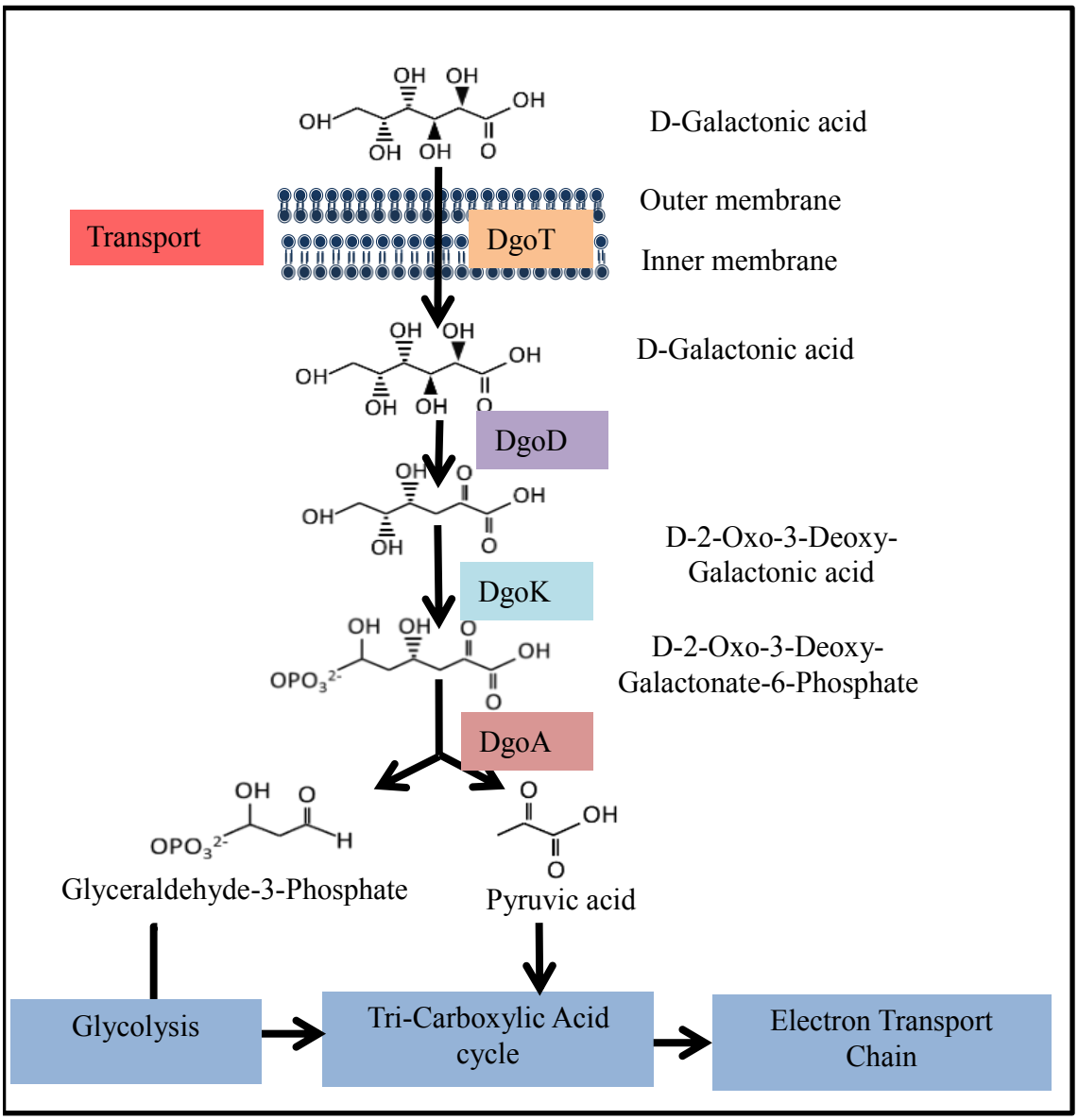

Fig. 1.6. Proposed pathway for D-Galactonate metabolism, adapted from [3].

Galactosemic patients who have problem in utilizing galactose from their diet, accumulate intermediates of galactose metabolism, D-galactonate and gactitol, in blood [15], liver, and kidney which ultimately end up in urine. It has been shown by microarray studies that *dgo* genes involved in D-galactonate metabolism are up-regulated in *E. coli* grown in human urine indicating that this carbon source is present in urine [6]. The above information suggests that galactosemic patients might be more prone to infections by bacteria that can use D-galactonate as carbon source. Hence, in-depth studies on D-galactonate metabolism are of medical relevance.

Considering that the involvement of *dgo* genes in D-galactonate metabolism has not been verified in candidate studies using deletion strains of *dgo* genes, and especially nothing is known about the regulatory aspects of D-galactonate metabolism, the main aims of this study were:

i) to verify the requirement of *dgo* genes in D-galactonate metabolism using *dgo* gene deletion strains.
ii) to determine whether *dgo* operon is induced by D-galactonate.
iii) to study the regulatory role of DgoR in D-galactonate metabolism.

Our bioinformatics studies predicted the binding site for a global transcriptional regulator, ArcA, in the cis element of *dgo* operon. One of the aims was to investigate whether ArcA is involved in regulation of *dgo* genes.

Chapter 2

Materials and Methods

2.1 Materials

2.1.1 Chemicals, Reagents and their Sources

Calcium D-Galactonate was ordered from MP Bio Medicals. Skimmed milk, LB, Tryptone, Yeast Extract and Bacteriological agar were purchased from BD Difco™. Primers were ordered from IDT. Oxalic Acid was from Rankem. $MgSO_4$, Na_2HPO_4, KH_2PO_4, NH_4Cl, KCl, D-gluconate, D-galactose, EDTA, Glycerol, Bromophenol blue, APS, TEMED, Imidazole and sodium citrate were purchased from Sigma. Glucose was purchased from Fisher scientific. EDTA, Acrylamide, NaCl, Tris and SDS were purchased from Affymetrix USB product. Glycine and Sodium Borate were purchased from Himedia. Glacial acetic acid, HCl and Methanol were purchased from Merck.

Antibiotics, Anti-Flag primary (M2) and secondary (anti-mouse IgG) antibodies were purchased from Sigma. Enzymes (CIP, Taq/Phusion DNA polymerase, restriction enzymes and T4 DNA ligase), Quick load DNA ladder, 100bp/1Kb DNA ladder, NEB Buffers, Protein ladder and dNTPs were purchased from New England Biolabs. Plasmid miniprep kit, PCR purification kit and gel extraction kit were purchased from Thermo Scientific. ECL chemiluminescence detection kit and Pre-stained protein ladder were purchased from Peirce. Hyperfilm™ ECL films were purchased from Amersham Nitrocellulose membrane was purchased from BioRad.

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	Wanner, B. L., (1983).{29}

DH5α	F- *endA1 glnV44 thi-1 recA1relA1 gyrA96 deoR nupG Φ80dlacZΔM15* Δ(*lacZYAargF*) U169, *hsdR17*(rK⁻ mK+), λ–	NEB	Hanahan,D.(1985) DNA Cloning, Vol.1,ed.Glover, D.M.(IRLPress, Oxford)pp 109-135.
BL21 (DE3)	F⁻ *ompT gal dcm lon hsdS$_B$(r$_B$⁻ m$_B$⁻)* λ(DE3 [*lacI lacUV5-T7* gene 1 *ind1 sam7 nin5*])	Agilent	Miroux B, Walker JE. J. Mol. Biol. (1996) 260, 289-298.
dgoR::kan	*dgoR::kan* in BW25113	Keio collection	Tomoyo Baba *et. al*., 2006
dgoK::kan	*dgoK::kan* in BW25113	Keio collection	Tomoyo Baba *et. al*., 2006
dgoA::kan	*dgoA::kan* in BW25113	Keio collection	Tomoyo Baba *et. al*., 2006
dgoD::kan	*dgoD::kan* in BW25113	Keio collection	Tomoyo Baba *et. al*., 2006
dgoT::kan	*dgoT::kan* in BW25113	Keio collection	Tomoyo Baba *et. al*., 2006
arcA::cam	*arcA::cam* in BW25113	ASKA knockout library	Unpublished
RC2148	*dgo::kan* in BW25113	Chaba Lab	Bhupinder Singh (unpublished)
RC3063	*dgoR* with 3X FLAG at C-terminus on chromosome in BW25113	Chaba lab	Yatendra Arya (unpublished)

2.1.3 Plasmids

Table 2.2 List of Plasmids

Plasmid Name	Description	Source	Reference
pACYC177	Vector, p15A ori, Amp^R, Kan^R	NEB	Chang A. and Cohen, S. (1978); Rose, R.E. (1988).
pACYC184	Vector, p15A ori, Cm^R, Tet^R	NEB	Chang A. and Cohen, S. (1978); Rose, R.E. (1988).
pCA24N	Vector, pBR322 ori, Cm^R	Mori lab	Kitagawa M et. al., (2005)
pBS3	*dgoR* cloned with its putative native promoter and ribosome binding site in NcoI-EcoRI sites of pACYC184	Chaba lab	Bhupinder Singh (Unpublished)
pBS10	*dgoR* sub cloned with its putative native promoter and ribosome binding site in BamHI site of pACYC177	Chaba lab	Bhupinder Singh (Unpublished)
6X-His-ArcA cloned in pCA24N	*arcA* cloned into pCA24N with a 6X His tag at the N-terminus	ASKA Overexpression plasmid library	Kitagawa M *et. al*. (2005)

2.1.4 Primers

Table 2.3 List of primers

Primers	Sequences	Purpose
SK1	CTGTTTAGCGCCTGATATCCC	Forward primer for *dgo* cis-

		element amplification
SK2	CGTAATGACAATGCGATCGG	Reverse primer for *dgo* cis-element amplification
BS38	GCAGGTCAGGGACTTTTGTAC	Forward primer for confirming *arcA::cam*
BS39	GAAGTTACAACGGACGATGAG	Reverse primer for confirming *arcA::cam*

2.1.5 Antibiotics

Ampicillin and Kanamycin stock as given in the table were made in autoclaved MQ water and were filter-sterilized using 0.22 µl filters (Millipore). Chloramphenicol was dissolved in absolute ethanol. Antibiotic stock solutions were stored at -20°C in aliquots of 500 µl.

Table 2.4 List of antibiotics and their concentration

Name of the antibiotics	Stock concentration	Working Concentration
Ampicillin	100 mg/ml	100 µg/ml
Kanamycin	30 mg/ml	30 µg/ml
Chloramphenicol	20 mg/ml	20 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 were dissolved in 1000 ml of milli-Q H_2O and autoclaved at 15lb/$inch^2$ 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 and poured into sterile petriplates (Tarsons). Antibiotics were added in molten agar media cooled to about 50°C. Plates were stored at 4°C till use.

2.1.7 M9 minimal media

Stocks of following M9 minimal media components were autoclaved separately and mixed in the laminar hood when needed to 1 X final concentration. Volume was made up with autoclaved MQ water.

Table 2.6 Composition of M9 minimal media

Composition	10 X M9 Salts	1 M $MgSO_4$	250 X vitamins
Working conc.	1 X	1 mM	1 X

A) 10 X M9 salts

The following components were first dissolved in 600 ml of milli-Q water and the volume was made up to 1000 ml. 10 X M9 salt stock solution was sterilized by autoclaving once all the components were dissolved completely.

Table 2.7 Composition of 10 X M9 salts

Composition	Na_2HPO_4	KH_2PO_4	NaCl	NH_4Cl
Amount	53 g	30 g	5 g	10 g

B) 250 X Vitamin stock

The following components were dissolved in 200 ml of deionized H_2O and filter-sterilized.

Table 2.8 Composition of vitamin stocks

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

2.1.8 M9 minimal solid media

Table 2.9 Composition of M9 minimal liquid media containing various carbon sources

Components	M9+ D-glucose	M9+ D-gluconate	M9+ D-galactose	M9+ D-galactonate	M9+ glycerol
10XM9 Salts	100 ml	100 ml	100 ml	100 ml	100 ml
1M Mg_2SO_4	1 ml	1 ml	1 ml	1 ml	1 ml
250X Vitamins	4 ml	4 ml	4 ml	4 ml	4 ml
20% D-glucose	10 ml	-	-	-	-
10% D-gluconate	-	20 ml	-	-	-
10% D-galactose	-	-	20 ml	-	-
100mM D-galactonate	-	-	-	100 ml	-
20% glycerol	-	-	-	-	10 ml

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2.1.9 M9 minimal liquid media

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

Components	M9 + D-glucose	M9 + D-gluconate	M9 + D-galactose	M9 + D-galactonate	M9 + glycerol
10 X M9 Salts	8 ml	8 ml	8 ml	8 ml	8 ml
1M Mg_2SO_4	80 µl (1 mM)	80 µl (1 mM)	80 µl (1mM)	80 µl (1mM)	80 µl (1 mM)
250X Vitamins	320 µl (1 X)	320 µl (1X)	320 µl (1X)	320 µl (1 X)	320 µl (1 X)
20% D-glucose	800 µl	-	-	-	-

10% D-gluconate	-	1.6 ml	-	-	-
10% D-galactose	-	-	1.6 ml	-	-
100mM D-galactonate	-	-	-	8 ml	-
20% Glycerol	-	-	-	-	1.6 ml
Autoclaved Water	Volume was made up to 40 ml	Volume was made up to 40 ml	Volume was made up to 40 ml	Volume was made up to 40 ml	Volume was made up to 40 ml
2X Molten Agar	40 ml	40 ml	40 ml	40 ml	40 ml

For preparing 80 ml M9 minimal agar media supplemented with various carbon sources, 40 ml 2 X 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. Plates were covered in paper and stored at 4^0C for 12-18 hours.

2.1.10 Composition of Buffers and Solutions used in Agarose Gel Electrophoresis

A) 50X TAE (Stock)

Table 2.11 Composition of 50 X TAE

Composition	Tris	Glacial acetic acid	0.5M EDTA(pH8.0)
Amount (L^{-1})	242 gm	57.1 ml	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 10 X stock was stored at room temperature.

B) 6 X DNA Loading Buffer (Stock)

Table 2.12 Composition of 6X DNA loading buffer

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

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

2.1.11 Composition of Buffers and Solutions used in SDS-PAGE

A) Acrylamide (30%)

Table 2.13 Composition of Acrylamide (30%)

Composition	Acrylamide	N,N-methylene bis acrylamide
Amount	30 g	0.8 g

The above composition was dissolved in 100 ml of milli-Q water and kept protected from light by storing in amber coloured bottle at 4^0C.

B) Lower Tris (for Resolving gel)

Table 2.14 Composition of lower Tris

Composition	Tris	10% SDS
Amount (for 100 ml)	18.17 g	4 ml

The above components were dissolved in 80 ml milli-Q water, pH was adjusted to 8.8 with HCl and volume was made up to 100 ml with milli-Q water. Solution was stored at room temperature.

C) Upper Tris (for Stacking gel)

Table 2.15 Composition of upper Tris

Composition	Tris	10% SDS
Amount (for 100 ml)	6.06 gm	4 ml

The above components were dissolved in 80 ml milli-Q water, pH was adjusted to 6.8 with HCl and volume was made up to 100 ml with milli-Q water. Solution was stored at room temperature.

D) 5 X SDS sample buffer

Table 2.16 Composition of 5 X SDS sample buffer

Composition	Tris-Cl (pH 6.8)	SDS	Glycerol	β-mercaptoethanol	DTT	Bromophenol Blue
Amount (for 10 ml)	2.5 ml	1 gm	5 ml	12.5%	77 mg	10 mg

All components except bromophenol blue and BME were mixed and dissolved by keeping in water bath set at 50^0C and then bromophenol was added and mixed. BME was added to sample buffer only at the time of preparing the samples. 5 X SDS sample buffer was split into 0.5ml aliquots and stored at -20^0C till use.

E) 10 X Tris-glycine-SDS Running Buffer (pH 8.3)

Table 2.17 Composition 10X Tris-glycine-SDS running buffer

Amount	SDS	Tris Base	Glycine
Composition	10 g	30.3 g	14.4 g

The above composition was mixed and volume was made up to 1000 ml with milli-Q water and stored at room temperature.

F) 15% Stacking and Resolving SDS-PAGE Gel

Table 2.18 Composition of 15% SDS-PAGE Gel

Composition	Amount for 1 gel	
	Stacking Gel	Resolving Gel
Water	1.4 ml	1.1 ml
Tris	630 µl of pH 6.8 (Upper Tris)	1.3 ml of pH 8.8 (Lower Tris)
30% Acrylamide	415 µl	2.5ml
10% SDS	25 µl	50 µl
10% APS	30 µl	50 µl

TEMED	3 µl	5 µl

G) Coomassie brilliant blue protein staining solution

Table 2.19 Composition of Coomassie brilliant blue protein staining solution

Composition	Methanol	Glacial acetic acid	Coomassie brilliant blue R
Amount (for 100 ml)	40 ml	10 ml	100 mg

The above composition was mixed and 50 ml of milli-Q water was added to make up the volume to 100ml. The staining solution was stored at room temperature.

H) De-staining solution

Table 2.20 Composition of de-staining solution

Composition	Methanol	Glacial acetic acid
Amount (for 100 ml)	40 ml	10 ml

The above composition was mixed and 50ml of milli-Q water was added to make up the volume to 100ml. The de staining solution was stored at room temperature.

2.2.12 Solution and Buffers for Western Blotting

A) Protein transfer buffer

Table 2.21 Composition of Protein transfer buffer

Composition	Glycine	Tris	Methanol
Concentration (L^{-1})	14.4 g	3 g	200 ml

Transfer buffer was stored at 4C till use. Transfer buffer can be reused for 4-5 times.

B) Tris-buffered saline-tween (TBST)

Table 2.22 Composition of Tris-buffered saline-tween (TBST)

Composition	Tris Base	NaCl
Amount	2.42 gm	8 gm

The above composition was first dissolved in 800 ml milli-Q water, 1 ml of Tween-20 was added, pH was adjusted to 7.6 with conc.HCl and final volume was made to 1 L. TBST was stored at room temperature till use.

C) Blocking milk solution:

A 5 % w/v skimmed milk was dissolved in TBST and stored at 4^0C till use.

2.1.13 Buffers and Solutions required For Purification of ArcA

A) 0.1M Potassium Phosphate Buffer

Table 2.23 Composition of 0.1 M potassium phosphate buffer

Composition	K_2HPO_4 (1 M)	KH_2PO_4 (1 M)
Amount (L^{-1})	61.5 ml	38.5 ml

The above composition was mixed in 800 ml milli-Q water, pH was adjusted to 7.0 and final volume was made up to 1000 ml with milli-Q water. The solution was stored at room temperature till use.

B) Lysis Buffer

Table 2.24 Composition of lysis buffer

Composition	Stocks	Working (30 ml)
Imidazole	5 M	60 µl
Potassium phosphate Buffer	0.1 M	15 ml
KCl	2.5 M	3.6 ml
100% Glycerol	100 %	1.5 ml

C) Wash Buffer

Table 2.25 Composition of wash buffer

Composition	Stocks	Working (30 ml)
Imidazole	5 M	120 µl
Potassium phosphate Buffer	0.1 M	15 ml
KCl	2.5 M	3.6 ml
100% Glycerol	100 %	1.5 ml

D) Elution Buffer

Table 2.26 Composition of Elution Buffer

Composition	Stocks	Working (30 ml)
Imidazol**e**	5 M	900 µl
Potassium phosphate Buffer	0.1 M	15 ml
KCl	2.5 M	3.6 ml
100% Glycerol	100 %	1.5 ml

E) 2 X Dialysis Buffer

Table 2.27 Composition of Dialysis Buffer

Composition	Stocks	Working (¯L)
Potassium phosphate Buffer	0.1 M	100 ml
KCl	2.5 M	240 ml
100% Glycerol	100 %	100 ml

250 ml of the Dialysis buffer was added with 7.5 ml, 3.75 ml and 0 ml of 5 M Imidazole and the volume was made up to 500 ml to get 75 mM, 37.5 mM and 0 mM Imidazole respectively.

2.1.14 Components for gel retardation assay

A) 40 % Acrylamide-Bisacrylamide solution

Table 2.28 Composition of 40 % Acrylamide-Bisacrylamide solution (50 ml)

Composition	Amount (for 50 ml)
Acrylamide	2 g
Bis-acrylamide	0.53 g

The above composition was mixed in 40 ml milli-Q water and finally volume was made up to 50ml with milli-Q water. The solution was stored in amber coloured bottle at 4^0C till use.

B) 4 X separating gel buffer

9.075 g Tris was dissolved in 30 ml milli-Q water, pH was adjusted to 8.8 with conc. HCl. 4 X separating buffer was stored at 4^0C till use.

C) 5 X Tris borate EDTA

Table 2.29 Composition of 5 X Tris borate EDTA

Composition (1L)	Stocks	Working (˙L)
Tris	54 g	100 ml
Boric acid	27.5 g	240 ml
EDTA (0.5M,pH 8.0)	20 ml	100 ml

The above composition was mixed in 700 ml milli-Q water, pH adjusted to 8.3 with conc.HCl and volume was made up to 1 L with milli-Q water. The solution was stored at room temperature.

D) 4 % Native PAGE (TBE)

Table 2.230 Composition of 4 % Native PAGE

Composition	Working (10ml $^{-1)}$
40 % acrylamide-bis acrylamide solution	1 ml
4 X separating buffer	2.5 ml
50% Glycerol	2.5 ml
Milli-Q water	4 ml
10% APS	50 µl
TEMED	10 µl

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 Transformation

A) TSS Transformation

TSS competent cells were prepared following the protocol as described in [7]. Cells from primary culture were inoculated in 25 ml LB to an initial OD_{600} of 0.01 and grown at 37°C, 220 rpm till OD_{600} reached 0.5. 10 ml of 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. 1.5 µl 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. Entire 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) Chemical transformation

1 µl of plasmid was added to 100 µl of chemically competent cells in a micro-centrifuge tube and kept on ice for 30 minutes. Cells were given heat shock at 42ºC for 45 seconds in a water bath and were immediately kept back on ice for 5 minutes. 500 µl LB was added to the cells and incubated at 37ºC for 90 minutes in a roller drum. 50 µl of cell suspension was spread on plates containing appropriate antibiotics. The plates were incubated at 37ºC for 16-18 hours.

2.2.3 Agarose Gel Electrophoresis

DNA samples were mixed with 6 X Bromophenol blue DNA loading dye (1 X final concentration) and 1 X TAE buffer. Samples were loaded in 1 % agarose gel in 1 X TAE buffer and run at 110 volts. The gel was observed using UV transilluminator or Gel documentation system from BioRad.

2.2.4 Transduction

P1 transduction was carried out according to the procedure mentioned in [8]. 1 ml of overnight culture was pelleted and re suspended in 1 ml solution containing 100 mM $MgSO_4$ and 10 mM $CaCl_2$. 100 µl of re-suspended 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, re-suspended in 100 µl LB containing Na citrate (10 mM) and incubated at 37°C for 1 hour in a 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. Two transductants were cultured and preserved as glycerol stocks.

2.2.5 Preparation of different carbon sources

A) Preparation of D-galactonate

D-galactonate was prepared following the published procedure [9]. For 1ml reaction, 520 mg Calcium D-galactonate (MP Biomedicals) and 126 mg Oxalic acid (Rankem) were added to 1 ml hot milli-Q water (~90°C) and mixed for 2-3 minutes. The milky reaction mixture was filtered using 0.22 µm syringe filter (Merck) and the clear filtrate was transferred to either 2 ml micro-centrifuge tube or 90 mm plates. The filtrate was cooled at -20°C for 5-6 hours. The filtrate formed crystals of D-galactonate upon cooling. D-galactonate crystals were air-dried and collected in an autoclaved micro-centrifuge tube. Few milligrams of D-galactonate was used for NMR analysis to check its purity. Finally, a stock of 100 mM D-galactonate was prepared in milli-Q water and filtered through a 0.22 µm syringe filter into a sterile centrifuge tube and stored at -20°C until use.

B) Preparation of other carbon sources

Preparation of various other carbon sources are described in materials section (2.1.6, 2.1.8 and 2.1.9)

2.2.6 Growth curve Assays

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 culture, 1 ml primary culture was centrifuged at 6000 rpm for 2 minutes and the pellet was re-suspended in 1 ml M9 minimal media. OD_{450} of cell suspension was measured by diluting it 20-fold in M9 minimal media. For secondary cultures, cell suspension was 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. log2 of OD_{450} values was plotted against time and slope of the linearly fit line was calculated. Reciprocal of the slope is the generation time.

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 was spotted on plates containing media: LB, M9 minimal media supplemented with one of the carbon sources- D-glucose, D-galactose, D-galactonate, glycerol, oleate or acetate). Plates were incubated at 37ºC and images were taken at different time intervals using Bio-Rad gel documentation system.

2.2.8 SDS-PAGE

SDS-PAGE was done following the protocol given in [17]. Samples were mixed with 5 X sample buffer so as to make 1 X concentration and boiled at 95°C for 5-7 minutes. The boiled samples were load on SDS-PAGE gel along with boiled protein ladder marker. Gel was run in SDS running buffer at 110 V. (Various composition were described on section 2.2.8)

2.2.9 Western Blotting

To check the expression of DgoR, *E. coli* strain carrying a C-terminally 3 X FLAG tagged *dgoR* on the chromosome was grown in M9 minimal media containing different C-sources. When OD_{450} of the culture reached 0.5, cells were harvested from 1ml culture by pelleting at 6000 rpm for 4 minutes. Cells were re suspended in 50 µl sample buffer for SDS-PAGE analysis. 10 µl of each sample was loaded on the gel and electrophoresed at 110 volts for 1 hr. Proteins were transferred to nitro-cellulose membrane using the BioRad mini trans blot apparatus. Transfer was carried out at 90 volts for 90 minutes at 4ºC. The membrane was incubated in blocking buffer (5% skim milk in TBST) overnight at 4ºC and washed with 10 ml of TBST four times for 15 minutes each. The membrane was incubated in 10 ml M2 anti-FLAG antibody (Sigma, 1:1000 dilution in TBST) for 2 hours followed by washing with TBST 4 times for 15 minutes each. The membrane was further incubated in 10 ml anti-mouse IgG (Pierce, 1:5000 dilution in TBST) for 1 hour followed by washing with TBST four times for 15 minutes each. Finally the membrane was treated with Super Signal West Dura Extended Duration substrate (Pierce) and exposed to X-ray film in dark room for 30 seconds. After exposure, the X-ray film was developed using X-ray film developing machine. The same blot was also analysed using LAS4000 machine (GE healthcare).

2.2.10 Purification of His-tagged ArcA protein by Cobalt-NTA chromatography

E. coli BL21 (DE3) strain carrying pCA24N plasmid with N-terminally 6X-His tagged *arcA* cloned under IPTG inducible promoter (ASKA plasmid library, [10]) was inoculated in LB supplemented with chloramphenicol (20µg/ml), and incubated

overnight. His tagged ArcA was purified following the protocol mentioned in [11] with slight modifications. 200 ml LB containing chloramphenicol was inoculated with overnight culture such that the initial OD_{600} was ~0.01. The culture was incubated at 37ºC, 220 rpm till OD_{600} reached 1.0. 1 ml culture was pelleted and stored for use as UN induced control. To the remaining culture, Isopropyl β-D-1-thiogalactopyranoside (IPTG) was added (final concentration 1mM) to induce the expression of 6X-His ArcA protein and again incubated at 37ºC, 220 rpm. After 90-120 minutes induced cells were harvested by pelleting the culture at 10,000 Xg, 4ºC for 10 minutes and stored at -80°C until use. Cells were thawed on ice and re-suspended in Lysis buffer (5ml/g pellet) containing 1 X Protease inhibitor (Pierce). Suspended cells were lysed by sonication (9 amplitude, 15 seconds pulse on and 25 second pulse off) for 20 minutes followed by centrifugation at 15000 Xg, 4ºC for 15 minutes to separate supernatant and cell lysate. After confirming that the protein was present in the supernatant by running a 15% SDS-PAGE gel, total protein in the supernatant was estimated by using Bradford reagent (Sigma). A calculated amount of Cobalt-NTA slurry (1ml for 10mg of protein) from Thermo Scientific was centrifuged at 1000 rpm for 1 minute to remove supernatant followed by washing with lysis buffer two times to get rid of 20% ethanol used as preservative for beads. Washed beads were incubated with supernatant for 90-120 minutes with shaking on Hula mixer at 4°C and passed through 10 ml gravity flow column (GE healthcare) twice and the flow through was collected. The column was washed with Wash buffer (double the bed volume) four times and washed fractions were collected. Finally, the protein was eluted using Elution Buffer. Volume of the elution buffer used was equal to the bed volume for first two fractions and half the bed volume for next six fractions. Once the protein was confirmed to be present in the elution fractions by SDS-PAGE, elution fractions with sufficient amount of 6X-His ArcA protein were pooled together. Protein was dialyzed at 4ºC using a 10 kDa dialysis tubing (Pierce) with four changes of 500 ml dialysis buffer (4 hours each) to get rid of imidazole in a step gradient (75 mM, 37.5 mM, 0 mM and 0 mM imidazole). Protein was quantified using Bradford reagent and stored at -80ºC in small aliquots.

2.2.11 PCR amplification of the cis-element of *dgo* operon

252 bp upstream region of *dgo* operon was PCR amplified with primers SK1 and SK2 from *dgoR* cloned in pACYC184 (pBS3, Bhupinder Singh, unpublished) using the following reaction mixture and PCR program

Table 2.30 Composition of PCR mix of *dgo* cis-element amplification

PCR component	Vol. added for 700 µl reaction
Water	454 µl
Phusion buffer	140 µl
dNTPs	14 µl
Template	1 µl
Phusion Polymerase	7 µl
Forward primer	35 µl
Reverse primer	35 µl
$MgCl_2$	14 µl

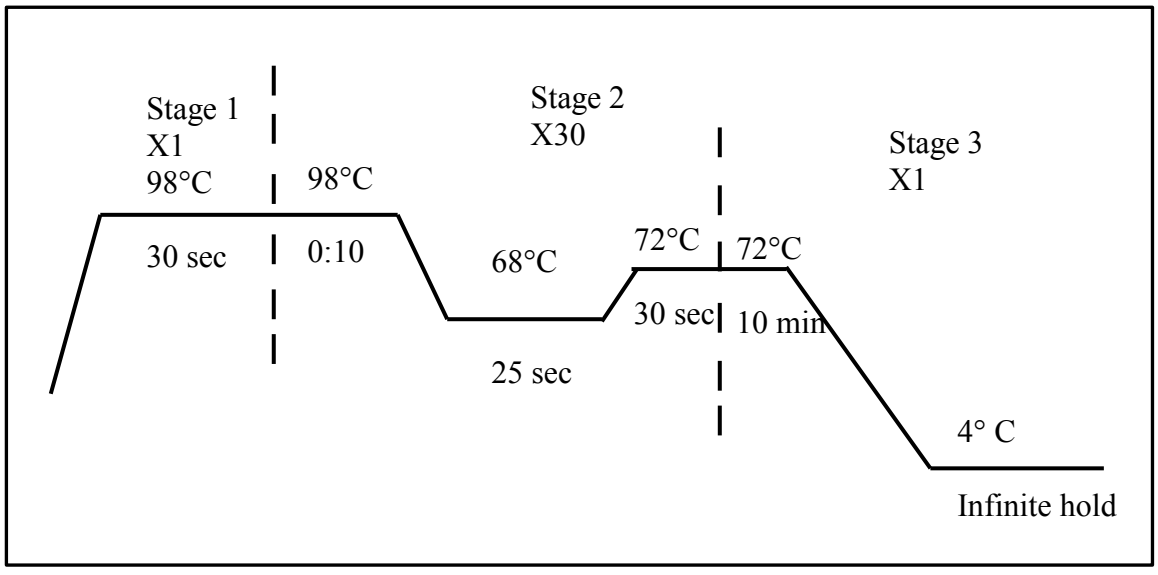


Fig. 2.1 PCR programme for amplification of cis-element of *dgo* operon.

2 µl of PCR amplified cis-element was run on 1% agarose gel to check amplification. Remaining PCR product was purified using PCR purification kit (Thermo

Scientific) according to procedure recommended by the manufacturer, and quantitated using nano drop (Nano view, GE healthcare).

2.2.12 Gel retardation assay to check binding of ArcA with the cis-element of *dgo* operon

In order to phosphorylate ArcA, 5 µg, 20 µg and 50 µg 6X-His ArcA protein was incubated with carbamoyl phosphate (50 mM final concentration) in a buffer containing 50 mM Tris-HCl, 50 mM KCl, 10 mM glycerol, 5 mM $MgCl_2$ and 0.5 mM EDTA for 1 hour in a reaction volume of 75 µl. 5 µg, 20 µg and 50 µg of either phosphorylated or unphosphorylated 6X-His ArcA was incubated with 500 ng PCR purified DNA (cis-element) in a 100 µl reaction containing 1 mM DTT, 4% glycerol, and 100 ng Salmon sperm DNA. The reaction mixture was incubated at 27ºC for 30 minutes in water bath. 14 µl of each sample was mixed with 6 µl of 6X Bromophenol DNA loading dye and 15 µl of the mixture was loaded in 4% Native PAGE gel and run at 90 volts for 2 hours. The gel was gently removed and stained with EtBr (0.5 µg/ml) in 1X TBE for 15 minutes and the gel was imaged using BioRad Gel Documentation system.

Chapter 3

Results and Discussion:

3. Results and Discussion

The current project aimed at understanding the role of *dgo* operon in D-galactonate metabolism and its regulation. In this direction, we first determined whether *dgo* genes (*dgoR, dgoK, dgoA, dgoD* and *dgoT*) that form a part of putative *dgo* operon exhibit any growth phenotype in minimal media containing D-galactonate as carbon source. We also checked the expression of C-terminally FLAG tagged DgoR from the chromosome in the presence of different carbon sources to determine if *dgo* operon is specifically induced by D-galactonate. Our bioinformatic analysis of the cis-element of *dgo* operon suggested a binding site for the global transcriptional regulator, ArcA. To investigate the regulatory role of ArcA in D-galactonate metabolism, the growth phenotype of *arcA* deletion strain was checked in minimal media supplemented with D-galactonate. We also performed a preliminary gel retardation assay to investigate whether ArcA binds to the cis-element of *dgo* operon.

3.1 *E. coli* BW25113 grows in M9 minimal media containing D-galactonate as the only carbon source

a) Preparation of D-galactonate.

Calcium D-galactonate was available commercially. However, calcium D-galactonate when added to M9 minimal media, at a concentration required to support the growth of *E. coli*, showed precipitation. Hence, D-galactonate was prepared by titration with oxalic acid as mentioned in methods (Section 2.25 A). The purity of D- galactonate preparation was determined by NMR spectroscopy. NMR spectra showed that D-galactonate was > 99% pure (Fig. 3.1). Purified D-galactonate was used in subsequent experiments.

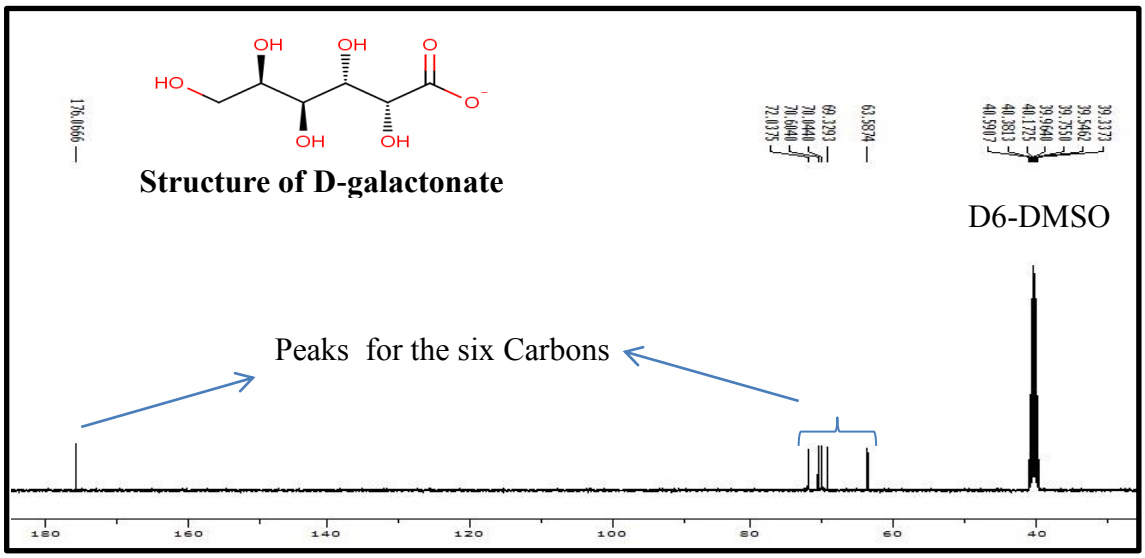
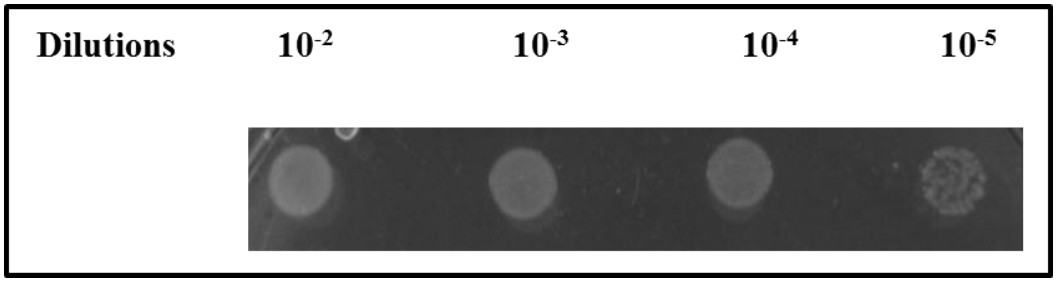


Fig. 3.1. NMR spectra of purified D-galactonate dissolved in deuterated DMSO.

b) D-galactonate can be used as a sole carbon source by WT *E. coli* in both solid and liquid media

To check the growth of *E. coli* on solid media, various dilutions of WT cells grown overnight were spotted on M9 minimal media containing D-galactonate (10 mM) as the only carbon source. Growth was observed after 24-26 hours (Fig. 3.2 A). To determine whether D-galactonate can be used as a carbon source in liquid media, WT strain was grown in M9 minimal media containing D-galactonate (10 mM) as the only carbon source and growth was monitored by measuring OD_{450} of the cultures at various time intervals. As shown in Fig. 3.2 B, WT strain showed growth in liquid media with a lag phase duration of ~10 hours and a generation time of ~100 minutes. These data show that D-galactonate can support the growth of WT *E. coli* in both solid and liquid media.

(A)



(B)

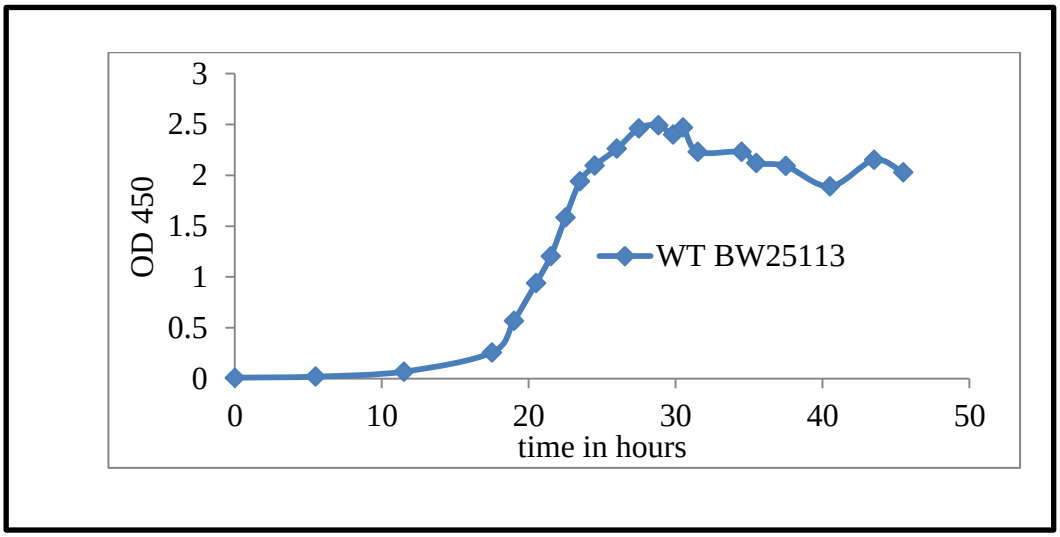
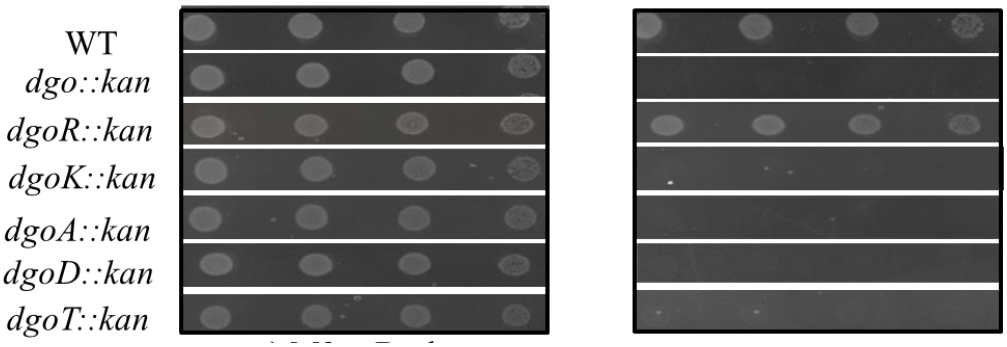


Fig. 3.2. WT *E. coli* grows in media containing D-galactonate as carbon source. A) Various dilutions of WT *E. coli* culture was spotted on M9 minimal media containing D-galactonate as carbon source. B) Growth curve of WT *E. coli* in M9 minimal media containing D-galactonate as carbon source.

3.2 Deletion of the predicted transporter for D-galactonate uptake and enzymes of D-galactonate metabolism show growth defect in media containing D-galactonate as carbon source.

Early studies carried out in 1970's [6 and 8] used chemical mutagenesis to isolate *E. coli* mutants unable to grow on media containing D-galactonate as the sole carbon source. These mutations were mapped and found to be arranged in a putative operon, D-galactonate operon (*dgo).* However, the growth phenotype of knockout strains carrying deletions in each individual *dgo* gene have not been reported. We obtained single knockouts of all the genes of the *dgo* operon from Keio library (kanamycin marked *E. coli* single-gene deletion library [18]. Strain carrying deletion of the entire *dgo* operon was constructed in our laboratory (Bhupinder Singh, unpublished) and was also used in the present study. To check which genes of *dgo* operon are required for D-galactonate metabolism, serial dilutions of various

deletion strains were spotted on M9 minimal media containing either D-glucose or D-galactonate as carbon source. In media containing D-glucose, all *dgo* deletion strains showed growth comparable to WT. However, knockout strains carrying deletion in genes encoding enzymes of D-galactonate metabolism (*dgoK*, *dgoD*, and *dgoA*), and predicted transporter for D-galactonate uptake (*dgoT*) did not grow in D-galactonate media, suggesting the requirement of these genes in D-galactonate metabolism. On the other hand, deletion of gene encoding the putative transcriptional regulator, *dgoR*, showed growth comparable to the WT strain.



A) M9 minimal media + D-glucose B) M9 minimal media + D-galactonate

Fig. 3.3. Spotting of *dgo* deletion strains in D-glucose and D-galactonate. *dgo* deletion strains do not show growth defect in media containing D-glucose, whereas, except *dgoR* deletion strain, deletion of every other *dgo* gene results in growth defect in D-galactonate media. *dgo* deletion strains were spotted on M9 minimal media plates containing A) D-glucose, or B) D-galactonate as carbon source and incubated at 37°C. Images were taken at various time intervals to observe the growth of bacteria.

3.3 DgoR is a negative regulator of D-galactonate metabolism in *E. coli*

a) Deletion of *dgoR* reduces the lag phase of *E. coli* grown in minimal media containing D-galactonate as carbon source

Since *dgoR* deletion strain did not show any growth phenotype on solid media, we assessed its phenotype in a growth curve experiment in liquid media. We

compared the growth profile of WT and *dgoR* deletion strain in M9 minimal media containing various carbon sources: D-glucose, D-gluconate, D-galactose and D-galactonate. *dgoR::kan* strain showed growth profile similar to WT in media containing D-glucose, D-gluconate and D-galactose (Fig. 3.4). However, in D-galactonate media, the *dgoR* deletion strain showed growth advantage over WT in terms of reduced lag phase duration (WT ~18 hours., and *dgoR*::*kan* ~10 hours.; Fig. 3.4). These data suggest that DgoR is a negative regulator of D-galactonate metabolism, which upon deletion leads to a constitutive expression of D-galactonate metabolic enzymes and transporter, leading to an early growth in D-galactonate media.

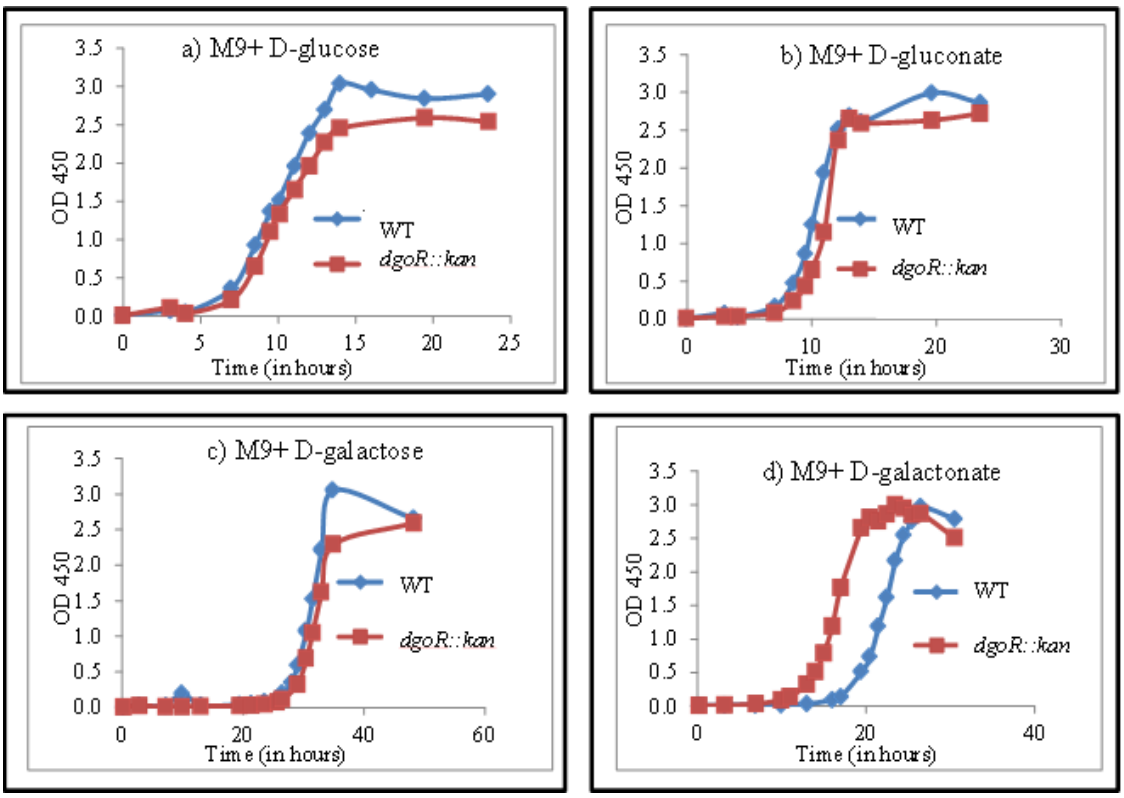


Fig. 3.4. *dgoR::kan* shows significant growth advantage over WT in media containing D-galactonate. Growth of WT and *dgoR* deletion strain in M9 minimal media containing a) D-glucose, b) D-gluconate, c) D-galactose, and d) D-galactonate as carbon source.

b) Growth advantage of *dgoR::kan* strain in D-galactonate is complemented by expressing DgoR from the plasmid.

To confirm that growth advantage of *dgoR::kan* over WT strain in terms of shortened lag phase is due to the function of DgoR protein and not due to cis-effect or polar effect of *dgoR* deletion on the downstream *dgo* genes, WT and *dgoR::kan* strains were transformed with empty pACYC177 plasmid or *dgoR* cloned in pACYC177 with its native putative promoter and ribosome binding site (pBS10, Bhupinder Singh, unpublished). Transformants were grown in minimal media containing D-glucose as control carbon source and D-galactonate as test carbon source. As shown in Fig 3.5, all the strains had a similar growth profile in media containing D-glucose as carbon source. On the other hand, in D-galactonate media, whereas, *dgoR::kan* strain carrying empty vector pACYC177 showed expected growth advantage in terms of shortened lag phase, the *dgoR::kan* strain transformed with pBS10 exhibited lag phase similar to that of wild type (with pACYC177 or pBS10). Thus, expression of DgoR from plasmid complements the phenotype of *dgoR* deletion strain in D-galactonate media thereby validating that the growth advantage of *dgoR::kan* in D-galactonate is due to the function of DgoR protein.

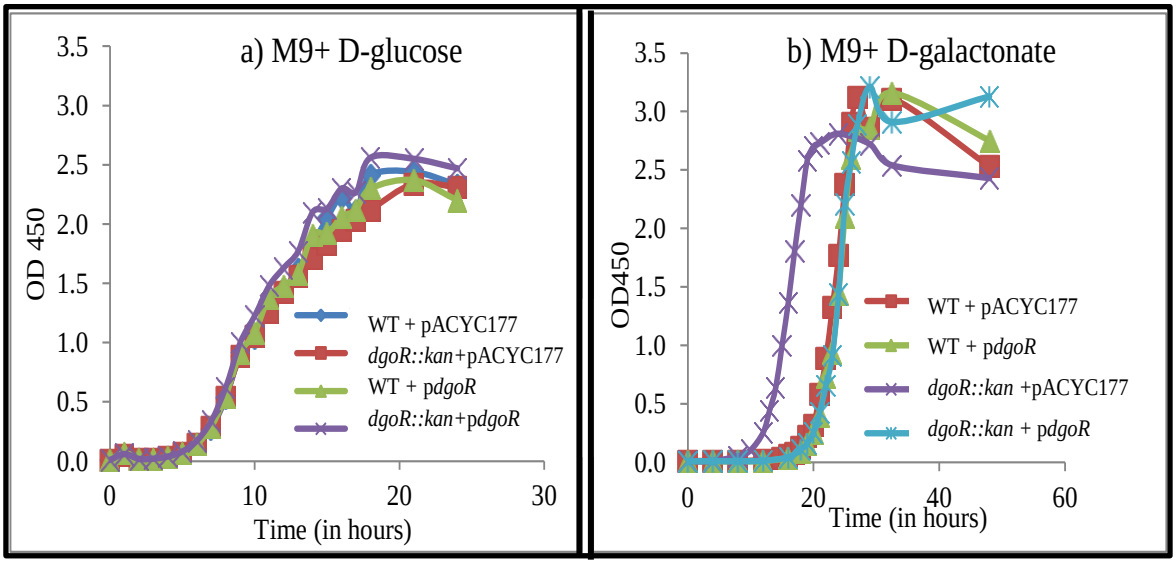


Fig. 3.5. Growth phenotype of *dgoR::kan* strain is complemented by DgoR expressed from plasmid in media containing D-galactonate. Growth of WT and *dgoR::kan* transformed with empty plasmid or *dgoR* cloned in plasmid (p*dgoR*) in M9 minimal media containing a) D-glucose and b) D-galactonate as carbon source.

3.4 DgoR is induced in minimal media containing D-galactonate as carbon source.

Since our above results indicated that *dgo* operon is involved in the metabolism of D-galactonate and its regulation, we investigated whether D-galactonate is the inducer of *dgo* operon. For this, we monitored the induction of DgoR by different carbon sources. WT and *dgoR-3X FLAG* (chromosomal *dgoR* tagged with 3X FLAG at the C-terminus: RC 3063, Yatendra Arya, unpublished) strains were grown in different carbon sources: glycerol, D-glucose, D-gluconate, D-galactose and D-galactonate. Cells were harvested from log phase cultures and processed for Western Blotting. Expression of DgoR-3X FLAG was detected using anti-FLAG antibody. Significant induction of DgoR was observed only in cells grown in the presence of D-galactonate (Fig. 3.6). These results show that D-galactonate is a specific inducer of *dgo* operon.

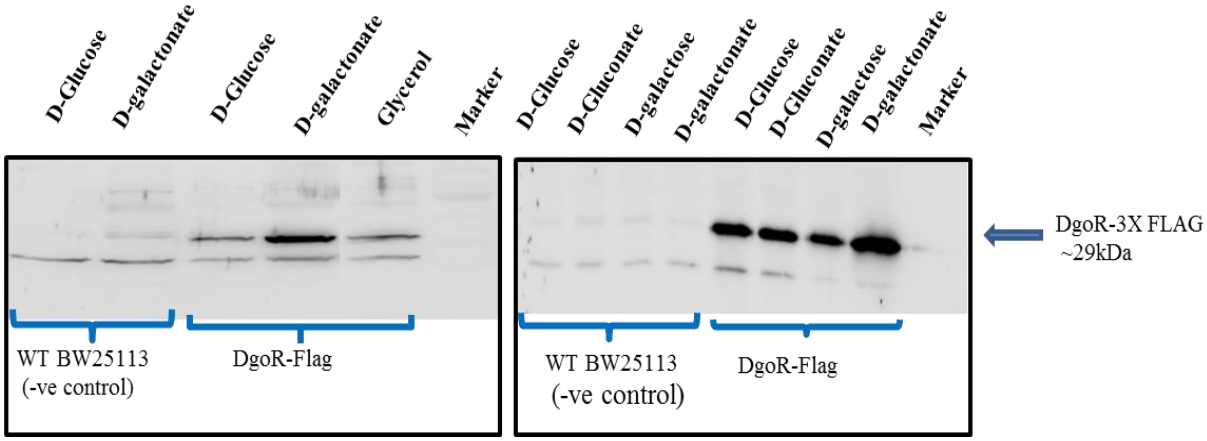


Fig 3.6. DgoR is induced in media containing D-galactonate as carbon source. WT and *dgoR-3X FLAG* (chromosomal *dgoR* tagged with 3X FLAG) were grown in different carbon sources. Cells were harvested and processed for Western blotting. Expression of DgoR-3X FLAG was detected by anti-FLAG antibody

3.5 Computational analysis to predict transcriptional regulator binding sites in the cis-element of *dgo* operon

Since DgoR is a putative transcriptional regulator, it has to bind DNA for regulating the transcription of *dgo* genes. Transcription factors are known to auto-regulate by binding to palindromic sequences, direct repeats or inverted repeats. As shown in Fig. 3.7, two inverted repeats are found in the cis element of *dgo* operon, which could be the binding sites of DgoR (highlighted in yellow). To predict additional transcription factor binding sites, ~250 bp upstream region of *dgo* operon was analysed by Virtual foot-printing. Besides putative DgoR binding site, a potential binding site for ArcA (Anaerobic response control), a global transcriptional regulator known to regulate various genes under microaerophilic conditions, was found in the cis-element of *dgo* operon. -10 and -35 boxes of promoter were also predicted using BPROM (highlighted in red).

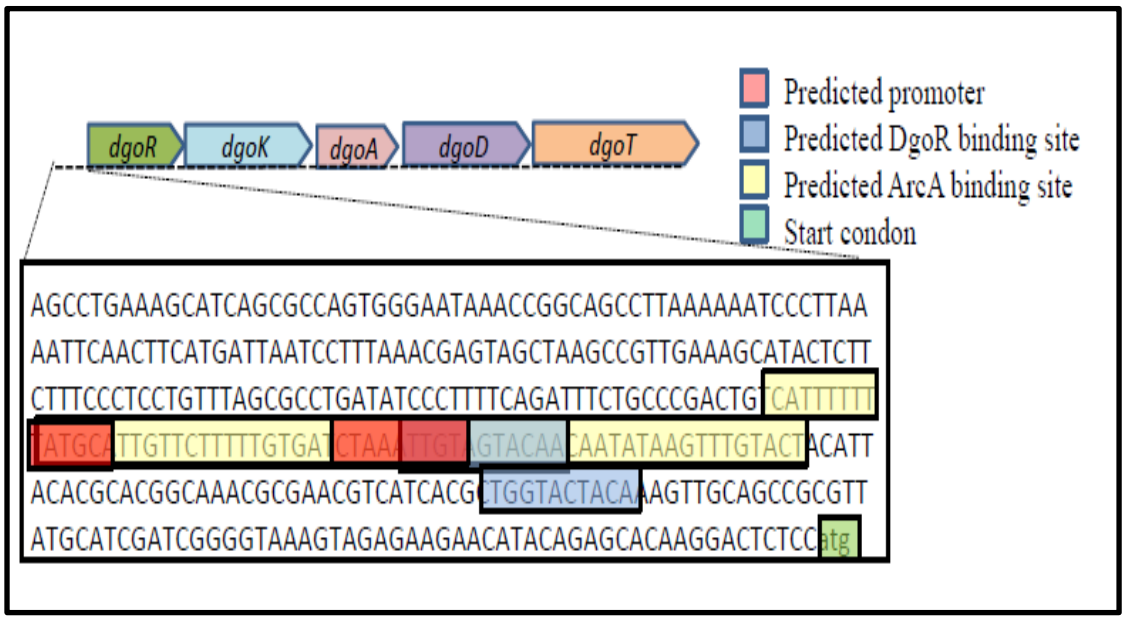


Fig. 3.7. Bioinformatic analysis of cis-element of *dgo* operon shows binding sites of DgoR and ArcA

3.6 ArcA may be a second negative regulator of D-galactonate metabolism

Since a potential ArcA binding site was found in the cis-element of *dgo* operon, we checked whether ArcA has some regulatory role in D-galactonate metabolism. In this direction, we compared the growth of WT, *dgoR::kan* and *arcA::cam* in M9 minimal media containing D- galactonate as carbon source. We found that, like *dgoR::kan*, *arcA::cam* strain has growth advantage over WT strain in terms of shortened lag phase (Fig. 3.8), but this reduction in lag phase was not as significant as observed for the *dgoR* deletion strain. The reduction in lag phase of *E. coli* upon deletion of *arcA* in D-galactonate media suggests that ArcA may be a second regulator of D-galactonate metabolism.

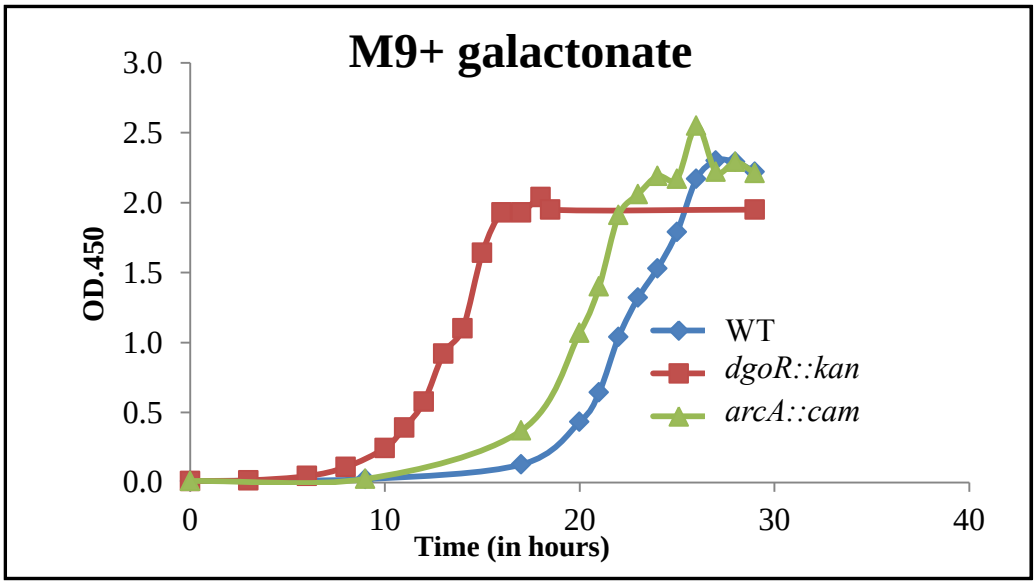


Fig. 3.8 Deletion of *arcA* shortens the lag phase of *E. coli* grown in media containing D-galactonate. Growth of WT, *dgoR::kan* and *arcA::cam* in M9 minimal media containing D- galactonate.

3.7 Purification of His-tagged ArcA protein

Since ArcA binding site was predicted in the cis element of *dgo* operon and also growth advantage of *arcA* deletion strain was observed in media containing D-

galactonate, we were interested in investigating whether ArcA binds to the cis-element. In this direction, 6X-Histidine tagged ArcA protein was expressed using a clone obtained from ASKA library (overexpression library of *E. coli*, [10] and purified by cobalt-NTA chromatography as described in methods (2.2.9). Various fractions collected during ArcA purification were run on 15% SDS PAGE and stained with coomassie brilliant blue. As shown in Figure 3.9, sufficient amount of ArcA (Mol. wt. ~29 kDa) protein was obtained in elution fractions. Since the protein has several contaminants, ArcA will be further purified by additional chromatography techniques. However, in our subsequent experiment (gel retardation assay), we have used ArcA that was purified only by Co-NTA chromatography.

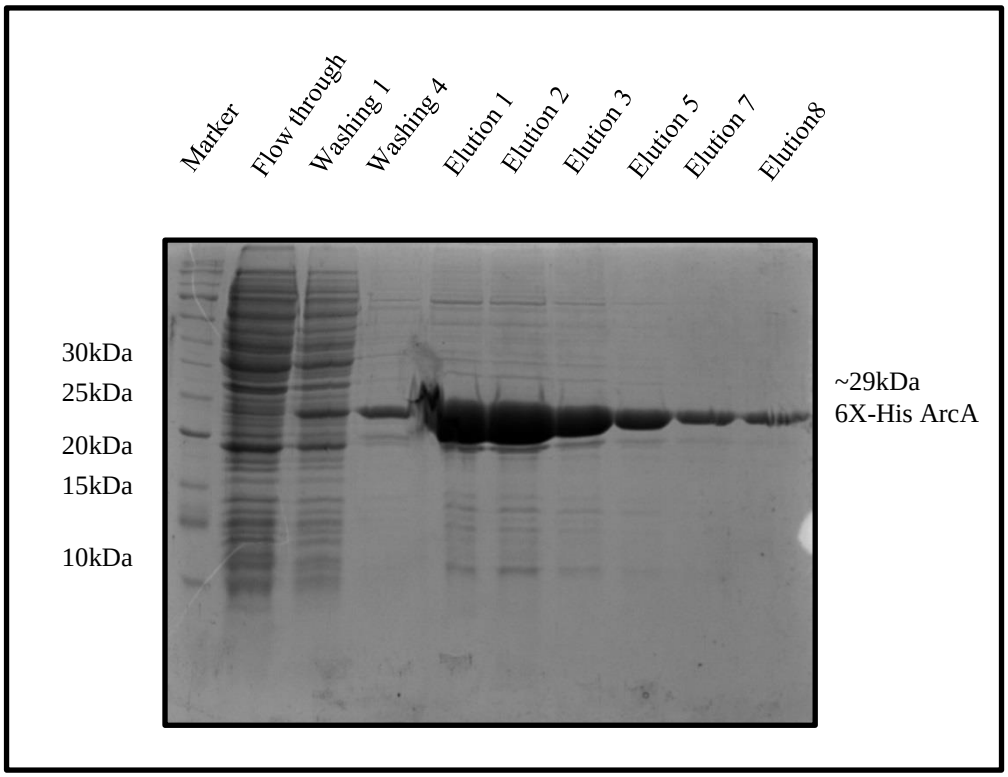


Fig. 3.9 Purification of 6X-His ArcA by Cobalt-NTA chromatography.

3.8 PCR amplification of cis-element of *dgo* operon

252 bp fragment of the cis element of *dgo* operon was amplified using pBS3 as template(Bhupinder, unpublished data) and SK1 and SK2 as forward and reverse

primer by PCR as described in methods (2.2.11), (Fig. 3.10). The amplified fragment was purified using PCR purification kit and used in gel retardation assay.

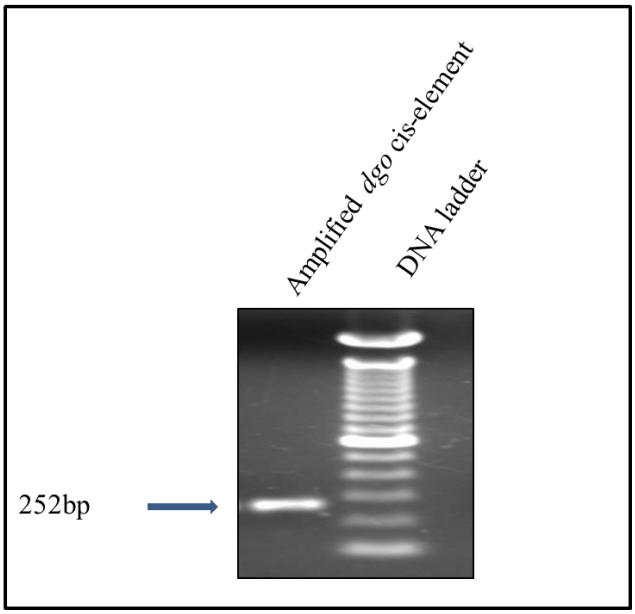


Fig. 3.10 PCR amplified fragments showing expected size of the *dgo* cis-element

3.9 Preliminary gel retardation assay suggests that phosphorylated ArcA binds to the cis-element of *dgo* operon

ArcA is known to bind its target DNA upon phosphorylation. Thus, to determine the binding of ArcA with the cis element of *dgo* operon, both unphosphorylated and phosphorylated ArcA was incubated with DNA fragment. Samples were loaded on 4% native PAGE. *dgo* cis-element alone (lane 10), ArcA protein (lane 2) and phosphorylated ArcA (lane 3) were run as controls. As seen in lane 2, ArcA alone showed signal for DNA, which indicates that ArcA purified by cobalt-NTA has DNA contamination. In lanes 7, 8 and 9 where unphosphorylated protein incubated with DNA was run, non-specific DNA bound to ArcA could be seen; however, there was no shift of cis element. Interestingly, in lanes 4, 5 and 6 where phosphorylated ArcA incubated with DNA was run, a shift of cis element DNA was observed. This shift was found to increase with increasing concentration of ArcA. Although these results are preliminary but they do suggest that phosphorylated ArcA binds to *dgo* cis element. Gel

retardation experiment will be repeated with pure preparation of ArcA and P^{32}-labeled DNA.

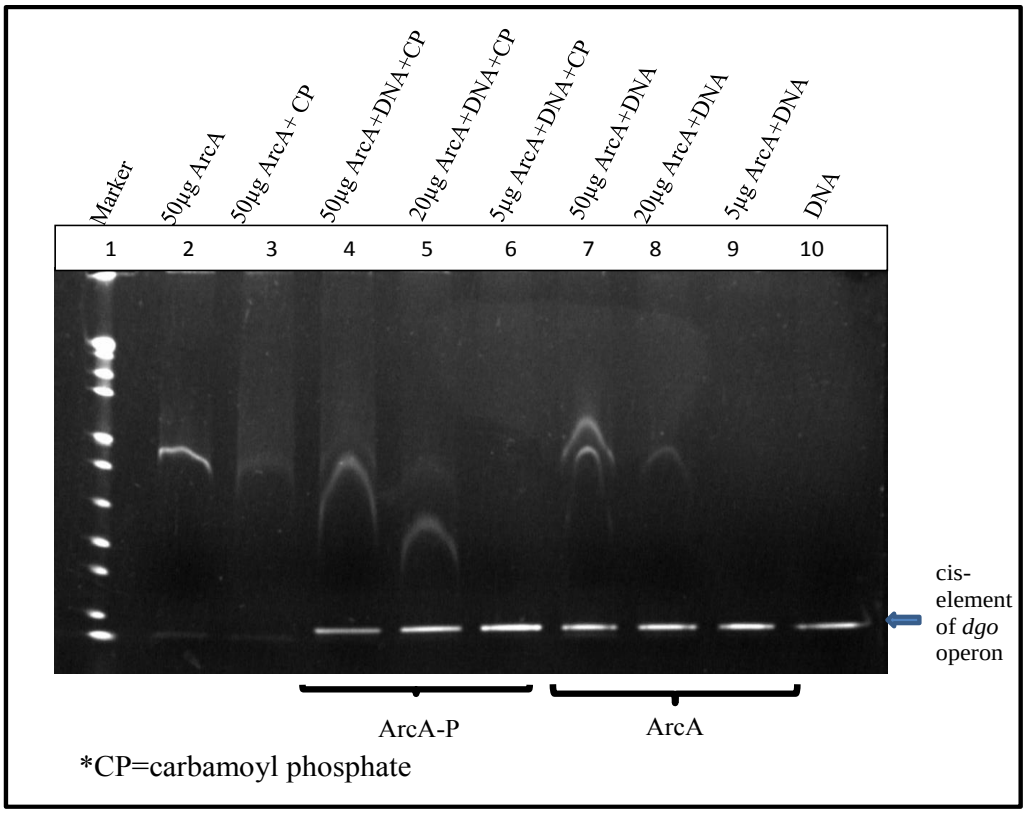


Fig 3.11 Preliminary Gel retardation assay shows binding of phosphorylated ArcA to cis-element of *dgoR* operon.

Summary and Future Goals

E. coli can utilize D-galactonate as carbon source. Metabolism of D-galactonate in this bacteria has been studied using classical mutagenesis techniques whereby mutants were isolated that were unable to utilize D-galactonate. Enzymatic analysis of crude cellular extracts of these mutants was carried out and thus a pathway for D-galactonate metabolism was proposed. However, none of the studies have used strains carrying clean deletions of *dgo* genes to validate the involvement of *dgo* genes in D-galactonate metabolism. Furthermore, nothing is known about the regulation of D-galactonate metabolism in *E. coli*. In the present study, using strains carrying clean deletion in genes encoding putative enzymes of D-galactonate metabolism (*dgoK* and *dgoD*), *dgoA* and predicted D-galactonate transporter (*dgoT*), we showed that these *dgo* genes are required for growth in D-galactonate media. Deletion of the putative transcriptional regulator, *dgoR*, did not show growth defect on solid media containing D-galactonate, however, there was significant reduction in lag phase of *dgoR* deletion strain in liquid media suggesting that DgoR is a negative regulator of *dgo* operon. The phenotype of *dgoR* deletion strain was complemented by expressing DgoR from a plasmid. In our future experiments, we will determine whether deletion of *dgoR* results in constitutive expression of Dgo enzymes and transporter. Transcription factors are known to auto-regulate by binding to palindromic sequences. Inverted repeat sequences are present in the cis-element of *dgo* operon. Future experiments will be aimed at determining the binding of DgoR to inverted repeat sequences present upstream of *dgo* operon. Of all the tested carbon sources, we observed that DgoR expression is induced only by D-galactonate, suggesting that D-galactonate is a specific inducer of *dgo* operon. We will further investigate whether D-galactonate induces *dgo* operon at a transcriptional level. Our bioinformatics analysis of the cis-element of *dgo* operon predicted the presence of binding sites for another transcriptional regulator, ArcA. Interestingly, *arcA* deletion also resulted in reduced lag phase of bacteria, suggesting that ArcA could be a second regulator of D-galactonate metabolism. In our preliminary gel retardation experiment using unlabelled DNA fragment, we observed binding of ArcA to the cis element of *dgo* operon. We will perform EMSA using radiolabelled DNA fragment, and pure DgoR and ArcA proteins, and investigate whether the binding of these transcription factors to the cis-element of *dgo* operon is co-operative or competitive.

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