CHAPTER 3

Materials and methods

3.1 Materials

3.1.1 Microbial Strains

All the microbial strains used in the study were either procured from culture collection or were obtained as gift from various authentic sources.

Table 3. 1: List of microbial strains used in the study

***E. coli* strains**	**Relevant genotype/Description**	**Reference/Source**
DH5α	*supE44 hsdR17 recA1 endA1gyrA96 thi-1*Δ(*lacIZYA argF*) *U169 relA1*?	lab collection
JM101	*glnV44 thi-1* Δ*(lac-proAB)* F'*[lacI*^{q}*ZΔM15 traD36 proAB*$^+$*]*	lab collection
MG1655	*λ*$^-$*, rph-1*	lab collection
MC4100 KP	*F*$^-$ *araD139 (argF-lac)U169 rpsL150 deoC1 relA1 thiA ptsF25 flbB5301 rbsR*	lab collection
DY330	W3110 Δ*lacU169 gal490 λcI857* Δ*(cro-bioA)*	Yu et al. 2000
CAG12182	*λ, cysI3152::*Tn*10kan, rph1*	Singer, M. *et al*,1989
JW2756-1	*Δ(araD-araB)567, ΔlacZ4787*(::rrnB-3), *λ*$^-$, *ΔrumA783*::*kan*, *rph-1*, *Δ(rhaD-rhaB)568, hsdR514*	Keio Collection, Baba *et al*, 2006
JW2757-1	*Δ(araD-araB)567, ΔlacZ4787*(::rrnB-3), *λ*$^-$, *ΔbarA784*::*kan*, *rph-1*, *Δ(rhaD-rhaB)568, hsdR514*	Keio Collection, Baba *et al*, 2006
JW2758-5	*Δ(araD-araB)567, ΔlacZ4787*(::rrnB-3), *λ*$^-$, *ΔgudD785*::*kan*, *rph-1*, *Δ(rhaD-rhaB)568, hsdR514*	Keio Collection, Baba *et al*, 2006
JW3617-1	*Δ(araD-araB)567, ΔlacZ4787*(::rrnB-3), *λ*$^-$, *ΔpyrE748*::*kan*, *rph-1*, *Δ(rhaD-rhaB)568, hsdR514*	Keio Collection, Baba *et al*, 2006
JW5437-1	*Δ(araD-araB)567, ΔlacZ4787*(::rrnB-3), *λ*$^-$, *ΔrpoS746*::*kan*, *rph-1*, *Δ(rhaD-rhaB)568, hsdR514*	Keio Collection, Baba et al, s2006
CF9240	MG1655 Δ*dksA*::Tn*10*tet	Cashel Lab (Brown *et al*, 2002)
CF17119	MG1655 Δ*rpoZ*::*Kan*	Cashel Lab (Brown *et al*, 2002)
HJT042	MG1655 *uvrY*::*Cat*	Tomenius *et al*,2005
KP1	JM101 pTE13 chromosomal integrant selected at 42°C	This study
KP1.1	JM101 Δ*rumA1 relA2009*	This study
KP1.2	JM101 *rumA*$^+$ segregant	This study
KP2	MC4100 *relA*$^+$ *cysI3152*::Tn*10kan* (transduction from CAG12182)	This study
KP3	MC4100 *relA1 cysI3152*::Tn*10kan* (transduction from CAG12182)	This study
KP4	MC4100 *relA*$^+$ *cysI*$^+$	This study
KP5	MC4100 *relA*$^+$ *ΔbarA784*::*KAN* (transduction from JW2757-1)	This study
KP6	MC4100 *relA*$^+$ *ΔgudD785*:: *KAN* (transduction from JW2758-5)	This study

KP7	MC4100 *relA2009* Δ*rumA1* by P1 transduction using KP1.1	This study
KP8	MC4100*relA*$^+$ *rumASalI*::*CAT*	This study
KP9	MC4100*relA*$^+$ *rumAMluI*::*CAT*	This study
KP10	MC4100*relA*$^+$ *rumA*$^+$ *barABglII*::*CAT*	This study
KP11	MC4100KP *spoT1ΔpyrE748*::*kan* (transduction from JW3617-1)	This study
KP12	KP4 Δ*rpoZ*::kan (transduction from CF2790)	This study
KP13	KP8Δ*rpoZ*::kan (transduction from CF2790)	This study
KP14	KP4 Δ*dksA*::Tn*10*tet (transduction from CF9240)	This study
KP15	KP8 *rpoB2015* spontaneous rif^R mutation	This study
KP18	KP4 Δ*rumA783*::*KAN* (transduction from JW2756-1)	This study
KP19	MC4100 *relA*::IS*2 rumASal*I::*CAT*	This study
KP22	MG1655 *relA1 rumA*$^+$ (transduction from KP3)	This study
KP23	MG1655 *relA2009* Δ*rumA1* (P1 transduction using KP1.1)	This study
KP24	MG1655 *relA*$^+$ *rumASalI*::*CAT*	This study
KP25	MG1655 *relA*$^+$ *rumAMluI*::*CAT*	This study
KP26	MG1655 *relA*$^+$ *rumA*$^+$ *barABglII*::*CAT*	This study
KP27	MG1655 *ΔbarA784*::*KAN* (transduction from JW2757-1)	This study
KP28	MG1655 *ΔgudD785*::*KAN* (transduction from JW2758-5)	This study
KP29	MG1655 *spoT1 ΔpyrE748*::*KAN* (transduction from KP11)	This study
KP30	KP24 *spoT1 ΔpyrE748*::*KAN* (transduction from KP11)	This study
KP31	MG1655 *spoT1pyrE*$^+$	This study
KP32	KP30 *spoT1 pyrE*$^+$	This study
KP33	MG1655 Δ*dksA*::Tn*10*tet (transduction from CF9240)	This study
KP34	KP24 Δ*dksA*::Tn*10*tet (transduction from CF9240)	This study
KP35	KP4 *spoT*$^+$ *ΔpyrE748*::*KAN* (transduction from JW3617-1)	This study
KP36	KP8 *spoT*$^+$ *ΔpyrE748*::*KAN* (transduction from JW3617-1)	This study
KP37	KP4 *spoT*$^+$ *pyrE*$^+$	This study
KP38	KP8 *spoT*$^+$ *pyrE*$^+$	This study
KP39	KP31 Δ*dksA*::Tn*10*tet	This study
KP40	MC4100KP Δ*dksA*::Tn*10*tet (transduction from CF9240)	This study
KP41	KP4 *ΔrpoS746*::*KAN* (transduction from JW5437-1)	This study
KP42	KP8 *ΔrpoS746*::*KAN* (transduction from JW5437-1)	This study
KP43	KP31 *ΔrpoS746*::*KAN* (transduction from JW5437-1)	This study
KP44	KP32 *ΔrpoS746*::*KAN* (transduction from JW5437-1)	This study

3.1.2 Plasmids and Constructs

Clones were constructed using plasmids available in lab collection. DH5α used as recipient for plasmid transformation.

Table 3. 2: List of plasmids and constructs used in the study

Plasmids	Description	Reference/Source
pBlueScriptKS	Cloning Vector Amp^r ColE1 replicon	Stratagene,USA
pBAD18Kan	Cloning Vector Kan^r ColE1 replicon	Guzman *et al.* 1995
pBBR1MCS2	Cloning vector Kan^r p15A replicon	Kovach *et al*. 1995
pACYC184	Cloning vector Cm^r, Tet^r	Bartolome *et al,*

		1991
pBR322	Cloning vector $Amp^{r,}$ Tet^{r}	Bolivar et al., 1977
pMAK705	Gene disruption vector, Ori^{ts} Cm^{r} pSC101 replicon	Hamilton et al. 1989
pKD46	Recombineering vector, Ori^{ts} Amp^{r} repA101 replicon	Datsenko and Wanner, 2000
pTE1	pBBR1MCS2 containing 7164 bp of *BamH*I genomic insert bearing full length *relA*, *rumA*, *barA*, and *gudD*	This study
pTE1ΔBgl	Removal of 310 bp of *Bgl*II DNA from pTE1 by digestion by RE *Bgl*II and intramolecular ligation.	This study
pKC1	Spontaneous $relA^{-}$ mutant of pTE1 plasmid	This study
pKC2	Spontaneous $relA^{-}$ mutant of pTE1 plasmid	This study
pTE2	pBBR1MCS2 plasmid containing *rumA* (1301 bp) + 586 bp upstream DNA and 1341 bp long *relA* DNA.	This study
pTE3	pBBR1MCS2 plasmid containing *rumA* (1301 bp) + 516 bp upstream DNA and 1368 bp long *relA* DNA.	This study
pTE4	Full length *relA* gene (2289 bp) cloned at *Eco*RI - *Kpn*I sites of pBAD18Kan.	This study
pTE1ΔAse	pTE1 derivative containing *relA* gene and upstream *rel*AP1 & *rel*AP2 promoters.	This study
pTE5	1529 bp *rumA* amplicon + 178 bp upstream *barA* DNA cloned at *Eco*RI – *Sac*I-*BamH*I of pBBR1MCS2.	This study
pTE6	3794 bp long contiguous segment bearing *relA* and *rumA* genes cloned at *Eco*RI - *Kpn*I fragment in pBAD18Kan.	This study
pTE7	2907 bp long contiguous segment bearing *relA* (NTD) and *rumA* genes cloned at *Eco*RI - *Pst*I fragment in pBAD18Kan.	This study
pTE8	3794 bp G251E mutant *relA* and *rumA* genes cloned in *Eco*RI-*Kpn*I of pBAD18Kan.	This study
pTE9	3794 bp C389P mutant *rumA* and *relA* genes cloned at *Eco*RI - *Kpn*I of pBAD18Kan.	This study
pTE11	Δ*rumA1* ORF (131 nucleotide 3' deletion) + 1173 bp of upstream DNA cloned at *BamH*I - *Pst*I sites of pBlueScriptKS vector.	This study
pTE12	pTE11 containing *relA* amplicon cloned at *Eco*RI - *Kpn*I sites.	This study
pTE13	Δ*rumA1*+ *relA* from pTE12 as *BamH*I - *Kpn*I insert cloned in pMAK705 at corresponding sites.	This study
pTE14	1529 bp *rumA* amplicon + 178 bp upstream *barA* DNA cloned at *Eco*RI - *Kpn*I of pBBR1MCS2	This study
pTE15	pTE14 containing *CAT* cassette cloned at unique *Sal*I site in *rumA*	This study
pTE16	pTE1ΔBgl containing *CAT* cassette cloned at unique *Mlu*I site in *rumA*	This study
pTE17	pTE1ΔBgl containing *CAT* cassette cloned at unique *Bgl*II site in *barA*	This study
pTE18	pBR322 $spoT^{+}$	This study
pTE18Δsal	*Sal*I restriction enzyme digestion of pTE18 plasmid followed by intramolecular ligation	This study
pTE18Δeco	*Eco*RI restriction enzyme digestion of pTE18 plasmid followed by intramolecular ligation	This study

3.1.3 Antibiotics

All antibiotics used were either procured from Sigma, U.S.A. or Hi-media, India

Table 3. 3: Antibiotics used in the study

Antibiotic	Solvent	Concentration (stock)
Ampicillin	Water	100 mg/ml
Chloramphenicol	Methanol	40 mg/ml
Daunomycin	Water	10 mg/ml
Erythromycin	70% Ethanol	100 mg/ml
Kanamycin	Water	100 mg/ml
Mitomycin	Water	10 mg/ml
Nalidixic acid	1N NaOH	30 mg/ml
Rifampicin	Methanol	50 mg/ml
Streptomycin	Water	100 mg/ml
Tetracycline hydrochloride	water	50 mg/ml
Trimethoprim	DMF	60 mg/ml

3.1.4 Chemicals and Biochemicals

Culture media including Luria broth, Luria agar, Tryptone, Yeast Extract, Agar-Agar and Nutrient Broth were obtained from **Hi-media**, India. Chemicals like Glucose, Magnesium Chloride, EDTA, Sodium Hydroxide Pellets, Sodium Chloride, Glacial Acetic Acid, Hydrochloric acid, and Sulfuric acid were purchased from **Qualigens**, India. Other chemicals, Tris Base (Molecular Biology grade), Potassium acetate, Sodium acetate, Phenol, Chloroform, Isopropanol, Isoamyl alcohol, Methanol, Agarose, Bromophenol blue and amino acids were obtained from **SRL** India. Sodium Dodecyl Sulphate and Sodium Citrate were from **S.D. fine chem**. Ltd, India. Serine hydroxamate, 3 amino - 1, 2, 4 triazol, Calcium Chloride, Agarose, Glycerol were purchased from **Sigma** Chemical Co., St. Louis, USA. Solvents like HPLC grade Acetonitrile, Chloroform, Methanol, Water were from **Merk** Research labroratories, USA. All other chemicals used in this study were also of analytical grade and obtained from local sources.

3.1.5 Enzymes

Restriction enzymes and T4 DNA Ligase were purchased from GBR Bangalore Genei, India New England Biolabs (UK); Thermo Fisher Scientific (Massachusetts, USA), Roche Molecular Biochemicals (Mannheim). Appropriate buffers and BSA provided with restriction enzymes were used. RNase was purchased from Sigma chemical Co., St. Louis, USA. DNAse kit was

procured from Invitrogen. XT-20 polymerse PCR kit from Bangalore Genei (Merk). RT-PCR kit was procured from life technologies (Invitrogen,).

3.1.6 Primers

Primers were designed using online free software - PRIMER3 (http://frodo.wi.mit.edu/). Oligonucleodites were procured from MWG-Biotech AG (Germany) and from IDT (California, USA)

Table 3. 4: List of Primers used in PCR studies

Primer Name	Primer sequence 5'→3'	Tm of primers (°C)
RumAF1	TTA*GAATTCGGATCC*AGTTGACGCTGCA	65.1
RumAF3	AAT*GGATCC*TGCTGCGTAGTGGGAA	64.6
RumARPst1	AAT*CTGCAG*GACCAGACCTGCCGAA	66.3
RumARKpn1	AAT*GGTACC*GACCAGACCTGCCGAA	66.3
RumARSac1	AAT*GAGCTCGGATCC*GACCAGACCTGCCGAA	72.2
RelAF	CTG*GAATTC*GCAGGTCTGGTCCCTA	66.3
RelAR	CGT*GGTACC*GAGCAAATTTCGGCCTA	66.4
BarAF	CGTGGTACCTTGGCGAGACTTTCTCA	66.4
C389ASDM1	TATCCCCGAACCCTGCAACGTTGGCTC	69.5
C389ASDM2	CAACGTTGCAGGGTTCGGGGATACATAAAC	68.1
G251E-5'[a]	GCGGAAGTGTATGAGCGTCCGAAACACATC	63.9
G251E-3'[a]	GATGTGTTTCGGACGCTCATACACTTCCGC	63.9
CATFSalI[b]	TTA*GTCGAC*ATGAGACGTTGATCGGCACG	68.1
CATRSalI[b]	AAT*GTCGAC*ATTCAGGCGTAGCACCAGGC	69.5
CATFMB[b]	TTA*ACGCGTAGATCT*ATGAGACGTTGATCGGCACG	64.3
CATRMB[b]	AAT*ACGCGTAGATCT*ATTCAGGCGTAGCACCAGGC	65.7
RpoZ Left	GTCAGGACAGCGAAGAGGTC	57.5
RpoZ Right	ATCACGTGCAACGAGATACG	55.1
RelART Left	ACTACCACGCGACCGTTATC	56.9
RelART Right	GGGCGACCAGATTGAAATTA	52.8
GAPDHRT Left	AGGTCTGATGACCACCGTTC	56.8
GAPDHRT Right	AGTAACCGGTTTCGTTGTCG	55.3

a (Gropp *et al.*, 2001) ; b (Poteete *et al.*, 2006)

3.1.7 Growth media used for culturing *E.coli*

Media, solutions for media, buffers, solutions for plasmid preparation, and other miscellaneous solutions, glass-wares and plastic-wares were sterilized by autoclaving at 15 lbs/ in^2 at 121ºC for 20 minute, unless otherwise specified.

Luria Agar (LA) - 35 gm/ 1000ml distilled water.

Luria Broth (LB) - 20 gm/ 1000ml distilled water.

Nutrient Broth (NB) - 8 gm / 1000ml distilled water

2X YT Medium for 1000ml:

To 900 ml of deionized water

Tryptone - 16gm

Yeast extract - 10 gm

Nacl - 5 gm

Adjust pH to 7.0 with 5N NaOH (few drops)

Soft Nutrient Agar (SNA) for 1000ml:

Difco powder - 8.0gm

Agar - 0.75%

pH adjust to 7.2

Agar- Agar

1.5 % Agar Agar Type I (Bact. Grade)

Z broth for 100ml

LB medium

$CaCl_2$ (0.5M) - 0.5ml

Z Agar

Same as Z Broth but with Luria Agar medium

Minimal Broth (MB) (Miller *et al.*, 1992) for 1000ml – (1X)

K_2HPO_4 - 10.5 gm

KH_2PO_4 - 4.5 gm

$(NH4)_2SO_4$ - 1 gm

Sodium citrate.$2H_2O$ - 0.5 gm

After autoclaving above salts, add Mg^{++} and other nutrients, which were autoclaved separately:

$MgSO_4.7H_2O$ - 1ml from 1 M stock solution

B1 (thiamine hydrochloride) - 0.5 ml from a 1% stock solution

Amino acids - 4 ml from 10mg/ml stock sol^n

Sugar - 10 ml from 20% stock sol^n

Minimal Agar Plate for 1000 ml

Same as minimal broth but with Agar - 15 g

3.1.8 Reagents for plasmid preparation

(Sambrook *et al,* 1989)

Alkaline lysis solution I (GTE)

50 mM glucose

25 mM Tris – Cl (pH 8.0)

10 mM EDTA (pH 8.0)

Prepare solution I from standard stocks of 2 M Glucose, 1M Tris – Cl (pH 8.0) and 0.8 M EDTA (pH 8.0) and make up the final volume with autoclaved D/W. (No need to autoclave the final solution).

Alkaline lysis solution II

0.2 N NaOH (freshly diluted from a 10 N stock)

1 % (w/v) SDS

Prepare solution II fresh and use at RT.

Phenol:chloroform (1:1)

Chloroform:Isoamylalcohol (24:1)

Alkaline lysis solution III

5 M potassium acetate	60.0 ml
Glacial acetic acid	11.5 ml
H_2O	28.5 ml

The resulting solution is 3 M with respect to potassium 5 M with respect to acetate.

Note: To be used cold.

10 X Tris EDTA (TE)

pH 8.0

100 mM Tris –Cl (pH 8.0)

10 mM EDTA (pH 8.0)

Sodium Acetate (3 M, pH 5.2)

Dissolve 408.3 g of sodium acetate.$3H_2O$ in 800 ml of H_2O. Adjust the pH to 5.2 with glacial acetic acid. Adjust the volume to 1 liter with water.

3.1.9 Solutions for agarose gel electrophoresis (Sambrook et al, 1989)

1.8-2.0% Agarose Gel with EtBr

Running buffer: 50X TAE

Tris-base	- 242g
Glacial acetic acid	- 57.1ml
0.5 M acetic acid (pH 8.0)	- 100ml
Distilled water	- 1000ml

50x TAE was diluted to 1X prior to use.

3.1.10 Reagents for SDS –PAGE and Western Blotting

Tris buffers

Prepare 200 ml each of the following two buffers

1.5 M Tris-HCl, pH 8.8 (for separating gel)

1.0 M Tris-HCl, pH 6.8 (for stacking gel)

Filter the buffers through 0.45 μm filters.

30% Acrylamide: bisacrylamide (Bio-Rad): 37.5:1, 2.6 %C

N,N,N',N'-Tetramethylethylenediamine (TEMED)

10% (w/v) Sodium dodecyl sulphate (SDS) in filtered distilled water (dH_2O)

10% (w/v) Ammonium persulfate solution (APS) in dH_2O: Prepare fresh for the day

Dual color and Broad Range SDS-PAGE protein molecular weight markers (Bio-Rad)

Urea-SDS sample buffer (Laemmli buffer)

62.5 mM Tris-HCl, pH 6.8

6 M urea (deionized using AG-X8 resin, Bio-Rad),

10% (v/v) glycerol

2% (w/v) SDS

0.00125% (w/v) bromophenol blue

Freshly added 5% (v/v) β-mercaptoethanol (β-ME)

Prepare solution without β-ME and store at ambient temperature.

Running buffer (5X)

125 mM Tris

960 mM glycine

0.5% (w/v) SDS

Dilute 200 ml of 5X running buffer with 800 ml dH_2O to make 1X and store both 5X and 1X at ambient temperature.

Hybond-C nitrocellulose membrane

Whatman chromatography filter paper (3 mm)

10X Transfer buffer

250 mM Tris,

1.92 M glycine

Dilute 400 ml of 10X transfer buffer with 2.8 L dH_2O and 800 ml methanol to make 1X (25 mM Tris, 192 mM glycine, 20% (v/v) methanol). Store 10X buffer at ambient temperature and 1X at 4°C.

Ponceau S: 0.1% (w/v) in 5% (v/v) acetic acid

1X Phosphate buffer saline (PBS)

Detection: Enhanced chemiluminescence (Immobilon Western, Millipore) and detection films XAR (Kodak).

3.1.11 Miscellaneous:

DNA loading Dye (6X)

10 mM Tris-HCl (pH 7.6), 0.25% bromophenol blue, 60% glycerol 60 mM EDTA.

RNase A

Dissolve RNase A at concentration of 10 mg/ml in 0.01 M Na-acetate (pH 5.2). Heat at 100°C for 15 minutes to remove DNAse contamination. Allow to cool slowly at RT. Adjust the pH by adding 0.1 volume of 1 M Tris-Cl (pH 7.4). Dispense in aliquots and store at -20 °C.

Table 3. 5: List of miscellaneous reagents

Solute	Solvent	Stock
Ethidium Bromide	Water	10mg/ml
IPTG	Water	100mM
X-gal	DMF	20mg/ml

3.1.12 Culture Maintenance and Growth conditions:

Maintenance of *E. coli* strains:

E.coli were grown at 37ºC on orbital shaker at 200 rpm (unless specified), stored for routine use in refrigerator as cultures on LA plates containing appropriate antibiotics. For long term storage, cultures were preserved in 20% glycerol at -20°C.

3 .2 Methods

3.2.1 DNA Isolation:

3.2.1.1 Isolation of plasmid from E. coli by Alkali lysis method (Sambrook et al, 1989):

10 ml of LB medium containing proper antibiotics were inoculated with single colony of transformed bacteria and incubated at 37 ºC for overnight with shaking at 200 rpm. The bacteria from culture were pellet down at 5000 rpm (Eppendorf - 5415R, F45-24-11) for 5 minute. Pellet

was resuspended in 1 ml chilled solution I (GTE) and vortex as necessary to fully resuspend bacteria. After 5 minute incubation, 2 ml of fresh RT solution II 0.2 N NaOH/1.0% SDS (alkaline SDS) added to the suspension. Immediately the solution was mixed very gently and 1.5 ml of ice-cold solution III (potassium acetate) was added to it. The contents of the tube were rotated very gently about 20-25 times and maintained on ice for 10 minute. The contents were centrifuged at 8000 rpm for 10 minute. The supernatant was taken and was treated first with equal amount of phenol-$CHCl_3$ and then spin at 5000 rpm for 5 minute. Aqueous phase was taken and equal volume of $CHCl_3$: isoamyl alcohol (24:1 ratio) was added, spin at 5000 rpm for 5 minute. Aqueous phase was taken and equal volume of iso-propanol was added. After gentle mixing it was incubated for 15 minute at RT. Pellet down at 8000 rpm for 15 minute. The pellet was washed twice with 70% ethanol. The tube was air dried and the pellet was dissolved in TDW.

3.2.1.2 Genomic DNA isolation of E.coli

50 ml of overnight grown culture was pellet down which was re-suspended in 5 ml GTE, add pinch of lysozyme and were incubated at 37°C for 15 minute followed by addition of 1 ml 0.5M EDTA; mixed gently and left for 5 minute. Added 1% SDS (final concentration) and incubated at 37°C until a drop of suspension on a microscopic slide was completely cleared by addition of a drop of 10 % SDS. To this mixture, add 5 ml of Tris-saturated phenol was added and mixed thoroughly at RT. 5 ml of $CHCl_3$ was added and shaked for 5-10 minute at RT. The aqueous phase was removed carefully, after spin (3000 rpm for 10 minute), using a 1 ml cut tip and re-extracted with phenol:$CHCl_3$ as above followed by two extraction with equal volume of $CHCl_3$. DNA was precipitated by adding 1/10 volume of 3M Na acetate and equal volume of iso-propanol, washed with 70% ethanol; air dry and resuspend in TDW.

3.2.2 Introduction of DNA into cells

3.2.2.1 Electroporation in E.coli

Preparation of Electro-competent cells:

Competent cells were prepared as described by Sambrook *et.al*, 1989, with minor modifications. Fresh overnight culture of E. coli was grown in LB and then 1% of this culture was added to 100ml of 2X YT. When the OD_{600} of culture was reached approximately between 0.4-0.6, rapidly flask was transferred on the ice water bath for 15-30 minute and centrifuged at 4000 (Hermle

Z323K) rpm for 10 minute at 4ºC. The pellet was resuspended in 40 ml of TDW, centrifuged at 4000 rpm for 10 minute at 4ºC., followed by resuspension in 30 ml of TDW, 20ml of 10% glycerol, followed by centrifugation each time at 4000 rpm for 10 minute at 4ºC. After decanting the 10% glycerol, the pellet was dissolved in the residual glycerol and aliquots (60µl) were stored in sterile microfuge tubes at -70ºC.

Electroporation:

An aliquot of frozen cells were thawed on ice. 100 ng DNA was mixed with the cells and kept on ice for 5 minute. This suspension was transferred into an electroporation cuvette (0.1cm width; Bio-Rad) and electroporated using the following pulse conditions; voltage, 1.8kV/ 2.5kV; resistance, 200Ω; capacitance, 25 µF, which gives a time constant of 4.5 to 5.5 milliseconds. The electroporated cells were diluted with 1 ml LB and kept at 37 ºC for a one hour for expression of antibiotic resistance. The culture was then plated on appropriate antibiotic containing LB plates, incubated at 37ºC for transformats to appear after 12 to 24 hours.

3.2.2.2 Transformation of E. coli using CaCl2: (Sambrook et al, 1989)

Fresh overnight grown cultures in LB was sub- cultured (1:10) in LB and grown up to an OD_{600} of 0.6. Cells were spin down at 5000 rpm for 5 minute at 4 ºC. Media was discarded till last drop and the pellet was resuspended in chilled 0.1 M $MgCl_2$ and the tube was kept on ice for 10 minute, spin for 5 minute at 5000 rpm at 4 ºC. Discard the Supernatant and resuspended the pellet in chilled 0.1M calcium chloride ($CaCl_2$). Kept the tube in ice for 10 minute, was centrifuged for 5 minute at 5000 rpm 4 ºC. The supernatant was discarded and , the pellet was resuspended in 200µl of $CaCl_2$. Incubate on ice for 45 minutes and add 5-7 µl of DNA, the content was mixed by tapping the tube, further incubated for 45 minute on ice. Give heat shock treatment to cells at 40-42ºC for 90 seconds, was rapidly transferred to ice bath. Add 1 ml LB and incubate for 1 hour at 37 ºC. After incubation period the tube was spin down for 5 minute at 3000 rpm, supernatant was discarded and the pellet was suspended in 200 µl drop. The culture was then plated on appropriate antibiotic containing LB plates, incubated at 30^{o}C. The transformants were counted after 12-24 hours.

3.2.3 Electrophoresis

3.2.3.1 Agarose gel electrophoresis

Agarose gel electrophoresis was carried out in a horizontal matrix of agarose with 1X TAE buffer as described by Sambrook *et al*., (1989). Lambda DNA digested with *Hind*III/ *Hind*III-*EcoR*I was run in parallel as a size standard. The bands were visualized using short wave UV light (302 nm) on a transilluminator.

3.2.4 Elution of DNA from agarose gel

The digested DNA containing the fragment to be eluted was separated on 0.7 % agarose gel. The bands corresponding to desired DNA fragment was excised out and chopped into small pieces, transferred in to 0.5 ml. microcentrifuge tube. With a hole at its bottom blocked by glass wool in TE, and placed into microcentrifuge tube of volume 1.5 ml. The assembly is kept at -20 ºC for 5-6 hrs and then spun after thawing at 12,000 rpm for 10 minute. The DNA was extracted with phenol, precipitated by ethanol, washed with 70 % ethanol, air dried and re suspended in small volume of water or TE.

3.2.5 Genomic DNA library preparation:

Partial digestions of genomic DNA were carried out for 2.5, 5, 7.5 and 10 minute time intervals at 37°C in thermocycler, using frequent cutter restriction enzyme *Sau3A*I. Reaction was stopped by immediately raising the temperature to 94ºC. Electrophoresis of reaction product with Lambda –Hind marker was carried out in 0.8% agarose gel. Gel corresponding to 5 – 9kb sized fragments was eluted from the gel and was subjected to overnight cracking in conventional glass wool assembly at -20 ºC. DNA was retrieved from gel by centrifugation (5000rpm, 5 minute, RT). The collected DNA sample was purified and used for ligation with pBR322 digested with *BamH*I. Ligation mixture was introduced in DH5α by electroporation.

3.2.6 PCR protocols

Primers were designed by the program PRIMER3 and ordered at either MWG or IDT. DNA was amplified by Polymerase Chain Reaction (PCR) using XT-20 DNA Polymerase according to manufacturer's instructions. In general, 50 µL reactions were set up consisting of 0.5 µM forward and reverse primer, (Table 3.4), 10 to 500 ng of template DNA, 1X XT-20A Buffer (final concentration), 200 µM of each dNTPs and 1 U enzyme. Reactions were conducted using

appropriate cycling programs (Table 3.6). Samples were analyzed subsequently by agarose gel electrophoresis

Table 3. 6: PCR Programme

Step	Time
Initial denaturation at 94°C	10 min.
denaturation at 94°C	30 sec.
Annealing (According to primer Tm value)	30 sec.
Extension at 72°C	1.5-2.5min/kb
Repeat 25 or 35 cycles	
Final extension at 72°C	10 min.
Hold at – 4°C	End

3.2.7 Western Blotting

The cell pellet was collected at log phase (0.6 O.D.) of growing culture and resuspend into the appropriate amount of Laemmli sample buffer (0.5-1 µg/1µl). Lysis was done by several pipetting (P200) and sonicated at 45% amplitude for 20 cycles. After centrifugation at 12000 g for 15 minute at 4°C, the supernatant was collected. Total protein content was quantified using Bradford assay (Bio-Rad Bradford Solution, USA). Protein was loaded (20µg) on a 10% SDS-Polyacrylamide gel and then electrophoretically transferred onto a Nitrocellulose membrane (Bio-Rad). The membrane was then incubated for 1 hour at room temperature in blocking buffer (TBS-T containing 5% skimmed milk) and further incubated overnight with the primary antibodies for monclonal RelA Antibody (1:2000;Santa Cruz) and β-galactosidase (1:5000; Novusbio) at 4°C. Membrane was then washed four times with PBS-T, and incubated with HRP-conjugated secondary antibodies (1:2500) for 1 hour. Finally, membrane was developed and visualized with Enhanced Chemiluminescence western blotting detection system by X-ray film development.

3.2.8 RNA isolation & semi-quantitative PCR

RNA was isolated from constructed *E.Coil* strains using the TRIzol reagent (Sigma Aldrich) as per manufacturer's instructions briefly mentioned here. 1ml of TRI Reagent solution was added directly to cells and collected lysed cells in 1.5ml tube allowed to stand at room temperature for 10 minute. At the end of incubation, the vials were centrifuged at 12,000Xg for 10 minute at 4°C. The tubes underwent incubation at room temperature for 10 minute after the

addition of 500µl of chloroform followed by centrifugation at 12,000 g for 10 minute. at 4°C. The aqueous phase formed was transferred to a fresh centrifuge tube. After addition of 500µl of isopropanol, the tubes were incubated at room temperature for 10 minute. and then centrifuged at 12,000 g for 10 minute at 4°C. Precipitated RNA formed a white pellet on the side and bottom of the tube. The RNA pellets were washed with 1 ml of 75% ethanol and centrifuged at 7,500 g for 5 minute. at 4°C. Ethanol was removed without disturbing the pellets, which were then air-dried for 3-5 minute. Quantity and quality of isolated RNA were assessed using spectrophotometer and samples with a ratio of A_{260}/A_{280} >1.8 processed for cDNA synthesis.

A reverse-transcription reaction was performed using 2µg RNA with reverse transcriptase in a 20µL reaction volume containing DEPC treated water (Life Technologist). PCR products were amplified using gene specific primers (Table 3.4). GAPDH was used as an endogenous control. The PCR products were analysed by electrophoresis on 2.0% agarose gels, and the gels were photographed after staining with ethidium bromide and intensities of the band were calculated by densitometric analysis using the Image J software.

3.2.9 Statistical analysis:

The results were analyzed using one-way analysis of variance (ANOVA) and students t-test to determine the level of significance. $p < 0.05$ was considered to be significant. Results were expressed as mean ± SEM. The statistical analysis was carried out by using the Graph Pad Prism 3.0 software.