CHAPTER 6

Addressing the physiological function of *rumA*.

6.1 Introduction

A complex ribonucleoprotein particle, the ribosome is composed of 23S, 16S, and 5S ribosomal RNAs and about 52 ribosomal proteins in *Escherichia coli*. The 16S and 23S ribosomal RNAs contain post-transcriptionally modified nucleotides that are derivatives of the four common nucleotides but none in 5S rRNA (Rozenski *et al.*, 1999, Agarwalla *et al.*, 2002). A common modification found in RNA is the methylation of nucleotides. *Escherichia coli* 16S rRNA contain 11 modifications of which 10 are methylated nucleotides, whereas 23S rRNA has a total of 25 modifications of which 14 are methylated (Rozenski *et al.*, 1999, Ofengand & Rudd, 2000) (Tscherne *et al.*, 1999, Smith *et al.*, 1994) The methylated nucleosides are clustered in the functionally important regions, the A- and P- tRNA binding sites for 16S rRNA, and the peptidyltransferase centre for 23S rRNA. Most of the modified nucleotides are conserved (Moore & Steitz, 2002), however in all living organisms the types of modification, the exact position of modification and the overall number of modified nucleotides are generally not very conserved (Connolly & Culver, 2009). Most of the methyltransferases responsible for formation of 24 methylated nucleotides found in *E. coli* rRNA have been characterized (Table 6.1).

Table 6. 1: Some examples of methylated nucleotides in rRNAs

rRNA	Position	Modifications	Enzyme (alternative name)	Reference
16S	1518 1519	$m^6{}_2A$	RsmA (KsgA)	Van Buul and van Knippenberg,1985
	967	m^5C	RsmB (YhdB, RrmB)	Tscherne et al., 1999a
	1207	m^2G	RsmC (YjjT)	Tscherne et al., 1999b
23S	745	m^1G	RlmA (YebH, RrmA)	Gustafsson and Persson, 1998
	2251	Gm	RlmB (YjfH)	Lovgren and Wilkstrom, 2001
	1939	m^5U	RlmD (YgcA, RumA)	Agarwalla et al., 2002
	747	m^5U	RlmC (YbjF, RumB)	Madsen et al., 2003
	2552	Um	RlmE (FtsJ, RrmJ)	Caldas et al., 2000a,b; Bugl et al., 2000

Posttranscriptional modifications are important because they impart a greater ability to the fully modified ribosomes to carry out protein synthesis as compared to unmodified ribosomes (Krzyzosiak *et al.*, 1987, Green & Noller, 1996). The modifications and modifying enzymes have been characterized; however the biological role of many is still unclear. In many cases, disruption or deletion of modifying enzyme does not affect growth rate; however the mutants are

out-competed by the wild-type strains in competition experiments, an indication that the modified bases confer a growth advantage to the bacteria (Gutgstell *et al.*, 2005). Persson B.C.et al, 1992 reported that *trmA*-encoding tRNA m^5U54 methyltransferase is essential for viability of cells (Persson *et al.*, 1992).

Our main focus is on RumA methyltransferase which modifies U1939 to m^5Uridine, as the importance of this modified nucleotide is being understood. In this chapter, we have attempted to unravel the unknown physiological relevance of RumA.

6.1.1 Genomic organization of *rumA*, *relA* and *barA*

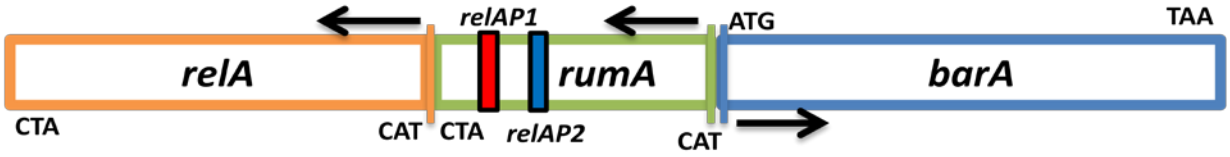


Figure 6. 1: Arrangement on *E coli* chromosome of *relA*, *rumA* and *barA* genes. The arrow indicates direction of transcription. The ORF is demarcated by ATG/TAC and TAA/ATC. The filled boxes represent *relA* promoters.

Genomic organization of *rumA*, *relA* and *barA* is quite unusual (Figure 6.1). *relA* gene is situated downstream of *rumA* gene and transcribed in the same direction as *rumA* with the major promoter (*relA*P1) located 178 bp upstream, and the second minor promoter (*relA*P2) 626 bp into *rumA* gene (Nakagawa *et al.*, 2006a). *barA* is situated upstream of *rumA* divergently transcribed in relation to *rumA* from promoter that also must be in *rumA* gene, though it is as yet not mapped. So the gene knockout strategy cannot be used to create the mutation in the chromosomal *rumA* without the knowledge of the promoter of the *barA* gene. Given that the promoters of *relA* are present in *rumA*, mutation in *rumA* may affect the expression of both *relA* and *barA*.

6.1.2 Coexistence of *relA* and *rumA*

An interesting coincidence came to be realized that *rumA* and *relA* genes are present together in the genome of *E. coli* and several members of *Enterobacteriaceae* family. The target of their functionality is also the same i.e, ribosome. Furthermore, “In the 70 S ribosome structure, the conserved loop containing U1939 tucks into the major groove at the end of the acceptor stem of the A-site tRNA, and hence it is strategically located for sensing the presence of uncharged

tRNA at this site in the ribosome. During amino acid starvation, the presence of uncharged tRNA at the A-site activates the ribosome bound RelA, resulting in the increased levels of the nucleotide (p)ppGpp, the mediator of the stringent response" - (Agarwalla *et al.*, 2002).

The significance of the coexistence of the two genes coexistence is yet to be appreciated; our observation may add a new dimension to the importance of their being together.

rumA mutant made by Persaud C. *et al*. shows little growth defect prompting *rumA* to be dispensable (Persaud *et al.*, 2010). This mutant has been made by deleting *rumA* ORF, making it *relA*$^-$ as well due to presence of *relA* promoters in *rumA* gene as discussed earlier (Figure 6.1). Since this *rumA* mutant represents deletion mutant, inactivation of *rumA* by insertional mutagenesis was attempted.

The results of this chapter are organized in four different parts. First part deals with construction of chromosomal *rumA* mutant through site directed mutagenesis and assessing its phenotype. Second part is also about construction of chromosomal *rumA* mutant but with different strategy i.e. recombineering - mediated insertion of chloramphenicol acetyl transferase (*CAT*) cassette in *rumA* gene. Third part is regarding the phenotypic characterization of *rumA* insertional mutant and fourth part is about regulation of *relA*.

6.2 Materials and Methods / Strategy

6.2.1 Growth Conditions

Bacterial cells were normally grown in Luria Bertani (LB) broth as well as in minimal broth (MB) at 37°C and 30°C with shaking. When necessary, media were supplemented with kanamycin (50µg/ml), ampicillin (50-100µg/ml), chloramphenicol (12-16µg/ml), Tetracycline (10 µg/ml), Streptomycin (100µg/ml) (Sigma-Aldrich, Himedia). DNA manipulations were carried out according to protocols described in (Sambrook, 1989).P1 transduction was carried out by method mentioned in A Short Course in Bacterial Genetics (Miller, 1992).

6.2.2 Bacterial strains and plasmids

Table 6. 2: Strains and plasmids used in this chapter

E. coli strains/Plasmids	Relevant genotype/Description	Reference/Source
Strains		
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*$^+$ *rumASal*I::*CAT*	This study
KP9	MC4100 *relA*$^+$ *rumAMlu*I::*CAT*	This study
KP10	MC4100 *relA*$^+$ *rumA*$^+$ *barABgl*II::*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*$^+$ *rumASal*I::*CAT*	This study
KP25	MG1655 *relA*$^+$ *rumAMlu*I::*CAT*	This study
KP26	MG1655 *relA*$^+$ *rumA*$^+$ *barABgl*II::*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
Plasmids		
pBlueScriptKS	Cloning Vector Amp^r ColE1 replicon	Stratagene,USA
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.*, 1989b)
pKD46	Recombineering vector, Ori^{ts} Amp^r repA101 replicon	Datsenko and Wanner, 2000
pKC1	Spontaneous *relA*$^-$ mutant of pTE1 plasmid	This study
pKC2	Spontaneous *relA*$^-$ mutant of pTE1 plasmid	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 *EcoR*I - *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 *EcoR*I - *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	*EcoR*I restriction enzyme digestion of pTE18 plasmid followed by intramolecular ligation	This study

Table 6. 3: List of Primers used in this work

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
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
RpoZRight	ATCACGTGCAACGAGATACG	55.1
RelART Left	ACTACCACGCGACCGTTATC	56.9
RelART Right	GGGCGACCAGATTGAAATTA	52.8
GAPDHRT Left	AGGTCTGATGACCACCGTTC	56.8
GAPDHRTRight	AGTAACCGGTTTCGTTGTCG	55.3

b (Poteete et al, 2006)

6.2.3 Construction of chromosomal C389P substitution mutation in *rumA* by SDM

6.2.3.1 Construction of vectors for the purpose of *rumA* gene mutation

Mutagenic primer pair (i) C389P-SDM1 – RumARPst1 generates 187 bp amplicon; whereas (ii) C389P-SDM2 – RumAF3 produces 2351 bp amplicon which consists of 1178 bp *rumA* DNA + 1173 bp of upstream *barA* DNA. These two amplicons were mixed, assembled and amplified in PCR using gene specific RumARPst1- RumAF3 primers. The 2518 bp amplicon was initially cloned at *BamH*I - *Pst*I sites of pBlueScriptKS vector to give rise to pTE11. 2289 bp *relA* amplicon was next cloned downstream of mutant *rumA* at *EcoR*I - *Kpn*I sites of pTE11 to obtain pTE12 construct. Δ*rumA1-relA* DNA from pTE12 was next cloned in temperature-sensitive plasmid, pMAK705 (Hamilton *et al.*, 1989a) at *BamH*I - *Kpn*I sites to generate pTE13.

pTE11 (Δ*rumA1*), pTE12 (Δ*rumA1+relA* ORF) and pTE13 (Δ*rumA1+relA* ORF in pMAK705) clones were confirmed by restriction digestion (Figure 6.2).

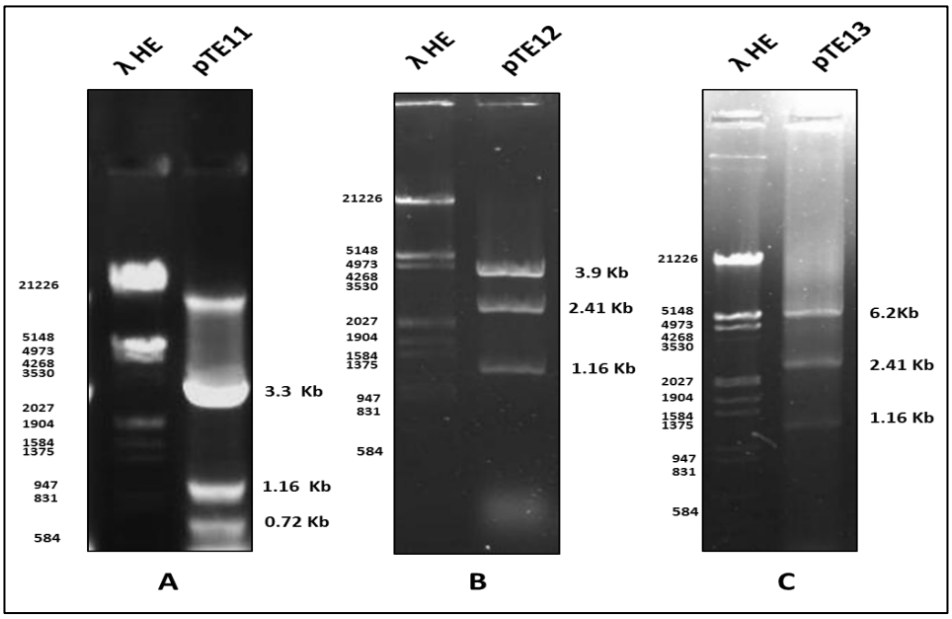


Figure 6. 2: Confirmation of clones by *Sal*I restriction enzyme digestion. (A) pTE11 (Δ*rumA1* in pBluescriptKS), **(B)** pTE12 (Δ*rumA1 relA* in pBluescriptKS) and **(C)** pTE13 (Δ*rumA1 relA* in pMAK705). λHE was used as M.W marker.

6.2.3.2 Construction of chromosomal Δ*rumA1* mutant KP1.1

We replaced the chromosomal *rumA* gene with the deletion allele using JM101(*recA*$^+$) by homologous recombination (Hamilton *et al.*, 1989a). With respect to C→P substitution mutation in *rumA* gene, there is 2518 bp mutant *rumA*/*barA* DNA and 2289 bp of *relA* DNA flanking the site of mutation. This ensures high possibility of unbiased integration/resolution events happening during double crossover homologous recombination. The plasmid was introduced by transformation into *recA*$^+$ strain JM101 at 30^0C. Transformed colony was amplified in 10 ml of LB containing CAM (LB/CAM) and spread on prewarmed LA/CAM plates at 10^{-4} dilutions and incubated at 44^0C. Stable plasmid integrants were selected at 44^0C at an efficiency of 2×10^{-4}/cell. This frequency of the cointegration event was estimated by dividing the number of cells growing at 44^0C by the number of viable cells at 30^0C after being plated at an equivalent dilution. Unlike the plasmid transformed cells at 30^0C which could be cured of plasmid borne CAM marker at 44^0C, the plasmid integrant selected at 44^0C could not be cured of CAM marker. One of the integrant's inoculum was amplified in the absence of selection pressure at 30^0C for thirty generations without antibiotic addition, diluted appropriately and plated at 44^0C for plasmid free cells to grow. Plasmid cured cells were screened by their being CAM-sensitive (90-99%). Genomic DNA was prepared from six independent plasmid-free segregants and PCR amplified

using RumARPst1 and RumAF1 primers. Four out of six segregants were wild type *rumA*$^{+}$ (KP1.2), and two were the Δ*rumA1* (KP1.1). It was possible to make this distinction in PCR and sequencing mutation was not required because wild type *rumA*$^{+}$ generates 1529 bp amplicon, whereas Δ*rumA1* deletion mutant allele produces 1397 bp product instead.

6.2.4 Determination of growth advantage of Δ*rumA1* mutant in growth competition experiment

The competition experiment was performed in LB media at 37°C. For the purpose of the experiment, isogenic wild type KP1.2 was tagged with miniTn*10* (Tet) transposon by the method described by Miller et al., (Miller, 1992). One of the insertion mutant of KP1.2 (KP1.2::Tn*10* miniTet) that exhibited the identical growth rates as the parental KP1.2 was used in coculturing experiments. Equal number of Tetracycline-sensitive KP1.1 mutant strain and Tetracycline-resistant wild type strain cells were inoculated in the same 10 ml of LB medium. At each 24 hrs growth cycle, the growth medium was diluted 1000-fold. At the same time, serial dilutions of the bacterial culture were made. Equal (100 µl) aliquots from the 10^{-6} diluted cultures were plated to LB agar and LB agar supplemented with Tetracycline (15 µg/ml). Plates were incubated at 37 °C overnight and number of colonies was counted and compared.

6.2.5 Cloning of *CAT* cassette in *rumA* gene at a plasmid level for creation of genomic *rumA*::*CAT* insertion mutant

1529 bp *rumA* amplicon generated using RumAF1-RumARKpnI primers was cloned in to the broad host range vector pBBR1MCS2 (Kovach *et al.*, 1995) at *Eco*RI-*Kpn*I sites (pTE14). Amplification of 824 bp *CAT* gene from pACYC184 vector (Bartolome et al, 1991) was carried out by 2 pairs of CAT primers. (1) CATFSalI and CATRSalI primers were used for cloning *CAT* amplicon into pTE14 vector at unique *Sal*I site to generate pTE15, (2) Using CATFMB and CATRMB primer pair, *CAT* amplicon was inserted either at *Mlu*I or *Bgl*II sites in pTE1ΔBgl to generate pTE16 and pTE17 constructs respectively. Insertion of *CAT* cassette at the plasmid level was confirmed by restriction digestion (Figure 6.3).

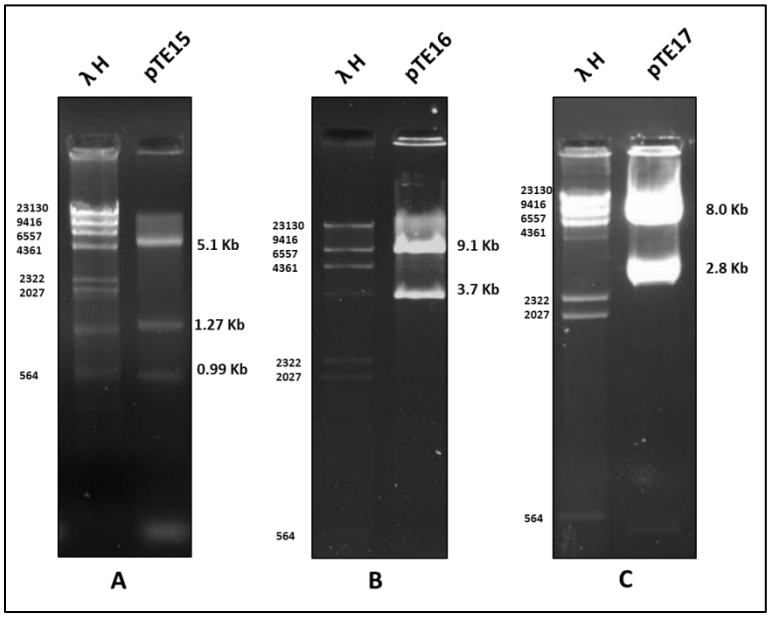


Figure 6. 3: Confirmation of *CAT* insertion in the plasmid by *EcoR*I restriction enzyme digestion. **(A**) pTE15 (*CAT* insertion at *Sal*I in *rumA* pBBR1MCS2), **(B)** pTE16 (*CAT* insertion at *Mlu*I in pTE1ΔBgl) and **(C)** pTE17 (*CAT* insertion at *Bgl*II in *barA* of pTE1ΔBgl). λH was used as M.W marker.

We replaced chromosomal *rumA* gene with the mutant allele by recombineering using *E. coli* strain DY330 (Yu *et al.*, 2000) and also using the pKD46 plasmid (Datsenko & Wanner, 2000) in MC4100 and MG1655 strains. The detailed method is described below.

6.2.5.1 Gene disruption by targeted chromosomal engineering of *E. coli* strain DY330

6.2.5.1.1 Linear DNA Preparation

PCR amplification of *CAT* cassette in *rumA* gene using RumAF1-RumARPst1 primers generated 2335 bp amplicon; whereas PCR amplification of *CAT* cassette in *barA* gene with RumAF3-BarAF primers generated 1669 bp amplicon. The amplicons were purified by gel elution method described in chapter 3, section 3.2.6

6.2.5.1.2 Induction of λ recombination functions: preparation of electro-competent cells and Electroporation

Preparation of electro-competent cells

For preparation of overnight grown cultures, single isolated colony was inoculated in 10 ml flask at 32°C and 1% of this culture were added in 50 ml or 100ml of 2xYT broth and grown at 32°C with shaking till the bacterial culture reached OD600 = 0.4–0.6. Induction was performed by

placing the flask in a water bath at 42°C with shaking (200 revolutions/min) for 10-12 min. After induction the flask transferred rapidly to the ice bath for 10 min. The cooled cultures were centrifuged for 10 min at 5500 rpm at 4°C. The cell pellet was suspended in 40 ml of ice-cold sterile water and was spun for 15 min at 4°C. The same procedure was repeated for two more times as described above and finally the cells were suspended in a 200ul of ice-cold sterile water. Aliquots of competent cells were stored in sterile microfuge tubes at -70ºC with 12 % glycerol present. Fresh competent cells give highest efficiencies of recombination and were used here.

Electroporation

Purified linear donor DNA (1–10µl; usually 1–100 ng) was mixed with competent cells in a final volume of 50 µl on ice. The mixture was pipetted into a precooled electroporation cuvette (0.1cm). Electroporation was performed by using a Bio-Rad Gene Pulser set at voltage-1.8 kV, resistance-200Ω; capacitance-25 µF, which gives a time constant of 4.5 to 5.5 milliseconds. 1ml of LB medium was immediately added to the electroporated cells and the cells were incubated for 1–2 h at 32°C before selecting for recombinants. Aliquots of the cells were spread on appropriate antibiotic containing LB plates and were incubated at 32°C

6.2.5.2 Gene disruption through Red helper plasmid – pKD46

First of all, a Red helper plasmid pKD46 was transformed in those strains where we wanted to disrupt a particular gene. The transformants carrying this plasmid were grown overnight in 10 ml LB and then 1% of this culture was inoculated and grown in 50ml/100ml of 2X YT with ampicillin and 1mM L-arabinose at 30°C. Culture was grown till OD 600 reach to ~0.6 and then kept on ice for 10 minute. Electrocompetent cells were made by washing the culture three times with sterile ice-cold 10% glycerol. The gel eluted linear DNA (10-100ng) was mixed with 50 µl competent cells and electroporation was done by using a Bio-Rad Gene Pulser. Instantly added 1 ml of LB to shocked cells and then incubated for 1 hr at 30°C. Half of the culture was spread on suitable antibiotic containing LB plate and incubated at 30°C for 24-48 hrs. The helper plasmid was cured by purifying the transformed colonies in the absence of helper plasmid encoded selection marker. The transformed colonies were purified nonselectively at 43°C and then tested for ampicillin sensitivity for loss of helper plasmid.

The mutations in each case were transduced into a fresh background of MC4100 and MG1655 through P1*vir*-mediated generalized transduction as described by Miller et al, 1992.

6.2.6 P1 lysate preparation

0.150 ml of overnight grown culture of the donor strain in LB broth was mixed with 10^7 pfu (75 µl) of P1 phage. Adsorption was allowed to occur at 37°C for 20 min and the lysate was prepared in one of the following ways:

6.2.6.1 Plate method

0.1 ml of the infection mix was dispensed in 2.5 ml of LB soft agar and poured on freshly prepared Z-agar plates and incubated at 37°C for overnight. Next day, 2 ml of Z-broth was added to each plate and incubation continued for 2 more hours. The Z-broth and soft agar layers were transferred into a 15 ml tarson tube and 1 ml of chloroform was added followed by vortexing vigorously for 30 seconds. After centrifuging down the debris, the clear supernatant was removed into a fresh sterile 15 ml tarson tube, treated again with chloroform and stored at 4°C.

6.2.6.2 Broth method

To 0.225 ml of infection mixture, 10 ml of Z-broth containing 0.2% glucose was added and incubated at 37°C with slow shaking until growth followed by the visible lysis of the culture occurred (approximately 4 hours). The lysate was treated with chloroform, centrifuged and the clear supernatant was stored at 4°C.

6.2.7 P1 Transduction

2 ml of freshly overnight culture of the recipient strain grown in Z-broth was mixed with 10^8 pfu of P1 (0.2 ml) and incubated at 37°C for 20 minutes to facilitate phage adsorption. The mixture was centrifuged, and unadsorbed phage particles in the pellet were removed by washing twice with citrate buffer and finally cells were suspended in 0.2 ml of N-saline. 0.1 ml of infected cells were plated on selective medium. In case of antibiotic selection, where phenotypic expression is required, either of two procedures followed: (a) The bacterial pellet after the initial centrifugation was resuspended in 10 ml of LB medium supplemented with 25mM Na citrate followed by centrifuged and repeat the same procedure. After 1 hr incubation at 37°C, the cells were recovered by centrifuged and plated directly onto appropriate antibiotic containing LB plates. (b) The mixture, after washing, was plated in soft agar on medium not containing selective antibiotic and incubated for 1-2 hrs at 37°C. Afterwards the appropriate antibiotic was added in a soft agar

overlay in quantities enough, after diffusion throughout the medium, to achieve the desired final concentration.

6.2.8 Assaying of *relA* phenotype

6.2.8. (a) 3-amino, 1, 2, 4-triazole (3-AT) it induces histidine starvation by acting as a competitive inhibitor of the imidazoleglycerol-phosphate dehydratase. This enzyme catalyses the sixth step of histidine production (Hilton, 1965). The sensitivity was determined by using M9 minimal medium supplemented with glucose (0.2%), all amino acids except histidine (4 µg/ml), adenine (1 mM), thiamine (1 mM), and AT (15 mM) (Silva & Benitez, 2006).

6.2.8. (b) SMG (serine, methionine and glycine) induces isoleucine starvation as serine is competitive inhibitor of threonine deaminase which catalyzed the 1st step of isoleucine biosynthesis. Serine, glycine, methionine (SMG) are one carbon amino acids and primary effect of SMG is to increase in level of THF (tetrahydrofolate) in cell which leads to inhibition of growth due to isoleucine starvation (Uzan *et al*.,1976). The sensitivity to inhibition by serine, methionine, glycine, (SMG) was tested with M9 glucose medium containing SMG (100 µg/ml each), adenine (50 µg/ml), thymine (50 µg/ml), and calcium pantothenate (1 µg/ml) (Silva & Benitez, 2006).

6.2.8. (c) Serine hydroxamate inhibits charging of seryl t-RNA resulting in auxotrophy for serine (Tosa & Pizer, 1971). Amino acid starvation was induced by addition of 0.5-1mg/ml serine hydroxamate in minimal medium containing 0.2% glucose.

Escherichia coli strains, MC4100 and MG1655, and their isogenic wildtype and mutant *relA* strains were streaked on 3-AT or SMG or SeOH plates. The plates were incubated for 24-36 hrs at 37°C.

Cultures of KP1.1 and isogenic wild type segregant KP1.2 were grown in M9 minimal broth to OD_{600} of 1.0. 1% of the cells were grown with two fold increasing concentration of serine hydroxamate in 2 ml of broth for 24 hr at 37°C.

6.2.9 Determining growth curve

Growth curve experiment was performed in either LB and/or Minimal glucose broth. Overnight grown cultures were inoculated at 1% density in 50ml LB flasks and/or 50ml Minimal glucose

broth. The inoculated flasks were kept on rotary shaker at 37°C and 25°C temperature. O.D.600 was measured at different time intervals. This experiment was repeated twice.

6.2.10 Quantitation of RelA Protein expression by Western blot

Western blot was performed to quantitate expression of RelA protein in each isogenic strain of MC4100 and MG1655. Different mutants of MG1655 and MC4100 were grown in different media (LB and MB) till culture reached 0.6 O.D. The culture was centrifuged and the pellet was resuspended in Laemmli buffer and sonicated. After sonication protein was estimated and loaded (20µg) on a 10% SDS-Polyacrylamide gel and then electrophoretically transferred onto a Nitrocellulose membrane (Bio-Rad). The blots were incubated overnight with the monclonal anti-RelA Antibody (1:2000; Santa Cruz) and β-galactosidase (1:5000; Novusbio) at 4°C. Anti-mouse/rabbit IgG conjugated with HRP (1:2500) was used as secondary antibody. Finally, membrane was developed and visualized with Enhanced Chemiluminescence western blotting detection system by X-ray film development.

6.2.11 RNA isolation and mRNA expression of *relA* gene by semi-quantitative RT PCR

RNA was isolated from each isogenic strain of MC4100 and MG1655 using the TRIzol reagent (Invirogen) as per manufacturer's instructions. Genomic DNA contaminated samples were treated with DNAseI kit (Invitrogen). A reverse-transcription into first strand c-DNA reaction was performed using 2µg RNA with MuLV reverse transcriptase in a 20µL reaction volume containing DEPC treated water (Invirogen) as per manufacturer's instructions. PCR product was amplified using gene specific primers (Table 6.3). GAPDH was used as an internal control and the PCR amplicon were analyzed by electrophoresis on 2.0% agarose gels and images were captured after staining with ethidium bromide and intensities of the band were calculated by densitometric analysis using the Image J software.

6.3 Results

6.3.1 Creation of chromosomal *rumA* mutant using two different strategies

The rationale of this study is to test the function of *rumA* in stringent response/*relA* regulation. The reasons for this thinking are twofold – (i) the strategically important location of U1939, the site which is modified by methylation by RumA protein in the ribosome - In the crystal structure of 70S ribosome complex with tRNA, U1939 is present in the conserved loop of 23S rRNA. This

loop is seen to tuck in to the major groove of tRNA at the A-site near the end of its acceptor arm (Yusupov *et al.*, 2001) , a condition apparently right for sensing the presence of uncharged tRNA at the A-site of the ribosome under nutrient starvation condition. (ii) Moreover, the two genes, *relA rumA* are contiguous on the genome of several gamma proteobacteria. In a study aimed at finding physiological role for *rumA*, Persaud *et al*., removed the complete ORF of *rumA* and in the process also removed the two promoters of *relA* which are internal to *rumA* gene. If *relA* were affected, say at the translational level, by the unmodified ribosomes produced in *rumA* mutant due to functions missing in the 5' UTR, the effect of the mutation would not be realized for *relA* transcript expressed from ectopic promoter. The effect of the *rumA* mutation on stringent response is thus not possible to be studied in the mutant. We therefore mutated *rumA* gene in two different ways; (i) by creating substitution mutation and (ii) by inserting antibiotic resistant cassette in the region of gene beyond the limits of the promoter(s) of *relA*.

6.3.1.1 Site directed mutagenesis of catalytic cysteine 389

6.3.1.1.1 Construction of *rumA1* mutation in plasmid pBluescriptKS (pTE11)

The structure of RumA protein is determined in two studies (Lee *et al.*, 2004, Lee *et al.*, 2005). The C-terminal domain of RumA contains catalytic amino acid cysteine C389 which is responsible for methylation at U1939 position of 23S rRNA. Hence, we decided to substitute the catalytic amino acid cysteine by site directed mutagenesis. We intended to substitute cysteine (ACA) codon with alanine (GCC) but due to our oversight it was altered to proline (CGG) codon. However this unintended Cys to Pro change would both compromise the conformation and function of the protein unlike the Cys to Ala alteration which would only affect function but not conformation. In the course of attempting PCR-mediated site directed mutagenesis of catalytic active site cysteine389, we noticed upon sequence analysis that the cloned mutant *rumA* DNA (in pTE11) not only contained the C389P substitution, but also an inadvertent deletion of 131 bp which corresponds to all but one template independent G nucleotide between primer binding sites for RumARPst1 and C389P-SDM1. The deleted DNA includes 115 bp coding portion for 38 aa of C-terminal of the RumA native protein and the rest (16 bp) being the intergenic DNA between *relA* and *rumA*. Removal of the stop codon of the *rumA* gene and an insertion of an extra G at the junction of the primer binding sites for RumARPst1 and C389P-SDM1 is expected to result in reading of the *rumA* message into the *relA* gene. Cloning of the

mutant Δ*rumA1* gene along with *relA* in either pBlueScript vector (pTE12) or in pMAK705 vector (pTE13) did not affect the Shine Dalgarno sequence of *relA* gene (Tedin & Bremer, 1992a), however, the cloning steps caused the duplication of the overlap sequence between the RelAF and RumARPst1. Deletion of the intervening DNA nevertheless, brought close the +1 transcription start site of *relA* gene, and thus the promoter from 177 (Nakagawa *et al.*, 2006b) to 71 bp. (Figure 6.4)

Wild type sequence (184 bp)

GACCAGACCTGCCGAACATCGGCAAATCGCAACTATTTAACGCGCGAGAA
AAGTACCATCGATTCCAGATGTCCCGTTGTGGGAACATATCCAGCATCGCC
AGTCGCGCAATGGTATATCCTGCTTTTAATAACGCTTCGCTATCCCAGCCAG
CGTTGCAGGGTTACAGGATACATAAACTAT

Δ*rumA1* mutant sequence (54 bp)

GACCAGACCTGCCGAAGGAGCCAGCGTTGCAGGTTCGGGGATACATAA
ACTAT

Figure 6. 4: Comparison between sequence of wild type *rumA* and Δ*rumA1* DNA. The color code describes the features in the sequence. ACA to CGG- Cys to Pro mutation, G +1 start site of *relA* gene, G – Extra nucleotide in the Δ*rumA1* mutant sequence, GACCAGACCTGCCGAAC – RumARpst1 primer, CTA- stop codon of *rumA* gene in wildtype gene and missing in the mutant sequence, Sequence shown in green (131 bp) is deleted in Δ*rumA1* sequence

We obtained only one clone and failed to generate a second SDM mutant of *rumA* gene in several attempts. We do not understand why. We think it is to do with the high copy number vector of pBlueScriptKS, since the SDM mutant of *rumA* could be constructed in pBAD18Kan vector at a later stage (See Chapter 4). We decided to continue to work with the mutation and used this clone for construction of chromosomal mutation of *rumA*. The deletion of 131 bp *rumA* DNA helped us analyse wild type versus Δ*rumA1* gene during the mutant construction experiment.

6.3.1.1.2 Construction of Δ*rumA1* chromosomal mutant using method for generating insertion/deletion in *E. coli* (Hamilton *et al*., 1989)

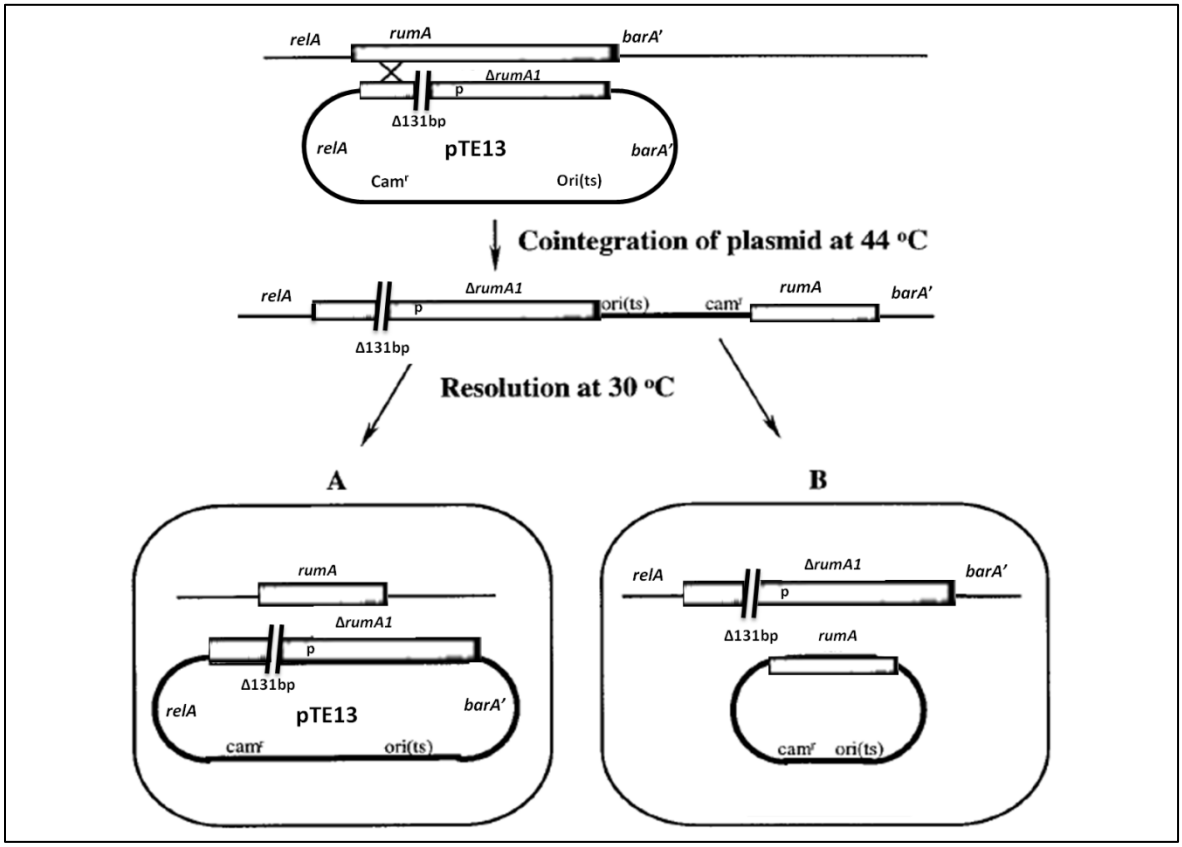


Figure 6. 5: Construction of chromosomal Δ*rumA1* mutant. Conditionally replicating plasmid, pTE13 (pMAK705 derivative) was integrated into the JM101 *recA*$^+$ strain via homologous recombination at nonpermissive temperature at 44°C. A second event is selected at permissive temperature of 30°C where the plasmid is resolved generating two variants. **(A)** resolved *rumA*$^+$ strain **(B)** the disrupted chromosomal Δ*rumA1* mutant.

The basis of this strategy is the use of pMAK705 vector which is a conditional replicating vector. It contains temperature-sensitive origin (ori^{ts}). Plasmid is episomal at permissive temperature of 30°C whereas it is cured completely in the absence of DNA that can let it integrate in the chromosome at nonpermissive temperature of 44°C. A homologous segment of DNA cloned in this vector can target corresponding DNA of the plasmid on the chromosome for generating insertion/deletion in *E. coli*. pTE13 (Δ*rumA1relA* pMAK705) was constructed for the purpose of allelic exchange. Δ*rumA1* was intended to be introduced into the chromosome using pTE13 plasmid (Figure 6.5). Transformed *E. coli* JM101 cells were grown at 30°C in 10 ml LB and appropriate dilution was plated on LA/CAM plate at 44°C. Cointegrate formation of pTE13 into the chromosome was obtained at an efficiency of 2×10^{-4}/cell at 44°C. 90-99% of cells were

plasmid free upon growth of the integrant at 30°C followed by curing at 44°C. Since the DNA flanking the Δ*rumA1* mutation is approximately of the same size, cointegrate formation/resolution will be unbiased with respect to the region of the DNA used for this event. Indeed we do see 40% of plasmid cured cells to be containing Δ*rumA1* mutation. The genomic DNA analyzed by PCR with *rumA* gene specific primers RumAF1-RumARPst1 was a testimony to this fact (Figure 6.6).

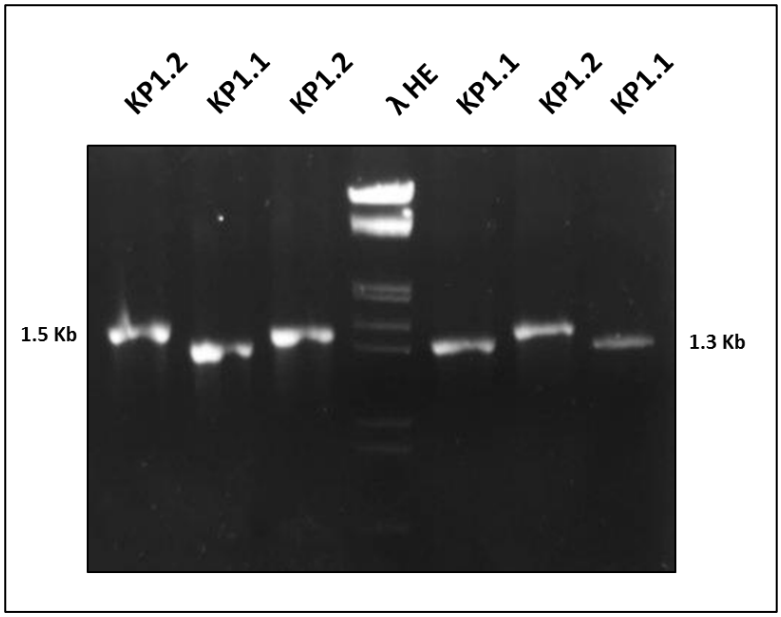


Figure 6. 6: Analysis of genomic DNA by PCR using RumAF1-RumARPst1 primers. Due to deletion of 131 bp in KP1.1 (Δ*rumA1*) PCR product size is 1.3 Kb while KP1.2 (wildtype) gives 1.5 Kb amplicon. λHE was used as M.W marker.

6.3.1.1.3 Chromosomal *rumA* mutation *(*Δ*rumA*) does not cause growth disadvantage in competition experiment

The deletion mutant, KP1.1, was used in competition experiment with isogenic wild type (KP1.2) cells tagged with tetracycline resistance marker. The cells of each of the strain were grown to 10^9cells/ml and mixed in 1:1 ratio. At the end of 50 generations of co-culturing, wild type and the mutant cells were enumerated by counting tetracycline resistant and sensitive cells. The 1:1 proportion of the mutant and wild type cells being recovered at the end of 50 generations of co culturing, and lack of any growth defect of the mutant [(generation time being same for the wild type and the *rumA* deletion mutant (Table 6.4)] means that the *rumA* gene is inessential and without any effect on growth rate of the mutant.

Table 6. 4: Growth competition between wildtype and Δ*rumA1* mutant in co-culture experiment

No. of generations	Total number of colonies on LA	Growth on (LA) KP1.1(Δ*rumA1*)	Growth on (LA+Tet) KP1.2
21	250	118	132
42	114	54	60
63	492	244	248

Persaud C.*et al*. created *rumA* mutant by deletion of *rumA* ORF; the mutant shows no growth defect and is also not changed in antibiotic resistance pattern. This result led them to pronounce *rumA* as dispensable. However, this *rumA* mutant strain is outcompeted by wild type strain suggests that this modification improves ribosome performance (Persaud *et al.*, 2010). In our study, we attempted site directed mutagenesis of catalytic amino acid Cys389 and isolated Δ*rumA1* allele which was also an inadvertent additional 131 bp deletion in *rumA* gene. This complex mutation was exchanged with the wild type gene. The mutant we obtained does not show any growth advantage in competition experiment. The apparent disparity between these two results could be due to undocumented genetic differences between the strains as explained in chapter 5. We did not investigate the reason of this disparity.

6.3.1.1.4 Δ*rumA* mutant is amorphic *relA* mutant too

Though the deletion is without the phenotype in relation to *rumA* gene, and that it did not include either the promoter or the start site of the *relA* gene, it did affect its expression. In effect, the inadvertent deletion of 131 bp brings the promoter *relA*P1 closer to *relA* gene - *relA*P1 which is 177 bp upstream of *relA* (Nakagawa *et al.*, 2006b) is now only 76 bp upstream. The Δ*rumA* mutant, KP1.1, is considerably sensitive to serine hydroxamate when compared to wild type strain, KP1.2 (Figure 6.7, A and B), suggesting deleted DNA to have, and hitherto unrealized, positive regulatory effect on *relA* expression. It is also equally possible that the cloning steps which caused the duplication of the overlap (14 nucleotides) sequence common between the RelAF and RumARPst1, generates stem and loop secondary structure that prevents the ribosome binding site on the *relA* mRNA being used for translation. Indeed the *relA* gene expression is detected at the level of transcript formation, as in RT-PCR the amounts of mutant *relA* are same as in wildtype strain, however the mutant RelA protein is undetectable in Western analysis. This could also be due to mutation in *relA* gene since XT-20 DNA polymerase (Banglore Genei) was

used during cloning of the *relA* gene (The gene sequence will confirm our prediction and is being done). The *relA* allele generated by this strategy is designated as a *relA2009*. As described in chapter 5, *relA2009* has a same phenotype as null allele of *relA* (Metzger *et al.*, 1989) and lacks any residual activity when compared to *relA1* mutation in MC4100 strain. The serine hydroxamate sensitivity of Δ*rumA* mutant KP1.1 is corrected, expectedly, by expression of *relA* gene under araP_{BAD} promoter (pTE4). Thus the Δ*rumA1* mutant, KP1.1 is amorphic *relA* mutant too.

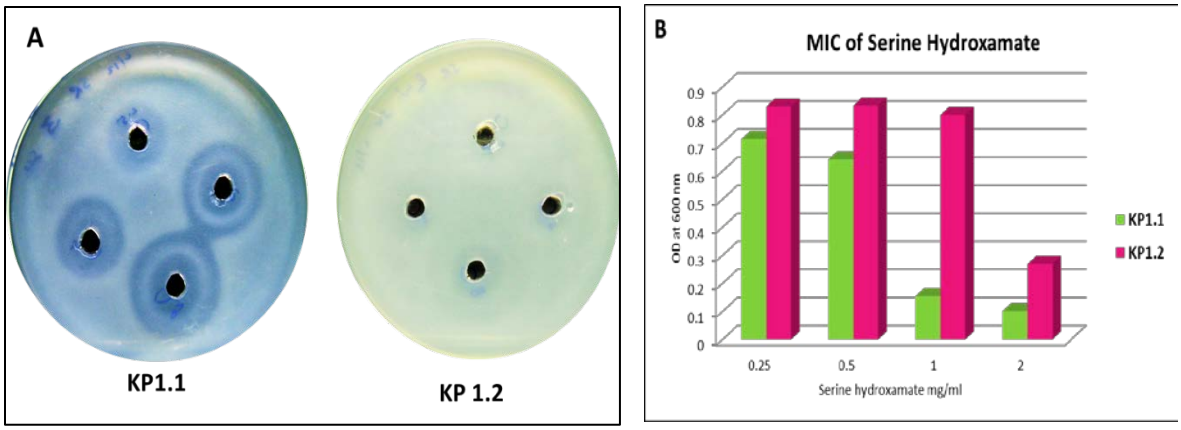


Figure 6. 7: Serine hydroxamate sensitivity of *relA2009* mutant. (**A**) Zone of inhibition by serine hydroxamate at different concentration on the lawns of KP1.1 (Δ*rumA1*) and KP1.2 (wildtype) strains. **(B)** MIC for serine hydroxamate for each of the two strains above.

6.3.1.2 Insertional mutation in *rumA* gene

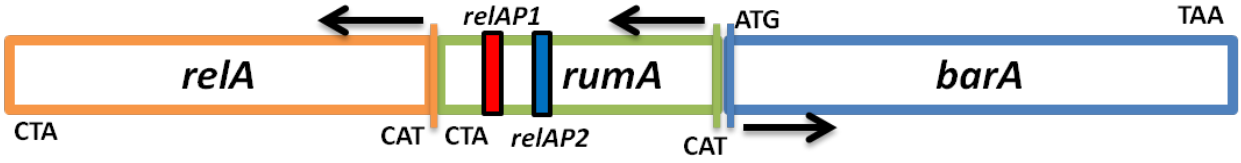


Figure 6.1: Arrangement on *E coli* chromosome of *relA*, *rumA* and *barA* genes. The arrow indicates direction of transcription. The ORF is demarcated by ATG/TAC and TAA/ATC. The filled boxes represent *relA* promoters.

The three genes, *rumA, relA & barA*, define independent transcription units, nevertheless, the intergenic distance between the ORFs, we believe, is too small to accommodate the promoter DNA of each of the genes; 56 bp to be precise, between ORFs of *rumA* and *barA* and 48 bp between *rumA* and *relA* genes, given that the +1 start site of neither *rumA* nor *barA* is mapped. *barA* is situated upstream of *rumA* divergently transcribed in relation to *rumA* from promoter that

also must be in *rumA* gene, though it is as yet not mapped. *relA* gene is situated downstream of *rumA* gene and transcribed in the same direction as *rumA* with the two σ-70 dependent promoters: first, the major promoter (*relA*P1) (Metzger *et al.*, 1988) located 178 bp upstream, and the second minor promoter (*relA*P2) 626 bp into *rumA* gene (Nakagawa *et al.*, 2006b). Transcription from the *relA*P1 promoter is constitutive and depends on a UP-element present 40 bp upstream of the transcription start site. In contrast *relAP*2 was inducible and was positively regulated by CRP which is activated by increased cAMP levels during glucose starvation (Nakagawa *et al.*, 2006b). Thus a gene knockout strategy cannot simply be used to create the mutation in the chromosomal *rumA* without the knowledge of the promoter of the *barA* gene, as a knockout mutation, depending on its position in the *rumA* gene can result in its effect on expression of either *relA* or *barA*. (Figure 6.1)

In this section, we describe an insertion of *CAT* cassette at *SalI* site which is 250 bp and at *MluI* site which is 688 bp upstream of *relAP2* promoter in *rumA* gene and at *Bgl*II site in *barA* gene used as a control to study physiological role of RumA.

6.3.1.2.1 Integration of *CAT* cassette into the chromosome using two different methods: (i) through recombineering in DY330 strain (ii) through pKD helper plasmid

6.3.1.2.1.1 Recombineering in DY330 strain (Yu et al., 2000)

The purpose of using DY330 strain is that it contains well characterized λ Red prophage recombination system which has an efficient and higher rate of recombination. The modified λ prophage harbors *red*-α, *red*-β (*bet*), *gam*, *exo* recombineering genes under the control of temperature sensitive λ cI857 repressor. The higher level synthesis of recombineering genes from p_L operon can be easily turned on at 42°C & off at 30°C for definite period of time to increase phage recombination. At 42°C λ is expressed and becomes more recombinogenic for assimilation of linear DNA into cells. Gam inhibits the RecBCD nuclease from attacking linear DNA, Exo and Beta contains recombination activity for that linear DNA. The most important feature of this system is that it requires short DNA homology upto 30-50 bp at the end of linear DNA. The other homologous recombination system in *E. coli recBC* derivatives has been used to generate recombinants; however the resultant recombinants are very rare and require ~1000 bp of homology of linear DNA (Oliner *et al.*, 1993, Takahashi *et al.*, 1993). After the recombination of

mutant allele in DY330 cells, the mutation has been transduced in any other strain background through P1 transduction.

6.3.1.2.1.2 Recombineering using pKD46 helper plasmid (Datsenko & Wanner, 2000)

pKD46 is a low copy number helper/portable plasmid containing Red recombinase system of λ. *gam*, *β* and *exo* are continuously expressed from arabinose inducible P_{ara} promoter. This helper plasmid can be easily driven out from the cell at 37°C as it has a temperature sensitive replicon (ori^{ts}). The advantage of this method is proficient recombination which uses as less as ~36 bp homology extension at the end of linear DNA and that gene disruption can be carried out in the strain background of one's choice.

Linear DNA was prepared for electrotransformation by PCR. (1) 2.3 kb *rumASal*I::*CAT* DNA and *rumAMlu*I::*CAT* DNA were amplified from pTE15 and pTE16 respectively using RumAF1-RumARSac1 primers (2) 1.6 kb *barABgl*II::*CAT* DNA was amplified from pTE17 using RumAF3-BarAF primer pair. Reciprocal recombination of each of the linear DNA with the host chromosome using two elegant homologous recombination systems of engineered DY330 strain and pKD46-helper plasmid was affected according to method described in section 6.2.5.1 and 6.2.5.2. Insertion mutation in each case was transferred in fresh strain background by P1 transduction. P1 lysate was prepared on DY330 mutants containing *rumASal*I::*CAT*, *rumAMlu*I::*CAT* and *barABgl*II::*CAT* insertions and transduced each into MC4100 and MG1655 strain background through P1 transduction.

rumA gene specific primers RumAF1-RumARSac1 were used for demonstrating the presence of *rumASal*I::*CAT* insertion in strains KP8, KP24 and *rumAMlu*I::*CAT* insertion in KP9, KP25 which generates 2335 bp amplicon (Figure 6.8). *barABgl*II::*CAT* (KP10, KP26) insertion yields 1669 bp amplicon in PCR using RumAF3-BarAF primers (Figure 6.8).

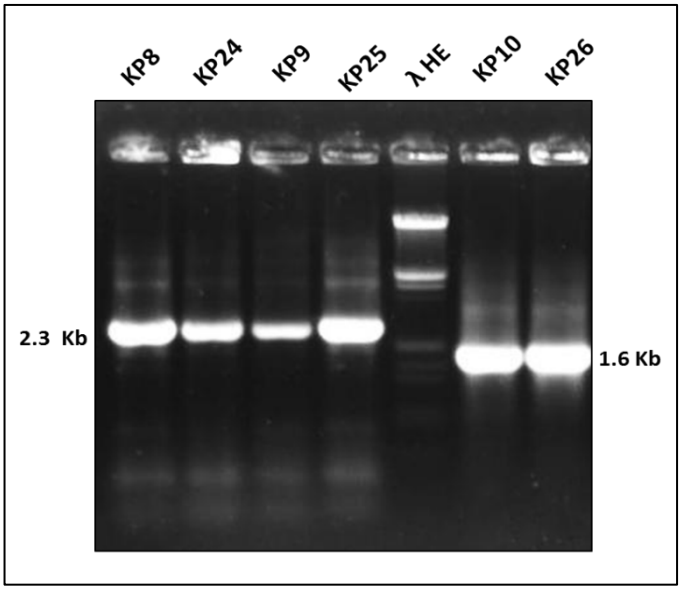


Figure 6. 8: Confirmation of *CAT* cassette insertion in *rumA* and *barA* by PCR amplification. KP8, KP9 and KP10 are derivatives of MC4100 containing *CAT* insertion in *rumA* at *Sal*I and *Mlu*I and in *barA* respectively. KP24, KP25 and KP26 represents MG1655 derivatives containing *CAT* insertion in *rumA* at *Sal*I and *Mlu*I and in *barA* respectively.

6.3.1.2.2 Determination of orientation of the *CAT* cassette in *rumA* gene

The orientation of *CAT* cassette was confirmed by PCR. The PCR reaction was performed on the genomic DNA isolated from *rumASal*I::*CAT* and *rumAMlu*I::*CAT* mutants. RumAF1 primer was tested with both CATFSalI/MB and CATRSalI/MB. The product of 1.4 Kb was generated only with primer pair RumAF1 (1) and CATRSalI (4) in *rumASal*I::*CAT* mutant not in *Mlu*I mutant (Figure 6.9, B). 1.0 Kb amplicon was produced with RumAF1 (1) and CATFMB (5) in *rumAMlu*I::*CAT* mutant and not with RumAF1 (1) and CATRMB (6) primer pair (Figure 6.9, C). This result indicates that *CAT* gene's transcription is in the same direction as that of *rumA* in *rumASal*I::*CAT* mutant. The orientation of *CAT* gene at *Mlu*I site of *rumA* is opposite to that of *rumA* gene's direction of transcription/translation (Figure 6.9, A).

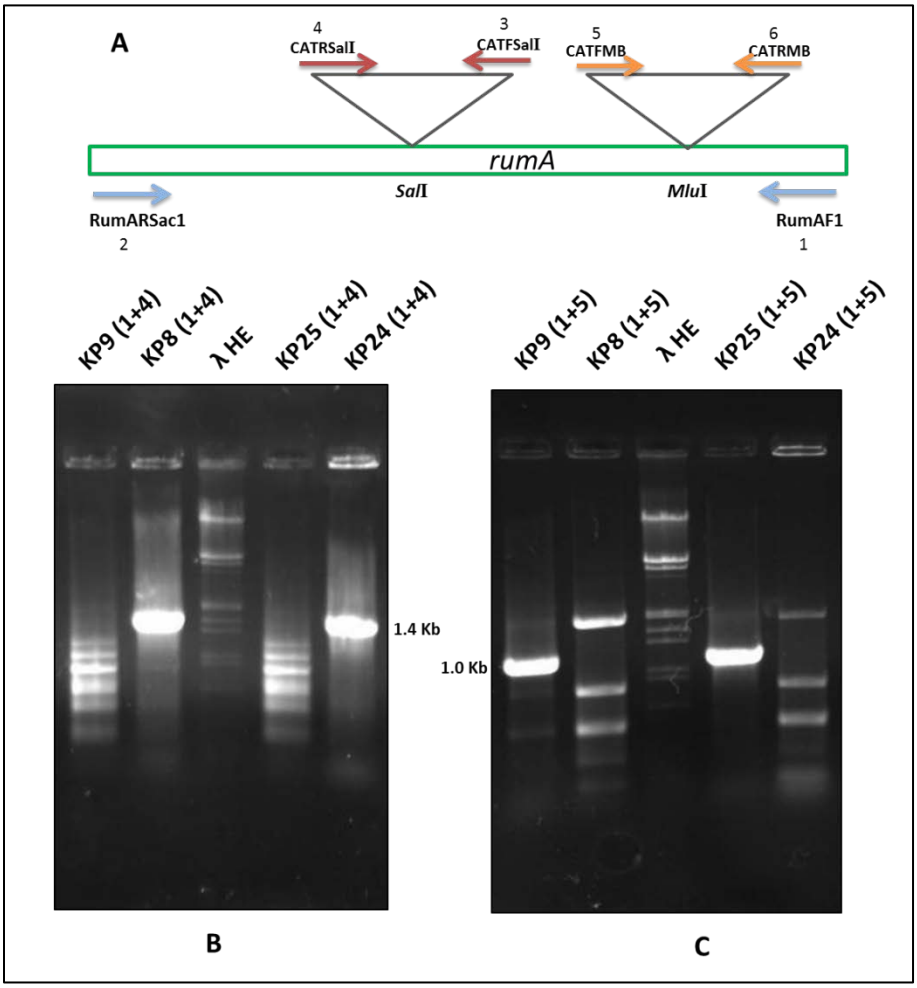


Figure 6. 9: Determination of orientation of *CAT* insertion in *rumA* gene at *SalI* and *MluI*. **(A)** Cartoon indicates the position of primers used in PCR. **(B)** The productive primer pair was 1+4 produced amplicon 1.4 Kb in *rumASal*I::*CAT* (KP8 and KP24). **(C)** The 1+5 productive primer pair generated 1.0 Kb amplicon in *rumAMlu*I::*CAT* (KP9 and KP25).

6.3.2 Phenotypic Characterization studies

6.3.2.1 Growth phenotype of *rumASal*I::*CAT* mutant of MC4100

*rumASal*I::*CAT*, *rumAMlu*I::*CAT* and *barABgl*II::*CAT* insertions were each transduced in MC4100 and MG1655 strains by P1 transduction using resistance to the drug chloramphenicol. *relA1* and *relA2009* Δ*rumA1* mutations were each introduced by P1 transduction in the same two strains using linked *cysI*::Tn*10*kan marker. The mutants of each of the two strains, for example, KP8 (MC4100*relA*$^+$ *rumASal*I::*CAT*), KP4 (MC4100 *relA*$^+$ *rumA*$^+$), MC4100KP (*relA1 rumA*$^+$) and KP7 (MC4100 *relA2009* Δ*rumA1*) panel of strains of MC4100 and similar panel constructed of MG1655 were tested for growth on nutrient rich medium (LA) as well as on nutrient poor medium (Minimal glucose medium) plates and grown at different temperatures.

Of all the mutants, we observed **striking growth phenotype** for KP8 (*MC4100relA*$^+$ *rumASal*I*::CAT*) strain. It exhibits severe growth retardation on nutrient poor minimal medium plate but less difference in growth on nutrient rich LA plate at 37°C. As discussed in chapter 5, we addressed the slow growth phenotype of KP4 (*relA*$^+$ *rumA*$^+$), KP5 (MC4100 *relA*$^+$ *ΔbarA784*::*KAN*), KP6 (MC4100 *relA*$^+$ *ΔgudD785*::*KAN*) and KP10 (MC4100 *barABgl*II::*CAT*) in comparison to MC4100KP strain, however, the KP8 mutant was unable to form colonies at least for ~36 hrs on minimal medium (Figure 6.10). We were surprised to observe that the growth phenotype of KP9 (*rumAMlu*I::*CAT*) was unaltered and same as that of KP4 on LA as well as on minimal medium (Figure 6.10).

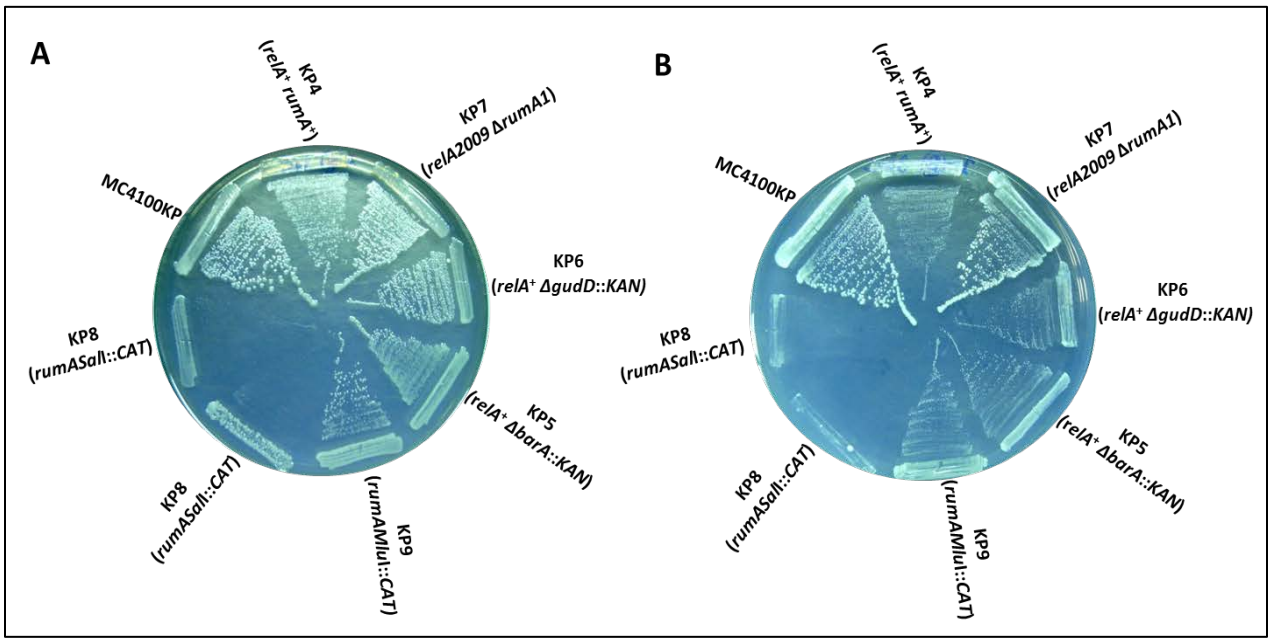
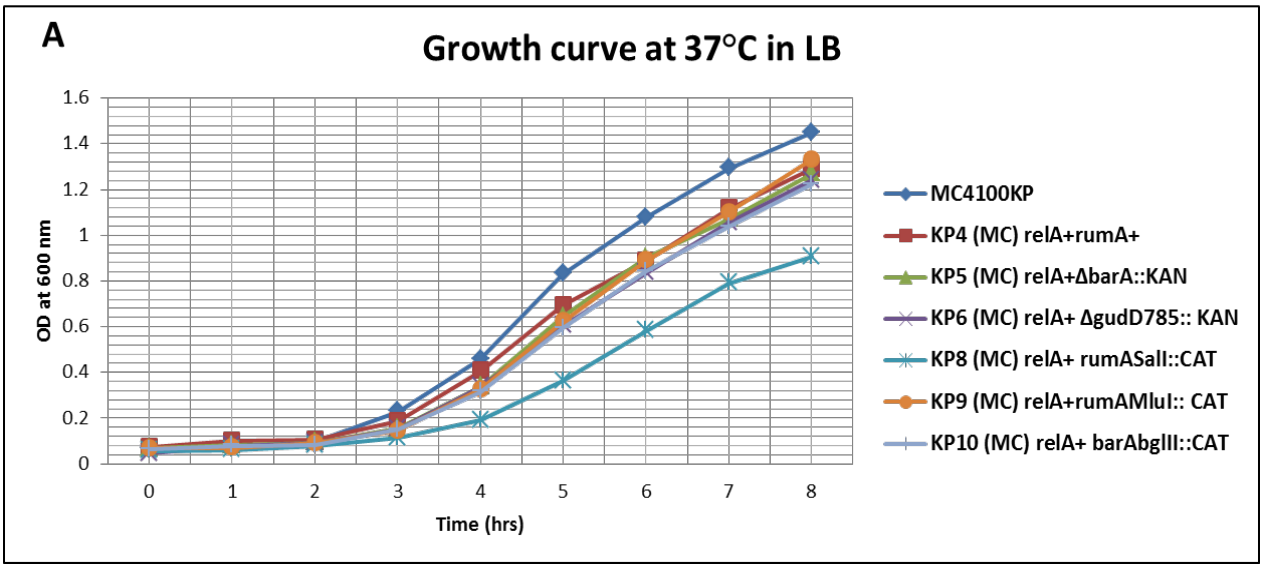


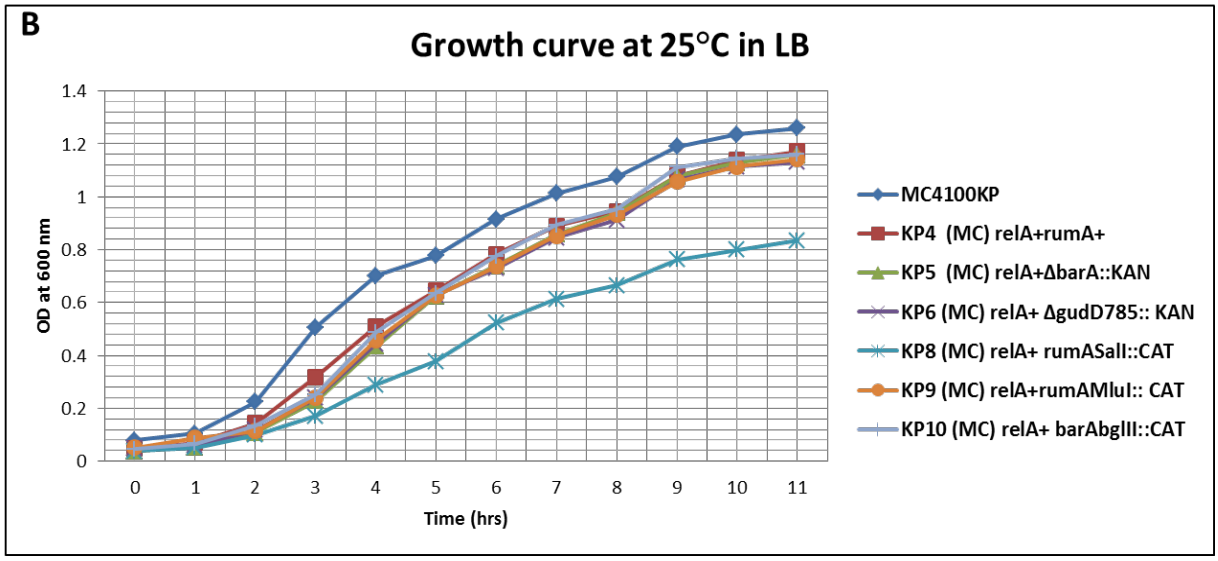
Figure 6. 10: Slow growth phenotype of *rumASal*I::*CAT* (KP8) mutant on minimal agar plates. The indicated strains were grown at 37°C **(A)** and at 30°C **(B)** for 24 hrs. Strains used here are MC4100 derivatives.

The *CAT* insertion either at *Sal*I or *Mlu*I site in *rumA* both disrupts ORF of *rumA*. Unlike *CAT* gene at *Sal*I site which almost splits the *rumA* gene into two halves, *CAT* gene at *Mlu*I site in *rumA* is present between 12-13 amino acids from the N-terminal end of RumA protein. We considered the possibility that the RumA Protein retains the activity; the precedence to this presumption is the *relA1* mutation retaining 1% of RelA activity. The IS*2* insertion in *relA* gene can be providing a possible Shine-Dalgarno sequence together with an AUG start codon and 8 additional amino acids that are in-frame with the carboxyl-terminal three-fourths of the RelA

protein (Metzger *et al.*, 1988). Examination of the sequence in the *CAT* gene from the end containing the stop codon of *CAT* gene represented by CATRMB primer revealed no ATG in the same reading frame as that of *rumA* ORF. Thus, RumA protein is likely to be nonfunctional in each of the two mutants. The fact that *rumAMlu*I::*CAT* mutant is not affected for growth indicates that *rumA* is dispensable for growth and viability. This result is further substantiated by the failure of *rumASal*I::*CAT* mutation to be complemented by pTE5 (pBBR1MCS2 *rumA*$^{+}$) plasmid. The inference of this result is that *CAT* insertion at *Sal*I site in *rumA* represents a *cis*-acting mutation affecting growth and not due to inactivation of *rumA* gene function. The implication of *rumASal*I::*CAT* mutation on growth is discussed below.

We measured steady state growth rates of different mutant derivatives of MC4100 in Minimal media at 37°C and in LB at 25°C and 37°C. It is important to note that KP8 mutant grows slow even in LB at 25°C and 37°C (Table 6.5).





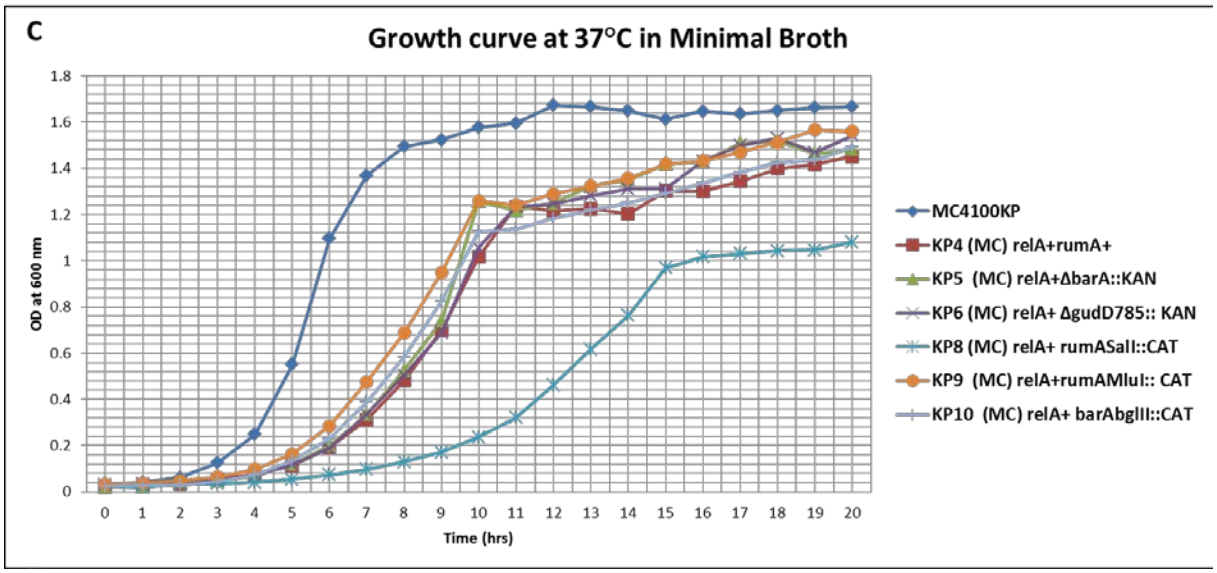


Figure 6. 11: Growth rate measurement in different media and temperature of *rumASal*I::*CAT* mutant and other derivatives. The growth curve of MC4100 strains was generated in LB at 37°C **(A)**, 25°C **(B)** and minimal at 37°C **(C)** by monitoring OD at 600 nm spectrophtometrically. The graphical data is an average of three independent experiments.

Table 6. 5: Doubling time and growth rate of MC4100 and its derivatives

Strains	Doubling time / Growth rate*		
	LB at 37°C	**LB at 25 °C**	**MB at 37°C**
MC4100KP	65.6 / 0.636	80.6 / 0.516	81.9 / 0.51
KP4	89.9 / 0.462	124 / 0.336	103 / 0.402
KP5	77.39/ 0.54	120 / 0.348	93.4 / 0.444
KP6	81.3/ 0.51	126 / 0.33	104 / 0.402
KP8	109.9/ 0.378	165 / 0.252	169 / 0.246
KP9	75.7 / 0.552	128 / 0.324	120 / 0.348
KP10	78 / 0.534	128 / 0.324	111 / 0.372

*Values represent the growth rates (μ, h^{-1}) of exponentially growing cells in LB and MB.

6.3.2.2 Growth phenotype of *rumASal*I::*CAT* mutant of MG1655

The growth defect phenotype credited to KP8 (MC4100*relA*$^+$ *rumASal*I::*CAT*) is not observed with KP24 (MG1655 *rumASal*I::*CAT*) under all conditions of growth. Other mutants in the panel of MG1655 like *relA2009* Δ*rumA1* and *relA1* mutants are unaffected for growth under all conditions. The growth of mutant strains is comparable to MG1655 parent. The reason behind the lack of phenotype is presence of *spoT*$^+$ allele in MG1655 which was also the important reason for ppGpp related difference between MG1655 and MC4100 strains (Chapter 5).

6.3.2.3 The effect of *barA* and *uvrY* mutation in KP4 (MC4100 *relA*$^+$)

The stringent response and carbon starvation systems are quiet integrated. BarA/UvrY TCS (two component system) has a positive effect on expression of small RNAs *csrB* and *csrC* which titrate *csrA* and indirectly translationally activate *relA* as it has multiple binding sites for *csrA* in the region of the RNA which includes SD and AUG (Edwards *et al.*, 2011).

The effect of transfer of mutations in *barA* and *uvrY* were tested in KP4. Each of the mutation did not affect growth of KP4 on minimal medium (data not shown). The growth on starvation plate was also unaffected. The phenotype of *rumASal*I::*CAT* mutant cannot be reproduced by *uvrY-barA* dependent *csrB/C* mediated effect on *csrA* which directly regulates translation of *relA* (Edwards *et al.*, 2011).

6.3.2.4 P1 transduction mediated exchange of *spoT* alleles between MC4100 and MG1655 backgrounds

(i) The *spoT1* allele of MC4100KP was first linked to *ΔpyrE748*::*KAN* (KP11) marker (~67% cotransduction) followed by its transfer into MG1655 and KP24 strains through P1 transduction

generating KP29 (*spoT1 ΔpyrE748*::*KAN*) and KP30 (*rumASal*I::*CAT spoT1 ΔpyrE748*::*KAN*) respectively. *pyrE*$^+$ derivatives (KP31 (*spoT1*) and KP32 (*spoT1 rumASal*I::*CAT*) of each of these two strains were constructed by P1 transduction by selection for growth on minimal medium in the absence of uracil.

(ii) Reciprocally, the *spoT*$^+$ allele of JW3617-1 was transferred into KP4 (MC4100 *relA*$^+$ *rumA*$^+$) and KP8 (*rumASal*I::*CAT*) strains producing KP35 (*spoT*$^+$ *ΔpyrE748*::*KAN*) and KP36 (*rumASal*I::*CAT spoT*$^+$ *ΔpyrE748*::*KAN*). The *pyrE*$^+$ derivatives are called respectively KP37 (*spoT*$^+$) and KP38 (*rumASal*I::*CAT spoT*$^+$).

6.3.2.4.1 Effect of exchange of *spoT* alleles on phenotypes of KP4 (MC4100 *relA*$^+$) and MG1655

The introduction of *spoT1* allele in MG1655 (KP31) was shown to increase basal level of ppGpp (Spira *et al.*, 2008) and is being monitored. The growth of KP31 as compared to KP4 (MC4100 *relA*$^+$ *rumA*$^+$) on minimal medium was not as retarded as was expected if the *spoT1* allele is wholly responsible for the slow growth of KP4 (Figure 6.12 A). On the contrary, *spoT*$^+$ transductant of KP4 (KP37) is same as MG1655 when compared for growth in minimal medium (Figure 6.12). This result is highly significant in indicating extra genetic mutations besides *spoT* being responsible for the differences between MC4100 and MG1655. A similar conclusion was arrived at when RpoS (stationary phase σ factor) levels were compared between *spoT*$^+$ derivative of MC4100 and MG1655. The protein amount of RpoS is lower in MG1655 background than in MC4100 (Spira *et al.*, 2008).

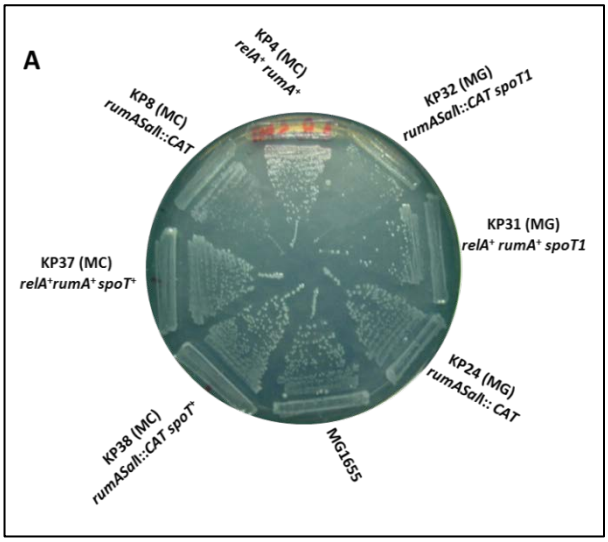
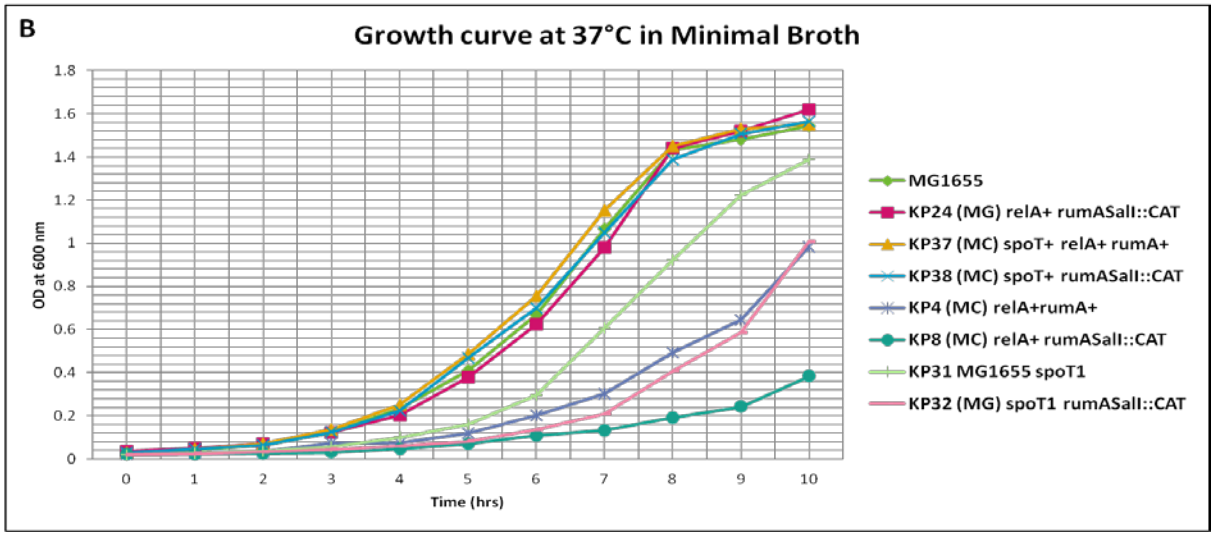


Figure 6. 12: Swapping of *spoT* alleles between MC4100 and MG1655 strains. (A) The growth on minimal agar plate of MG1655 *spoT1* and MC4100 *spoT*$^{+}$ derivatives **(B)** The growth curve of same set of strains used in the plate.



6.3.2.4.2 Effect of exchange of *spoT* alleles on *rumASalI::CAT* phenotype in MC4100 and MG1655 background

As discussed above, the growth of KP24 (MG1655 *rumASal*I::*CAT*) was unaltered on minimal medium in comparison to that of KP8 (MC4100*relA*$^{+}$ *rumASal*I::*CAT*). The growth of KP32 (MG1655 *spoT1 rumASal*I::*CAT*) on minimal medium was not as much retarded as that of KP8 (Figure 6.12 A), suggesting that one of the reason behind this phenotype is *spoT1* allele of MC4100. KP32 was significantly impaired in growth in comparison to KP31 (MG1655 *spoT1*) but not as severely as KP8. Extragenic mutational differences between the two backgrounds strains could be implicated here.

Introduction of *spoT*$^+$ allele in KP4 and KP8 generated KP37 (MC4100 *spoT*$^+$) and KP38 (MC4100 *spoT*$^+$ *rumASal*I::*CAT*). Each of these two strains grew same as MG1655 and its derivatives on minimal medium (Figure 6.12 A).

The growth phenotype on minimal plate was confirmed by measuring growth rates of the mutants following swapping (Figure 6.12 B; Table 6.6)

Table 6. 6: Doubling time and growth rate of different mutants

Strains	Doubling time	Growth rate*
MG1655	84.04	0.492
KP24	95.5	0.438
KP4	108	0.384
KP8	141	0.294
KP31	98.3	0.426
KP32	135	0.306
KP37	78.5	0.528
KP38	77.3	0.540

*Values represent the growth rates (μ, h^{-1}) of exponentially growing cells in MB at 37°C

In summary, reciprocal exchange of *spoT1* and *spoT*$^+$ alleles between MC4100, MG1655 and their derivatives suggest that MC4100 background potentiates the effect of *spoT1* mutation; in contrast *spoT*$^+$ effect is same in both MC4100 and MG1655 background. It appears that extragenic mutation(s) in MC4100 is(are) relevant to metabolism of intracellular ppGpp pools. SpoT protein is proficient in hydrolysis of ppGpp, can reduce the pool in MC4100 which is otherwise high even when *relA*$^-$ (Spira *et al.*, 2008) (the measurement of ppGpp are being done). It would be interesting to find out and identify 'the other genetic effects' on ppGpp pool besides *relA* and *spoT*. There is basis for speculating/proposing extragenic involvement in ppGpp pool control. *relX* and *relS* mutation have been identified to affect accumulation of ppGpp (Pao & Gallant, 1978, Ball *et al.*, 1979). In the context of the result above, we seem to have a strategy to identify/clone the 'extragenenic genes'. Genomic library of KP31 (MG1655 *spoT1*) could be used to select/identify the cloned DNA's ability to suppress/correct the severe slow growth defect of KP8 (MC4100 *relA*$^+$ *rumASal*I::*CAT*). Transfer of *spoT1* across different strains results in increase in ppGpp levels; conversely ppGpp levels are low in *spoT*$^+$ strain. The fact that effect of *rumASal*I::*CAT* on growth is *spoT* allele dependent - s*poT1* is required for manifestation of phenotype while *spoT*$^+$ allele abolishes it means that growth inhibition effect is ppGpp

dependent. We prove that this is indeed the case with isolation of extragenic inactivating suppressor mutation in *relA* which relieves the slow growth phenotype.

6.3.2.5 Assessment of *relA* phenotype of *rumA*::*CAT* mutation in MC4100 and MG1655 background

The *relA* phenotype of KP4 (MC4100 *relA*$^+$) and MG1655 have been described in detail in chapter 5. It is interesting to note that KP4 grows better on minimal plate supplemented with 3-AT (Figure 6.13 A) than on minimal alone (Figure 6.10 B). MG1655 does not show any distinct difference in the growth on minimal plate with and without supplementation of 3-AT.

Growth of KP8 (MC4100 *relA*$^+$ *rumASal*I::*CAT*) mutant was extremely slow and unaffected by whether the minimal plate contained 3-AT supplementation or not (Figure 6.13 A). It is as if the stringent response is not expressed. Growth is apparently frozen on minimal agar as well as on amino acid starvation plate.

The *relA* phenotype in terms of growth on 3-AT plate of *rumASal*I::*CAT* derivative of MG1655 (KP24) is same as of MG1655.

Following reciprocal swapping of *spoT* allele between MC4100, MG1655 and their derivatives the *relA* phenotype was assayed of these mutants. Growth of KP31 (MG1655 *spoT1*) is almost the same on minimal (Figure 6.12 A) as well as minimal plate containing 3-AT (Figure 6.13 B). Interestingly, KP32 (MG1655 *spoT1 rumASal*I::*CAT*) and KP8 growth characteristics are comparable on 3-AT supplemented minimal plates (Figure 6.13 B).

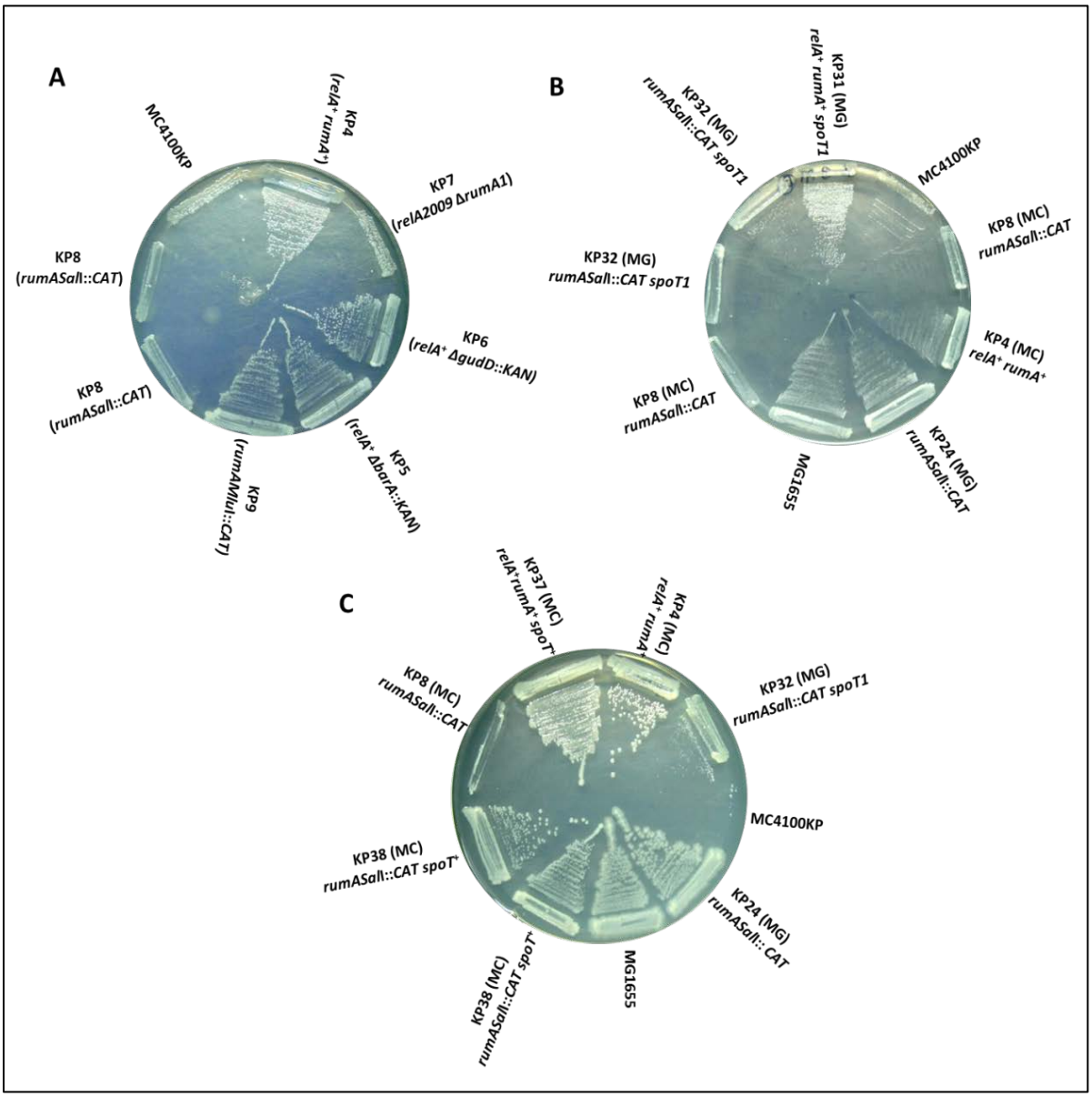


Figure 6. 13: Assessment of *relA* phenotype. The growth was monitored of different mutants on minimal agar plates supplemented with 3-AT. **(A)** Phenotype of *relA* and *rumA* mutants of MC4100**, (B)** Growth of MG1655 *spoT1* allele and their derivatives. **(C)** Growth of MC4100 *spoT*$^{+}$ and their derivatives

6.3.2.6 Isolation of spontaneous suppressors of slow growth phenotype of *rumASal*I::*CAT* mutant

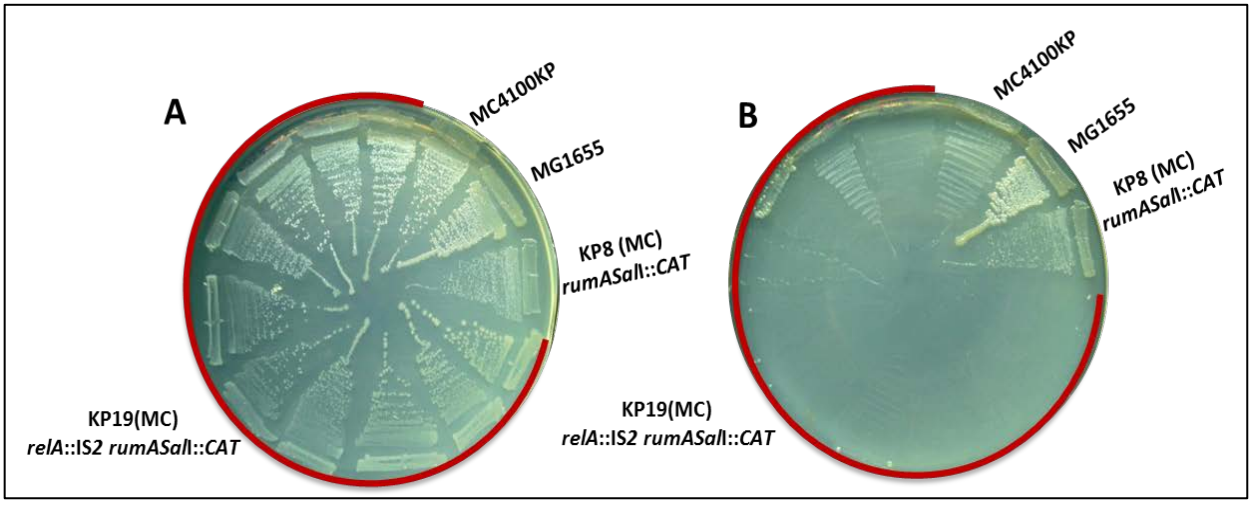


Figure 6. 14: Growth of suppressors of *rumASal*I::*CAT* mutant. (A**)** on minimal agar plate and **(**B**)** on minimal agar plate containing 3-AT. The red line encircling the sectors shows growth of suppressors.

*rumASal*I::*CAT* mutant's growth was affected severely on minimal medium to the extent that this growth defect could be used in selection. Fast growing colonies were often seen growing on the plate within 24 hours of incubation. We assayed their *relA* phenotype on 3-AT plate. The fast growing colonies almost always had mutation in *relA* and won't grow on starvation plate (Figure 6.14 B). These suppressors grow normally as MC4100 (*relA1*) on minimal medium (Figure 6.14, A). Thus these suppressors are indeed extragenic and were phenotypically *relA*$^-$. Further, we transduced *relA1* mutation from MC4100KP to KP8 (*rumASal*I::*CAT*) in P1 transduction using the linked *cysI*::Tn*10kan*. The Kan^r transductants were plated on minimal plate supplemented with cysteine and chloramphenicol. One out of 10,000 transductants was *relA1*, fast growing and unable to grow on starvation plate (3-AT/SMG) (Figure 6.14, B). Presence of *relA1* (*relA*::IS*2*) and *rumASal*I::*CAT* mutations was confirmed by PCR (data not shown).

6.3.2.7 KP8 (MC4100 *relA*$^+$ *rumASal*I::*CAT*) cannot be transformed by multiple copy of *relA*$^+$ plasmid

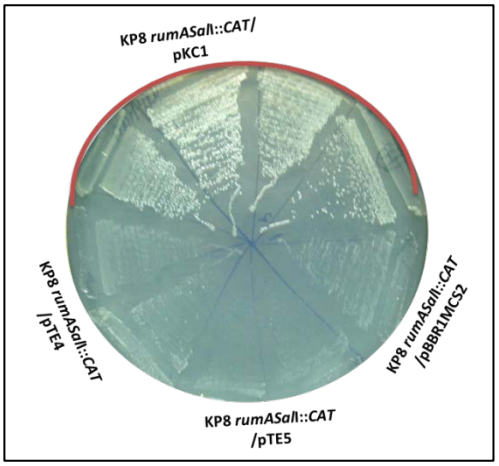
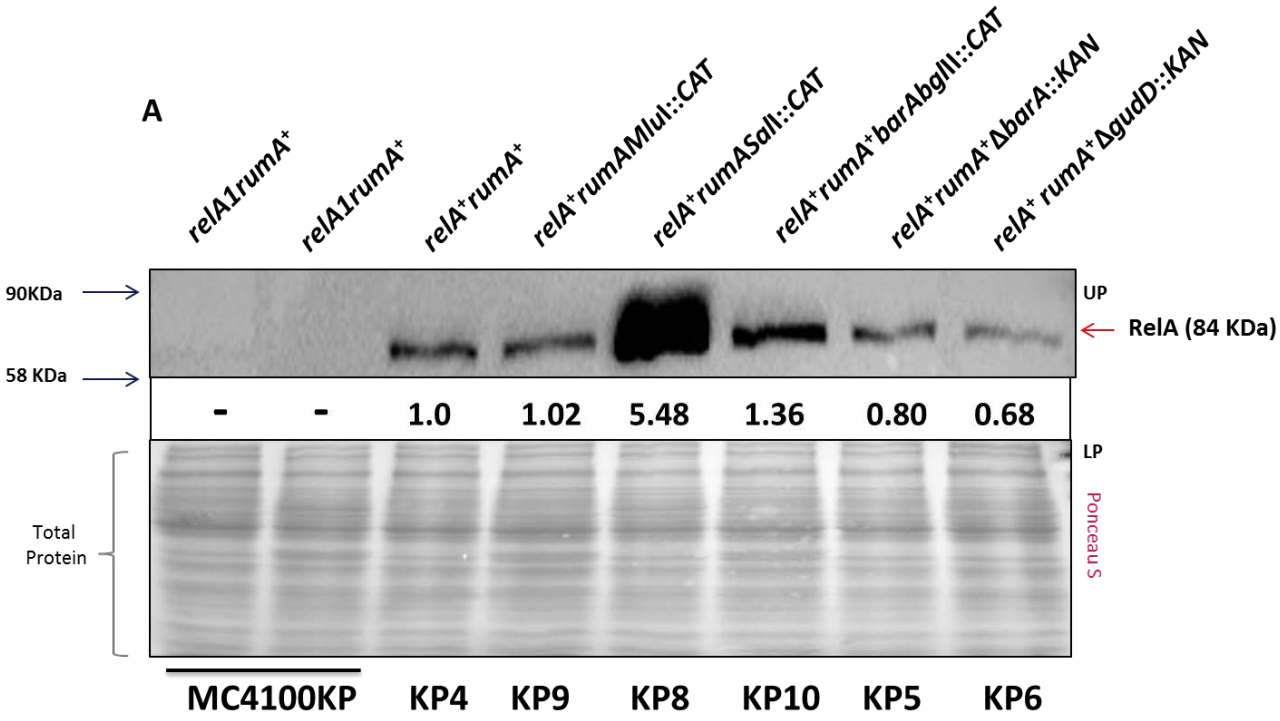


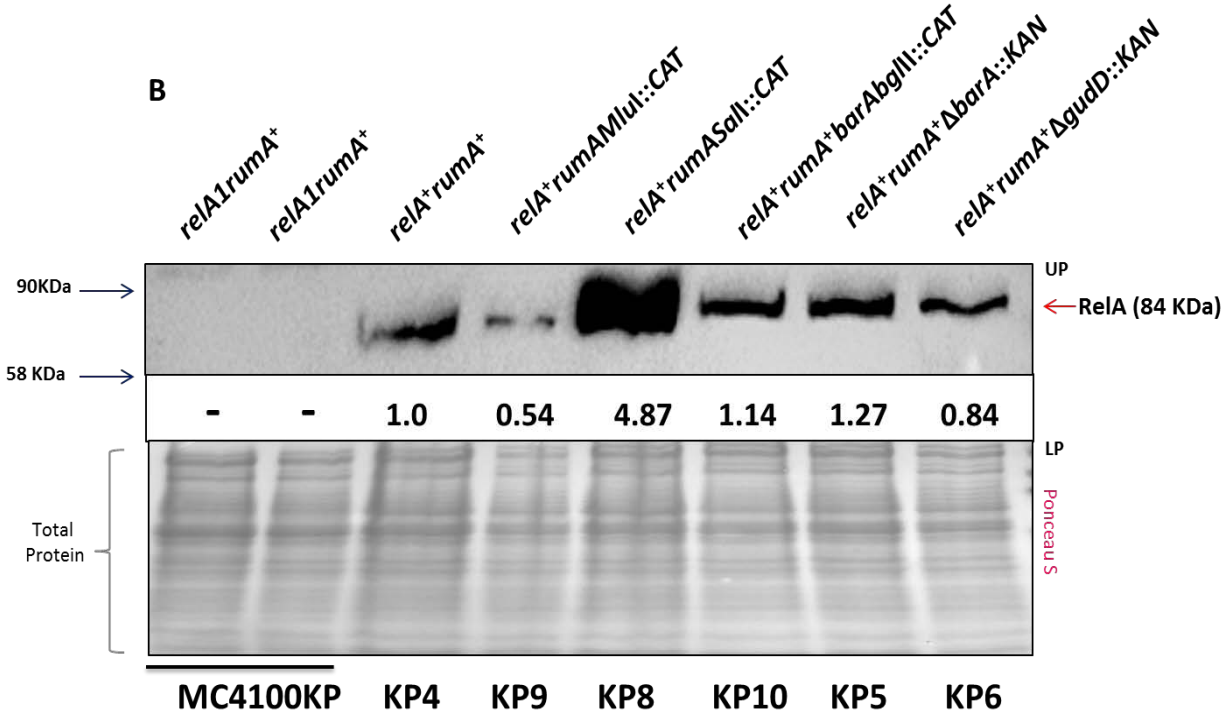
Figure 6. 15: Complementation of *rumASal*I::*CAT* mutant by pKC1 plasmid. The red line encircling shows growth of pKC1 transformants on minimal agar plate.

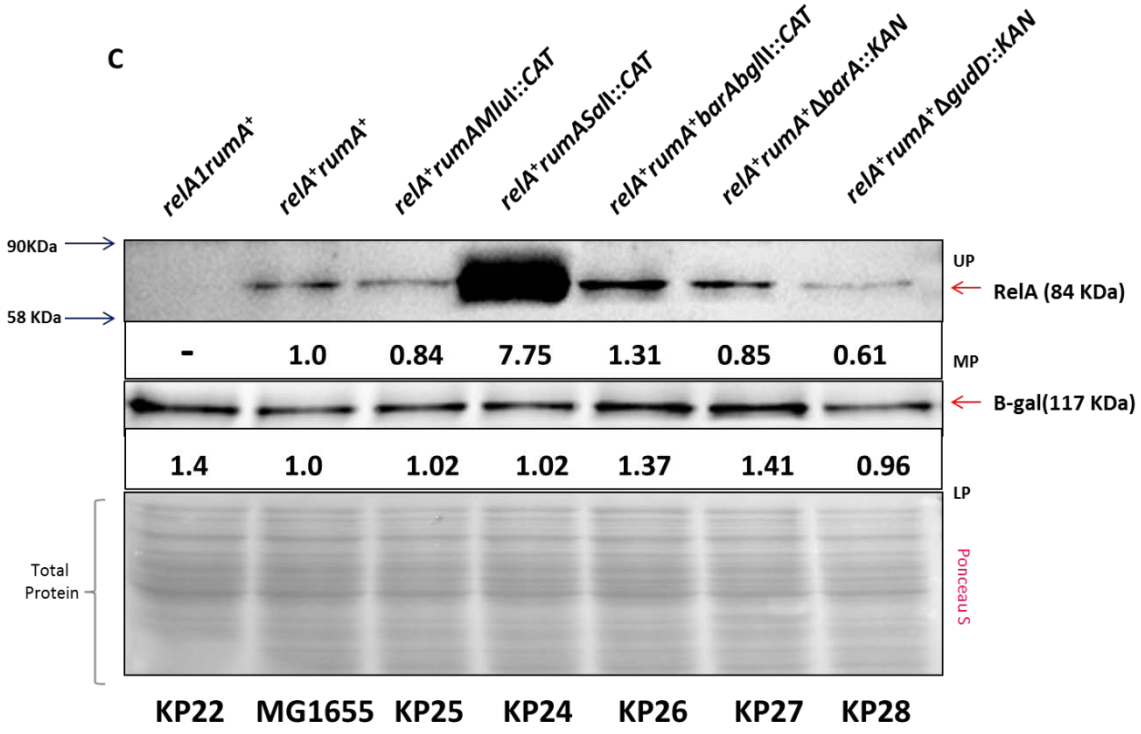
The multiple copy of *relA*$^+$ plasmids, (a) pTE1ΔBgl (*relA*$^+$ *rumA*$^+$ in pBBR1MCS2); (b) pTE1ΔAse (*relA*$^+$ in pBBR1MCS2) and (c) pTE6 (*relA*$^+$ *rumA*$^+$ in pBAD18Kan), were not able to form colonies in KP8 after transformation. The first former two plasmids is a derivative of low copy (~8copies/cell) pBBR1MCS2, whereas latter plasmid is derivative of pBAD18Kan (~20copies/cell). The *relA* DNA was confirmed to be wild type *relA* by DNA sequencing. None of the other strains has problems maintaining these plasmids including KP4 (MC4100 *relA*$^+$). The rare spontaneous KP8 transformants of pTE1ΔBgl that grew to form colonies harbored mutation in a *relA* gene of the plasmid. Two mutant plasmids, pKC1 and pKC2 were further analysed. Unlike pTE1ΔBgl, pKC1 and pKC2 were able to retransform KP8, revoke the growth defect of KP8 (Figure 6.15), and did not complement MC4100 *relA1* mutation. DNA sequence of *relA* and *rumA* genes present on two plasmids, pKC1 and pKC2 was determined. (i) The *relA* gene in pKC1 plasmid has a -2 frameshift mutation in the early region i.e 246 bp away from the ATG of the *relA* sequence (Appendix I) The mutation is expected to disrupt the reading frame of the protein. However, in the BLAST analysis of the sequence, -1 frameshift 12 nucleotide downstream of -2 mutation restores the reading frame. However, RelA protein was not detected in the Western blot of pKC1 plasmid (see below, Figure 6.17). (ii) DNA sequence of *relA* gene in pKC2 contained two substitutions. Since protein is likely to be full length in the latter case and non-functional, we did not use pKC2 in the Western analysis, also because it will be difficult to distinguish the signal for RelA protein arising from genomic copy and plasmid RelA.

6.3.2.8 Measurement of RelA protein by Western analysis

The result that the slow growth phenotype of KP8 (*rumASal*I::*CAT*) insertion mutant is suppressed by *relA*$^{-}$ mutation indicates that the slow growth of KP8 is due to over production/over activation of RelA upon steady state growth in nutrient poor medium which increases ppGpp levels to the extent that it inhibits growth. We thus surmise that level of RelA protein is presumably increased in the insertion mutant. We measured RelA protein amounts in different strains under different conditions of growth in Western Blot using anti-RelA antibody. From the results of densitometric analysis of more than three independent blots, it is clear that amount of RelA protein is 6-7 fold more than the protein amounts in other strains. Importantly, we found RelA protein to be increased in *rumASal*I::*CAT* mutant of both MC4100 (*relA*$^{+}$) and MG1655 background under all conditions of growth (Figure 6.16, A; B; C and D). Notwithstanding this increased amounts, as indicated earlier, the reason of lack of phenotype in MG1655 *rumASal*I::*CAT* mutant is the wild type *spoT*$^{+}$ gene.




B
relA1rumA+
relA1rumA+
relA+rumA+
relA+rumAMlui::CAT
relA+rumASalI::CAT
relA+rumA+barAbglII::CAT
relA+rumA+ΔbarA::KAN
relA+rumA+ΔgudD::KAN
90KDa
58 KDa
UP
RelA (84 KDa)
- - 1.0 0.54 4.87 1.14 1.27 0.84
LP
Ponceau S
Total Protein
MC4100KP KP4 KP9 KP8 KP10 KP5 KP6


C
relA1rumA+
relA+rumA+
relA+rumAMlui::CAT
relA+rumASalI::CAT
relA+rumA+barAbglII::CAT
relA+rumA+ΔbarA::KAN
relA+rumA+ΔgudD::KAN
90KDa
58 KDa
UP
RelA (84 KDa)
- 1.0 0.84 7.75 1.31 0.85 0.61
MP
B-gal(117 KDa)
1.4 1.0 1.02 1.02 1.37 1.41 0.96
LP
Ponceau S
Total Protein
KP22 MG1655 KP25 KP24 KP26 KP27 KP28

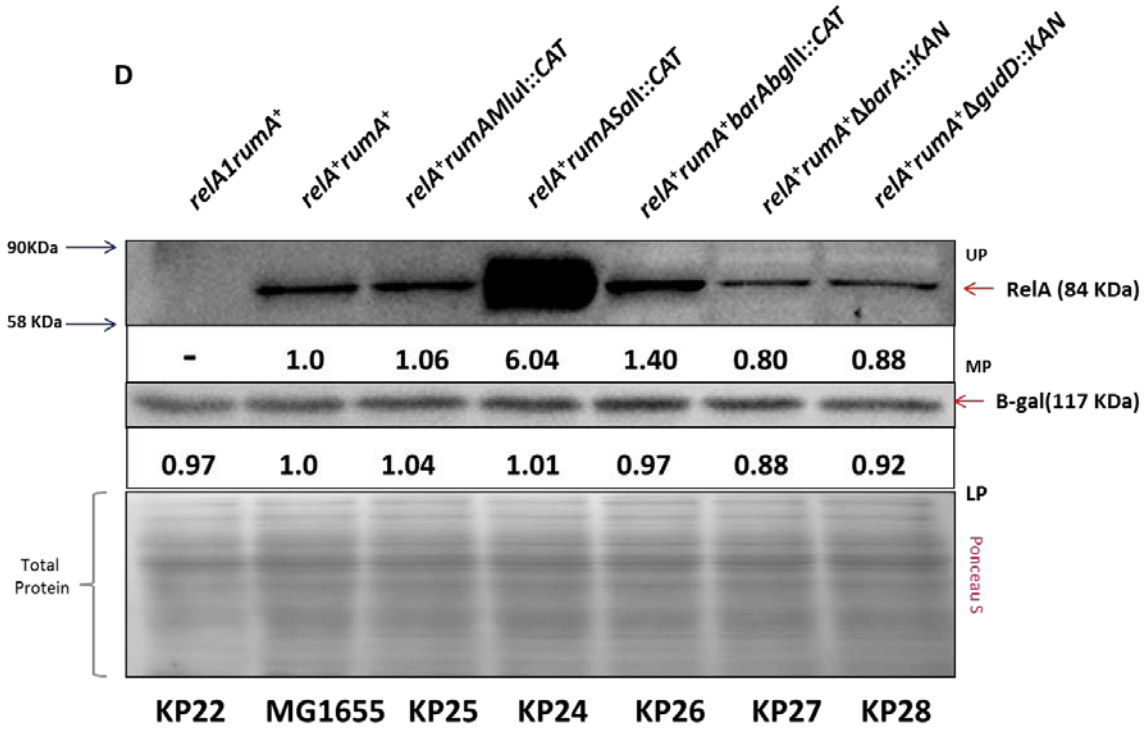


Figure 6. 16: Quantification of RelA protein of KP8 (*rumAsal*I::*CAT*) mutant by Western blotting. RelA protein as measured in MC4100 derivatives grown in LB **(A)** and in MB **(B)** at 37°C; in MG1655 derivatives in LB **(C)** and in MB **(D)**. Upper panel (UP) of each group of strains indicate immunoblot developed by anti-RelA antibody and lower panel (LP) indicate ponceau stained gel. Fold differences in protein levels of KP8 relative to wildtype represents average of the three independent experiments and indicated against each lane. In immunoblots of MG1655 strains **(C** and **D)** the middle panel (MP) shows β-galactosidase protein as internal protein control being detected by anti-β-gal antibody**.**

The slow growth defect of KP8 was suppressed by pKC1 plasmid (*relA*$^-$ *rumA*$^+$) (Figure 6.15). This is correlated quite well with the amount of RelA protein in KP8 being strikingly less in the presence of pKC1 DNA (Figure 6.17), presumably a reason for correction of growth defect of KP8. pKC1 and pKC2 plasmids clearly complemented the growth defect of *rumASal*I::*CAT* mutant most probably due to reduction in RelA protein level, atleast in case of pKC1. The RelA protein levels are comparable to that in single copy state of *relA*$^+$ gene. Unexpectedly, growth on 3-AT plate was same as that of KP8 mutant. We do not understand the reason why stringent response is irreparable. Though not true for pKC1, pKC2 plasmid which contains two substitution mutations, again in the early region of *relA*, can produce functional C-terminal

product capable of oligomerization with functional RelA protein expressed from the genomic copy causing titration of excess of RelA and inactivating it (Gropp *et al.*, 2001).

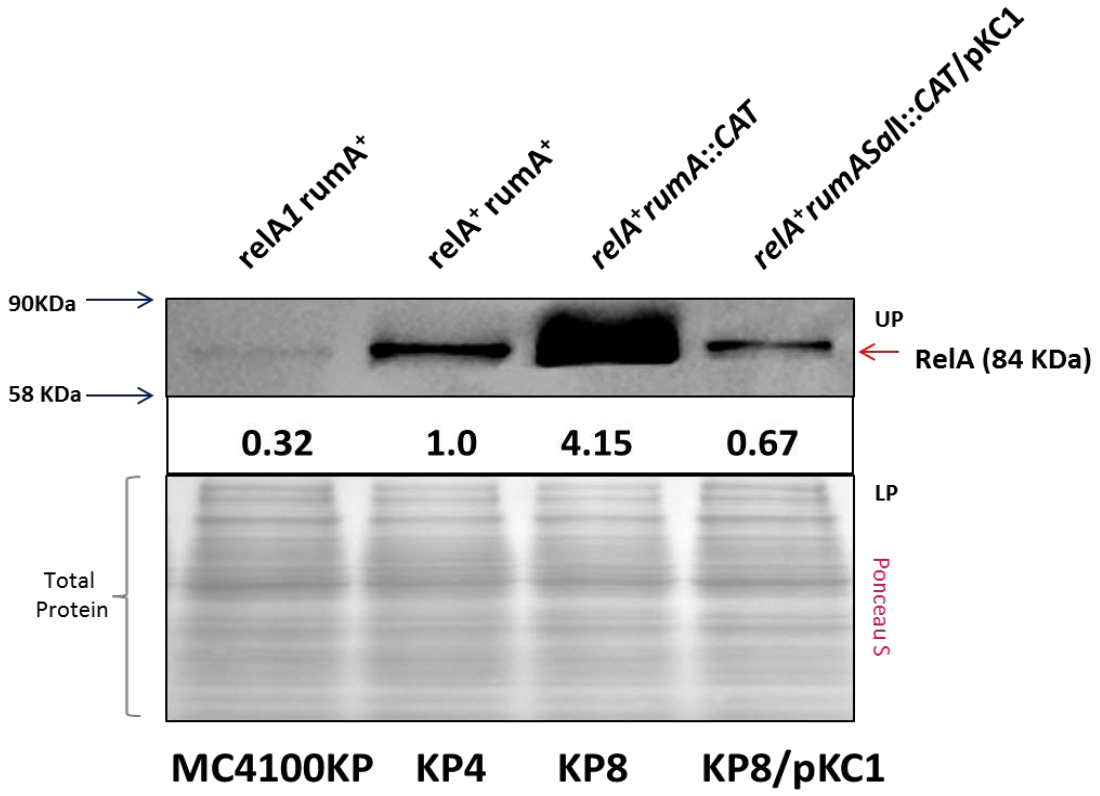


Figure 6. 17: Measurement of RelA protein in pKC1 transformants of KP8. Upper panel (UP) indicates immunoblot developed with anti-RelA antibody and lower panel (LP) indicates Ponceau stained gel. The fold differences in the protein levels indicated against each lane relative to wildtype represents average of three independent experiments.

6.3.2.9 Quantitation of *relA* transcript

The *relA* mRNA levels is quantitated from different strains by RT-PCR and compared to that in *rumASal*I::*CAT* mutant under growth in LB and MB. There is no significant difference in the mRNA levels which are unaltered in all the strains used in this study (Figure 6.18 A and B). Though insignificant, we observed that the *relA* transcript abundance is consistently more in cells grown in MB than in LB. Also the transcript level though insignificant was reproducibly high in *rumASal*I::*CAT* mutant when compared to that in other strains. However this small increase cannot account for fold increase (~7 fold) in protein level. The primer pair for RT PCR of *relA* gene is designed from the DNA corresponding to its C-terminal portion. We observed that the transcript amounts are unchanged in *relA1* strain which is contrary to substantially reduced

amounts of *relA* detected in Northern blot using the probe for same C-terminal region (Metzger *et al.*, 1989). It is noteworthy that in Δ*rumA* mutant (KP18) which lacks *rel*AP1/P2 promoters, produces *relA* transcript in amounts comparable to *relA* expressed from its own promoter (Figure 6.18 A and B). Thus the insertion of *CAT* cassette in *rumA* at *Sal*I influences translation and not transcription of *relA*.

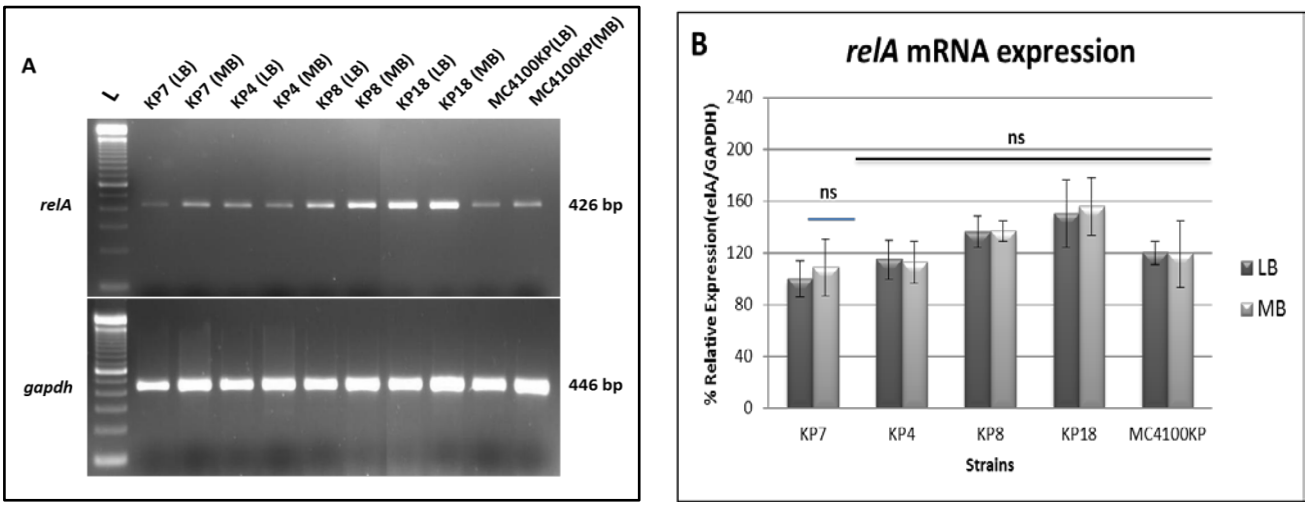


Figure 6. 18: *relA* mRNA transcript quantitated by RT-PCR. **(A)** Upper panel shows 426 bp *relA* amplicon generated using RelA RT primers (Table 6.3) corresponding to C-terminal portion. Lower panel represents RT-PCR of internal control of *gapdh* mRNA. 100 bp ladder used as M.W marker**. (B)** The abundance of *relA* transcript was measured by densitometry analysis as an average of three independent experiments and normalized with gapdh internal control. Data presented as Mean ± SEM of n=3. p-value **≤ 0.005 as compared to KP7; p-value ; ns > 0.05 as compared to KP7(LB) & KP7(MB).

6.3.2.10 *spoT*$^+$ as a suppressor of lethality of multiple copy of *relA*$^+$ in KP8 (MC4100 *relA*$^+$ *rumASal*I::*CAT*)

To find out the reason of the lethality of *rumASal*I::*CAT* mutant against multiple copy of *relA*$^+$, genomic library of *E. coli*, MG1655 in pBR322 vector was prepared and transformed into *rumASal*I::*CAT* mutant. The genomic pool was transduced with pTE1ΔBgl plasmid. From this library we found ~3 big size colonies which grew fast. Retransformation of KP8 by DNA isolated from each of the three clones reproduced the growth phenotype of suppressing lethality of *relA*$^+$ plasmid, pTE1ΔBgl. We further confirmed that the cloned DNA suppressed the growth defect of KP8 mutant on minimal medium, and also restored the *relA* phenotype in that the growth on 3-AT plate of transformant of *rumASal*I::*CAT* mutant was same as that of a *relA*$^+$ strain (Figure 6.19 A and B). This result is important in that it indicates that the sequence of *relA* gene in *rumASal*I::*CAT* mutant is unaltered and without mutations. The apparent irrevocable

stringent response defect is not due to mutation in *relA* gene compromising the protein function. Inability of pKC1/pKC2 to complement the stringent response defect of KP8 raised the possibility of *relA* gene containing unidentified mutations.

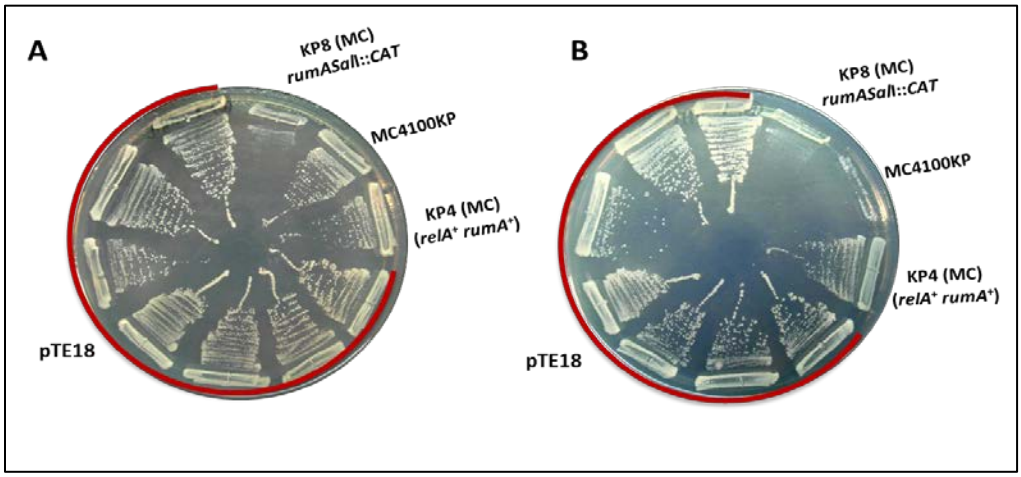
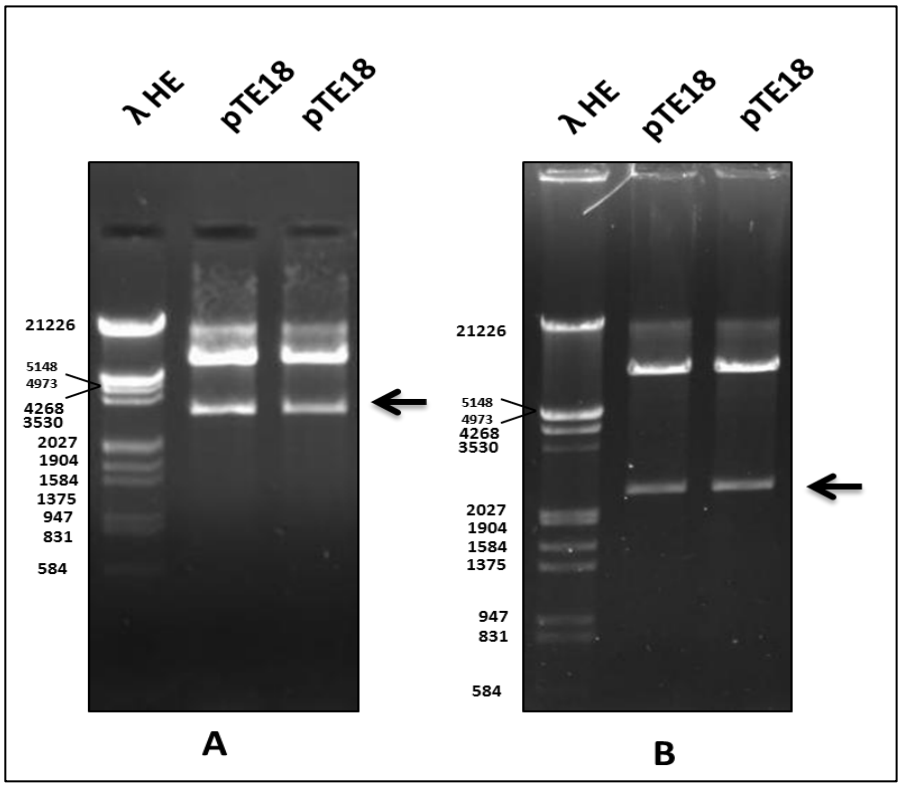


Figure 6. 19: Cloned *spoT*$^+$ gene suppresses slow growth defect of KP8 (*rumASal*I::*CAT*). **(A)** Growth on minimal agar and **(B)** on starvation plate (Minimal agar + 3-AT) of Plasmid transformants of pTE18 (complete *spoT*$^+$ gene).

The restriction digestion pattern of cloned DNAs has been checked by different restriction enzymes (Figure 6.20 A and B). The three clones were not independent and were siblings are clear from the same restriction digestion pattern of their DNA. The plasmid was named as pTE18 (pBR322 *spoT*$^+$).



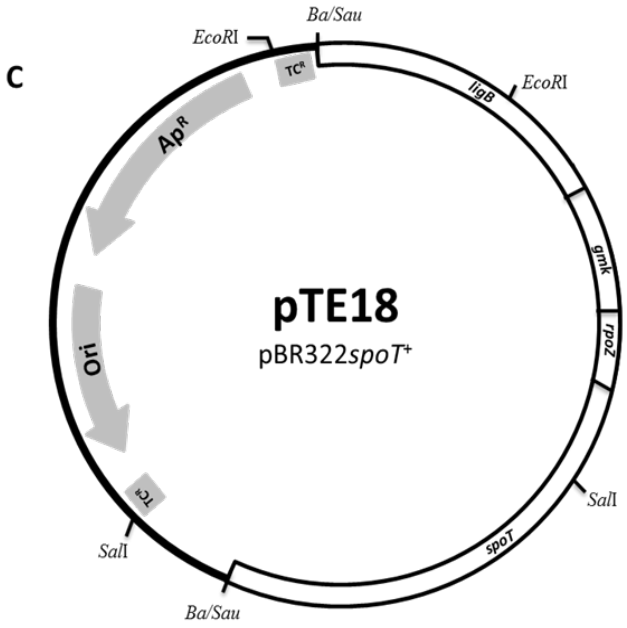


Figure 6. 20: Analysis of pTE18 by restriction enzymes (A) *Sal*I, **(B)** *Eco*RI. The insert size generated are indicated by arrow. λHE was used as M.W marker. **(C)** Schematic diagram of relevant restriction enzyme sites in the cloned *spoT* gene in pBR322 vector (pTE18).

The characteristic restriction sites in the *spoT* DNA were present in the pTE18 clone. For example *Sal*I site is present in the *spoT* gene whereas *Eco*RI site is present in a flanking region (*ligB*) (Figure 6.20 C). We thus removed the DNA between each of the restriction sites of the plasmids and the corresponding sites in the insert generating pTE18Δsal and pTE18Δeco derivatives. The growth and stringent response phenotype of KP8 was rescued by pTE18Δeco but clearly not by pTE18Δsal (Figure 6.21 A and B). The *spoT* specific primers were able to generate amplicon with pTE18 (Figure 6.22). One of the clones was sequenced; BLAST analysis of the sequence revealed presence of *spoT* gene.

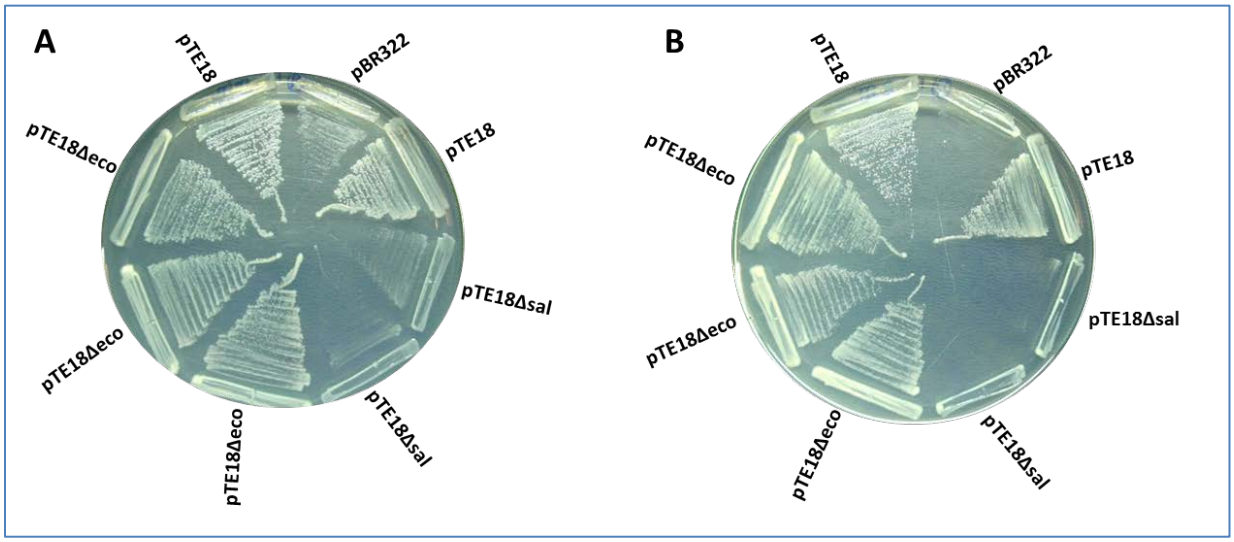


Figure 6. 21: **Ability of the derivatives of pTE18 (*spoT*$^+$ in pBR322) plasmid to suppresses slow growth defect of KP8 (*rumASal*I::*CAT*). (A)** Growth on minimal agar and **(**B**)** on starvation plate (Minimal agar + 3-AT) of plasmid transformants of pTE18, pTE18Δsal and pTE18Δeco.

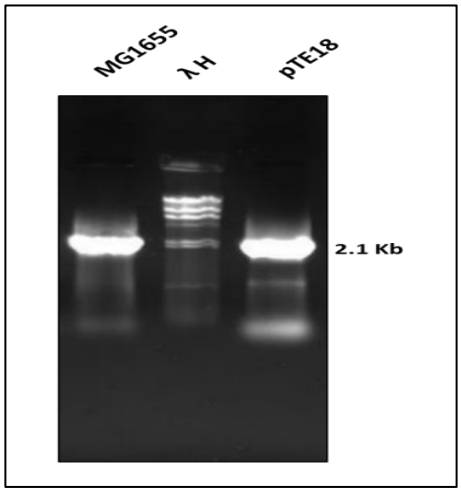


Figure 6. 22: Confirmation of *spoT* gene in pTE18 by PCR. Genomic DNA of MG1655 used as positive control.

We assumed that the severely reduced growth phenotype of *rumASal*I::*CAT* mutant is due to the increased level of ppGpp synthesis and that hydrolytic function of SpoT protein is responsible for reduction of ppGpp to normal levels characteristic of wild type strain required for growth rate as well as stringent control of ppGpp synthesis.

6.3.2.11 Role of transcriptional factors relevant to stringent response

6.3.2.11.1 DksA

ppGpp is involved in both the stringent response and growth rate regulation. ppGpp is a negative regulator of rRNA synthesis (Cashel & Gallant, 1969) and importantly this effect is augmented by *dksA*, a transcription factor directly binds to RNAP - acts synergistically with ppGpp on transcription initiation - to play a key role in regulation of rRNA (Paul *et al.*, 2004), Recently it is revealed that DksA is a transcription elongation factor, protects elongating RNAP against stalling on ribosomes (Zhang *et al.*, 2014).

We reasoned that if slow growth phenotype of *rumASal*I::*CAT* is possibly oversynthesis of ppGpp, and that DksA is mediator of ppGpp effect, null mutant of *dksA* would suppress the slow growth phenotype. On the contrary, we observed that upon introduction of Δ*dksA*::Tn*10*tet mutation (from CF9240) into KP8 strain caused synthetic lethality - Δ*dksA*::Tn*10*tet could not be introduced in P1 transduction into *rumASal*I::*CAT* mutant (Figure 6.23 C) while transductants could be obtained in all other strains including KP4 (MC4100 $relA^+$ $rumA^+$). *dksA* transductants of KP4 were however small size and slow growing (Figure 6.23 B). The same observations were reproduced with KP32 (MG1655 *spoT1 rumASal*I::*CAT*) and KP31 (MG1655 *spoT1*) strains (Figure 6.23 F and E respectively).

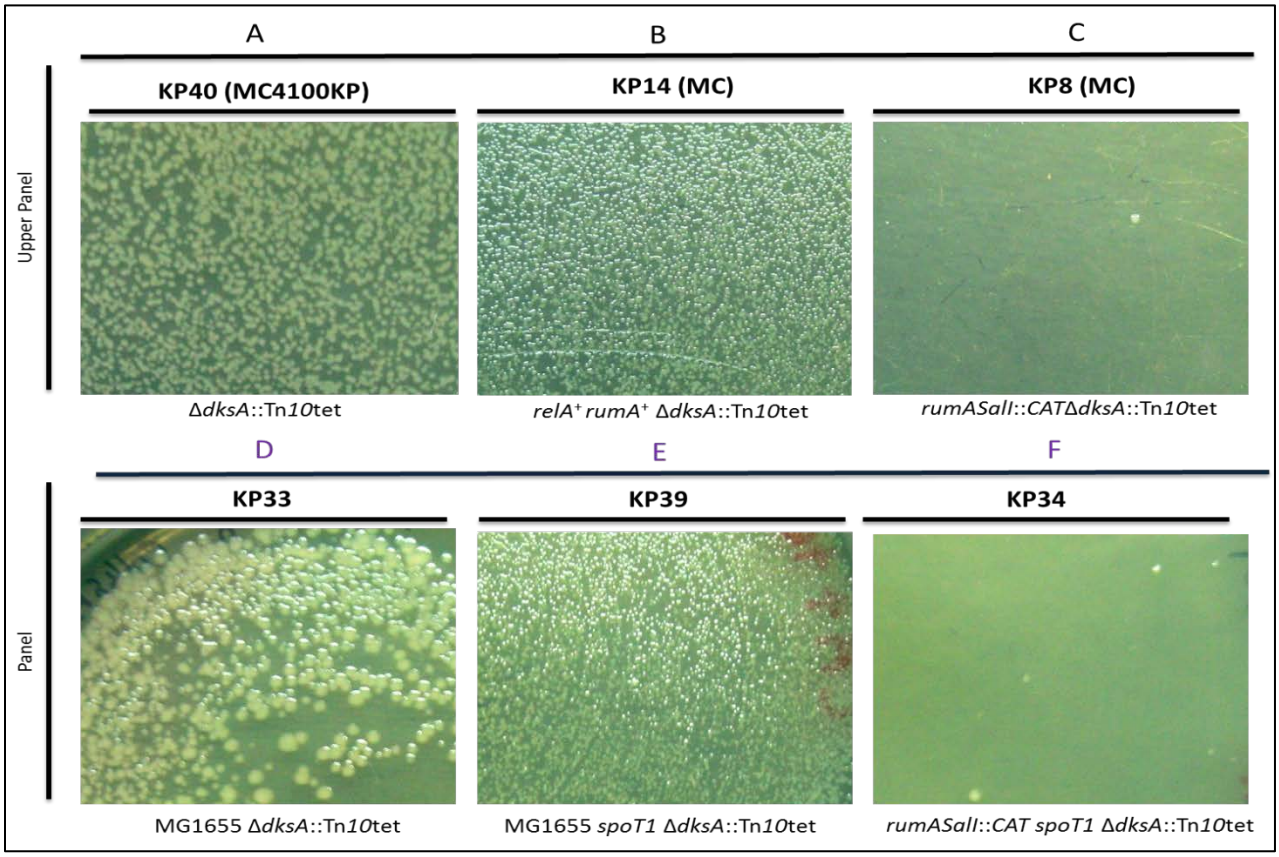


Figure 6. 23: Δ*dksA rumASal*I::*CAT* double mutants do not form colonies. In Upper panel **(A,B,C)** Δ*dksA*::Tn*10*tet mutation was introduced by P1 transduction into mutants of MC4100 background. Lower panel **(D,E,F)** shows Δ*dksA*::Tn*10*tet transductants of MG1655 strains. Note that C and F have no transductants colonies grow.

We have also transferred Δ*dksA*::Tn*10*tet in MG1655 and KP24 (MG1655 *rumASal*I::*CAT*) strains through P1 transduction. RelA protein was quantitated by Western blot in KP33 (MG1655 Δ*dksA*::Tn*10*tet) and KP34 (KP24 Δ*dksA*::Tn*10*tet) in cells grown in LB. The *dksA* mutation did not alter the amount of RelA protein content in KP33 and KP34 (Figure 6.24).

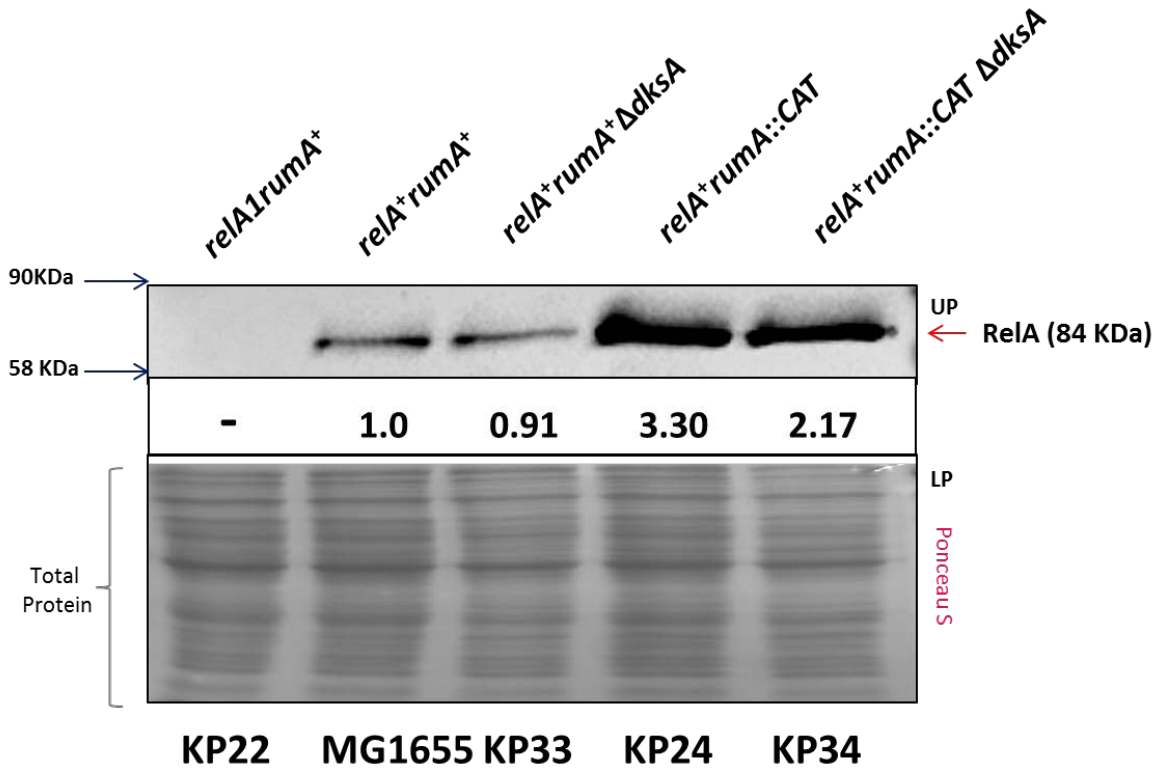


Figure 6. 24: Quantification of RelA protein in *dksA rumASal*I::*CAT* mutants. Cell free lysate of MG1655 derivatives, KP33 (MG) and KP34 (MG *rumASal*I::*CAT*) were assayed. Upper panel (UP) shows immunoblot probed with anti-RelA antibody and Lower panel indicates Ponceau S stained gel.

Though *dksA* mutation's effect on translational regulation of *rpoS* is described by (Brown *et al.*, 2002) but mechanism still not understood, synthetic lethality between *dksA* and *rumASal*I::*CAT* mutation is not involved in effect on translational regulation of *relA* as amount of protein is same in both *dksA*$^+$ and Δ*dksA* mutant. This experiment is carried out in cells grown in LB medium and using MG1655 strain because in minimal medium *dksA* mutant exhibits polyauxotrophy and that *dksA* mutation in KP8 (MC4100 *relA*$^+$ *rumASal*I::*CAT*) is lethal. Importantly the RelA protein amounts are the same irrespective of strain background (MG1655 and MC4100), with and without phenotype and in cells grown in LB and minimal medium.

6.3.2.11.2 RpoZ

RpoZ is ω sigma factor - smallest subunit of bacterial RNAP and ubiquitously present in bacteria (Burgess, 1969). *rpoZ* maps at 82 min upstream of *spoT* situated in the same operon as *spoT* (Gentry & Burgess, 1989). The role of ω sigma factor as is understood today is to cause sensitization of RNAP holoezyme to ppGpp (Vrentas *et al.*, 2005, Mathew & Chatterji, 2006).

Apparent lack of phenotype of *rpoZ* deletion mutant (Gentry *et al.*, 1991) is due to a similar function of DksA in mediating ppGpp effects (Vrentas *et al.*, 2005). Chatterji, et al. (2007), have shown the effect of ω subunit on *relA* gene (Chatterji *et al.*, 2007). By the same logic that we used for testing *dksA* mutation on *rumASal*I::*CAT* phenotype, we also tested *rpoZ* effect.

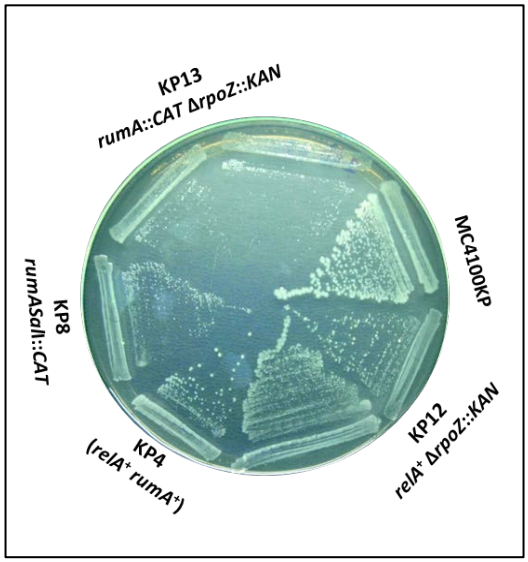


Figure 6. 25: Effect of Δ*rpoZ*::*Kan* mutation. Δ*rpoZ*::*kan* mutation does not correct growth defect of KP4 and KP8

Δ*rpoZ*::*Kan* (CF17119) strain has insertion of *kan* with least polar effect on downstream *spoT* gene. We performed amplification of Δ*rpoZ*::*kan* allele with RpoZ Left and RpoZ right primers (primers were outside of the *rpoZ* ORF) which gives 1421 bp product. Integration of Δ*rpoZ*::*Kan* gene was caused in MC4100 genome by strategy of recombineering using pKD46-helper plasmid. Then *rpoZ* mutation was transduced into KP8 (MC4100 *relA*$^+$ *rumASal*I::*CAT*) and KP4 (MC4100 *relA*$^+$ *rumA*$^+$) strains through P1 transduction. We find that growth of *rpoZ* null mutant KP13 (KP8 Δ*rpoZ*::*Kan*) is not significantly affected compared to KP8. Similarly, KP12 (KP4 Δ*rpoZ*::*Kan*) and KP4 strains had an unchanged growth pattern on different growth media (Figure 6.25). Thus *rpoZ* mutation has no effect on growth in the strains used for comparison. The reason for lack of phenotype associated with ω mutation could be also the reason for lack of phenotype of *rumASal*I::*CAT* mutant and that is *dksA* having the masking effect due to the similar function as ω (Gentry *et al.*, 1991, Vrentas *et al.*, 2005).

6.3.2.11.3 RpoS

RpoS is a stationary phase sigma factor also called σ^S or σ^{38}, important for stress regulated and stationary phase gene expression. The protein responds to various environmental signals regulated by several mechanisms both at transcription and translational level. One important regulator of *rpoS* is ppGpp which affects *rpoS* at translational level (Brown *et al.*, 2002). Spira *et al.*,.(2008) showed that deletion of *rpoS* corrects growth defect of MC4100 which contains approximately 2 fold higher levels of ppGpp and RpoS protein (Spira *et al.*, 2008). Increase in *rpoS* during log phase of growth may cause precocious stress response induction; deletion of *rpoS* indeed proved this effect on growth to be true in that it relieves slow growth atleast in KP4 (MC4100 *relA*$^+$). We reasoned the conditions which raise ppGpp levels like overexpression of *relA* from heterologous promoter (Schreiber *et al.*, 1991) or *relA*$^-$ strains containing hydrolytic activity defective *spoT* alleles (Sarubbi *et al.*, 1988) should be relieved of slow growth by mutation in *rpoS*. This proposal was tested in the context of slow growth phenotype of KP8 (MC4100 *relA*$^+$ *rumASal*I*::CAT*) mutant. Δ*rpoS*::*KAN* allele was transduced into KP4 (MC4100 *relA*$^+$), KP8 (MC4100 *relA*$^+$ *rumASal*I*::CAT*), KP31 (MG1655 *spoT1*) and KP32 (MG1655 *rumASal*I::*CAT spoT1*). The growth of Kan^r transductants of each of the strain was measured on minimal agar plate, *rpoS* mutation suppressed only marginally growth defect of KP8 and KP32 (Figure 6.26 A), As *rpoS* mutation cannot affect ppGpp levels as *relA*$^-$ mutation does, which is also suppressor of growth of *rumASal*I::*CAT* mutant, high levels of ppGpp are rather the cause of slow growth.

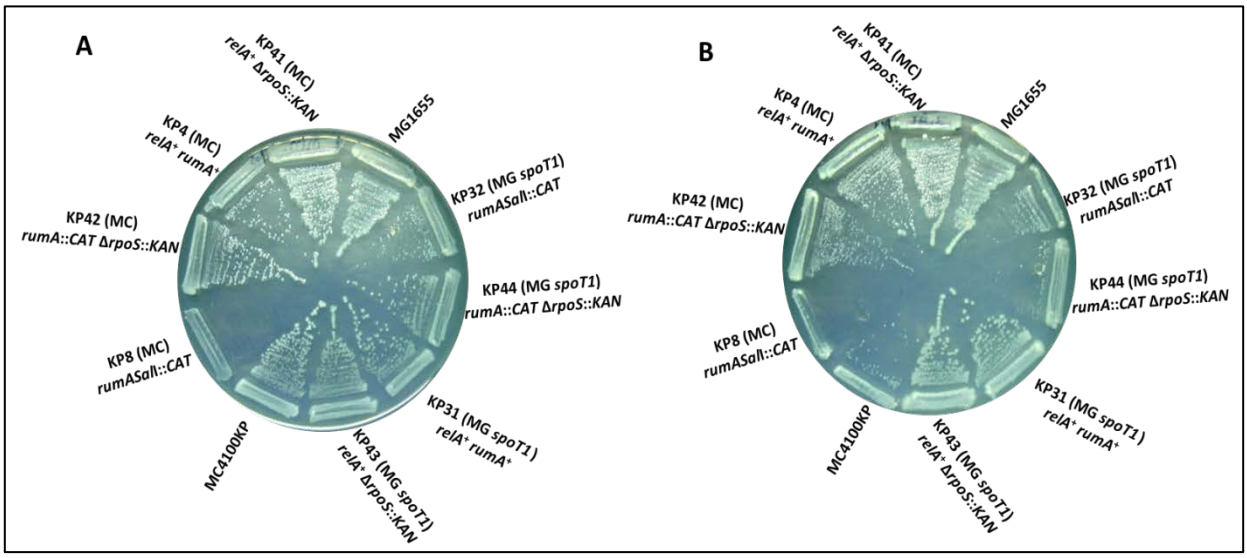


Figure 6. 26: Δ*rpoS*::*KAN* mutation effect on growth of KP8 (*rumASal*I::*CAT*). **(**A**)** Growth of the indicated strains on minimal agar plate; **(B)** on starvation plate (minimal agar + 3-AT).

Assaying for the RelA phenotype of different mutants on 3-AT supplemented plate produced the interesting result. Growth of KP41 (MC4100 *relA*$^{+}$*ΔrpoS746*::*KAN*) was distinctly fast and same as KP43 (MG1655 *spoT1 ΔrpoS746*::*KAN*) unlike that of KP4. However KP8 growth was slow and partially repaired comparable to KP4. KP44 (MG1655 *rumASal*I::*CAT spoT1 ΔrpoS746*::*KAN*) and KP32 grew to the same extent (Figure 6.26 B).

6.3.2.11.4 RpoB

RpoB is a β subunit of RNA polymerase. ppGpp inhibitory effect on transcription is by its direct binding to RNA polymerase and potentiating effect by DksA protein. Previous study reported that increased level of ppGpp in cells shows growth retardation and this detrimental effect can be relieved by *rpoB* mutation (Tedin & Bremer, 1992b). Similarly many of the *rpoB* mutations suppress polyauxotrophy of ppGpp^{0} and *dksA* mutation (Brown *et al.*, 2002). Since RelA protein is overexpressed in *rumASal*I::*CAT* mutant, leading presumably to increase in intracellular level of ppGpp (being quantitated), *rif* ^{R} mutation in *rpoB* could suppress the growth defect of KP8 similar to the toxic effects of *relA* overexpression and thus the ppGpp levels being relieved by *rpoB* mutations. Thus we assumed *rpoB* mutations to act as suppressor of growth defects of KP8 strain.

We isolated spontaneous Rif ^{R} mutation of KP8 strain by plating 2 x 10^{8} cells on LA plate supplemented with 250 µg/ml of rifampicin. Rif ^{R} were obtained at the frequency of 2.5 x 10^{-7} /cell. Only one mutant of Rif ^{R} mutants was able to grow on minimal agar unlike the parent KP8. The mutant that grew on minimal agar normally was unable to do so on 3-AT plate. (Figure 6.27).

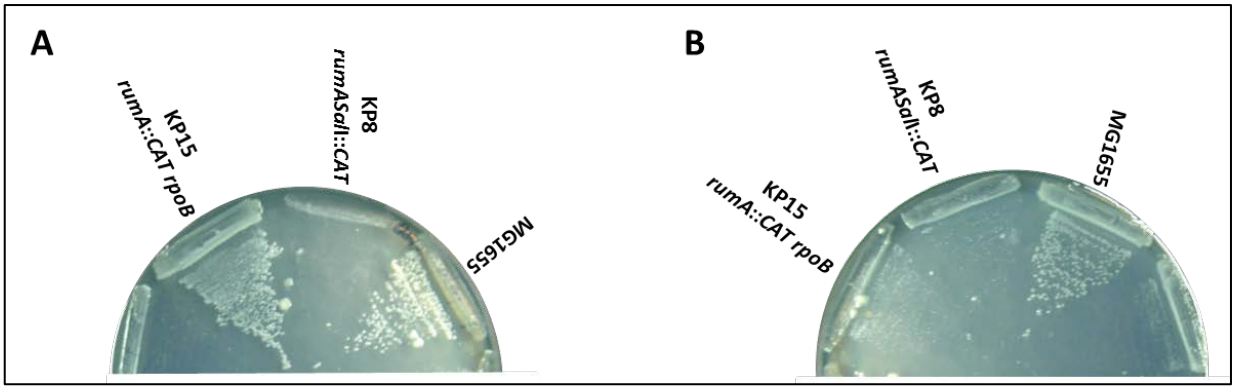


Figure 6. 27: Effect of *rif* ^{R} mutation in *rpoB* on growth of KP8 (*rumASal*I::*CAT*). **(A)** growth on minimal plate and **(B)** the stringent response plate.

The mutant KP15 (*rpoB2015*) is most probably insensitive to ppGpp levels achieved by nutrient downshift but not to levels raised by stringent response which are high enough to inhibit growth. The mutant described by (Tedin & Bremer, 1992b) KT13 isolated as overcoming the inhibitory effect of *relA* gene has similar properties.

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