CHAPTER 7

Summary and Conclusion

We describe here construction of mutation in *rumA* gene in order to understand relevance of its genomic position and function with respect to *relA,* given that the nucleotide U1939 in 23S rRNA methylated by RumA is positioned to sense uncharged t-RNA binding to ribosomes (Yusupov *et al.*, 2001), a requirement for eliciting a stringent response. Results obtained with mutant *rumA* gene which has both site specific substitution of C1939P and also a spontaneous deletion of 131 nucleotides from C terminal-encoding region of *rumA*, indicated that the *rumA* gene is in effect inessential for growth and viability of the organism. The same inference was reinforced with insertion of *CAT* cassette at *Mlu*I site of *rumA* and also in the studies of (Persaud *et al.*, 2010).

Interestingly *CAT* insertion 881 bp upstream of *relA* gene at *Sal*I site in *rumA* is associated with the range of novel phenotypes whose implication are being understood and yet to be realized in its entirety. Firstly, the growth of mutant is almost absent on minimal medium (no growth for 36 hrs at 37°C) to the extent that spontaneous suppressors of growth defect can be selected for. Often fast growing suppressors overtake the mutant culture.

Secondly, growth on starvation plate is indeed inimitable not described till now for any *relA*/*spoT* mutant. The growth on either 3-AT containing plate, causing starvation for histidine, or SMG supplementation, causing isoleucine limitation is same as that on minimal medium, best described as being 'suspended or frozen'. This is in striking contrast to KP4 (MC4100 *relA$^+$*) which grows slightly better than *rumASal*I::*CAT* mutant on minimal medium, nevertheless grows even better on SMG/3-AT plate (Figure 6.10 A and B). Second site mutations in following genes reverse either the slow growth or slow growth and 'suspended' stringent response phenotype of the *rumASal*I*::CAT* mutant. Characterization of the suppressors provided valuable insight into the reason of slow growth of *rumASal*I::*CAT* mutant.

(i)   Loss of function mutation in *relA* rescues the growth defect of *rumASal*I::*CAT* mutant (KP8): Several independent variants of *rumASal*I*::CAT* mutant that exhibit normal growth in minimal medium, like MG1655, has second mutation in *relA* gene, rendering it inactive. Thus the *relA$^-$ rumASal*I*::CAT* double mutant is rescued of the growth defect on glucose

minimal agar, however scores phenotypically RelA$^-$ on stringent response (3-AT supplemented minimal agar) plate. The inference of this result is that insertion of *CAT* gene in *rumA* DNA at spacing unique *Sal*I site results in *relA* overexpression or overactivation upon nutrient downshift, presumably resulting in increased production of ppGpp and inversely and adversely affecting growth, a result borne out by Western blot of RelA protein (see below).

(ii)   Functional *spoT*$^+$ gene suppresses both slow growth and 'suspended' stringent response defects of *rumASal*I::*CAT* mutant: A large effect of the slow growth phenotype of KT8 (MC4100*relA*$^+$ *rumASal*I::*CAT*) mutant is due to *spoT1* mutation, the reason also why the *rumASal*I::*CAT* insertion did not produce any phenotype in MG1655 background (Figure 6.12), though there is an elevated level of RelA protein. This was confirmed in reciprocal swapping experiments of *spoT* alleles, *spoT1* and *spoT*$^+$ between MC4100 and MG1655 respectively. There was a complete linkage of the slow growth phenotype and *spoT1* mutation. KP32 (MG1655 *rumASal*I::*CAT spoT1*) grew strikingly at reduced rate but not as drastically as KT8 (Figure 6.12). On the contrary, the growth of *spoT*$^+$ KP38 (MC4100 *relA*$^+$ *spoT*$^+$ *rumASal*I::*CAT*) is equal to that of MG1655 or KP24 (MG1655 *rumASal*I::*CAT*). Genetic mutations besides that in *relA*/*spoT* are implicated to be important for aggravation of the growth phenotype in MC4100 background, a conclusion also reached in other studies (Laffler & Gallant, 1974, Sokawa *et al.*, 1975, Spira *et al.*, 2008). We believe the genetic differences are relevant to ppGpp metabolism. *relX* and *relS* mutation have earlier been shown to affect ppGpp levels and explain the physiological differences between certain strains of *E. coli* (Pao & Gallant, 1978, Engel, 1979). *spoT1* allele is a weak allele of *spoT* with presumably ppGpp hydrolysis impairment since *spoT1* mutants have raised intracellular level of ppGpp even in the absence of *relA* gene; cause slight impairment in growth in *relA*$^+$ background e.g MG1655 (Fiil *et al.*, 1977) but growth retardation is quite severe when KP4 (MC4100 *relA*$^+$) strain is constructed (Chapter 5, section 5.3). More severe defective alleles of *spoT* have been isolated as restoring 3-AT resistance in *relA*$^-$ mutants with intracellular pools of ppGpp varying in tenfold range (Fiil *et al.*, 1977, Sarubbi *et al.*, 1988). Growth of *rumASal*I::*CAT* derivatives, KP8 and KP32 borders being slightly better than *relA*$^-$ strain on SMG/3-AT plate. The fact that *spoT*$^+$ strain of *rumASal*I::*CAT* mutant is fully *relA*$^+$, expressing stringent response normally, grows on SMG like any other *relA*$^+$ strain argues against *relA* gene as containing mutations. Furthermore, introduction of cloned *spoT*$^+$ gene (pTE18 and

pTE18ΔEco) rescues the growth defect phenotype including that of starvation condition, strongly implies high level of ppGpp in the mutant. Quantitation of ppGpp is underway.

The basis of the growth phenotype of *rumA*::*CAT* mutant is thus described: The relative difference between uncharged to charged tRNA in the cell resulting from nutrient downshift is presumably enough to cause a large disproportionate increase in ppGpp in the *rumA*::*CAT* mutant as the amounts of RelA are significantly and constitutively high under all conditions of growth in the mutant. Starvation-induced further increase in ppGpp is rather toxic to the bacteria so as to curtail growth notwithstanding the beneficial effects of high ppGpp amounts required for overcoming starvation by increased expression of amino acid biosynthetic genes. The growth phenotype is just short of being described RelA$^-$. Only SpoT$^+$ reduced the high levels of ppGpp as expected, reaffirming the fact that ppGpp levels are indeed high in the *rumA*::*CAT* mutant to affect growth substantially.

(iii)   *rif*$^{\text{R}}$ mutation in *rpoB* gene corrects growth phenotype of KP8 (MC4100 *relA*$^+$ *rumASal*I::*CAT*) mutant: The growth reversal effect of *relA*$^-$ suppressor mutation and of *spoT*$^+$ gene strongly suggests elevated levels of ppGpp as the reason of slow growth of *rumASal*I::*CAT* mutant. Intracellular pools of ppGpp are inversely related to growth rate of the strain, high levels cause reduction in growth due to inhibition of stable RNA synthesis by directly binding to RNA polymerase (Cashel, 1996, Chatterji *et al.*, 1998, Reddy *et al.*, 1995, Ross *et al.*, 2013). ppGpp insensitive *rif*$^{\text{R}}$ mutations in *rpoB* gene have been isolated to suppress multiple auxotrophic requirement of ppGpp^0 strain (Xiao *et al.*, 1991, Murphy & Cashel, 2003). They were also isolated as relieving severe growth inhibition caused by overexpression of *relA* from heterologous *lac* promoter (Tedin & Bremer, 1992). Independent evidence that intracellular pools are indeed high in the mutant was obtained in this study with the isolation of Rif$^{\text{R}}$ mutants that have overcome the growth defects. *Rif*$^{\text{R}}$ mutation rescues the slow growth of *rumASal*I::*CAT* mutant on minimal glucose medium but did not repair the 'frozen' growth phenotype on starvation plate (Figure 6.27).

The results are not unexpected. Isolation of an ideal ppGpp insensitive mutation in RNA polymerase is unlikely as ppGpp is required to modulate RNA polymerase binding to different sigma factors for proper expression of different growth phases and adaptation responses (Jishage *et al.*, 2002). If the intracellular levels of ppGpp are indeed high in the mutant affected by nutrient downshift and enhanced further by amino acid starvation, *rpoB* mutations can

presumably be obtained correcting growth rate defect; being insensitive to starvation-induced high level of ppGpp may be representing an ideal insensitive mutation.

(iv)   Null *rpoS* mutation reversed marginally growth phenotypes of *rumASal*I::*CAT* mutant but not its stringent response defect: In the comparative study of strains MC4100 and MG1655, Spira *et al*., (2008) showed that MC4100TF expresses *rpoS* at higher level than MG1655 (Spira *et al.*, 2008). This is partly due to *spoT1* mutation that causes less degradation and thus accumulation of ppGpp in MC4100TF/BS. They proposed that as a result of high levels of ppGpp, σ^S competes effectively with σ^{70} causing shift in the balance of gene expression towards expression of stress response/stationary phase genes at the cost of genes involved in growth. This phenomenon is coined the term SPANC (self-protection and nutritional capability) balance. The two strains MG1655 and MC4100 represents the two extremes of the balance- MC4100 [We find KP4 (MC4100 *relA*$^+$) is rather the extreme than MC4100 (*relA1*)] is better adapted to stress and invest less in growth (thus growth is slow even on LA) when compared to MG1655 which grows fast on all media is however less tolerant to different stresses.

ppGpp is an important small molecule, among others, required for synthesis of RpoS in stationary phase (Lange *et al.*, 1995). High levels of ppGpp signal nutrient starvation, slowdown growth, and herald onset of stationary phase in normally growing culture, a condition for expressing stationary phase program. Precocious production of ppGpp in exponential phase of growth induces *rpoS* production. Similarly *rpoS* overexpression has been effected by heterologous *ara*p$_{BAD}$ promoter or by overexpression of regulatory RprA sRNA. However, this is without an overt phenotype on growth, though the stationary phase genes under the regulation of RpoS sigma factor are indeed expressed in exponential phase of growth (Karen K. Carter, 2011). The corrective effect of *rpoS* mutations is not mediated through altering the concentration of ppGpp in the cell (Spira *et al.*, 2008). RpoS protein overexpressed by heterologous promoter or sRNA manipulation (Karen K. Carter, 2011) does not affect growth, thus it was expected that *rpoS* alone would not have a large corrective effect in the presence of high levels of ppGpp. Indeed, *rpoS* mutation had only a small effect (if at all) on growth of the *rumA*::*CAT* mutant, however growth of KP4 (MC4100 *relA*$^+$) is almost restored to normal in the double mutant.

It appears that the effects of ppGpp and *rpoS* on growth are mutually exclusive. The growth inhibition under starvation condition overwhelms growth repair function of *rpoS/rpoB* (rifR) mutation(s).

The *rumASal*I::*CAT* strain which we constructed has fairly high levels of RelA protein synthesized constitutively under all conditions of growth in both MG1655/MC4100 background. Phenotype of *relA* overexpressing strain is described only in the context of cloned *relA* DNA synthesizing *relA* from *lac* inducible promoter under IPTG control (Schreiber *et al.*, 1991). However, pALS10 *relA*⁺ plasmid containing cells grow slow even without IPTG in LB medium, submaximal concentration of IPTG cause complete growth inhibition due to synthesis of very high ppGpp (Tedin & Bremer, 1992).

Few mutations are described in literature to effect *relA* regulation. CsrA has been reported to effect *relA* translation due to binding of protein to *csrA* binding elements upstream of SD/AUG of *relA* mRNA (Edwards *et al.*, 2011). The effect we observed with *rumASal*I::*CAT* mutation is also translational and presumably independent of *csrA* involvement for more than one reason. The effect would be independent of promoter DNA; secondly *uvrY/barA* mutations would indirectly reduce the effect of *csrA* mediated regulation (Edwards *et al.*, 2011). *csrA* binding sites are in multiple copies, five of them have been noted in the sequence upstream of *relA* start codon in the transcript produced from the major P1 promoter. Multiple base substitution mutation unlinked to *rumA::CAT* insertion in the *csrA* binding sites are required to strongly effect *csrA* mediated regulation of translation of *relA*. We observed a far upstream DNA 256 bp upstream of P2 promoter and 704 bp upstream of P1 promoter to affect *relA*'s translation. *csrA* mediated effect cannot be explained in a transcript specific manner. In addition *barA/uvrY* mutation did not affect *relA* expression noticeably. Nonetheless, the translational regulation we observed is also difficult to explain in the context of P1/P2 promoter initiated transcription; given that the region in DNA in question is upstream of each of the two promoters. Invoking a third promoter may be required to explain the effect we study.

ppGpp is synthesized by two proteins RelA and SpoT, the former required mostly for stringent response and the latter for growth rate dependent production. Affecting ppGpp synthesis by each of the two processes led to interesting consequences that was important for revealing the concentration range that characterizes growth rate and stringent response. Changes in the

concentration of ppGpp in micromolar range occur during growth rate regulation whereas it is required in millimolar range for activation of amino acid biosynthetic genes during stringent response. The segregation of the range under which the two conditions - stringent and growth response operate is though not strict one. Conditions or mutation in *relA*/*spoT* can affect the concentration with interesting repercussions. For example, mutation in the hydrolytic function of *spoT* results in raised ppGpp levels, many of these mutations are not possible to be transduced into *relA*$^+$ strain. These strains still exhibit, though not strictly linear, inverse relationship between growth rate and ppGpp levels and inverse exponential relationship with *rrnP*1 promoter (Fiil *et al.*, 1977, Sarubbi *et al.*, 1988). Moreover, the concentration of ppGpp is high enough to permit growth on 3-AT plate in the range required for histidine biosynthetic operon; apparently meaning that *spoT* can bypass *relA* in stringent response. RelA protein mediated hypersynthesis of ppGpp is not through mutation but by heterologous promoter use; there are no mutations described we are aware of that cause stable altered expression of *relA*. However a study is worth mentioning here (Lee, 2010). Using error prone PCR, mutation in ppGpp synthesis domain have been generated in the study that stably increase, irrespective of growth medium, by ten-fold, intracellular ppGpp levels. There is no further study carried out using the mutant gene regarding growth rate and stringent response. In the study described here, we find that constitutive elevation of RelA by 7 fold in cells grown in different media causing over synthesis of ppGpp under nutrient downshift condition to the extent that in the presence of hydrolysis-defective *spoT1* mutation, curtails growth drastically. Growth retardation is due to ppGpp accumulation is obvious as hydrolysis proficient *spoT*$^+$ protein corrects the growth defect completely indicating the growth defect being mediated by ppGpp. The possible interpretation of result is that increased expression of *relA* upsets the growth rate regulation and also the stringent response, implying a possible indirect role for *relA* in growth rate regulation.

Synthetic lethality of *dksA* and *rumASal*I::*CAT* mutation: With more evidence gathering, there is shift in our understanding of *dksA* function. It is appreciated as being more than transcriptional cofactor; isolated first as suppressor of *dnaK*, it is proposed to be involved in replication restart to prevent head on collision between transcription and replication and DNA repair through its transcriptional effect (Tehranchi *et al.*, 2010). It is well understood for its functional role in potentiating effect of ppGpp in response to nutritional stress and growth rate

regulation, as *dksA* null mutant behaves similar to ppGpp^0 mutant. Synthetic lethality of the combination of *dksA* and *rumASal*I::*CAT* mutations seemed counterintuitive. If ppGpp overproduction is the cause of slow growth in *rumASal*I::*CAT* mutant as a result of increased RelA protein levels, and that growth inhibitory effect of ppGpp is mediated by *dksA*, it was anticipated that mutation in *dksA* would act to suppress the slow growth defect. The *dksA* mutant exhibits relaxed phenotype - RNA to protein ratio unaffected by growth rate affecting condition and by nutrient starvation. Instead, the synthetic lethality was the phenotype of combining two mutations *dksA* and *rumASal*I::*CAT*. The lethality effect was same both in MC4100 and MG1655 background. The barrier to strain differences was obliterated with this combination of two mutations, though *rumASal*I::*CAT* mutant derivative, KP32 (MG1655 *spoT1*) is less severely affected for growth in comparison to KP8 (MC4100 *relA*$^+$). If the finding that higher levels of ppGpp destabilize RNAP complex on P1 promoter even when it is in competition resistant state (Vrentas *et al.*, 2005) can be extrapolated *in vivo*, *dksA* potentiating ppGpp effect on transcription can be obviated. Elevated concentration of ppGpp has same effect on transcription as ppGpp mediating effect through DksA at normal concentration range seen during nutritional shiftdown/stringent response. ppGpp effect on growth may become independent of *dksA* at very high concentration. The converse has already been shown.In ppGpp^0 mutant, overexpression of *dksA* suppresses several of phenotypes like polyauxotrophy. The *in vitro* reflection of this phenotype is the concentration dependent effect of DksA alone and in combination with ppGpp on RNA polymerase P1 promoter (Paul *et al.*, 2004, Vrentas *et al.*, 2005). As indicated earlier, we are unaware of mutation described in literature to cause increased synthesis of *relA*. The condition of ectopic regulatable*lac/ara* promoters have been used to cause high RelA production Thus *dksA* mutation effect under *relA* overexpression has not been tested.

The *dksA* null mutation's lethality associated with introduction of *rumASal*I::*CAT* mutant can be hypothesized as follows. In light of advancement of our understanding of *dksA*, particularly its role in transcription elongation (Zhang *et al.*, 2014), relieving transcription-replication collision and thus DNA repair (Tehranchi *et al.*, 2010), it can be postulated that under high levels of ppGpp, similar to amino acid starvation condition, there is transcription-replication block, unresolved collision between replication machinery and transcription proteins, and inability to repair the DNA block due to down-regulation of SOS response genes, all cumulatively lead to

lethality (Tehranchi *et al.*, 2010, Trautinger *et al.*, 2005). Testing the effect of introduction of *recA* mutation in *rumA*::*CAT* mutant could prove if the line of thinking is correct. More work is needed to test and refine this model.

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