# CHAPTER-1

# Introduction & Review of Literature

## 1.1 Antibiotics

"Antibiotics are literally 'against life'- are typically antibacterial drugs, interfering with some structure or process that is essential to bacterial growth or survival without harm to the eukaryotic host harboring the infecting bacteria." - (Walsh, 2000).

### 1.1.1 Targets of antibiotics

Most of the antibiotics hit upon only three targets: the ribosome (which consists of 50S and 30S subunits), cell wall synthesis and DNA gyrase or DNA topoisomerase (Figure 1.1). Examples of targets of antibiotics are the following: actinomycin D (RNA synthesis), chloramphenicol (bacterial protein synthesis), cycloserine (peptidoglycan synthesis), nalidixic acid and novobiocin (bacterial DNA synthesis), rifampin (bacterial RNA synthesis), cycloheximide (eucaryotic proteinsynthesis), daunomycin (fungal RNA synthesis), mitomycin C (DNA synthesis), polyoxin D (fungal cell wall chitin synthesis), and cerulenin (fatty acid synthesis) (Lewis, 2013).

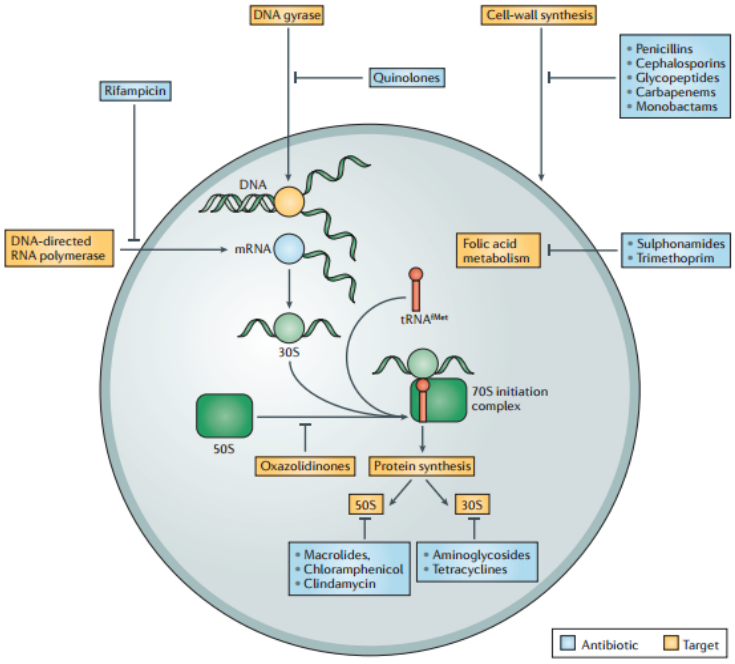


**Figure 1. 1: Targets of antibiotics.** Schematic figure shows that exploited targets are only three- the ribosome, cell wall synthesis and DNA gyrase or DNA Topoisomerase (Lewis, 2013).

## 1.2 Antibiotic resistance/tolerance

Much is written about rising and spread of antibiotic resistance among different human pathogens. Over the millennia, bacteria are exposed to numerous noxious chemical compounds (including antimicrobial drugs) and to various environmental challenges. To defeat these challenges, bacteria have developed different escaping strategies (Wright, 2007, Davies & Davies, 2010, Davies *et al.*, 2006, Davies, 1997).

Antimicrobial resistance can be acquired or natural (Intrinsic).

### 1.2.1 Acquired Resistance

Acquired resistance evolve via genetic alterations in the bacteria's own genome (Normark & Normark, 2002), by horizontal acquisition of resistance genes which are situated on various types of mobile DNA elements (i.e. plasmids or transposons) or by recombination of foreign DNA into the chromosome (by transduction and conjugation) (Davies, 1997). A trait acquired by bacteria as a result of the rate of spontaneous mutations in the chromosome are transferred vertically as the bacteria replicates (Martinez & Baquero, 2000).

### 1.2.2 Intrinsic Resistance

Intrinsic resistance is a natural resistance/tolerance to antimicrobial agents possessed by majority population of a bacterial species to antibiotics (Cox & Wright, 2013, Rodionov & Ishiguro, 1995) showed that overproduction of ppGpp inhibited the synthesis of peptidoglycan and phospholipid as well as exhibited penicillin tolerance. In addition to the intrinsic resistance mediated by the bacterial outer membrane and active efflux (Nikaido, 2001a, Piddock, 2006a), studies have shown that a surprising number of additional genes and genetic loci also contribute to intrinsic susceptibility (Blake & O'Neill, 2013, Fajardo *et al.*, 2008, Gomez & Neyfakh, 2006, Liu *et al.*, 2010). *"Combined, these different elements encompass the 'intrinsic resistome' and reveal that this phenomenon is more complex than originally anticipated"*(Cox & Wright, 2013).

#### 1.2.2.1 Mechanisms of Intrinsic Resistance

(i) Outer-membrane permeability:

The bacteria have a cell membrane which provides a barrier between the external environment and bacterial cytoplasm. The flexibility and permeability of the membrane is imparted by lipid bilayer component (Vance & Vance, 2008). Bacteria live in an environment where they have to

encounter with numerous noxious compounds. For self-protection against these toxic compounds bacteria have additional external structures which provide as a permeability barrier (Nikaido, 2001b).

Gram positive bacteria has a thick outer peptidoglycan layer with teichoic acid polymers and covalently bound proteins providing tensile strength and osmotic stability (Clark *et al.*, 2009, Schäffer & Messner, 2005). It has been reported that the coarse meshwork of peptidoglycan has a large permeability threshold and small molecules of up to 30-57 kDa easily penetrate, which is the molecular basis for the relative intrinsic susceptibility of Gram positive organisms to many antibiotics (Scherrer & Gerhardt, 1971, Randall *et al.*, 2013).

Conversely, Gram negative bacteria are intrinsically resistance due to fine molecular sieve called the outer membrane (OM) (Vaara, 1992) which is surrounded by comparatively thin peptidoglycan layer, consists of an extraordinary composition of lipid molecules Lipid-A-covalently bonded to polysaccharide units and studded with a variety of proteins called porins which provides an additional mechanism in uptake of essential nutrients (Benz & Bauer, 1988) and also these porin channels restrict influx of various antibiotics (Ochs *et al.*, 1999, Olesky *et al.*, 2006, Parr *et al.*, 1987, Ruiz *et al.*, 2003). Porins retard the influx of drugs by a number of mechanisms including size limitations (Decad & Nikaido, 1976), hydrophobicity and charge repulsion (Nikaido *et al.*, 1983, Cowan *et al.*, 1992). The OM alone does not provide considerable level of antibiotic resistance; it requires second contributor, such as the *Psuedomonas aeruginosa* periplasmic β-lactamase - the retardation in influx of drugs is beneficial for inducible β-lactamase which is inherently present in *P. aeruginosa* (Hancock & Brinkman, 2002) and inactivates β-lactams as it pass into the periplasm, contributes intrinsic resistance of *P. aeruginosa* to β -lactam antibiotics or active efflux (Nakae *et al.*, 1999).

(ii) Multi-Drug Resistance (MDR) efflux pumps

Efflux pumps are present in all the organisms' chromosome as well as those that do not produced antibiotics (Van Bambeke *et al.*, 2000). These pumps are able to efflux either specifically one type of molecule or a range of diverse classes of molecules. The pumps involve in transportation of numerous compound can be associated with multidrug resistance (MDR). It is likely that resistance to antibiotics mediated by active efflux is an accidental by-product of the

physiological role-export toxic molecules that have been produced by the host (such as Bile salts) - exhibited by such pumps. This observation pinpoints that this mechanism is for survival of bacteria in natural habitat rather than evolved for evading antibiotics (Piddock, 2006b). It was reported first time for tetracycline resistance through efflux pump in 1970s (Levy & McMurry, 1978, McMurry *et al.*, 1980) and since then, active efflux of a plethora of antibiotics has been well documented in many Gram-positive and Gram-negative bacteria.

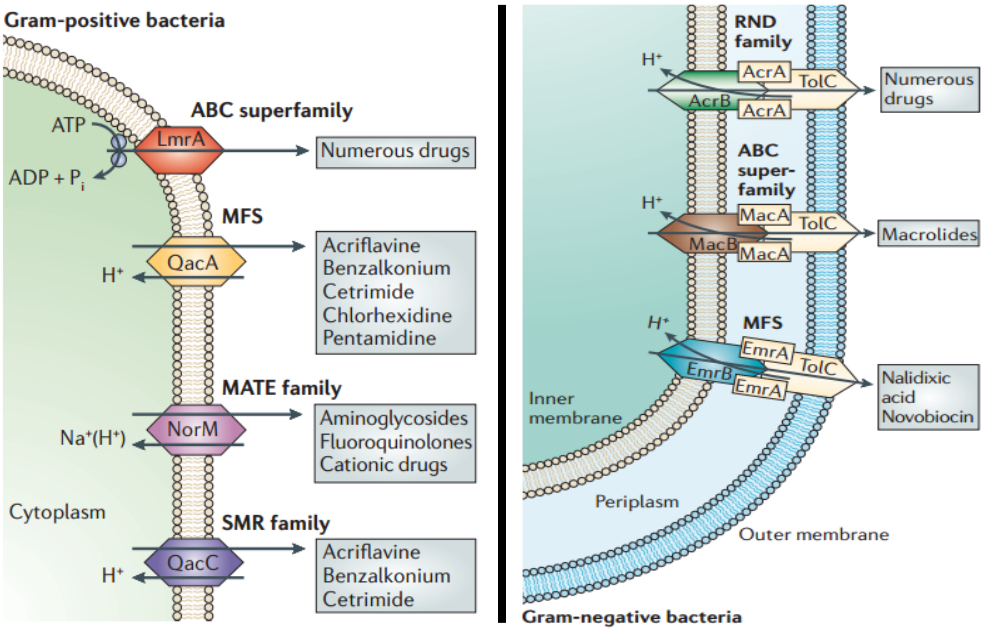


**Figure 1. 2**: **Multidrug-resistance efflux pumps of Gram negative and Gram positive of bacteria.** This figure represents structure and membrane location of five families of Efflux pumps and their individual proteins (Piddock, 2006b).

There are distributions of five families of efflux pump proteins within the bacterial membrane: the ATP binding cassette (ABC), the major facilitator (MF), the multidrug and toxic-compound efflux (MATE), the small multidrug resistance (SMR), and the resistance-nodulation-division family(RND) (Webber & Piddock, 2003, Piddock, 2006b, Pagès *et al.*, 2005). Efflux Pump requires an energy as it is an 'active' process. All the families use Proton Motive Force except one family i.e. ABC family, which hydrolyze ATP and give the energy for export of molecules (Paulsen *et al.*, 1996). Among all the families mostly intrinsic resistance exhibited by the RND family of efflux pump in Gram negative bacteria (Marquez, 2005), i.e. In E coli, the AcrAB-TolC RND tripartite efflux pump has a resistance to broad range of antibiotics like tetracyclines, fluoroquinolones, β-lactams and the macrolides. TolC, a protein channel, can interact with MFS

transporters (for example, EmrAB of *E. coli*) and ABC-superfamily transporters (for example, MacAB of *E. coli*) (Figure 1.2) (Piddock, 2006b). Also in P. aeruginosa, the tripartite efflux system MexAB-OprM (RND family) is constitutively expressed and gives intrinsic resistance to structurally dissimilar antibiotics like β-lactams and fluoroquinolones (Rice, 2006).

The high level of intrinsic resistance, bestowed alone by neither active efflux (Webber & Piddock, 2003) nor OM of bacteria, of Gram-negative bacteria has been traditionally accredited to a synergistic relationship between both the low permeability of the OM and active detoxification system such as efflux pump or periplasmic inactivating enzymes; all those maintain fine balance of antibiotic concentration between influx and efflux.

(iii) Additional elements of the 'intrinsic resistome'

Apart from above these two mechanisms for intrinsic resistance, a group of scientists' studies have recently revealed that this phenotype also entails additional chromosomally encoded elements. Utilizing comprehensive transposon tagged mutant libraries in different organisms such as *Escherichia coli* (Girgis *et al.*, 2009, Liu *et al.*, 2010, Tamae *et al.*, 2008), *Pseudomonas aeruginosa* (Alvarez-Ortega *et al.*, 2010, Breidenstein *et al.*, 2008, Fajardo *et al.*, 2008, Schurek *et al.*, 2008), *Acinetobacter baylyi* (Gomez & Neyfakh, 2006) and in recent *Staphylococcus aureus* (Blake & O'Neill, 2013) demonstrated the existence of genetic mutations in an unexpected plethora of genes, either sensitizes or elevates innate resistance of the organisms, which code for proteins involved in various cellular and metabolic pathways. This intrinsic resistance phenomenon is not due to the constant exposure of antibiotic by which bacteria is adapted but rather a characteristic phenotype dependent on the intricate metabolic networks of each bacterial species. In objective 1, our study was focused on understanding the basis of multiple copies of two genes, *relA* and *rumA* conferring multiple antibiotic tolerance phenotype.

## 1.3 Escherichia coli

*E. coli* is a widely studied model organism commonly used in microbiology and genetics to study bacterial physiology, gene regulation, metabolism, signal transduction, cell wall structure and function. It was one of the first organisms to have its genome sequenced (the complete genome of *E. coli* K12- MG1655). The research work of the present study has been carried out on *E.coli* K-12 strains.

## 1.4 Bacterial global regulatory system

As Charles Darwin noted, "*it is not the strongest of the species that survives, nor the most intelligent, but the one most responsive to change*."

Bacteria have the ability to survive in numerous environmental stresses like nutritional deprivation, heat stress, cold stress, acid stress, osmotic stress, and oxidative stress etc. They ought to cope with repeated bouts of feast and famine in their natural environment otherwise they might be eliminated. Bacteria sense these environmental changes through complex, interconnected regulatory circuits; and thus accordingly coordinate physiological processes and evolve. The global regulatory systems defined by existence of a regulatory gene that responds to a physiological imbalance or stress by coordinating the expression of a variety of genetically unlinked target loci. The regulator mechanism as well as the signals provoking regulation consists of a varied and growing list including proteins operating as repressors, inducers, or alternative sigma factors and DNA promoter enhancing elements, often acting in concert with regulatory nucleotides (Neidhardt *et al.*, 1987, Hoopes & McClure, 1987). Frequently, second messenger signaling molecules are used by bacteria in the form of modified nucleotides to efficiently counter nutrient limitations and environmental stresses. These second messengers are quickly synthesized and diffused, and their synthesis and degradation are strictly regulated. Cyclic AMP (cAMP), the most well studied second messenger, serves to alert cells to the status of the available carbon source, while others, such as a guanosine tetraphosphate (ppGpp) and cyclic di-GMP (c-di-GMP), are synthesized in response to a variety of conditions. These types of regulation, express one set of genes, while repressing others, through transmission of integrated environmental cues to produce an appropriate response which involve global changes resulting in physiological and metabolic alterations. Among all the adaptive responses, a 'Stringent Response' has remained the subject of active interest and is the most studied global regulatory system as its role in growth and control of gene expression is vital.

## 1.5 The Stringent response

Amino acid starvation of *E.coli* results in reorientation of its cellular metabolism that conserves energy for survival, known as the "stringent response". SR (stringent response) is a highly conserved stress response (Cashel, 1996, Potrykus & Cashel, 2008) and characterized by

repression of transcription of stable RNAs (i.e rRNA and tRNA) which are required for rapid growth and concomitant upregulation of genes involved in amino acid biosynthesis, nutrient acquisition & stress survival. This adaptive mechanism was first noticed as the bewildering ability of bacterial cells to restrict stable RNA accumulation to amino acid starvation (Sands & Roberts, 1952). The suppression of this phenotype in which bacteria do not show this response and elicit the continuous synthesis of stable RNAs, was termed as "relaxed mutant" (Borek (Borek *et al.*, 1956), later this mutation was genetically mapped to RNA control locus ($RC^{rel}$) called as *relA* (Stent & Brenner, 1961). Stent and Brenner first described SR as inhibition of translation coupled with reduction in transcription and leads to global changes in transcriptome of starved cells.

In pursuit of effectors of this response, over 45 years ago, Cashel and Gallent visualized two spots on two dimensional thin layer chromatography from nucleotide extracts of *E.coli* that could be associated with stress provoked by amino acid starvation, which they dubbed "magic spots" (Cashel & Gallant, 1969). The magic spots were derivative of GTP or GDP later recognized as hyperphosphorylated guanosine derivatives ppGpp (guanosine 5'-diphosphate, 3'-diphosphate) and pppGpp (guanosine 5'-triphosphate, 3'-diphosphate) collectively abbreviated to here as ppGpp, a small nucleotide alarmone that is hallmark of the stringent response and common in eubacteria and plants (Mittenhuber, 2001). Although initially ppGpp is engendered as a SR to amino acid starvation, then the term SR has been expanded to other stresses and nutritional limitation including carbon (Hernandez & Bremer, 1991, Murray & Bremer, 1996, Cashel, 1996), iron (Vinella *et al.*, 2005) fatty acid (Seyfzadeh *et al.*, 1993, Gong *et al.*, 2002) and phosphate (Spira *et al.*, 1995) starvation also accumulate cellular ppGpp and exert the regulatory effect. Ultimately SR reorganizes cellular resources towards adaptation to a semi dormant state in challenging environmental condition for survival (Potrykus & Cashel, 2008, Dalebroux & Swanson, 2012).

### 1.5.1 Involvement of RelA and SpoT in metabolism of ppGpp

The ppGpp levels are regulated by two classes of enzymes: monofunctional synthetase enzyme known as RelA which is encoded by the *relA* gene (Metzger *et al.*, 1988) and bifunctional synthetase/hydrolase enzyme SpoT or RSH (RelA/SpoT homologue). Both RelA and SpoT are

present in *E.coli* and other γ-proteobacteria species but its paralogue is exceptionally absent in *Neisseria* and *Bordetella* species, which belong to the β-proteobacteria (Mittenhuber, 2001).

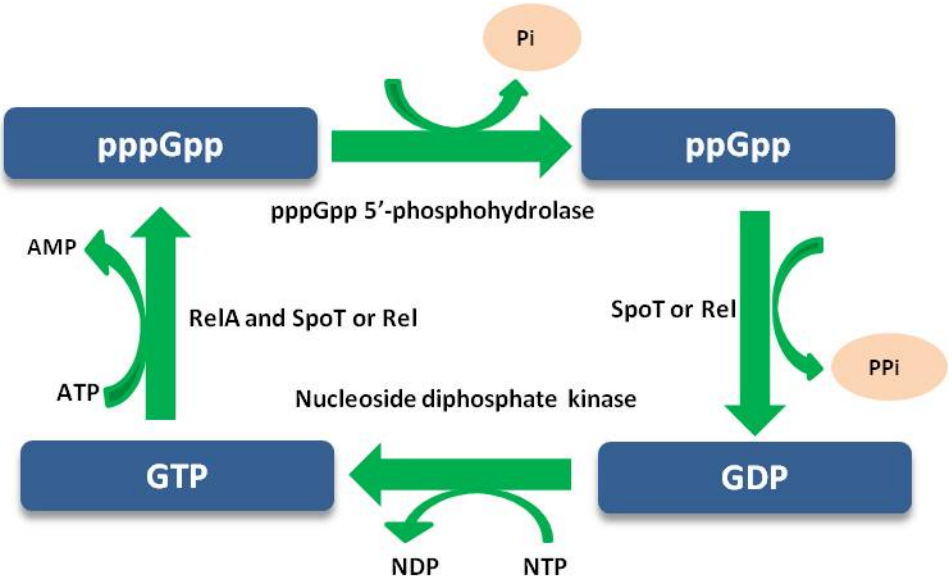


**Figure 1. 3**: **Schematic diagram of synthesis and degradation of (p)ppGpp.** The enzymes involved are represented by *relA*, *spoT*, (p)ppGpp 5'- phosphohydrolase (*gpp*), nucleoside 5'-diphosphate kinase (*ndk*) (Adapted from Cashel et al, 1996).

Figure 1.3 explains the synthesis & degradation of ppGpp. ppGpp synthesis driven by these enzymes catalyze, a pyrophosphoryl group transfer of the of the β,γ-phosphates from ATP to the ribose 3'OH of either GTP or GDP (Haseltine & Block, 1972, Cochran & Byrne, 1974, Hernandez & Bremer, 1991, Xiao *et al.*, 1991). The half-life of the product pppGpp of this reaction is only 6 seconds (Weyer *et al.*, 1976) and then rapidly converted to ppGpp by third enzyme pppGpp 5'-phosphohydrolase (Gpp) (Keasling *et al.*, 1993, Weyer *et al.*, 1976) yet an important enzyme for this metabolic cycle (Mechold *et al.*, 2013). Recent study has suggested that ppGpp is ~10 times more potent than (p)ppGpp with respect to regulation of growth rate, RNA/DNA ratios, rRNAP1 promoter transcription inhibition, threonine operon promoter activation and RpoS induction by *in vivo* and *in vitro* experiments (Mechold *et al.*, 2013). Hence, ppGpp is the main molecular effector that accumulates during stringent response and bind to RNA polymerase. Albeit SpoT enzyme has weak synthetase activity and possesses manganese-dependent phosphohydrolase activity, degrading accumulated pool of pppGpp or ppGpp to GTP or GDP and pyrophosphate (PPi) (De Boer *et al.*, 1977, Heinemeyer & Richter, 1977, Ny &

Björk, 1977, An *et al.*, 1979, Hernandez & Bremer, 1991, Murray & Bremer, 1996) and (Cashel, 1996) after recovery from starvation. Also SpoT is thought to be involved in maintenance of (p)ppGpp during the steady state of bacterial growth (Sarubbi *et al.*, 1988). The ppGpp levels shoots within a few seconds during starvation condition, at highest level after 10-15 minutes and then drops to a new steady state value which is 10-20 fold higher than the basal level of growing bacteria (Lagosky & Chang, 1980, Cashel & Gallant, 1969, Fiil *et al.*, 1972). The concentration of GTP level decrease upto 50% in proportion to increase in ppGpp level during the starvation condition (Gallant *et al.*, 1970, Fiil *et al.*, 1977).

Historically, the RelA and SpoT enzyme of β & γ proteobacteria gave their name to the RelA-SpoT homologue RSH protein family (Atkinson *et al.*, 2011). It has been thought that paralogues *relA* and *spot* genes have evolved separately via gene duplication of an ancestral Rel protein found in gram positive bacteria (Mittenhuber, 2001). Although RelA, SpoT and RSH proteins have extensive homology of amino acid sequence (Metzger *et al.*, 1989), RelA protein contains only synthetic activity due to absence of His-Asp doublet amino acid in conserved histidine-aspartate(HD) domain of metal dependent phosphohydrolase found in SpoT and RSH proteins (Aravind & Koonin, 1998, Heinemeyer & Richter, 1978). The synthetase and hydrolase domains overlap within N- terminal portion of SpoT and RSH proteins (Gentry & Cashel, 1996, Avarbock *et al.*, 2005). X-ray crystallography study of the N-terminal fragment of $Rel_{seq}$ (RSH in *Streptococcusdysgalactiae* subsp *equisimilis*) have demonstrated ligand-binding-induced conformational change in mechanism of two monomers which reciprocally regulate two antagonistic active sites (hydrolase-OFF/synthetase-ON and hydrolase-ON/synthetase-OFF), thus preventing futile cycling of ppGpp synthesis and hydrolysis (Mechold *et al.*, 2002, Hogg *et al.*, 2004).

Rel-like gene is absent in obligately parasitic organisms and archaea (Mittenhuber, 2001) but four functional long *rsh* genes are present in plant Arabidopsis thaliana genome (Mizusawa *et al.*, 2008) and also in pea chloroplast (Tozawa & Nomura, 2011). ppGpp is produced in chloroplast and inhibits RNA polymerase activity *in vitro* after wounding or applying physical stress (Takahashi *et al.*, 2004). Most other organisms contain single bifunctional RSH protein, either synthetase domain known as Rel or RelA (Mittenhuber, 2001) or hydrolase domain (HD), unlike γ proteobacteria and other few exceptions. *Streptococcus mutants* (Lemos *et al.*, 2007),

*Enterococcus faecilis* (Abranches *et al.*, 2009), *Bacillus subtilis* (Nanamiya *et al.*, 2008) and *Vibrio cholerae* (Das *et al.*, 2009) all encode one or more monofunctional, RelA like synthetase fragments, abbreviated as small alarmone synthetases (SASs), in addition to single RSH protein. Firmicute bacteria have RelP, RelQ proteins also known as Yjbm and Ywac, respectively and *Vibrio cholerae* has RelV protein which lacks both the hydrolase and the regulatory C-terminal domains, but by sensing extracellular inputs basal level of ppGpp synthesis have been observed in these organisms (Lemos *et al.*, 2007) which is unique from RelA and SpoT. Generally bacteria contains only HD domain which has not been reported yet, however HD domain is identified only in metazoan which contains Mesh1 RSH protein (Sun *et al.*, 2010).

In summary, the intracellular concentration of (p)ppGpp is adjusted by synthetic activity of RelA and hydrolytic activity of SpoT or RSH proteins, which are modulated in response to distinct nutrient stimuli and thus, coordinate global transcription patterns of organisms.

#### 1.5.1.1 ppGpp synthetaseI- RelA

*relA* is first gene encoding the amino acid starvation response of *E.coli* which has been cloned, sequenced and characterized (Metzger *et al.*, 1988). *relA* gene has been transcribed from two promoters -*rel*AP1 and *rel*AP2 - located 178 bp and 626 bp upstream of the *relA* translational start site respectively. The house-keeping sigma factor σ70 is involved in regulation of both these two promoters. The promoter *rel*AP1 is constitutive promoter that is active during all growth phases which is dependent on an UP stream-like sequence. It is AT-rich sequence which located 40 bp upstream of the transcriptional start-site and thus, enhances promoter recognition by RNA polymerase. The *rel*AP2 promoter is inducible promoter as it was transiently induced at the transition state between the exponential growth phase and the stationary phase and also to be regulated by CRP region which is centered 61bp upstream from the transcriptional start site (Nakagawa *et al.*, 2006, Metzger *et al.*, 1988).

RelA protein initially known as stringent factor (SF) (Cashel & Gallant, 1969) encodes 744 amino acids with a 84 KDa molecular mass. Structural studies of RelA consists of two functional domain: 1) N- terminal catalytic domain consists of 455 amino acids, encoding ribosome independent, constitutive (p)ppGpp synthetase. Introduction of mutation in NTD impaired its ability to synthesize ppGpp and thus lost its binding ability to both ATP and GTP (Gropp *et al.*,

2001). This truncated protein was labile with half-life of 7.5min,while full length RelA protein was stable with a half-life of 2-3h hrs (Schreiber *et al.*, 1991) ; 2) The C-terminal domain contains 456 to 744 amino acids, that controls the activity of RelA. Two independent studies demonstrated that CTD point mutations and deletion abrogate activation of RelA in *E.coli* and the RSH homologue ($Rel_{Mtb}$) in Mycobacterial tuberculosis (Gropp *et al.*, 2001, Avarbock *et al.*, 2005). The CTD is also required for oligomerization of RelA and $Rel_{Mtb}$. This led us to a conclusion that regulatory function of the CTD is in transmitting activation signal from the ribosome to the N- terminal catalytic domain and it possibly involves oligomerization.

In normal growing bacterial cells, deacylated tRNA constitutes approximately 15% of the total tRNA, the majority of which is present in ribosome bound state. Upon amino acid starvation condition, the fraction of deacylated tRNA can rise up to 80% of the total tRNA (Yegian *et al.*, 1966). The shortage of aminoacylated tRNA due to large pools of free deacylated tRNA, enables decylated tRNA to bind empty acceptor A- site of 50S ribosome in the presence of a cognate codon, the protein synthesis is stalled and ribosome bound RelA is activated to synthesize (p)ppGpp (Haseltine & Block, 1973, Ramagopal & Davis, 1974).

The precise mechanisms of RelA mediated (p)ppGpp synthesis is not fully understood. Initial studies demonstrated RelA binding to 70S ribosome for production of (p)ppGpp is necessary (Ramagopal & Davis, 1974, Richter, 1976, Richter *et al.*, 1975) and binding is enhanced by the presence of a poly (U) – mRNA (Wagner & Kurland, 1980). Apart from deacylated tRNA, RelA activation is dependent on N-terminal segment of L11, 50S ribosomal protein (Friesen *et al.*, 1974, Yang & Ishiguro, 2001, Wendrich *et al.*, 2002). Interestingly, recent studies proposed that RelA binding to the ribosome is governed primarily by mRNA but independent of ribosomal protein L11 or deacylated tRNA (Wendrich *et al.*, 2002). Some 30 years later a model was proposed (Figure 1.4) for mechanism of RelA mediated (p)ppGpp synthesis which involves RelA in detection of blocked ribosomes with extended 3' end of them RNA. Upon (p)ppGpp synthesis RelA, but not deacylated tRNA, is released from the ribosome and RelA 'hops' between blocked ribosomes which would correlate the amount of (p)ppGpp synthesized to the number of blocked ribosomes within the cell (Wendrich *et al.*, 2002). In post stress condition, aminoacylated tRNAs are replenished, can easily replace deacylated tRNAs, rescuing blocked ribosomes as amino acylated tRNAs have much higher affinity for the acceptor site of the

ribosomes (Schilling-Bartetzko *et al.*, 1992). The intracellular concentration of RelA is low i.e 110 molecules RelA/cell. *E.coli* cell carries approximately 1500 ribosomes thus RelA is associated with only about 5% of them (Pedersen & Kjeldgaard, 1977). This is much less than what would be expected. Further RelA activity is modulated by positive allosteric feedback mechanism by its product i.e (p)ppGpp (Shyp *et al.*, 2012).

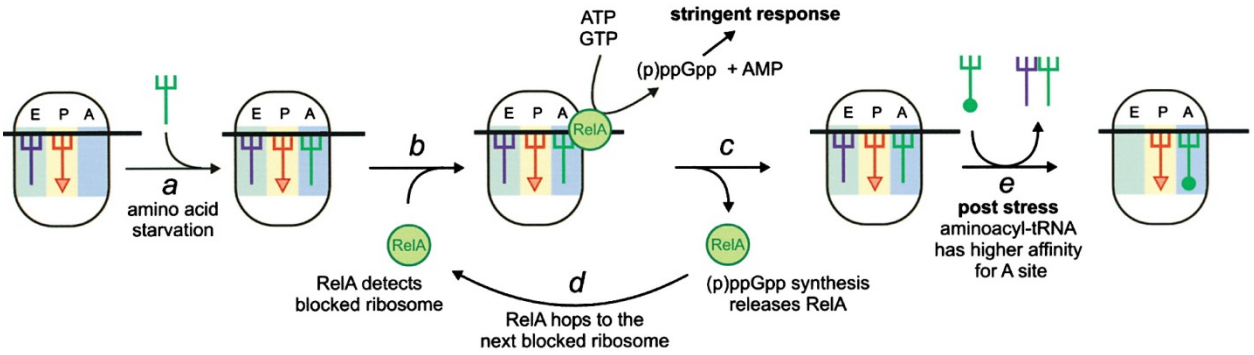


**Figure 1. 4: A "hop" model for mechanism of RelA mediated (p)ppGpp synthesis.** (a) during amino acid starvation condition, uncharged t-RNA binds to A-site of the ribosome and block the ribosome (b) RelA detects blocked ribosome with a 3' extension of the mRNA. (c) synthesis of (p)ppGpp from ATP and GTP and RelA releases from blocked ribosome but not decaylated tRNA (d) RelA "hops" to the next blocked ribosome, and the synthesis of (p)ppGpp is repeated. (e) After post-stress conditions an aminoacylated tRNA has a higher affinity for the A site of ribosome enables dislocation of the deacylated tRNAs, which rescues blocked ribosomes and reactivates translation (Wendrich *et al.*, 2002) .

Recently, novel single molecule in vivo investigations of stringent response indicated that RelA is tightly bound on the ribosome during non-starved condition but on induction of starvation condition RelA rapidly dissociates from the ribosome and perform the multiple round of catalysis termed as 'extended hopping' model (English *et al.*, 2011).

### Genetic factors required for regulating *relA*

A model of regulation of *relA* has been proposed by Edward et al, (2011), they explained the Csr regulatory system fine tunes the stringent response (Edwards *et al.*, 2011). *csrA* is a carbon storage regulator and small dimeric RNA binding protein which binds to leader sequence of *relA* and repressed its expression, there by inhibited ppGpp accumulation. *csrA* has modest/negligible effect on *dksA* and *spoT* expression, this effect has been masked by negative autoregulation of DksA. ppGpp and DksA activate transcription of small noncoding RNAs, CsrB and CsrC by 10-fold (Edwards *et al.*, 2011). CsrA indirectly activates *csrB* and *csrC* expression via the BarA–

UvrY TCS (Suzuki *et al.*, 2002, Weilbacher *et al.*, 2003), in turn, CsrB and CsrC RNAs sequester and antagonize CsrA (Weilbacher *et al.*, 2003, Liu *et al.*, 1997). Thus csr system negatively regulated stringent response.

**1.5.1.2 ppGpp synthetaseII- SpoT**

In β and γ proteobacteria there is a second pathway for ppGpp production which is SpoT dependent pathway. Some mutation or conditions significantly affect the accumulated pppGpp and ppGpp ratio (Cashel & Gallant, 1969). In mutant strain large quantities of ppGpp accumulate and the quantities of pppGpp stayed scarcely detectable which was originally called 'spotless phenotype' and after that it was mapped to the locus on *E.coli* chromosome by Laffler and Gallant (Laffler & Gallant, 1974) later termed as "SpoT". SpoT was identified to be a (p)ppGpp synthetase II (Hernandez & Bremer, 1991). SpoT is a bifunctional enzyme that possesses both synthetic and hydrolytic activity, but synthetic activity is weak than to RelA (Xiao *et al.*, 1991). The *spoT* gene sequence has been characterized (Sarubbi *et al.*, 1989) which encodes 702 amino acids with a molecular mass of 80 KDa and is located at 82 minutes on *E.coli* chromosome. ppGpp-3'-pyrophosphatase (ppGppase) activity resides in first 203 amino acids and overlapping region containing residues 63-374, which is involved in ppGpp synthetic activity (Gentry & Cashel, 1996). In *E coli relA* deleted strain, substitution of residue Asp293 in SpoT could not accumulate ppGpp, thus suggested that Asp293 residue is crucial for its activity (Fujita *et al.*, 2002). The mechanism of ppGpp degradation has been well elucidated (Johnson *et al.*, 1979). In an early study a group of scientists has reported that hydrolytic activity of SpoT repressed by uncharged tRNA and cofractionates with the ribosome (Heinemeyer & Richter, 1977, Richter, 1980, Sy, 1977); Gantry and Cashel study showed that SpoT protein is localized in cytosolic fraction of cell (Gentry & Cashel, 1995) while recent report showed that SpoT copurifies with pre-50S ribosome particle (Jiang *et al.*, 2007).

Interestingly, SpoT has been known to sense unique signals those not sensed by RelA, which include Carbon, fatty acid, iron and phosphate starvation (Battesti & Bouveret, 2006, Seyfzadeh *et al.*, 1993, Vinella *et al.*, 2005, Spira *et al.*, 1995) and exerted (p)ppGpp synthesis.

An example of fatty acid starvation is illustrated here : In *E.coli*, upon perturbations in fatty acid metabolism, a physical interaction between SpoT and acyl carrier protein (ACP), central cofactor

in fatty acid and lipid metabolism, has been shown to act as a signal for SpoT dependent ppGpp synthesis (Battesti (Battesti & Bouveret, 2006). Subsequent study showed that the ACP and SpoT interaction restricted to bacteria containing both RelA and SpoT and to ACP protein encoded by genes located in fatty acid synthesis operons (Battesti & Bouveret, 2009), emphasizing that bacteria have evolved different mechanisms of (p)ppGpp regulation. A working model explained how ACP regulates switch between SpoT- dependent ppGpp hydrolysis and synthesis activity (Figure 1.5).

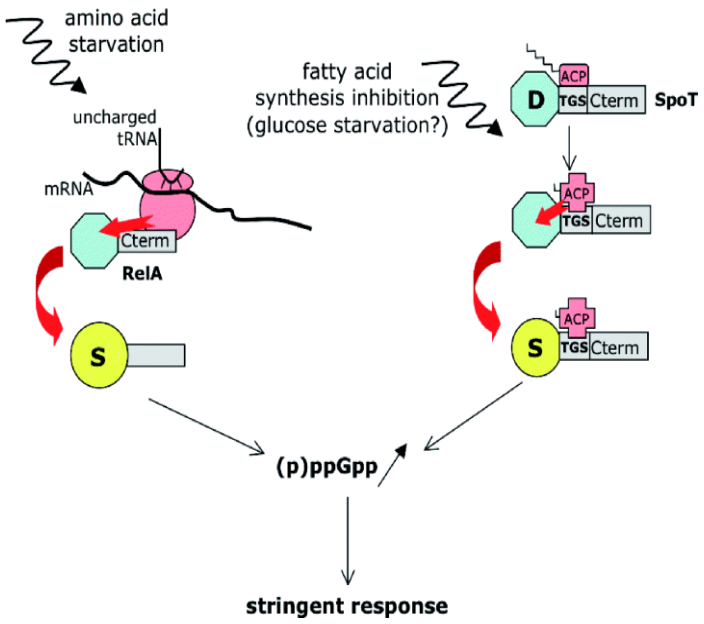


**Figure 1. 5: A switch model for ACP mediated ppGpp synthesis from SpoT.** Fatty acid starvation triggers conformational changes in ACP transduced to SpoT, favouring the (p)ppGpp synthesis activity upon degradation (Battesti & Bouveret, 2006).

In *E.coli* and *V.cholarae*, GTP binding protein Obg (also known as CgtA (GTPase) directly interact with SpoT (Wout *et al.*, 2004, Raskin *et al.*, 2007) and alters ppGpp levels in exponential growth (Jiang *et al.*, 2007, Raskin *et al.*, 2007), by promoting the hydrolase activity of SpoT; thereby maintaining low ppGpp levels in normal growth condition. This study requires further verification as the effects of Obg on ppGpp pool is quantitatively minor.

A strain lacking, both RelA and SpoT proteins are not able to produce any ppGpp, referred to as a $ppGpp^0$ strain. Such strains are unable to grow in minimal media as these strains become auxotrophs for amino acids (Xiao *et al.*, 1991). This phonotype is most probably due to the lack

of induction of promoters for amino acid biosynthesis as these operons are positively regulated in ppGpp dependent manner (Cashel, 1996). $ppGpp^0$ strain continuously accumulate stable RNA and ribosomal protein in starvation condition, this response is termed as a 'relaxed phenotype'.

### 1.5.2 Pivotal role of global regulator - (p)ppGpp

It has been suggested that ppGpp binds to β and β' subunit of RNA polymerase identified by crosslinking and crystal structure study (Chatterji *et al.*, 1998, Toulokhonov *et al.*, 2001, Artsimovitch *et al.*, 2004), however recent study showed that (p)ppGpp bind to a site at the interface between β' and ω subunits (Ross *et al.*, 2013). ppGpp is a global regulator, mediates global transcriptional switch from highly expressed genes required for growth to amino acid biosynthetic operons to genes required for stress resistance and for homeostasis during slow/non-growth. ppGpp can act both as positive and negative regulator of transcription. Some of $\sigma^{70}$ – dependent genes involved in cell proliferation and growth are negatively regulated (Barker *et al.*, 2001) and genes required for survival of bacteria are positive regulated by ppGpp (Nyström, 2004)

The regulatory effects of ppGpp on main cellular processes of bacteria are summarized in figure 1.6 and lists.

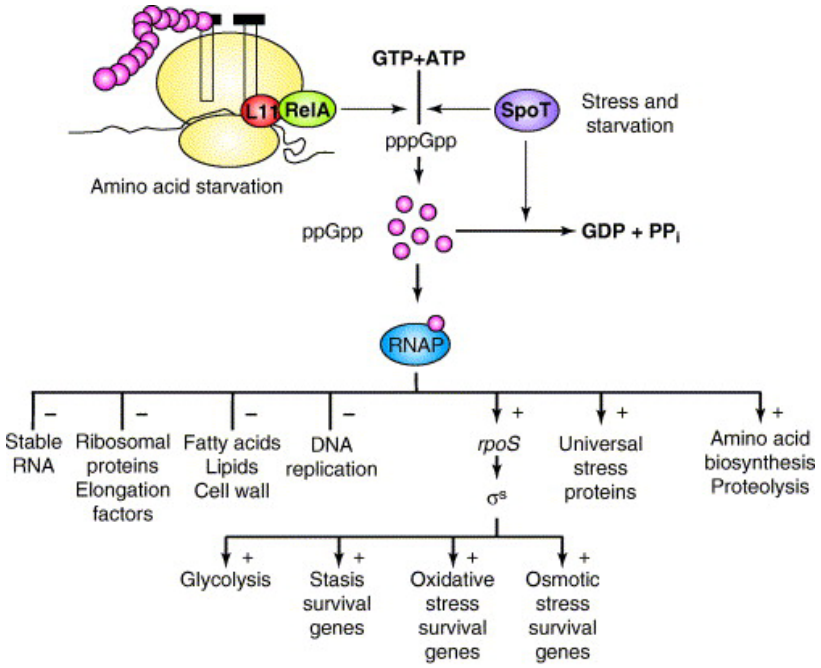

**Figure 1. 6: ppGpp and its' effect on global gene expression.** ppGpp binds RNAP and redirects transcription from growth-related genes to genes involved in stress resistance and starvation survival (Magnusson *et al.*, 2005).

Following lists are about down-regulation and upregulation of physiological activities during stress condition:

**Down regulation**

1. Inhibition of stable RNA transcription.
2. Inhibition of initiation and elongation of DNA replication.
3. Down-regulation of protein synthesis.
4. Inhibition of cell wall synthesis.
5. Cell cycle inhibition.
6. Inhibition of nucleotide biosynthesis, phospholipid synthesis, oxidative metabolism, metabolite transport, cell motility, cell morphology etc.

**Up regulation**

1. Amino acid biosynthesis
2. Long term persistance and virulence
3. Universal stress protein synthesis
4. Synthesis of sigma factor
5. Antibiotic resistance
6. Toxin/antitoxin system
7. Carbohydrate metabolism
8. Chaperones and proteolysis system.

### 1.5.2.1 DksA- potentiator of ppGpp

DksA (DnaK suppressor A) was originally described as a multiple copy suppressor of the temperature sensitive growth and filamentous phenotype of a *dnaK* (encoding Hsp70) mutant (Kang & Craig, 1990). *dksA* gene has been cloned, sequenced and characterized (Vassylyeva *et al.*, 2004). DksA is a stable, small 17 KDa protein and contains 151 amino acids encoded by nonessential gene in bacteria (Kang & Craig, 1990). Since then, DksA is involved in many pleiotropic effects within cell including, generating profound changes in amino acid biosynthesis (Kang & Craig, 1990), cell division (Yamanaka *et al.*, 1994), chaperonin function (Paul *et al.*, 2004), quorum sensing in *Psuedomonas aeruginosa* (Jude *et al.*, 2003, Branny *et al.*, 2001)

Virulence in *Salmonella*, *Shigella flexeneri* and *Legionella pneumophila* (Turner *et al.*, 1998, Sharma & Payne, 2006, Dalebroux *et al.*, 2010) and in increased resistance to reactive oxygen species in the *Salmonella* (Henard *et al.*, 2010). Though the levels of stable DksA protein remain constant under log- and stationary-phase growth conditions and different growth rate (Chandrangsu *et al.*, 2011, Brown *et al.*, 2002, Paul *et al.*, 2004), it is playing important role in stringent response. DksA was shown to augment regulation by ppGpp of both the inhibition of rRNA transcription (Paul *et al.*, 2004) and activation of amino acid biosynthetic promoters (Paul *et al.*, 2005).

Structural position of DksA has been found close to ppGpp on RNAP (Figure 1.7). In *E. coli* DksA, GreA and GreB proteins bind to the secondary channel of RNAP which is both the entry point for nucleotide triphosphate precursors (NTPs) and the exit point for backtracked RNA (Nickels & Hochschild, 2004, Perederina *et al.*, 2004, Rutherford *et al.*, 2007). X-ray crystallography study of DksA revealed a coiled-coil globular domain with two highly conserved aspartic acid (Asp) residue at the fingertip (Vassylyeva *et al.*, 2004, Perederina *et al.*, 2004).

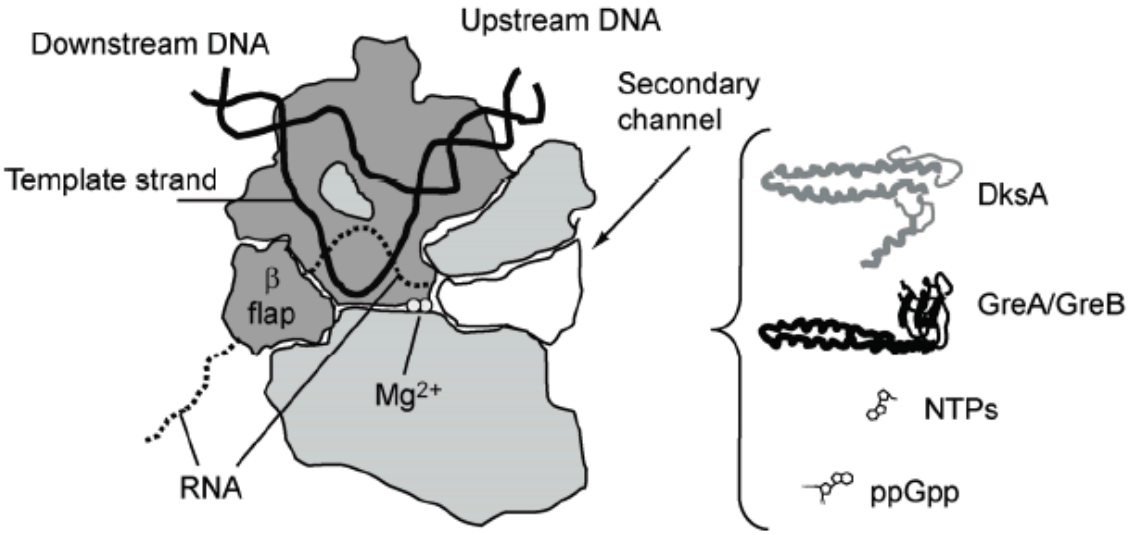


**Figure 1. 7: Structure of the RNAP core enzyme with the main channel containing and the secondary channel** (Nickels & Hochschild, 2004).

The structure of DksA is similar to GreA and GreB despite no sequence homology between them (Perederina *et al.*, 2004). GreA and GreB are transcriptional elongation factors, also cleavage factors, activate RNAP by cleaving backtracked RNA during elongation arrest and that is able to

facilitate production of RNA synthesis (Hsu *et al.*, 1995) and two conserved residues of Gre factors coordinate $Mg^{+2}$ ion required for hydrolysis of the backtracked RNA (Laptenko *et al.*, 2003). GreA/B are structurally similar to DksA, might have possibility that they have similar roles in transcription. Overexpressed GreA antagonizes the negative effects of DksA, independently of ppGpp, on *rrn*P1 transcription initiation in vivo, but shows the modest effect on rRNA promoters *in vitro* (Potrykus *et al.*, 2006). In contrast, GreB mimics the negative effect of DksA on *rrn* expression in vitro but the low concentration of GreB does not reproduce measurable effect in vivo. Furthermore, Gre factors neither are unable to mimic positive effects of DksA on amino acid biosynthetic promoters nor rescues the ability of RNAP lacking the ω subunit to respond to ppGpp (Rutherford *et al.*, 2007).

To elucidate the mechanism of DksA on RNAP, the study revealed that the two independent mutations in *dksA*, L15F and N88I, bypassed the requirement for ppGpp and suppressed the inability of *(*Δ*relA*Δ*spoT*) $ppGpp^0$ cells growth on minimal media lacking amino acid. The mutants increased DksA activity both *in vivo* and *in vitro* (Blankschien *et al.*, 2009). In a conjunction with above study, the report suggested that affinity of DksA to RNAP plays an important role in its function, as DksA has higher affinity for free RNAP versus RNAP in an open complex (Lennon *et al.*, 2009).

In *Mycobacteria tuberculosis* CarD is present, which is homolog of DksA. CarD interacts with different site of RNAP than DksA, however, generates stringent response upon starvation and controls rRNA transcription same as *E.coli* (Stallings *et al.*, 2009).

**1.5.2.2 ppGpp and DksA**

The suppressors of *relAspoT* double mutants ($ppGpp^0$ cells) in *E.coli* provided insights into the role of ppGpp function. These majority suppressors mapped to β and β' subunits of RNAP, named the genes as *rpoB* and *rpoC* respectively (Bartlett *et al.*, 1998, Barker *et al.*, 2001, Murphy & Cashel, 2003, Trautinger & Lloyd, 2002, Szalewska-Palasz *et al.*, 2007) and some suppressors at lower frequency mapped to *rpoD* (Hernandez & Cashel, 1995). The mutations in RNAP further weaken the - interaction with stringently controlled promoters, mimicking the stringent response (Zhou & Jin, 1998). Similarly Δ*dksA* suppressors obtained, mapped them in

RNAP, inferring that DksA hinders conformational changes in RNAP and DNA during transition from closed complex ($RP_C$) to initiation complex ($RP_I$) (Rutherford *et al.*, 2009).

ppGpp and DksA have been demonstrated to directly interact with RNA polymerase and reduce the half life of open complex which influence transcription in a promoter-dependent mechanism (Barker & Gourse, 2001, Paul *et al.*, 2004). The *rrn* promoters form intrinsically instable open complex during transcription initiation and are very sensitive to further destabilization, inhibited by DksA and ppGpp (Zhou & Jin, 1998, Barker & Gourse, 2001, Paul *et al.*, 2004). The other promoters have long-lived open complexes with RNAP can be positively regulated by ppGpp and DksA as RNAP clears the promoter before destabilization. The negatively regulated rRNA promoters are featured by the presence of a suboptimal -35 hexamer, a suboptimal (extended) -10 hexamer, asuboptimal spacer length (16 bp) and a GC rich discriminator region (Haugen *et al.*, 2006). A GC-rich discriminator sequence located between the -10 and the transcription start site and suboptimal sequences for sigma factor recognition (Travers, 1980, Travers, 1984, Josaitis *et al.*, 1995, Park, 2002). DksA and ppGpp have been involved in open complex collapse in concert and independent of each other (Barker & Gourse, 2001, Paul *et al.*, 2004, Rutherford *et al.*, 2009) but a conflicting report suggested that DksA does not contribute to open complex collapse at the *rrn* promoter (Potrykus *et al.*, 2006). The varying concentration of ppGpp and iNTPs, but not DksA (as mentioned earlier, remain constant in all growth condition) are the modulators of rRNA transcription (Murray *et al.*, 2003, Dalebroux *et al.*, 2010).

Direct positive regulation of ppGpp and DksA has not been studied in detail than negative regulation. However, the positive effects of ppGpp and DksA areexertedby both direct and indirect mechanisms. DksA and ppGpp activate increased rate of an isomerization ($k_i$) step on the pathway to open complex formation, thus promoting transcription of some amino acid promoters (Paul *et al.*, 2005). According to a hypothesis, increase in free RNAP concentration can be obtained by decreasing that amount of RNAP involved in transcription of stable RNA promoters (Barker *et al.*, 2001, Paul *et al.*, 2005). Also high levels of free core RNAP could indirectly promote alternative sigma factor-dependent gene regulation by allowing competition of alternative sigma factors (e.g. $\sigma^S$ ,$\sigma^E$ ,$\sigma^N$) (Jishage *et al.*, 2002) . A study has also given evidence that RpoS-dependent promoters are not efficiently transcribed in a $ppGpp^0$ strain even if RpoS is present in high concentration. This may occur because of a decrease in level of free RNAP core

in absence of ppGpp (Kvint *et al.*, 2000). Many studies have demonstrated that *in vivo* ppGpp and/or DksA are needed for alternative sigma factor-dependent transcription. They have also shown that mutated RNAP mimics stringent response and allows for efficient competition of sigma factor in the absence of ppGpp accumulation (Jishage *et al.*, 2002, Laurie *et al.*, 2003) (Bernardo *et al.*, 2006, Szalewska-Palasz *et al.*, 2007, Costanzo *et al.*, 2008), which suggests that the stringent response not only employs direct but also indirect mechanisms to alter global transcription.

ω subunit of bacterial RNAP, also known as RpoZ has been found to be involved in stringent response. ω subunit is involved in the assembly of RNAP, helps in proper folding of the β' subunit and directs the association of β' with the $\alpha_2\beta$ subunits (Gentry *et al.*, 1993, Ghosh *et al.*, 2001, Mukherjee *et al.*, 1999).

Initial study showed that the ω protein which was encoded in the same operon as *spoT* (Gentry & Burgess, 1989), was required for RNAP sensitivity to ppGpp in vitro (Igarashi *et al.*, 1989) in contrast another study demonstrated that it was not necessary for stringent response in vivo (Gentry *et al.*, 1991). Another group of researchers proved that on the addition of ω, RNAP could regain sensitivity to ppGpp *in vitro*, which inferred that ω is required for an appropriate RNAP response to ppGpp. It was found that DksA can rescue RNAP sensitivity to ppGpp in the absence of the ω subunit in vitro, and this cleared the conflict between the *in vivo* and *in vitro* studies (Vrentas *et al.*, 2005). This is the most likely explanation for the ability of *rpoZ* mutants to retain sensitivity to ppGpp *in vivo*; however, in-depth studies still need to be done for further clarification regarding the role that ω has on the ppGpp-responsiveness of RNAP.

#### 1.5.2.3 Regulatory targets of ppGpp and DksA

The effects of ppGpp and DksA on bacterial physiology are quite broad. Some researchers linked stringent response to growth rate control (Bremer & Dennis, 1996). Apart from regulating metabolic processes, ppGpp and DksA also play an active role in regulating a number of cellular processes involved in survival under various environmental stresses and are also responsible for colonization and virulence in pathogenic bacteria.

*rpoS* is the master regulator of general stress response (Lange & Hengge-Aronis, 1991) and regulate hundreds of genes in both stationary and exponential phase which are involved in amino

acid biosynthesis, oxidative stress, osmotic shock (Nyström, 2004, Weber *et al.*, 2005). The major role of *rpoS* is in adaptability and survival of cells during abnormal condition. High level of ppGpp increases the competition of $\sigma^S$ and $\sigma^{32}$ with $\sigma^{70}$ for binding to core RNAP. Growth pattern is dependent on competition of sigma factors and their ppGpp dependent binding to RNAP and its synthesis (Jishage *et al.*, 2002). One of the important regulatory mechanisms for *rpoS* is stringent response in *E. coli* which induces expression of the stationary phase sigma factor, RpoS (Gentry *et al.*, 1993), and both ppGpp and DksA appear to show effects at multiple levels. *rpoS* transcription is ppGpp dependent (Lange *et al.*, 1995). It has been shown that basal expression of *rpoS* is effected by ppGpp, although *rpoS* expression is slightly delayed in a Δ*relA*Δ*spoT*strain (Hirsch & Elliott, 2002). DksA is also required for the ppGpp-dependent activation of *rpoS* at the translational level; however, it is unclear if this effect is direct or indirect (Hirsch & Elliott, 2002, Brown *et al.*, 2002). ppGpp also controls RpoS protein stability.

ppGpp and DksA exert their effects on DNA replication during amino acid starvation (Levine *et al.*, 1991). In *B. subtilis*, replication elongation is inhibited by ppGpp which directly inhibits primase, an essential component of the replication machinery, and decreases the ability to recruit RecA to replication forks (Wang *et al.*, 2007). In *E. coli*, replication initiation and chromosome segregation is blocked by ppGpp and these effects require both Dam and SeqA (Ferullo & Lovett, 2008). Independently of ppGpp, DksA, along with GreA, GreB and TraR, inhibits stalled transcription complexes from interfering with replication, likely by promoting transcriptional elongation (Tehranchi *et al.*, 2010). A recent study supports a model in which DksA directly aids transcription elongation to coordinate the replication and transcription machineries to prevent generation of DNA lesions by replication blockage (Zhang *et al.*, 2014).

### 1.5.3 BarA ("bacterial adaptive responses")

*barA* gene has been identified as a multicopy suppressors of deletion mutant of *envZ*, which is the sensor domain of EnvZ-OmpR two-component signal transduction system (Nagasawa *et al.*, 1992). A membrane associated 102 kDa protein, the BarA has both the ‘sensor kinase’ as well as the ‘response regulator’ domains. It is the tripartite histidine sensor kinase of the BarA/UvrY two-component signal transduction system and initiates a His-Asp-His phosphorelay in response to accumulation of acetate, the physiological stimulus for BarA activity (Edwards *et al.*, 2011). BarA phosphorylates its cognate response regulator UvrY and BarA/UvrY two-component

system is required for expression of the two non-coding small RNAs, CsrB and CsrC (Pernestig *et al.*, 2001, Suzuki *et al.*, 2002). BarA is thought to be involved in bacterial adaptive response in E. coli as BarA-UvrY system is important for switching between glycolytic and gluconeogenic carbon sources and controls Csr system (Pernestig *et al.*, 2003).

## 1.6 Growth rate regulation and ppGpp

Growth rate regulation in bacteria is first described by Maalaoe and his colleague (Kjeldgaard *et al.*, 1958). They observed that cell mass and RNA level can be affected during transition between different growth rates either by nutrient up-shift from a minimal medium to rich medium or conversely, by nutrient down-shift. Hence, the RNA synthesis is the first to respond in various media and can be either accelerating or decelerating rapidly; simultaneously but delayed relative to RNA synthesis, rate of synthesis of other macromolecules like DNA and protein, during periods of changing growth rates (Neidhardt & Fraenkel, 1961, Maaløe & Kjeldgaard, 1966). During rapid growth in *E. coli* cells, the synthesis of ribosome is the cell's single largest expenditure of biosynthetic energy. During this condition, the cells contain 70,000 ribosomes which are composed of ~ 50 ribosomal proteins (r-proteins) and 3 ribosomal RNAs (rRNAs). The translational rate of the ribosomes in the cells altered at different growth rates. Hence the number of ribosome synthesis must be regulated in proportional to growth rate to meet the cell's demand for proper level of protein synthesis (Bremer & Dennis, 1996, Nomura *et al.*, 1984). The rate limiting step of ribosome synthesis is the synthesis of rRNA. Since 50 years, the growth rate regulation with a focus on the control of ribosomal RNA transcription is one of the most interesting subjects intensely scrutinized by many microbial physiologists.

The intracellular concentration of ppGpp is inversely correlated with growth rate (Ryals *et al.*, 1982, Bremer & Dennis, 1996) and it is tempting to propose that ppGpp might have an important role in growth rate control similar to stringent response.

Several models have been proposed for growth rate dependent regulation of transcription initiation of rRNA, are basically divided into two categories: ribosome feedback models and ppGpp models.

### 1.6.1 Ribosome feedback model

This model proposed by Nomura(Jinks-Robertson *et al.*, 1983) and suggest that excess amount of ribosomes inhibit the rRNA transcription by feedback mechanism. Further study suggested

that pool of ATP and GTP (act as a feedback signals to ribosome promoters) are exhausted in excessive ribosome translation; due to this reason transcription from *rrn* promoters inhibited as these promoters required high amount of initiating NTPs (ATP and GTP) for efficient transcription (Gaal *et al.*, 1997), but controversial report have mentioned that concentration of NTPs cannot be changed with growth rate (Petersen & Møller, 2000). The feedback signal in this model remains ambiguous.

### 1.6.2 ppGpp model

This model is further divided into two models RNAP partitioning model and RNAP sequestering model (passive model). Regulation of both the model is dependent upon concentration of RNAP and ppGpp in cell.

#### (i) RNAP partitioning model

According to this model RNAP exists in two forms, a ppGpp bound form and a free form. If intracellular concentration of ppGpp high (slow growth), it bound to RNAP which direct RNAP away from *rrn* promoters. When the ppGpp levels are low (fast growth), free form of RNAP allocated to *rrn* promoters and is able to initiate transcription needed for growth (Travers, 1980, Ryals *et al.*, 1982).

#### (ii) RNAP sequestering model (passive model)

This model is adapted from passive control mechanism examined by Maaloe. This model suggested that the initiation of transcription rate from *rrn* promoters is dependent upon free form of RNAP (Jensen & Pedersen, 1990) and concentration of free RNA polymerase are inversely correlated with the level of ppGpp, where ppGpp sequesters RNAP in the elongation cycle (Krohn & Wagner, 1996, Sørensen *et al.*, 1994, Zhang *et al.*, 2002).

From above these two models, it was uncertain and contentious topic whether or not the global regulator ppGpp is the major determinant for growth rate control. Recently Cashel and his group's study revealed that *relA spoT* double mutant ($ppGpp^0$) cells grow slowly and showed RNA/DNA, RNA/protein and ratio similar to fast growing cells. The proper growth rate dependent regulation is abolished in cells lacking ppGpp or DksA (Potrykus *et al.*, 2011). Interestingly, they have also shown that overproduction of DksA is able to substitute for ppGpp in growth rate control. In summary, ppGpp, synergistically acting through DksA, is the major determinant of growth rate control according to the nutritional status of the cell.

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