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REVIEW

ppGpp: Stringent Response and Survival

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Adaptation to any undesirable change in the environment dictates the survivability of many microorganisms, with such changes generating a quick and suitable response, which guides the physiology of bacteria. During nutritional deprivation, bacteria show a stringent response, as characterized by the accumulation of (p)ppGpp, resulting in the repression of stable RNA species, such as rRNA and tRNA, with a concomitant change in colony morphology. However, genes involved in amino acid biosynthesis become over-expressed to help bacteria survive under such conditions. The survivability of pathogenic bacteria inside a host cell also depends upon the stringent response demonstrated. Therefore, an understanding of the physiology of stringent conditions becomes very interesting in regulation of the growth and persistence of such invading pathogens.

Keywords: Rel protein, stringent response, (p)ppGpp, omega subunit

Organisms have the ability to adapt to their environment, and those that do not show such skills face the risk of being eliminated. The hallmark for bacterial survival under a changing environment is its adaptability under different stressful situations.

The response originating due to an undesirable change in the environment, "stress", is a result of several biological reactions, most of which occur at the genetic level. A bacterium, be it pathogenic or non-pathogenic, can face several stress conditions over it entire life, such as lack of food and oxygen or the presence of excess salts or inhibitory agents, such as drugs, all of which can affect normal bacterial growth. However, a special class of adaptive response exhibited by bacteria under nutritional stress is called "Stringent response", which has remained the subject of active interest over many years due to its role in growth and the control of gene expression in organisms (Cashel *et al.*, 1996).

However, additional complexities arise in the case of pathogenic microorganisms; which unlike non-pathogenic organisms, as they even have to fight with the host defense system for survival. Therefore, pathogenic bacteria devise strategies to evade the host immune system. Mycobacterium is one such organism that has the ability to quite effectively survive inside the host, with stringent response appearing to play an important role (Primm *et al.*, 2000; Dahl *et al.*, 2003). Tuberculosis, the disease caused by *M. tuberculosis*, is a result of the survivability of the bacteria inside the human body, even under stress conditions (Parrish *et al.*, 1998; Manabe and Bishai, 2000). However, not all members of this genus are pathogenic. *Mycobacterium smegmatis* is a non-pathogenic organism, which can be grown under laboratory conditions and is closely related to *M. tuberculosis* in its basic biology (Ojha *et al.*, 2000; Reyrat and Kahn, 2001). Therefore, this organism provides a good model to study the stringent response shown by *M. tuberculosis* inside the host system (Gupta *et al.*, 2002; Mayuri *et al.*, 2002).

Stringent Response

One of the factors that provide the bacterium with an ability to survive under hostile conditions is the synthesis of an unusual guanosine nucleotide, termed GDP 3'-diphosphate or GTP 3'-diphosphate, which are collectively called (p)ppGpp (Cashel, 1975; Chatterji and Ojha, 2001). In 1969, Cashel and Gallant first discovered that bacteria accumulate these molecules during nutrient starvation. The protein responsible for the synthesis of (p)ppGpp is RelA/SpoT

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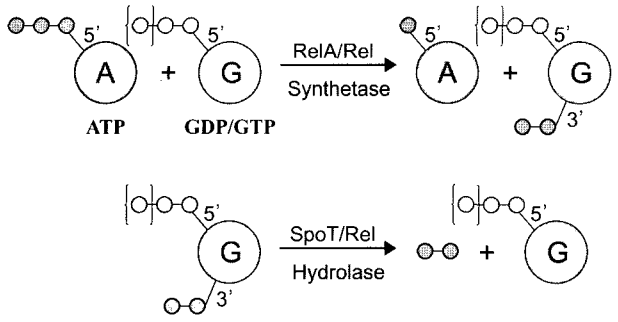


Fig. 1. Representation of the mechanisms of synthesis and hydrolysis of (p)ppGpp by Rel or RelA/SpoT. The former reaction occurs in the presence of Mg^{2+}; whereas, the latter occurs in the presence of Mn^{2+}. Phosphates have been depicted as small circles. **A** stands for Adenosine ribonucleoside and **G** represents Guanosine ribonucleoside.

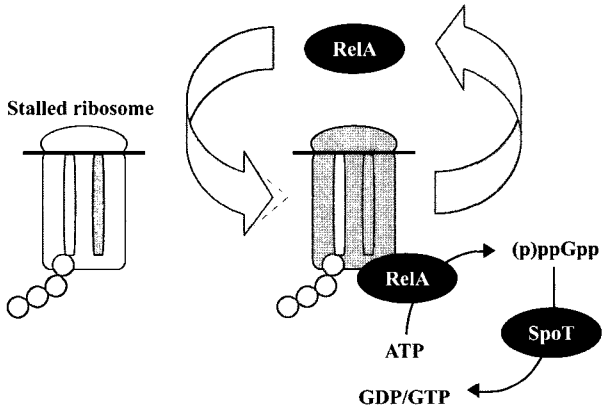


Fig. 2. Representation of the mechanism of action of Rel or RelA and SpoT. An actively translating ribosome stalls upon entry of an uncharged tRNA (shown in red), which signals the Rel or RelA to bind to ribosome, causing the synthesis of (p)ppGpp. RelA or Rel can then transfer from this ribosome to another stalled ribosome. Conversely, SpoT causes the degradation of (p)ppGpp when it is not required.

or Rel, which carries out the transfer of a pyrophosphate moiety from ATP to the 3' hydroxyl position of the ribose of GDP/GTP in a reaction occurring in the presence of Mg^{2+} (Fig. 1) (Cashel, 1974; Cashel, 1975; Avarbock *et al.*, 2000).

Extensive research is being carried out to study the mechanism of the synthesis and hydrolysis of ppGpp. Two proteins, RelA and SpoT, are expressed by Gram negative bacteria, which together help to maintain the level of ppGpp in the cell (Chatterji and Ojha, 2001). The synthesis activity of RelA occurs in the presence of Mg^{2+}; whereas, the SpoT protein mainly carries out its pyrophosphatehydrolase activity in the presence of manganese (Xiao *et al.*, 1991), but also has mild synthesis activity (Johnson *et al.*, 1979; Xiao *et al.*, 1991). However, so far RelA has shown no hydrolysis activity, which is probably due to the absence of the hydrolysis or HD domain present in metal-dependent hydrolase enzymes (Aravind and Koonin, 1998). Earlier studies have suggested that RelA protein from Gram negative organisms, like *E. coli* and *Streptomyces coelicolor*, is dependent on ribosomes and uncharged tRNA for its activity (Haseltine and Block, 1973; Martinez-Costa *et al.*, 1998). It has been proposed that during amino acid starvation, an increase in the ratio of deacylated to acylated tRNA occurs. The translating machinery can then obtain a deacylated tRNA at it's A-site, thus aborting the translation. RelA is able to recognize such a stalled ribosome and binds to it, resulting in the synthesis of ppGpp (Fig. 2) (Wendrich *et al.*, 2002).

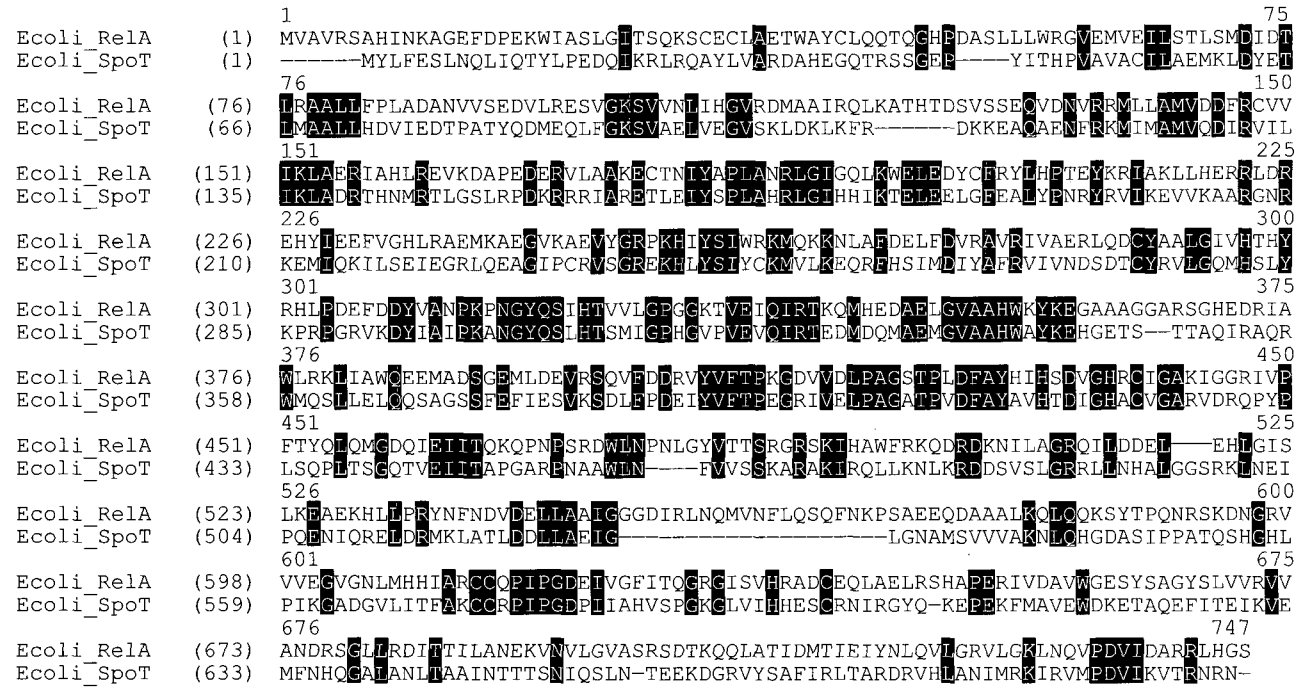
Physically and functionally, RelA protein from *E. coli* can be divided into N-terminal and C-terminal domains, abbreviated as NTD and CTD. The CTD of RelA has been shown to be involved in oligomerization, which in turn regulates the activity of RelA. Moreover, the over-expression of CTD in $RelA^+$ cells poses a negative effect on the accumulation of (p)ppGpp during amino acid starvation (Gropp *et al.*, 2001).

Rel from Gram positive organisms, like *M. tuberculosis* and *Streptococcus equisimilis*, do not require a ribosome and uncharged tRNA for their activity. However, in the presence of such factors, the synthesis activity increases several fold (Mechold *et al.*, 1996; Avarbock *et al.*, 1999; Avarbock *et al.*, 2000; Mechold *et al.*, 2002; Avarbock *et al.*, 2005). Similar results have been obtained in the case of *M. smegmatis* (unpublished data).

By protease digestion of the native Rel protein from *S. equisimilis*, the whole protein has been shown to be composed of two domains interconnected by a flexible protease sensitive hinge (Mechold *et al.*, 2002). It was proposed that the N-terminal domain contains both synthesis and hydrolysis activities, with the C-terminal domain playing a regulatory role by interacting with the N-terminal domain, so that only one activity remains, with the other being switched off. Also, the deletion of the C-terminal domain increases the synthesis activity of the Rel protein several fold, which at the same time makes it non-responsive to a ribosome, mRNA and tRNA (Mechold *et al.*, 2002). Similarly, in *M. smegmatis*, deletion of the C-terminal domain resulted in higher synthesis activity, which was unaffected by uncharged tRNA (unpublished data).

A similar observation has been made with the Rel protein from *M. tuberculosis*. The synthesis activity of Rel protein has been shown to be greatly increased in the presence of a complex containing mRNA-ribosome-tRNA, collectively termed RAC complex. More-

A



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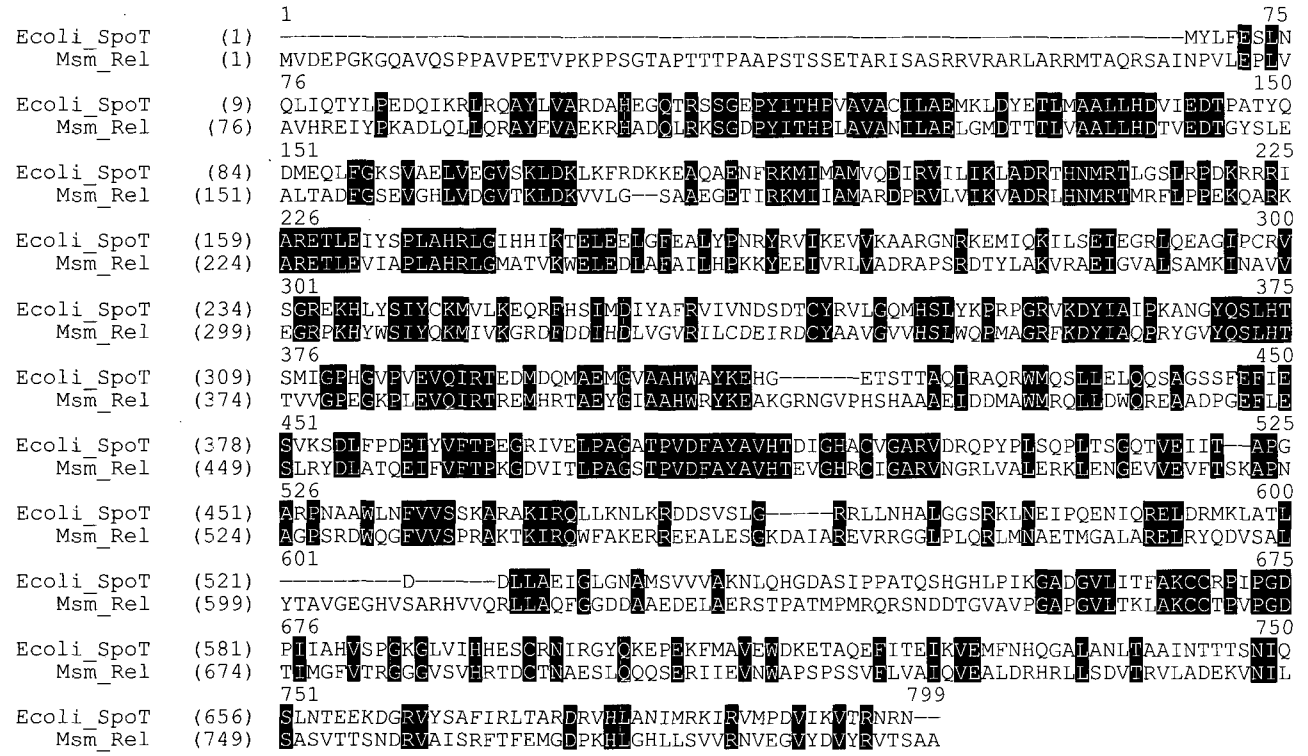

Fig. 3B. A, pairwise alignment of the RelA and SpoT of *E. coli.* B, pairwise alignment of SpoT of *E. coli* and Rel of *M. smegmatis*. The entire protein sequence alignment is shown, with identical residues represented with a black background.

over, the hydrolysis activity decreases significantly in the presence of RAC (Avarbock *et al.*, 2000). In a recent study by the same group, Rel_{Mtb} was shown to form a trimer *in vitro*. Deleting the C-terminal domain results in a monomer and trimer mixture, where the trimer dissociates to the monomer upon substrate addition (Avarbock *et al.*, 2005). It was therefore, proposed that the conversion of the trimer to the monomer, and vice-versa, regulates the protein activity *in vivo* (Avarbock *et al.*, 2005).

Together with these observations, it is quite evident that in Gram negative bacteria, RelA protein catalyzes the synthesis of (p)ppGpp in the presence of uncharged tRNA upon binding to a ribosome. This hydrolysis predominantly occurs due to SpoT protein, with the two acting together to maintain a constant cellular (p)ppGpp level. In Gram positive organisms, since the basal level activity occurs even in the absence of RAC, it is difficult to understand exactly how the activity of Rel is regulated.

Evolution of Rel

It has been suggested that the *relA* and *spoT* genes of Gram negative bacteria evolved from the duplicated *rel*-like gene of Gram positive bacteria (Mittenhuber, 2001). By pairwise alignment, the RelA and SpoT of *E. coli* only have 29% identity (Fig. 3A); whereas, *E. coli* SpoT and *M. smegmatis* Rel have 35% identity (Fig. 3B). Therefore, it appears that the Rel protein of *M. smegmatis* is similar to the SpoT protein of *E. coli*, as both possess synthesis and hydrolysis activities. RelA protein does not possess a distinct HD domain; and therefore, does not carry out hydrolysis due to the substitutions in that region (Mittenhuber, 2001). It has also been shown, using a BLAST search of the *B. subtilis* Rel sequence against obligate parasites, such as, *Treponema pallidum*, *Rickettsia prowazekii* and *Archaea* (Cellini *et al.*, 2004), that *rel*-like genes are absent in these genomes. Therefore, it appears that during the process known as reductive evolution, *rel*-like genes are deleted while adapting to their intracellular life (Mittenhuber, 2001).

In the case of the eubacteria, *Helicobactor pylori*, by monitoring the accumulation of 16S rRNA, even upon amino acid starvation, it showed a relaxed phenotype and minimal synthesis of (p)ppGpp (Scoarughi *et al.*, 1999). Therefore, this presents the first observed case of a wild type eubacteria with a relaxed phenotype. *H. pylori* also showed no *relA* like gene in a homology search of the genome using the *E. coli relA* gene sequence as a reference. However, some similarity was shown with the *spoT* gene, which explains the appearance of (p)ppGpp. Since the amount of (p)ppGpp produced upon amino acid starvation was minimal, the authors argued that other possible mechanisms may govern the growth rate control and mediate the stringent response in the absence of (p)ppGpp (Scoarughi *et al.*, 1999).

The finding that plants possess *relA/spoT* like genes suggests the presence of bacterial like transcription apparatus within plant cells. The presence of bacterial-type RNA Polymerase subunits in the chloroplasts also gives strength to the idea of (p)ppGpp synthesis in plants (van der Biezen *et al.*, 2000; Takahashi *et al.*, 2004).

ppGpp: A reflex response of bacteria

ppGpp is known to have a wide array of effects on the physiology of bacteria. Extensive research on the study of the functional significance of (p)ppGpp has been performed (Svitil *et al.*, 1993; Garza *et al.*, 2000; Sun *et al.*, 2001). Repression of the transcription of stable RNA species, like tRNA and rRNA, and the upregulation of transcription of genes coding the enzymes involved in amino acid biosynthesis are some of the effects posed as a reflex during stringent conditions (Chatterji and Ojha, 2001). ppGpp plays a significant role in the survival of bacteria in different environmental conditions. However, in this review, our attention was focused on certain properties in bacteria that are affected by ppGpp, and which have not previously been comprehensively documented. These functions include the long term persistence, virulence, symbiosis and production of antibiotics, etc. (Fig. 4), each of which will be discussed here.

(p)ppGpp affects the long-term persistence of M. tuberculosis

Although Rel is absent in certain obligatory parasites, its presence is essential in bacteria, such as *M. tuberculosis*. It has been shown that the Rel protein is required for the long term survival of *M. tuberculosis* inside the host. Deletion of the *rel* gene was found to severely affected the persistence of the bacterium in a liquid culture (Primm *et al.*, 2000) as well as in a mouse model (Dahl *et al.*, 2003).

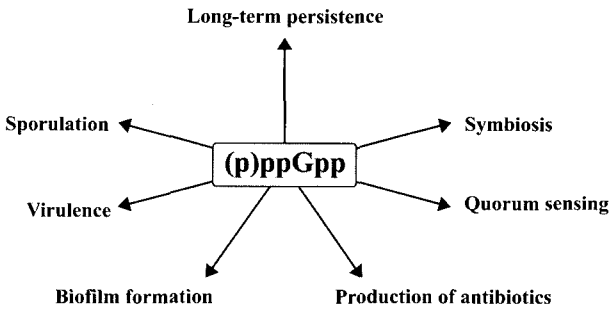

Fig. 4. Diagram depicting the various physiological aspects in bacteria affected by (p)ppGpp.

Colony morphology is affected by the over production of (p)ppGpp

A remarkable difference in the growth of the *M. smegmatis rel* knockout strain compared with the wild type has previously been observed. Moreover, the deletion of *rel* was also shown to affect the long term persistence of *M. smegmatis* in a liquid culture (Mathew *et al.*, 2004; Dahl *et al.*, 2005). It was also found that the over expression of *rel* in *M. smegmatis* resulted in spherical shaped colonies with a smooth surface (Ojha *et al.*, 2000). Furthermore, the appearance of a new polar GPL was shown in a carbon starved culture of *M. smegmatis*, a condition in which the synthesis of (p)ppGpp has also been noted (Ojha *et al.*, 2000; Mukherjee *et al.*, 2005).

(p)ppGpp is involved in quorum sensing in Pseudomonas aeruginosa

In *Pseudomonas aeruginosa*, a human pathogen, quorum sensing (a cell density dependent factor) has been shown to be activated by the expression of the *relA* gene, with the ectopic expression making it cell density independent (van Delden *et al.*, 2001; Erickson *et al.*, 2004). The quorum sensing in this bacteria is mediated by the activation of two transcription regulators, LasR and RhlR, and two other molecules, 3-oxo-C_{12}-homoserine lactone and C_4 homoserine lactone, which function as autoinducers for these transcription regulators. Moreover, these molecules also act as intercellular signals. Therefore, these experiments, suggested that a stringent response is a mediator of quorum sensing in *P. aeruginosa* (van Delden *et al.*, 2001; Erickson *et al.*, 2004).

Recently, it has been shown that changes in cell envelope properties also trigger a stringent response. In an interesting study on *P. aeruginosa*, deletion of the *lptA* gene that codes for an enzyme in the phospholipid biosynthesis pathway, resulted in an increased level of (p)ppGpp in the cell (Baysse *et al.*, 2005).

Symbiosis in Rhizobia is mediated by (p)ppGpp

Rhizobia are known to form colonies or nodules in legumes during periods of nitrogen limitation. Very recently, ppGpp has been shown to be required for nodulation and deletion of the *rsh* gene (*relA/spoT* homologue) in *R. etli*, rendering bacteria incapable of forming colonies (Calderon-Flores *et al.*, 2005; Moris *et al.*, 2005). The *rsh* gene was shown to produce (p)ppGpp in Δ*relA* and Δ*spoT* mutants of *E. coli*. Moreover, nitrogen fixation was strongly reduced in plants nodulated by *rel* mutants of *R. etli* (Calderon-Flores *et al.*, 2005).

In a different study on *Sinorhizobium meliloti*, Gram negative plant symbiont bacteria, with a mutation in the *rel* gene, were found to be incapable of displaying a stringent response, and also showed overproduction of the succinoglycan required for host infection (Wells and Long, 2002). However, mutation in the *rpoB* and *rpoC* genes restored the deletion effect of *relA*. *S. meliloti* has also been shown to have only one *relA/spoT* homologue (Wells and Long, 2002). Using a database analysis, this was found to be true for several other alpha-proteobacteria, such as *A. tumefaciens*, *M. loti* and *C. crescentus*; thus, questioning the existing notion that two genes regulate the stringent response in Gram negative bacteria (Wells and Long, 2002).

Sporulation in Myxococcus sp. is initiated by (p)ppGpp

Myxococcus xanthus, a social bacterium, shows high levels of (p)ppGpp on carbon or amino acid starvation. Earlier reports have suggested that an increased level of (p)ppGpp is responsible for initiating development of the fruiting body (Harris *et al.*, 1998). Increasing the cellular level of (p)ppGpp by the ectopic expression of *relA* of *E. coli* resulted in the expression of specific genes responsible for the development. These results suggest the involvement of (p)ppGpp in the development of *M. xanthus* (Harris *et al.*, 1998; Crawford and Shimkets, 2000).

Virulence in bacteria is influenced by (p)ppGpp

(p)ppGpp has been shown to have direct implications in the virulence, pathogenesis and survival of microorganisms inside the host. Pathogens, such as *Vibrio cholerae* and *Salmonella*, and opportunistic pathogens, like *Pseudomonas* and *Legionella*, were also reported to have their virulence controlled by ppGpp (Hammer and Swanson, 1999; Haralalka *et al.*, 2003; Pizarro-Cerda and Tedin, 2004). *Campylobactor jejuni*, a well known human pathogen, shows upregulation of *spoT* gene during infection of human epithelium, which is also coexpressed with the virulence factor(s). A stringent response in this bacteria is required for its survival in the stationary phase and under low CO_2/high O_2 conditions; moreover, it also provides resistance to rifampicin (Gaynor *et al.*, 2005).

Down regulation of virulence factors in the absence of the *relA* gene was observed in the case of *Vibrio cholerae*. An increased level of (p)ppGpp was observed under conditions where the virulence genes were induced (toxin-coregulated pilus and cholera toxin); whereas, mutants lacking *relA* failed to produce these virulence factors (Haralalka *et al.*, 2003).

The virulence of a pathogen is also governed by its ability to adhere to the host surface, which in turn produces a biofilm, with ppGpp reported to play a crucial role in this biofilm formation. *Listeria monocytogenes*, a known human pathogen, which produces listeriosis defectiveness in *relA*, lost its ability to ad-

here to the model surface and produce listeriosis in murine models (Taylor *et al.*, 2002).

In *Salmonella typhimurium*, a strain with mutations in the *relA* and *spoT* genes, the lack of pGpp synthesis make it highly attenuated *in vivo*. Two transcription activators, *hilA* and *invF*, are required for the pathogenicity in *Salmonella*. The expressions of these factors were greatly reduced in a *relA* and *spoT* deleted strain (Song *et al.*, 2004). Moreover, only the *spoT* mediated synthesis of ppGpp was sufficient for the expression of virulent genes (Pizarro-Cerda and Tedin, 2004). Therefore, this clearly shows the requirement of ppGpp for the virulence in *Salmonella*. Conversely, *relA* alone was found to be responsible for (p)ppGpp synthesis in *Neisseria gonorrhoeae* during nutritional stress (Fisher *et al.*, 2005). An adjustment of the ppGpp levels was also observed in the case of *P. aeruginosa* during infection in Drosophila (Erickson *et al.*, 2004).

(p)ppGpp affects the production of antibiotics

To survive in an adverse environment, and obtain an edge in the competition with other organisms, some bacteria synthesize antibiotics and secondary metabolites. It has been observed that the level of ppGpp in the cells affects the production of antibiotics and that the *relA* gene plays a role in the production of the antibiotics undecylprodigiosin and actinorhodin in *Streptomyces coelicolor*. Disruption of *relA* in *S. coelicolor* abolishes the production of these antibiotics under conditions of nitrogen limitation, which is otherwise, unaffected (Chakraburtty and Bibb, 1997). Conversely, deletion of the *relA* gene in *Streptomyces antibioticus* showed unconditional abrogation of the production of actinomycin (Hoyt and Jones, 1999). Similarly, the production of bacilysin by *Bacillus subtilis* is regulated by both ppGpp and GTP (Inaoka *et al.*, 2003). *relA* has also been shown to regulate the production of cephamycin C in *Streptomyces clavuligerus* (Jin *et al.*, 2004).

(p)ppGpp in Plants

Similarly to bacteria, plants also encounter stresses due to environmental fluctuations, including salt, UV radiation, acid and alkali, pathogen infection and drought etc. Extensive studies have been performed to gain an understanding of the complex signal transduction network in order to combat these stresses.

The functional homologue of *relA/spoT* or *RSH* has been reported in *Arabidopsis thaliana* which could complement the loss of *relA* in *E. coli*. The interaction of *At-RSH* with the plant defense system indicates the role of (p)ppGpp in cell signaling (van der Biezen *et al.*, 2000), but the role of (p)ppGpp has largely been overlooked in the plants.

Recently, Ochi and coworkers showed the presence of this alarmone molecule in chloroplasts; and interestingly, the levels of (p)ppGpp were observed to be elevated during stresses, such as heat shock, acidity, heavy metals and UV irradiation, as well as due to an abrupt change in light intensities. Treatment with plant hormones known to play a critical role in signal transduction also increased the amount of ppGpp, suggesting its involvement in systemic plant signaling towards the stress response (Takahashi *et al.*, 2004).

Effect of ppGpp on RNA Polymerase: Mediation of Stringent Response

In order to mediate its effect on the transcription apparatus in a bacterial cell, ppGpp binds to the β and β' subunits of RNA polymerase. Earlier reports, using aminonapthalenesulfonate (AmNS)-ppGpp as a fluorescent substrate, have shown that the β subunit encoded by the *rpoB* gene is the binding site for ppGpp (Reddy *et al.*, 1995). Subsequently, with the use of chemical cross-linking of azido-ppGpp to the C-terminal of the β subunit, it was clearly indicated that the meka-ppGpp binds to the β subunit of RNA polymerase (Chatterji *et al.*, 1998). However, using a smaller ppGpp analog, 6-thio-ppGpp, it was found that the β' subunit encoded by *rpoC* gene is the target for ppGpp binding (Toulokhonov *et al.*, 2001). These conflicting results can be explained by the fact that the two analogs have different properties, and the N-terminus of β' and C-terminus of β are spatially close, as observed in the crystal structure of RNA polymerase from *T. aquaticus* (Zhang *et al.*, 1999).

Recently, the crystal structure of RNA polymerase from *Thermus thermophilus* complexed with ppGpp appeared, which clearly showed the binding of ppGpp to the β and β' subunits (Artsimovitch *et al.*, 2004). It also described the possible mechanism by which ppGpp exerts a negative effect on the transcription from certain promoters suggesting the presence of a Cytosine base in the non-template strand of DNA results in the ppGpp mediated transcription inhibition by base-pairing with the G of ppGpp; thereby, lowering the life time of the open complex. It further explained the differences in the level of inhibition from the two rRNA promoters, *rrnB* and *rrnD*. Although, this alone explained the effect of ppGpp on transcription, the possibility of the involvement of accessory factors able to enhance its effect cannot be ruled out.

One such factor that enhances the effect of ppGpp is DksA, a protein coded by the *dksA* gene of *E. coli*. In a recent study, a modeled structure of the DksA-ppGpp-RNA polymerase complex showed that the DksA protein mediates the effect of ppGpp in the

transcription inhibition (Perederina *et al.*, 2004). *In vitro* experiments with purified DksA suggested that it greatly amplifies the ppGpp mediated inhibition of rRNA promoters (Paul *et al.*, 2004; Paul *et al.*, 2005). A recent study on RNA polymerase showed that the ω subunit, coded by the *rpoZ* gene, of RNA polymerase is required for ppGpp to mediate its effect. This has been reported earlier (Igarashi *et al.*, 1989), but raised controversy in the literature, because *rpoZ*-deleted *E. coli* responded to stringency (Gentry and Burgess, 1989). It was subsequently shown that the ω subunit of RNA polymerase was not necessary for the *in vivo* stringent response (Gentry *et al.*, 1991). It has now been reported that RNA polymerase lacking the ω subunit showed a ppGpp mediated stringent response in the presence of DksA protein (Vrentas *et al.*, 2005).

The competition for sigma factors is another mechanism able to regulate gene expression, with ppGpp having been shown to play a significant role (Jishage *et al.*, 2002). ppGpp has been shown as being required for both the production of σ^S as well as for the induction of the σ^S dependent promoters. Furthermore, mutations in the RNA polymerase β subunit relieved the σ^S dependent promoters of their requirement for ppGpp in order to be expressed (Kvint *et al.*, 2000).

New Insights from the Crystal Structure of Rel

Nearly three decades after its discovery, the first crystal structure of Rel from *Streptococcus equisimilis* was published, although this was not a full-length protein, but the catalytic centers responsible for both synthesis and hydrolysis activities were clearly shown (Hogg *et al.*, 2004). The N-terminal half of the Rel_{Seq} protein contains two distinct domains, each harboring an active site for catalysis. A central 3-helix bundle interconnects the two domains and the crystal structure, resolved at a resolution of 2.1 Å, showed two distinct conformations that corresponded to the two activities of Rel. For the synthesis activity to occur, the conformation was termed Hydrolase OFF/Synthetase ON, and for the hydrolysis activity to occur, the conformation was said to be Hydrolase ON/Synthetase OFF. The two active sites were found to be 30 Å apart and to contain bound nucleotides. An unusual guanosine nucleotide, GDP-2':3' cyclic monophosphate, was found in one of these active sites. Rel_{Seq} was found to be structurally homologous to Human phosphodiesterase and Human DNA polymerase Beta.

Detailed study on the Rel_{Seq} structure showed an "Intra-molecular cross-talk" caused one of the activities to be predominant, thus, avoiding a futile cycle of ppGpp synthesis and hydrolysis. The presence of a substrate in one of the active sites results in disruption of the other, therefore, avoiding the simultaneous catalysis from both the sites (Hogg *et al.*, 2004). Thus, this model suggested regulation of the two antagonistic activities, resulting in the synthesis and hydrolysis of (p)ppGpp.

Characterization of *Rel* Promoter Element

The promoter for the *rel* gene from *M. tuberculosis* has recently been characterized, with several interesting properties observed (Jain *et al.*, 2005). Mycobacteria are known to have only an extended −10 region instead of a clear cut −35 box (Bashyam *et al.*, 1996). Here, the promoter was found to be specific for Mycobacteria and was not expressed in *E. coli*. Moreover, using site directed mutagenesis, the −10 promoter region was identified as TATCCT, which matches with TAyGAT (where y = pyrimidine), the −10 promoter consensus sequence in *M. tuberculosis* (Mulder *et al.*, 1997). The distance between the +1 transcription start and the −10 promoter region was also observed to be only 2 nucleotides in length, which is a very unique case for prokaryotes, with such a short distance has never been reported before.

Concluding Remarks

So far, the *rel* gene in Gram positive organisms is clearly known to be responsible for the synthesis and hydrolysis of (p)ppGpp. However, the two activities are performed by two different proteins in Gram negative bacteria. A small signaling molecule controls several events in the life of a bacterium. Depending upon the environment in which the bacteria live, (p)ppGpp plays different roles, including sporulation, biofilm formation, symbiosis and virulence etc. However, all the evidence points to one notion, this being that an organism must survive under unfavourable conditions. Under conditions of nutrient deprivation, Rel (or RelA) synthesizes an alarmone molecule, (p)ppGpp, which modifies the physiology of the bacterium to such an extent to allow survival under these circumstances.

One interesting point to be noted is that most of the genes are repressed, resulting in a limited pool of protein molecules. Thus, some mechanism(s) must exist to increase the stability of these proteins so they will remain functional for a much longer time in the same environment. Further studies in this area will need to be performed to help explore the complex network behind the survival of bacteria under hostile conditions.

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References

Aravind, L. and E.V. Koonin. 1998. The HD domain defines a new superfamily of metal-dependent phosphohydrolases. *Trends Biochem Sci.* 23, 469-472.

Artsimovitch, I., V. Patlan, S. Sekine, M.N. Vassylyeva, T. Hosaka, K. Ochi, S. Yokoyama and D.G. Vassylyev. 2004. Structural basis for transcription regulation by alarmone ppGpp. *Cell* 117, 299-310.

Avarbock, A., D. Avarbock, J.S. Teh, M. Buckstein, Z.M. Wang and H. Rubin. 2005. Functional regulation of the opposing (p)ppGpp synthetase/hydrolase activities of Rel_{Mtb} from *Mycobacterium tuberculosis*. *Biochemistry* 44, 9913-9923.

Avarbock, D., A. Avarbock and H. Rubin. 2000. Differential regulation of opposing Rel_{Mtb} activities by the aminoacylation state of a tRNA.ribosome.mRNA.Rel_{Mtb} complex. *Biochemistry* 39, 11640-11648.

Avarbock, D., J. Salem, L.S. Li, Z.M. Wang and H. Rubin. 1999. Cloning and characterization of a bifunctional RelA/SpoT homologue from *Mycobacterium tuberculosis*. *Gene* 233, 261-269.

Bashyam, M.D., D. Kaushal, S.K. Dasgupta and A.K. Tyagi. 1996. A study of mycobacterial transcriptional apparatus: identification of novel features in promoter elements. *J. Bacteriol.* 178, 4847-4853.

Baysse, C., M. Cullinane, V. Denervaud, E. Burrowes, J.M. Dow, J.P. Morrissey, L. Tam, J.T. Trevors and F. O'Gara. 2005. Modulation of quorum sensing in *Pseudomonas aeruginosa* through alteration of membrane properties. *Microbiology* 151, 2529-2542.

Calderon-Flores, A., G. Du Pont, A. Huerta-Saquero, H. Merchant-Larios, L. Servin-Gonzalez and S. Duran. 2005. The stringent response is required for amino acid and nitrate utilization, nod factor regulation, nodulation, and nitrogen fixation in *Rhizobium etli*. *J. Bacteriol.* 187, 5075-5083.

Cashel, M. 1974. Preparation of guanosine tetraphosphate (ppGpp) and guanosine pentaphosphate (pppGpp) from *Escherichia coli* ribosomes. *Anal. Biochem.* 57, 100-107.

Cashel, M. 1975. Regulation of bacterial ppGpp and pppGpp. *Annu. Rev. Microbiol.* 29, 301-318.

Cashel, M., D.R. Gentry, V.J. Hernandez and D. Vinella. 1996. The stringent response, p.1458-1496. *In* F.C. Neidhardt, R. Curtiss III, J.L. Ingraham, E.C.C. Lin, K.B. Low, B. Magasanik, W.S. Reznikoff, M. Riley, M. Schaechter and H.E. Umbarger (eds.), *Escherichia coli* and *Salmonella typhimurium*: Cellular and Molecular Biology. 2nd ed. ASM Press, Washington, D.C.

Cellini, A., G.L. Scoarughi, P. Poggiali, I. Santino, R. Sessa, P. Donini and C. Cimmino. 2004. Stringent control in the archaeal genus *Sulfolobus*. *Res. Microbiol.* 155, 98-104.

Chakraburtty, R. and M. Bibb. 1997. The ppGpp synthetase gene (*relA*) of *Streptomyces coelicolor* A3(2) plays a conditional role in antibiotic production and morphological differentiation. *J. Bacteriol.* 179, 5854-5861.

Chatterji, D., N. Fujita and A. Ishihama. 1998. The mediator for stringent control, ppGpp, binds to the beta-subunit of *Escherichia coli* RNA polymerase. *Genes Cells* 3, 279-287.

Chatterji, D. and A.K. Ojha. 2001. Revisiting the stringent response, ppGpp and starvation signaling. *Curr. Opin. Microbiol.* 4, 160-165.

Crawford, E.W., Jr. and L.J. Shimkets. 2000. The stringent response in *Myxococcus xanthus* is regulated by SocE and the CsgA C-signaling protein. *Genes Dev.* 14, 483-492.

Dahl, J.L., K. Arora, H.I. Boshoff, D.C. Whiteford, S.A. Pacheco, O.J. Walsh, D. Lau-Bonilla, W.B. Davis and A.G. Garza. 2005. The *relA* homolog of *Mycobacterium smegmatis* affects cell appearance, viability, and gene expression. *J. Bacteriol.* 187, 2439-2447.

Dahl, J.L., C.N. Kraus, H.I. Boshoff, B. Doan, K. Foley, D. Avarbock, G. Kaplan, V. Mizrahi, H. Rubin and C.E. Barry, 3rd. 2003. The role of Rel_{Mtb}-mediated adaptation to stationary phase in long-term persistence of *Mycobacterium tuberculosis* in mice. *Proc. Natl. Acad. Sci. USA* 100, 10026-10031.

Erickson, D.L., J.L. Lines, E.C. Pesci, V. Venturi and D.G. Storey. 2004. *Pseudomonas aeruginosa relA* contributes to virulence in *Drosophila melanogaster*. *Infect. Immun.* 72, 5638-5645.

Fisher, S.D., A.D. Reger, A. Baum and S.A. Hill. 2005. RelA alone appears essential for (p)ppGpp production when *Neisseria gonorrhoeae* encounters nutritional stress. *FEMS Microbiol. Lett.* 248, 1-8.

Garza, A.G., B.Z. Harris, B.M. Greenberg and M. Singer. 2000. Control of *asgE* expression during growth and development of *Myxococcus xanthus*. *J. Bacteriol.* 182, 6622-6629.

Gaynor, E.C., D.H. Wells, J.K. MacKichan and S. Falkow. 2005. The *Campylobacter jejuni* stringent response controls specific stress survival and virulence-associated phenotypes. *Mol. Microbiol.* 56, 8-27.

Gentry, D., H. Xiao, R. Burgess and M. Cashel. 1991. The omega subunit of *Escherichia coli* K-12 RNA polymerase is not required for stringent RNA control *in vivo*. *J. Bacteriol.* 173, 3901-3903.

Gentry, D.R. and R.R. Burgess. 1989. *rpoZ*, encoding the omega subunit of *Escherichia coli* RNA polymerase, is in the same operon as *spoT*. *J. Bacteriol.* 171, 1271-1277.

Gropp, M., Y. Strausz, M. Gross and G. Glaser. 2001. Regulation of *Escherichia coli* RelA requires oligomerization of the C-terminal domain. *J. Bacteriol.* 183, 570-579.

Gupta, S., S.B. Pandit, N. Srinivasan and D. Chatterji. 2002. Proteomics analysis of carbon-starved *Mycobacterium smegmatis*: induction of Dps-like protein. *Protein Eng.* 15, 503-512.

Hammer, B.K. and M.S. Swanson. 1999. Co-ordination of *Legionella pneumophila* virulence with entry into stationary phase by ppGpp. *Mol. Microbiol.* 33, 721-731.

Haralalka, S., S. Nandi and R.K. Bhadra. 2003. Mutation in the *relA* gene of *Vibrio cholerae* affects *in vitro* and *in*

vivo expression of virulence factors. *J. Bacteriol.* 185, 4672-4682.

Harris, B.Z., D. Kaiser and M. Singer. 1998. The guanosine nucleotide (p)ppGpp initiates development and A-factor production in *Myxococcus xanthus*. *Genes Dev.* 12, 1022-1035.

Haseltine, W.A. and R. Block. 1973. Synthesis of guanosine tetra- and pentaphosphate requires the presence of a codon-specific, uncharged transfer ribonucleic acid in the acceptor site of ribosomes. *Proc. Natl. Acad. Sci. USA* 70, 1564-1568.

Hogg, T., U. Mechold, H. Malke, M. Cashel and R. Hilgenfeld. 2004. Conformational antagonism between opposing active sites in a bifunctional RelA/SpoT homolog modulates (p)ppGpp metabolism during the stringent response. *Cell* 117, 57-68.

Hoyt, S. and G.H. Jones. 1999. *relA* is required for actinomycin production in *Streptomyces antibioticus*. *J. Bacteriol.* 181, 3824-3829.

Igarashi, K., N. Fujita and A. Ishihama. 1989. Promoter selectivity of *Escherichia coli* RNA polymerase: omega factor is responsible for the ppGpp sensitivity. *Nucleic Acids Res.* 17, 8755-8765.

Inaoka, T., K. Takahashi, M. Ohnishi-Kameyama, M. Yoshida and K. Ochi. 2003. Guanine nucleotides guanosine 5'-diphosphate 3'-diphosphate and GTP co-operatively regulate the production of an antibiotic bacilysin in *Bacillus subtilis*. *J. Biol. Chem.* 278, 2169-2176.

Jain, V., S. Sujatha, A.K. Ojha and D. Chatterji. 2005. Identification and characterization of *rel* promoter element of *Mycobacterium tuberculosis*. *Gene* 351, 149-157.

Jin, W., H.K. Kim, J.Y. Kim, S.G. Kang, S.H. Lee and K.J. Lee. 2004. Cephamycin C production is regulated by *relA* and *rsh* genes in *Streptomyces clavuligerus* ATCC27064. *J. Biotechnol.* 114, 81-87.

Jishage, M., K. Kvint, V. Shingler and T. Nystrom. 2002. Regulation of sigma factor competition by the alarmone ppGpp. *Genes Dev.* 16, 1260-1270.

Johnson, G.S., C.R. Adler, J.J. Collins and D. Court. 1979. Role of the *spoT* gene product and manganese ion in the metabolism of guanosine 5'-diphosphate 3'-diphosphate in *Escherichia coli*. *J. Biol. Chem.* 254, 5483-5487.

Kvint, K., A. Farewell and T. Nystrom. 2000. RpoS-dependent promoters require guanosine tetraphosphate for induction even in the presence of high levels of sigma(s). *J. Biol. Chem.* 275, 14795-14798.

Manabe, Y.C. and W.R. Bishai. 2000. Latent *Mycobacterium tuberculosis*-persistence, patience, and winning by waiting. *Nat. Med.* 6, 1327-1329.

Martinez-Costa, O.H., M.A. Fernandez-Moreno and F. Malpartida. 1998. The *relA/spoT*-homologous gene in *Streptomyces coelicolor* encodes both ribosome-dependent (p)ppGpp-synthesizing and -degrading activities. *J. Bacteriol.* 180, 4123-4132.

Mathew, R., A.K. Ojha, A. Karande and D. Chatterji. 2004. Deletion of the *rel* gene in *Mycobacterium smegmatis* reduces its staionary phase survival without altering the cell-surface associated properties. *Curr. Sci.* 86, 149-153.

Mayuri, G. Bagchi, T.K. Das and J.S. Tyagi. 2002. Molecular analysis of the dormancy response in *Mycobacterium smegmatis*: expression analysis of genes encoding the DevR-DevS two-component system, Rv3134c and chaperone alpha-crystallin homologues. *FEMS Microbiol. Lett.* 211, 231-237.

Mechold, U., M. Cashel, K. Steiner, D. Gentry and H. Malke. 1996. Functional analysis of a *relA/spoT* gene homolog from *Streptococcus equisimilis*. *J. Bacteriol.* 178, 1401-1411.

Mechold, U., H. Murphy, L. Brown and M. Cashel. 2002. Intramolecular regulation of the opposing (p)ppGpp catalytic activities of Rel(Seq), the Rel/Spo enzyme from *Streptococcus equisimilis*. *J. Bacteriol.* 184, 2878-2888.

Mittenhuber, G. 2001. Comparative genomics and evolution of genes encoding bacterial (p)ppGpp synthetases/hydrolases (the Rel, RelA and SpoT proteins). *J. Mol. Microbiol. Biotechnol.* 3, 585-600.

Moris, M., K. Braeken, E. Schoeters, C. Verreth, S. Beullens, J. Vanderleyden and J. Michiels. 2005. Effective symbiosis between *Rhizobium etli* and *Phaseolus vulgaris* requires the alarmone ppGpp. *J. Bacteriol.* 187, 5460-5469.

Mukherjee, R., M. Gomez, N. Jayaraman, I. Smith and D. Chatterji. 2005. Hyperglycosylation of glycopeptidolipid of *Mycobacterium smegmatis* under nutrient starvation: structural studies. *Microbiology* 151, 2385-2392.

Mulder, M.A., H. Zappe and L.M. Steyn. 1997. Mycobacterial promoters. *Tuber. Lung Dis.* 78, 211-223.

Ojha, A.K., T.K. Mukherjee and D. Chatterji. 2000. High intracellular level of guanosine tetraphosphate in *Mycobacterium smegmatis* changes the morphology of the bacterium. *Infect. Immun.* 68, 4084-4091.

Parrish, N.M., J.D. Dick and W.R. Bishai. 1998. Mechanisms of latency in *Mycobacterium tuberculosis*. *Trends. Microbiol.* 6, 107-112.

Paul, B.J., M.M. Barker, W. Ross, D.A. Schneider, C. Webb, J.W. Foster and R.L. Gourse. 2004. DksA: a critical component of the transcription initiation machinery that potentiates the regulation of rRNA promoters by ppGpp and the initiating NTP. *Cell* 118, 311-322.

Paul, B.J., M.B. Berkmen and R.L. Gourse. 2005. DksA potentiates direct activation of amino acid promoters by ppGpp. *Proc. Natl. Acad. Sci. USA* 102, 7823-7828.

Perederina, A., V. Svetlov, M.N. Vassylyeva, T.H. Tahirov, S. Yokoyama, I. Artsimovitch and D.G. Vassylyev. 2004. Regulation through the secondary channel-structural framework for ppGpp-DksA synergism during transcription. *Cell* 118, 297-309.

Pizarro-Cerda, J. and K. Tedin. 2004. The bacterial signal molecule, ppGpp, regulates *Salmonella* virulence gene expression. *Mol. Microbiol.* 52, 1827-1844.

Primm, T.P., S.J. Andersen, V. Mizrahi, D. Avarbock, H. Rubin and C.E. Barry, 3rd. 2000. The stringent response of *Mycobacterium tuberculosis* is required for long-term survival. *J. Bacteriol.* 182, 4889-4898.

Reddy, P.S., A. Raghavan and D. Chatterji. 1995. Evidence for a ppGpp-binding site on *Escherichia coli* RNA polymerase: proximity relationship with the rifampicin-binding domain. *Mol. Microbiol.* 15, 255-265.

Reyrat, J.M. and D. Kahn. 2001. *Mycobacterium smegmatis*: an absurd model for tuberculosis? *Trends. Microbiol.* 9, 472-474.

Scoarughi, G.L., C. Cimmino and P. Donini. 1999. *Helicobacter pylori*: a eubacterium lacking the stringent response. *J. Bacteriol.* 181, 552-555.

Song, M., H.J. Kim, E.Y. Kim, M. Shin, H.C. Lee, Y. Hong, J.H. Rhee, H. Yoon, S. Ryu, S. Lim and H.E. Choy. 2004. ppGpp-dependent stationary phase induction of genes on *Salmonella* pathogenicity island 1. *J. Biol. Chem.* 279, 34183-34190.

Sun, J., A. Hesketh and M. Bibb. 2001. Functional analysis of *relA* and *rshA*, two *relA/spoT* homologues of *Streptomyces coelicolor* A3(2). *J. Bacteriol.* 183, 3488-3498.

Svitil, A.L., M. Cashel and J.W. Zyskind. 1993. Guanosine tetraphosphate inhibits protein synthesis *in vivo*. A possible protective mechanism for starvation stress in *Escherichia coli*. *J. Biol. Chem.* 268, 2307-2311.

Takahashi, K., K. Kasai and K. Ochi. 2004. Identification of the bacterial alarmone guanosine 5'-diphosphate 3'-diphosphate (ppGpp) in plants. *Proc. Natl. Acad. Sci. USA*. 101, 4320-4324.

Taylor, C.M., M. Beresford, H.A. Epton, D.C. Sigee, G. Shama, P.W. Andrew and I.S. Roberts. 2002. *Listeria monocytogenes relA* and *hpt* mutants are impaired in surface-attached growth and virulence. *J. Bacteriol.* 184, 621-628.

Toulokhonov, II, I. Shulgina and V.J. Hernandez. 2001. Binding of the transcription effector ppGpp to *Escherichia coli* RNA polymerase is allosteric, modular, and occurs near the N terminus of the beta'-subunit. *J. Biol. Chem.* 276, 1220-1225.

van Delden, C., R. Comte and A.M. Bally. 2001. Stringent response activates quorum sensing and modulates cell density-dependent gene expression in *Pseudomonas aeruginosa*. *J. Bacteriol.* 183, 5376-5384.

van der Biezen, E.A., J. Sun, M.J. Coleman, M.J. Bibb and J.D. Jones. 2000. *Arabidopsis* RelA/SpoT homologs implicate (p)ppGpp in plant signaling. *Proc. Natl. Acad. Sci. USA* 97, 3747-3752.

Vrentas, C.E., T. Gaal, W. Ross, R.H. Ebright and R.L. Gourse. 2005. Response of RNA polymerase to ppGpp: requirement for the omega subunit and relief of this requirement by DksA. *Genes. Dev.* 19, 2378-2387.

Wells, D.H. and S.R. Long. 2002. The *Sinorhizobium meliloti* stringent response affects multiple aspects of symbiosis. *Mol Microbiol.* 43, 1115-1127.

Wendrich, T.M., G. Blaha, D.N. Wilson, M.A. Marahiel and K.H. Nierhaus. 2002. Dissection of the mechanism for the stringent factor RelA. *Mol. Cell* 10, 779-788.

Xiao, H., M. Kalman, K. Ikehara, S. Zemel, G. Glaser and M. Cashel. 1991. Residual guanosine 3',5'-bispyrophosphate synthetic activity of *relA* null mutants can be eliminated by *spoT* null mutations. *J. Biol. Chem.* 266, 5980-5990.

Zhang, G., E.A. Campbell, L. Minakhin, C. Richter, K. Severinov and S.A. Darst. 1999. Crystal structure of *Thermus aquaticus* core RNA polymerase at 3.3 A resolution. *Cell* 98, 811-824.