Shortage of nutrients in bacteria: The stringent response

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In nature, bacteria adopt different strategies to survive for long period under hostile conditions. When nutrients are limiting, it appears now that there may be a common pathway which controls the maintenance of a dormant stage in bacteria till fresh nutrients are available again. Stringent response is one of the many responses that bacteria exhibit faced with nutrient starvation, characterized by the production of nucleotide polyphosphate and arrest in stable RNA synthesis. The commonality of mechanism in stringent response in bacteria and the importance of dormant stage biology of pathogenic microorganism have generated a lot of interest in recent time and form the base of this review.

When rapidly growing *E. coli* cells are subjected to amino acid starvation, a panoply of metabolic and physiological changes occur that constitute an adaptational response to the altered extracellular conditions. An important conservative aspect of this response, is the rapid curtailment of rRNA and tRNA (stable RNA) synthesis leading to the lower rates of ribosome formation and eventually, protein synthesis. This adaptational response is termed as stringent response and is mediated through the production of the nucleotide guanosine 3′-5′-bisphosphate (ppGpp). Thus, ppGpp seems to serve as an 'emergency break' to stop production of protein synthesizing machinery when substrates for protein synthesis are in short supply. However, basal level synthesis of ppGpp is always necessary for the fine control of growth rate[1]. Conservation of energy by fine tuning of growth rate (by inhibition of stable rRNA and tRNA synthesis) and protection of cells from changing environment are the two main features for stringent response. The production and maintenance of ppGpp under stress are controlled by the genes *relA* and *spoT*. There is a strong homology between the two enzymes produced by these two genes from different species. In short, stringent response can be represented as shown in Figure 1.

Relation among nutritional deficiency, growth rate and cellular morphology

Individual and multiple nutrient deficiency (carbon, nitrogen, phosphorous) cause an enhanced production of ppGpp which is reflected in slowing down of growth rate and concomitant alteration in cellular morphology. Bacterial cells behave differently upon exposure to different kinds of nutritional shortage. The carbon-starved *V. cholerae* S14 cells show abrupt cessation of growth, well-defined stationary phase, and changes in shape from rod to coccoid. The nitrogen-starved cells were characterized by the absence of well-defined stationary phase and they were converted to thin filamentous morphological form, which may indicate that some late or intermediate stage of cell division is blocked in nitrogen-starved *vibrio* species S14. Phosphorus-starved cells were characterized by an extended deceleration phase and the cells were swollen and elongated after prolonged starvation[2] (Figure 2).

Basic mechanism of synthesis and degradation of ppGpp

E. coli has two ppGpp synthetases, PSI and PSII encoded by *relA* and *spoT* genes. The *spoT* gene also encodes a ppGpp hydrolase. Amino acid starvation leads to the accumulation of uncharged cognate tRNA. When the ratio of charged to uncharged tRNA falls below a critical threshold level, occupation of the vacant mRNA codon at the ribosomal A site by uncharged cognate tRNA leads to stalling of peptide chain elongation, and synthesis of the nucleotides pppGpp and ppGpp from GTP and ATP in an idling reaction involving the *relA* product, 'pppGpp-synthetase-I' and magnesium. The pentaphosphate pppGpp thus produced is converted to ppGpp by the enzyme 5′ phosphohydrolase. The carboxyterminal domain of pppGpp-synthetase-I is involved in ribosome binding, and the ribosome-independent amino terminal domain is involved in pppGpp synthetase-I activity[7–11].

PSII is a highly unstable enzyme, not associated with ribosomes and its activity is negatively controlled by cellular amino acid pool[12,13]. PSII-mediated accumulation of pppGpp responds to fluctuations in the intracellular energy pools[14], and is thought to be more significant during changes in growth rates. The basal level of ppGpp during exponential growth is provided by PSII, whose control helps fine tune ribosome synthesis in a given medium to optimize energy utilization, and thereby the growth rate. Bacterial strains showing deletions/mutations for both *relA* and *spoT* genes do not accumulate ppGpp[15], they are, however, auxotrophic for certain

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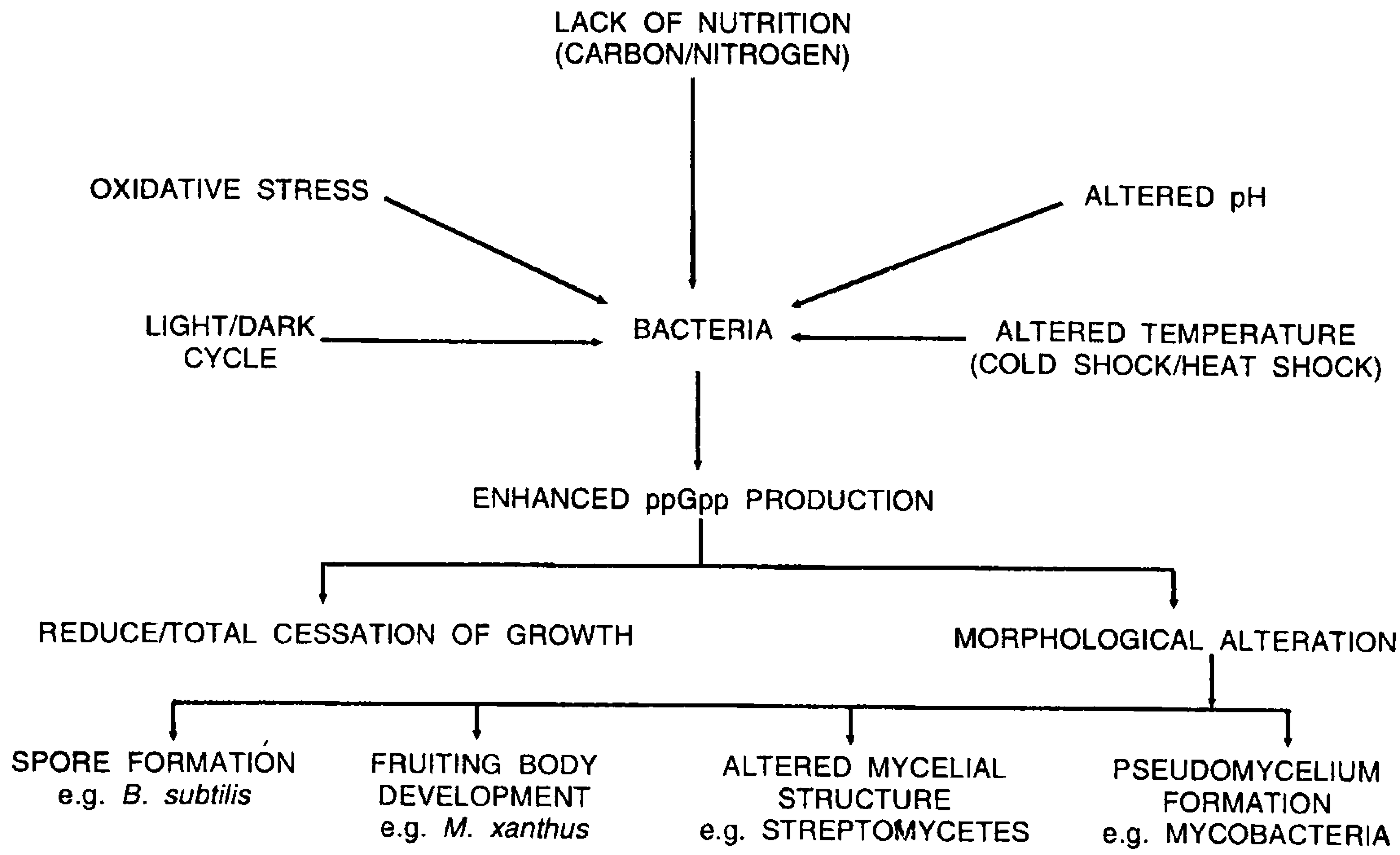

Figure 1. Schematic representation of the protection of bacterial cells against changing environment.

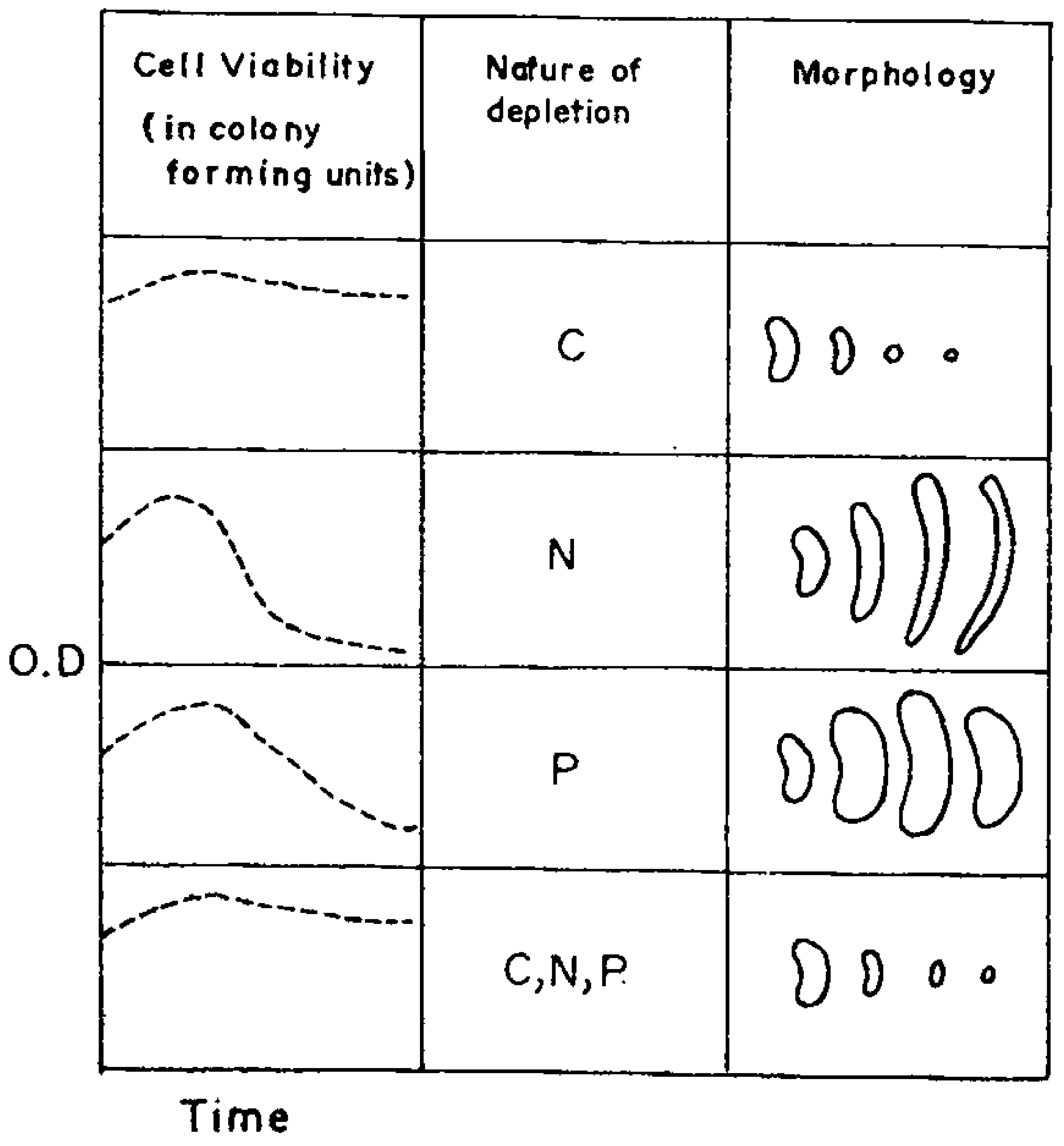

Figure 2. Changes during starvation for multiple as well as for individual nutrients in *vibrio* sp. S14-CNP, multiple nutrient; -C, carbon; -N, nitrogen; -P, phosphorus. Left column: cell viability (in colony formation unit); right column: schematic diagram of cellular morphology as shown by phase-contrast microscopy.

amino acids in minimal media, which show positive regulation by ppGpp.

The basal level of ppGpp during exponential growth depends on the balanced activity of pppGpp-synthetase-II and ppGpp hydrolase. In contrast to the apparently, unstable pppGpp synthetase-II activity (PSII), the ppGpp hydrolase is a stable enzyme[16-18]. Some recent studies further claimed that like pppGpp synthetase-II, ppGpp hydrolase also is not associated with ribosomes. It appears that the nascent *spoT* polypeptide might be subjected to a modification that either gives it PSII activity and renders it unstable or vice versa produces a stable ppGpp hydrolase from an unstable *PSII*[6]. Uncharged tRNA inhibit degradation activity *in vivo* and that is the basis of often-studied ppGpp accumulation during carbon source downshift[19]. Lack of carbon source, affects both ppGpp synthesis and its degradation[6].

Inducers and inhibitors of ppGpp synthesis

Besides the *in vivo* and *in vitro* effects of different natural inducers (nutritional starvation, temperature shifts, etc.), several antibiotics/other chemicals are presently available which also help to control the cellular level of ppGpp either by induction or inhibition of ppGpp synthesis.

The antibiotic mupirocin produces cellular effects similar to those of 'isoleucine starvation' by preventing the charging of tRNA due to inhibition of isoleucyl tRNA synthetase in *E. coli*[3,4], *Staphylococcus aureus*[5] and other organisms. Similarly, generation of pppGpp also occurs with indolmycin which produces conditions similar to 'tryptophan starvation'. Polymixin, gramicidin, alcohol and detergents have shown similar activity, whereas piconilic acid addition results in *spoT* gene-dependent, *relA*-independent ppGpp accumulation that is probably due to manganese ion chelation, since the effects of piconilic acid are reversible by Mn^{2+} addition. Sodium azide (an uncoupler of oxidative phosphorylation), increases ppGpp level by causing energy starvation[6].

A number of antibiotic inhibitors for ppGpp synthesis are presently available. The detailed mechanism of action of these antibiotics is yet to be discovered, although it is thought to be due to the inhibition of protein synthesis. The most important of these kinds of antibiotics are ribosome inhibitors chloramphenicol (binds to the ribosomal A site in *E. coli*), trimethoprim (an inhibitor of tetrahydrofolate reductase, hence blocking synthesis of fmet tRNA), and fusidic acid (inhibits the ribosomal synthetic reaction). Alteration in the *relA*-dependent synthesis of ppGpp synthetase-I (due to alterations in cellular level of ribosomes) may be one of the major factors for the decreased synthesis of pppGpp by some antibiotics. Substantial inhibitory activity can also be seen by oxytetracyclin and puromycin.

Mechanism of growth rate control by ppGpp

Two contrasting models have been proposed to explain the mechanism of growth rate control – the ribosome feedback model and ppGpp-dependent model.

The ribosome feedback model proposes that the rate of tRNA synthesis is independent of ppGpp and is governed by a feedback mechanism sensitive to the translational capacity of the cell[20,21].

The ppGpp-dependent model proposes that ppGpp binds β-subunit of RNA polymerase[22,23]. Both *in vitro* and *in vivo*, this altered RNA polymerase has reduced affinity for the promoters of stable RNA (rRNA and tRNA) genes. So, ribosome synthesis decreases when the level of ppGpp increases. When synthesis of new ribosomes stops in response to ppGpp, the concentration of active ribosome decreases. This will tend to increase the amount of free RNA polymerase able to make new rRNA. Thus, the steady state is suspended as a compromise between the number of ribosomes and ribosome growth rate[24–26].

Both models have inherent drawbacks and consensus on how growth rate control is achieved, remains elusive. In a recent work, Gaal *et al.* have suggested that the concentration of initiating nucleotides is the main controller of growth rate of *E. coli*[27]. They proposed a model for *homeostatic control* of ribosome synthesis by NTP sensing.

Based on their work with wild-type strain as well as a *rpoB* mutant strain at stringently and nonstringently controlled promoters, Zhou *et al.* proposed that destabilization of the initiation complexes and reduction in superhelicity at the stringently-controlled promoters are responsible for stringent response. ppGpp could affect either directly the stability of the initiation complexes of stringent promoters or indirectly by decreasing the transcription activity in general[28].

ppGpp in other organisms

Although our knowledge on stringent response and ppGpp production centres around *E. coli*, recent evidences have shown the presence of similar pathways in other organisms. Homologues of *E. coli*, *relA* and *spoT* genes and the synthesis of pppGpp in response to carbon and/or amino acid starvation have been reported in many organisms[29].

Cyanobacteria: *Anacystis nidulans* (a photosynthetic cyanobacteria) shows accumulation of ppGpp when the bacteria is shifted from light to dark. It is assumed that decreased level of amino acids in the dark phase is the main stimulus for the synthesis of ppGpp. Use of carbonylcyanide-M-chlorophenyl-hydrazone (an uncoupler) or treatment of cells with L-methionine-DL-sulfoximine (an inducer of nitrogen starvation) causes large-scale accumulation of ppGpp. This proved that energy/ nutrient starvation causes accumulation of ppGpp which in turn controls the metabolic activities of the bacteria[30,31].

Staphylococci: These are gram-positive bacteria and most of them are pathogenic. One recent observation has shown that mupirocin-induced *Staphylococci* behave similarly like isoleucine-starved *E. coli* and produce a high amounts of stringent nucleotides. Another important observation during *Staphylococci* stringent response is that the ratio of pppGpp to ppGpp in *Staphylococci* is reversed as compared to that *E. coli*, where pppGpp is produced at lower concentrations than ppGpp. Probably, low efficiency of the enzyme, 5′-phosphohydrolase (which converts pppGpp to ppGpp) is responsible for the accumulation of pppGpp. Biological implication of such a control in *Staphylococci* is yet to be studied[32].

Streptococci: *Streptococcus equisimilis* behaves like *E. coli* under nutrient starvation. The most important observation in *Streptococci* is that they accumulate an unknown phosphorylated compound that comigrate with ppGpp under standard two-dimensional thin layer chromatographic conditions. Unlike ppGpp, this compound did not adsorb the charcoal (used to separate ppGpp) and did not accumulate appreciably during isoleucine starvation. Like ppGpp, the unknown compound did accumulate during energy source deprivation. The biological implication of this newly discovered compound is yet to be analysed[33].

Bacillus subtilis: This is one of the well-studied sporulating bacteria. A high level of pppGpp was detected from stationary phase (sporulating stage) cells of *B. subtilis*. However, carbon/nitrogen deprived cells showed high amounts of ppGpp much before stationary phase, with concomitant onset of sporulation. Work on

B. subtilis suggested that ppGpp may be one of the most important factors which is involved directly/indirectly with initiation of sporulation[34,35]. However, biology of sporulation in *B. subtilis* has been worked out in detail[36] which involves stage-specific involvement of σ-factors, without any participation of ppGpp.

Streptomyces: In *Streptomyces* (mycelial soil bacteria) several studies have noted a correlation between ppGpp synthesis and the onset of antibiotic production and morphological differentiation. In *S. coelicolor* M145, deletion of *relA* drastically affects morphological differentiation and inhibits antibiotic production[37].

Myxococcus xanthus: Amino acids or carbon limitation are sufficient to initiate fruiting body development in *M. xanthus*. *M. xanthus* cells could monitor their capacity for protein synthesis as a sensitive indicator of the availability of nutrients by means of PSI-controlled ppGpp level[38]. Introduction of *E. coli relA* gene under the control of light-induced car QRS promoter in *M. xanthus* leads to excessive production of ppGpp even under nutrient enrichment condition, and *M. xanthus* cells immediately undergo fruiting body development. This developmental regulation of gene expression by ppGpp suggests that *M. xanthus* cells can assess their nutritional status by monitoring the internal availability of amino acids through ppGpp levels[39].

In a recent study, the same group[29] identified a new factor in *M. xanthus*, which they named 'A factor', and they observed that its intracellular production has a positive correlation with the accumulation of ppGpp. 'A factor' consists of a set of amino acids released by starving cells that are used at low concentration to assess the cell density[40].

Mycobacteria: *Mycobacterium tuberculosis* is one of the most successful bacterial parasites of humans, infecting over one-third of the population of the world and causing 10 million new cases of tuberculosis (TB) annually. *M. tuberculosis* has the capacity to infect an individual early in life, remains dormant for decades and then resurface in later life[41]. The factors which are believed to increase the risk of reactivation of TB are HIV infection, malnutrition, some pathophysiological conditions like cancer, diabetes and chronic renal insufficiency, and immunosuppressive drug therapy[42]. Although the reactivated mycobacteria have immense role in disease transmission and fatality, there is no information of the mechanism of dormancy of mycobacteria, especially during nutritional starvation. As ppGpp is one of the major growth rate controllers of the cell, one could speculate that ppGpp may have direct or indirect role in maintaining dormancy of mycobacterial cells inside the macrophages. In fact, we demonstrated that mycobacteria can produce ppGpp under nutritional starvation and that ppGpp is involved in morphological differentiation and probably in dormancy.

It has been demonstrated by us that *Mycobacterium smegmatis* produces guanosine tetraphosphate (ppGpp) in response to stationary phase entry and carbon starvation. While it is not known whether *M. smegmatis* contains *relA* or *spoT* homologues which play important roles in ppGpp metabolism in *E. coli*, a related species

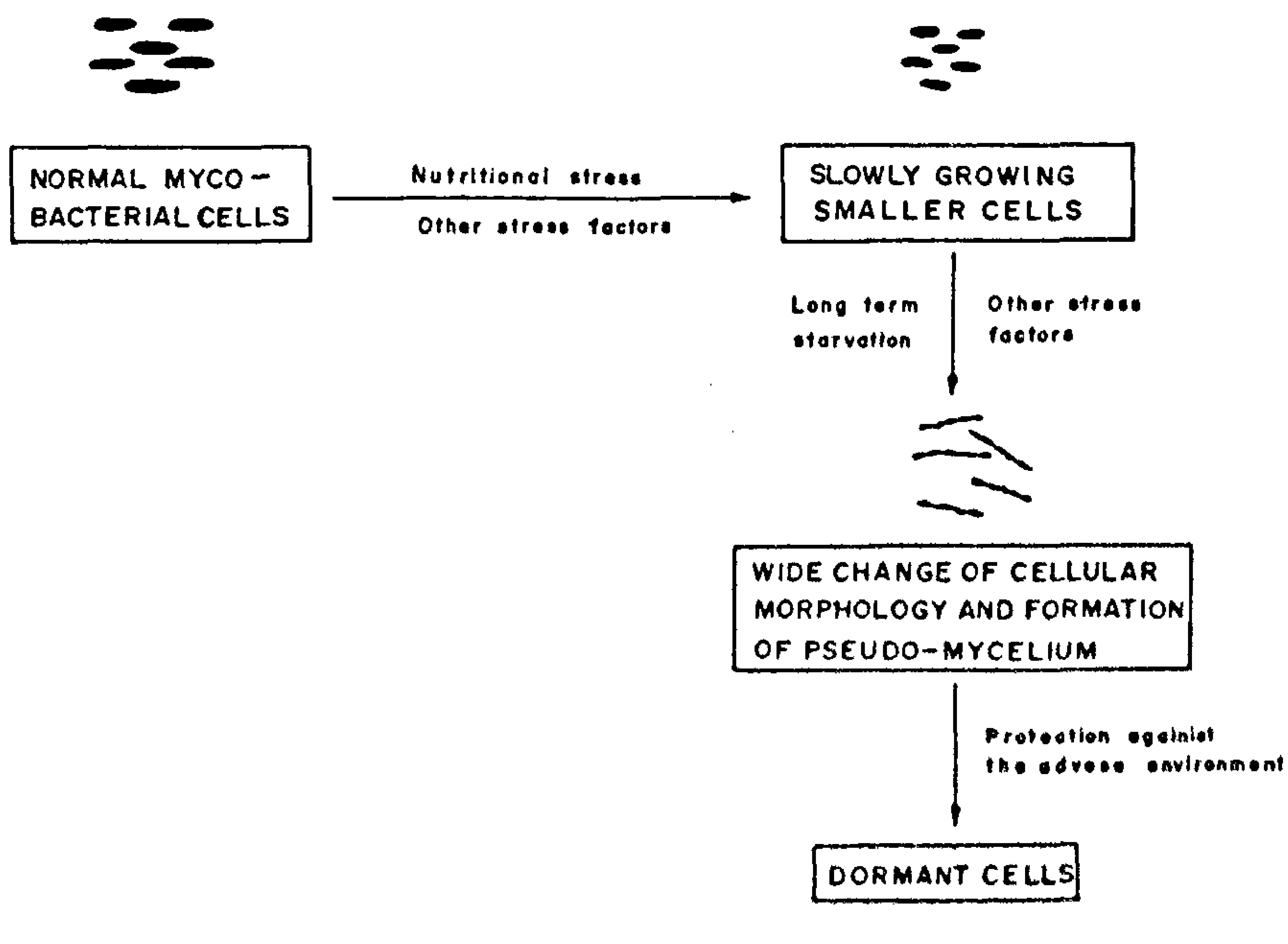

Figure 3. Schematic representation of the formation of pseudomycelium by nutrient starved mycobacterial cells. We assume that pseudomycelial cells are the dormant mycobacterial cells which helps to protect themselves against the adverse environment.

M. tuberculosis, whose complete genome has now been sequenced contains only a single *relA/spoT* homologue. *In vitro* culture of *M. smegmatis* in carbon source deficient Middlebrook 7H9 medium (minimal medium) resulted in the synthesis of ppGpp in levels detectable by thin layer chromatography. However, basal level synthesis of ppGpp was also noticed when *M. smegmatis* was grown in enriched medium. Phase-contrast microscopy of the cells grown in minimal medium indicated a reduction in size or the formation of fused chain of cells (like pseudomycelium) depending on the type of medium. These morphological alterations may be related to enhanced ppGpp production and dormancy of mycobacteria under starvation (Mukherjee *et al.*, unpublished) (Figure 3).

Stringent response has also been discovered in some other organisms like *Vibrio cholerae*[43], *Salmonella typhimurium*[44–46], *Bacillus stearothermophilus*[47,48], and *Bacillus brevis*[49–51].

All these bacteria basically follow the same pattern exhibited by *E. coli.*

Importance of the work on stringent response

Some recent observations indicate that ppGpp may have wide variety of functions in bacterial growth apart from ribosome synthesis or growth rate control. It is involved in a variety of biological functions like peptidoglycan synthesis[52–54], control of cell cycle[55], antibiotic production[37], osmoregulation, NH_4^+ assimilation[56] and DNA replication[57,58]. Studies on stringent regulation will be helpful in better understanding of the above physiological functions and their biological implications. Moreover, our recent work shows that ppGpp has a very important role in maintaining the dormancy of bacteria. Our results document significant ppGpp production in *M. smegmatis* coincident with the onset of stationary phase and alteration in bacterial morphology. This coincidence of enhanced ppGpp production and morphological alteration led us to predict that there may be a correlation between enhanced ppGpp production and morphological alteration which may have a role in mycobacterial dormancy. As the biology of dormant mycobacteria is poorly understood, more efforts are necessary to elucidate this point.

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