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Mini review

Recent functional insights into the magic role of (p)ppGpp in growth control

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ABSTRACT

Rapid growth and survival are two key traits that enable bacterial cells to thrive in their natural habitat. The guanosine tetraphosphate and pentaphosphate [(p)ppGpp], also known as "magic spot", is a key second messenger inside bacterial cells as well as chloroplasts of plants and green algae. (p)ppGpp not only controls various stages of central dogma processes (replication, transcription, ribosome maturation and translation) and central metabolism but also regulates various physiological processes such as pathogenesis, persistence, motility and competence. Under extreme conditions such as nutrient starvation, (p)ppGpp-mediated stringent response is crucial for the survival of bacterial cells. This mini-review highlights some of the very recent progress on the key role of (p)ppGpp in bacterial growth control in light of cellular resource allocation and cell size regulation. We also briefly discuss some recent functional insights into the role of (p)ppGpp in plants and green algae from the angle of growth and development and further discuss several important open directions for future studies.

Contents

1. Introduction

To thrive in the natural living niche, bacterial cells should be capable of rapidly proliferating under favorable conditions and

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persisting under harsh environments. To maximize fitness, bacterial cells need to balance the two key traits: rapid growth (reproduction) and stress tolerance [1–3]. Given that bacterial cells frequently undergo various stressful conditions in nature (e.g., nutrient deprivation, osmotic shock, high temperature, oxidative stress and low/high pH conditions) [4,5], the adaptive response of bacteria to stress is a central topic of microbiology. The guanosine tetraphosphate and pentaphosphate [(p)ppGpp] (“magic spot”), discovered over 50 years ago, is a key second messenger inside bacterial cells as well as chloroplast of plants and green algae [6–9]. It was initially found to be dramatically induced inside *Escherichia coli* during amino acid starvation [7]. Later, it was found that (p)ppGpp could be strongly induced by various other types of stressful conditions. This phenomenon, collectively referred to as stringent response, lies at the core of bacterial stress response system [7,10–13]. Under extreme conditions such as nutrient starvation, (p)ppGpp-mediated stringent response re-shapes the global gene expression pattern of bacteria and shuts down various central-dogma processes including replication, transcription, ribosome assembly/maturation and translation (initiation/elongation) as well as transcription-translation coordination [6–8,11,14–17], further facilitating the survival of bacterial cells. Since (p)ppGpp has an incredibly diverse set of targets *in vivo* [12,18], it also participates in regulating many important physiological processes of bacteria such as pathogenesis, antibiotic resistance/tolerance, persistence, motility and competence [8,10,19].

Besides the capability of stress tolerance, rapid growth is another core trait that determines bacterial fitness. As remarked by Francois Jacob, “the dream of every cell is to become two cells”[20]. Rapid growth is a core property of bacterial cells and many kinds of eukaryotic cells such as yeast and tumor cells [21,22]. The characterization of microbial growth constitutes an important chapter of the classical textbook of microbiology. The exponential stage of bacterial cells, as the best-defined stage in bacterial life cycle [23–25], is crucial for the propagation and expansion of bacterial species and thus constitutes an indispensable part of bacterial fitness. Given that the nutrient quality and availability (e.g., carbon, ammonium, amino acid and other growth factors) are often highly-fluctuating in the natural habitats of bacterial cells [26–30], bacteria must be able to adapt to different exponential growth stages with varied growth rates. In this sense, growth control reflects a fundamental aspect of the design principles of bacterial systems and is also a highly active field in the recently emerging systems and quantitative biology [22,25,31].

It is known that bacterial cells manage to maintain a basal level of (p)ppGpp during exponential growth stage [32–36] which is proposed to be crucial for bacterial physiology considering that (p)ppGpp^0 strains of model bacterium *E. coli* and *B. subtilis* are amino acid auxotrophic and are incapable of growing in minimal medium [33]. Recent studies have shown that the basal-level (p)ppGpp plays a crucial role in regulating bacterial growth from the angle of both cellular resource allocation and cell size homeostasis, which provide new insights into the function of (p)ppGpp. Here we review these recent progresses regarding the relation between (p)ppGpp and growth rate control and further highlight several future directions of this field.

2. Growth control of bacterial cells

It should be noted that the term of “growth” has different meanings between unicellular microbial cells and multicellular organisms. In the former case, “growth” equates with “reproduction” while in the latter case, “growth” denotes the increase of size and is physically separated from the process of reproduction. The population growth of bacterial cells contains two major parts: mass accumulation and number increase (Fig. 1) [25,32,37]. Mass accumulation denotes the biosynthesis of macromolecules such as protein, RNA, DNA and lipid [22,31,32]. Given that protein accounts over half of the dry mass and its synthesis consumes two thirds of the overall cellular energy budget [38], protein and ribosome synthesis (rRNA accounts for 80–90 % of total cellular RNA) lie at the core of biomass accumulation [22,24,31]. On the other hand, biomass accumulation is tightly coordinated with cell cycle progression to fulfill the binary fission process in a “adder” manner (individual cells add a constant size between birth and division, irrespective of the birth size) so that the number of individual cells in the population also increases exponentially [25,27,39–43]. The cell size homeostasis of bacteria could then be achieved during balanced growth in a specific nutrient condition.

Modern quantitative characterization of bacterial growth was initiated by Jacques Monod, who won the Nobel Prize in 1965 with François Jacob and André Lwoff for the groundbreaking finding of the genetic regulation of bacteria [25,37,44]. As remarked by Monod, “the growth of bacterial cultures, despite the immense complexity of phenomena to which it testifies, generally obeys relatively simple laws, which makes it possible to define certain quantitative characteristics of growth cycle” [45]. Monod proposed one of the earliest influential examples of coarse-grained modeling of the bacterial growth physiology by showing that the exponential growth rate of bacteria has a Michaelis–Menten dependence on the concentration of the growth-limiting substrate (e.g., lactose), while the yield was proportional to the amount of substrate available [23,25,45]. Monod’s follow-up studies on “diauxic growth” further inspired him to elucidate the genetic regulation of *lac* operon in *E. coli* [44]. In 1950s, the Ole Maaløe group in Copenhagen [“Copenhagen school” [46]] published a milestone paper of microbial growth physiology, establishing the empirical dependence of cell size and chemical composition (especially DNA content) on the bacterial growth rate under various nutrient conditions [47]. Ten years later, in 1968, Charles Helmstetter and Stephen Cooper derived a quantitative formula describing the relation of DNA content per cell with cell cycle and growth rate, known as “Helmstetter-Cooper theory” [48]. They introduced the concept of overlapping rounds of replication, in which a new round of DNA replication could be initiated before the end of previous round of replication. This concept explains the phenomenon of multi-replication fork in fast-growing cells and how bacteria manage to grow with a generation time that is shorter than the time required for chromosome replication (C-period). At the same year, by introducing the concept of “initiation mass” (the cell mass per replication origin at the onset time of replication initiation), Donachie formulated the quantitative equation linking cell size, cell cycle with growth rate [49]. The two works of Helmstetter & Cooper and Donachie provided a phenomenological explanation of the empirical growth rate-dependence of cell size and DNA content observed in Schaechter *et al* [47]. Meanwhile, in 1960s, people began to realize the central role of ribosome and protein synthesis in growth control from the linear relation of ribosome content with growth rate under different nutrient conditions [50–52]. This classical bacterial growth law was rationalized by Maaløe and colleagues in terms of the demanding for more actively translating ribosomes for protein synthesis to achieve faster growth rate when the rate of translational elongation by ribosomes was largely saturated [46]. Besides this, the compositions of many other cellular components as well as the kinetic parameters of cellular processes as a function of growth rate became known in some detail with the extraordinary efforts from various quantitative bacterial physiologists [32].

With the emergence of systems biology and new technologies (e.g., “omics” approaches, microfluidics), the research on microbial growth physiology has gained a revival since 2000s in order to seek

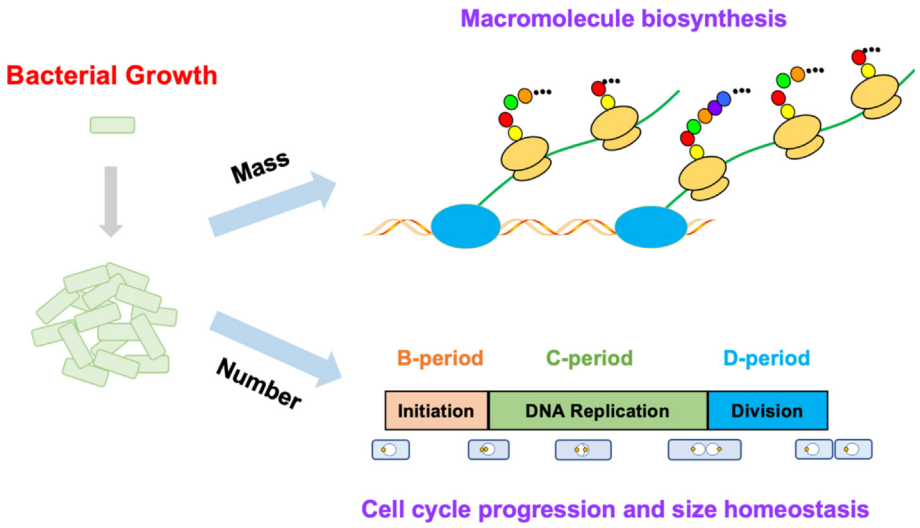

Fig. 1. A global view of bacterial growth. Bacterial growth includes two major parts: biomass accumulation and number increase of individual cells. Biomass accumulation denotes the biosynthesis of macromolecules such as DNA, RNA, protein and lipid. Among them, protein synthesis lies at the core of biomass growth as protein accounts for most of the cellular biomass and its synthesis consumes two thirds of the overall cellular energy budget. The number increase of individual cells requires the tight coordination of biomass growth with cell cycle progression, which ensures cell size homeostasis. The cell cycle of bacteria generally includes three stages: the time between the birth of a new cell to the initiation of chromosome replication (B-period), chromosome replication stage (C-period) and the cell division stage (D-period). Note that B-period only exists in slow-growing cells for which the generation time is longer than C + D period.

a deeper quantitative understanding of phenomena on the whole population level as well as the single-cell level. For example, at the population level, a coarse-grained model of proteome resource allocation can quantitatively describe and predict the interdependence of gene expression and growth rate under various growth conditions [53–57]. At single-cell level, the "adder" phenomenological model could satisfactorily explain how bacteria maintain the homeostasis of cell size [39,40]. Recent studies have shown that (p)ppGpp controls both proteome resource allocation and cell size, further playing a central role in bacterial growth control, for which we will highlight below.

2.1. (p)ppGpp and bacterial growth in light of cellular resource allocation

Protein synthesis lies at the core of bacterial biomass accumulation. Considering the finite cellular resources, bacterial cells manage to balance the investment on different proteome sectors to ensure optimal proteome resource allocation under different conditions [23,24]. Recent quantitative studies have revealed a profound role of proteome resource allocation in controlling microbial growth [53,54,58–61]. In the bacterial proteome, two major sectors are crucial for supporting exponential growth: metabolic proteins (including both catabolic and anabolic proteins), ribosomal & translation-affiliated protein (such as EF-Tu, EF-G) [56,62]. The former one is responsible for the uptake of external nutrients and further processing of metabolic precursors into amino acids while the later one polymerizes amino acids into functional proteins. Given that all the proteome components including ribosomes themselves (consists of rRNA and over 50 ribosomal proteins) are synthesized by ribosomes, ribosome synthesis lies at the core of proteome resource allocation [22,31]. The tight relation between growth rate and proteome resource allocation is manifested by a trade-off between metabolic proteins and ribosomes across nutrient conditions [24,54,56]. In rich medium where most amino acids and growth factors are available, bacteria could maximize the ribosome synthesis to achieve rapid growth. In poor nutrient conditions, however, bacteria must allocate a substantial fraction of ribosomes for synthesizing metabolic proteins to facilitate nutrient uptake and processing. As a result, the fraction of ribosomes for making themselves (r-proteins) substantially drops, resulting in lower ribosome content and slow growth. A coarse-grained model of proteome resource allocation developed by Hwa group could fully account for the above phenomenon and further quantitively connect proteome resource allocation with growth rate with a few phenomenological parameters [23,53].

Mechanistically, recent studies have shown that (p)ppGpp signalling pathway is crucial for bacteria to implement such a clever strategy of optimal resource allocation under different nutrient conditions. (p)ppGpp directly inhibits rRNA synthesis while activates amino acid biosynthesis via binding to RNA polymerase (RNAP) synergistically with DksA in *E. coli* [15,16]. Under different nutrient conditions, the cellular (p)ppGpp pool of *E. coli* is negatively correlated with growth rate, further leading to the linear relation between ribosome content and growth rate (Fig. 2A) [24,32,63]. An increase in the (p)ppGpp pool under nutrient limitation could reduce ribosome synthesis and facilitate the synthesis of metabolic proteins such as amino acid biosynthetic proteins [64]. It has recently been shown that a non-optimum (p)ppGpp pool inhibits the growth of *E. coli* due to sub-optimal resource allocation in which increasing the (p)ppGpp pool limits ribosome synthesis while decreasing the (p)ppGpp pool limits the synthesis of metabolic proteins due to unnecessary overaccumulation of ribosomes [65]. In this sense, maintaining an optimum cellular (p)ppGpp pool in a specific nutrient condition allows *E. coli* to balance its investment on ribosomes and metabolic proteins so that optimal growth status could be achieved. This finding could also explain the amino acid auxotrophy of the *E. coli* (p)ppGpp^0 strain as this strain is devoid of growth control of ribosome synthesis [33], and thus the overaccumulation of ribosomes severely compromises the proteome investment on metabolic proteins, which are indispensable for bacteria to grow in minimal medium.

A further question is how bacteria cells sense the external nutrient condition and further convert the nutrient signal to a suitable (p)ppGpp pool in order to achieve the growth rate control of ribosome synthesis and proteome resource allocation. In a very recent study of Hwa group [63)], they showed that *E. coli* could fine-tune

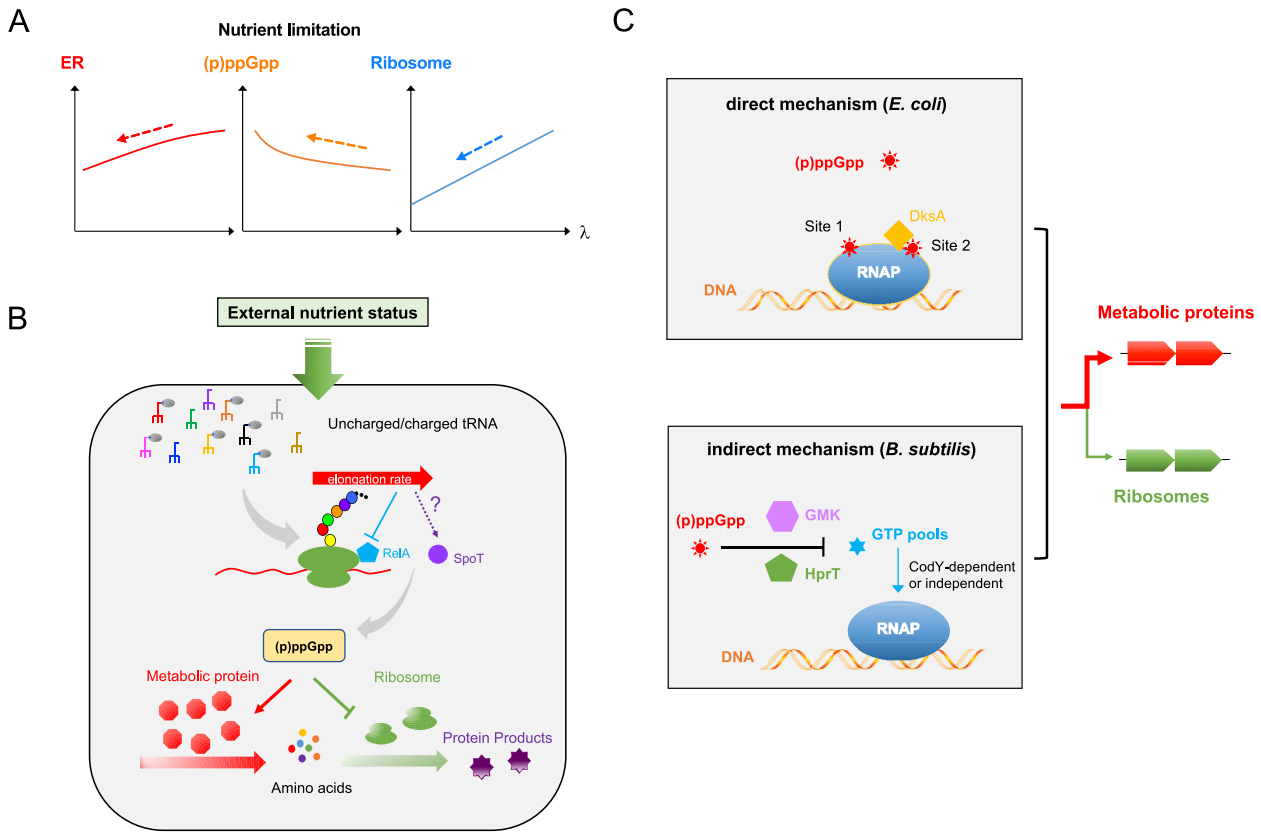

Fig. 2. Mechanistic relation between (p)ppGpp and growth control from the view of resource allocation. (A) The correlation of translation elongation rate (ER), (p)ppGpp pools and ribosome content with growth rate under nutrient limitation for *E. coli* cells. **(B)** The direct sensing mechanism of ER that triggers (p)ppGpp signaling in *E. coli*. The external nutrient status affects the intracellular status of uncharged/charged tRNA pools, further affects the ER of ribosomes. A slow-down of ER can stimulate the activity of RelA (p)ppGpp synthetase (or presumably inhibits the SpoT hydrolase activity), further leading to an increase in the intracellular (p)ppGpp pools. (p)ppGpp exerts its growth-control effects via inhibiting ribosome synthesis but activating the synthesis of metabolic proteins. Note that here we only describe the direct-sensing mechanism of (p)ppGpp synthesis. In principle, (p)ppGpp signaling could also be induced via other RelA/SpoT-dependent mechanisms under various conditions. See comprehensive reviews in Ref. [90,91]. **(C)** Direct mechanism versus indirect mechanism of (p)ppGpp. In *E. coli* cells, (p)ppGpp regulates transcription through directly acting on RNA polymerase (site 1 and site 2) synergistically with DksA (site 2). In *B. subtilis*, (p)ppGpp takes effect indirectly via inhibiting the biosynthesis of GTP, which acts as the initiating NTP of rRNA transcription and also participates in regulating amino acid biosynthesis via either a CodY-dependent and -independent mechanism. Nevertheless, the ultimate regulatory outcomes of *E. coli* and *B. subtilis* are similar with each other from a phenomenological view. In both cases, (p)ppGpp inhibits the ribosome synthesis while activates the biosynthesis of related key metabolic proteins (e.g., amino acid biosynthesis), further playing a central role in growth control of bacteria.

its cellular (p)ppGpp pool via a strategy of direct-sensing of the translation elongation rate (ER). They first found that ER dropped substantially due to the shortage of amino acid pool during carbon diauxic transition. Strikingly, an abrupt increase in the cellular (p)ppGpp pool of *E. coli* occurred simultaneously and was inversely and linearly correlated with the change of ER. The abrupt increase of the (p)ppGpp level during carbon diauxic transition (short-term carbon starvation) largely depends on RelA, as has also been observed in the case of fatty acid starvation (by uncharged $tRNA^{lys}$ due to lysine depletion) [13]. These findings suggest that RelA-mediated (p)ppGpp synthesis is activated not only under amino acid starvation (by uncharged tRNA) [7] but also under other types of stress. Further analysis showed that the linear relationship between ER and (p)ppGpp during diauxie shift originated from the dependence of RelA [(p)ppGpp synthetase] activity on the ratio of dwelling and translocating ribosomes. Recent structure biology studies and early biochemical studies have shown that the (p)ppGpp synthetase activity of RelA is activated when the ribosome is in the dwelling state [66–69]. Therefore, a lower ER (a longer dwelling time of ribosome) could in principle stimulate the RelA activity and further up-regulate the (p)ppGpp pool. In addition, the authors found that the inverse relation of (p)ppGpp and ER still held well during exponential growth under both nutrient limitation and sublethal levels of chloramphenicol and further overlapped with the relation established during carbon diauxic shift. Based on these observations, the authors propose that a change of nutrient source first affects ER via altering the intracellular amino acids and tRNA pools; the change of ER is then directly sensed by cells to be transformed into (p)ppGpp signaling to achieve the growth rate control of ribosome synthesis and proteome resource allocation (Fig. 2A and 2B). Taken together, these studies provide a self-consistent picture of how *E. coli* cells manage to achieve the growth rate control of ribosome synthesis and proteome resource allocation via fine-tuning the cellular (p)ppGpp pool.

Beyond the control of the ribosome synthesis, recent studies have also suggested an additional growth-related function of (p)ppGpp by increasing the inactive ribosome fraction of bacteria during slow growth. It is known that bacterial cells manage to maintain a basal level of inactive ribosomes during very slow growth [70]. Such a strategy of ribosome reserve allows slow-growing cells to quickly adapt to a sudden improvement in the growth condition [71]. Recent studies have shown that (p)ppGpp could increase the inactive ribosome fraction via various mechanisms. For example, it could interfere with the translation initiation process by targeting translation initiation factor-2 (IF2) [72,73]. (p)ppGpp could also positively regulate the expressions of various ribosome hibernation factors such as Rmf, Hpf, and RaiA, further inactivating the ribosomes [63]. On the other hand, it has also been found that *relA*-deficient strain harboring more, but slower translating (more

pronounced stalling) ribosomes than the wild type strain during nitrogen-limited chemostat conditions, suggesting that (p)ppGpp could modulate the transition of ribosomes from the translation initiation to elongation [74].

2.2. (p)ppGpp and bacterial growth in light of cell size regulation

As mentioned above, another key aspect of bacterial growth is the increase of cell number, for which bacterial cells must coordinate biomass accumulation with cell cycle progression to achieve size homeostasis [20,25,27,75]. The size of bacteria is tightly coupled with growth rate and cell cycle progression by the following empirical equation [41]: $S = S_0 \times 2^{C+D/\tau}$, in which S denotes the average cell size, S_0 denotes the initiation mass (or "unit cell"), which largely keeps constant and only changes mildly under different growth conditions [42], C + D is the time required for cell cycle progression including the period of chromosome replication (C period) and the division period (D period) and τ denotes the generation time. Therefore, without the change of other parameters, an increase of bacterial growth rate (smaller τ) or a delay of cell cycle progression (larger C + D) can both lead to increased cell size [41].

Three recent studies have shown that (p)ppGpp also has a profound role in regulating cell cycle progression and size homeostasis [76–78]. Vadia *et al* identified the crucial role of fatty acid synthesis in determining the cell size of *E. coli, B. subtilis* and *Saccharomyces cerevisiae* [78]. They found that the downregulation of fatty acid synthesis is responsible for the negative effect of (p)ppGpp on cell size. In addition, (p)ppGpp could coordinate the lipid synthesis with other anabolic processes to maintain the cell envelope integrity. Fernández-Coll *et al* investigated the chromosome initiation, cell cycle progression and cell size of (p)ppGpp^0 strain across different nutrient conditions [76]. It has long been known that the *ori/ter* ratio of *E. coli* increases substantially with increasing growth rates due to the occurrence of overlapping rounds of chromosome replication during fast growth [20]. However, the authors found that the positive correlation between *ori/ter* and growth rate largely disappeared in (p)ppGpp^0 strain, for which, the *ori/ter* ratio was maintained at a high level even during slow growth. Meanwhile, being in contrast to the case of wild type strain for which initiation mass remains largely constant across conditions, the initiation mass of (p)ppGpp^0 strain during slow growth (poor conditions) is much larger than that during fast growth (rich condition) as well as the values of wild type strain. These observations suggest that (p)ppGpp inhibits and delays the initiation of chromosome replication. In addition, the C-period of (p)ppGpp^0 strain during slow growth is much longer than that of wild type strain at a similar growth rate, suggesting that the elongation process of chromosome replication is also regulated by (p)ppGpp. With increased initiation mass and prolonged cell cycle progression, the cell size of (p)ppGpp^0 strain is still maintained at a high value during slow growth, being much larger than that of wild type strain. As a result, the positive growth rate-dependence of cell size found on wild type strain also disappears on (p)ppGpp^0 strain. Collectively, all these observations suggest a fundamental role of (p)ppGpp in regulating bacterial cell size and cell cycle[76].

The regulation of (p)ppGpp of the cell size could originate from two mechanisms: (i) the hierarchical mechanism in which the regulation of cell size results from a secondary effect of (p)ppGpp on bacterial growth; (ii) direct mechanism in which (p)ppGpp could directly regulates cell size in separation from its regulation of cell growth. Combining the Mesh1[a (p)ppGpp hydrolase from *Drosophila*] and RelA* perturbation system, Büke *et al* has recently investigated the effect of systematic perturbation of the cellular (p)ppGpp pool on the cell size of *E. coli* [77]. They found that while the maximal growth rate of *E. coli* was reached only at a suitable (p)ppGpp level, the cell size (including both added size and the steady-state birth size) increased monotonically with the decrease of ppGpp level regardless of growth rate. As a result, cells of different sizes could have similar growth rates with each other in certain conditions. In other words, cell size could be decoupled from growth rate under (p)ppGpp perturbation. In addition, added size responds very rapidly to the change of ppGpp level, aided by transiently accelerated or delayed divisions in a fashion that is independent from the alteration of growth rate. Therefore, these observations suggest that (p)ppGpp is a direct regulator of cell size and a key coordinator of cell cycle and growth rate.

2.3. (p)ppGpp and bacterial growth in light of Bacillus subtilis

Currently, studies on the regulation of (p)ppGpp of bacterial growth are still largely limited to *E. coli*. However, there are enough evidences showing that (p)ppGpp signaling of *B. subtilis* differs significantly from that of *E. coli*. The growth control of ribosome synthesis has also been observed in *B. subtilis*, which results from (p)ppGpp-mediated inhibition of rRNA synthesis as well [79]. However, in contrast to the case of *E. coli* where (p)ppGpp inhibits rRNA synthesis via directly acting on RNA polymerase synergistically with DksA, (p)ppGpp functions on rRNA synthesis indirectly via inhibiting the biosynthesis of GTP (the initiating NTP (iNTP) of rRNA transcription) in *B. subtilis* (Fig. 2C) [15,16,79]. The lack of direct effect of (p)ppGpp on RNAP in *B. subtilis* is likely due to: first, the *B. subtilis* RNAP lacks the critical residues in β′ and ω that form Site 1 [the (p)ppGpp binding site] of proteobacterial RNAPs, and second, a lack of DksA homolog in *B. subtilis* [15,16].

Mechanistically, the regulation of (p)ppGpp of GTP pool originates from its inhibitory effect on two major GTP biosynthesis enzymes including GMK (guanylate kinase, which converts GMP to GDP) and HprT (which converts hypoxanthine to IMP and guanine to GMP) [36]. Such a mechanism is crucial for maintaining cellular GTP homeostasis and viability under nutrient starvation. The (p)ppGpp^0 strain of *B. subtilis* suffers severe viability loss during nutrient starvation due to uncontrollable over-accumulation of GTP [36]. Importantly, the basal level of (p)ppGpp inside *B. subtilis* during exponential growth (10–20 μM) is enough to result in a ~50 % inhibition of the activities of GMP and HprT [36], and therefore, such a mechanistic origin should be directly related to (p)ppGpp-mediated regulation of GTP homeostasis and further rRNA synthesis during exponential growth.

A further question is the relation between GTP pool and exponential growth in *B. subtilis*, which is important to elucidate the (p)ppGpp-mediated growth control strategy in *B. subtilis*. Although with different mechanistic origins, one common phenomenon of (p)ppGpp signaling shared by *E. coli* and *B. subtilis* is the amino acid auxotrophy of (p)ppGpp^0 strain. Kriel *et al* showed that the growth of *B. subtilis* (p)ppGpp^0 strain strongly required the supplementation of multiple amino acids such as leucine, isoleucine, valine, methionine, and threonine [80]. They found that the amino acid auxotrophy of (p)ppGpp^0 strain could be rescued by reducing the over-accumulated cellular GTP pool resulting from a lack of (p)ppGpp control. The downregulation of GTP pool could activate the expression of many amino acid biosynthesis pathways in either CodY-dependent or -independent manner. In this sense, (p)ppGpp in *B. subtilis* could indirectly activate the amino acid biosynthesis via controlling GTP pools, being with the same logic of its regulation of rRNA transcription.

Bittner *et al* further studied the quantitative relation between GTP pool and growth rate in *B. subtilis* [81]. They studied the effect of altering intracellular GTP pool on the growth rate of *B. subtilis* in (p)ppGpp^0 genetic background so that GTP biosynthesis could be uncoupled from the regulation of (p)ppGpp. A series of mutations were introduced into the promoter or the coding region of *guaB* (en-

coding IMP dehydrogenase) to achieve titratable levels of GTP. Strikingly, GTP pool was positively and almost linearly correlated with growth rate for these mutant strains. The relation between GTP pool and growth rate was further supported by external supplementation of guanosine (GUO), during which the growth of the *B. subtilis* (p)ppGpp^0 *guaB*1 strain could be significantly accelerated with increasing levels of GUO. Therefore, at a certain range, increasing the GTP levels could positively modulate the exponential growth rate of *B. subtilis*. However, an excess level of GTP could ultimately be toxic and lead to cell death. This work suggests that (p)ppGpp signaling is crucial for maintaining an appropriate GTP pool, further enabling *B. subtilis* to maximize the growth rate. Overall, although the mechanisms of (p)ppGpp regulation in *E. coli* (direct effect on RNAP) and *B. subtilis* (indirect effect on the biosynthesis of GTP) differ significantly from each other, the ultimate regulatory outcomes of them are similar with each other from a phenomenological view. In both cases, (p)ppGpp inhibits ribosome synthesis while activates amino acid biosynthesis, further playing a central role in growth control of both species (Fig. 2C).

3. Beyond bacteria: (p)ppGpp in plant and algae as well as metazoan

Chloroplast originates from the engulfment of cyanobacterium by eukaryotic cells over one billion years ago according to the endosymbiosis theory. It has recently been found that (p)ppGpp regulation is also maintained in the chloroplasts of plants and green algae (9) and (p)ppGpp participates in regulating the growth and development of plants and algae. Four chloroplast-targeted RSH enzymes have been identified in plants such as *Arabidopsis* (RSH1, RSH2, RSH3, and CRSH/RSH4)(9). Overproduction of (p)ppGpp in the chloroplast of *Arabidopsis* causes metabolite reduction, dwarf chloroplasts and significant inhibition of plastidial transcription and translation, thus lowering photosynthesis capacity and inhibiting plant growth, but meanwhile, improving the resistance of plants to nutrient stress [82,83]. Similarly, in some other organisms such as *Phaeodactylum tricornutum*, (p)ppGpp accumulation also results in reduced photosynthesis capacity and proliferation rates, further promoting algae to enter into a quiescent-like state with reduced ageing [84]. Therefore, the two major physiological functions of (p)ppGpp signalling: growth inhibition and stress adaptation, are shared across species from bacterial cells to green algae as well as higher plants. Interestingly, (p)ppGpp also affects the size of chloroplast with (p)ppGpp accumulation reducing the chloroplast size while (p)ppGpp shortage increasing the chloroplast size [82,83], mimicking the effect of (p)ppGpp on the cell size of bacteria as mentioned above.

The detailed mechanism of (p)ppGpp regulation in plants and green algae remains poorly understood. In *Arabidopsis*, (p)ppGpp could regulate plastid gene expression through reducing both the quantity of chloroplast transcripts and chloroplast-encoded proteins such as RubisCO (Ribulose-1,5-bishosphate carboxylase) component, and moreover, inhibit rRNA and tRNA synthesis via acting on the bacterial-like plastid encoded polymerase (PEP) and two nucleus-encoded polymerases (NEPs)(9) [82,83,85]. In addition, a very recent study on *P. tricornutum* has also shown that (p)ppGpp accumulation causes the down-regulation of many chloroplast-encoded proteins with links to photosynthesis (e.g., components of PSI, PSII complex and the Calvin cycle) and chloroplast translation (e.g., several chloroplast ribosomal proteins) [84]. In contrast, the expressions of many types of chaperones and proteases are strongly induced, which are related to preventing protein aggregation and misfolding [84]. These observations again support that (p)ppGpp promotes the transition of the plants and algae from growth mode to survival (stress adaption) mode. The chloroplast effectors of (p)ppGpp signaling in plants are still controversial and not conclusive. It is suggested that a *Bacillus subtilis*-like GTP inhibitory mechanism might be responsible for the (p)ppGpp's effect on chloroplasts transcription as it has been found that the chloroplastic guanylate kinase enzymes from *Oryza* and *Arabidopsis* are as sensitive to inhibition by (p)ppGpp *in vitro* as the *B. subtilis* guanylate kinase [86]. Moreover, being similar with *B. subtilis*, it is known that GTP also acts as the initiating NTP for the chloroplast rRNA operon [83]. However, this picture contradicts with recent studies which found that (p)ppGpp overproduction did not affect GTP pools in both plants and algae [84,87]. Therefore, it is clear that more work is needed to identify the real effectors and targets of (p)ppGpp inside chloroplasts.

A (p)ppGpp hydrolase, Mesh1 has been identified in metazoa including both *Drosophila melanogaster* and *Homo Sapiens* for a dozen of years [88]. Recently, a low level of (p)ppGpp has been successfully detected in metazoa including both *D. melanogaster* and human cells. The accumulation of (p)ppGpp induces metabolic changes, cell death and ultimately lethality in *Drosophila* [89]. Much more work is required to reveal more details of (p)ppGpp signaling in metazoa including how it is synthesized, its regulatory mechanism and physiological relevance.

4. Outlook of future directions

4.1. Cell size and cell cycle regulation by (p)ppGpp

Studies on *E. coli* have revealed a profound role of (p)ppGpp in regulating various aspects of cell cycle and cell size of bacteria including chromosome initiation/elongation, initiation mass, adder sizer and cell division process. It is possible that (p)ppGpp has multiple downstream targets that coordinate together to exert its impact on cell cycle and cell size regulation. However, much more work is needed to identify the downstream effectors of (p)ppGpp that are responsible for mediating such a global regulation. Moreover, it is also unclear whether and how (p)ppGpp regulates cell size in other species such as *Bacillus*, *Cyanobacteria* and *Mycobacteria*.

4.2. Nutrient sensing and (p)ppGpp signalling in bacteria

Direct sensing of translation elongation rate (ER) has recently been shown to be an important nutrient-sensing mechanism of *E. coli* to modulate cellular (p)ppGpp pool to achieve growth-rate control [63]. However, the underlying mechanistic picture is still not complete. During exponential growth, RelA protein is not required for such a sensing mechanism in *E. coli*. Therefore, it is conceivable that SpoT could also sense ER and further modulate the synthesis/degradation of (p)ppGpp in *E. coli* during exponential growth, the mechanism of which remains to be an open question. In addition, other nutrient-sensing mechanisms could also be involved. For example, the synthetase activity of SpoT could be stimulated by the binding of acyl carrier protein (ACP), which senses the fatty acid status of the cell (probably via the acylation degree of ACP) [90–92]. In addition, Rsd protein could induce the hydrolase activity of SpoT during carbon downshift in a phosphotransferase system-dependent (PTS-dependent) manner [90,91,93]. It remains challenging to distinguish the involvements of different mechanisms under different circumstances. More broadly, does a similar nutrient-sensing mechanism also exist in other bacterial species such as *B. subtilis* and *Mycobacteria* species?

4.3. The regulatory mechanism of (p)ppGpp signaling in chloroplast

It is now known that (p)ppGpp in the chloroplast participates in regulating the growth, development and stress adaptation of plants

and green algae. However, the regulation of chloroplast gene expression by (p)ppGpp remains poorly understood. The direct mechanism of binding to RNA polymerase and the indirect mechanism via inhibiting GTP biosynthesis of (p)ppGpp action have been demonstrated for *E. coli* (proteobacteria model) and *B. subtilis* (firmicutes model), respectively. Further studies are required to identify the effectors and targets of (p)ppGpp inside chloroplast to elucidate how exactly (p)ppGpp modulates the gene expression in chloroplasts.

4.4. The function and mechanism of (p)ppGpp signaling in metazoa

The fields of (p)ppGpp regulation in metazoa are still on the initial stage with many open questions. Does (p)ppGpp really play an important role in the growth, development and survival of animals? How is it synthesized and metabolized? How does (p)ppGpp regulate gene expression in metazoan cells? Ultimately, we expect to see a full picture of (p)ppGpp signaling across species from bacteria, plants and algae to metazoa and further elucidate its ecological and evolutionary relevance.

Credit author statement

Manlu Zhu: Conceptualization, materials collection, writing and revision. **Xiongfeng Dai:** Conceptualization, materials collection, writing and revision.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Authors' contributions

Zhu M and Dai X conceptualized the work. All the authors participated in providing related references and discussing related content. Mu H, Han F, Zhu M and Dai X wrote the papers.

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