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REVIEW PAPER

Some Aspects of the Cell Biology of the Bacterial Stationary Phase: Programmed Cell Death and Its Regulation by Guanosine Tetraphosphate

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Abstract—This paper discusses (1) programmed cell death, a phenomenon typical of the stationary phase of bacteria occurring under unfavorable conditions, (2) its pleiotropic regulation by guanosine tetraphosphate, and (3) the conception of "addiction module," a specific genetic system responsible for the cell choice between survival and death under unfavorable conditions. The shortcomings of the proposed interpretation of the problem at hand are considered and the necessity of their further investigation is substantiated.

Key words: bacteria, stationary phase, cell biology, programmed cell death, regulation, guanosine tetraphosphate.

INTRODUCTION TO THE PROBLEM

The investigation of the bacterial stationary phase goes back to the 1930s, when Kluyver and Perquin developed and first described the technique of submerged cultures ([1] as found in [2, 3]). For at least half a century, the investigations of such kind were centered on the physiology of stationary-phase batch cultures and their analogues, i.e., continuous cultures grown at low rates under the conditions of severe limitation by one of the nutrients present in the cultivation medium [2]. Studied were physiological parameters in a conventional sense, such as the dynamics of biomass and population, the consumption of substrates, the secretion of metabolic products, respiration, and the effect of various physicochemical conditions on the growth parameters and the metabolic activity of cells. Populational processes in the stationary phase were considered, as a rule, in relation to spore formation [3].

During the last decade, there has been a tendency to treat such processes as the expression of genes, the conversion of macromolecules, the transduction of signals, and, particularly, the responses of bacteria to various shocks and stresses [4–6] in terms of bacterial physiology. Recently, however, these processes, as well as some others (e.g., signal reception, the control of metabolic fluxes, and the operation of intracellular regulators), have been properly placed in the science of cell biology (relevant discussion may be found, for instance, in [7]).

The present paper, like the previous one [8], will consider the stationary phase of bacteria just in terms of cell biology, taking into account the fact that the stationary phase is a sophisticated phenomenon, which includes several organizational levels of cells, different global regulatory systems, and the expression of various genetic programs.

The previous review [8] was devoted to the cell response to general stress, i.e., to various shocks and stresses, including those which initiate the transition of bacteria to the stationary phase. Great attention was given to the alternative σ-subunit of the RpoS RNA polymerase and the specific protein regulator UspA of *Escherichia coli*, which control certain regulons and are activated under starvation conditions. At the same time, some other important aspects of the cell and population biology of the bacterial stationary phase remained beyond the scope of that review.

The aim of the present paper is to fill this gap, with particular emphasis on programmed cell death and the function of the pleiotropic regulator guanosine tetraphosphate.

PROGRAMMED CELL DEATH

General consideration. The phenomenon of programmed cell death ("suicide") has long been investigated with reference to embryogenesis and morphogenesis in higher organisms [9–11], for which this phenomenon is known as apoptosis [9–14]. This term is also used with respect to yeasts [12–14] but not with respect to prokaryotic organisms, in which the mechanisms of programmed death are very specific.

The phenomenon of programmed death has long been recognized for bacteria. For instance, it was found that the bacterial cells that were transformed with certain plasmid vectors remain viable during subsequent

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replication if they retain the vector. But if the transformed cells lose the vector as a result of segregation, they rapidly die. Similar segregational death was observed for natural plasmid-bearing bacterial strains. This particular case of programmed bacterial death was considered by Yarmolinsky [15].

An analysis of the segregational death of bacteria and the apoptosis of yeasts, as well as of some other related phenomena, such as morphogenetic processes associated with the formation of spores and fruiting bodies, allows some suggestions important for further discussion to be made. Namely,

(1) microorganisms benefit from the death of a portion of their population;

(2) there is an adaptive mechanism that provides for the viability of a portion of a microbial population provided that the other portion of the population will die;

(3) programmed cell death may be triggered by the very population heterogeneity;

(4) some stationary-phase phenomena, such as the partial autolysis of stationary-phase cultures, may result from programmed bacterial death [15] and not from random metabolic alterations.

The mechanisms of the stabilization–programmed death of bacteria may be different. One of the possible mechanisms was proposed by Yarmolinsky [15] and Naito *et al.* [16], who studied mutants defective in restriction endonucleases and the restriction–modification DNA methylase of plasmid Plk137 and found that it is the proportion between these enzymes that may be responsible for programmed cell death. In normal *E. coli* cells bearing this plasmid, DNA is protected from the action of restriction endonucleases by the methylation of susceptible sites with the plasmid DNA methylase. In the mutants lacking the plasmid, DNA is no longer protected by the methylase and is digested by the remaining restriction endonucleases, which results in cell death. This mechanism may be treated as a "selfish behavior" of the plasmid, since plasmidless cells are excluded by plasmid-bearing cells [15, 16].

The conception of "addiction module." Within the framework of his general conception of the stabilization–programmed death of bacteria [15], Yarmolinsky postulated the existence of a specific genetic module, the so-called "addiction module," which is responsible for the continuous maintenance in cells of a gene that is not necessary for their normal functioning and, under certain conditions, may even cause their death. Based on the data of Naito *et al.* [16], Yarmolinsky suggested that this module should contain two genes, one of which encodes a factor (for instance, a restriction endonuclease) that is toxic to the cell and is neutralized by the product (as DNA methylase) of the second gene. These factors are usually called toxin and antitoxin. Under normal conditions, the high expression of the second gene results in antitoxin domination. However, when a cell loses the addiction module, the remaining toxin, which is more abundant and stable than the antitoxin, kills the cell.

The diversity of addiction modules. In 1944, Bigger showed that even the long-term incubation of a suspension of staphylococci with penicillin leaves a portion of the population viable ([17] as found in [18]). The survived cells were unlikely to be defective, since their portions in cultures occurring under different conditions were the same. Nor were the survived cells mutant, since their offspring cells were also heterogeneous with respect to penicillin resistance. The cells behaved as though they could choose between their survival and death under the action of the antibiotic, a situation typical of an addiction module. Similar phenomena were observed in other bacteria for other detrimental factors, such as UV radiation and severe thymine starvation [18].

As for the mechanism of the action of the addiction module, there are several relevant hypotheses. Along with the aforementioned hypotheses of Yarmolinsky [15] and Naito *et al.* [16], there is also the hypothesis of Gerdes *et al.* [19], who suggested that the product of the *sok* gene of the *hok–sok* system carried by plasmid R1 is a lethal antisense RNA, which plays the role of the unstable (nuclease-susceptible) component of the addiction module.

The addiction module can be carried by various plasmids [20] and phages. For instance, the *phd–doc* module is known to stabilize prophage P1 [21]. The *rexB* gene of bacteriophage lambda is an antideath gene [22].

Chromosomal addiction modules. A search for genes controlling the death of *E. coli* cells allowed Moyed and Bertrand [23] to reveal three independent *hip* (high-persistence) chromosomal loci, whose mutation increased the percentage of antibiotic-resistant cells by three orders [23]. The following discussion will be restricted to the chromosomal addiction modules, leaving aside the addiction modules of mobile extrachromosomal genetic elements.

One of the best studied and important (due to its interaction with global regulatory systems) chromosomal addiction modules of bacteria is *mazEF* [24], also known as *chpA* [25]. In the nucleotide sequence, this module is homologous to the *pemIK* module of plasmid R100. The *mazEF* genes are situated downstream the *relA* gene, which controls the stringent response of cells to a deficiency of nitrogen in the medium, i.e., the global system of metabolic rearrangement under nitrogen starvation conditions. Accordingly, the *mazEF* genes belong to the *relA* operon [24]. Moreover, they are identical to the *relBE* genes of this operon [26]. The investigation of the products of the *mazEF* genes showed that MazE is a labile protein which protects bacterial cells from the lethal action of the stable MazF protein [27]. To remain viable, the cells must maintain a high level of expression of the *mazEF* addiction module.

PROGRAMMED CELL DEATH AND ITS REGULATION BY GUANOSINE TETRAPHOSPHATE

General information on guanosine tetraphosphate–mediated regulation. As mentioned above, the *relA* operon controls the global regulatory system of the stringent response [28]. The product of the *relA* gene (RelA) is the synthetase of the universal regulator 3',5'-bis(guanosyl)pyrophosphate (guanosine tetraphosphate, ppGpp), also called ppGpp synthetase I (PS I). There is also a second synthetase of ppGpp, SpoT (PS II). The RelA protein (stringent factor) is localized at the A site of ribosome and is activated under amino acid deficiency. In wild-type strains, the amino acid deficiency is induced by the exhaustion of nitrogen sources in the medium, due to which they transit to the stationary phase. RelA tests this state by detecting nonacylated tRNA in ribosomes [28–30] and responds to it by synthesizing ppGpp through guanosine pentaphosphate (pppGpp) with the use of GTP and the energy and the phosphate groups of ATP.

The location of the second phosphatase, SpoT, is not conclusively established. It may be associated with the ribosome or may occur in the cytosol [30]. Unlike RelA, SpoT is activated when cell growth is limited by the deficiency of carbon sources. It is likely that SpoT is directly activated by intermediates of the synthesis of membrane fatty acids, which accumulate in cells when their growth is limited [28–30].

Spira *et al.* reported that the synthesis of guanosine tetraphosphate may also be activated by the deficiency of inorganic phosphate in the medium [31] and depends on SpoT. Therefore, ppGpp is synthesized when cells transit to the stationary phase, which precedes the phase of cell death and the formation of resting forms. Let us analyze in more detail the role of ppGpp in the stationary phase control.

Guanosine tetraphosphate is a powerful pleiotropic regulator of the metabolism of cells whose growth is limited by some factors. The role of ppGpp is to compensate for the action of these factors. This regulatory system has been described not only for many bacteria but also for actinomycetes [32]. Moreover, analogous stringent control systems were revealed in yeasts [33], although their mechanisms differ from those found in bacteria [34]. Therefore, ppGpp may be a universal regulator in prokaryotes. At least, there is no doubt that guanosine tetraphosphate is one of the major regulators in *E. coli* cells, especially those which grow under limitation conditions or occur in the stationary phase [29, 35–37]. The regulatory action of ppGpp is either activating or inhibiting. Inhibited are glycolysis, respiration, and the synthesis of ribosomal proteins, translational factors and other components of the protein-synthesizing apparatus, lipids, nucleotides, and peptidoglycans. Activated are the transport of amino acids and some carbohydrates, the synthesis of necessary amino acids and the excretion of those which are unnecessary at a given time, the expression of the *lac* gene, some other genes of carbohydrate metabolism, and some genes of the *Pho* regulon that control phosphate uptake in *E. coli*, the proteolysis of unnecessary proteins, the cleavage and mobilization of compounds required for biosyntheses and reserve substances, and the hydrolysis of stable RNA [28, 31, 36–42]. These processes are regulated at several levels, including DNA transcription and the modulation of enzymatic activities [28, 29, 36–38]. Guanosine tetraphosphate is also involved in the regulation of secondary syntheses and morphogenesis, as shown for *Streptomyces coelicolor* A(3)2 [43].

Furthermore, guanosine tetraphosphate controls some populational processes. In *E. coli*, it coordinates growth rate in accordance with the availability of nutrients by controlling DNA replication and cell division [44], as well as influences the frequency of mutations [45]. In *Pseudomonas aeruginosa*, ppGpp activates the quorum sensing system and modulates the expression of genes depending on the concentration of cells in the medium [46]. During the formation of the *P. aeruginosa* biofilm, guanosine tetraphosphate, together with the AlgR 2 protein, is involved in the coordination of alginate synthesis [47]. In this case, AlgR 2 may mediate the transduction of the signals generated by guanosine tetraphosphate in response to the onset of the stationary phase and other conditions necessary for biofilm formation.

It should be noted that the concentration of guanosine tetraphosphate in cells correlates with their growth rate, but the relationship between these parameters is not functional [47, 48]. The dynamics of ppGpp in cells may be determined by the specificity of the carbon sources used or the occurrence of stressful conditions, as was shown for pseudomonads grown on toxic substituted phenols [49]. Sce and Shingler showed that guanosine tetraphosphate directly controls the P_o promoter of the DmpR operon responsible for the degradation of the phenols. This operon is transcribed by an RNA polymerase with the alternative σ^{54} subunit (which is specific for nitrogen metabolism and the metabolism of aromatic compounds), but not by the major bacterial RNA polymerase [49]. It is known that transcriptional regulation by guanosine tetraphosphate involves its interaction with the β-subunit of RNA polymerase [50]. The ability of ppGpp to interact with the different σ subunits of RNA polymerase indicates that ppGpp may be involved in the regulation of the slow growth of bacteria under various stressful conditions, in which these subunits are also involved.

It should be noted that some authors do not recognize ppGpp as a bacterial growth regulator [51].

Guanosine tetraphosphate and programmed cell death. The coexpression of the *mazEF* addiction module genes is inhibited by high concentrations of ppGpp in cells [27]. Since the *mazE* gene product is more labile than the *mazF* gene product, the balance between

them is upset, and the prevailing concentrations of the MazF protein kill the cell. Thus, the program of cell suicide is implemented by the stringent response system through the addiction module. The deficiency of the stabilizing MazE protein is caused by protease ClpA, which is involved in the control of regulatory and stress proteins and is most likely activated in the stationary phase. The role of ppGpp in this process remains unclear, although there is evidence that ppGpp controls the P_2 promoter of the *mazE* and *mazF* genes [27] and blocks the function of RNA polymerase at the level of the initiation of transcription [52]. The finding that ppGpp is slightly toxic to mutants having deletions in the *mazEF* module or possessing the impaired *clpA* gene opens a new way for elucidating the role of ppGpp in programmed cell death. This role is not simple, as is evident from the data of Sat *et al.* [53], who showed that the antibiotics rifampicin, chloramphenicol, and spectinomycin, known as the inhibitors of DNA transcription and/or translation, can trigger the *mazEF* lethality.

It should be noted that some authors sometimes interpret the common death of cells under the action of unfavorable factors as the phenomenon of bacterial suicide. For instance, Aldsworth *et al.* [54] called the death of cells induced by active oxygen species accumulated in response to various shocks and growth-limiting conditions as cell suicide. It is also difficult to suggest the involvement of any addiction modules in the lysis of cells under the action of quinolone mutagens [55]. These mutagens enhance the synthesis of the SOS proteins RecA, LexA, and SulA, the latter of which inhibits the division of cells and activates their autolysis. The cell death induced by various unfavorable factors is a special problem of the cell biology of bacteria and will not be considered here.

CONCLUSION

When discussing the role of programmed cell death in the physiology, cell biology, and the population biology of bacteria, it seems necessary to emphasize that researchers have long overestimated the significance of stochastic processes in the developmental biology of bacterial cultures. The idea that the heterogeneity of a genetically homogeneous microbial population and the death of some cells under the action of unfavorable factors are due to random fluctuations in the environmental conditions and metabolic alterations in these cells corresponds to one of the postulates of the general theory of ecology and evolution: those organisms die that are the weakest and the least adapted.

The conception of programmed cell death, which states that cells occurring under unfavorable conditions have a choice to survive or to die, refutes this postulate and shows that some of the general principles of the theory of ecology and evolution are inapplicable to bacteria and that the adaptive responses of a microbial population based on stochastic processes cannot provide for its survival. The microbial population, as an organism, must control the death and the life of each of its cells.

It should be noted that there are still many unclear points in the phenomenon of programmed cell death. What signal causes a cell to choose between death and life? Guanosine tetraphosphate cannot obviously be such a signal, since it will otherwise be difficult to explain the different responses of particular cells to external factors. Those researchers may be right who suggest that programmed cell death is merely caused by the genetic mechanisms of self-destruction, which are activated by stress factors in some cells in accordance with the random distribution of the adverse effect of these factors among the cells [54, 55]. Then the postulated ability of cells to choose between life and death becomes a beautiful legend. Is there a relation between the viable but not culturable state of bacteria [56] and their programmed death? May the transition to this state be programmed? These and many other relevant questions need further comprehensive studies.

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