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review

Bacterial Stationary-Phase Evolution

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Summary

Metagenomics and advances in molecular biology methods have enhanced knowledge of microbial evolution, metabolism, functions, their interactions with other organisms and their environment. The ability to persist and adapt to changes in their environment is a common lifestyle of 1 % of the known culturable bacteria. Studies in the variety of species have identified an incredible diversity of bacterial lifespan. The holy grail of molecular biology is to understand the integrated genetic and metabolic patterns of prokaryotic organisms like the enteric bacterium *Escherichia coli*. The usual description of *E. coli* life cycle comprises four phases: lag, logarithmic, stationary, and death phase, omitting their persistence and evolution during prolonged stationary phase. During prolonged stationary/starvation period, in batch bacterial culture, selected mutants with increased fitness express growth advantage in stationary phase (GASP), which enables them to grow and displace the parent cells as the majority population. The analyses of growth competition of Gram-negative and/or Gram-positive mixed bacterial cultures showed that GASP phenomenon can result in four GASP phenotypes: strong, moderate, weak or abortive. Bacterial stress responses to starvation include functions that can increase genetic variability and produce transient mutator state, which is important for adaptive evolution.

Key words: evolution, *Escherichia coli*, prolonged stationary phase, growth advantage in stationary phase

Introduction

Numerous microorganisms that surround us have evolved various strategies that allow them to inhabit almost any environmental niche on Earth. A wealth of knowledge including basic concepts of molecular genetics has been acquired by studying the cells of *Escherichia coli* under laboratory conditions (*1*,*2*). When *E. coli* cells are inoculated in a batch culture medium, a bacterial population is formed which goes through several distinct growth phases (*3*–*6*). Once adapted to the medium during lag phase (Fig. 1, phase 1), the cells enter the exponential growth phase (Fig. 1, phase 2), during which they grow and divide at a maximal rate for the species under the existing conditions. After the available nutrients are exhausted, due to depletion of nutrients and the accumulation of waste products, the bacterial culture enters stationary growth phase (Fig. 1, phase 3), and the majority of parental cells die during death phase (Fig. 1, phase 4). In fact, scarce natural environment, in which bacteria can be found, is very similar to stationary growth phase conditions during laboratory cultivation. On entry into stationary growth phase, nonsporulating bacteria respond to the starvation conditions by undergoing a series of morphological and physiological changes (*7*). Two outcomes are possible for bacterial cultures in this phase. First, failing to adapt to new conditions, a cell population enters death phase, and second, a cell population evolves mechanisms enabling it to rapidly adapt to stress conditions and remain viable during prolonged station-

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ary growth phase, up to 5 years (*8*,*9*), with the help of periodical appearance of new growth advantage stationary phase (GASP) mutants, from GASP 1 to GASP n+1 (Fig. 1, phase 5).

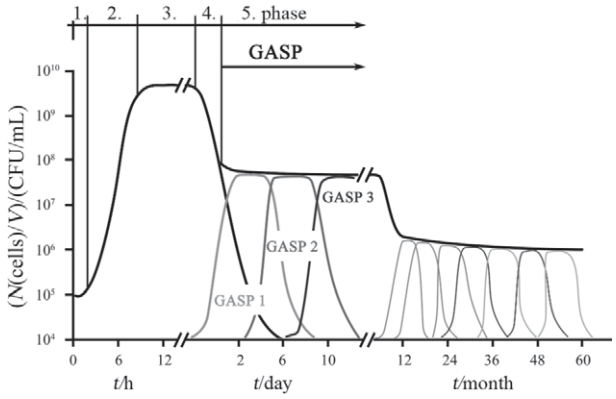

Fig. 1. Growth curve of a batch non-sporulating bacterial culture in the plot of time *vs.* N(cells)/V (1 % inoculum). The growth curve is characterized by five distinct stages: 1. lag phase, 2. log or exponential phase, 3. stationary phase, 4. death phase, and 5. prolonged stationary phase with the appearance of GASP mutants, successively GASP 1, GASP 2, GASP 3, *etc.*, which was measured until the sixtieth month. The scheme is modified from Nester *et al.* (*4*), Finkel *et al.* (*8*) and Bačun-Družina (*9*)

The bacterial ability to colonize every niche in the natural environment is not only due to their metabolic flexibility, but more importantly due to their capacity for adaptation to different stresses (*10–12*). This review will discuss highly dynamic nature of bacterial cells and their evolution during prolonged starvation with the appearance of various GASP mutants.

Bacterial Gene Expression

Basic enterobacterial biology studies on gene regulation have been dominant through the last five decades. Along with the complete sequence of the *E. coli* genome, there are new analyzing tools that permit monitoring of both the transcription and translation of each individual gene. In order to understand gene regulation in *E. coli*, three hierarchical levels of control have been suggested: (*i*) global control of chromosome structure, (*ii*) global control of regulons and stimulons, and (*iii*) operon-specific controls. Activity at the first level of control is coordinated with local superhelical density and topological structure of the whole chromosome. In cooperation with operon-specific controls, the second level of control is mediated by site-specific proteins, which regulate often overlapping groups of metabolically related operons in response to metabolic or environmental signals (*13*). The last level is controlled by less abundant regulatory proteins that respond to operon-specific signals and bind in a site-specific manner to one or few sites in order to regulate single operons. Certain important processes such as the response to starvation stress are controlled within multiple levels of gene regulation, so RpoS regulon overlaps extensively with other global response networks in *E. coli*. This regulation is described in more detail in the section Bacterial Stress Response to Starvation.

Bacterial RNA polymerase (RNApol) is the principal enzyme of gene expression and regulation. It catalyzes the first step in gene expression – transcription of structural genes as templates for constructing mRNA and noncoding RNA. There are two forms of RNA polymerase: the core enzyme and complete holoenzyme. Core RNA polymerase can bind to DNA and catalyze the synthesis of RNA but it has no specificity. This form of enzyme will bind to non-specific DNA but it cannot recognize promoters. The core enzyme has four polypeptide subunits: alpha (α), beta (β), beta' (β') and omega (ω) in the stoichiometry $\alpha_2\beta\beta'\omega$. Two α subunits assemble the enzyme and recognize regulatory factors. β has the polymerase activity (catalyzes the synthesis of RNA), which includes chain initiation and elongation. β' nonspecifically binds to DNA, and ω restores denatured RNA polymerase to its functional form *in vitro*. Transcription is a cyclic process that can be divided into three major steps – promoter DNA binding and RNA chain initiation, processive RNA chain elongation, and termination. The first step of the cycle requires the presence of one of the promoter-specific sigma (σ) subunits that form a holoenzyme together with the core. The sigma factor reduces the affinity of RNApol for nonspecific DNA while increasing the specificity for certain promoter regions, depending on the sigma factor (*14*,*15*). Each subunit is encoded by a certain gene and has a specific size (Table 1; *16–20*). When not in use, RNA polymerase binds to low-affinity sites to allow rapid exchange for an active promoter site when one opens.

Table 1. The subunits of *E. coli* RNA polymerase and their properties

Subunit	M_r/kDa	Gene	Function
α	36511	*rpoA*	required for assembly of the enzyme; interacts with some regulatory proteins; involved in catalysis (*16*)
β	150616	*rpoB*	involved in catalysis, chain initiation and elongation (*17*)
β'	155159	*rpoC*	binds to the DNA template (*18*)
σ^D	70263	*rpoD*	directs enzyme to the promoter of the genes expressed in the exponential growth phase (*19*)
ω	10237	*rpoZ*	required to restore denatured RNA polymerase *in vitro* to its fully functional form (*20*)

The number of sigma factors varies between bacterial species. Particularly, *E. coli* has at least seven sigma factors, and each of them is activated in response to different environmental conditions (*21*). Sigma factors are distinguished by their characteristic molecular mass and their function (Table 2; *1*,*19*,*22–26*). For example, the principal sigma factor σ^{70} refers to the subunit with a molecular mass of 70 kDa. The primary σ^{70} factor recognizes promoters of the growth-related and housekeeping genes expressed in the exponential phase of bacterial growth (*15*,*27*). Subunit σ^{54} is present all the time and it is required for the expression of many genes that are involved in nitrogen metabolism. Furthermore, subunit σ^{32}

Table 2. The sigma factors of *E. coli* and their specific functions

Sigma factor	Gene	Function
σ^{70}	*rpoD*	transcription of most genes during the exponential phase (*19*)
σ^{54}	*rpoN*	nitrogen-regulated gene transcription (*22*)
σ^{38}	*rpoS*	gene expression during the starvation and stationary phase (*23*)
σ^{32}	*rpoH*	heat-shock gene transcription (*24*)
σ^{28}	*rpoF*	expression of flagellar and chemotaxis genes (*25*)
σ^{24}	*rpoE*	response to the extracytoplasmic and extreme heat stress (*1*)
σ^{19}	*fecI*	regulation of the *fec* genes for iron dicitrate transport (*26*)

is required for the expression of heat-shock genes, *i.e.* genes that are only expressed when the cell is exposed to a high temperature and it must make special proteins in order to survive, while σ^{38} appears only when cells enter the stationary phase of growth (Fig. 2, *28*). It is responsible for transcription of all of the genes whose products are required during starvation.

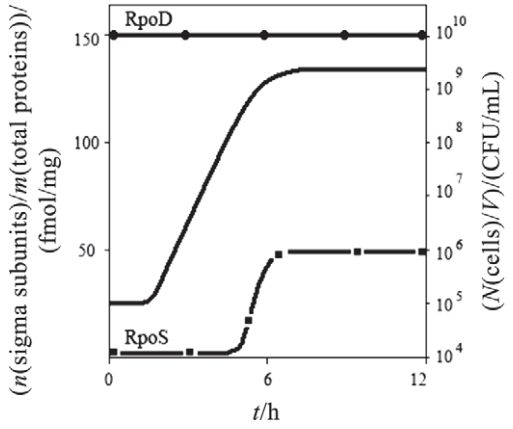

Fig. 2. Concentration of sigma subunits RpoD and RpoS during *E. coli* growth in batch culture. Values for proteins RpoD and RpoS are used from Ishihama (*28*)

In addition, proteomic analysis of cells lacking small nucleotide guanosine-3',5'-bis(diphosphate), ppGpp, has shown that it acts as a global regulator of gene expression that helps bacteria survive in limited environments (*15,29,30*). This nucleotide is produced not only in response to amino acid limitation but also in response to many different kinds of nutrient limitations and circumstances that cause growth arrest. Nucleotide ppGpp generates a redirection of transcription favouring genes important for starvation survival, growth, adaptation, secondary metabolism, persistence, cell division, motility, biofilms, development, competence and virulence (*31,32*). Furthermore, the use of ppGpp by *E. coli* cells in order to mediate a variety of starvation responses is regulated in part by modulating σ^S levels (*33*). The studies have shown that the activity of the alternative sigma factors σ^S, σ^N and σ^H increases in a ppGpp-dependent manner following the entry into stationary phase (*34,35*). These findings could lead to the hypothesis that the ppGpp--signalling pathway provides a means by which different moduli can be activated in concert to provide a coordinate response to nutritional depletion (*32,36*).

The content of core RNA polymerase components (α, β, β' and ω subunits) is relatively constant in a cell, as well as level in the expression of housekeeping genes; however, the elevation of RpoS protein (or σ^S) levels under starvation or extreme stress conditions is followed by constant stable level of RpoD protein (Fig. 2) (*14,37*). Furthermore, the general stress sigma factor σ^S and the vegetative σ^D are highly related and recognize the same core promoter elements (*38*).

RpoS-dependent gene expression leads to a semi--differentiated state with high levels of protection of cellular integrity in stressful environments. During exponential growth, the general stress response is poorly induced and RpoD (σ^D) does not need to compete for RNA polymerase components. This pattern of decision making is fine under the extremes of feast and famine, but bacteria also need to face intermediate situations in many natural settings. For example, under nutrient limitation with low levels of glucose and ppGpp, the *rpoS* expression is elevated, but high level of RpoD protein is constant (Fig. 2). Jishage and Ishihama (*39*) identified in *E. coli* Rsd protein (regulator of sigma D), which has been proposed to function as a σ^{70}-specific anti-σ factor. Mitchell *et al.* (*40*) support a model in which the role of Rsd is primarily to sequester σ^{70}, thereby increasing the levels of RNA polymerase containing the alternative σ^{38} factor. Although the role of σ^{38} is understood, the control of σ^S and other sigma factors as well as their competition for the RNA polymerase (RNAP) core enzyme are still an open field with many questions.

Another interesting observation is the evolution of *rpoS* mutations found in natural populations of enterobacteria which contain a high frequency of polymorphisms in this gene (*41*) as well as the fact that some bacterial strains have a higher propensity for accumulating *rpoS* mutations than others (*42,43*). The competition between the σ-factors RpoS and RpoD was proposed as a reason for the enrichment of *rpoS* mutations in bacterial populations. The accumulation of *rpoS* mutations in stationary--phase batch cultures (*44*) and in steady-state glucose--limited chemostat populations (*45*) of *E. coli* could be explained by the characteristic of bacterial cell to improve nutrient scavenging by expression of RpoD-dependent genes (*41*).

Bacterial Stress Response to Starvation

Two most common models of life in the microbial world are feast and famine. Bacterial cells have evolved systems that enable them to survive in environments which lack essential building blocks for growth, including carbon, nitrogen, and phosphorus. When a particular nutrient becomes limiting, *E. coli* increases the production of proteins that compensate for the limiting nutrient (*46*). The master regulator of stress response, RpoS, controls the promoter regions of scavenging regulons such

as Cap (catabolite activator protein)/Crp (cyclic AMP [cAMP] receptor protein), which allows for the use of alternative carbon sources, and the two-component regulatory systems PhoR/PhoB and NtrB/NtrC, which control scavenging for phosphorus and nitrogen, respectively. Both the Cap/Crp and Ntr systems oversee nutrient status through intracellular metabolites in such a manner that Cap/Crp recognizes cAMP, while NtrC responds to glutamine (*47*,*48*). Furthermore, the Pho regulon monitors inorganic phosphate levels *via* the activity of the two low-affinity P_i transporters, PitA and PitB, and the high-affinity Pst transport system (*49*,*50*). In all three cases, if scavenging fails, the cells starve and enter stationary phase.

As previously mentioned, the entry of *E. coli* into starvation/stationary phase is regulated by σ^S subunit, which can positively or negatively affect the expression of over 500 genes (*48*,*51*,*52*). The main determining factor for the induction of σ^S is the amount of nutrients and its regulation occurs at the levels of transcription, translation, proteolysis and protein activity. Protein RpoS regulates transcription, directly or indirectly, of genes whose products render the cell resistant to many different stresses, such as starvation, pH downshift, hyperosmolarity, or nonoptimal high or low temperature (*53*). RpoS-regulated proteins alter every compartment in the cell, including both membranes and peptidoglycan (*38*), as well as the morphology and induction of functions that allow adhesion and biofilm formation (*48*,*54–56*). Hengge in her reviews (*38*,*51*) explained how the fine-tuned regulatory mechanisms of σ^S operate at four levels: (*i*) an activation of *rpoS* transcription that requires the general stress alarmone ppGpp; (*ii*) an activation of *rpoS* translation, which involves a structural rearrangement of *rpoS* mRNA, the RNA chaperone Hfq and several small regulatory RNAs; (*iii*) a reduction of σ^S proteolysis; and (*iv*) several factors that support σ^S-RNAP holoenzyme ($E\sigma^S$) formation such as Crl, the anti-σ^{70} factor Rsd and ppGpp. Examining the regulation of RpoS in *E. coli* in minimal media, Dong and Schellhorn (*57*) found that about 400 genes were RpoS-dependent and suggested that RpoS might play a role in nutrient recycling and proper protein folding by mediating the expression of chaperones and proteases for the survival during starvation. Starvation for carbon, phosphorus, or nitrogen in *E. coli* is sensed differently, and the activation of the RpoS regulon occurs in a unique way. In the cells starved for carbon or phosphorus, RpoS protein levels increase because degradation stops or translation of *rpoS* mRNA increases, respectively. However, RpoS levels change little in nitrogen-starved cells, but its activity increases. By influencing different elements of the pathway, each starvation signal activates RpoS regardless of the status of starvation level or excess of the other nutrients. Thus, the signals act independently and do not interfere with each other.

Moreover, three feast/famine regulatory proteins (FFRPs), which comprise a diverse family of transcription factors, have been detected in the genome of *E. coli* that coded: leucine-responsive regulatory protein (Lrp), asparagine synthase C gene product (AsnC) and putative transcriptional regulator YbaO (*58*). In many cases depending on extracellular leucine availability, Lrp activates or represses the transcription of a number of related genes in *E. coli* (*58–60*). The Lrp regulon includes genes responsible for stress response, outer membrane proteins (OMPs), amino acid biosynthesis and degradation, small molecule transport, carbon and energy metabolism, pilus synthesis, macromolecular biosynthesis, and gene regulators (*59*,*61*) as well as the repression of *rpoS* gene (*62*). Furthermore, the observations suggest that Lrp may play a major role in stationary-phase transitions: (*i*) Lrp levels vary with growth phase, being the lowest in the late exponential phase when ppGpp levels are low (*63*), so Lrp-repressed genes should show a burst of expression during the transition to stationary phase; (*ii*) mutations in *lrp* confer a growth advantage in stationary phase (GASP) phenotype (*44*); and (*iii*) the transcription of the stationary phase-induced gene *osmY* is regulated by Lrp and it was noted that Lrp appears to control additional stationary phase-induced genes (*64*). Furthermore, Lrp expression is induced by guanosine tetraphosphate, ppGpp (*63*). Lintner *et al.* (*65*) showed by the microarray analyses that Lrp, influences the expression of nearly 400 genes, at least 70 of which are directly controlled. The regulatory action of Lrp on target genes is often modulated by the binding of the effector molecule leucine and it affects Lrp, whose activity could be enhanced, reversed, or unaffected (*59*,*60*). Interestingly, when *E. coli* cells are sensing rich nutrition with a high concentration of leucine in the environment, their metabolism is changed by terminating autotrophic pathways and activating absorption of nutrients *via* the cell membrane, thereby shifting to a more heterotrophic mode. Heretofore, exact molecular mechanisms for regulating the previously mentioned genes are not fully understood. However, Cho *et al.* (*66*) described an integrative analysis of various types of genome-scale data to comprehensively understand the design principles of a global transcription factor, Lrp, in *E. coli*.

Hence, cells are able to respond to an environmental change as a population and make a coordinated transition into stationary phase. A stationary phase can be described as a spore-like state, in which cells manage to survive long periods of time by using a minimal amount of nutrients and by enduring many assaults from the environment before continuing to grow once the conditions improve and available nutrients are replenished.

Expression of GASP Phenotype

Genetic variability and natural selection of beneficial mutations permit the adaptation to newly created conditions, and are highly significant for bacterial evolution. Particularly, some investigations have been focused on the entry of bacteria into starvation phase and on their long-term survival under these stress conditions. The ability of the bacterium *E. coli* to grow during prolonged stationary phase in the absence of a carbon source is referred to as growth advantage in stationary phase, or the GASP phenomenon (*67–71*). Kolter's investigating team (*7*) has found that *E. coli* bacterial cultures can persist in the prolonged stationary phase for the period of up to 5 years, only with the addition of water. Under starvation conditions, GASP phenotype cells show better ability to adapt and displace their wild-type parents as

the majority (*44*). These GASP mutants can scavenge poor nutrient sources formed by the dead cells. The surviving population is highly dynamic and is a constant source of genetic diversity (Fig. 1, phase 5). Within such population, GASP mutants with better adaptability emerge throughout the starvation period, so that every new generation of GASP mutants competes with the old one. Particularly, in a typical GASP competition experiment, cells from old bacterial culture (*e.g.* 10-day-old or other period) are inoculated as a numerical minority, such as 1:10, 1:100, 1:1000 or 1:10000 by volume, into a younger cell culture (1-day-old or some other age).

The first identified *E. coli* GASP mutation was an allele of *rpoS* (*44*). These mutants are referred to as G1 (GASP 1) strains, as they have a GASP phenotype when competing against their wild-type (G0) parent (Fig. 3, *72*). *E. coli* isolated from an aged culture of the *rpoS* strain expresses the GASP phenotype when competing against its G1 parent and was thus designated a G2 strain (*73*). This strain, G2, is designated *sgaA*, *sgaB*, and *sga*C due to three mutations (*72*). The *sgaB* mutation was identified as an allele of *lrp*, encoding the leucine-responsive regulatory protein, demonstrating that a published *lrp* null allele also confers a GASP phenotype. Moreover, microarray analysis suggested that the loss of Lrp function could enhance scavenging capabilities; in addition to increased catabolism of serine, glycine, and threonine, *lrp* mutants have increased expression of enzymes involved in gluconeogenesis and the pentose phosphate pathway, and in the tolerance of acid and osmotic stresses (*61*).

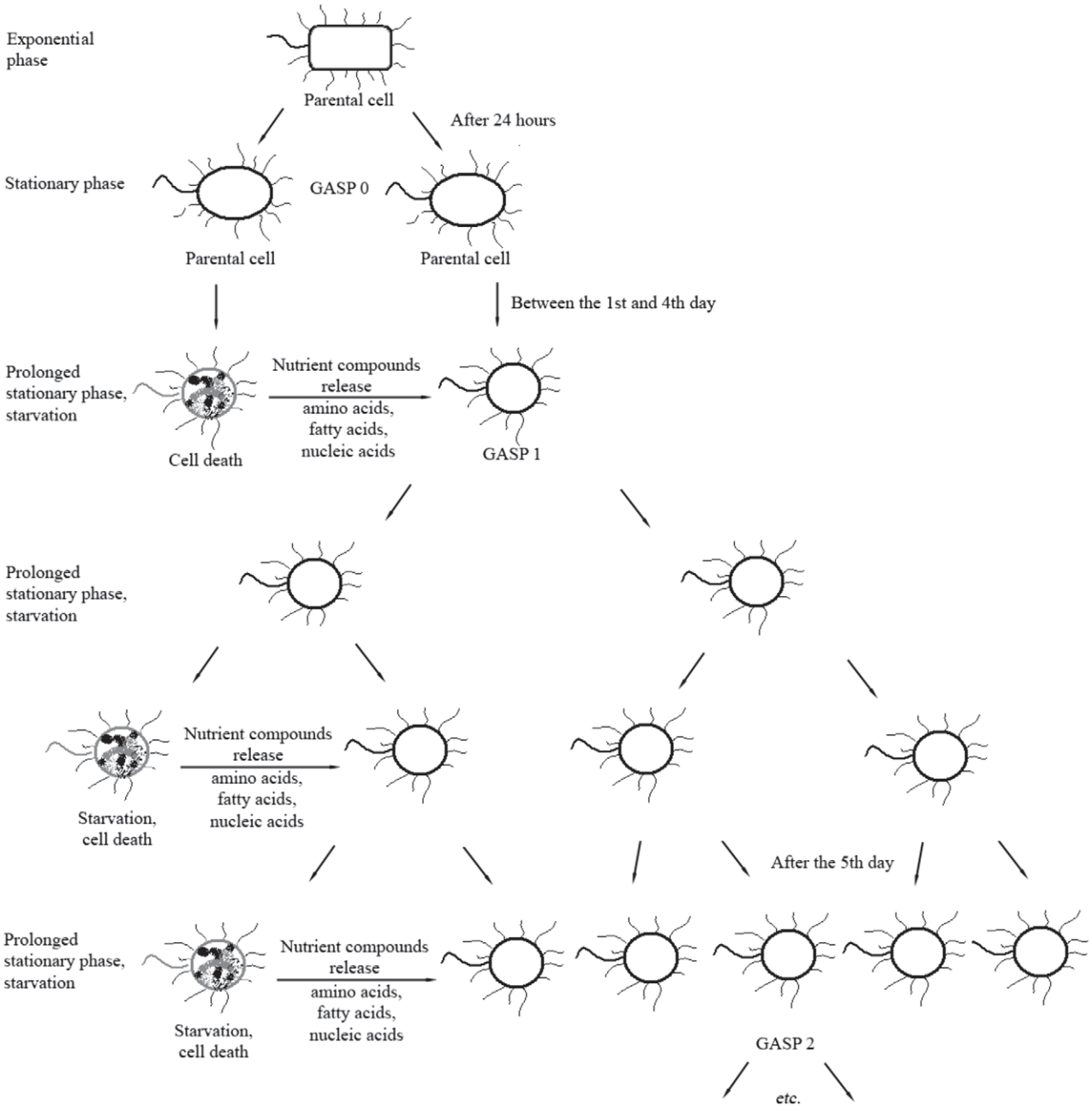

Fig. 3. A model for the appearance of GASP 1 and GASP 2 mutants. The scheme is modified from Zinser and Kolter (*72*)

Although the appearance of GASP mutants is a highly dynamic process, only some GASP mutants were characterized and the discovery of a number of different point mutations in the various GASP generations is expected (Fig. 1). Until today two generations of GASP mutants, G1 and G2, have been genetically identified when competing against their wild-type (G0) and G1 parent, respectively. When conditions are unpropitious, G0 streak introduces one mutation into its genome yielding G1 mutant, and if starvation conditions persist, additional mutations are introduced in G1 strain, and a G2 generation of mutants appears. As previously stated, the first GASP mutation identified was an allele of *rpoS*, which bears a 46-base-pair duplication, resulting in a reduced gene expression (*44*). It has been detected that the attenuated *rpoS* gene in other G1 GASP mutants includes all kinds of point mutations such as transversions, transitions, and frameshift mutations (*74*). A reduced expression of the *rpoS* gene is connected with an increased level of the RpoD. Genes participating in the catabolism of amino acids are under control of σ^D subunit; hence mutations of the *rpoS* gene increase the catabolism of amino acids and thus accelerate the growth of G1 mutant on amino acids released by the dead cells.

As previously mentioned, the G2 generation of GASP phenotype is due to three mutations, determined as *sgaA*, *sgaB*, and *sgaC*. The *sgaB* mutation was identified as an allele of *lrp* gene, encoding the leucine-responsive regulatory protein. The *lrp* gene encodes the transcription factor which regulates the expression of many genes participating in the metabolism and transport of amino acids, and it is induced during the transition into the stationary growth phase. The Lrp is a dimeric protein which can be both an activator and a repressor. Its activity in a cell is regulated by the amount of intracellular leucine. This protein regulates the metabolism of amino acids by increasing anabolism and reducing catabolism. It has been detected that in G2 there is a three-base-pair deletion 5'-GGA-3' in the *lrp* gene resulting in an emergence of a protein that has a glycine amino acid deletion at position 39 in the DNA-binding domain. The consequence of the mutation is the formation of a protein which bears a different conformation in the region responsible for DNA binding, making it unable to bind to DNA. This causes a non-activation of genes which are under the control of this protein. A mutation in this region allows for better fitness of GASP mutants in the starvation phase. Strains with GASP-conferring *lrp* mutations proliferate at the expense of less-fit cells, in part because such mutations enhance the ability to grow on certain amino acids. Another mutation, leading to the formation of GASP G2 mutants, occurs in the *sgaA* gene. This mutation has been identified as a genomic rearrangement which is the consequence of sequence insertion IS*5*, causing the activation of the *ybeJ-gltJKL-ybeK* operon and the inactivation of the *cstA* gene. The rearrangement takes place in two steps involving an insertion of IS*5* element, which is 103 bp upstream of the *ybeJ*. First, the new IS*5* element is transposed into the regulatory region of the *cstA* gene between the promoter and the Crp region. The *cstA* gene encodes an olygopeptide permease, and it is induced during stationary growth phase by a specific activation *via* CRP region. Second, a homologous recombination takes place between the inserted and an old IS*5* element resulting in an inversion in the bacterial genome. The consequence of this rearrangement is transposition of the *cstA* gene and the *ybeJ-gltJKL-ybeK* operon, which is the only mutation detected within the *sgaA* gene in GASP mutants. The activated *ybeJ-gltJKL-ybeK* operon increases the cell capacity for transport and growth on amino acids as a sole carbon source (*6*).

Consequently, mutations in *rpoS* and *lrp* genes make global shifts in metabolism and physiology, very often with a coordinated effect such as enhanced catabolism of multiple amino acids in G1 and G2 mutants. Therefore, while altering a single activity may increase fitness, altering many activities simultaneously by altering the function of a global regulator may result in an even higher fitness gain. Hence, beneficial mutations in global regulators may be selected over other mutations when bacteria are exposed to new environments. This hypothesis is supported by reports that clinical and soil isolates as well as laboratory cultures of *E. coli* (*75–77*) and *Salmonella enterica* (*78–80*) have extensive allele variation of *rpoS*, indicating that there is considerable selective pressure acting on the *rpoS* locus in the natural world environments.

The Coexistence of GASP Mutants

In the competition experiments bacterial populations are distinguished by using neutral chromosome markers for resistance to streptomycin (StrR) or nalidixic acid (NalR). Experiments to determine whether two initially isogenic populations of *E. coli* bacteria could evolve in different ways in a completely identical environment (*67*) were conducted. Two isogenic Str^R cultures, Str^R-1 and Str^R-2, and one Nal^R culture were cultivated for 30 days. After 30 days, the cells of old Nal^R culture were mixed separately with the old Str^R-1 and old Str^R-2 culture. The cells of Str^R-1 culture overtook the Nal^R culture very quickly, while the cells of Nal^R culture kept up fairly well with the Str^R-2 culture. This experiment has shown that two initially isogenic populations generate different mutations during 30 days of separated cultivation, which enables them to survive under unfavourable conditions.

In the culture going through a long-term stationary phase, there is a continuous selection and overtaking of novel GASP mutants. Experiments have shown not only that 10-day-old cultures outcompete the young culture cells, but also that cells isolated from a 20-day-old culture outcompete the cells of a 10-day-old culture, as well as that mutants from a 30-day-old culture show competitive advantage compared to the cells of a 20-day-old culture (*67*). These experiments have shown that in cultures incubated for a long period of time, there is a constant selection for ever more fitness of the mutants, and that these cultures are highly dynamic, regardless of the fact that their viable count seems constant.

Finkel and Kolter (*67*) observed the morphological variability of the colonies on the Luria-Bertani (LB) plates that were taken from a 150-day-old mixed culture. Different pattern was determined in morphologically different cultures during restriction fragment length polymorphism analysis. In the work by Bačun-Družina *et al.* (*71*) electrophoretic karyotypes of genomic DNA of 1- and 10-day-old wild-type *E. coli* cells were compared with

those resistant to nalidixic acid or streptomycin. Significant chromosomal rearrangements were determined within 10-day-old antibiotic-resistant *E. coli* mutants, whose colonies were not morphologically different.

Four Types of GASP Phenotype

During the growth of bacterial cells in prolonged stationary phase after the initial appearance of GASP alleles of *rpoS*, novel GASP mutations continuously appear. Four types of GASP phenotype have been observed in mixed bacterial cultures: strong, weak, moderate and abortive (Fig. 4; *81*,*82*). At strong GASP phenotype (Fig. 4, class 1), a significantly smaller cell number of the aged minority culture, compared to the young culture, remains unchanged only briefly. After a few days, the aged bacterial cell culture, which contains favourable mutations due to better adjustment to deprived conditions during long-term growth and starvation, overtakes the young culture. Old culture viable count is stable at about 10^6 CFU/mL, while the young culture cells gradually die out (Fig. 4, type 1). Defined weak and abortive GASP phenotypes are related to the mutants pol II, pol IV and pol V, which in their functional form code for SOS-induced DNA polymerases. Out of the five polymerases present in *E. coli*, three are induced as part of the SOS regulon in response to DNA damage (*83*). Polymerases V and IV, encoded by *umuDC* and *dinB* genes respectively, belong to the Y-family of polymerases, which copy DNA with low fidelity, *i.e.* error-prone polymerases (*84*), while 'error--free' polymerase II that copies DNA with high fidelity comes from the B-family of polymerases. In dividing cells, Pol V is responsible for generating substitution mutations targeted at DNA damage sites (*85*). Pol II has a key role in rescuing stalled replication forks on damaged DNA (*86*), but as opposed to pol V, pol II-catalysed replication restart occurs without any measurable increase in mutational load. Pol IV is primarily involved in generating simple, nontargeted frameshift mutations (*87*), and in that way it helps in rescuing replication forks stalled on the undamaged DNA. Error-prone SOS polymerases play a significant role in creating genetic variability, which will enable the selection of favourable mutations under the stressful conditions. Yeiser *et al.* (*81*) noticed that *polB*, *dinB* and *umuDC* genes transcribe during stationary phase in the absence of extracellular DNA-damaging agents, which means that these polymerases are highly important for a long-term survival, because they take part not only in damage repair but also in creating genetic diversity in the stationary phase. Experiments in which *pol* mutants are mixed with the wild type as well as with

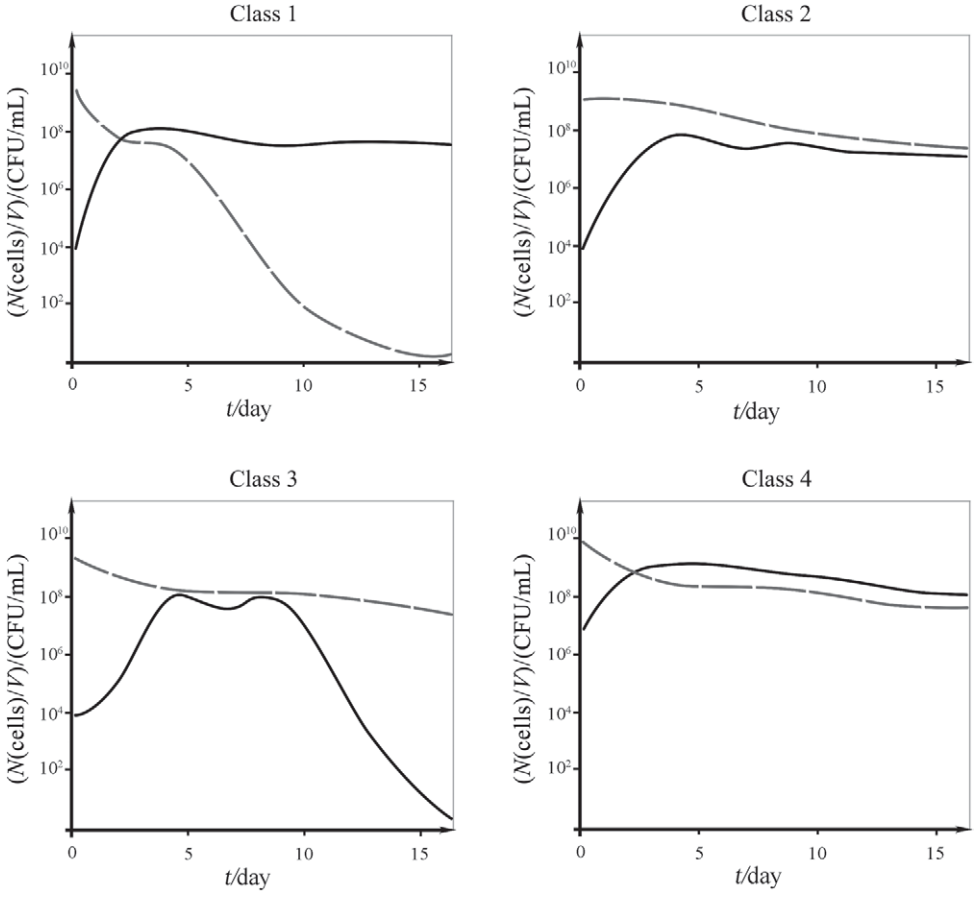

Fig. 4. Four classes of GASP phenotype: class 1 – strong phenotype, class 2 – weak phenotype, class 3 – abortive phenotype, class 4 – moderate phenotype. When cells from a 10-day-old bacterial culture (full line) at the beginning as minority competed with 1-day--old bacterial cells (broken line) as the majority, one of the presented GASP phenotype classes can appear. Growth curve of a batch bacterial culture in the plot of time *vs.* *N*(cells)/*V*. Scheme is modified from Yeiser *et al.* (*81*) and Bačun-Družina *et al.* (*82*)

each other have shown that SOS-*pol* mutants do not have strong GASP phenotype compared to their wild-type parent, but they almost always show strong GASP phenotype compared to other *pol* mutants, with the exception of the aged *pol V* mutant culture that did not acquire strong GASP phenotype in the culture with young *pol IV* mutant culture. Weak GASP phenotype is characterized by the ability of old cells to increase their number in the competitive growth, and to maintain equal number of old and young cells (10^6 CFU/mL) in the culture during 14-day growth (Fig. 4, class 2). During abortive GASP phenotype (Fig. 4, class 3), aged population cells show growth advantage over the young cells at the beginning, and their number in the population increases for a short period of time, after which they are completely eliminated by the parent cells. Moderate type of GASP phenotype (Fig. 4, class 4) was discovered while monitoring competitive growth of the 10-day-old enterobacteria and 1-day-old *Lactobacillus brevis* cells. After a few days, aged cells in the mixed culture slightly overtook the young *Lactobacillus* cells and their count of about 10^8 CFU/mL remained stable during 25 days (*82*).

Evolutionary Dynamics of GASP Mutants

Among others in evolutionary microbiology, a branch of mathematics named game theory is applied, which uses various models to study interactions with formalized driving force structures (games). Two defined game theories, Prisoner's dilemma and Chicken game, have been used to interpret the appearance of GASP mutants in specific period of starvation during prolonged stationary phase. Cells stopping their growth and division, and also showing a significantly reduced metabolic activity and increased stress resistance are the main characteristics of the bacterial batch culture that has just entered stationary phase. As previously mentioned, the activity of RpoS protein is controlled by collaboration of numerous intracellular and extracellular signals, excreted by the cell, that are highly important modulators of the expression of genes responsible for the response of the population to the environmental conditions. The principal factors in the induction of stationary phase are the amount of nutrients and the number of cells that use them. By coordinating extracellular signals of these two factors, the cells can respond as a population to the changed environmental conditions and accomplish a coordinated transition into stationary phase. However, as any system in nature depending on the coordination, this transition is vulnerable to the activity of the cheaters, who do not perform a typical response like the rest of the population, and profit henceforth. It was suggested that the appearance of GASP mutants could be described by the evolutionary game theory called Prisoner's dilemma (*88*). GASP mutants are capable of reinitiating their growth by scavenging nutrients released by the dying cells, which can be considered 'cheating'. These mutants-cheaters ignore the signals that keep the wild-type population in the nondividing state and they profit by growing, either by not inducing the starvation response, or they resume vegetative growth by reversing this response prematurely. Since σ^S subunit is the master regulator of entering the stationary phase, it is to be expected that the majority of mutants, which have the ability to ignore the stationary phase, will be the mutants in the gene of that global regulator. The results of Vulić and Kolter (*88*) show that the survival advantage of the GASP mutants, which is reflected in its overall increased number of viable counts, is the greatest when it is rare in the wild-type population and decreases when it becomes more abundant. The death rate of the wild-type population is increased with the higher number of the GASP mutants present, which means that the presence and growth of GASP subpopulation has a negative effect on the survival of the wild type. These results show that the strategy of the GASP mutants, within the Prisoner's dilemma model game, is seen as cheating the cooperative wild-type cells. Even though the increased GASP mutant frequency has a negative effect on its survival advantage, GASP mutant overtakes the entire population; this is also because in any ratio its survival ability is greater than that of the wild type. In other words, from the point of view of the GASP mutant, cheating pays off. Prisoner's dilemma model has been used with various biological phenomena. Because of its simplicity, Prisoner's dilemma is used as a measurement of fitness (*89–91*). Vulić and Kolter (*88*) succeeded in applying this model for description of GASP phenomenon. It is important to mention that later GASP mutant takeovers are incomplete, unlike the early ones. Therefore, it has been suggested that, as opposed to the first dynamic appearance of GASP mutants which can be described as Prisoner's dilemma, later GASP mutant takeovers should be described with another game theory known as the Chicken game (*92*). In the latter case, the damage *vs*. advantage ratio is such that it prevents the cheater from taking the culture over completely, resulting in a mixed population. Numerous natural bacterial isolates, which possess variations in *rpoS* gene, suggest that the above described population takeovers are quite common in nature. However, the question here is how *rpoS* gene, which is prone to changes and elimination by GASP mutants, managed to survive the evolution as a functional gene. The answer is simple, since the regular *rpoS* gene is obtained and renewed by gene horizontal transfers. Three different ways provide bacteria with the access to foreign genetic material: transformation, conjugation and transduction occurring under particular conditions in every bacterium. The presence of cheaters in the bacterial population leads to the assumption that the existence of such mutants in populations of nondividing cells is highly probable, *e.g.* in multicellular organisms cancerous cells can be considered as cheaters that reinitiate growth (*93*).

Conclusion

Studies of cell traits and various stress responses in bacterial cultures during prolonged starvation in stationary phase have given a new insight into bacterial interactions and the evolution of cell diversity in chemostat, in batch, and in natural populations. In any of these environments, innate random cell divergence occurs constantly. Excellent example are GASP mutants with increased fitness which appear in waves that can sweep through the bacterial culture as it ages (Fig. 1) (*70,74,94*). Endogenous molecular mechanisms, such as SOS response

and down-regulation of enzymes (because of deletions in genes *mutS* or *mutL*), which are responsible for mismatch repair, elevate mutation rates leading to hypermutable state and may result in stress-induced mutagenesis in bacteria (for review see *12*,*95*,*96*). Resulting mutants in bacterial population are able to change rapidly and persistently over many generations under any selective environment conditions such as nutritional deprivation, temperature shift, DNA damage, and exposure to antibiotics. Furthermore, other endogenous mechanisms may enhance gene diversity through movement of insertion sequences and transposable elements, as well as chromosomal rearrangements such as insertion, deletion, duplication and amplification. Many of the mentioned molecular mechanisms keep advantage for accelerated production of GASP mutants during prolonged starvation. It is important to note that the knowledge of GASP phenomenon serves as a valuable model to understand the interaction and dynamics of bacterial population as well as the incurrence of adaptive mutations. Such learning may give more insight into elucidation of bacterial pathogenicity, increased antibiotic resistance, spontaneous adaptation of remediation bacteria, persistence of food contaminants, biofilm formation, quorum sensing, and aging.

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