REVIEW ARTICLE

Stationary phase in gram-negative bacteria

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Abstract

Conditions that sustain constant bacterial growth are seldom found in nature. Oligotrophic environments and competition among microorganisms force bacteria to be able to adapt quickly to rough and changing situations. A particular lifestyle composed of continuous cycles of growth and starvation is commonly referred to as feast and famine. Bacteria have developed many different mechanisms to survive in nutrient-depleted and harsh environments, varying from producing a more resistant vegetative cell to complex developmental programmes. As a consequence of prolonged starvation, certain bacterial species enter a dynamic nonproliferative state in which continuous cycles of growth and death occur until 'better times' come (restoration of favourable growth conditions). In the laboratory, microbiologists approach famine situations using batch culture conditions. The entrance to the stationary phase is a very regulated process governed by the alternative sigma factor RpoS. Induction of RpoS changes the gene expression pattern, aiming to produce a more resistant cell. The study of stationary phase revealed very interesting phenomena such as the growth advantage in stationary phase phenotype. This review focuses on some of the interesting responses of gram-negative bacteria when they enter the fascinating world of stationary phase.

Introduction

Many environments in the biosphere are oligotrophic, setting the stage for evolution in conditions of near-starvation or fluctuating nutrient availability (Morita, 1997). Consider for example the oceans, wherein the average organic carbon content varies from $1\,mg\,L^{-1}$ in surface waters to $0.5\,mg\,L^{-1}$ in the deep sea (Kurath & Morita, 1983), extremely low concentrations by comparison with the $10\,g\,L^{-1}$ content of typical rich media used in laboratories. When nutrient concentrations are insufficient to sustain the requirements for steady growth, bacteria, which have an extraordinary ability to endure in the absence of nutrients, confront such demanding conditions by entering a state named stationary phase. As low nutrients and harsh conditions are prevalent in natural environments, the exhaustion of resources commonly forces bacteria to remain in stationary phase (Kolter *et al.*, 1993). In fact, it is estimated that 60% of the earth's biomass is composed of resting microorganisms (Gray *et al.*, 2004). Starvation survival is defined as the ability to withstand long periods without energy-yielding substrates. Bacteria have adapted different subsistence strategies to keep cells viable for long periods of time. Many species of gram-positive bacteria produce dormant spores in response to starvation. By contrast, many gram-negative bacteria develop resistance cells without dormancy. In both cases, if nutrients become available again, cells will resume growth until exhaustion of nutrients, entering again in a stationary phase period. The continuous alternation of growth and nongrowth cycles has been linked to a feast and famine lifestyle (Almiron *et al.*, 1992; Kolter *et al.*, 1993).

In this review bacterial growth is considered an increase in cell number in a population, which occurs through cell growth and division. Plotting the viability (measured as $CFU\,mL^{-1}$) for several days of an *Escherichia coli* bacteria growing in optimal laboratory conditions (rich media, 37 °C and agitation) reveals a characteristic growth pattern comprising five phases (Fig. 1) (Finkel, 2006). Even though some minor aspects (length of the lag phase, time needed to reach stationary phase and the total number of cells in the population) of the chart represented in Fig. 1 may vary depending on several factors, such as the bacterial species or the growth conditions, the general tendency is similar. When cells enter a new habitat and face different nutritional

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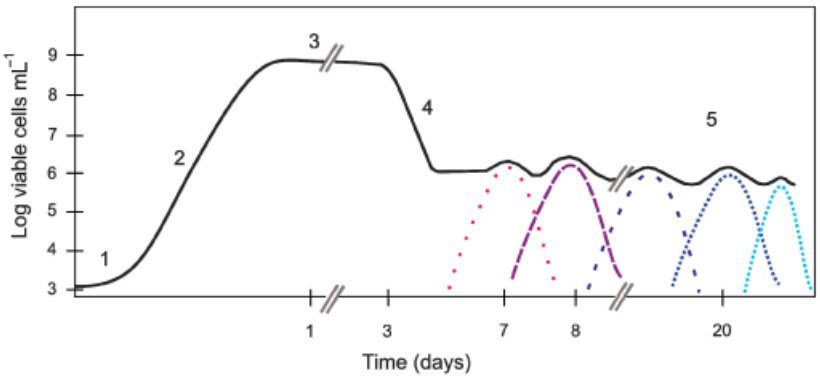


Fig. 1. Stages of bacterial growth. *Escherichia coli* cells growing in optimal laboratory conditions, rich media, 37 °C with aeration. 1, lag phase; 2, exponential phase; 3, stationary phase; 4, death phase; 5, long-term stationary phase. Dotted coloured lines represent the continuous growth and taking over of the different mutants that appear within a deep stationary phase population (GASP cycles) (Zambrano & Kolter, 1996; Finkel, 2006). Figure adapted by permission from Macmillan Publishers Ltd, from Finkel (2006), copyright 2006. http://www.nature.com/nrmicro

conditions they experiment with what is known as *lag phase* (phase 1). This phase is characterized by a metabolic reprogramming of the cell that enables it to thrive in the current environment, and the length of the lag phase is determined by several factors, such as the bacterial species, the shifting environmental parameter, and also the length of time that the cells have undergone in starved conditions before the new settings (Pin & Baranyi, 2008). Once cells are adapted to the new settings they start to grow and divide exponentially, a state known as *exponential phase* (phase 2). Cells divide asexually by binary fission at a constant rate. The growth rate of a bacterial population is the number of doublings per hour. This rate varies depending on the milieu conditions, being slower in nutrient-poor conditions and faster in nutrient-rich ones. For example: *E. coli* cells growing at 37 °C in a nutrient-rich medium divide every 20 min. As exponentially growing bacteria exhaust nutrient sources, cells enter into *stationary phase* (phase 3) in which no increase in cell number is observed. In gram-negative bacteria the starvation response triggers the alternative sigma factor RpoS, which controls up to 10% of the *E. coli* genes, genes that prepare the cell for survival in crude settings (Lacour & Landini, 2004; Weber *et al.*, 2005). Stress cross-protection often occurs; for example, the bacterial starvation response also provides protection against osmotic stress (Jenkins *et al.*, 1990) and temperature stress (Givskov *et al.*, 1994). Nutrient-depleted stationary-phase cultures accumulate waste products as a consequence of bacterial metabolism, which eventually leads to a *death phase* (phase 4), where the number of viable cells declines exponentially, reversing the pattern of growth during exponential phase. *Escherichia coli* cells enter death phase in Luria–Bertani (LB) media after approximately 3 days of continuous incubation (Finkel, 2006). Stochastic death or programmed cell death (PCD) has been postulated to be responsible for this lost of viability, but this has not been corroborated for either. In this phase, 90–99% of the population dies. As the majority of the cells in the population die, they release nutrients to the exhausted media that can be exploited by survivors. Viability can then remain constant for months or even years in what has been called *long-term stationary phase* (phase 5) (Finkel, 2006). Quite surprisingly, it has been shown that this prolonged stationary phase is a cryptic dynamic period (Zambrano & Kolter, 1996). Successive rounds of growth advantage in stationary phase (GASP) phenotypes, mutants with a better fitness to scavenge for nutrients than the parental strain, appear within the bacterial population, derived from multiple population shifts within the same culture. However, the balance between dying and growing cells provides a dynamic equilibrium where the final output is the stable viability of the population.

It is important to bear in mind that these five phases of bacterial growth have been defined from laboratory batch cultures using a model organism. The time microorganisms spend in any of these five phases in nature is not known, although this will probably depend on the species and on the characteristics of the ecosystem. Having said that, this laboratory approach has proved an excellent system that has helped the scientific community to broaden the view of bacterial physiology and to realize that the stationary phase is in fact a very interesting period of bacterial life.

To obtain a global view of how gram-negative bacteria endure famine in nature we have structured this review following a similar pattern of the growth curve depicted in Fig. 1 (Finkel, 2006). We start with the gene regulation mechanisms that control the onset of stationary phase (growth stage 3). Then, we consider the physiological adaptations that take place within cells to cope with nutrient limitation, followed by the death phase (growth stage 4) and the interesting phenomena associated to late stationary-phase cultures (growth stage 5): GASP phenotype, viable but nonculturable state (VBNC) and stationary phase

contact-dependent inhibition (SCDI). Finally, we finish by analysing different processes by which genetic diversity is generated in stationary-phase cells.

Different levels of regulation at the onset of stationary phase

To ensure survival under changing conditions, bacteria have evolved signalling cascades to regulate gene expression (Cases *et al.*, 2003). Adaptive responses include a series of genetic switches that control many metabolic changes. The entrance in stationary phase is a very well-regulated process with a sigma factor and many regulators involved. Environmental signals, through different mechanisms, feed into the action of transcriptional regulators and sigma factors that direct the activity of RNA polymerase towards gene expression. Most of the regulatory mechanisms of gene expression are complex and involve many regulatory links. A good example of this complexity is the regulation of the stationary-phase sigma factor RpoS, which governs both entrance into stationary phase and stress resistance. A general description of sigma factors is given below, followed by a brief description of the action of transcriptional regulators, sRNAs, proteases, and the phenomenon known as stringent response, which all together coordinate entrance into stationary phase.

Sigma factors

The RNA polymerase holoenzyme is formed by a multisubunit core ($\alpha_2\beta\beta'\omega$) complexed with a σ subunit (Mooney *et al.*, 2005). The σ component plays a crucial role in promoter recognition and transcription initiation (Ishihama, 2000). Therefore, a direct way to dramatically switch the expression profile is by replacement of the sigma subunit in RNA polymerase. In *E. coli*, most housekeeping gene promoters are recognized by σ^{70}, whereas promoters of specific regulons, stationary phase and stress response are recognized by alternative sigma factors (Gruber & Gross, 2003).

Sigma factors, based on structural and functional criteria, can be clustered within the σ^{70}-family or the σ^{54}-family. A precise description of the differences between these two families can be found elsewhere (Paget & Helmann, 2003). The σ^{70} family is further divided into various groups. *Group 1*, including σ^{70} itself, comprises sigma factors essential for cell growth, whereas *group 2* includes sigma factors closely related to σ^{70} but not essential for bacterial growth. *Group 3* sigma factors control heat shock response, flagellar biosynthesis and sporulation. Members of *group 4* comprise extracytoplasmic function, which is involved in protection from extracellular stresses (Paget & Helmann, 2003). The number of different sigma factors ranges from one type in *Mycoplasma* sp. and seven in *E. coli*, to 63 in *Streptomyces coelicolor*. The presence of a larger number of sigma factors in a genome is generally correlated with a more complex lifestyle of the organism, suggesting that sigma factors are a key to adaptive capacity and bacterial development (Cases *et al.*, 2003; Gruber & Gross, 2003).

Within the cytoplasm, different sigma subunits compete for binding a limited available amount of RNA polymerase core enzyme (Ishihama, 2000). Shifting patterns of sigma factor gene expression fine-tune the relative concentrations of the different sigma factors in the cytoplasm. Accordingly, the onset of stationary phase is marked by alterations in the relative abundance of specific sigma factors (Table 1). In gram-negative bacteria the two principal sigma factors responsible for stationary-phase survival and damage protection are σ^S (the product of the *rpoS* gene) and σ^{32} (encoded by *rpoH*) (Fredriksson & Nystrom, 2006).

σ^{32} is a sigma factor with an important role in bacterial survival during growth arrest (Fredriksson & Nystrom, 2006). It induces the expression of the heat shock regulon, which increases the production of chaperones and proteases to deal with the oxidative damage caused by the presence of aberrant proteins (carbonylated) (Fredriksson *et al.*, 2005).

σ^S is included within group 2 of the σ^{70}-family (Gross *et al.*, 1992). The *E. coli rpoS* gene was known for a long time (Loewen & Triggs, 1984) before it was described as a sigma factor-coding gene and named as such (Lange & Hengge-

Table 1. Variation of different sigma factors in stationary phase

Sigma factor	Main function	Variation in stationary phase	References
RpoD (σ^{70})	Housekeeping	No variation in Ec Threefold decreased in Ps	Yuste *et al.* (2006)
RpoF (σ^{28})	Synthesis of *Flagella* and chemotaxis	No variation in Ps and Ec	Yuste *et al.* (2006)
RpoN (σ^{54})	Nitrogen metabolism motility	No variation in Ps or Ec	Yuste *et al.* (2006)
RpoH (σ^{32})	Heat shock	No variation in Ps or Ec	Yuste *et al.* (2006)
RpoE (σ^{24})	Extra cytoplasmic stress	Fivefold increase in Ec	Costanzo & Ades (2006)
RpoS (σ^{38})	Stationary phase and stress resistance	Three- to fourfold increase in Ps and Ec	Yuste *et al.* (2006)
ECF sigma factors	Extracellular functions	Decrease in Ec No variation in Ps	Maeda *et al.* (2000) Yuste *et al.* (2006)

Ec, *Escherichia coli*; Ps, *Pseudomonas putida*; ECF, extracytoplasmic function.

Aronis, 1991b). Later it was described in other enteric bacteria species (Martinez-Garcia *et al.*, 2001). Although it is expressed during exponential growth, its concentration significantly increases at the onset of stationary phase or under stress conditions (Lange & Hengge-Aronis, 1994; Hirsch & Elliott, 2005).

Studies of RpoS regulation have revealed a very complex mechanism, extending to transcription, translation and post-translational controls, all tightly coordinated in response to several stress signals (Hengge-Aronis, 2002). Among these stress conditions, low levels of carbon, nitrogen or phosphorus, as well as amino acid starvation, trigger RpoS synthesis (Gentry *et al.*, 1993; Hengge, 2008). Increased RpoS levels during stationary phase are due to stronger *rpoS* transcription (Hengge-Aronis, 2002), better efficiency of translation (Majdalani *et al.*, 2001) and an increase in protein stability (Lange & Hengge-Aronis, 1994). Figure 2 shows a detailed scheme with all the stages and molecules involved in the regulation of RpoS. An extensive review on the signal transduction and regulatory mechanisms involved in the control of RpoS can be found elsewhere (Hengge-Aronis, 2002; Hirsch & Elliott, 2005; Hengge, 2008).

The overarching role of RpoS production is to make stationary cells more adaptable and resistant to challenging situations. RpoS is responsible for the expression of 10% of *E. coli* genes (Weber *et al.*, 2005).

Regulators

The leucine-responsive regulatory protein (Lrp) is a dimeric protein that acts as global transcriptional regulator whose expression level is inversely related to the growth rate. It is positively regulated by ppGpp (Landgraf *et al.*, 1996). In some cases, the activity of Lrp can be modulated, positively or negatively, by leucine (Calvo & Matthews, 1994). Lrp is broadly conserved in bacteria and can be either a repressor or an activator depending on the promoter sequence of the regulated gene (Zinser & Kolter, 2000). Lrp affects more than 400 *E. coli* genes and nearly three-quarters of the stationary-phase induced genes, including those involved in response to nutrient limitation, high concentrations of organic acids, and osmotic stress (Tani *et al.*, 2002). In general, the purpose of Lrp activity is to coordinate the cellular metabolism with the nutritional environmental state (Landgraf *et al.*, 1996). Specifically, Lrp increases anabolism of amino acids while decreasing catabolism (Zinser & Kolter, 2000). In fact, during transition to stationary phase, some of the induced proteins prepare the cell to mobilize internal nutrient reserves and to metabolize fermentation products (Tani *et al.*, 2002). An *lrp* mutation affecting the DNA-binding domain of the regulator confers the GASP, partly because such mutations enhance the ability of that strain to grow faster on certain amino acids (Zinser & Kolter, 2000).

The integration host factor, commonly known as IHF, is a histone-like protein that contributes to genome organization. It binds conserved DNA sequences, producing up to 180° DNA bending, thus facilitating distal interactions and as a consequence gene expression (Mangan *et al.*, 2006). IHF shows a growth phase-dependent concentration, increasing at the onset of stationary phase (Ditto *et al.*, 1994). IHF contributes to the regulation of some *E. coli* stationary phase genes, such as curli-producing genes (Gerstel *et al.*, 2003), *dps* (Altuvia *et al.*, 1994) and *osmY* (Colland *et al.*, 2000). IHF also seems to be important for the expression of virulence genes during stationary phase in *Salmonella enterica* serovar Typhimurium (Mangan *et al.*, 2006).

In addition, small noncoding RNAs (sRNAs) have been observed to have implications for the regulation of stationary phase (Gottesman, 2005). sRNAs modulate translation and stability of specific target mRNAs. More than 60 species of sRNAs have been identified in *E. coli* so far, some of them involved in regulation of stress response (Gottesman, 2005). sRNAs are often between 80 and 100 nucleotides in length and some require the binding of the bacterial RNA chaperone Hfq (Gottesman, 2005). Hfq binds single-stranded AU-rich regions and can stabilize sRNA molecules as well as stimulate formation of sRNA–mRNA pairs, producing an inhibitory effect on either its translation or in the stability of the target mRNA. Figure 2 shows the effect of sRNAs on the expression of the stationary phase sigma factor RpoS. The small RNAs DsrA and RprA stimulate RpoS translation (Majdalani *et al.*, 1998, 2001). Under conditions without stress, the 5′-UTR region of *rpoS* mRNA folds, blocking its ribosome-binding site. DsrA and RprA bind the same 5′-leader region, unfolding the mRNA in a way that ribosomes can access it, therefore activating its translation. Both sRNAs are induced under different conditions: DsrA I is induced at low temperatures (Sledjeski *et al.*, 1996), whereas RprA expression depends on the Rcs phosphorelay system (RcsC–RcsD–RcsB) (Majdalani & Gottesman, 2005). The actual signal that activates the RcsC sensor protein is still unknown (Majdalani & Gottesman, 2005). In contrast, the sRNA OxyS, expressed in response to oxidative stress, represses RpoS translation by sequestering the RNA chaperone Hfq (Zhang *et al.*, 1998).

Other examples of sRNA regulators acting in stationary phase are *micA* and *rybB*, both involved in outer membrane permeability (Johansen *et al.*, 2006). The outer membrane provides the first cellular contact with the environment and requires an accurate control of its composition for cell survival. Both *micA* and *rybB* are σ^E-dependent and together with Hfq are proposed to cause antisense RNA-inhibiting translation. *rybB* sRNA controls the production of two outer membrane proteins, OmpC and OmpW, whereas *micA* sRNA triggers the mRNA decay of the outer membrane protein OmpA (Guillier *et al.*, 2006; Johansen *et al.*, 2006).

Another step in stationary phase regulation is provided by macromolecule turnover. In fact, proteolysis of regulators and sigma factors is an important mechanism in the control of gene expression. Clp ATP-dependent protease is crucial in the post-translational regulation of RpoS (Zhou & Gottesman, 1998). It is composed of two components, ClpP, a serine-protease with proteolytic activity, and an ATPase, which confers substrate specificity (Butler *et al.*, 2006). The ClpP subunit is a highly conserved protein among prokaryotes and eukaryotes (Maurizi *et al.*, 1990). The regulator subunits ClpA and ClpX belong to the Clp/Hsp100 family of ATPases and both associate with ClpP in gram-negative bacteria (Butler *et al.*, 2006). Among multiple cellular functions, ClpXP degrades RpoS in growing cells (Fig. 2) (Zhou & Gottesman, 1998).

The stringent response

Cells respond to amino acid starvation by downregulating rRNA biosynthesis, ribosomal proteins and DNA replication, and upregulating the levels of RpoS, stress protein and amino acid biosynthesis (Magnusson *et al.*, 2005). This phenomenon is known as the stringent response and it is mediated by the accumulation of hyperphosphorylated

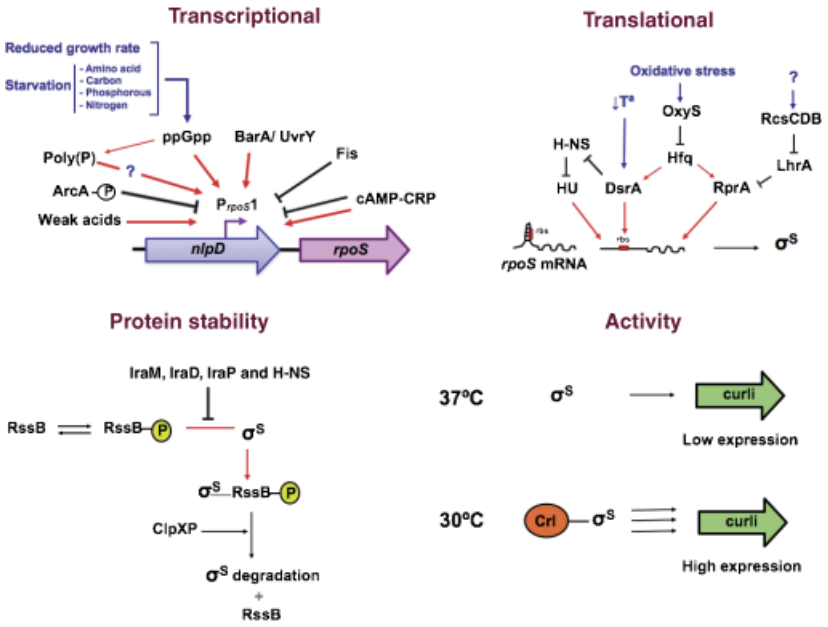


Fig. 2. Regulation of RpoS in *Escherichia coli*. (1) Transcriptional. Several regulators have been described to affect *rpoS* transcription from the stationary promoter (P*rpoS*1), which lies within the *nlpD* gene in *E. coli* (Lange *et al.*, 1995). The alarmone ppGpp induces *rpoS* expression under several starvation conditions (Hengge-Aronis, 2002). The membrane sensor kinase BarA modulates *rpoS* transcription during exponential phase (Hengge-Aronis, 2002). Early experiments showed that weak acids, such as benzoate, acetate or propionate, influence *rpoS* expression (Schellhorn & Stones, 1992; Hengge-Aronis, 2002). The function of cAMP-CRP seems to be growth phase-dependent, repressing *rpoS* transcription in exponential growth while activating it in stationary phase (Hengge-Aronis, 2002). It has been shown that inorganic polyphosphate [poly(P)] somehow stimulates *rpoS* expression (Shiba *et al.*, 1997). Fis represses *rpoS* transcription during exponential phase. However, Fis concentration is greatly reduced in stationary phase (Hirsch & Elliott, 2005). Phosphorylated ArcA represses *rpoS* transcription. The Arc system (ArcB–ArcA–RssB) allows cells to integrate the levels of oxygen and energy supplies together through the autophosphorylation of the sensor kinase ArcB, which phosphorylates RssB and ArcA (Mika & Hengge, 2005). (2) Translational. DsrA, a small regulatory RNA that requires the chaperone protein Hfq, positively regulates translation of RpoS at low temperature in rich medium ($\leq$ 30 °C) by unfolding a hairpin bend in the 5′-UTR region of the *rpoS* mRNA (Sledjeski *et al.*, 1996). DsrA negatively regulates translation of H-NS, which represses RpoS synthesis (Lease *et al.*, 2004). The Rcs phosphorelay can activate RpoS translation in two ways: (i) by direct stimulation of the sRNA RprA (Majdalani *et al.*, 2002) and (ii) by repression of the RprA repressor LrhA (Peterson *et al.*, 2006). Moreover, OxyS sRNA negatively regulates *rpoS* mRNA translation by sequestering Hfq or by forming a translationally incompetent ternary complex with Hfq and *rpoS* mRNA (Zhang *et al.*, 1998). *rpoS* mRNA translation initiation is positively controlled by HU (Balandina *et al.*, 2001). (3) Protein stability. RpoS is highly unstable in growing cells, whereas its stability increases dramatically upon starvation or under stress conditions. Degradation of RpoS is controlled by the phosphoryl acceptor protein RssB, and by the protease ClpXP (Zhou *et al.*, 2001) under carbon and/or energy limitation (Mandel & Silhavy, 2005). Small antiadaptor proteins (IraP, IraM and IraD) stabilize RpoS during different stress conditions by interaction with RssB: IraP stabilizes under phosphate starvation, and IraM inhibits RssB in low magnesium concentrations, and IraD after DNA damage (Bougdour *et al.*, 2006, 2008; Merrikh *et al.*, 2009). Moreover, H-NS seems to regulate an RssB inhibitor (Zhou & Gottesman, 2006). (4) Activity. In *E. coli* the small regulatory protein Crl increases the activity of RpoS (Pratt & Silhavy, 1998). Crl accumulates in stationary phase cells at 30 °C but not at 37 °C (Bougdour *et al.*, 2004). Crl in coordination with the RNA polymerase with RpoS specifically regulates the expression of at least 40 genes in deep stationary phase at 30 °C (Lelong *et al.*, 2007), being one of the curli operon *csgBA* (Pratt & Silhavy, 1998). Activity figure modified from Bougdour *et al.* (2004). $\rightarrow$, activation; $\top$, inhibition.

guanine nucleotides, guanosine 3′,5′-bispyrophosphate, abbreviated as *ppGpp* (Cashel *et al.*, 1996). ppGpp is a key factor in bacterial physiology because it responds rapidly to diverse stresses, shutting down growth and priming cellular defensive and adaptive processes (Magnusson *et al.*, 2005; Srivatsan & Wang, 2008). Proteins RelA and SpoT are both in charge of adjusting ppGpp concentration within the cell (Cashel *et al.*, 1996). RelA (ppGpp synthetase I) only synthesizes ppGpp, whereas SpoT can either produce ppGpp (ppGpp synthetase II) or degrade it (ppGpp hydrolase) (Gentry & Cashel, 1996). RelA and SpoT are induced by different environmental settings. Whereas RelA senses amino acid starvation (Cashel *et al.*, 1996), SpoT recognizes carbon (Xiao *et al.*, 1991), phosphorus (Spira *et al.*, 1995), iron (Vinella *et al.*, 2005) and fatty acid scarcity (Seyfzadeh *et al.*, 1993).

RelA works in association with ribosomes and is activated by the presence of uncharged tRNAs in the ribosomal A site. Once activated, it catalyses the transfer of pyrophosphate from ATP to GTP/GDP to synthesize ppGpp (Wendrich *et al.*, 2002).

In the case of SpoT, RelA maintains a basal hydrolase activity under favourable growth conditions, promoting a rapid turnover of ppGpp (Murray & Bremer, 1996). In contrast, carbon, phosphate, iron and fatty acid starvation conditions inhibit the hydrolase activity and induce the synthetase activity. An appealing mechanism shows that SpoT is associated to an acyl carrier protein to sense fatty acid levels (and hypothesizes that might also be responsible for sensing carbon levels); when these are low, it triggers ppGpp synthesis (Battesti & Bouveret, 2006).

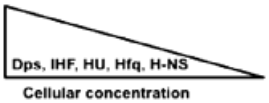
ppGpp binds the β-subunits of RNA polymerase, affecting promoter specificity and thus altering the expression of more than 80 different genes (Barker *et al.*, 2001a, b; Chang *et al.*, 2002). In addition, ppGpp together with the anti-sigma factor Rsd helps σ^S to out-compete σ^{70} for RNA polymerase binding, thus causing a decay in the expression of the σ^{70}-dependent genes (Jishage *et al.*, 2002). When environmental conditions become favourable, the ppGpp levels decrease and the stringent response is reversed. The stringent response has been shown to play a significant role in processes as different as biofilm, quorum sensing (QS) in *Pseudomonas aeruginosa* (van Delden *et al.*, 2001), antibiotic production in *Streptomyces antibioticus* (Hoyt & Jones, 1999), virulence regulation in *Legionella pneumophila* (Bachman & Swanson, 2001), and the intrinsic antibiotic resistance in *E. coli* (Greenway & England, 1999).

Adaptations to stationary phase entrance: making a resistant cell

Stationary phase entry is characterized by the accumulation of RpoS (Hengge-Aronis, 2002). This alternative sigma factor affects, directly or indirectly, the expression of 10% of the *E. coli* genes (Lacour & Landini, 2004; Weber *et al.*, 2005). Therefore, RpoS influences the entire cellular physiology. Analysis of RpoS-dependent genes revealed a consensus extended -10 promoter sequence of 5′-TCTA TACTTAA-3′ (Weber *et al.*, 2005). Briefly, genes that belong to the RpoS regulon are involved in morphological changes of the cell, resistance to various stress conditions (e.g. oxidative stress, heat shock, osmotic stress, near-UV irradiation or pH changes), metabolic processes, virulence and the GASP phenotype (Hengge-Aronis, 1996; Martinez-Garcia *et al.*, 2001; Raiger-Iustman & Ruiz, 2008). Table 2 presents a summary of the most relevant changes produced in a cell during stationary phase.

In addition, bacteria in stationary phase undergo morphological adaptations. Cells are smaller as the result of two processes, reductive division and dwarfing (Nystrom, 2004). Reductive division increases the surface/volume ratio, producing spherical cells (Nystrom, 2004). Also, the expression of the transcriptional regulator BolA, which controls the

Table 2. Major changes observed during stationary phase, death phase and long-term stationary phase. See text for details

	Cellular changes in stationary phase
Morphological	Smaller and spherical cells
	More resistant and rigid cell envelope
Nucleoid	Condensation of the nucleoid as certain histone-like proteins increase their concentration
	
Metabolic	Stringent response
	Repression of aerobic metabolism
	Increase fermentative enzymes expression
	Production of RMF (ribosome modulating factor)
	Drop in protein synthesis while increase peptidases/proteases synthesis
Transcriptional	Change of sigma factors affinity: σ^S, σ^E
	Adjustments of global regulators:
	Lrp
	IHF
	sRNAs
Translational	100S ribosome dimers (inactive)
	Decrease protein synthesis
	Increase proteases and peptidases synthesis
Others	Increased resistance against physical and chemical stresses
	Synthesis of quorum sensing molecules
	Production of secondary metabolites
	Programmed cell death (PCD)
	GASP phenotype
	Mutator phenotype
	Viable but nonculturable (VBNC) state
	Stationary phase contact-dependent inhibition (SCDI)

penicillin-binding proteins PBP5 and PBP6, and the β-lactamase AmpC are partly responsible for this coccoid morphology (Santos *et al.*, 2002). The expression of *bolA* in *E. coli* is mainly controlled by RpoS (Lange & Hengge-Aronis, 1991a), but it can also be induced by several types of stress (Santos *et al.*, 1999), although the concrete roles of BolA may differ in different bacterial species (Koch & Nybroe, 2006).

Dwarfing is a form of self-digestion and is the result of degradation of endogenous cell material, especially from the cytoplasmic and the outer membranes (Nystrom, 2004).

A hallmark of stationary phase adaptation is the production of cell envelopes for an effective protection of the cells against different assaults (Huisman *et al.*, 1996). The transformation to an enhanced barrier includes extensive changes in all structures of the cell envelope: outer membrane, periplasm, peptidoglycan and the inner membrane (Huisman *et al.*, 1996).

The concentration of lipopolysaccharides increases in the exterior surface of the outer membrane. There is also a reduction in the amount of protein in the outer membrane (Allen & Scott, 1979), together with an increased cross-linking of the outer membrane lipoproteins with the peptidoglycan layer (Huisman *et al.*, 1996). The periplasm accumulates membrane-derived oligosaccharides, such as trehalose, which function as osmoprotectants (Huisman *et al.*, 1996). The peptidoglycan layer (a strong and elastic polymer that serves as the stress-bearing component of the bacterial cell wall) increases in thickness (Mengin-Lecreulx & van Heijenoort, 1985). Recently, Lam *et al.* (2009) described the synthesis of D-amino acids during stationary phase and their role in modifying the peptidoglycan layer, by their incorporation into the peptidoglycan polymer and repression of the peptidoglycan synthesis.

The inner membrane undergoes multiple changes. A decrease in monounsaturated fatty acids is accompanied by an increase of polyunsaturated fatty acids (Huisman *et al.*, 1996). Also, unsaturated fatty acids are converted into cyclopropyl derivatives, and the ratio between phosphatidylglycerol and phosphatidylethanolamine increases as cells approach stationary phase (Cronan, 1968; Huisman *et al.*, 1996). As a consequence of all these changes the inner membrane presents a highly ordered structure with a reduced fluidity (Nystrom, 2004).

Alterations in the cell envelope occur together with changes in the cytoplasm. The nucleoid becomes condensed to protect the DNA. Nucleoid condensation requires Dps (DNA-binding protein from starved cells), a nonspecific DNA-binding protein that preferentially acts during starvation (Fig. 3). This protein is present in over 130 bacterial species, its expression being σ^{70}-dependent under oxidative stress by OxyR (LySR-transcriptional regulator), and RpoS-dependent during starvation (Almiron *et al.*, 1992; Nair & Finkel, 2004). Upon its induction in stationary phase, Dps becomes the most abundant protein in *E. coli* (Almiron *et al.*, 1992). Dps proteins form a ring-like dodecamer structure of 90 Å diameter, which upon DNA binding, and in the presence of Mg^{2+}, forms a highly ordered and stable nucleoprotein complex called biocrystal, which results in a compacted nucleoid (Wolf *et al.*, 1999; Frenkiel-Krispin *et al.*, 2001). Dps also shows a significant structural homology to ferritins, which have the double function of sequestering ferrous iron and reducing the formation of oxidative radicals formed within the cell through the Fenton reaction ($Fe^{2+}+H_2O_2 \rightarrow Fe^{3+}+OH+OH^-$), as well as ferroxidase activity, which helps to neutralize toxic peroxides through iron mineralization ($2Fe^{2+}+H_2O_2+H_2O \rightarrow 2FeOOH+4H^+$) (Ilari *et al.*, 2002). The global protective role of Dps

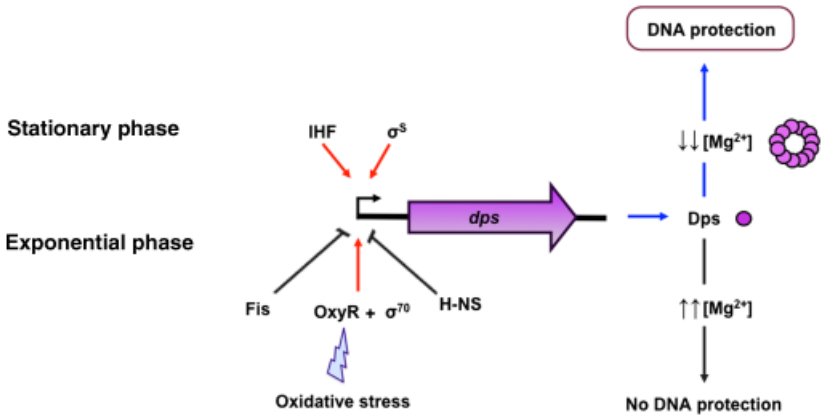


Fig. 3. The role of Dps in stationary phase. In exponential phase, *dps* is expressed by σ^{70} and downregulated by the nucleoid-associated proteins Fis and H-NS (Grainger *et al.*, 2008; Schnetz, 2008). OxyR exerts a positive regulation as a response to oxidative stress. During stationary phase, *dps* transcription is σ^S-dependent (Altuvia *et al.*, 1994). Dps proteins form a ring-like dodecamer structure with a 90 Å diameter, which in the presence of Mg^{2+} binds the chromosome, forming a highly ordered and stable nucleoprotein complex called biocrystal. Biocrystal protects the DNA from several damaging agents (Wolf *et al.*, 1999; Frenkiel-Krispin *et al.*, 2004).

against various stresses (starvation, oxidative damage, UV and γ-irradiation, thermal stress and pH) seems to be performed through a combination of its functions – DNA–Dps cocrystallization, iron chelation and ferroxidase activity – together with its ability to affect the regulation of gene expression (Ilari *et al.*, 2002; Nair & Finkel, 2004).

Starved microorganisms slow their growth rate dramatically and reduce protein synthesis (about 20%) and levels of rRNA and tRNA compared with cells in exponential growth (Reeve *et al.*, 1984). The activity of transport systems and the metabolism of carbohydrates, amino acids and phospholipids are decreased as well (Brown *et al.*, 2002). On the other hand, protein turnover increases fivefold in famished *E. coli* cells, as many of the proteins synthesized in the early stages of starvation are proteases and peptidases (Groat *et al.*, 1986). Mutations that reduce peptidase activity drastically reduce survival in stationary phase (Reeve *et al.*, 1984). The fact that cells lacking functional ClpAP or ClpXP proteases exhibit an accelerated die-off during extended stationary phase further supports the role of protein degradation in famine survival (Weichart *et al.*, 2003). This protein turnover facilitates *de novo* protein synthesis in the absence of an exogenous carbon source (Shaikh *et al.*, 2009). The amino acids required for this protein synthesis are provided by peptidase-dependent autophagy (Reeve *et al.*, 1984).

Dimerization of the ribosomes (a process also known as 'hibernation stage'; Yoshida *et al.*, 2002) could be responsible for the observed decrease in the rate of protein translation. Dimerization requires a ribosome modulation factor that covers the peptidyl transferase domain and the entrance of the peptide exit tunnel, blocking ribosomal translation activity (Wada *et al.*, 2000). Expression of *rmf* is induced by ppGpp during stationary phase (Yoshida *et al.*, 2004). Other proteins also participate in this process: YhbH, which promotes and stabilizes ribosome dimer formation; YfiA, which prevents 70S ribosome subunit dimer formation; a stationary phase-induced ribosome-associated protein (Ueta *et al.*, 2005); and a hibernation-promoting factor (Yoshida *et al.*, 2009). Dimerized ribosomes could represent a way to store ribosomes during periods of translational inactivity. Reversion of dimerization occurs within 2 min of the addition of nutrients, and protein synthesis resumes in 6 min (Yoshida *et al.*, 2004).

During entry into stationary phase the expression of the FadR regulon increases (Farewell *et al.*, 1996). The expression of the *fad* operon is controlled by the repressor FadR. The *fad* operon is required for growth using long-chain fatty acids as carbon source, with a suggested role in providing carbon and energy during digestion of endogenous membrane constituents (Farewell *et al.*, 1996). Fatty acids generated from degradation of membrane lipids during the dwarfing process could be scavenged by the activity of acyl-CoA synthetase (a product of the *fadD* gene belonging to the FadR regulon) to generate acyl-CoA, which would be metabolized by the β-oxidation enzymes (*fadBA*, *fadE*, *fadFG* and *fadH*), generating carbon and energy (Nystrom, 2004).

Moreover, an increased synthesis of glycolysis enzymes, pyruvate formate lyase, phospho-transacetylase and acetate kinase, together with a lower production of TCA enzymes mediated by the three-component response regulator ArcB/ArcA/RssB is observed in stationary phase (Nystrom, 2004). These changes in catabolic activities during stationary phase appear to be of importance, as *arcA* strains do not perform the early reductive division that occurs during starvation and lose viability (Nystrom *et al.*, 1996). Repression of aerobic metabolism upon stationary phase may prevent an uncontrolled use of endogenous reserves during autophagy and it could also be a defence mechanism in starvation against the damaging effects of reactive oxygen species produced by the respiratory chain (Nystrom, 2004).

Stationary phase is associated *in vitro* with high cell densities where the production of QS molecules reaches its maximum. QS is a cooperative behaviour that coordinates gene expression in a cell density-dependent manner, as bacteria leak signalling molecules into the milieu, which accumulate depending on cell numbers. When the signal concentration reaches a threshold, cells detect it and trigger a specific response (Keller & Surette, 2006). QS is involved in the transition to stationary phase and in other processes, such as biofilm formation and virulence (Lazazzera, 2000). In particular, in *P. aeruginosa*, RpoS controls QS gene expression at the onset of stationary phase (Schuster *et al.*, 2004). A wide variety of molecules are used as extracellular signals, such as *N*-acyl-homoserine lactones (Eberhard *et al.*, 1981), autoinducer-2 (Schauder *et al.*, 2001), diketopiperazines (Holden *et al.*, 1999) and the *Pseudomonas* quinolone signal (Pesci *et al.*, 1999).

During stationary phase a wide collection of secondary metabolites, antibiotics and toxins, are synthesized. Microcins McjA and MccA are produced in an RpoS-dependent manner in stationary phase (Duquesne *et al.*, 2007). Microcins are very small bacteriocins with a strong antibacterial activity against closely related bacteria and they are believed to be involved in the fight between intestinal microbiota and foreign enterobacteria. Although the implications of these microcins during stationary phase still remain unknown, as their expression occurs mainly during this stage, their role could be of importance. Something similar happens with growth-dependent bacteriocins, such as colicin K, which is highly active during stationary phase, but its significance during this phase remains to be assessed (Kuhar & Zgur-Bertok, 1999).

RpoS controls the expression of virulence determinants in several important human pathogens. It induces the

expression of the *spv* plasmidic virulence genes in *S.* Typhimurium (Fang *et al.*, 1992). In addition, RpoS is important for the effective intestinal colonization of *Vibrio cholerae* (Merrell *et al.*, 2000) and it is critical for the low-pH survival of *Shigella flexneri* (Small *et al.*, 1994). Besides, in *P. aeruginosa* RpoS controls production of exotoxin A, the phenazine pyocyanin and the siderophore pyoverdin (Suh *et al.*, 1999).

Bacterial death phase

The lost of viability observed after a few days in stationary phase could be the result of stochastic cellular death or a consequence of an altruistic death response of part of the population to provide food to the few survivors.

Cellular degeneration in stationary phase could be due to oxidative damage. The levels of oxidized proteins increase in stationary phase cultures (Dukan & Nystrom, 1998). The accuracy of the ribosomes seems to be responsible for these higher levels of protein oxidation in nonproliferating cells (Ballesteros *et al.*, 2001). Progressive accumulation of damaged molecules in starved cells would then eventually lead to bacterial death. Besides, it has been shown that *E. coli* cells experience ageing, as daughter cells that inherit the old pole do not have an equal fitness relative to the ones with the newly synthesized pole (Stewart *et al.*, 2005).

On the other hand, under certain unfavourable conditions, cells initiate a programme that ends in their own death, suicide from an anthropocentric point of view. In eukaryotes, apoptosis is activated during developmental processes, and also clears damaged cells (Danial & Korsmeyer, 2004). In a similar way, programmed bacterial death is also crucial in multiple bacterial developmental processes, such as sporulation in *Bacillus subtilis* (Lewis, 2000; Gonzalez-Pastor *et al.*, 2003) and formation of fruiting bodies in Myxobacteria (Nariya & Inouye, 2008). Moreover, certain environmental stresses, among which is starvation, induce bacterial death mediated by toxin–antitoxin (TA) modules (Engelberg-Kulka *et al.*, 2006). TA systems, also known as addiction modules, were first described in low copy plasmids, such as the *ccd* mechanism of plasmid F (Hiraga *et al.*, 1986), and the *hok*/*sok* genes (Gerdes *et al.*, 1986) and the *parD* system (Bravo *et al.*, 1987), both of plasmid R1. These discoveries opened up the TA field in bacteria. Plasmidic TA systems are thought to be acquired through horizontal gene transfer, clearly contributing to maintenance of vertical plasmid inheritance by selectively killing plasmid-free daughter cells (postsegregational killing) (Gerdes *et al.*, 1986; Hiraga *et al.*, 1986; Bravo *et al.*, 1987). TAs were later found to be widespread in the genomes of Bacteria and Archaea and probably contributed to bacterial evolution (Pandey & Gerdes, 2005). The genetic organization of a typical TA module comprises an operon of two closely linked genes, one encoding a stable toxin and the other codifying its cognate antitoxin, which neutralizes the deleterious effect of the toxin. In some cases the antitoxin molecule is a labile small protein (type II TA systems), whereas in others it is a small antisense RNA that binds the toxin mRNA (type I TA systems). In both types, the toxin is a protein (Van Melderen & Saavedra De Bast, 2009). Some chromosomal TA systems could be integrated into the host regulatory networks, allowing cells to cope with various stresses (e.g. *mazF-mx* and *hipBA*) (Keren *et al.*, 2004). Even though several chromosomally encoded TA systems have been described, such as the *relBE* operon, which is induced by nutritional starvation (Christensen *et al.*, 2001), and the *hipBA*, which affects cell survival in oxidative stress (Kawano *et al.*, 2009), understanding of bacterial PCD is based mainly on the study of the *E. coli mazEF* operon (Fig. 4) (Engelberg-Kulka *et al.*, 2006). The *mazEF* operon comprises MazF, a stable endoribonuclease toxin, and MazE, a labile antitoxin. Stress conditions, such as amino acid starvation and DNA damage, repress expression of the *mazEF* genes and as a consequence the toxin accumulates due to the rapid degradation of the antitoxin MazE by a serine-protease (Aizenman *et al.*, 1996). Therefore, the free MazF toxin cleaves mRNA, preferentially at 5′-(U/A)CA-3′ sites, inhibiting translation (Zhang *et al.*, 2003; Munoz-Gomez *et al.*, 2004). However, some authors report that MazF produces a reversible stasis phenomenon instead of causing cell death (Buts *et al.*, 2005; Gerdes *et al.*, 2005). Interestingly, a dual regulatory role for MazF has been reported: it promotes translation inhibition of most proteins while simultaneously enhancing the production of a specific group of small proteins (< 20 kDa). The majority of these small proteins cause cell death, although some are necessary for the survival of a small subpopulation (Amitai *et al.*, 2009). Moreover, it has been shown that once *mazEF* is triggered by a certain stress, it only produces death in cell-dense cultures. This means that *mazEF*-mediated death is a population-dependent phenomenon requiring the presence of a QS extracellular pentapeptide (Kolodkin-Gal *et al.*, 2007). In this way, death of the bulk cell population would provide nutrients for the leftover cells. Survivors will resume cell division when conditions are propitious (Engelberg-Kulka *et al.*, 2006).

The biological function of genome-encoded TA modules is still the subject of intense debate. The two lines of thought are based on the effect elicited by the toxin; that it causes the death of a part of the population, allowing survivors to feed with debris released from dead cells (Amitai *et al.*, 2009), or acts as a growth modulator, transforming a cell into a quiescent structure that would allow bacteria to thrive in troublesome settings (Gerdes *et al.*, 2005). In addition, other roles have been proposed, such as antiaddiction modules acting as a defence mechanism against foreign DNA

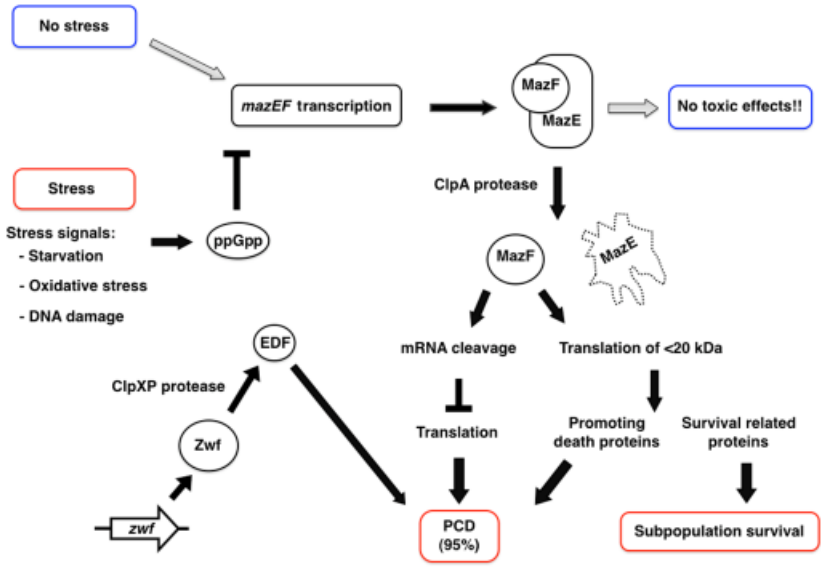


Fig. 4. PCD: the *mazEF*-mediated cell death model. Arrows in black represent the model in the presence of PCD induction. Arrows in grey represent the model without stress, and therefore no induction of PCD. The gene *zwf* (glucose-6-phosphate dehydrogenase) is thought to be the precursor of the QS-induced extracellular pentapeptide (Kolodkin-Gal & Engelberg-Kulka, 2008). PCD model based on Engelberg-Kulka *et al*. (2006), Kolodkin-Gal *et al*. (2007) and Amitai *et al*. (2009).

(Saavedra De Bast *et al*., 2008), or genome stabilization systems that avoid the deletion of big dispensable chromosome regions (Rowe-Magnus *et al*., 2003).

Long-term stationary phase-related phenomena

Microorganisms can sometimes respond to adversity in surprising ways. There are various interesting phenomena associated to continued starvation, such as the GASP phenotype, the VBNC state, and the SCDI.

GASP phenotype

Bacterial populations can evolve and adapt to become diverse niche specialists, even in seemingly homogeneous environments. For instance, one source of this diversity arises from the activities of bacteria themselves. The GASP is the ability of aged cells (cells isolated after 10 days in stationary phase) to take over young cells (cells that just have entered stationary phase). The GASP phenotype is caused by stable mutations that confer an advantageous ability to grow during starvation, and it can either replace the parental population (Zambrano *et al*., 1993) or coexist with it (Rozen *et al*., 2009). During stationary phase a continuous cycling of growth and death in starved cultures occurs due to the rise and propagation of mutants with greater fitness than the parental strain. Stationary phase cell cultures reveal that populations are indeed highly dynamic during this phase (Zambrano & Kolter, 1996).

The first described GASP phenotype (G_I, the parental strain being G_0) was caused by a single *rpoS*-down mutation (Zambrano *et al*., 1993). The mutation was the result of a 46-base pair duplication in the 3′ end of *rpoS*, producing a protein in which the four last C-terminal amino acids are replaced by 39 new amino acids. The mutated RpoS shows a reduction in its activity, attenuating the expression of the *rpoS* regulon, whereas null *rpoS* mutations do not confer the GASP phenotype (Zambrano *et al*., 1993). The physiological role of this *rpoS*-down mutation has been hypothesized to be due to pleiotropic effects (Zinser & Kolter, 2004). A plausible explanation of why *rpoS*-down mutations are involved in GASP acquisition could be that a reduction in RpoS activity may unbalance the competition among sigma factors for the RNA polymerase. Cells growing in LB cultures for a long time face two main stresses, lack of nutrients and basification of the milieu (Farrell & Finkel, 2003). The sigma factor RpoD regulates glucose scavenging and RpoN ammonia assimilation and amino acid uptake, as well as protecting the cells against alkaline stress. In summary, cells with *rpoS*-down mutations will favour the presence of RNA polymerases with RpoD and RpoN as sigma factors, and thus have a better fitness than RpoS wild-type strains (Farrell & Finkel, 2003).

Other loci involved in the subsequent GASP cycle (G_{II}) have been identified. In this case, three additional mutations were responsible for the G_{II} (Zinser & Kolter, 1999). One of the mutations was mapped to the *lrp* gene encoding the leucine-responsive global regulator (Zinser & Kolter, 2000). The mutation responsible was caused by an in-frame 3-base

pair deletion originating a protein lacking a conserved glycine residue (G39) within the helix-turn-helix domain. Interestingly, this loss-of-function mutation behaves as dominant negative, meaning that the stable nonfunctional Lrp can still form dimers with a wild-type monomer, blocking its activity. Dominant negative mutations have the ability to change the cell physiology as soon as the mutated allele is produced (no need for the wild-type product to disappear), which seems very important under starvation situations.

lrp mutant cells, in addition to the *rpoS*-down mutation allele, show an enhanced ability over the parental cells to scavenge amino acids released by dead cells, in particular serine, threonine and alanine (Zinser & Kolter, 1999); however, although important, this enhanced amino acid utilization is not sufficient to explain all the gain in fitness (Zinser & Kolter, 2000).

The second mutation in G_{II} was caused by a genomic rearrangement produced by two IS5 insertion sequences and designated IN(*cstA*::IS*5*-IS*5D*) (Zinser *et al.*, 2003). First, an IS5 transposition event inactivates the *cstA* gene, whose product is an oligopeptide permease. Secondly, an inversion between this IS5 and another IS5 at *c.* 60 kb away, activates the *ybeJ-gltJKL* five-gene operon. Four genes of the operon are annotated as an ABC-type transporter for aspartate and glutamate. Therefore an activation of these genes would increase the amino acid uptake potential of the G_{II} GASP mutant (Zinser *et al.*, 2003).

Surprisingly, the third mutation (*sgaC*) has not been characterized as yet (Zinser & Kolter, 2000).

Additionally, *E. coli rpoS*-down mutants upon prolonged incubation have also been described to activate the cryptic *bgl* operon, which is involved in the uptake and use of the aryl-β-glucosides salicin and arbutin. The activation of the *bgl* operon possesses a GASP relative to the parental strain (*bgl*$^-$) by an unknown mechanism (Madan *et al.*, 2005). The activation is caused by a point mutation in the CRP–cAMP binding site in the regulatory region of the operon, although different methods of activating the *bgl* operon (IS*1* insertion in *bglR*) also produced the GASP phenotype (Madan *et al.*, 2005).

A recently published experiment showed that two ecotypes (S and L), evolved from a parental *E. coli* strain after a long-term evolution experiment, were able to coexist through frequency-dependent interactions (Rozen *et al.*, 2009). Coexistence within a niche arises as a result of differential exploitation of the resources. In this case, S cells evolved a GASP phenotype, specialized in cannibalizing the debris released by the lysis of L cells, and thus favouring coexistence with L cells. The molecular differences of the resulting ecotypes are associated with divergence in the activity of RpoS: S cells displayed no detectable activity, whereas L cells showed an increased activity relative to the ancestral genotype. However, the *rpoS* gene itself presented no mutations, and therefore an RpoS regulator is hypothesized to be affected (Rozen *et al.*, 2009).

Other works have described reduction of RpoS activity either by a decrease in its expression downwards, such as the transposition of an IS*10*R element in the promoter region of *rpoS* in *Enterobacter cloacae* (Martinez-Garcia *et al.*, 2003a), or by mutation of one of its multiple regulators (Kojic & Venturi, 2001). However, whether these alterations confer a GASP phenotype remains to be clarified.

In contrast, elimination of the GASP phenotype, by an unknown mechanism, was observed with knock-out mutants in the genes *nuoA* and *nuoB*, which encode the NADH dehydrogenase I subunits (Zambrano & Kolter, 1993). As an interesting curiosity, it has been shown that high magnetic fields eliminate the GASP phenotype (Okuno *et al.*, 2001).

Moreover, processes of GASP acquisition through *rpoS*-independent pathways have been observed in other enterobacteria (Martinez-Garcia *et al.*, 2003b), although the particular mechanism involved was not characterized.

The GASP phenomenon is not only restricted to *E. coli* laboratory isolates; it has also been observed in other enterobacteria such as *E. cloacae*, *Salmonella enterica*, *Shigella dysenteriae* and *Providencia stuartii* (Martinez-Garcia *et al.*, 2003b), in other gram-negative bacteria such as *Pseudomonas aureofaciens* (Silby *et al.*, 2005) and *Pseudomonas putida* (Tark *et al.*, 2005), *V. cholerae* (Paul *et al.*, 2004) and even in gram-positives *Mycobacterium smegmatis* (Smeulders *et al.*, 1999), *Staphylococcus aureus*, *Bacillus globigii* and *Enterococcus faecalis* (Finkel *et al.*, 2000). Interestingly GASP also has been reported in mixed cultures of *E. coli* and *S. enterica*, two related enterobacterial species (Bacun-Druzina *et al.*, 2007). It has also been found in Eukarya (Gray *et al.*, 2004).

The appearance of the GASP phenotype in such a wide range of conditions, through different pathways and in multiple bacterial species, indicates that it is a generalized phenomenon of the microbial world under starvation periods (Finkel *et al.*, 2000).

Although GASP mutants are competitors with the wild-type strain in shared spaces with nonrenewable resources, they do not necessarily wage a battle for dominance at the cost of extinction of the less-fit strain; sometimes they cooperate to maximize fitness (long-term total productivity) via spatial segregation (Keymer *et al.*, 2008).

VBNC

Certain bacterial species under prolonged starvation lose their culturability, failing to grow under standard laboratory conditions, but remain viable. This phenomenon is known as VBNC state and is a survival strategy in response to adverse environmental conditions (starvation, temperature, salinity, pH) (Na *et al.*, 2006; Lleo Mdel *et al.*, 2007). VBNC

has been observed in more than 50 bacterial species so far, and it would appear that there is no single molecular mechanism behind it (Hayes & Low, 2009). This VBNC state is characterized by a low metabolic activity and by morphological changes characteristic of stationary phase bacteria, or dormant stages (Oliver, 2005; Amako *et al.*, 2008). Surprisingly, not much is known about the molecular mechanisms that contribute to dormancy and later to recovery from this state (Tufariello *et al.*, 2006). Resuscitation from the dormant state, with resumption of cell division, has been achieved with some species under favourable environmental conditions (Oliver, 2005; Coutard *et al.*, 2007; Zhong *et al.*, 2009). The resuscitated cells can still be pathogenic, and therefore VBNC represents an important risk not only to human health but for combating pests or for the food preservation industry. There is a health risk not only because dormant pathogenic bacteria could act as a reservoir for new infections, but because they might not be detectable using culturable sampling.

SCDI

An interesting phenomenon named SCDI has been described recently (Lemonnier *et al.*, 2008). After serial culture passage experiments with an *E. coli* K-12 strain (Δ*mutS*), the authors found emerging strains that appeared to kill or inhibit growth of the parent strain. This inhibition ability seems to lie in several single-base nonsynonymous substitutions within the *glgC* gene, which codes for ADP-glucose pyrophosphorylase, a regulatory protein involved in glycogen synthesis. All evolved strains overproduced glycogen, a necessary condition for the SCDI phenomenon. Although SCDI and GASP share some characteristics, the authors claim that these processes are functionally and genetically distinct (Lemonnier *et al.*, 2008). However, the underlying mechanism of cell inhibition by SCDI is still not known. Nonetheless, SCDI as well as GASP are of great importance as they could be useful in the development of new antimicrobial agents (Lemonnier *et al.*, 2008).

Genetic diversity in stationary phase

Genetic diversity can be acquired by gaining or losing genes or alleles due to random mutation, recombination and horizontal gene transfer. As the majority of point mutations are either detrimental or neutral, microorganisms have evolved mechanisms to keep mutation rates as low as possible (Drake *et al.*, 1998). However, many *E. coli* natural isolates present high mutation rates during stress situations (Bjedov *et al.*, 2003).

In the late 1980s a controversial paper, 'The origin of mutants', published in *Nature*, described 'directed mutations' (Cairns *et al.*, 1988). This paper challenged the random mutation dogma of Luria & Delbrück (1943), suggesting that mutations in nondividing *E. coli* cells can be specifically directed by environmental stimuli (Cairns *et al.*, 1988). Those nonrandom mutations that relieve bacteria from the nonlethal selection allowing their growth were named adaptive mutations (Cairns *et al.*, 1988). Further experiments proved that adaptive mutations were not directed, as many other mutations also accumulate in the process (Foster, 2005). Stress-induced mutagenesis involves mutations generated by a group of mechanisms when cells are subjected to harmful situations (Galhardo *et al.*, 2009). Because microorganisms, in nature, spend more of their life under stress conditions, these stress-induced mutations could be an important way to generate genetic diversity, upon which natural selection will act to select the fittest mutant for a specific environmental condition (Loewe *et al.*, 2003). Mechanisms for generating variation under harsh conditions is not a phenomenon restricted to prokaryotes, as eukaryotes also present a system, through inactivation of the chaperone Hsp90, to uncover the already present genetic diversity within the population (Rutherford & Lindquist, 1998).

Different models were proposed to explain stress-induced mutagenesis in prokaryotes, such as the SOS response or the induction of the RpoS regulon (Saint-Ruf & Matic, 2006). Here we are going to focus only on the RpoS-dependent mechanism. Although the expression of RpoS-dependent genes aims to protect the cell, it is also responsible for increasing the mutation rate in stress situations by repressing the methyl-mismatch repair (MMR) and inducing the error-prone DNA polIV (Saint-Ruf & Matic, 2006). The MMR system, comprising genes *mutS*, *mutL* and *mutH*, controls the accuracy of DNA replication, repairing postreplicative errors and inhibiting recombination between different DNA segments, and inducing a mutator phenotype upon its inactivation (Oliver *et al.*, 2002). In stationary phase the expression of *mutS* and *mutH* is repressed by RpoS and Hfq, therefore, decreasing the activity of the MMR system (Tsui *et al.*, 1997). Furthermore, in stationary phase RpoS induces the expression of the error-prone DNA polymerase PolIV, encoded by *dinB* (Saint-Ruf & Matic, 2006). PolIV belongs to the Y-family of DNA polymerases; widespread in prokaryotes and eukaryotes, PolIV lacks $3' \rightarrow 5'$ exonuclease activity and has the ability to copy damaged DNA (Goodman, 2002). It is thought to be responsible for 85% of the adaptive mutations described so far (Goodman, 2002). PolIV has also been related to mutagenesis in long-term-starved populations of *P. putida* (Tegova *et al.*, 2004). Also, it has been reported that mutant cells lacking any of the SOS DNA polymerases (PolIV, PolV and PolII) do not acquire the GASP phenotype (Yeiser *et al.*, 2002).

Another stress-induced mutagenesis process that depends on RpoS is caused by transposition events (Kivistik *et al.*,

2007). Certain environmental stresses trigger these events (Kretschmer & Cohen, 1979; Eichenbaum & Livneh, 1998; Lamrani *et al.*, 1999). For example, the transposition during stationary phase of Tn*4652*, dependent on RpoS and IHF, promotes the appearance of phenol-degrading cells by activation of a promoter-less phenol-degrading operon (Kasak *et al.*, 1997; Kivistik *et al.*, 2007).

As RpoS is responsible for stationary phase survival and for the increase of the mutation rate during stress situations, it is interesting to note that mutations in this alternative sigma factor could be propitious in specific situations. Alteration of sigma factor concentrations could, apart from decreasing the mutation rate, also unbalance the RpoD/RpoS ratio, providing a trade-off between nourishment and stress resistance (Notley-McRobb *et al.*, 2002; King *et al.*, 2004). This is probably why different *rpoS* alleles are found not only in laboratory strains but also in clinical isolates, such as the shiga-like toxin-producing *E. coli*, an important enteric pathogen (Waterman & Small, 1996), and in *S.* Typhimurium (Sutton *et al.*, 2000). Multiple studies in *E. coli* show an important *rpoS* variability within different strains (Ivanova *et al.*, 1992; Zambrano *et al.*, 1993; Jishage & Ishihama, 1997; Atlung *et al.*, 2002). All of these sequence analyses showed that *rpoS* is a highly polymorphic locus. When the growth conditions are propitious, for example rich medium, mutations can accumulate in *rpoS*, somehow provoking a domestication of environmental or clinical strains (Davidson *et al.*, 2008). Moreover, it has been found that the frequency of base substitutions and the generation of large increases in deletions in long-term-starved populations of *rpoS*-deficient *P. putida* cells, may be because *rpoS*-deficient cells are less protected against damage caused by ROS (Tarassova *et al.*, 2009).

Nonetheless, the role of stress-induced mutations is still a matter of controversy, as some studies point out that if a system is based on an increased mutation rate under stress conditions, this would reduce its long-term fitness (Roth *et al.*, 2006). Although the fact remains that stress-induced mutagenesis happens, the evolutionary significance of these mutations remains an open discussion.

Conclusions and perspectives

As shown in this review, stationary phase, including death stage and long-term stationary phase, represents a very complex state regulated by a variety of environmental and physiological cues. The signals leading to activation of this phase are usually confused or mixed with other concurrent processes, such as general stress responses or biofilm formation, creating an extra difficulty for the study of stationary phase. Regulatory mechanisms of all of these cellular processes could suppose a complex interplay, the result of which would depend on the concrete environmental circumstances and the particular type of bacteria, even at strain level. Therefore, this review has highlighted only some of the different mechanisms adopted by gram-negative bacteria to survive starvation, and we should not forget that other systems could contribute differently.

In natural environments, bacteria face long periods of nutrient deprivation and strong competition for nutrients together with other stresses, so microorganisms need to adapt rapidly to thrive in nature. A fast way of altering the gene expression pattern to coordinate environmental adaptation behaviours is through the expression of alternative sigma factors and regulators. The entrance into stationary phase is dictated by RpoS, involved not only in the physiological changes observed but also in stress resistance, secondary metabolism, GASP and, probably, PCD. However, many discrepancies can be found in the literature, probably due to the complexity involved in the integration of all the variables involved.

Microbial populations suffer genetic adaptations under environmental stresses by mechanisms that lead to a high mutation rate. As a consequence, mutagenic processes occurring in stressed bacteria are translated into the acquisition of genetic variability important in the development of antibiotic-resistance, colonization of new hosts, pathogenesis and even in the acquisition of new catabolic capabilities. On the other hand, transient, noninherited bacterial resistance to bactericidal agents or antibiotics of a small part of the population is the result of the suppression of important cell functions. Growth rate is therefore the decisive factor in the transition of the cells to the persister state (Samuilov *et al.*, 2008).

A better knowledge of the stationary phase physiology is of importance not only in medicine, but also in biotechnology. During this phase bacteria produce secondary metabolites that offer interesting industrial applications. Among these compounds we find antibiotics and bacteriocins (microcins and colicin K), whose potent activity associated with their narrow spectrum make them particularly attractive; different enzymes (lipases, proteases); and even polymers, such as the biodegradable thermoplastic poly-β-butyric acid.

Although our knowledge of stationary phase has improved in the last decade, there are still elusive questions that remain unresolved, such as how specific environmental signals are sensed or transmitted, how cells are interconnected to respond to them, and what is the biological role of TA systems in the death phase (death vs. dormancy).

A complete understanding of how bacterial stationary phase is induced and regulated could help us to understand better the complete life cycle of microorganisms, the complex processes of ageing, survival and mutation under harsh conditions. A better knowledge of the stationary state and the metabolites produced in it would facilitate its

manipulation for our own benefit. Stationary phase physiology therefore remains an exciting challenge for basic and applied research.

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