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
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

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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
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 multi-subunit core (σ2ββ′ω) 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 σ70, 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 σ70-family or the σ32-family. A precise description of the differences between these two families can be found elsewhere (Paget & Helmann, 2003). The σ70 family is further divided into various groups. Group 1, including σ70 itself, comprises sigma factors essential for cell growth, whereas group 2 includes sigma factors closely related to σ70 but not essential for bacterial growth. Group 3 sigma factors control heat shock response, flagellar biosynthesis and sporulation. Members of group 4 comprise extractoytoplasmic 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 Streptomycyes 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 σ5 (the product of the rpoS gene) and σ32 (encoded by rpoH) (Fredriksson & Nystrom, 2006).

σ32 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).

σ5 is included within group 2 of the σ70-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

<table>
<thead>
<tr>
<th>Sigma factor</th>
<th>Main function</th>
<th>Variation in stationary phase</th>
<th>References</th>
</tr>
</thead>
<tbody>
<tr>
<td>RpoD (σ70)</td>
<td>Housekeeping</td>
<td>No variation in Ec</td>
<td>Yuste et al. (2006)</td>
</tr>
<tr>
<td>RpoF (σ28)</td>
<td>Synthesis of Flagella and chemotaxis</td>
<td>Threefold decreased in Ps</td>
<td>Yuste et al. (2006)</td>
</tr>
<tr>
<td>RpoN (σ32)</td>
<td>Nitrogen metabolism motility</td>
<td>No variation in Ps and Ec</td>
<td>Yuste et al. (2006)</td>
</tr>
<tr>
<td>RpoH (σ32)</td>
<td>Heat shock</td>
<td>No variation in Ps or Ec</td>
<td>Yuste et al. (2006)</td>
</tr>
<tr>
<td>RpoE (σ52)</td>
<td>Extra cytoplasmic stress</td>
<td>No variation in Ps or Ec</td>
<td>Yuste et al. (2006)</td>
</tr>
<tr>
<td>RpoS (σ70)</td>
<td>Stationary phase and stress resistance</td>
<td>Fivefold increase in Ec</td>
<td>Costanzo &amp; Ades (2006)</td>
</tr>
<tr>
<td>ECF sigma factors</td>
<td>Extracellular functions</td>
<td>Three- to fourfold increase in Ps and Ec</td>
<td>Yuste et al. (2006)</td>
</tr>
</tbody>
</table>

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 (Collard 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 $r_{E}^{E}$-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 tRNA 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

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**Fig. 2.** Regulation of RpoS in *Escherichia coli*. (1) Transcriptional. Several regulators have been described to affect rpoS transcription from the stationary promoter (P_{rpoS1}), 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 (Schelhorn & 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 *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 (Schelhorn & 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 ( \( \leq 30^\circ C \)) by unfolding a hairpin bend in the 5' UTR region of the rpoS mRNA (Siedjeski 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 (Bougourdou et al., 2006, 2008; Merrick 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 (Bougourdou 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 (Leelong et al., 2007), being one of the curli operon csgBA (Pratt & Silhavy, 1998). Activity figure modified from Bougourdou et al. (2004). →, activation; ⊲, 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 σ70 to out-compete σ70 for RNA polymerase binding, thus causing a decay in the expression of the σ70-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'-TCTTAA-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-Justman & 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

<table>
<thead>
<tr>
<th>Category</th>
<th>Change in Stationary Phase</th>
<th>Death Phase</th>
<th>Long-Term Stationary Phase</th>
</tr>
</thead>
<tbody>
<tr>
<td>Morphological</td>
<td>Smaller and spherical cells</td>
<td>More resistant and rigid cell envelope</td>
<td></td>
</tr>
<tr>
<td>Nucleoid</td>
<td>Condensation of the nucleoid as certain histone-like proteins increase their concentration</td>
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<td></td>
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<tr>
<td>Metabolic</td>
<td>Stringent response</td>
<td>Repression of aerobic metabolism</td>
<td>Increase fermentative enzymes expression</td>
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<td></td>
<td></td>
<td></td>
<td>Production of RMF (ribosome modulating factor)</td>
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<td></td>
<td></td>
<td></td>
<td>Drop in protein synthesis while increase peptidases/proteases synthesis</td>
</tr>
<tr>
<td>Transcriptional</td>
<td>Change of sigma factors affinity: σS, σ70</td>
<td>Adjustments of global regulators: Lrp, IHF, sRNAs</td>
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<tr>
<td>Translational</td>
<td>100S ribosome dimers (inactive)</td>
<td>Increase protein synthesis</td>
<td>Increase proteases and peptidases synthesis</td>
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<tr>
<td>Others</td>
<td>Increased resistance against physical and chemical stresses</td>
<td>Synthesis of quorum sensing molecules</td>
<td>Production of secondary metabolites</td>
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<td>Programmed cell death (PCD)</td>
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<td>GASP phenotype</td>
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<td>Mutator phenotype</td>
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<td>Viable but nonculturable (VBNC) state</td>
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<td>Stationary phase contact-dependent inhibition (SCDI)</td>
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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 phosphorylcholine 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 σ70-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 Mg²⁺, 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 (Fe²⁺ + H₂O₂ → Fe³⁺ + OH⁻ + OH⁻), as well as ferroxidase activity, which helps to neutralize toxic peroxides through iron mineralization (2Fe²⁺ + H₂O₂ + H₂O → 2FeOOH + 4 H⁺) (Ilari et al., 2002). The global protective role of Dps...
against various stresses (starvation, oxidative damage, UV and \(\gamma\)-irradiation, thermal stress and pH) seems to be performed through a combination of its functions – DNA–Dps cocystallization, iron chelation and ferrooxidase activity – together with its ability to affect the regulation of gene expression (Iliari 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; YfA, 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 \(\beta\)-oxidation enzymes (fadBA, fadE, fadFG and fadH), generating carbon and energy (Nystrom, 2004).

Moreover, an increased synthesis of glycolysis enzymes, pyruvate formate lyase, phospho-transacylase 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 (Schauer 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 loss 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 aging, 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 antiadiction modules acting as a defence mechanism against foreign DNA.
(Saavedra De Bast et al., 2008), or genome stabilization systems that avoid the deletion of big dispensable chromo-
some 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 (G₁, the parental strain being G₀) 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 (Gᵢ) have been identified. In this case, three additional mutations were responsible for the Gᵢ (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 **GII** was caused by a genomic rearrangement produced by two IS5 insertion sequences and designated **IN**(csta::IS5-SSD5) (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 IS 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 **GII** GASP mutant (Zinser et al., 2003).

Surprisingly, the third mutation (**sguC**) 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**<sup>+</sup>) 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 (**IS1** 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 **IS10**R element in the promoter region of **rpoS** in **Enterobacter cloacae** (Martínez-García 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 **muoA** and **muoB**, which encode the NADH dehydrogenase I subunits (Zambreno & 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 (Martínez-García 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** (Martínez-García 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 (ΔmutS), 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 polymerase 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′ → 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.,
Certain environmental stresses trigger these events (Kretschmer & Cohen, 1979; Eichenbaum & Livneh, 1998; Lamrani et al., 1999). For example, the transposition during stationary phase of Tn4652, 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., 1999). For example, the transposition during stationary phase of Tn4652, 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).

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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