«(p)ppGpp and its role in bacterial persistence: New challenges»

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ABSTRACT

Antibiotic failure is not only due to the development of resistance by pathogens, but it can also often be explained by persistence and tolerance. Persistence and tolerance can be included in the “persistent phenotype”, with high relevance for clinics. Two of the most important molecular mechanisms involved in tolerance and persistence are toxin-antitoxin (TA) modules and signaling via guanosine pentaphosphate/tetraphosphate (ppGpp, also known as “magic spot”). (ppGpp is a very important stress alarmone which orchestrates the stringent response in bacteria; hence, (ppGpp is produced during amino acid or fatty acid starvation by proteins belonging to the RelA/SpoT homologs family (RSH). However, (ppGpp levels can also accumulate in response to a wide range of signals, including oxygen variation, pH downshift, osmotic shock, temperature shift or even exposure to darkness. Furthermore, the stringent response is not only involved in responses to environmental stresses (starvation for carbon sources, fatty acids, phosphate or heat shock), but it is also used in bacterial pathogenesis, host invasion, antibiotic tolerance and persistence. Given the exhaustive and contradictory literature surrounding the role of (ppGpp in bacterial persistence, and with the aim of summarizing what is known so far about the “magic spot” in this bacterial stage, this review provides new insights into the link between the stringent response and persistence. Moreover, we review some of the innovative treatments that have (ppGpp as a target, which are in the spotlight of the scientific community as candidates for effective anti-persistence agents.
1. Introduction

Antimicrobial resistance crisis is a serious health problem worldwide. During the past fifty years, very few new anti-infective molecules have been discovered (1). Hence, microbial pathogens have been able to accumulate molecular mechanisms enabling them to counteract antibiotics.

Nonetheless, there are more antibiotic evasion strategies other than resistance that are of great interest, such as persistence and tolerance. Persisters are a subpopulation of cells that are non-growing, non-replicative, dormant bacteria that exhibit transient high levels of tolerance to antibiotics without affecting their MICs (2-4). Once the drug pressure is removed, these persisters can rapidly regrow, thus returning to an antibiotic sensitive state. Moreover, the persistent state can be maintained for hours up to days before persisters revert to an antibiotic-sensitive cell type, resuming growth under drug-free conditions (5).

The term “triggered persistence” has been recently coined to indicate a form of persistence that is induced by particular signals, such as starvation and nutrient transitions, acid- and oxidant-stress, DNA damage, subinhibitory concentrations of antibiotics and intracellular infections (6).

Similar to persisters, tolerant cells are populations of bacteria that can also overcome antibiotic therapy. Tolerance allows cells to temporarily counteract the lethal consequences of high doses of antibiotics, maintaining their vital processes slowed (4, 7, 8). Also, tolerant bacteria arise when the whole population slows its growth (e.g., stationary-phase) whereas persister bacteria are a small subpopulation of the population (4).

Both tolerant and persistent bacteria can be included in the “persistent phenotype”, which has high relevance in clinics because: (i) there is evidence that persistent cells are responsible for relapses of infections, which is common in tuberculosis, cystic fibrosis and Lyme disease (9, 10); (ii) antibiotic therapy does not effectively work against these types of infections; (iii) persisters are responsible for the majority of biofilm-associated infections (11, 12) and (iv) they are associated with better survival of bacteria inside macrophages (13). Furthermore, persister cells can also survive in immune-compromised patients and in patients in whom antibiotics do not effectively kill pathogenic bacteria, as they might deploy immune-evasion strategies (14).

Differences between resistance, persistence and tolerance have been established; nonetheless, there are also relationships among these bacterial populations which are worthy of consideration. Despite evidence showing that tolerance and persistence to antibiotics promote the evolution of resistance in bacteria (8, 14, 15) both mechanisms are currently underestimated by the scientific and medical communities.
There are several molecular mechanisms involved in bacterial persistence and tolerance to antibiotics, reviewed by Trastoy and colleagues (2018), which include: (p)ppGpp network; toxin-antitoxin (TA) system; the quorum sensing (QS) system; drug efflux pumps; reactive oxygen species (ROS); the SOS response; and RpoS (sigma factor of stationary phase) (7).

(p)ppGpp orchestrates the stringent response (SR) in bacteria, thus it is produced during nutrient stress (such as amino acid or fatty acid starvation) by proteins belonging to the RelA/SpoT Homologs family (RSH) (16). In *E. coli*, RelA is the (p)ppGpp synthetase I or GTP-pyrophosphokinase that synthesizes (p)ppGpp from GTP/GDP and ATP, whereas SpoT is a bifunctional (p)ppGpp synthetase II or pyrophosphohydrolase (17). However, (p)ppGpp levels in bacteria do not depend exclusively on nutrient availability (18), since it can also accumulate in response to a wide range of signals, including oxygen variation (19), pH downshift (20), osmotic shock, temperature shift (21) or even exposure to darkness (22). Furthermore, the SR is not only involved in responses to environmental stresses (starvation for carbon sources, fatty acids, phosphate or heat shock), but also in bacterial pathogenesis (23), host invasion (24), antibiotic tolerance and persister cell formation (25).

Given the exhaustive and contradictory literature surrounding the role of (p)ppGpp in bacterial persistence, and with the aim of summarizing what is known so far about the “magic spot” in this bacterial stage, this review provides new insights into the link between the SR and persisters. Finally, we have reviewed and discussed some of the innovative treatments that have (p)ppGpp as a target, which are in the spotlight of the scientific community as candidates of effective anti-persistence agents.

2. (p)ppGpp as a key regulator

It was in 1969 that Cashel and Gallant described for the first time guanosine penta/tetraphosphate (26). During these fifty years, many functions have been attributed to this alarmone as it plays a key role in the physiology of bacteria, mainly controlling energetic metabolism (26, 27) but also their virulence and immune evasion (26). Despite being widely studied in the model organism *E. coli*, (p)ppGpp behaves differently in other species and its regulation changes among phylogenetically-related bacterial groups (27). In *E. coli*, the accumulation of (p)ppGpp causes the differential expression of approximately 500 genes, as it activates RpoS and RpoE (the stress response sigma factor for misfolded proteins in the periplasm) (28). Also in *E. coli*, (p)ppGpp directly inhibits DNA primase (29), and is thought to inhibit the synthesis of rRNA, which also affects translation globally, by regulating the transcription of the ribosomal modulation factor (Rmf) (30). More specifically, in response to stresses such as amino acid starvation, in Gram negative bacteria (p)ppGpp binds to the RNA polymerase inducing an allosteric signal to the catalytic Mg$^{2+}$ site, which severally decreases
transcription causing a global re-wiring of the gene expression profile. Taken together, all
these changes lead to dormancy or slow growth for most cells (Figure 1) (31, 32).
Most Gram-positive bacteria possess only one long RSH, named Rel, which has both (p)ppGpp
synthetase and hydrolase activities (33, 34) together with a short RSH, named SAS (Small
Alarmone Synthetase) or SAH (Small Alarmone Hydrolase) (35). Nonetheless, the existence of a
diverse population of (p)ppGpp synthetases and hydrolases in bacteria has been demonstrated
(35-38).
There is evidence that (p)ppGpp is related to antibiotic tolerance and persister cell formation
(25, 39-42). For example, increased levels of (p)ppGpp inhibit negative supercoiling of DNA in
E. coli, thus preventing DNA replication and transcription, resulting in tolerance towards
ofloxacin and quinolones (Figure 1) (43, 44). Persister cells tend to form in biofilms, in which
the bacterial cells are embedded in a three-dimensional matrix that provides protection during
pathogenesis and other conditions. Thus, it is logical that bacteria in biofilms encounter limited
access to nutrients and therefore display the SR (45). Consistently, different groups have
shown that multidrug tolerance of P. aeruginosa and E. coli grown in biofilms depends on
(p)ppGpp (45-47). Also, in 2014, Helaine and colleagues performed an experiment where they
studied the invasion of mouse macrophages by Salmonella enterica, and they observed that
(p)ppGpp production by bacteria residing in acidified vacuoles of macrophages was required
for persistence (13).
3. (p)ppGpp as a mediator in bacterial growth.
Accumulation of (p)ppGpp promotes transcriptional alterations in the bacterial cell, such as
general repression of rapid growth genes and activation of genes involved in amino acid
biosynthesis and survival to stress (29). Therefore, loss of (p)ppGpp in some conditions (for
example minimal medium) leads to amino acid auxotrophy of the whole population and an
increased survival due to high tolerance. Similarly, many other conditions or mutations that
decrease bacterial growth rate have been shown to induce the same tolerant phenotype (48).
Several authors suggested that, at least in Salmonella, there was no specific molecular
pathway underlying bacterial persistence, but that slow growth was the main factor to induce
persistence (49, 50). Regarding the involvement of (p)ppGpp in bacterial growth, it has been
shown that relA spoT double null mutants of E. coli, completely depleted of (p)ppGpp, are
more elongated than wild-type cells (51). LpxC, a key enzyme that catalyzes one of the first
steps in the synthesis of lipopolysaccharide (LPS) in E. coli, is degraded during slow growth but
stabilized when cells grow faster (Figure 1) (52). Hence, in 2013 it was reported that in relA
spot double null mutants, there was a deregulation of LpxC degradation, resulting in rapid
proteolysis in fast-growing cells and stabilization during slow growth, in opposition to the
normal state where (p)ppGpp is present (52). In 2015, Yamaguchi and colleagues found that elevated levels of (p)ppGpp led to inhibition of bacterial growth by interfering with the FtsZ protein assembly in *Salmonella paratyphi* A (53). FtsZ is a protein essential for the prokaryotic cell division that needs to form linear structures and has a GTP binding site; however, increased levels of (p)ppGpp (20-fold higher than the required GTP levels) causes FtsZ to form helical structures unable to form the Z-ring at the division site (53), which impairs the division of the bacterial cell and, therefore, the population growth. In short, high levels of (p)ppGpp can promote persistence by halting growth in a subpopulation (even in a rich-nutrient medium), while absence of this alarmone can contribute to tolerance (by preventing the whole population to appropriately handle a nutritional stress).

4. Association between other molecular mechanisms and (p)ppGpp in persistence

According to Trastoy and colleagues (7), some of the molecular mechanisms that have been related to bacterial persistence are TA systems, QS and secretion systems, efflux pumps, SOS system and ROS response (Figure 2A). We focus only on those where a link between (p)ppGpp or SR and persistence has been reported: TA systems, efflux pumps and ROS systems (Figure 2B):

4.1 TA systems

The persistent state actually describes many different growth-arrested physiologies and it has been associated, during years, with the activity of TA systems, at least partially (54, 55). TA systems are a module of two genes encoding a stable toxin and a usually unstable antitoxin which is degraded under stress conditions; however, bound antitoxin to toxin is not likely the source of free toxin since the two proteins are bound tightly (56) (Figure 2A and B). Once unbound toxin is produced, the harmful toxin slows growth, maintains plasmids (57), inhibits phage (58) and induces biofilm formation (59, 60). TA systems are widely distributed in pathogenic bacteria and found in the bacterial chromosome, plasmids and bacteriophages (61, 62).

In 1983, Moyed and Bertrand identified the first persistence gene related to increased survival in presence of ampicillin in *E. coli* (63). They discovered a high persistence, gain of function mutation, named hipA7; hipA encodes the toxin of the HipA/HipB TA system. Similarly, the second link between TA systems and persistence was reported by Aizenman and colleagues in 1996, when they observed that (p)ppGpp was required to activate MazF toxicity, the toxin of the MazF/MazE TA system (64). In 2003, Korch and colleagues studied the role of HipAB TA system in persistence, showing that the ability of *E. coli* to survive to a prolonged exposure to penicillin was due to two mutations in the non-toxic hipA7 allele (65). Both mutations, G22S and D291A, were required for the full range of phenotypes associated with high persistence,
increasing 1,000- to 10,000-fold the frequency of persisters. Moreover, in this study they also demonstrated that relA knockouts diminished the high persistent phenotype in hipA7 mutants, and that relA spoT knockouts completely eliminated this high persistence, suggesting that hipA7 facilitates the establishment of the persistent state by inducing (p)ppGpp synthesis (65). This result was confirmed in 2011 by Nguyen and colleagues in P. aeruginosa, where a relA spot double mutant led to a decrease of 68-fold in persistence (45). Interestingly, Correia and colleagues demonstrated that HipA exhibits a eukaryotic serine-threonine kinase activity, required for both the inhibition of cell growth and the stimulation of persister cells (66). In 2009, Schumacher and colleagues suggested that HipA inhibited cell growth by the phosphorylation of the essential Elongation Factor Tu (EF-Tu), involved in translation (67). Notwithstanding, in 2013, Germain and colleagues challenged the previous model in which HipA inactivated translation by the phosphorylation of the EF-Tu, and proposed a novel paradigm in which HipA inactivates the glutamyl-tRNA-synthetase (GltX) by phosphorylation of the conserved Ser239 near the active center (68). In addition, the authors claimed that this phosphorylation inhibited aminoacylation, which halts translation and induces the SR by the generation of “hungry” codons at the ribosomal A site. Thus, RelA binds to the ribosome, is activated, and increases (p)ppGpp levels that inhibit translation, transcription, replication and cell-wall synthesis, thereby leading to slow growth, multidrug tolerance, and persistence (Figure 1) (47, 68-70).

In this context, further studies have been conducted to unravel the association of TA systems and (p)ppGpp in persistence. In 2012, Gerdes and Maisonneuve reported that the removal of 10 mRNAse-encoding TA loci of E. coli led to a dramatic decrease of persistence in the presence of the antibiotic (71). A similar phenomenon was observed with a mutant lacking Lon protease, which indicated that TA systems and Lon protease were somehow correlated and both implicated in the persistence of E. coli (72). Since many antitoxins of E. coli were degraded by Lon protease, Gerdes and Maisonneuve hypothesized that HipB was one of the targets. In E. coli, Lon can be activated by polyphosphate (PolyP), synthesized by polyphosphate kinase (PPK) and degraded by an exopolyphosphatase (PPX). PPX is inhibited by (p)ppGpp, which leads to an increase in PolyP. Hence, these authors claimed that high levels of (p)ppGpp associated with persistence inhibited PPX, thus allowing PolyP to activate Lon protease, which would degrade the HipB antitoxin. Furthermore, they suggested that PolyP functions as an intracellular signaling molecule controlled by the SR, and (p)ppGpp reprograms the cells to survive starvation. (71). In 2014, these authors completed this model by adding one additional step, which is a speculative positive-feedback loop that ensures even more synthesis of (p)ppGpp (42). Thus, the model predicts that the degradation of HipB enables free HipA to...
8 phosphorylate GltX and inhibits charging of tRNA-glu. When uncharged tRNAs enter the ribosomal A site, RelA-dependent synthesis of (p)ppGpp is triggered (Table 1 and Figure 1) (42). Even though this model became very influential, the authors retracted the key articles in 2018, claiming that the apparent inhibition of persistence in the multiple-deletion strain was due to an inadvertent lysogenisation with the bacteriophage Φ80, a contaminant that caused artefacts in their experiments (73). In addition, there were conflicting reports for the (p)ppGpp/PolyP/Lon persistence model, such as that Lon protease is not activated but deactivated by PolP (74), or not required for persistence (Table 1 and Figure 2B) (75).

In 2019, Pontes and Groisman studied the implication of TA modules and (p)ppGpp in the persistence of Salmonella and they revealed that low cytoplasmic Mg$^{2+}$ induced tolerance to antibiotics independently of (p)ppGpp and TA modules (Table 1) (49). In fact, a relA spoT double mutant of Salmonella, unable to produce (p)ppGpp, exhibited similar tolerance to antibiotics after growing in low Mg$^{2+}$ than the wild-type strain. The same phenomenon occurred with the mutant strain lacking 12 TA systems (Δ12TA). However, when the antibiotic treatment was added at neutral pH, they saw five- to eightfold fewer persisters compared to WT both in the Δ12TA strain and in double mutant relA spoT (49). Nonetheless, when they deleted one single TA module found that this mutation had no effect in persistence (49) (Figure 2B).

Recently, Song and Wood (2020), claimed that TA systems were not involved in the formation of persistent bacteria (76). The many contradictory observations in diverse experimental setups lead to no clear conclusive understanding of the implications of TA systems in persistence and further work is needed.

### 4.2 Efflux Pumps

Efflux pumps are proteic complexes that allow bacteria to draw out intracellular toxins or antibiotic molecules. Some genes encoding efflux pumps are upregulated in cells that constitute biofilms, which are composed mainly by non-growing, persistent cells (77). This upregulation in persister cells can be triggered by different signals, such as ROS response, QS and (p)ppGpp (78).

The first data that linked SR with efflux pumps were apported by Wang and colleagues, who observed that a ppx2 (encoding the exopolyphosphatase that degrades poly(P)) knockout mutant of M. tuberculosis (where poly(P) and (p)ppGpp accumulate) exhibited increased levels of several efflux genes, including iniA, iniB, mmpL10 and Rv2459 (Figure 2B). Notwithstanding, the authors concluded that the element which contributed the most to isoniazid tolerance in this mutant was the changes in the cell wall thickness, as they limited the diffusion of polar molecules such as isoniazid (79).
Some years later, in 2018, Ge and colleagues described that a glucose/galactose transporter of *H. pylori*, Hp1174, functions as an efflux pump and is highly expressed in biofilm-forming and MDR *H. pylori* strains. This transporter, encoded by the *gluP* gene, is upregulated by SpoT (78). A *H. pylori* mutant lacking *gluP* gene and its product Hp1174 constituted an unstructured biofilm whose matrix was damaged. As this study revealed that SpoT enzyme upregulates Hp1174 in persistent biofilm-forming cells, and provided that the transcription of this gene is controlled by an alternative sigma factor (σ^54), they hypothesized that SpoT may upregulate the expression of *gluP* by a σ^54-dependent transcription.

Finally, Pu and colleagues demonstrated that β-lactam-induced *E. coli* persisters exhibited less cytoplasmic drug accumulation because of an enhanced efflux activity compared to non-persister cells. Combining time-lapse imaging and mutagenesis techniques, scientists determined a positive correlation between *tolC* expression and arising of *E. coli* persisters (80).

### 4.3 ROS response

The reactive oxygen species (ROS) are produced as a natural response to the normal metabolism of the oxygen and perform important functions in cell signaling and homeostasis. However, when cells are exposed to environmental pressure, such as antibiotics, UV, or heat pressure, ROS levels can increase; this increase can cause damages in the DNA, lipids and proteins, which subsequently leads to cell death. Like all molecular mechanisms, ROS are subject to regulation (Figure 2B). Among the molecules capable of eliminating ROS, we find enzymes, such as superoxide dismutase (SOD) and catalase, as well as antioxidant agents, such as glutathione and vitamin C. However, when an increase in ROS levels occurs due to an imbalance between the production and elimination mechanisms, cells are said to be subject to oxidative stress (7).

The relationship between ROS response and persistent bacteria are widely described in the literature. Nguyen and colleagues, in 2011, showed that the survival of multidrug-tolerant persisters in biofilms of *P. aeruginosa* was largely dependent on catalase or SOD enzymes, which are under the control of the (p)ppGpp signaling (45). Along the same lines, the study of Khakimova and colleagues (2013) demonstrated that the SR regulates catalases, likely through a complex interplay of regulators (81) (Figure 2A and B). Furthermore, they also demonstrated that H_2O_2 and antibiotic tolerance were the result of a balance between prooxidant stress and antioxidant stress (81). Similarly, the work of Molina-Quiroz and colleagues (2018), gave more evidence of the relationship between oxidative stress and bacterial survival to antibiotics. In this study, the authors demonstrated the impact of ROS on the generation of persister cells, exposing the cultures of a WT strain and its corresponding mutant lacking the cAMP synthase adenylate cyclase (ΔcyaA) under the antibiotic pressure of ampicillin in the presence and
absence of oxygen. For both strains, they observed a 100-fold increase of ampicillin survival in absence of oxygen compared to the strain under aerobic conditions. This study concludes that the damage that ROS cause in the DNA was regulated by cAMP, a negative regulator of persistence in uropathogenic E. coli (82).

5. PRDP: (p)ppGpp ribosome dimerization persister model

Song and Wood, proposed a novel model in which the alarmone (p)ppGpp would generate persister cells by inactivating ribosomes via the Rmf and the hibernation promoting factor (Hpf) (Figure 1 and 2B, Table 1) (83). Among their findings, the following should be highlighted: (i) E. coli persisters contain a large fraction of inactivated 100S ribosomes; (ii) Rmf and Hpff induced persistence, and the inactivation of these proteins increased the single cell persister resuscitation, and (iii) (p)ppGpp did not affect the single-cell persister resuscitation. In another work it was reported that (p)ppGpp induced the Hpf, converting the 90S ribosomes into 100S ribosomes, and that overproduction of Rmf and Hpf increased persistence as well as reduced single cell resuscitation (84). Furthermore, authors based their theory on the fact that (p)ppGpp inhibits the ribosome-associated GTPase Era, essential in the assembling of ribosomal 30S subunits in Staphylococcus aureus (85). Hence, a connection between (p)ppGpp and persistence via ribosome dimerization was demonstrated.

In 2019, Libby and colleagues showed that there was an enormous variability in sasA expression (the gene encoding SasA in B. subtilis) among bacterial cells, linking a higher expression of sasA with an increase in antibiotic survival (86). (p)ppGpp synthetases in B. subtilis, such as SasA, are important in ribosome dimerization, as YwaC induces the transcription of yyyD gene, whose product, YvyD protein, is essential for the dimerization of 70S ribosomes (87). 70S dimers are similar to the above-mentioned 100S ribosomes in E. coli, therefore translationally inactive. These results agree with the PRDP model, where (p)ppGpp would induce bacterial persistence by promoting ribosome dimerization and compromising the translation inside the cells (83).

6. Anti-persisters treatments with (p)ppGpp as a target

Most antibiotics used in clinics target active metabolic processes. Therefore, bacteria that exhibit a reduction in metabolism and growth rate, such as tolerant or persistent cells, are not a target for the classic antibiotics. In the literature, a few reviews summarizing some of the most useful strategies to combat persistent infectious diseases can be found (2, 88, 89).

As the activation of the SR leads to the shutdown of nearly all metabolic processes and the entrance into a state of dormancy, an interesting therapeutic approach to combat persistent infections is the inhibition of the SR network. Hobbs and Boranson (2019) reviewed the recent attempts that have been made to design and discover inhibitors of the SR, and they concluded...
that there are currently two approaches: (i) inhibition of (p)ppGpp synthetases by using (p)ppGpp analogs and (ii) inhibition of (p)ppGpp accumulation by using protein inhibitors (90).

i. Inhibition of (p)ppGpp synthetases by using (p)ppGpp analogs

Several in vitro studies using double relA spoT null mutants in E. coli have shown that this bacterium lacks the (p)ppGpp and therefore has significantly reduced persistence to antibiotics. In this context, some compounds that inhibit the (p)ppGpp production (thus SR), had been developed to abolish persistence. Relacin, one of these compounds, was first designed in 2012 by Wexselblatt and colleagues, and was shown to inhibit Rel-mediated (p)ppGpp synthesis, leading to the death of B. subtilis with an estimated IC_{50} of 200 μM (Table 2) (91). Importantly, relacin also prevented the formation of spores and biofilm in this species. Before bacterial death, relacin also induced a prolonged exponential phase. In 2017, Syal and colleagues performed a slight modification of relacin and found that cells of Mycobacterium smegmatis treated with this molecule were not able to establish any biofilm and were elongated, showing exactly the same phenotype as a rel- mutant (92). Interestingly, this relacin-derived compound lacked toxicity with human red blood cells and has good permeability into the human lung epithelial cells. One of the most persistent pathogens, M. tuberculosis, could be potentially targeted with this (p)ppGpp inhibitor if its evaluation in humans turns to be effective and safe (Table 2).

In order to find other inhibitors of Rel protein, Dutta and colleagues (2019) performed a high-throughput screening approach, using Rel from M. tuberculosis and a novel (p)ppGpp synthetase assay, based on detection of AMP released after Rel catalyzes the transfer of pyrophosphate groups from ATP to GTP/GDP (93). This screening led to the identification of the most potent Rel inhibitor to date, the compound X9, which exhibited an IC_{50} of ~15 μM against purified Rel. At 4 μM, when M. tuberculosis was nutrient-starved, it enhanced its susceptibility against isoniazid (Table 2). Even if the molecular mechanism by which X9 inhibits Rel is not fully understood yet, this compound displays the most potent activity of any Rel inhibitor to date.

ii. Inhibition of (p)ppGpp accumulation

A second approach to design anti-persistence strategies that target (p)ppGpp would be the inhibition of the accumulation of this alarmon. Biofilms are very important in the establishment and maintenance of many infections caused by pathogenic bacteria, therefore some cationic peptides with anti-biofilm abilities have been tested and proposed to act via disruption of the SR (11, 90).

The 1018 peptide (VRLIVAVRIWRR-NH2) is a small, synthetic, L-amino acid peptide derived from a bovine host defense peptide. De la Fuente and colleagues (2014) first described that
1018 marks (p)ppGpp for degradation, exhibiting potent activity against biofilms produced by Gram-positive (S. aureus) and Gram-negative bacteria (E. coli, P. aeruginosa, K. pneumoniae or A. baumannii), but not in planktonic cultures (94). They also observed that the 1018 peptide prevented the biofilm formation and degraded the pre-formed biofilm (as old as 2 days). As an overproduction of (p)ppGpp leads to resistance to the 1018 peptide, the authors suggested that this peptide specifically targeted SR (Table 2). The same research group generated another small peptide, called 1037, and observed its effects on biofilm formation and swarming motility in P. aeruginosa, Burkholderia cenocepacia and Gram-positive Listeria monocytogenes (Table 2) (95). They observed that 1037 reduced flagella-dependent swimming in P. aeruginosa PA14, P. aeruginosa PAO1, and B. cenocepacia; consistently, transcriptomic analysis revealed that, in the presence of 1037, several genes related to flagella were downregulated by 2- to 3-fold. This is interesting because flagella are involved in biofilm formation and swarming motility, both significantly inhibited by the action of 1037 in the tested species (95).

Notwithstanding, the study of Andresen and colleagues in 2016 rejected the idea that 1018 peptide specifically targets the SR alarmone (p)ppGpp (96). Furthermore, they observed that in P. aeruginosa this peptide showed anti-bacterial activity both in planktonic and in biofilm-derived cells.

Interestingly, Allison and colleagues published an innovative article in 2011 where they killed E. coli and S. aureus persisters combining different metabolites with aminoglycosides (97). They reported that glucose, pyruvate, mannitol or fructose significantly increased the PMF; this leads to a higher uptake of the aminoglycoside and the consequent killing of the persisters, either in vitro and in a mouse model carrying a catheter colonized by uropathogenic E. coli. In conclusion, these findings mean that some of these PMF-stimulating metabolites might be a good adjuvant to aminoglycoside to treat persistent, chronic infections (97).

7. Discussion

We have summarized the functions of (p)ppGpp regarding its role as a global transcription and translation regulator of metabolism, slow growth and dormancy, nutrient starvation, different kinds of stress, virulence, tolerance to antibiotics, persister cell formation and even persistence inside macrophages. However, an accurate role of this alarmone in persistence has not been determined yet. Clearly, evidence relates this molecule to the persistent phenotype, based on its dominant role in the stress response of bacteria. The diversity among the conclusions obtained by laboratories around the globe raised the question of whether persisters in phylogenetically close organisms are produced through different pathways (49, 98). Nevertheless, this seems unlikely as the SR is a universal, highly
conserved network in many phyla, and all microbes use it to protect themselves against different types of stress. The relevance of the role of the ATP in the antibiotic persistence is revealed as tolerant cells slow down their metabolism and persistent cells are quiescent. Pontes and Groisman (2019) showed that *Salmonella* pre-exposed to chloramphenicol resisted the killing by bactericidal antibiotics (49). However, contradictory results have been obtained from different groups indicating that ATP does not control persistence (49, 99, 100), or even that persister cell formation is based on reducing ATP (101-104).

The results of Pontes and Groisman (2019) (49), agree with the findings of both Hobby (105) and Bigger (106) and with many other researchers who have shown that deliberate induction of bacteriostasis promotes antibiotic tolerance (15). Whereas deliberate induction of bacteriostasis overrides bacterial control of growth, it remains to be explored what mechanisms promote growth arrest in individual cells. They showed that *Salmonella* persisters emerged as a result of slow growth alone and transitory disturbances to core activities, regardless of the underlying physiological process. They also performed studies with *Salmonella* mutants lacking 12 TA modules and observed their implication in persistence in some conditions (49). Kaldalu and colleagues (2019) claimed that there was no specific molecular mechanism involved in persistence but this latter was simply produced by slow growth of bacteria (50).

It remains unclear if efflux pumps and the SOS-system have a real link with (p)ppGpp. Despite being important molecular mechanisms widely studied in pathogenic bacteria, there is still few literature linking (p)ppGpp metabolism with these mechanisms, and it is even trickier the association of these to persistence; a proof of that is the article of Ge and colleagues, where they claim that bifunctional SpoT enzyme up-regulates the Hp1174 efflux pump of *H. pylori*, contributing to biofilm formation (78). Regarding the role of ROS-system in persistence, different research groups have demonstrated that (p)ppGpp and the SR regulate the expression of antioxidant enzymes, e.g SOD or catalases, in order to avoid the intracellular accumulation of ROS (82). Even if the intermediate regulators involved in this pathway need further research, this opens the door to anti-persister therapies targeting SR (45, 81).

Traditionally, the persistence of the bacteria has been widely attributed to TA systems, as Chowdhury and colleagues published in 2016, where they claimed that persister cells can form in the absence of (p)ppGpp (although at much-reduced levels), mainly due to the effect of production of any toxic protein (75). Nevertheless, Dr T. K. Wood, reported some years later an essential role of (p)ppGpp in the establishment of persistence via induction of dimerization of...
ribosomes. In this new model, called PRDP and proposed by Song and colleagues in 2020, there is evidence of a direct role of the magic spot in the persistent phenotype (83).

An interesting issue is the individual variability within a population of cells regarding their tolerance to antibiotics. Whether this heterogeneity is regulated or, on the contrary, is an unavoidable consequence of stochastic fluctuations, remains unknown. In 2004, Balaban and colleagues showed that spontaneous persisters are rare, suggesting that they were not stochastically produced (3). Similarly, the PRDP model also suggests that both persistence and resuscitation are sophisticatedly-regulated by ribosome content and their activation status (83). Finally, another hint of the regulation of SR was proposed by Libby and colleagues (2019), based on the fact that SasA in B. subtilis has multiple sites of phosphorylation, which can explain the cell-to-cell variability in sasA expression (86, 87). These differences may be the reason for the physiologically relevant variability in (p)ppGpp levels and shed some light into the heterogeneity within a bacterial population and their phenotypic variability.

There are some arguments in favor of the PRDP model: one is that Hpf, which converts 90S ribosomes into inactivated 100S ribosomes in E. coli, is highly conserved in most bacteria (84); secondly, other (p)ppGpp synthetases found in B. subtilis are essential for ribosome dimerization in Gram-positive bacteria, generating translationally inactive ribosomes associated with persistence (87).

In this review we have contrasted different models that have been proposed over many years all of them aiming at answering the same question: what is the precise role of (p)ppGpp and SR in bacterial persistence? Definitely, after comparing all those models we can conclude that uncontrolled variables such as contaminants (as Φ80 phage), particular setups that differ from lab to lab, artifacts that mislead to conclusions, changes in the tested strains and, in short, different experimental conditions can be some of the underlying reasons to explain the controversy around this question.

The current lack of effective antibiotics against multi-drug resistant, persister and tolerant pathogens leads the urgency to develop new antibacterial treatments, as the anti-persistent treatments targeting the (p)ppGpp network. The inhibitors of (p)ppGpp synthetases are good candidates as antimicrobial agents, because of their high efficacy in avoiding biofilm formation (91), in the loss of the persistent phenotype and even in the prolongation of exponential phase, when bacteria replicate more actively, being more susceptible to antibiotics. This also opens the door to the possibility of a combination of therapies (inhibitors of (p)ppGpp synthetase with antibiotics) and to the establishment of potential synergies. Even if no effect of the Rel inhibitor relacin was observed in E. coli, the authors suggested that this could be due to the inability of relacin to penetrate Gram negative cells and reach its target. However,
agents as polymyxins can destabilize the outer membrane in Gram negatives facilitating the entrance of therapeutic molecules, e.g., relacin, or endolysins (108). The absence of known (p)ppGpp synthetases in mammalian cells and the specificity of these inhibitors for the Rel protein, make this protein a good candidate as an antibacterial agent.

The second strategy of inhibition of (p)ppGpp accumulation, supported by the 1018 and 1037 peptides, exhibited promising anti-persistent activities as they specifically targeted biofilm-forming cells and had no effect on planktonic cells (94, 95). According to the few studies that focus on inhibiting the SR as an anti-persistent therapeutic approach, we can consider this as an emerging field; therefore, further research and financial investment are needed to efficiently prevent persistent, chronic and life-threatening infections.

8. Conclusion

The emergence of contradictory models about the involvement of the “magic spot” in bacterial persistence highlights the need to deepen the studies in this field. In summary, one potential strategy to fight persistent infectious disease resides in specifically targeting SR or (p)ppGpp of pathogenic bacteria, but further knowledge is necessary to provide a better understanding of the complexity of bacterial persistence, as well as its implications in clinics.
Legends

Figure 1. Physiological pathways regulated by (p)ppGpp and the stringent response in *E. coli*.

Some of the most common human opportunistic pathogens are represented, such as biofilm-forming *P. aeruginosa*, intestinal *E. coli* and skin-borne opportunistic pathogen *S. aureus*. Focusing on *E. coli*: 1) increased levels of (p)ppGpp induce transcription of RpoS, sigma factor for the stationary phase, and RpoE, the sigma factor that regulates the expression of genes related to misfolded proteins; 2) (p)ppGpp inhibits DNA primase thus the replication of the chromosome; 3) (p)ppGpp also inhibits transcription of rRNA, affecting the general translation; 4) (p)ppGpp also deregulates LpxC, an enzyme catalyzing the first step of LPS formation; 5) (p)ppGpp binds to RNAP (RNA polymerase) regulating the transcription of many genes; 6) according to certain models, HipA toxin would phosphorylate glutamyl-tRNA-synthetase (GltX), inactivating it and therefore impairing aminoacylation. Empty tRNAs then trigger the stringent response: RelA associates to ribosome and synthesizes (p)ppGpp from GTP/GDP + ATP; 7) (p)ppGpp can directly inhibit negative supercoiling of DNA in *E. coli*, associated with resistance to quinolones; 8) (p)ppGpp induces the transcription of the ribosome modulating factor (Rmf) and hibernation promoting factor (Hpf), which play a role in ribosome dimerization, typical from persister cells. (p)ppGpp is also involved in immune evasion, virulence and human pathogenesis.

Figure 2. A. Molecular mechanisms underlying bacterial persistence. B. Different models explaining the involvement of (p)ppGpp in persistence and representative publications.

Table 1. Summary of the main ppGpp models.

Table 2. Summary of the main treatments having ppGpp as a target.
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AUTHOR CONTRIBUTIONS

O.P., L.B., I.B., L.F-G., A.A., M.L., wrote the manuscript; G.B., R.C., R.G-C., T.K.W., M.T., supervised and revised the manuscript.

TRANSPARENCY DECLARATIONS

All authors declare no conflict of interest.
REFERENCES


Table 1.

<table>
<thead>
<tr>
<th>Year</th>
<th>Journal</th>
<th>Author</th>
<th>Model</th>
<th>References</th>
</tr>
</thead>
<tbody>
<tr>
<td>2016</td>
<td>Scientific Reports</td>
<td>Chowdhury N., Kwan B.W, and Wood T.K.</td>
<td>The formation of persister cells in E. coli K-12 strain MG1655 is attributed to production of any toxic protein (e.g., MazF, RelB and YafO) and (p)ppGpp is not essential but increases persistence by 1000X.</td>
<td>(75)</td>
</tr>
<tr>
<td>2019</td>
<td>Science Signalling</td>
<td>Pontes M.H. and Groisman E.A.</td>
<td>Low cytoplasmatic Mg²⁺ induces S. typhimurium tolerance to antibiotic independently of (p)ppGpp and TA modules. However, (p)ppGpp reduces antibiotic tolerance under certain conditions.</td>
<td>(49)</td>
</tr>
<tr>
<td>2020</td>
<td>Biochemical and Biophysical Research Communication</td>
<td>Song S. and Wood T.K.</td>
<td>(p)ppGpp generates persister cells directly by inactivation of ribosomes via Rmf and Hpf.</td>
<td>(83)</td>
</tr>
</tbody>
</table>

Rmf: ribosome modulation factor, Hpf: hibernation promoting factor. *Some of these publications have been retracted due to the contamination with the Φ80 bacteriophage causing artefacts in the results.
Table 2.

<table>
<thead>
<tr>
<th>Year</th>
<th>Author</th>
<th>Strategy</th>
<th>Mechanism of action</th>
<th>References</th>
</tr>
</thead>
<tbody>
<tr>
<td>2012</td>
<td>Wexselblatt and colleagues</td>
<td>(p)ppGpp analogs inhibit Rel protein: relacin</td>
<td>Relacin produced death of B. subtilis with an IC_{50}=200 μM, prevention of sporulation and biofilm formation and induction of a prolonged exponential phase.</td>
<td>(91)</td>
</tr>
<tr>
<td>2012</td>
<td>De la Fuente-Núñez and colleagues</td>
<td>Inhibition of (p)ppGpp accumulation: 1037 peptide</td>
<td>1037 reduced expression of flagella-associated genes that favor biofilm establishment in P. aeruginosa and B. cenocepacia. It also reduced the swarming motility.</td>
<td>(95)</td>
</tr>
<tr>
<td>2014</td>
<td>De la Fuente-Núñez and colleagues</td>
<td>Inhibition of (p)ppGpp accumulation: 1018 peptide</td>
<td>1018 marked (p)ppGpp for degradation*: broad anti-biofilm activity against Gram positive and negative bacteria and lack of effect for planktonic cultures.</td>
<td>(94)</td>
</tr>
<tr>
<td>2017</td>
<td>Syal and colleagues</td>
<td>(p)ppGpp analog to inhibit Rel protein: modification of relacin</td>
<td>Impairing of biofilm formation by M. smegmatis and arising of elongated cells. Lack of toxicity, good permeability to human lung epithelial cells.</td>
<td>(92)</td>
</tr>
<tr>
<td>2019</td>
<td>Dutta and colleagues</td>
<td>(p)ppGpp analog to inhibit Rel protein: compound X9</td>
<td>Highest inhibitory activity against Rel protein: IC_{50} of ~15 μM against purified Rel of M. tuberculosis. Enhancement of susceptibility against isoniazid.</td>
<td>(93)</td>
</tr>
</tbody>
</table>

*Andresen and colleagues rejected this hypothesis two years later, questioning its specificity for (p)ppGpp and for biofilm-forming cells (96).