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

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35 **ABSTRACT**

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

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55 **1. Introduction**

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

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

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

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

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

84 Differences between resistance, persistence and tolerance have been established;
85 nonetheless, there are also relationships among these bacterial populations which are worthy
86 of consideration. Despite evidence showing that tolerance and persistence to antibiotics
87 promote the evolution of resistance in bacteria (8, 14, 15) both mechanisms are currently
88 underestimated by the scientific and medical communities.

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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

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124 transcription causing a global re-wiring of the gene expression profile. Taken together, all
125 these changes lead to dormancy or slow growth for most cells (Figure 1) (31, 32).
126 Most Gram-positive bacteria possess ~~only~~ one long RSH, named Rel, which has both (p)ppGpp
127 synthetase and hydrolase activities (33, 34) together with a short RSH, named SAS (Small
128 Alarmone Synthetase) or SAH (Small Alarmone Hydrolase) (35). Nonetheless, the existence of a
129 diverse population of (p)ppGpp synthetases and hydrolases in bacteria has been demonstrated
130 (35-38).
131 There is evidence that (p)ppGpp is related to antibiotic tolerance and persister cell formation
132 (25, 39-42). For example, increased levels of (p)ppGpp inhibit negative supercoiling of DNA in
133 *E. coli*, thus preventing DNA replication and transcription, resulting in tolerance towards
134 ofloxacin and quinolones (Figure 1) (43, 44). Persister cells tend to form in biofilms, in which
135 the bacterial cells are embedded in a three-dimensional matrix that provides protection during
136 pathogenesis and other conditions. Thus, it is logical that bacteria in biofilms encounter limited
137 access to nutrients and therefore display the SR (45). Consistently, different groups have
138 shown that multidrug tolerance of *P. aeruginosa* and *E. coli* grown in biofilms depends on
139 (p)ppGpp (45-47). Also, in 2014, Helaine and colleagues performed an experiment where they
140 studied the invasion of mouse macrophages by *Salmonella enterica,* and they observed that
141 (p)ppGpp production by bacteria residing in acidified vacuoles of macrophages was required
142 for persistence (13).

143 **3. (p)ppGpp as a mediator in bacterial growth.**

144 Accumulation of (p)ppGpp promotes transcriptional alterations in the bacterial cell, such as
145 general repression of rapid growth genes and activation of genes involved in amino acid
146 biosynthesis and survival to stress (29). Therefore, loss of (p)ppGpp in some conditions (for
147 example minimal medium) leads to amino acid auxotrophy of the whole population and an
148 increased survival due to high tolerance. Similarly, many other conditions or mutations that
149 decrease bacterial growth rate have been shown to induce the same tolerant phenotype (48).
150 Several authors suggested that, at least in *Salmonella*, there was no specific molecular
151 pathway underlying bacterial persistence, but that slow growth was the main factor to induce
152 persistence (49, 50). Regarding the involvement of (p)ppGpp in bacterial growth, it has been
153 shown that *relA spoT* double null mutants of *E. coli*, completely depleted of (p)ppGpp, are
154 more elongated than wild-type cells (51). LpxC, a key enzyme that catalyzes one of the first
155 steps in the synthesis of lipopolysaccharide (LPS) in *E. coli,* is degraded during slow growth but
156 stabilized when cells grow faster (Figure 1) (52). Hence, in 2013 it was reported that in *relA*
157 *spot* double null mutants, there was a deregulation of LpxC degradation, resulting in rapid
158 proteolysis in fast-growing cells and stabilization during slow growth, in opposition to the

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159 normal state where (p)ppGpp is present (52). In 2015, Yamaguchi and colleagues found that
160 elevated levels of (p)ppGpp led to inhibition of bacterial growth by interfering with the FtsZ
161 protein assembly in *Salmonella paratyphi* A (53). FtsZ is a protein essential for the prokaryotic
162 cell division that needs to form linear structures and has a GTP binding site; however,
163 increased levels of (p)ppGpp (20-fold higher than the required GTP levels) causes FtsZ to form
164 helical structures unable to form the Z-ring at the division site (53), which impairs the division
165 of the bacterial cell and, therefore, the population growth. In short, high levels of (p)ppGpp
166 can promote persistence by halting growth in a subpopulation (even in a rich-nutrient
167 medium), while absence of this alarmone can contribute to tolerance (by preventing the whole
168 population to appropriately handle a nutritional stress).

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

170 According to Trastoy and colleagues (7), some of the molecular mechanisms that have been
171 related to bacterial persistence are TA systems, QS and secretion systems, efflux pumps, SOS
172 system and ROS response (Figure 2A). We focus only on those where a link between (p)ppGpp
173 or SR and persistence has been reported: TA systems, efflux pumps and ROS systems (Figure
174 2B):

175 4.1 TA systems

176 The persistent state actually describes many different growth-arrested physiologies and it has
177 been associated, during years, with the activity of TA systems, at least partially (54, 55). TA
178 systems are a module of two genes encoding a stable toxin and a usually unstable antitoxin
179 which is degraded under stress conditions; however, bound antitoxin to toxin is not likely the
180 source of free toxin since the two proteins are bound tightly (56) (Figure 2A and B). Once
181 unbound toxin is produced, the harmful toxin slows growth, maintains plasmids (57), inhibits
182 phage (58) and induces biofilm formation (59, 60). TA systems are widely distributed in
183 pathogenic bacteria and found in the bacterial chromosome, plasmids and bacteriophages (61,
184 62).

185 In 1983, Moyed and Bertrand identified the first persistence gene related to increased survival
186 in presence of ampicillin in *E. coli* (63). They discovered a high persistence, gain of function
187 mutation, named *hipA7; hipA* encodes the toxin of the HipA/HipB TA system. Similarly, the
188 second link between TA systems and persistence was reported by Aizenman and colleagues in
189 1996, when they observed that (p)ppGpp was required to activate MazF toxicity, the toxin of
190 the MazF/MazE TA system (64). In 2003, Korch and colleagues studied the role of HipAB TA
191 system in persistence, showing that the ability of *E. coli* to survive to a prolonged exposure to
192 penicillin was due to two mutations in the non-toxic *hipA7* allele (65). Both mutations, G22S
193 and D291A, were required for the full range of phenotypes associated with high persistence,

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194 increasing 1,000- to 10,000-fold the frequency of persisters. Moreover, in this study they also
195 demonstrated that *relA* knockouts diminished the high persistent phenotype in *hipA7* mutants,
196 and that *relA spoT* knockouts completely eliminated this high persistence, suggesting that
197 *hipA7* facilitates the establishment of the persistent state by inducing (p)ppGpp synthesis (65).
198 This result was confirmed in 2011 by Nguyen and colleagues in *P. aeruginosa,* where a *relA*
199 *spot* double mutant led to a decrease of 68-fold in persistence (45). Interestingly, Correia and
200 colleagues demonstrated that HipA exhibits a eukaryotic serine-threonine kinase activity,
201 required for both the inhibition of cell growth and the stimulation of persister cells (66). In
202 2009, Schumacher and colleagues suggested that HipA inhibited cell growth by the
203 phosphorylation of the essential Elongation Factor Tu (EF-Tu), involved in translation (67).
204 Notwithstanding, in 2013, Germain and colleagues challenged the previous model in which
205 HipA inactivated translation by the phosphorylation of the EF-Tu, and proposed a novel
206 paradigm in which HipA inactivates the glutamyl-tRNA-synthetase (GltX) by phosphorylation of
207 the conserved Ser^{239} near the active center (68). In addition, the authors claimed that this
208 phosphorylation inhibited aminoacylation, which halts translation and induces the SR by the
209 generation of "hungry" codons at the ribosomal A site. Thus, RelA binds to the ribosome, is
210 activated, and increases (p)ppGpp levels that inhibit translation, transcription, replication and
211 cell-wall synthesis, thereby leading to slow growth, multidrug tolerance, and persistence
212 (Figure 1) (47, 68-70).
213 In this context, further studies have been conducted to unravel the association of TA systems
214 and (p)ppGpp in persistence. In 2012, Gerdes and Maisonneuve reported that the removal of
215 10 mRNAse-encoding TA loci of *E. coli* led to a dramatic decrease of persistence in the
216 presence of the antibiotic (71). A similar phenomenon was observed with a mutant lacking Lon
217 protease, which indicated that TA systems and Lon protease were somehow correlated and
218 both implicated in the persistence of *E. coli* (72). Since many antitoxins of *E. coli* were
219 degraded by Lon protease, Gerdes and Maisonneuve hypothesized that HipB was one of the
220 targets. In *E. coli*, Lon can be activated by polyphosphate (PolyP), synthesized by
221 polyphosphate kinase (PPK) and degraded by an exopolyphosphatase (PPX). PPX is inhibited by
222 (p)ppGpp, which leads to an increase in PolyP. Hence, these authors claimed that high levels of
223 (p)ppGpp associated with persistence inhibited PPX, thus allowing PolyP to activate Lon
224 protease, which would degrade the HipB antitoxin. Furthermore, they suggested that PolyP
225 functions as an intracellular signaling molecule controlled by the SR, and (p)ppGpp reprograms
226 the cells to survive starvation. (71). In 2014, these authors completed this model by adding one
227 additional step, which is a speculative positive-feedback loop that ensures even more synthesis
228 of (p)ppGpp (42). Thus, the model predicts that the degradation of HipB enables free HipA to

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229 phosphorylate GltX and inhibits charging of tRNA-glu. When uncharged tRNAs enter the
230 ribosomal A site, RelA-dependent synthesis of (p)ppGpp is triggered (Table 1 and Figure 1)
231 (42). Even though this model became very influential, the authors retracted the key articles in
232 2018, claiming that the apparent inhibition of persistence in the multiple-deletion strain was
233 due to an inadvertent lysogenisation with the bacteriophage Φ80, a contaminant that caused
234 artefacts in their experiments (73). In addition, there were conflicting reports for the
235 (p)ppGpp/PolyP/Lon persistence model, such as that Lon protease is not activated but
236 deactivated by PolP (74), or not required for persistence (Table 1 and Figure 2B) (75).
237 In 2019, Pontes and Groisman studied the implication of TA modules and (p)ppGpp in the
238 persistence of *Salmonella* and they revealed that low cytoplasmic Mg^{2+} induced tolerance to
239 antibiotics independently of (p)ppGpp and TA modules (Table 1) (49). In fact, a *relA spoT*
240 double mutant of *Salmonella*, unable to produce (p)ppGpp, exhibited similar tolerance to
241 antibiotics after growing in low Mg^{2+} than the wild-type strain. The same phenomenon
242 occurred with the mutant strain lacking 12 TA systems (Δ12TA). However, when the antibiotic
243 treatment was added at neutral pH, they saw five- to eightfold fewer persisters compared to
244 WT both in the Δ12TA strain and in double mutant *relA spoT* (49). Nonetheless, when they
245 deleted one single TA module found that this mutation had no effect in persistence (49)
246 (Figure 2B).
247 Recently, Song and Wood (2020), claimed that TA systems were not involved in the formation
248 of persistent bacteria (76). The many contradictory observations in diverse experimental
249 setups lead to no clear conclusive understanding of the implications of TA systems in
250 persistence and further work is needed.

251 4.2 Efflux Pumps

252 Efflux pumps are proteic complexes that allow bacteria to draw out intracellular toxins or
253 antibiotic molecules. Some genes encoding efflux pumps are upregulated in cells that
254 constitute biofilms, which are composed mainly by non-growing, persistent cells (77). This
255 upregulation in persister cells can be triggered by different signals, such as ROS response, QS
256 and (p)ppGpp (78).
257 The first data that linked SR with efflux pumps were apported by Wang and colleagues, who
258 observed that a *ppx2* (encoding the exopolyphosphatase that degrades poly(P)) knockout
259 mutant of *M. tuberculosis* (where poly(P) and (p)ppGpp accumulate) exhibited increased levels
260 of several efflux genes, including *iniA*, *iniB*, *mmpL10* and *Rv2459* (Figure 2B). Notwithstanding,
261 the authors concluded that the element which contributed the most to isoniazid tolerance in
262 this mutant was the changes in the cell wall thickness, as they limited the diffusion of polar
263 molecules such as isoniazid (79).

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264 Some years later, in 2018, Ge and colleagues described that a glucose/galactose transporter of
265 *H. pylori*, Hp1174, functions as an efflux pump and is highly expressed in biofilm-forming and
266 MDR *H. pylori* strains. This transporter, encoded by the *gluP* gene, is upregulated by SpoT (78).
267 A *H. pylori* mutant lacking *gluP* gene and its product Hp1174 constituted an unstructured
268 biofilm whose matrix was damaged. As this study revealed that SpoT enzyme upregulates
269 Hp1174 in persistent biofilm-forming cells, and provided that the transcription of this gene is
270 controlled by an alternative sigma factor (σ^{54}), they hypothesized that SpoT may upregulate
271 the expression of *gluP* by a σ^{54}-dependent transcription.
272 Finally, Pu and colleagues demonstrated that β-lactam-induced *E. coli* persisters exhibited less
273 cytoplasmic drug accumulation because of an enhanced efflux activity compared to non-
274 persister cells. Combining time-lapse imaging and mutagenesis techniques, scientists
275 determined a positive correlation between *tolC* expression and arising of *E. coli* persisters (80).
276 4.3 ROS response
277 The reactive oxygen species (ROS) are produced as a natural response to the normal
278 metabolism of the oxygen and perform important functions in cell signaling and homeostasis.
279 However, when cells are exposed to environmental pressure, such as antibiotics, UV, or heat
280 pressure, ROS levels can increase; this increase can cause damages in the DNA, lipids and
281 proteins, which subsequently leads to cell death. Like all molecular mechanisms, ROS are
282 subject to regulation (Figure 2B). Among the molecules capable of eliminating ROS, we find
283 enzymes, such as superoxide dismutase (SOD) and catalase, as well as antioxidant agents, such
284 as glutathione and vitamin C. However, when an increase in ROS levels occurs due to an
285 imbalance between the production and elimination mechanisms, cells are said to be subject to
286 oxidative stress (7).
287 The relationship between ROS response and persistent bacteria are widely described in the
288 literature. Nguyen and colleagues, in 2011, showed that the survival of multidrug-tolerant
289 persisters in biofilms of *P. aeruginosa* was largely dependent on catalase or SOD enzymes,
290 which are under the control of the (p)ppGpp signaling (45). Along the same lines, the study of
291 Khakimova and colleagues (2013) demonstrated that the SR regulates catalases, likely through
292 a complex interplay of regulators (81) (Figure 2A and B). Furthermore, they also demonstrated
293 that H_2O_2 and antibiotic tolerance were the result of a balance between prooxidant stress and
294 antioxidant stress (81). Similarly, the work of Molina-Quiroz and colleagues (2018), gave more
295 evidence of the relationship between oxidative stress and bacterial survival to antibiotics. In
296 this study, the authors demonstrated the impact of ROS on the generation of persister cells,
297 exposing the cultures of a WT strain and its corresponding mutant lacking the cAMP synthase
298 adenylate cyclase (Δ*cyaA*) under the antibiotic pressure of ampicillin in the presence and

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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 Hpf 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 *yvyD* 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

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334 that there are currently two approaches: (i) inhibition of (p)ppGpp synthetases by using
335 (p)ppGpp analogs and (ii) inhibition of (p)ppGpp accumulation by using protein inhibitors (90).

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

337 Several *in vitro* studies using double *relA spoT* null mutants in *E. coli* have shown that this
338 bacterium lacks the (p)ppGpp and therefore has significantly reduced persistence to
339 antibiotics. In this context, some compounds that inhibit the (p)ppGpp production (thus SR),
340 had been developed to abolish persistence. Relacin, one of these compounds, was first
341 designed in 2012 by Wexselblatt and colleagues, and was shown to inhibit Rel-mediated
342 (p)ppGpp synthesis, leading to the death of *B. subtilis* with an estimated IC_{50} of 200 µM (Table
343 2) (91). Importantly, relacin also prevented the formation of spores and biofilm in this species.
344 Before bacterial death, relacin also induced a prolonged exponential phase. In 2017, Syal and
345 colleagues performed a slight modification of relacin and found that cells of *Mycobacterium*
346 *smegmatis* treated with this molecule were not able to establish any biofilm and were
347 elongated, showing exactly the same phenotype as a *rel*$^-$ mutant (92). Interestingly, this
348 relacin-derived compound lacked toxicity with human red blood cells and has good
349 permeability into the human lung epithelial cells. One of the most persistent pathogens, *M.*
350 *tuberculosis*, could be potentially targeted with this (p)ppGpp inhibitor if its evaluation in
351 humans turns to be effective and safe (Table 2).

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

361 ii. Inhibition of (p)ppGpp accumulation

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

367 The 1018 peptide (VRLIVAVRIWRR-NH2) is a small, synthetic, L-amino acid peptide derived
368 from a bovine host defense peptide. De la Fuente and colleagues (2014) first described that

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369 1018 marks (p)ppGpp for degradation**,** exhibiting potent activity against biofilms produced by
370 Gram-positive (*S. aureus*) and Gram-negative bacteria (*E. coli, P. aeruginosa, K. pneumoniae* or
371 *A. baumannii*), but not in planktonic cultures (94). They also observed that the 1018 peptide
372 prevented the biofilm formation and degraded the pre-formed biofilm (as old as 2 days). As an
373 overproduction of (p)ppGpp leads to resistance to the 1018 peptide, the authors suggested
374 that this peptide specifically targeted SR (Table 2). The same research group generated
375 another small peptide, called 1037, and observed its effects on biofilm formation and
376 swarming motility in *P. aeruginosa*, *Burkholderia cenocepacia* and Gram-positive *Listeria*
377 *monocytogenes* (Table 2) (95). They observed that 1037 reduced flagella-dependent swimming
378 in *P. aeruginosa* PA14, *P. aeruginosa* PAO1, and *B. cenocepacia*; consistently, transcriptomic
379 analysis revealed that, in the presence of 1037, several genes related to flagella were
380 downregulated by 2- to 3-fold. This is interesting because flagella are involved in biofilm
381 formation and swarming motility, both significantly inhibited by the action of 1037 in the
382 tested species (95).
383 Notwithstanding, the study of Andresen and colleagues in 2016 rejected the idea that 1018
384 peptide specifically targets the SR alarmone (p)ppGpp (96). Furthermore, they observed that in
385 *P. aeruginosa* this peptide showed anti-bacterial activity both in planktonic and in biofilm-
386 derived cells.
387 Interestingly, Allison and colleagues published an innovative article in 2011 where they killed *E.*
388 *coli* and *S. aureus* persisters combining different metabolites with aminoglycosides (97). They
389 reported that glucose, pyruvate, mannitol or fructose significantly increased the PMF; this
390 leads to a higher uptake of the aminoglycoside and the consequent killing of the persisters,
391 either in vitro and in a mouse model carrying a catheter colonized by uropathogenic *E. coli*. In
392 conclusion, these findings mean that some of these PMF-stimulating metabolites might be a
393 good adjuvant to aminoglycoside to treat persistent, chronic infections (97).

394 **7. Discussion**

395 We have summarized the functions of (p)ppGpp regarding its role as a global transcription and
396 translation regulator of metabolism, slow growth and dormancy, nutrient starvation, different
397 kinds of stress, virulence, tolerance to antibiotics, persister cell formation and even persistence
398 inside macrophages. However, an accurate role of this alarmone in persistence has not been
399 determined yet. Clearly, evidence relates this molecule to the persistent phenotype, based on
400 its dominant role in the stress response of bacteria.
401 The diversity among the conclusions obtained by laboratories around the globe raised the
402 question of whether persisters in phylogenetically close organisms are produced through
403 different pathways (49, 98). Nevertheless, this seems unlikely as the SR is a universal, highly

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404 conserved network in many phyla, and all microbes use it to protect themselves against
405 different types of stress.
406 The relevance of the role of the ATP in the antibiotic persistence is revealed as tolerant cells
407 slow down their metabolism and persistent cells are quiescent. Pontes and Groisman (2019)
408 showed that *Salmonella* pre-exposed to chloramphenicol resisted the killing by bactericidal
409 antibiotics (49). However, contradictory results have been obtained from different groups
410 indicating that ATP does not control persistence (49, 99, 100), or even that persister cell
411 formation is based on reducing ATP (101-104).
412 The results of Pontes and Groisman (2019) (49), agree with the findings of both Hobby (105)
413 and Bigger (106) and with many other researchers who have shown that deliberate induction
414 of bacteriostasis promotes antibiotic tolerance (15). Whereas deliberate induction of
415 bacteriostasis overrides bacterial control of growth, it remains to be explored what
416 mechanisms promote growth arrest in individual cells. They showed that *Salmonella* persisters
417 emerged as a result of slow growth alone and transitory disturbances to core activities,
418 regardless of the underlying physiological process. They also performed studies with
419 *Salmonella* mutants lacking 12 TA modules and observed their implication in persistence in
420 some conditions (49). Kaldalu and colleagues (2019) claimed that there was no specific
421 molecular mechanism involved in persistence but this latter was simply produced by slow
422 growth of bacteria (50).
423 It remains unclear if efflux pumps and the SOS-system have a real link with (p)ppGpp. Despite
424 being important molecular mechanisms widely studied in pathogenic bacteria, there is still few
425 literature linking (p)ppGpp metabolism with these mechanisms, and it is even trickier the
426 association of these to persistence; a proof of that is the article of Ge and colleagues, where
427 they claim that bifunctional SpoT enzyme up-regulates the Hp1174 efflux pump of *H. pylori,*
428 contributing to biofilm formation (78). Regarding the role of ROS-system in persistence,
429 different research groups have demonstrated that (p)ppGpp and the SR regulate the
430 expression of antioxidant enzymes, e.g SOD or catalases, in order to avoid the intracellular
431 accumulation of ROS (82). Even if the intermediate regulators involved in this pathway need
432 further research, this opens the door to anti-persister therapies targeting SR (45, 81).
433 Traditionally, the persistence of the bacteria has been widely attributed to TA systems, as
434 Chowdhury and colleagues published in 2016, where they claimed that persister cells can form
435 in the absence of (p)ppGpp (although at much-reduced levels), mainly due to the effect of
436 production of any toxic protein (75). Nevertheless, Dr T. K. Wood, reported some years later an
437 essential role of (p)ppGpp in the establishment of persistence via induction of dimerization of

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438 ribosomes. In this new model, called PRDP and proposed by Song and colleagues in 2020,
439 there is evidence of a direct role of the magic spot in the persistent phenotype (83).
440 An interesting issue is the individual variability within a population of cells regarding their
441 tolerance to antibiotics. Whether this heterogeneity is regulated or, on the contrary, is an
442 unavoidable consequence of stochastic fluctuations, remains unknown. In 2004, Balaban and
443 colleagues showed that spontaneous persisters are rare, suggesting that they were not
444 stochastically produced (3). Similarly, the PRDP model also suggests that both persistence and
445 resuscitation are sophisticatedly-regulated by ribosome content and their activation status
446 (83). Finally, another hint of the regulation of SR was proposed by Libby and colleagues (2019),
447 based on the fact that SasA in *B. subtilis* has multiple sites of phosphorylation, which can
448 explain the cell-to-cell variability in *sasA* expression (86, 87). These differences may be the
449 reason for the physiologically relevant variability in (p)ppGpp levels and shed some light into
450 the heterogeneity within a bacterial population and their phenotypic variability.
451 There are some arguments in favor of the PRDP model: one is that Hpf, which converts 90S
452 ribosomes into inactivated 100S ribosomes in *E. coli*, is highly conserved in most bacteria (84);
453 secondly, other (p)ppGpp synthetases found in *B. subtilis* are essential for ribosome
454 dimerization in Gram-positive bacteria, generating translationally inactive ribosomes
455 associated with persistence (87).
456 In this review we have contrasted different models that have been proposed over many years
457 all of them aiming at answering the same question: what is the precise role of (p)ppGpp and SR
458 in bacterial persistence? Definitely, after comparing all those models we can conclude that
459 uncontrolled variables such as contaminants
460 (as Φ80 phage), particular setups that differ from lab to lab, artifacts that mislead to
461 conclusions, changes in the tested strains and, in short, different experimental conditions can
462 be some of the underlying reasons to explain the controversy around this question.
463 The current lack of effective antibiotics against multi-drug resistant, persister and tolerant
464 pathogens leads the urgency to develop new antibacterial treatments, as the anti-persistent
465 treatments targeting the (p)ppGpp network. The inhibitors of (p)ppGpp synthetases are good
466 candidates as antimicrobial agents, because of their high efficacy in avoiding biofilm formation
467 (91), in the loss of the persistent phenotype and even in the prolongation of exponential
468 phase, when bacteria replicate more actively, being more susceptible to antibiotics. This also
469 opens the door to the possibility of a combination of therapies (inhibitors of (p)ppGpp
470 synthetase with antibiotics) and to the establishment of potential synergies. Even if no effect
471 of the Rel inhibitor relacin was observed in *E. coli*, the authors suggested that this could be due
472 to the inability of relacin to penetrate Gram negative cells and reach its target. However,

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473 agents as polymyxins can destabilize the outer membrane in Gram negatives facilitating the
474 entrance of therapeutic molecules, e. g., relacin, or endolysins (108). The absence of known
475 (p)ppGpp synthetases in mammalian cells and the specificity of these inhibitors for the Rel
476 protein, make this protein a good candidate as an antibacterial agent.
477 The second strategy of inhibition of (p)ppGpp accumulation, supported by the 1018 and 1037
478 peptides, exhibited promising anti-persistent activities as they specifically targeted biofilm-
479 forming cells and had no effect on planktonic cells (94, 95). According to the few studies that
480 focus on inhibiting the SR as an anti-persistent therapeutic approach, we can consider this as
481 an emerging field; therefore, further research and financial investment are needed to
482 efficiently prevent persistent, chronic and life-threatening infections.

483 **8. Conclusion**

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

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

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

530 **Table 1**. Summary of the main ppGpp models.

531 **Table 2.** Summary of the main treatments having ppGpp as a target.

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553 **AUTHOR CONTRIBUTIONS**

554 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.,
555 supervised and revised the manuscript.

556 **TRANSPARENCY DECLARATIONS**

557 All authors declare no conflict of interest

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569 **REFERENCES**

570 1. Lewis K. 2020. The Science of Antibiotic Discovery. Cell 181:29-45.
571 2. Dewachter L, Fauvart M, Michiels J. 2019. Bacterial Heterogeneity and Antibiotic
572 Survival: Understanding and Combatting Persistence and Heteroresistance. Mol Cell
573 76:255-267.
574 3. Balaban NQ, Merrin J, Chait R, Kowalik L, Leibler S. 2004. Bacterial persistence as a
575 phenotypic switch. Science 305:1622-5.
576 4. Brauner A, Fridman O, Gefen O, Balaban NQ. 2016. Distinguishing between resistance,
577 tolerance and persistence to antibiotic treatment, p 320-30, Nat Rev Microbiol, vol 14,
578 England.
579 5. Girgis HS, Harris K, Tavazoie S. 2012. Large mutational target size for rapid emergence
580 of bacterial persistence. Proc Natl Acad Sci U S A 109:12740-5.
581 6. Balaban NQ, Helaine S, Lewis K, Ackermann M, Aldridge B, Andersson DI, Brynildsen
582 MP, Bumann D, Camilli A, Collins JJ, Dehio C, Fortune S, Ghigo JM, Hardt WD, Harms A,
583 Heinemann M, Hung DT, Jenal U, Levin BR, Michiels J, Storz G, Tan MW, Tenson T, Van
584 Melderen L, Zinkernagel A. 2019. Definitions and guidelines for research on antibiotic
585 persistence. Nat Rev Microbiol 17:441-448.
586 7. Trastoy R, Manso T, Fernández-García L, Blasco L, Ambroa A, Pérez Del Molino ML, Bou
587 G, García-Contreras R, Wood TK, Tomás M. 2018. Mechanisms of Bacterial Tolerance
588 and Persistence in the Gatrointestinal and Respiratory Environments. Clin Microbiol
589 Rev 31.
590 8. Windels EM, Michiels JE, Van den Bergh B, Fauvart M, Michiels J. 2019. Antibiotics:
591 Combatting Tolerance To Stop Resistance. MBio 10.
592 9. Zhang Y. 2014. Persisters, persistent infections and the Yin-Yang model. Emerg
593 Microbes Infect 3:e3.
594 10. Lewis K. 2007. Persister cells, dormancy and infectious disease. Nat Rev Microbiol
595 5:48-56.
596 11. Lebeaux D, Chauhan A, Létoffé S, Fischer F, de Reuse H, Beloin C, Ghigo J-M. 2014. pH-
597 mediated potentiation of aminoglycosides kills bacterial persisters and eradicates in
598 vivo biofilms. The Journal of infectious diseases 210:1357-1366.
599 12. Wood TK, Knabel SJ, Kwan BW. 2013. Bacterial persister cell formation and dormancy.
600 Appl Environ Microbiol 79:7116-21.
601 13. Helaine S, Cheverton AM, Watson KG, Faure LM, Matthews SA, Holden DW. 2014.
602 Internalization of Salmonella by macrophages induces formation of nonreplicating
603 persisters. Science 343:204-8.
604 14. Fisher RA, Gollan B, Helaine S. 2017. Persistent bacterial infections and persister cells.
605 Nat Rev Microbiol 15:453-464.
606 15. Windels EM, Michiels JE, Fauvart M, Wenseleers T, Van den Bergh B, Michiels J. 2019.
607 Bacterial persistence promotes the evolution of antibiotic resistance by increasing
608 survival and mutation rates. Isme j 13:1239-1251.
609 16. Potrykus K, Cashel M. 2008. (p)ppGpp: still magical? Annu Rev Microbiol 62:35-51.
610 17. Ronneau S, Hallez R. 2019. Make and break the alarmone: regulation of (p)ppGpp
611 synthetase/hydrolase enzymes in bacteria. FEMS Microbiol Rev 43:389-400.
612 18. Irving SE, Corrigan RM. 2018. Triggering the stringent response: signals responsible for
613 activating (p)ppGpp synthesis in bacteria. Microbiology 164:268-276.
614 19. Glass TL, Holmes WM, Hylemon PB, Stellwag EJ. 1979. Synthesis of guanosine tetra-
615 and pentaphosphates by the obligately anaerobic bacterium *Bacteroides*
616 *thetaiotaomicron* in response to molecular oxygen. J Bacteriol 137:956-62.
617 20. Wells DH, Gaynor EC. 2006. *Helicobacter pylori* initiates the stringent response upon
618 nutrient and pH downshift. J Bacteriol 188:3726-9.

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619 21. Gallant J, Palmer L, Pao CC. 1977. Anomalous synthesis of ppGpp in growing cells. Cell
620 11:181-5.
621 22. Hood RD, Higgins SA, Flamholz A, Nichols RJ, Savage DF. 2016. The stringent response
622 regulates adaptation to darkness in the cyanobacterium *Synechococcus elongatus*.
623 Proc Natl Acad Sci U S A 113:E4867-76.
624 23. Dalebroux ZD, Svensson SL, Gaynor EC, Swanson MS. 2010. ppGpp conjures bacterial
625 virulence. Microbiol Mol Biol Rev 74:171-99.
626 24. Geiger T, Francois P, Liebeke M, Fraunholz M, Goerke C, Krismer B, Schrenzel J, Lalk M,
627 Wolz C. 2012. The stringent response of *Staphylococcus aureus* and its impact on
628 survival after phagocytosis through the induction of intracellular PSMs expression.
629 PLoS Pathog 8:e1003016.
630 25. Rodionov DG, Ishiguro EE. 1995. Direct correlation between overproduction of
631 guanosine 3',5'-bispyrophosphate (ppGpp) and penicillin tolerance in *Escherichia coli*. J
632 Bacteriol 177:4224-9.
633 26. Cashel M, Gallant J. 1969. Two compounds implicated in the function of the RC gene of
634 *Escherichia coli*. Nature 221:838-41.
635 27. Hauryliuk V, Atkinson GC, Murakami KS, Tenson T, Gerdes K. 2015. Recent functional
636 insights into the role of (p)ppGpp in bacterial physiology. Nat Rev Microbiol 13:298-
637 309.
638 28. Costanzo A, Ades SE. 2006. Growth phase-dependent regulation of the
639 extracytoplasmic stress factor, sigmaE, by guanosine 3',5'-bispyrophosphate (ppGpp). J
640 Bacteriol 188:4627-34.
641 29. Gaca AO, Colomer-Winter C, Lemos JA. 2015. Many means to a common end: the
642 intricacies of (p)ppGpp metabolism and its control of bacterial homeostasis. J Bacteriol
643 197:1146-56.
644 30. Shimada T, Yoshida H, Ishihama A. 2013. Involvement of cyclic AMP receptor protein in
645 regulation of the rmf gene encoding the ribosome modulation factor in *Escherichia*
646 *coli*. J Bacteriol 195:2212-9.
647 31. Durfee T, Hansen AM, Zhi H, Blattner FR, Jin DJ. 2008. Transcription profiling of the
648 stringent response in *Escherichia coli*. J Bacteriol 190:1084-96.
649 32. Liu K, Bittner AN, Wang JD. 2015. Diversity in (p)ppGpp metabolism and effectors. Curr
650 Opin Microbiol 24:72-9.
651 33. Wendrich TM, Marahiel MA. 1997. Cloning and characterization of a relA/spoT
652 homologue from *Bacillus subtilis.* Mol Microbiol 26:65-79.
653 34. Mittenhuber G. 2001. Comparative genomics of prokaryotic GTP-binding proteins (the
654 Era, Obg, EngA, ThdF (TrmE), YchF and YihA families) and their relationship to
655 eukaryotic GTP-binding proteins (the DRG, ARF, RAB, RAN, RAS and RHO families). J
656 Mol Microbiol Biotechnol 3:21-35.
657 35. Atkinson GC, Tenson T, Hauryliuk V. 2011. The RelA/SpoT homolog (RSH) superfamily:
658 distribution and functional evolution of ppGpp synthetases and hydrolases across the
659 tree of life. PLoS One 6:e23479.
660 36. Nanamiya H, Kasai K, Nozawa A, Yun CS, Narisawa T, Murakami K, Natori Y, Kawamura
661 F, Tozawa Y. 2008. Identification and functional analysis of novel (p)ppGpp synthetase
662 genes in *Bacillus subtilis*. Mol Microbiol 67:291-304.
663 37. Das B, Pal RR, Bag S, Bhadra RK. 2009. Stringent response in *Vibrio cholerae*: genetic
664 analysis of spoT gene function and identification of a novel (p)ppGpp synthetase gene.
665 Mol Microbiol 72:380-98.
666 38. Lemos JA, Lin VK, Nascimento MM, Abranches J, Burne RA. 2007. Three gene products
667 govern (p)ppGpp production by *Streptococcus mutans*. Mol Microbiol 65:1568-81.
668 39. Goodell W, Tomasz A. 1980. Alteration of *Escherichia coli* murein during amino acid
669 starvation. J Bacteriol 144:1009-16.

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670 40. Kusser W, Ishiguro EE. 1985. Involvement of the relA gene in the autolysis of
671 *Escherichia coli* induced by inhibitors of peptidoglycan biosynthesis. J Bacteriol
672 164:861-5.
673 41. Joseleau-Petit D, Thévenet D, D'Ari R. 1994. ppGpp concentration, growth without
674 PBP2 activity, and growth-rate control in *Escherichia coli*. Mol Microbiol 13:911-7.
675 42. Maisonneuve E, Gerdes K. 2014. Molecular mechanisms underlying bacterial
676 persisters. Cell 157:539-48.
677 43. Viducic D, Ono T, Murakami K, Susilowati H, Kayama S, Hirota K, Miyake Y. 2006.
678 Functional analysis of spoT, relA and dksA genes on quinolone tolerance in
679 *Pseudomonas aeruginosa* under nongrowing condition. Microbiol Immunol 50:349-57.
680 44. Amato SM, Orman MA, Brynildsen MP. 2013. Metabolic control of persister formation
681 in *Escherichia coli.* Mol Cell 50:475-87.
682 45. Nguyen D, Joshi-Datar A, Lepine F, Bauerle E, Olakanmi O, Beer K, McKay G, Siehnel R,
683 Schafhauser J, Wang Y, Britigan BE, Singh PK. 2011. Active starvation responses
684 mediate antibiotic tolerance in biofilms and nutrient-limited bacteria. Science 334:982-
685 6.
686 46. Bernier SP, Surette MG. 2013. Concentration-dependent activity of antibiotics in
687 natural environments. Front Microbiol 4:20.
688 47. Maisonneuve E, Castro-Camargo M, Gerdes K. 2013. (p)ppGpp controls bacterial
689 persistence by stochastic induction of toxin-antitoxin activity. Cell 154:1140-1150.
690 48. Tuomanen E, Cozens R, Tosch W, Zak O, Tomasz A. 1986. The rate of killing of
691 *Escherichia coli* by beta-lactam antibiotics is strictly proportional to the rate of
692 bacterial growth. J Gen Microbiol 132:1297-304.
693 49. Pontes MH, Groisman EA. 2019. Slow growth determines nonheritable antibiotic
694 resistance in. Sci Signal 12.
695 50. Kaldalu N, Tenson T. 2019. Slow growth causes bacterial persistence. Sci Signal 12.
696 51. Xiao H, Kalman M, Ikehara K, Zemel S, Glaser G, Cashel M. 1991. Residual guanosine
697 3',5'-bispyrophosphate synthetic activity of relA null mutants can be eliminated by
698 spoT null mutations. J Biol Chem 266:5980-90.
699 52. Schäkermann M, Langklotz S, Narberhaus F. 2013. FtsH-mediated coordination of
700 lipopolysaccharide biosynthesis in *Escherichia coli* correlates with the growth rate and
701 the alarmone (p)ppGpp. J Bacteriol 195:1912-9.
702 53. Yamaguchi T, Iida K, Shiota S, Nakayama H, Yoshida S. 2015. Elevated guanosine 5'-
703 diphosphate 3'-diphosphate level inhibits bacterial growth and interferes with FtsZ
704 assembly. FEMS Microbiol Lett 362:fnv187.
705 54. Bokinsky G, Baidoo EE, Akella S, Burd H, Weaver D, Alonso-Gutierrez J, García-Martín
706 H, Lee TS, Keasling JD. 2013. HipA-triggered growth arrest and β-lactam tolerance in
707 *Escherichia coli* are mediated by RelA-dependent ppGpp synthesis. J Bacteriol
708 195:3173-82.
709 55. Page R, Peti W. 2016. Toxin-antitoxin systems in bacterial growth arrest and
710 persistence. Nat Chem Biol 12:208-14.
711 56. Song S, Wood TK. 2020. Toxin/Antitoxin System Paradigms: Toxins Bound to Antitoxins
712 Are Not Likely Activated by Preferential Antitoxin Degradation. Advanced Biosystems
713 4:1900290.
714 57. Ogura T, Hiraga S. 1983. Mini-F plasmid genes that couple host cell division to plasmid
715 proliferation. PNAS 80:4784-4788.
716 58. Pecota DC, Wood TK. 1996. Exclusion of T4 Phage by the *hok/sok* Killer Locus from
717 Plasmid R1. J Bacteriol 178:2044-2050.
718 59. Kim Y, Wang X, Ma Q, Zhang X-S, Wood TK. 2009. Toxin-antitoxin systems in
719 *Escherichia coli* influence biofilm formation through YjgK (TabA) and fimbriae. J
720 Bacteriol 191:1258-1267.

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721 60. Ren D, Bedzyk LA, Thomas SM, Ye RW, Wood TK. 2004. Gene expression in *Escherichia*
722 *coli* biofilms. Appl Microbiol Biotechnol 64:515-24.
723 61. Yang QE, Walsh TR. 2017. Toxin-antitoxin systems and their role in disseminating and
724 maintaining antimicrobial resistance. FEMS Microbiol Rev 41:343-353.
725 62. Fernández-García L, Blasco L, Lopez M, Bou G, García-Contreras R, Wood T, Tomas M.
726 2016. Toxin-Antitoxin Systems in Clinical Pathogens. Toxins (Basel) 8.
727 63. Moyed HS, Bertrand KP. 1983. hipA, a newly recognized gene of *Escherichia coli* K-12
728 that affects frequency of persistence after inhibition of murein synthesis. J Bacteriol
729 155:768-75.
730 64. Aizenman E, Engelberg-Kulka H, Glaser G. 1996. An *Escherichia coli* chromosomal
731 "addiction module" regulated by guanosine [corrected] 3',5'-bispyrophosphate: a
732 model for programmed bacterial cell death. Proc Natl Acad Sci U S A 93:6059-63.
733 65. Korch SB, Henderson TA, Hill TM. 2003. Characterization of the hipA7 allele of
734 *Escherichia coli* and evidence that high persistence is governed by (p)ppGpp synthesis.
735 Mol Microbiol 50:1199-213.
736 66. Correia FF, D'Onofrio A, Rejtar T, Li L, Karger BL, Makarova K, Koonin EV, Lewis K. 2006.
737 Kinase activity of overexpressed HipA is required for growth arrest and multidrug
738 tolerance in *Escherichia coli.* J Bacteriol 188:8360-7.
739 67. Schumacher MA, Piro KM, Xu W, Hansen S, Lewis K, Brennan RG. 2009. Molecular
740 mechanisms of HipA-mediated multidrug tolerance and its neutralization by HipB.
741 Science 323:396-401.
742 68. Germain E, Castro-Roa D, Zenkin N, Gerdes K. 2013. Molecular mechanism of bacterial
743 persistence by HipA. Mol Cell 52:248-54.
744 69. Magnusson LU, Farewell A, Nyström T. 2005. ppGpp: a global regulator in *Escherichia*
745 *coli.* Trends Microbiol 13:236-42.
746 70. Srivatsan A, Wang JD. 2008. Control of bacterial transcription, translation and
747 replication by (p)ppGpp. Curr Opin Microbiol 11:100-5.
748 71. Gerdes K, Maisonneuve E. 2012. Bacterial persistence and toxin-antitoxin loci. Annu
749 Rev Microbiol 66:103-23.
750 72. Maisonneuve E, Shakespeare LJ, Jørgensen MG, Gerdes K. 2011. Bacterial persistence
751 by RNA endonucleases. Proc Natl Acad Sci U S A 108:13206-11.
752 73. Anonymous. 2018. Retraction for Maisonneuve et al., Bacterial persistence by RNA
753 endonucleases. Proc Natl Acad Sci U S A 115:E2901.
754 74. Osbourne DO, Soo VWC, Konieczny I, Wood TK. 2014. Polyphosphate, cyclic AMP,
755 guanosine tetraphosphate, and c-di-GMP reduce in vitro Lon activity. Bioengineered
756 5:264-268.
757 75. Chowdhury N, Kwan BW, Wood TK. 2016. Persistence Increases in the Absence of the
758 Alarmone Guanosine Tetraphosphate by Reducing Cell Growth. Sci Rep 6:20519.
759 76. Song S, Wood TK. 2020. Toxin/Antitoxin System Paradigms: Toxins Bound to Antitoxins
760 Are Not Likely Activated by Preferential Antitoxin Degradation. Adv Biosyst
761 4:e1900290.
762 77. Alav I, Sutton JM, Rahman KM. 2018. Role of bacterial efflux pumps in biofilm
763 formation. J Antimicrob Chemother 73:2003-2020.
764 78. Ge X, Cai Y, Chen Z, Gao S, Geng X, Li Y, Jia J, Sun Y. 2018. Bifunctional Enzyme SpoT Is
765 Involved in Biofilm Formation of *Helicobacter pylori* with Multidrug Resistance by
766 Upregulating Efflux Pump Hp1174 (Antimicrob Agents Chemother 62.
767 79. Chuang YM, Bandyopadhyay N, Rifat D, Rubin H, Bader JS, Karakousis PC. 2015.
768 Deficiency of the novel exopolyphosphatase Rv1026/PPX2 leads to metabolic
769 downshift and altered cell wall permeability in *Mycobacterium tuberculosis*. mBio
770 6:e02428.

Downloaded from http://aac.asm.org/ on July 28, 2020 at Penn State Univ

AAC Antimicrobial Agents and Chemotherapy

Accepted Manuscript Posted Online

771 80. Pu Y, Zhao Z, Li Y, Zou J, Ma Q, Zhao Y, Ke Y, Zhu Y, Chen H, Baker MAB, Ge H, Sun Y,
772 Xie XS, Bai F. 2016. Enhanced Efflux Activity Facilitates Drug Tolerance in Dormant
773 Bacterial Cells. Mol Cell 62:284-294.
774 81. Khakimova M, Ahlgren HG, Harrison JJ, English AM, Nguyen D. 2013. The stringent
775 response controls catalases in *Pseudomonas aeruginosa* and is required for hydrogen
776 peroxide and antibiotic tolerance. J Bacteriol 195:2011-20.
777 82. Molina-Quiroz RC, Silva-Valenzuela C, Brewster J, Castro-Nallar E, Levy SB, Camilli A.
778 2018. Cyclic AMP Regulates Bacterial Persistence through Repression of the Oxidative
779 Stress Response and SOS-Dependent DNA Repair in Uropathogenic. mBio 9.
780 83. Song S, Wood TK. 2020. ppGpp ribosome dimerization model for bacterial persister
781 formation and resuscitation. Biochem Biophys Res Commun 523:281-286.
782 84. Prossliner T, Skovbo Winther K, Sørensen MA, Gerdes K. 2018. Ribosome Hibernation.
783 Annu Rev Genet 52:321-348.
784 85. Wood A, Irving SE, Bennison DJ, Corrigan RM. 2019. The (p)ppGpp-binding GTPase Era
785 promotes rRNA processing and cold adaptation in *Staphylococcus aureus*. PLoS Genet
786 15:e1008346.
787 86. Libby EA, Reuveni S, Dworkin J. 2019. Multisite phosphorylation drives phenotypic
788 variation in (p)ppGpp synthetase-dependent antibiotic tolerance. Nat Commun
789 10:5133.
790 87. Tagami K, Nanamiya H, Kazo Y, Maehashi M, Suzuki S, Namba E, Hoshiya M, Hanai R,
791 Tozawa Y, Morimoto T, Ogasawara N, Kageyama Y, Ara K, Ozaki K, Yoshida M, Kuroiwa
792 H, Kuroiwa T, Ohashi Y, Kawamura F. 2012. Expression of a small (p)ppGpp synthetase,
793 YwaC, in the (p)ppGpp(0) mutant of *Bacillus subtilis* triggers YvyD-dependent
794 dimerization of ribosome. Microbiologyopen 1:115-34.
795 88. Yan J, Bassler BL. 2019. Surviving as a Community: Antibiotic Tolerance and Persistence
796 in Bacterial Biofilms. Cell Host Microbe 26:15-21.
797 89. Pacios O, Blasco L, Bleriot I, Fernandez-Garcia L, Gonzalez Bardanca M, Ambroa A,
798 Lopez M, Bou G, Tomas M. 2020. Strategies to Combat Multidrug-Resistant and
799 Persistent Infectious Diseases. Antibiotics (Basel) 9.
800 90. Hobbs JK, Boraston AB. 2019. (p)ppGpp and the Stringent Response: An Emerging
801 Threat to Antibiotic Therapy. ACS Infect Dis 5:1505-1517.
802 91. Wexselblatt E, Oppenheimer-Shaanan Y, Kaspy I, London N, Schueler-Furman O, Yavin
803 E, Glaser G, Katzhendler J, Ben-Yehuda S. 2012. Relacin, a novel antibacterial agent
804 targeting the Stringent Response. PLoS pathogens 8:e1002925-e1002925.
805 92. Syal K, Flentie K, Bhardwaj N, Maiti K, Jayaraman N, Stallings CL, Chatterji D. 2017.
806 Synthetic (p)ppGpp Analogue Is an Inhibitor of Stringent Response in Mycobacteria.
807 Antimicrob Agents Chemother 61.
808 93. Dutta N, Klinkenberg L, Vázquez M-J, Segura-Carro D, Colmenarejo G, Ramón F,
809 Rodriguez-Miquel B, Mata-Cantero L, Porras-De Francisco E, Chuang Y-M, Rubin H,
810 Lee J, Eoh H, Bader J, Perez-Herran E, Mendoza-Losana A, Karakousis P. 2019.
811 Inhibiting the Stringent Response Blocks *Mycobacterium tuberculosis* Entry Into
812 Quiescence and Reduces Persistence, vol 5(3). Science advances.
813 94. de la Fuente-Núñez C, Reffuveille F, Haney EF, Straus SK, Hancock RE. 2014. Broad-
814 spectrum anti-biofilm peptide that targets a cellular stress response. PLoS Pathog
815 10:e1004152.
816 95. de la Fuente-Núñez C, Korolik V, Bains M, Nguyen U, Breidenstein EB, Horsman S,
817 Lewenza S, Burrows L, Hancock RE. 2012. Inhibition of bacterial biofilm formation and
818 swarming motility by a small synthetic cationic peptide. Antimicrob Agents Chemother
819 56:2696-704.
820 96. Andresen L, Tenson T, Hauryliuk V. 2016. Cationic bactericidal peptide 1018 does not
821 specifically target the stringent response alarmone (p)ppGpp. Sci Rep 6:36549.

Downloaded from http://aac.asm.org/ on July 28, 2020 at Penn State Univ

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Antimicrobial Agents and Chemotherapy
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822 97. Allison KR, Brynildsen MP, Collins JJ. 2011. Metabolite-enabled eradication of bacterial
823 persisters by aminoglycosides. Nature 473:216-20.
824 98. Bhaskar A, De Piano C, Gelman E, McKinney JD, Dhar N. 2018. Elucidating the role of
825 (p)ppGpp in mycobacterial persistence against antibiotics. IUBMB Life 70:836-844.
826 99. Cameron DR, Shan Y, Zalis EA, Isabella V, Lewis K. 2018. A Genetic Determinant of
827 Persister Cell Formation in Bacterial Pathogens. J Bacteriol 200.
828 100. Svenningsen MS, Veress A, Harms A, Mitarai N, Semsey S. 2019. Birth and
829 Resuscitation of (p)ppGpp Induced Antibiotic Tolerant Persister Cells. Sci Rep 9:6056.
830 101. Dörr T, Vulić M, Lewis K. 2010. Ciprofloxacin causes persister formation by inducing the
831 TisB toxin in *Escherichia coli*. PLoS Biol 8:e1000317.
832 102. Conlon BP, Rowe SE, Gandt AB, Nuxoll AS, Donegan NP, Zalis EA, Clair G, Adkins JN,
833 Cheung AL, Lewis K. 2016. Persister formation in *Staphylococcus aureus* is associated
834 with ATP depletion. Nat Microbiol 1:16051.
835 103. Shan Y, Brown Gandt A, Rowe SE, Deisinger JP, Conlon BP, Lewis K. 2017. ATP-
836 Dependent Persister Formation in *Escherichia coli*. MBio 8.
837 104. Cheng HY, Soo VW, Islam S, McAnulty MJ, Benedik MJ, Wood TK. 2014. Toxin GhoT of
838 the GhoT/GhoS toxin/antitoxin system damages the cell membrane to reduce
839 adenosine triphosphate and to reduce growth under stress. Environ Microbiol
840 16:1741-54.
841 105. Hobby GL, Meyer K, Chaffee E. 1942. Observations on the Mechanism of Action of
842 Penicillin. Exp Biol Med 50:281-285.
843 106. W. BJ. 1944. Treatment of staphylococcal infections with penicillin by intermittent
844 sterilisation. , vol 244 p497–500. L*ancet*.
845 107. Fraikin N, Goormaghtigh F, Van Melderen L. 2020. Type II Toxin-Antitoxin Systems:
846 Evolution and Revolutions. J Bacteriol 202.
847 108. Blasco L, Ambroa A, Trastoy R, Bleriot I, Moscoso M, Fernández-Garcia L, Perez-
848 Nadales E, Fernández-Cuenca F, Torre-Cisneros J, Oteo-Iglesias J, Oliver A, Canton R,
849 Kidd T, Navarro F, Miró E, Pascual A, Bou G, Martínez-Martínez L, Tomas M. 2020. In
850 vitro and in vivo efficacy of combinations of colistin and different endolysins against
851 clinical strains of multi-drug resistant pathogens. Sci Rep 10:7163.
852 109. Schumacher MA, Balani P, Min J, Chinnam NB, Hansen S, Vulic M, Lewis K, Brennan RG.
853 2015. HipBA-promoter structures reveal the basis of heritable multidrug tolerance.
854 Nature 524:59-64.

855

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Year	Journal	Author	Model	References
2014	Cell	Maisonneuve E. and Gerdes K.	(p)ppGpp induces persistence by activating TA loci via PolyP and Lon protease in *E. coli* K-12 strain MG1655.	(42, 71-73)* (3, 65, 109)
2016	Scientific Reports	Chowdhury N., Kwan B.W, and Wood T.K.	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.	(75)
2019	Science Signalling	Pontes M.H. and Groismann E.A.	Low cytoplasmatic Mg^{2+} induces *S. typhimurium* tolerance to antibiotic independently of (p)ppGpp and TA modules. However, (p)ppGpp reduces antibiotic tolerance under certain conditions.	(49)
2020	Biochemical and Biophysical Research Communication	Song S. and Wood T.K.	(p)ppGpp generates persister cells directly by inactivation of ribosomes via Rmf and Hpf.	(83)

Table 1.

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

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

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863 **Table 2.**

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

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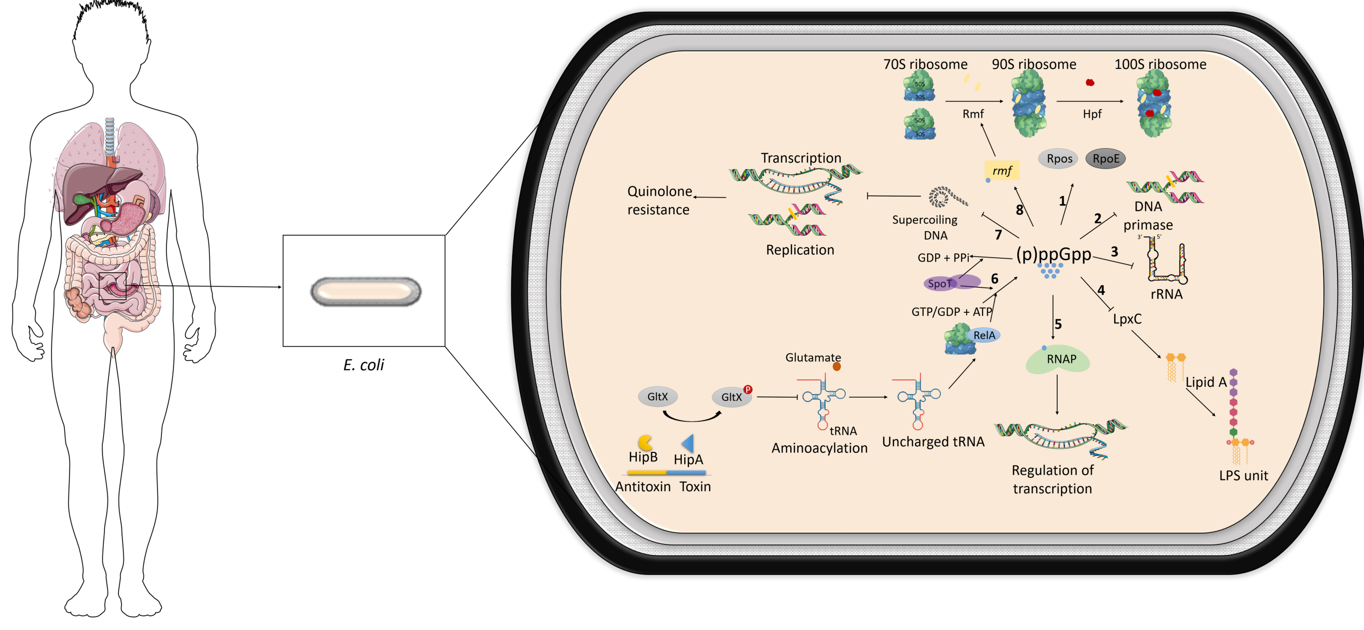

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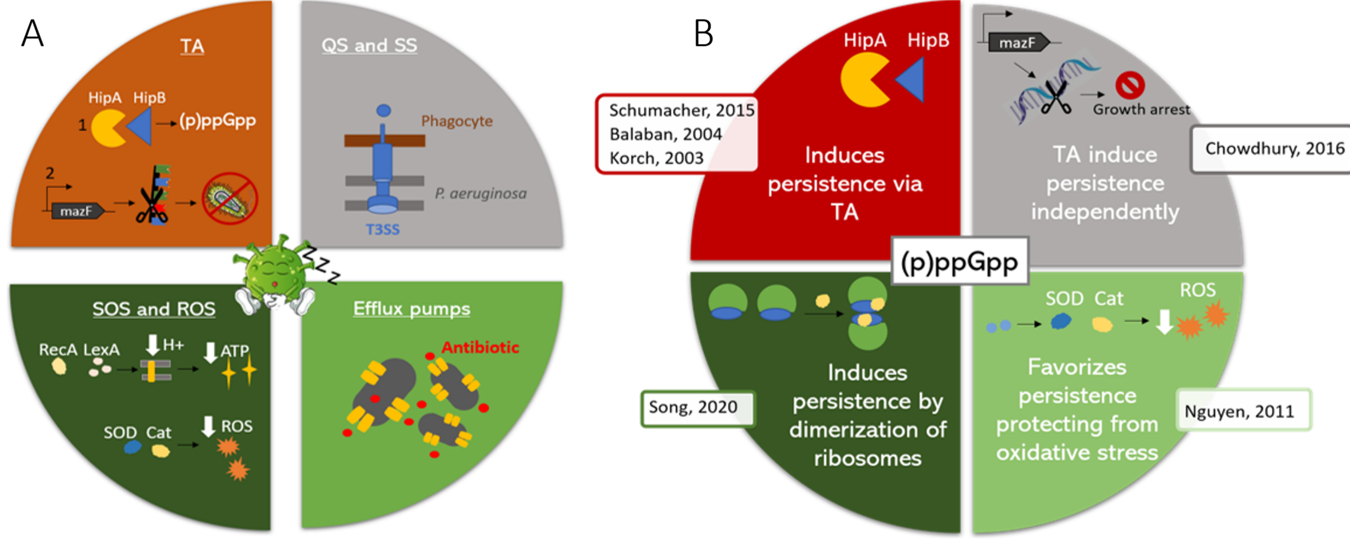

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