Forming and Waking Dormant Cells: The ppGpp Ribosome Dimerization Persister Model

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ABSTRACT

Procaryotes starve and face myriad stresses. The bulk population actively resists the stress, but a small population weatherst the stress by entering a resting stage known as persistence. No mutations occur, and so persisters behave like wild-type cells upon removal of the stress and regrowth; hence, persisters are phenotypic variants. In contrast, resistant bacteria have mutations that allow cells to grow in the presence of antibiotics, and tolerant cells survive antibiotics better than actively-growing cells due to their slow growth (such as that of the stationary phase). In this review, we focus on the latest developments in studies related to the formation and resuscitation of persister cells and propose the guanosine pentaphosphate/tetraphosphate (henceforth ppGpp) ribosome dimerization persister (PRDP) model for entering and exiting the persister state.

REVIEW

Persister cells: dormant and ubiquitous. Hobby et al. (Hobby et al., 1942) first noted that penicillin does not kill about 1% of Staphylococcus aureus cells and also showed non-dividing (dormant cells) were penicillin resistant; hence, convincing evidence was provided immediately that persister cells are metabolically inactive. Two years later, Bigger (Bigger, 1944) named these same surviving S. aureus cells “persisters” and corroborated the 1942 findings of Hobby et al. by showing with three experiments that penicillin does not kill non-dividing cells. After 62 years, a ribosomal reporter was used to show persister cells have low metabolic activity (Shah et al., 2006).

Although the original literature strongly suggested persisters are dormant, there has been some controversy and confusion about their metabolic state. For example, the Brynildsen group (Orman and Brynildsen, 2013) claimed, most likely by incorrectly interpreting FACS data (Wood et al., 2013), that persisters were not necessarily dormant, then three years later concluded that persisters were dormant (Henry and Brynildsen, 2016). Critically, with Salmonella enterica, it was shown again recently that persisters stem primarily from slow growth (Pontes and Groisman, 2019).

Although they are not formed in the exponential phase, persisters are found in the stationary phase and in biofilms (Lewis, 2007; Lewis, 2008) since cells are stressed. All bacterial cells tested to date form persisters (Van den Bergh et al., 2017) and this includes pathogens; when these dormant cells revive, they likely reconstitute infections (Defrain et al., 2018). Since all bacterial cells starve (Schmidt, 2012),
Persistence is a general phenotype (Kim et al., 2018a). We have found, based on antibiotic tolerance, morphology, resuscitation rates, and lack of metabolic activity, that the cells capable of resuscitation that are part of the “viable but non-culturable” population generated from starvation (Xu et al., 1982) are persister cells (Kim et al., 2018a). Therefore, due to its ubiquity, persistence is arguably one of the most important metabolic states, and insights on eradicating persisters is important for disease (e.g., tuberculosis, cystic fibrosis, ear infections) and agriculture (Martins et al., 2018).

**Studying persister cells.** Since persister cells are found naturally at very low concentrations, from 0.0001 to 1% (Van den Bergh et al., 2017), some groups have studied slowly-growing cells rather than persister cells, such as those created by a nutrient shift (Amato et al., 2013; Amato and Brynildsen, 2014; Amato and Brynildsen, 2015; Radzikowski et al., 2016) and those of the stationary stage (Orman and Brynildsen, 2015). This results in incorrectly attributing the characteristics of slowly-growing (tolerant) cells to persister cells. Another difficulty is that persister cells can respond to nutrients immediately, so adding fresh medium (e.g., during cell washing steps) causes the persister cells to resuscitate (Kim et al., 2018b).

Since persister cells are dormant and cells are 50% protein, we reasoned inactivating ribosomes should increase persistence. Hence, we (Kwan et al., 2013) devised methods to convert nearly all of the exponentially-growing cells into persisters by pretreating with (i) rifampicin to stop transcription, (ii) with tetracycline to stop translation, or (iii) with carbonyl cyanide m-chlorophenylhydrazone (CCCP) to stop translation by eliminating ATP production. These pretreatment methods may be used to convert nearly all the exponentially-growing cells into a population of persister cells, resulting in a 10,000-fold increase in persistence (Kwan et al., 2013). By increasing the population of persister cells dramatically, mechanistic insights into their formation and resuscitation may be made. Furthermore, these methods produce *bona fide* persister cells as they have been verified eight ways: multi-drug tolerance, immediate change from persister to non-persister in the presence of nutrients, dormancy via flow cytometry with the metabolic dye redox sensor green, dormancy via a lack of resuscitation on gel pads that lack nutrients (some exponential cells divide under these conditions), no change in MIC compared to exponential cells, no resistance phenotype, similar morphology to ampicillin-induced persisters, and similar resuscitation as ampicillin-induced persisters (Kim et al., 2018b). Critically, our methods have been validated for both *Pseudomonas aeruginosa* and *S. aureus* (Grassi et al., 2017) and have been verified to date by six independent groups (Grassi et al., 2017; Cui et al., 2018; Narayanaswamy et al., 2018; Sulaiman et al., 2018; Tkhilaishvili et al., 2018; Pu et al., 2019).

Similar to pretreating with rifampicin, tetracycline, and CCCP, fluoroquinolones may be used to increase
persistence by inducing the SOS response via DNA strand breaks (Dörr et al., 2009). Also, pre-treatment with oxidative stress or acid stress increases persistence 12,000-fold (Hong et al., 2012) and increasing the toxicity of a toxin such as MqsR of the MqsR/MqsA TA system increases persistence dramatically (Hong et al., 2012). By using these methods to increase the concentration of persister cells, it was determined that bacterial persistence increases when the cells are less fit to mount an active fight against an environmental stress (Hong et al., 2012). Also, these methods led to the insight that indole decreases persistence in Escherichia coli (Hu et al., 2015; Kwan et al., 2015). From this realization, halogenated indoles such as 5-iodoindole, 4-fluoroindole, 7-chloroindole, and 7-bromoindole were identified that effectively kill E. coli and S. aureus persister cells (Lee et al., 2016).

**Persister cells form by inactivating ribosomes via ppGpp.** With few exceptions, the alarmone ppGpp is central for the mechanism of how cells become persistent. Originally, ppGpp had an important role in the study of the first mutation related to persistence, hipA7. In E. coli, the gain-of-function set of mutations in toxin gene hipA known as hipA7 (causing substitutions G22S and D291A in HipA) of the HipA/HipB TA system increased persistence 1,000 fold (Moyed and Bertrand, 1983) due to reduced antitoxin HipB binding (Schumacher et al., 2015). These substitutions render the HipA7 variant not toxic (Korch et al., 2003), so the link of TA systems to persistence from the use of this variant is problematic.

In addition, ppGpp has been linked to the MazF/MazE TA system since ppGpp is required to activate MazF toxicity (Aizenman et al., 1996). However, there is a trend to distance persistence from intracellular TA systems since a credible mechanism relating ppGpp to the activation of TA systems has not been discerned. Specifically, the model in which Lon protease is activated by polyphosphate (which builds up as ppGpp is activated) and then degrades antitoxins to activate toxins has been retracted (Maisonneuve et al., 2018). Also, two other manuscripts relating persistence to the deletion of 10 type II TA systems (2018) and relating persistence to HipA (2019) have been retracted. Basically, data from other laboratories disputed these findings (Van Melderen and Wood, 2017) including (i) polyphosphate inactivates Lon protease in vitro, rather than activates Lon activity (Osbourne et al., 2014), (ii) there is little connection between Lon and persistence (Shan et al., 2015; Chowdhury et al., 2016a), (iii) degradation of YefM antitoxin of the YoeB/YefM TA system is independent of ppGpp and polyphosphate (Ramisetty et al., 2016), and (iv) degradation of antitoxins RelB and MazE is independent of ppGpp (Van Melderen and Wood, 2017). Also, the TA system deletion strain had 79 coding mutations beyond those related to the 10 TA systems (Shan et al., 2017), and the strain was contaminated with phage (Harms et al., 2017). Note, in contrast to intracellular TA systems, Lon protease and ppGpp are implemented for creating persister cells via contact-dependent
growth inhibition (Ghosh et al., 2018).

Further evidence of the absence of a link between TA systems and persistence is that of another *E. coli* strain with 10 type II TA systems deleted (but with a large genomic inversion) which showed TA systems are not related to persistence (Goormaghtigh et al., 2018). Also, deletion of 12 TA systems in *S. enterica* had no effect on persistence (Pontes and Groisman, 2019). Furthermore, using single cells and a verified *E. coli* strain with 10 type II TA systems deleted, ATP concentrations and TA systems did not influence persistence (Svenningsen et al., 2019), and ppGpp was confirmed as important for persister formation (Svenningsen et al., 2019). However, the authors used a RpoS-mCherry proxy for ppGpp that may not be accurate (Goormaghtigh et al., 2018). Of course, high concentrations of persister cells may be formed by producing nearly any toxic protein in *E. coli* (Chowdhury et al., 2016c), so production of toxin MqsR of the MqsR/MqsA TA system (Ren et al., 2004; Brown et al., 2011; Wang et al., 2011; Wang et al., 2013) has been used to increase persistence by 14,000-fold for *E. coli* (Hong et al., 2012). However, use of these non-physiological levels of toxins does not mean cells use toxins of TA systems for persistence. Instead, the primary physiological roles of TA systems (to date) seem more likely to be (Song and Wood, 2018) (i) inhibiting phage (Pecota and Wood, 1996), (ii) maintaining genetic elements such as plasmids (Ogura and Hiraga, 1983), (iii) reducing metabolism as a response to stress (Gerdes, 2000; Wang et al., 2011), and (iv) forming biofilms (González Barrios et al., 2006; Kim et al., 2009).

Since the link between ppGpp and TA systems is problematic, we reasoned that it may be possible to directly relate ppGpp to persistence based on its dominant role in the stress response; we also utilized the realization that reducing ribosome activity is the critical step for persistence (Kwan et al., 2013). Upon experiencing myriad stresses (e.g., nutrient, antibiotic, or oxidative stress), most cells mount an active response against the stress by altering replication, transcription, and translation via ppGpp (Gaca et al., 2015). ppGpp slows replication by inhibiting DNA primase (Gaca et al., 2015), and ppGpp reduces translation by decreasing ribosome synthesis (Shimada et al., 2013). ppGpp also directly controls the activity of several enzymes; for example, ppGpp inhibits GTPases (Gaca et al., 2015). ppGpp changes transcription by activating RpoS (sigma^{S}, the stress response sigma factor for the stationary phase) and RpoE (sigma^{E}, the stress response sigma factor for misfolded proteins in the periplasm) (Dalebroux and Swanson, 2012). Notably, the physiological roles of ppGpp continue to expand, such as inhibition of the synthesis of purine nucleotides (Wang et al., 2019), regulation of purine homeostasis through activation of nucleosidase PpnN (Zhang et al., 2019), inhibition of the ribosome-associated GTPase Era that is important for assembling 30S ribosome subunits (Wood et al., 2019a), and binding the GTPase HflX that activates 100S ribosomes (Zhang et al., 2018).
In contrast to the vast majority of cells that mount an active response to stress, the sub-population of cells that become persisters may be created when ppGpp turns off an excessive number of ribosomes by activating the expression of the small (55 aa) ribosome modulation factor (RMF) (Izutsu et al., 2001), which converts active 70S ribosomes into inactive 100S ribosomes (Wada et al., 1995) via an inactive 90S dimer complex (Ueta et al., 2005). Also, ppGpp induces the hibernation promoting factor the hibernation promoting factor (Hpf) (95 aa) which converts 90S ribosomes into 100S ribosomes and is highly conserved as it is found in most bacteria and some plants (Prossliner et al., 2018). This dimerization is essential for survival in the stationary phase (Yoshida et al., 2018). Hence, we reasoned that ppGpp directly modulates persistence through its direct inactivation of ribosomes. In agreement with this hypothesis, we found overproduction of RMF and Hpf increase persistence as well as reduce single-cell resuscitation while ppGpp has no effect on single-cell resuscitation (Song and Wood, 2019a).

We have termed this mechanism the ppGpp ribosome dimerization persister (PRDP) model (Fig. 1) in which persister cells form not through TA systems but by the direct control of ribosome activity by ppGpp. In support of this model, E. coli cells have 100 times less persistence in the absence of ppGpp (Chowdhury et al., 2016a), and higher ppGpp levels increases the persistence of single cells by 1,000-fold (Svenningsen et al., 2019).

Another model for persister cell formation is based on reducing ATP (Dörr et al., 2010; Cheng et al., 2014; Conlon et al., 2016; Shan et al., 2017; Cameron et al., 2018) or uncoupling the membrane potential with compounds like CCCP to reduce ATP (Kwan et al., 2013). However, conflicting results have been obtained from other labs suggesting ATP does not control persistence (Pontes and Groisman, 2019; Svenningsen et al., 2019).

**Persister cell resuscitation is heterogeneous and is based on ribosome content.** Single cell analysis has revealed that most persister cells do not resuscitate spontaneously, but instead, wake upon sensing fresh nutrients (Yamasaki et al., 2019). This is in opposition to the ‘Scout Model’ (Epstein, 2009) in which it was proposed (without data) that persister cells wake spontaneously and periodically to see if it is safe to grow.

Upon waking, persister resuscitation is heterogeneous and includes five phenotypes: (i) immediate division, (ii) immediate elongation followed by division, (iii) immediate elongation but no division, (iv) delayed elongation/division, and (v) no growth (Kim et al., 2018b). Critically, this heterogeneity in resuscitation is due to the different levels of active ribosomes: by using a validated green fluorescence...
protein reporter of ribosome levels, we observed that higher ribosome levels result in faster waking (Kim et al., 2018b). Hence, cells with low levels of ribosomes have to increase their ribosome levels to a threshold value, then they begin to divide or elongate (Kim et al., 2018b). The varying ribosome levels result from cell-to-cell differences in the numbers of ribosomes in exponentially-growing cells upon ceasing their metabolic activity (Kim et al., 2018b).

This heterogeneity in persister cell resuscitation (Kim et al., 2018b) has been corroborated by several groups. For example, using *E. coli* and time-lapse microscopy, Şimşek and Kim (Şimşek and Kim, 2019) followed 12,800 of what appears to be persister cells (or stationary cells, it is not clear which they used since it is not clear if the stationary-phase cells that they used were pre-treated with ampicillin to eliminate non-persisters) to find that most cells wake immediately and that their rejuvenation probability decays exponentially, resulting in lag time before resuscitation from 0 to over 1000 min. Pu et al. (Pu et al., 2019), also found that single persister cells wake heterogeneously (from less than 12 h to 40 h), but their explanation based on the degree of protein refolding required for their resuscitation is unlikely as it would require an enzyme that could re-nature a thousand different proteins (Wood et al., 2019b). In addition, Goormaghtigh and Van Melderen (Goormaghtigh and Van Melderen, 2019) found persister cells wake heterogeneously and frequently include elongation.

In addition, when persisters resuscitate and begin dividing, their growth rate is the same as exponential cells; hence, once the formerly-dormant cell commences division, it is fully recovered (Kim et al., 2018b). Also, it is important to note that residual stress may be detrimental to sustained resuscitation in that cells with residual ampicillin antibiotic fail to resuscitate (Kim et al., 2018b).

**Persister cells resuscitate by activating dormant ribosomes.** It has been proposed that toxins are inactivated and in this way persister cells may revive. However, as with the model that relies on TA systems for persister cell formation, there is little data that support this. For example, it was suggested that the peptidyl-tRNA hydrolase Pth counteracts toxin TacT in *S. Typhimurium*, “explaining how bacterial persisters can resume growth”; however, there were no resuscitation data to validate this claim (Cheverton et al., 2016). Similarly, it was claimed that deactivation of polymerized (active) HokB toxin by monomerization controls persister waking; however, single-cell observations were not made but instead delays in resuscitation were estimated from growth data (Wilmaerts et al., 2019) for this type I system. Also, the key features for the HokB system have been discerned from non-physiological levels of HokB (i.e., from overproduction of HokB) (Wilmaerts et al., 2018; Wilmaerts et al., 2019), and there is no persister phenotype for deleting *hokB* (Verstraeten et al., 2015). Critically, for type II TA systems,
there is little data for the paradigm that antitoxins are degraded from TA complexes and cells revive; reactivation of toxins bound to antitoxins is unlikely given that the antitoxin is bound tightly to the toxin in most cases (for type II TA systems).

Since there is little evidence of toxins of TA systems being inactivated to revive cells, the simplest model for persister cell resuscitation is re-activation of ribosomes. Based on single-cell observations (Song and Wood, 2019a; Yamasaki et al., 2019), we propose the PRDP model (Fig. 1) holds for persister resuscitation: after ppGpp generates persister cells directly by inactivating ribosomes via increased production of RaiA (inactivates 70S ribosomes) (Prossliner et al., 2018), production of the ribosome modulation factor (RMF), and production of Hpf. Once the stress dissipates and the cells have fresh nutrients, inactive 100S ribosomes reactivate by the ribosome resuscitation factor HflX (Gohara and Yap, 2018) which will no longer be inactivated by ppGpp (Zhang et al., 2018) and repressed by cAMP (Lin et al., 2011); HflX also rescues ribosomes from mRNA (Zhang et al., 2015). In support of this, we found RaiA, RMF, and Hpf increase persistence and reduce single-cell persister resuscitation and that RMF increases 100S ribosome formation in persister cells (Song and Wood, 2019a). In addition, we found producing HflX increases single-cell waking (Yamasaki et al., 2019). Furthermore, the resuscitating cells sense the fresh nutrients by chemotaxis and phosphotransferase membrane proteins (Yamasaki et al., 2019), and transport of nutrients reduces the level of secondary messenger cAMP through enzyme II A (Yamasaki et al., 2019); this reduction in cAMP levels stimulates ribosome resuscitation and rescue (Yamasaki et al., 2019). The waking persister cells also immediately commence chemotaxis toward nutrients (Yamasaki et al., 2019). Therefore, persister cells wake by sensing nutrients via membrane receptors, and this external waking signal is conveyed via the secondary messenger cAMP to inactive ribosomes which are used by the persisters to wake and begin chemotaxis to acquire nutrients (Yamasaki et al., 2019).

Corroborating the ribosome model for persister cell resuscitation (Song and Wood, 2019a), a 10,000-member library of druglike compounds was screened to identify compounds that increase persister cell resuscitation and 2-[(2-(4-bromophenyl)-2-oxoethyl)thio]-3-ethyl-5,6,7,8-tetrahydro[1]benzothieno[2,3-d]pyrimidin-4(3H)-one (BPOET) was identified that stimulates persister cell resuscitation by activating ribosomes via pseudouridine modification of 23S rRNA by pseudouridine synthase RluD (Song and Wood, 2019b); hence, ribosomes are modified to facilitate persister resuscitation. Also, 100S ribosome recovery is extremely rapid and occurs in less than one minute (Prossliner et al., 2018), which matches our data indicating some persister cells wake immediately (Kim et al., 2018b).
**Perspectives.** Implications of the PRDP model include that persister cell formation is an elegantly-regulated response to stress rather than a bet-hedging (“stochastic”) process. Experimental data support this idea in that spontaneous persisters are rare (Balaban et al., 2004) but external stress (e.g., antibiotics, hydrogen peroxide, acid) converts nearly the whole population into persister cells (Dörr et al., 2009; Hong et al., 2012; Kwan et al., 2013). In addition, the PRDP model suggests resuscitation is also an elegant response to improved environmental conditions rather than spontaneous. Since all cells starve and need deep resting states, it is logical that cells utilize elegant pathways for both persister cell formation and resuscitation.

Also, clearly there is something more than just stress that makes cells become persistent. For example, all stationary-phase cells are starving but only a small percentage become persisters. The PRDP model suggests that a small population of stressed cells has a larger number of their ribosomes inactivated so this heterogeneity in ribosome dimerization is perhaps the “switch” that makes cells persistent.

Remaining questions include whether the PRDP model is general; i.e., holds for other species, and whether it is relevant for clinical isolates. The PRDP model is likely applicable to myriad stresses since RMF dramatically increases persistence with for stress from ampicillin (Song and Wood, 2019a), ciprofloxacin (Song and Wood, 2019a), netilmicin (Tkachenko et al., 2017), gentamicin (McKay and Portnoy, 2015), acid (El-Sharoud and Niven, 2007), osmotic stress (Shcherbakova et al., 2015) and nutrient limitation in the stationary phase (Yamagishi M, 1993; Bubunenko et al., 2007). Furthermore, since RMF is conserved in bacteria (Prossliner et al., 2018) and Hpf is even more widely distributed (Akiyama et al., 2018), there is the possibility that the PRDP model may be applicable for the formation of the persister state of many strains. For example, like *E. coli*, the persistence of *P. aeruginosa* also requires ppGpp (Nguyen et al., 2011) and so it would be interesting to test further whether ribosome dimerization also controls persister cell formation and resuscitation in this strain. It is already established that both Hpf and ppGpp (but not RMF) are necessary for the long term survival of *P. aeruginosa* during starvation by preserving ribosomes (Akiyama et al., 2017) and that ppGpp plays a role in *hpf* expression (Akiyama et al., 2018). However, a preliminary report indicates that after treating with ciprofloxacin, high persister variants are present in cystic fibrosis patents, but mutations related to ppGpp were not found (Mojsoska et al., 2019).

Since persister cells are formed in the absence of ppGpp but at much lower levels (Chowdhury et al., 2016a), there must be other means to activate Rmf and Hpf without ppGpp, which leads to the formation of inactive and dimerized ribosomes. For example, cAMP, which is generated upon starvation (e.g.,
glucose depletion), induces rmf (Shimada et al., 2013) and raiA (Prossliner et al., 2018), and plays a role in the formation of persister cells (Kwan et al., 2015) (Fig. 1). In addition, cAMP represses hflX (Lin et al., 2011). Hence, cAMP is as likely to be as important as ppGpp for persistence. Critically, there may be several mechanisms for forming persister cells, but the net result is that ribosomes are inactivated.

New compounds will indubitably be identified for killing persister cells since it now possible to create large libraries of bona fide persister cells in a facile manner (Kwan et al., 2013; Kim et al., 2018b). For example, by screening a 10,000-member library of druglike compounds, we identified that the indole derivative, 5-nitro-3-phenyl-1H-indol-2-yl-methylamine hydrochloride (NPIMA) (Song et al., 2019), kills E. coli persister cells better than 5-iodoindole (Lee et al., 2016) and better than the DNA crosslinking agent cisplatin (Chowdhury et al., 2016b; Cruz-Muñiz et al., 2016). NPIMA also eliminated P. aeruginosa persister cells and had activity on the representative Gram positive pathogen, S. aureus (Song et al., 2019). The mode of action of NPIMA with both Gram-positive and Gram-negative bacteria was determined to be via membrane damage which leads to lysis. Critically, understanding the steps for persister formation and resuscitation (Fig. 1) will provide additional targets for fighting persistence.

There are also parallels being discovered between bacterial persisters and cancer cells that are recalcitrant to known treatments (Vallette et al., 2019). Dormancy for cancer cells occurs when the growth of the cells is temporarily stopped before initiating a new primary tumor that may be even more aggressive the original tumor. Hence, insights gleaned from bacterial persisters may be relevant for treating cancer.

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Fig. 1. Schematic of the ppGpp ribosome dimerization persister (PRDP) model. Stress such as nutrient limitation, osmotic stress, and acid stress induces the stringent response which results in ppGpp formation by RelA/SpoT and generation of cAMP (e.g., upon glucose depletion via the phosphorylated glucose phosphotransfer enzyme, EIIA-P) in *E. coli*. ppGpp induces *hpf*, and both ppGpp and cAMP together induce *raiA* and *rmf*. RaiA inactivates 70S ribosomes, RMF converts 70S ribosomes into inactive 90S ribosomes, and Hpf converts inactive 90S ribosomes into 100S ribosomes. Moreover, ppGpp binds HflX to likely inactivate it, and cAMP represses *hflX*. Upon addition of nutrients and removal of the stress, cAMP decreases (due to unphosphorylated EIIA) which stimulates HflX production; HflX dissociates 100S ribosomes into active 70S ribosomes and growth resumes. Since persister cells form in the absence of ppGpp (although at much reduced levels), cAMP by itself and perhaps other mechanisms activate RMF and Hpf as well.
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ABSTRACT

Procaryotes starve and face myriad stresses. The bulk population actively resists the stress, but a small population weathers the stress by entering a resting stage known as persistence. No mutations occur, and so persisters behave like wild-type cells upon removal of the stress and regrowth; hence, persisters are phenotypic variants. In contrast, resistant bacteria have mutations that allow cells to grow in the presence of antibiotics, and tolerant cells survive antibiotics better than actively-growing cells due to their slow growth (such as that of the stationary phase). In this review, we focus on the latest developments in studies related to the formation and resuscitation of persister cells and propose the guanosine pentaphosphate/tetraphosphate (henceforth ppGpp) ribosome dimerization persister (PRDP) model for entering and exiting the persister state.

REVIEW

Persisters: dormant and ubiquitous. Hobby et al. (Hobby et al., 1942) first noted that penicillin does not kill about 1% of Staphylococcus aureus cells and also showed non-dividing cells were penicillin resistant; hence, convincing evidence was provided immediately that persister cells are metabolically inactive. Two years later, Bigger (Bigger, 1944) named these same surviving S. aureus cells “persisters” and corroborated the 1942 findings of Hobby et al. by showing with three experiments that penicillin does not kill non-dividing cells. After 62 years, a ribosomal reporter was used to show persister cells have low metabolic activity (Shah et al., 2006).

Although the original literature strongly suggested persisters are dormant, there has been some controversy and confusion about their metabolic state. For example, the Brynildsen group (Orman and Brynildsen, 2013) claimed, most likely by incorrectly interpreting FACS data (Wood et al., 2013), that persisters were not necessarily dormant, then three years later concluded that persisters were dormant (Henry and Brynildsen, 2016). Critically, with Salmonella enterica, it was shown again recently that persisters stem primarily from slow growth (Pontes and Groisman, 2019).

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a general phenotype (Kim et al., 2018a). We have found, based on antibiotic tolerance, morphology, resuscitation rates, and lack of metabolic activity, that the cells capable of resuscitation that are part of the “viable but non-culturable” population generated from starvation (Xu et al., 1982) are persister cells (Kim et al., 2018a). Therefore, due to its ubiquity, persistence is arguably one of the most important metabolic states, and insights on eradicating persisters is important for disease (e.g., tuberculosis, cystic fibrosis, ear infections) and agriculture (Martins et al., 2018).

Studying persister cells. Since persister cells are found naturally at very low concentrations, from 0.0001 to 1% (Van den Bergh et al., 2017), some groups have studied slowly-growing cells rather than persister cells, such as those created by a nutrient shift (Amato et al., 2013; Amato and Brynildsen, 2014; Amato and Brynildsen, 2015; Radzikowski et al., 2016) and those of the stationary stage (Orman and Brynildsen, 2015). This results in incorrectly attributing the characteristics of slowly-growing (tolerant) cells to persister cells. Another difficulty is that persister cells can respond to nutrients immediately, so adding fresh medium (e.g., during cell washing steps) causes the persister cells to resuscitate (Kim et al., 2018b).

Since persister cells are dormant and cells are 50% protein, we reasoned inactivating ribosomes should increase persistence. Hence, we (Kwan et al., 2013) devised methods to convert nearly all of the exponentially-growing cells into persisters by pretreating with (i) rifampicin to stop transcription, (ii) with tetracycline to stop translation, or (iii) with carbonyl cyanide m-chlorophenylhydrazone (CCCP) to stop translation by eliminating ATP production. These pretreatment methods may be used to convert nearly all the exponentially-growing cells into a population of persister cells, resulting in a 10,000-fold increase in persistence (Kwan et al., 2013). By increasing the population of persister cells dramatically, mechanistic insights into their formation and resuscitation may be made. Furthermore, these methods produce bona fide persister cells as they have been verified eight ways: multi-drug tolerance, immediate change from persister to non-persister in the presence of nutrients, dormancy via flow cytometry with the metabolic dye redox sensor green, dormancy via a lack of resuscitation on gel pads that lack nutrients (some exponential cells divide under these conditions), no change in MIC compared to exponential cells, no resistance phenotype, similar morphology to ampicillin-induced persisters, and similar resuscitation as ampicillin-induced persisters (Kim et al., 2018b). Critically, our methods have been validated for both Pseudomonas aeruginosa and S. aureus (Grassi et al., 2017) and have been verified to date by six independent groups (Grassi et al., 2017; Cui et al., 2018; Narayanaswamy et al., 2018; Sulaiman et al., 2018; Tkhiilaishvili et al., 2018; Pu et al., 2019).

Similar to pretreating with rifampicin, tetracycline, and CCCP, fluoroquinolones may be used to increase
persistence by inducing the SOS response via DNA strand breaks (Dörr et al., 2009). Also, pre-treatment with oxidative stress or acid stress increases persistence 12,000-fold (Hong et al., 2012) and increasing the toxicity of a toxin such as MqsR of the MqsR/MqsA TA system increases persistence dramatically (Hong et al., 2012). By using these methods to increase the concentration of persister cells, it was determined that bacterial persistence increases when the cells are less fit to mount an active fight against an environmental stress (Hong et al., 2012). Also, these methods led to the insight that indole decreases persistence in *Escherichia coli* (Hu et al., 2015; Kwan et al., 2015). From this realization, halogenated indoles such as 5-iodoindole, 4-fluoroindole, 7-chloroindole, and 7-bromoindole were identified that effectively kill *E. coli* and *S. aureus* persister cells (Lee et al., 2016).

**Persister cells form by inactivating ribosomes via ppGpp.** With few exceptions, the alarmone ppGpp is central for the mechanism of how cells become persistent. Originally, ppGpp had an important role in the study of the first mutation related to persistence, *hipA*7. In *E. coli*, the gain-of-function set of mutations in toxin gene *hipA* known as *hipA*7 (causing substitutions G22S and D291A in HipA) of the HipA/HipB TA system increased persistence 1,000 fold (Moyed and Bertrand, 1983) due to reduced antitoxin HipB binding (Schumacher et al., 2015). These substitutions render the HipA7 variant not toxic (Korch et al., 2003), so the link of TA systems to persistence from the use of this variant is problematic. In addition, ppGpp has been linked to the MazF/MazE TA system since ppGpp is required to activate MazF toxicity (Aizenman et al., 1996).

However, there is a trend to distance persistence from intracellular TA systems since a credible mechanism relating ppGpp to the activation of TA systems has not been discerned. Specifically, the model in which Lon protease is activated by polyphosphate (which builds up as ppGpp is activated) and then degrades antitoxins to activate toxins has been retracted (Maisonneuve et al., 2018). Also, two other manuscripts relating persistence to the deletion of 10 type II TA systems (2018) and relating persistence to HipA (2019) have been retracted. Basically, data from other laboratories disputed these findings (Van Melderen and Wood, 2017) including (i) polyphosphate inactivates Lon protease in vitro, rather than activates Lon activity (Osbourne et al., 2014), (ii) there is little connection between Lon and persistence (Shan et al., 2015; Chowdhury et al., 2016a), (iii) degradation of YefM antitoxin of the YoeB/YefM TA system is independent of ppGpp and polyphosphate (Ramisetty et al., 2016), and (iv) degradation of antitoxins RelB and MazE is independent of ppGpp (Van Melderen and Wood, 2017). Also, the TA system deletion strain had 79 coding mutations beyond those related to the 10 TA systems (Shan et al., 2017), and the strain was contaminated with phage (Harms et al., 2017). **Note, in contrast to intracellular TA systems, Lon protease and ppGpp are implemented for creating persister cells via contact-dependent growth inhibition (Ghosh et al., 2018).**
Further evidence of the absence of a link between TA systems and persistence is that of another *E. coli* strain with 10 type II TA systems deleted (but with a large genomic inversion) which showed TA systems are not related to persistence (Goormaghtigh et al., 2018). Also, deletion of 12 TA systems in *S. enterica* had no effect on persistence (Pontes and Groisman, 2019). Furthermore, using single cells and a verified *E. coli* strain with 10 type II TA systems deleted, ATP concentrations and TA systems did not influence persistence (Svenningsen et al., 2019), and ppGpp was confirmed as important for persister formation (Svenningsen et al., 2019). However, the authors used a RpoS-mCherry proxy for ppGpp that may not be accurate (Goormaghtigh et al., 2018). Of course, high concentrations of persister cells may be formed by producing nearly any toxic protein in *E. coli* (Chowdhury et al., 2016c), so production of toxin MqsR of the MqsR/MqsA TA system (Ren et al., 2004; Brown et al., 2011; Wang et al., 2011; Wang et al., 2013) has been used to increase persistence by 14,000-fold for *E. coli* (Hong et al., 2012). However, use of these non-physiological levels of toxins does not mean cells use toxins of TA systems for persistence. Instead, the primary physiological roles of TA systems (to date) seem more likely to be (Song and Wood, 2018) (i) inhibiting phage (Pecota and Wood, 1996), (ii) maintaining genetic elements such as plasmids (Ogura and Hiraga, 1983), (iii) reducing metabolism as a response to stress (Gerdes, 2000; Wang et al., 2011), and (iv) forming biofilms (González Barrios et al., 2006; Kim et al., 2009).

Since the link between ppGpp and TA systems is problematic, we reasoned that it may be possible to directly relate ppGpp to persistence based on its dominant role in the stress response; we also utilized the realization that reducing ribosome activity is the critical step for persistence (Kwan et al., 2013). Upon experiencing myriad stresses (e.g., nutrient, antibiotic, or oxidative stress), most cells mount an active response against the stress by altering replication, transcription, and translation via ppGpp (Gaca et al., 2015). ppGpp slows replication by inhibiting DNA primase (Gaca et al., 2015), and ppGpp reduces translation by decreasing ribosome synthesis (Shimada et al., 2013). ppGpp also directly controls the activity of several enzymes; for example, ppGpp inhibits GTPases (Gaca et al., 2015). ppGpp changes transcription by activating RpoS (sigmaS, the stress response sigma factor for the stationary phase) and RpoE (sigmaE, the stress response sigma factor for misfolded proteins in the periplasm) (Dalebroux and Swanson, 2012). Notably, the physiological roles of ppGpp continue to expand, such as inhibition of the synthesis of purine nucleotides (Wang et al., 2019), regulation of purine homeostasis through activation of nucleosidase PpnN (Zhang et al., 2019), inhibition of the ribosome-associated GTPase Era that is important for assembling 30S ribosome subunits (Wood et al., 2019a), and binding the GTPase HflX that activates 100S ribosomes (Zhang et al., 2018).
In contrast to the vast majority of cells that mount an active response to stress, the sub-population of cells that become persisters may be created when ppGpp turns off an excessive number of ribosomes by activating the expression of the small (55 aa) ribosome modulation factor (RMF) (Izutsu et al., 2001), which converts active 70S ribosomes into inactive 100S ribosomes (Wada et al., 1995) via an inactive 90S dimer complex (Ueta et al., 2005). Also, ppGpp induces the hibernation promoting factor the hibernation promoting factor (Hpf) (95 aa) which converts 90S ribosomes into 100S ribosomes and is highly conserved as it is found in most bacteria and some plants (Prossliner et al., 2018). This dimerization is essential for survival in the stationary phase (Yoshida et al., 2018). Hence, we reasoned that ppGpp directly modulates persistence through its direct inactivation of ribosomes. In agreement with this hypothesis, we found overproduction of RMF and Hpf increase persistence as well as reduce single-cell resuscitation while ppGpp has no effect on single-cell resuscitation (Song and Wood, 2019a). We have termed this mechanism the ppGpp ribosome dimerization persister (PRDP) model (Fig. 1) in which persister cells form not through TA systems but by the direct control of ribosome activity by ppGpp. In support of this model, *E. coli* cells have 100 times less persistence in the absence of ppGpp (Chowdhury et al., 2016a), and higher ppGpp levels increases the persistence of single cells by 1,000-fold (Svenningsen et al., 2019).

Another model for persister cell formation is based on reducing ATP (Dörr et al., 2010; Cheng et al., 2014; Conlon et al., 2016; Shan et al., 2017; Cameron et al., 2018) or uncoupling the membrane potential with compounds like CCCP to reduce ATP (Kwan et al., 2013). However, conflicting results have been obtained from other labs suggesting ATP does not control persistence (Pontes and Groisman, 2019; Svenningsen et al., 2019).

**Persister cell resuscitation is heterogeneous and is based on ribosome content.** Single cell analysis has revealed that most persister cells do not resuscitate spontaneously, but instead, wake upon sensing fresh nutrients (Yamasaki et al., 2019). This is in opposition to the ‘Scout Model’ (Epstein, 2009) in which it was proposed (without data) that persister cells wake spontaneously and periodically to see if it is safe to grow.

Upon waking, persister resuscitation is heterogeneous and includes five phenotypes: (i) immediate division, (ii) immediate elongation followed by division, (iii) immediate elongation but no division, (iv) delayed elongation/division, and (v) no growth (Kim et al., 2018b). Critically, this heterogeneity in resuscitation is due to the different levels of active ribosomes: by using a validated green fluorescence protein reporter of ribosome levels, we observed that higher ribosome levels result in faster waking (Kim et al., 2018b). Hence, cells with low levels of ribosomes have to increase their ribosome levels to a threshold value, then they begin to divide or elongate (Kim et al., 2018b). The varying ribosome levels result from cell-to-cell
differences in the numbers of ribosomes in exponentially-growing cells upon ceasing their metabolic activity (Kim et al., 2018b).

This heterogeneity in persister cell resuscitation (Kim et al., 2018b) has been corroborated by several groups. For example, using *E. coli* and time-lapse microscopy, Şimşek and Kim (Şimşek and Kim, 2019) followed 12,800 of what appears to be persister cells (or stationary cells, it is not clear which they used since it is not clear if the stationary-phase cells that they used were pre-treated with ampicillin to eliminate non-persisters) to find that most cells wake immediately and that their rejuvenation probability decays exponentially, resulting in lag time before resuscitation from 0 to over 1000 min. Pu et al. (Pu et al., 2019), also found that single persister cells wake heterogeneously (from less than 12 h to 40 h), but their explanation based on the degree of protein refolding required for their resuscitation is unlikely as it would require an enzyme that could re-nature a thousand different proteins (Wood et al., 2019b). In addition, Goormaghtigh and Van Melderen (Goormaghtigh and Van Melderen, 2019) found persister cells wake heterogeneously and frequently include elongation.

In addition, when persisters resuscitate and begin dividing, their growth rate is the same as exponential cells; hence, once the formerly-dormant cell commences division, it is fully recovered (Kim et al., 2018b). Also, it is important to note that residual stress may be detrimental to sustained resuscitation in that cells with residual ampicillin antibiotic fail to resuscitate (Kim et al., 2018b).

**Persister cells resuscitate by activating dormant ribosomes.** It has been proposed that toxins are inactivated and in this way persister cells may revive. However, as with the model that relies on TA systems for persister cell formation, there is little data that support this. For example, it was suggested that the peptidyl-tRNA hydrolase Pth counteracts toxin TacT in *S. Typhimurium*, “explaining how bacterial persisters can resume growth”; however, there were no resuscitation data to validate this claim (Cheverton et al., 2016). Similarly, it was claimed that deactivation of polymerized (active) HokB toxin by monomerization controls persister waking; however, single-cell observations were not made but instead delays in resuscitation were estimated from growth data (Wilmaerts et al., 2019) for this type I system. Also, the key features for the HokB system have been discerned from non-physiological levels of HokB (i.e., from overproduction of HokB) (Wilmaerts et al., 2018; Wilmaerts et al., 2019), and there is no persister phenotype for deleting *hokB* (Verstraeten et al., 2015). Critically, for type II TA systems, there is little data for the paradigm that antitoxins are degraded from TA complexes and cells revive; reactivation of toxins bound to antitoxins is unlikely given that the antitoxin is bound tightly to the toxin in most cases (for type II TA systems).
Since there is little evidence of toxins of TA systems being inactivated to revive cells, the simplest model for persister cell resuscitation is re-activation of ribosomes. Based on single-cell observations (Song and Wood, 2019a; Yamasaki et al., 2019), we propose the PRDP model (Fig. 1) holds for persister resuscitation: after ppGpp generates persister cells directly by inactivating ribosomes via increased production of RaiA (inactivates 70S ribosomes) (Prossliner et al., 2018), production of the ribosome modulation factor (RMF), and production of Hpf. Once the stress dissipates and the cells have fresh nutrients, inactive 100S ribosomes reactivate by the ribosome resuscitation factor HflX (Gohara and Yap, 2018) which will no longer be inactivated by ppGpp (Zhang et al., 2018) and repressed by cAMP (Lin et al., 2011); HflX also rescues ribosomes from mRNA (Zhang et al., 2015). In support of this, we found RaiA, RMF, and Hpf increase persistence and reduce single-cell persister resuscitation and that RMF increases 100S ribosome formation in persister cells (Song and Wood, 2019a). In addition, we found producing HflX increases single-cell waking (Yamasaki et al., 2019). Furthermore, the resuscitating cells sense the fresh nutrients by chemotaxis and phosphotransferase membrane proteins (Yamasaki et al., 2019), and transport of nutrients reduces the level of secondary messenger cAMP through enzyme IIA (Yamasaki et al., 2019); this reduction in cAMP levels stimulates ribosome resuscitation and rescue (Yamasaki et al., 2019). The waking persister cells also immediately commence chemotaxis toward nutrients (Yamasaki et al., 2019). Therefore, persister cells wake by sensing nutrients via membrane receptors, and this external waking signal is conveyed via the secondary messenger cAMP to inactive ribosomes which are used by the persisters to wake and begin chemotaxis to acquire nutrients (Yamasaki et al., 2019).

Corroborating the ribosome model for persister cell resuscitation (Song and Wood, 2019a), a 10,000-member library of druglike compounds was screened to identify compounds that increase persister cell resuscitation and 2-[(2-(4-bromophenyl)-2-oxoethyl)thio]-3-ethyl-5,6,7,8-tetrahydro[1]benzothieno[2,3-d]pyrimidin-4(3H)-one (BPOET) was identified that stimulates persister cell resuscitation by activating ribosomes via pseudouridine modification of 23S rRNA by pseudouridine synthase RluD (Song and Wood, 2019b); hence, ribosomes are modified to facilitate persister resuscitation. Also, 100S ribosome recovery is extremely rapid and occurs in less than one minute (Prossliner et al., 2018), which matches our data indicating some persister cells wake immediately (Kim et al., 2018b).

**Perspectives.** Implications of the PRDP model include that persister cell formation is an elegantly-regulated response to stress rather than a bet-hedging (“stochastic”) process. Experimental data support this idea in that spontaneous persisters are rare (Balaban et al., 2004) but external stress (e.g., antibiotics, hydrogen peroxide, acid) converts nearly the whole population into persister cells (Dörr et al., 2009; Hong
et al., 2012; Kwan et al., 2013). In addition, the PRDP model suggests resuscitation is also an elegant response to improved environmental conditions rather than spontaneous. Since all cells starve and need deep resting states, it is logical that cells utilize elegant pathways for both persister cell formation and resuscitation.

Also, clearly there is something more than just stress that makes cells become persistent. For example, all stationary-phase cells are starving but only a small percentage become persisters. The PRDP model suggests that a small population of stressed cells has a larger number of their ribosomes inactivated so this heterogeneity in ribosome dimerization is perhaps the “switch” that makes cells persistent.

Remaining questions include whether the PRDP model is general; i.e., holds for other species, and whether it is relevant for clinical isolates. The PRDP model is likely applicable to myriad stresses since RMF dramatically increases persistence with for stress from ampicillin (Song and Wood, 2019a), ciprofloxacin (Song and Wood, 2019a), netilmicin (Tkachenko et al., 2017), gentamicin (McKay and Portnoy, 2015), acid (El-Sharoud and Niven, 2007), osmotic stress (Shcherbakova et al., 2015) and nutrient limitation in the stationary phase (Yamagishi M, 1993; Bubunenko et al., 2007). Furthermore, since RMF is conserved in bacteria (Prossliner et al., 2018) and Hpf is even more widely distributed (Akiyama et al., 2018), there is the possibility that the PRDP model may be applicable for the formation of the persister state of many strains. For example, like E. coli, the persistence of P. aeruginosa also requires ppGpp (Nguyen et al., 2011) and so it would be interesting to test further whether ribosome dimerization also controls persister cell formation and resuscitation in this strain. It is already established that both Hpf and ppGpp (but not RMF) are necessary for the long term survival of P. aeruginosa during starvation by preserving ribosomes (Akiyama et al., 2017) and that ppGpp plays a role in hpf expression (Akiyama et al., 2018). However, a preliminary report indicates that after treating with ciprofloxacin, high persister variants are present in cystic fibrosis patents, but mutations related to ppGpp were not found (Mojsoska et al., 2019).

Since persister cells are formed in the absence of ppGpp but at much lower levels (Chowdhury et al., 2016a), there must be other means to activate Rmf and Hpf without ppGpp, which leads to the formation of inactive and dimerized ribosomes. For example, cAMP, which is generated upon starvation (e.g., glucose depletion), induces rnf (Shimada et al., 2013) and raiA (Prossliner et al., 2018), and plays a role in the formation of persister cells (Kwan et al., 2015) (Fig. 1). In addition, cAMP represses hflX (Lin et al., 2011). Hence, cAMP is as likely to be as important as ppGpp for persistence. Critically, there may be several mechanisms for forming persister cells, but the net result is that ribosomes are inactivated.

New compounds will indubitably be identified for killing persister cells since it now possible to create large...
libraries of *bona fide* persister cells in a facile manner (Kwan et al., 2013; Kim et al., 2018b). For example, by screening a 10,000-member library of druglike compounds, we identified that the indole derivative, 5-nitro-3-phenyl-1H-indol-2-yl-methylamine hydrochloride (NPIMA) (Song et al., 2019), kills *E. coli* persister cells better than 5-iodoindole (Lee et al., 2016) and better than the DNA crosslinking agent cisplatin (Chowdhury et al., 2016b; Cruz-Muñiz et al., 2016). NPIMA also eliminated *P. aeruginosa* persister cells and had activity on the representative Gram positive pathogen, *S. aureus* (Song et al., 2019). The mode of action of NPIMA with both Gram-positive and Gram-negative bacteria was determined to be via membrane damage which leads to lysis. Critically, understanding the steps for persister formation and resuscitation (Fig. 1) will provide additional targets for fighting persistence.

There are also parallels being discovered between bacterial persisters and cancer cells that are recalcitrant to known treatments (Vallette et al., 2019). Dormancy for cancer cells occurs when the growth of the cells is temporarily stopped before initiating a new primary tumor that may be even more aggressive the original tumor. Hence, insights gleaned from bacterial persisters may be relevant for treating cancer.

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Stress such as nutrient limitation, osmotic stress, and acid stress induce the stringent response which results in ppGpp formation by RelA/SpoT and generation of cAMP (e.g., upon glucose depletion via the phosphorylated glucose phosphotransfer enzyme, EIIA-P) in *E. coli*. ppGpp induces *hpf*, and both ppGpp and cAMP together induce *raiA* and *rmf*. RaiA inactivates 70S ribosomes, RMF converts 70S ribosomes into inactive 90S ribosomes, and Hpf converts inactive 90S ribosomes into 100S ribosomes. Moreover, ppGpp binds HflX to likely inactivate it, and cAMP represses *hflX*. Upon addition of nutrients and removal of the stress, cAMP decreases (due to unphosphorylated EIIA) which stimulates HflX production; HflX dissociates 100S ribosomes into active 70S ribosomes and growth resumes. Since persister cells form in the absence of ppGpp (although at much reduced levels), cAMP by itself and perhaps other mechanisms activate RMF and Hpf as well.

**Fig. 1. Schematic of the ppGpp ribosome dimerization persister (PRDP) model.** Stress such as nutrient limitation, osmotic stress, and acid stress induces the stringent response which results in ppGpp formation by RelA/SpoT and generation of cAMP (e.g., upon glucose depletion via the phosphorylated glucose phosphotransfer enzyme, EIIA-P) in *E. coli*. ppGpp induces *hpf*, and both ppGpp and cAMP together induce *raiA* and *rmf*. RaiA inactivates 70S ribosomes, RMF converts 70S ribosomes into inactive 90S ribosomes, and Hpf converts inactive 90S ribosomes into 100S ribosomes. Moreover, ppGpp binds HflX to likely inactivate it, and cAMP represses *hflX*. Upon addition of nutrients and removal of the stress, cAMP decreases (due to unphosphorylated EIIA) which stimulates HflX production; HflX dissociates 100S ribosomes into active 70S ribosomes and growth resumes. Since persister cells form in the absence of ppGpp (although at much reduced levels), cAMP by itself and perhaps other mechanisms activate RMF and Hpf as well.