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Combatting Bacterial Persister Cells†

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ABSTRACT

Most bacterial cells lead lives of quiet desperation in biofilms, combatting stress; yet, their prevalence attests to their ability to alter gene regulation to cope with myriad insults. Since biofilm bacteria are faced with starvation and other environmental stress (e.g., antibiotics from competitors, oxidative stress from host immune systems), it behooves them to be able to ramp down their metabolism in a highly-regulated manner and enter a resting state known as persistence to weather stress. Hence, persister cells are metabolically dormant cells that arise predominantly as a response to stress through elegant gene regulation that includes toxin/antitoxin systems. In this review, an analysis is made of the genetic pathways that lead to persistence, of cell signaling via the interspecies and interkingdom signal indole that leads to persistence, and the means found to date for combatting these cells which are frequently tolerant to a range of antibiotics.

Keywords: persister cells, cell signaling, antimicrobial tolerance

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INTRODUCTION

Persister cells are tolerant to multiple antibiotics (Lewis 2010) and other stresses. Unlike resistant cells, persisters do not grow in the presence of antibiotics and arise without undergoing genetic change. Developing treatments for these antibiotic-tolerant cells is urgent since they are a major cause of recurring/chronic biofilm infections such as those associated with cystic fibrosis (Mulcahy et al. 2010), and microbial infections are the leading cause of death worldwide (Rasko and Sperandio 2010).

Persister cells withstand various stresses by becoming metabolically dormant. Persister cells were first described by Hobby et al. (Hobby et al. 1942) with *Staphylococcus aureus*; they found that 1% of the cells were not killed by penicillin and showed first that penicillin was ineffective against non-growing (cold) cells. The main result that these *S. aureus* persister cells are dormant was corroborated three ways by Bigger (Bigger 1944) by showing that (i) stopping cell growth by lowering the culture temperature generated more persisters to penicillin, (ii) halting growth by removing nutrients generated more persisters, and (iii) reducing growth by adding boric acid at concentrations where it was known to be bacteriostatic generated more persisters.

Subsequent research has confirmed that persister cells are metabolically inactive or have vastly reduced metabolism; for example, Shah et al. (2006) fractionated metabolically-active cells from those with much reduced metabolism by using a ribosomal promoter fused to a GFP reporter to show that the metabolically-inactive cells were more tolerant to the fluoroquinolone ofloxacin. These results were extended by Kwan et al. (2013) who demonstrated that persister cells lack protein synthesis to become tolerant to antibiotics; persistence was increased dramatically by pre-treating cells with (i) rifampin to stop transcription, (ii) with tetracycline to halt translation, and (iii) with carbonyl cyanide *m*-chlorophenyl hydrazine to halt ATP synthesis. With these three pre-treatments, nearly all of the bacterial population was converted to persister cells from an initial population of 0.01% (a 10,000-fold increase in persister cells).

In stark contrast to decades of research showing persister cells are dormant, others have argued that persister cells are metabolically active. However, the claims of metabolic activity in persister cells have not been compelling (Wood et al. 2013). For example, Orman and Brynildsen (2013) utilized FACS in an attempt to determine whether persister cells were active; however, they likely carried over high numbers of dormant

persister cells into their FACS and persister cell assays, and the number of cells they used to make their conclusions were much less than the likely errors in their technique. In the study of Wakamoto et al. (2013), they claimed that cells surviving lethal treatment with the prodrug isoniazid were metabolically active; however, isoniazid requires activation by catalase KatG, so the metabolically-active cells tolerant to isoniazid were simply cells with low levels of KatG activity; therefore, the metabolic activity observed for cells surviving isoniazid is not indicative of metabolic activity in persister cells.

Furthermore, since persister cell formation is likely a well-regulated response to external stress, metabolism is of course altered as the cells proceed to the dormant state; hence, this change in metabolism on the way to the persister state is not evidence that persister cells themselves are not dormant. Critically, a key area to explore is how persisters wake; i.e., what elements of metabolism must remain intact to enable cells to perhaps monitor their environment and to resume growth.

Toxin/antitoxin systems as the origin of persister cells. Persister cells are absent from exponentially-growing cells (Keren et al. 2004a; Orman and Brynildsen 2013) where cells grow robustly but are as high as 1% in stationary-phase cultures and in biofilms (Lewis 2007; Lewis 2008), where cells incur various forms of stress (Stewart and Franklin 2008). Although they may be generated stochastically as a result of fluctuations in gene expression and protein levels within individual cells of an isogenic population (Balaban et al. 2004), it appears the more germane mechanism is that persister cells arise from elegant gene regulation as a result of environmental influence, as first shown by Bigger (1944) and now by others (Dörr et al. 2010; Hu et al. 2015; Kwan et al. 2015c; Kwan et al. 2013; Möker et al. 2010; Vega et al. 2012). For example, antibiotics are readily encountered by bacteria in the environment, and ciprofloxacin, rifampicin, and tetracycline induce persistence (Dörr et al. 2010; Kwan et al. 2013); hence, extracellular compounds that induce stress increase persistence dramatically and reveal that a subpopulation of cells respond to their environment by becoming dormant.

The best model for the induction of persistence (i.e., dormancy) as a stress response is that the cells respond to stress by activating toxin/antitoxin (TA) systems whose primary role in cell physiology is to reduce metabolism under stressful conditions (Wang and Wood 2011). For example, MqsR/MqsA TA system of *Escherichia coli* protects the cell during bile acid stress in the gastrointestinal tract (Kwan et al. 2015b) and protects the cells from oxidative stress by controlling stress response sigma factor RpoS (Wang et al. 2011).

Toxin MqsR reduces metabolism by cleaving nearly all the cellular mRNA (Brown et al. 2009; González Barrios et al. 2006), and the MqsR/MqsA TA systems was first identified as important for biofilm formation (Ren et al. 2004). The genes encoding TA systems occur in pairs with the antitoxin masking the action of the toxin until a stress activates the toxin (usually by deactivating the antitoxin) such as the activation of toxins upon phage attack (Hazan and Engelberg-Kulka 2004; Pecota and Wood 1996). Toxins are misnamed as they are not external products that affect competitors or host cells but instead are intracellular proteins that reduce metabolism frequently by reducing protein production by reducing transcription, translation, and ATP production. It is now clear that fitness of bacteria is enhanced if they are able to reduce their metabolism during stress. For example, membrane toxin GhoT (Wang et al. 2012) reduces cell growth by decreasing ATP production and the proton motive force during antibiotic stress to make the cell more fit (Cheng et al. 2014), and membrane toxin OrtT reduces cell growth by reducing ATP during the stringent response (e.g., nutrient stress) to make the cell more fit (Islam et al. 2015).

The role of TA systems in persister cells was first established in 1983 through the HipA/HipB TA system (Moyed and Bertrand 1983). After chemical mutagenesis, the gain of function *hipA7* mutation was identified in *E. coli* that leads to increased tolerance to ampicillin (Moyed and Bertrand 1983) as a result of activated HipA; this increased activity was first suggested as probably due to reduced antitoxin binding as suggested by the structure HipA-HipB-DNA complex (Schumacher et al. 2009). Paradoxically, the HipA7 variant (G22S and D291A) is nontoxic (Korch et al. 2003), and the many persister-related experiments using this variant are not studying an active toxin. The HipA toxin was initially thought to inactivate the translation factor EF-Tu by phosphorylating it (Schumacher et al. 2009); however, it was found later that that HipA phosphorylates the active center of the aminoacyl-tRNA synthetase GltX to inhibit translation (Germain et al. 2013). Furthermore, another study utilizing the structure of the HipB-$(HipA)_2$-HipB complex has suggested that toxicity of the HipA7 variant stems from exposure of the HipA active site due to the G22S substitution; i.e., this substitution prevents HipA dimerization and exposes the HipA active site (Schumacher et al. 2015). However, although the *hipA7* mutations increase persistence and are found in antibiotic-tolerant *E. coli* strains that cause urinary tract infections, HipA toxin activity after this G22S substitution remains to be shown (Schumacher et al. 2015).

Additional evidence of the key role of TA systems in persister cell formation was provided in 2004 when

DNA microarrays showed that the TA systems YafQ/DinJ, RelE/RelB, and MazF/MazE were induced in persister cells, and production of toxin RelE led to as much as a 10,000-fold increase in persistence (Keren et al. 2004b). In 2006, DNA microarrays were used again and toxin gene *mqsR* was the most induced gene for persister cells (Shah et al. 2006). Other TA-related genes with differential transcription included *dinJ*, *yoeB,* and *yefM* (Shah et al. 2006). Critically, deleting several individual TA loci has been shown to reduce persistence: *mqsR* and *mqsRA* (Kim and Wood 2010; Luidalepp et al. 2011), *tisAB/istR* (Dörr et al. 2010), and *yafQ* (Harrison et al. 2009). Therefore, it is not necessary to reduce several TA loci simultaneously to see an impact on persistence as has been claimed (Maisonneuve et al. 2011) after these seminal initial reports of deleting single TA loci and the corresponding reduction in persistence. Hence, TA systems are intimately associated with persistence.

It has been proposed that the mechanism of activating toxins of TA systems to create persister cells is through production of polyphosphate which is proposed to activate Lon protease; activated Lon then degrades antitoxins, which would result in activated toxins (Maisonneuve et al. 2013). However, *in vitro*, Lon is not activated by polyphosphate but instead is deactivated by it (Osbourne et al. 2014), and deletions in *lon* have no effect on persistence with aminoglycosides or β-lactams (Shan et al. 2015). Furthermore, deleting *ppk*, which encodes the enzyme that produces polyphosphate, has little impact on persistence with aminoglycosides (Shan et al. 2015). Therefore the link between polyphosphate and toxin activation via Lon protease is controversial.

Since overproduction of every toxin to date increases persistence (Keren et al. 2004b; Kim and Wood 2010), random mutagenesis of a toxin gene (*mqsR*) to select for a more toxic RNase toxin was performed to determine how toxins increase persistence (Hong et al. 2012). Two amino acid replacements were sufficient to increase the stability of toxin MqsR, thereby increasing both its toxicity and the number of persister cells it generated. Transcriptomics showed that the MqsR variant increased persistence by digesting more readily mRNA for acid resistance, multidrug resistance, and osmotic resistance. Therefore, the more toxic MqsR *increases* persistence by *decreasing* the ability of the cell to respond to antibiotic stress through its RpoS-based regulation of acid resistance, multidrug resistance, and osmotic resistance systems. Paradoxically, cells that are less able to withstand stress are more likely to become persister cells (Hong et al. 2012).

In summary, a working model (**Fig. 1**) for the role of TA systems in the formation of persister cells is based

on a spectrum of activity toxins in cells: some cells mount an active response to cellular stress by using such tools as toxin RNases to remove old transcripts to allow the cell to focus on newly transcribed messages triggered by activation of alternate sigma factors such as RpoS whose activity is derepressed by inactivating antitoxins such as MqsA (Wang et al. 2011) and DinJ (Hu et al. 2012). Other cells choose to wait out the stress by becoming dormant as an extreme activation of toxins. To achieve dormancy, they halt protein production by inactivating nearly all mRNA and by reducing ATP as a result of toxin membrane damage. Upon cessation of the stress, these cells revive and reconstitute the biofilm.

Indole cell signaling and persistence. Given the prominent role of TA systems in the formation of persister cells, an investigation using system biology methods was initiated to gain additional insights into how persister cells form, with the surprising result that two independent lines of investigation led to the realization that the interkingdom signal indole (**Fig. 2**) is involved in persister cell formation (Hu et al. 2015; Kwan et al. 2015c). Indole production is widespread as it is produced by at least 27 different genera that produce tryptophanase (TnaA) (Lee et al. 2007b); tryptophanase converts tryptophan into indole. Indole was discovered as a signal in *E. coli* since it activates *gabT* and *astD* (Wang et al. 2001), and then it was shown to be a quorum-sensing signal (Lee et al. 2007a). It appears *E. coli* has at least two quorum-sensing systems in that indole is the primarily signal at low temperatures and autoinducer-2 is the primary signal at higher temperatures in the gastrointestinal tract (Lee et al. 2008). Indole also is an interspecies signal since it reduces the pathogenicity of cells that do not synthesize it (Chu et al. 2012; Hidalgo-Romano et al. 2014; Lee et al. 2009a) and influences the biofilm formation of other cells (Lee et al. 2007b); for example, indole reduces the virulence of *Pseudomonas aeruginosa* by repressing the *mexGHI-opmD* multidrug efflux pump and the genes involved in the synthesis of pyocyanin (*phz* operon), 2-heptyl-3-hydroxy-4(1*H*)-quinolone (PQS) signal (*pqs* operon), pyochelin (*pch* operon) and pyoverdine (*pvd* operon) which results in reduced pyocyanin, rhamnolipid, PQS and pyoverdine and enhanced antibiotic resistance (Lee et al. 2009a). Remarkably, indole also acts as an interkingdom signal since it is one of the first compounds made by commensal bacteria in the gastrointestinal tract and shown to be beneficial; it tightens epithelial cell junctions and thereby prevents invasion of pathogens (Bansal et al. 2010; Shimada et al. 2013). Its resemblance to human and plant hormones has also led to speculation that indole is the archetype for cell hormones (Lee et al. 2007b). Moreover, indole was shown initially to increase antibiotic

resistance by activating efflux pumps (Hirakawa et al. 2005; Kobayashi et al. 2006), and later to increase the antibiotic resistance of neighboring cells (Lee et al. 2010). Therefore, indole is an important interspecies and interkingdom signal.

Since toxins of TA systems are responsible for persister cell formation and the *dinJ/yafQ* locus is induced in persister cells (Keren et al. 2004b), YafQ of the YafQ/DinJ *E. coli* TA system was investigated for insights on how toxin YafQ generates persister cells (Hu et al. 2015). Under stress, YafQ alters metabolism by cleaving transcripts with in-frame 5'-AAA-G/A-3' sites (Prysak et al. 2009), and YafQ increases persistence with multiple antibiotics including the β-lactam ampicillin (1000-fold) and ciprofloxacin (43-fold) (Hu et al. 2015). By investigating changes in the proteome, it was found that YafQ reduces tryptophanase levels (TnaA mRNA has 16 putative YafQ cleavage sites) (Hu et al. 2015). Consistently, YafQ also reduces TnaA mRNA levels. Tryptophanase is activated in the stationary phase by the stationary-phase sigma factor RpoS, which is also reduced dramatically upon production of YafQ. Corroborating the results with TnaA, indole levels are reduced by producing YafQ, and adding indole reduces persistence. Also, deleting *tnaA* increases persistence and adding tryptophan (to increase indole levels) decreases persistence. Also, YafQ production had a much smaller effect on persistence in a strain unable to produce indole. Therefore, toxin YafQ increases persistence by reducing the cue indole (**Fig. 3**), and TA systems were once again related to cell signaling as they were previously with autoinducer 2 (González Barrios et al. 2006).

Given that the population of persisters increases in biofilms and that cyclic diguanylate (c-di-GMP) is an intracellular signal that increases biofilm formation, it was explored whether c-di-GMP has a role in bacterial persistence (Kwan et al. 2015c). After examining the effect of 30 genes from *E. coli* related to diguanylate cyclases that synthesize c-di-GMP and phosphodiesterases that breakdown c-di-GMP, DosP (direct oxygen sensing phosphodiesterase) was identified as increasing persistence by over a thousand fold (Kwan et al. 2015c). Using both transcriptomic and proteomic approaches, the mechanism by which DosP increases persistence was determined to be by decreasing tryptophanase activity and indole levels. Critically, although DosP is a well-known c-di-GMP phosphodiesterase, the decrease in tryptophanase activity was found to be a result of its degradation of another cyclic nucleotide signal, cyclic adenosine monophosphate (cAMP). Corroborating this result, the reduction of cAMP via CpdA, a cAMP-specific phosphodiesterase, increased persistence and reduced

indole levels similarly to DosP (Kwan et al. 2015c). Therefore, phosphodiesterase DosP increases persistence by reducing the interkingdom signal indole via reduction of the global regulator cAMP (**Fig. 3**). Note these results with cAMP are in opposition to an earlier study which proposed that cAMP increases persistence by stimulating RelA expression to produce the stringent response alarmone ppGpp (Amato et al. 2013), which was previously implicated in multiple persister formation pathways (Wood et al. 2013).

Since indole increases antibiotic resistance by enhancing antibiotic efflux (Hirakawa et al. 2005; Kobayashi et al. 2006), a likely mechanism for the rise in persistence upon the reduction in indole concentrations (Hu et al. 2015; Kwan et al. 2015c) is that the cells become less resistant (due to reduced antibiotic efflux) and thereby less fit to withstand antibiotic stress. This interpretation of the reverse relation between fitness and persistence fits earlier results based on producing a more toxic toxin (Hong et al. 2012). This inverse relation of indole and persistence, found via the independent studies using toxin YafQ and cAMP-degrading DosP (Hu et al. 2015; Kwan et al. 2015c), is in opposition to another report in which indole was reported to increase persistence (Vega et al. 2012); however, the difference in the role of indole may be related to the manner in which indole is added to the cells during the persistence assay. Moreover, the inverse relationship between indole and persistence is reasonable since indole reduces biofilm formation in *E. coli* in rich medium (Domka et al. 2007; Domka et al. 2006; Lee et al. 2007a; Lee et al. 2007b; Lee et al. 2009b) and is reduced in biofilms (Domka et al. 2007). Since persister cells are more prevalent in biofilms (Lewis 2007), cells should reduce indole signaling in order to increase persistence in biofilms.

Combatting persister-based infections. Persisters, due to their state of metabolic dormancy, have high tolerance against all traditional antibiotics (e.g., fluoroquinolones, aminoglycosides, and β-lactams), which are mainly effective against actively-growing cells. Therefore, approaches that should be effective against persister cells should focus on compounds that enter the cell without active transport and should be able to kill the cells without utilizing any of the cell's machinery, which is probably inactive due to its resting state. The ability of the compounds to penetrate the biofilm matrix should also be considered. To test the compounds against persister cells, which are notoriously difficult to study due to their low percentage of the cell population, techniques such as pretreating cells with rifampicin (or inducing toxins) should be used to increase the population of persister cells (Kwan et al. 2013) so that the compounds may be tested directly on persister cells. It is also important to

evaluate new therapies using cells cultured in a clinically-relevant manner such as in wound models, animal models, and as viable but non-culturable bacteria (VBNC), which are related to persister cells (Ayrapetyan et al. 2015).

As to combatting persister cells, the approaches can be broken into those that (i) try to kill persister cells while they sleep, (ii) those that try to wake persister cells and then treat them with traditional antibiotics, and (iii) those that prevent the formation of persister cells. A recent success in killing persister cells while they sleep makes use of the FDA-approved, anti-cancer drug mitomycin C (**Fig. 2**) produced by *Streptomyces caespitosus*, which eradicates persister cells through a growth-independent mechanism (Kwan et al. 2015a). Critically, mitomycin C can enter the cell passively and then spontaneously crosslink DNA without relying on cell activity; indeed, mitomycin C was found to crosslink the DNA of cells in the persister state (Kwan et al. 2015a). Mitomycin C eradicates cells growing exponentially and in the stationary phase as well as grown planktonically and in biofilms and is effective in both rich and minimal media (Kwan et al. 2015a). Additionally, mitomycin C kills the persister cells of a broad range of bacterial species, including commensal *E. coli* K-12 as well as the pathogenic species *E. coli* O157:H7 (EHEC), *S. aureus*, *P. aeruginosa*, and *Vibrio cholerae*. Furthermore, mitomycin C was shown to be effective in reducing an EHEC infection in a nematode *Caenorhabditis elegans* animal model and was also effective in a wound model against a consortium of *S. aureus* and *P. aeruginosa*, which frequently occur together in wounds (Kwan et al. 2015a). Mitomycin C also had activity against VBNC bacteria (Kwan et al. 2015a). Therefore, mitomycin C is the first broad-spectrum compound capable of eliminating persister cells and has already been approved for human use (Kwan et al. 2015a). Corroborating this initial success, mitomycin C has also been shown to be effective against persister cells of the spirochete *Borrelia burgdorferi*, the causative agent of Lyme disease (Sharma et al. 2015).

One caveat for mitomycin C is that it is somewhat toxic and was used at 10 μg/mL to combat bacterial persisters (at five times the MIC which is common in persister cell research) (Kwan et al. 2015a). In comparison, 0.5-2.0 μg/mL is used for various cancer treatments (Bradner 2001) and topical dosages have been safely applied at concentrations up to 400 μg/mL (Shields et al. 2002). Therefore, mitomycin C may be more appropriate for wound treatments while oral doses may be problematic.

Another success in combatting persister cell infections by killing the cells as they sleep utilizes the

spontaneous degradation of proteins by activating ClpP protease via the antibiotic acyldepsipeptide ADEP4 (**Fig. 2**) (Conlon et al. 2013). This method is effective since protein degradation can be made to be a spontaneous process; i.e., one that does not depend on cellular activity: ADEP4 removes the requirement for ATP with ClpP and makes the enzyme a nonspecific protease. ADEP4 increased protein degradation in *S. aureus* stationary cells (persister cells were not tested) and was effective against *S. aureus* infections in a mouse model and effective against *S. aureus* biofilms. However, ADEP4 is not effective on its own and must be combined with other antibiotics like rifampicin (Conlon et al. 2013). Also, ClpP null mutants arise rapidly which would allow the cells to escape the effects of this treatment.

By first screening 85,000 compounds that prevent the death of *C. elegans* infected with methicillin-resistant *S. aureus* (MRSA) followed by screening the 101 hits for cell permeabilization of MRSA persister cells via Sytox Green, NH125 (1-hexadecyl-2-methyl-3-(phenylmethyl)-1H-imidazolium iodide) was identified that is effective in killing MRSA by permeabilizing its membrane (Kim et al. 2015). This compound was also effective in causing biofilm dispersal. The nematode screen that was utilized has the advantage that only compounds that are non-toxic are identified.

Pyrazinamide (**Fig. 2**), an analog of nicotinamide, is used in short course chemotherapy (along with isoniazid, ethambutol, and rifampin) for *Mycobacterium tuberculosis* infections and kills sleeping persister cells by corrupting the trans-translation process that recovers stalled ribosomes (Shi et al. 2011). Pyrazinamide probably enters cells passively and is converted to pyrazinoic acid which binds RpsA, a key protein in trans translation. Use of pyrazinamide has reduced tuberculosis treatments from nine to 12 months to six months. The same group has also had success in killing the persister cells of *Borrelia burgdorferi* which causes Lyme disease by combining three antibiotics, the lipopeptide daptomycin, the beta-lactam cefoperazone, and tetracycline-class doxycycline (Feng et al. 2015). Daptomycin was identified by screening a FDA-approved drug library of 1524 antibiotics that have activity against stationary phase *B. burgdorferi* persisters using a high-throughput SYBR Green I/propidium iodide assay, but has little activity on actively-growing cells so combining it with antibiotics that kill actively-growing *B. burgdorferi* cells proved effective in eradicating the most persistent microcolony cells of *B. burgdorferi*.

Linear cationic antimicrobial peptides (**Fig. 2**) containing Trp/Arg-repeats have also been used to kill *E. coli*

persisters and reduce their numbers by up to 1000 at 80 μM (Chen et al. 2011). These compounds were also effective for killing planktonic and biofilm cells, and caused biofilm dispersal. Antimicrobial peptides disrupt the cell membrane and have activity against bacteria, viruses, and fungi (Izadpanah and Gallo 2005). In a clever strategy utilizing antimicrobial peptides, a 12 aa antimicrobial peptide has been tethered to the aminoglycoside tobramycin (Pentobra, **Fig. 2**) so that the tobramycin moiety allows the hybrid compound to enter persister cells and then the combination of the translation-inhibiting antibiotic and peptide kills *E. coli* and *S. aureus* persister cells (up to a million-fold reduction in cell numbers) (Schmidt et al. 2014).

Since persister cells are easily and rapidly awakened by fresh medium, adding sugars and glycolysis intermediates (e.g., mannitol **Fig. 2**, glucose, fructose, pyruvate) also wakes persister cells, and the resulting growing cells are sensitive to aminoglycoside antibiotics (Allison et al. 2011) (see supplemental data where "potientiating" compounds induced growth, even under poor growth conditions in 96 well plates). Persister cells may also be awakened by compounds not used as nutrients; for example, by adding *cis*-2-decenoic acid (Marques et al. 2014) (**Fig. 2**). *cis*-2-Decenoic acid is an extracellular signal from *P. aeruginosa* that causes biofilms to disperse (Davies and Marques 2009). In persister cells, *cis*-2-decenoic acid causes a sudden burst in protein synthesis, and when combined with ciprofloxacin, the cells that are awakened by *cis*-2-decenoic acid are killed by ciprofloxacin, resulting in a 3000-fold reduction for *P. aeruginosa* persister cells from planktonic cultures and a reduction of one million-fold for biofilm-derived persisters (Marques et al. 2014).

Another compound that wakes persister cells was found by screening 6800 chemicals from a random chemical library, 3-(4-(4-methoxyphenyl)piperazin-1-yl)piperidin-4-yl biphenyl-4-carboxylate (Kim et al. 2011) (**Fig. 2**). This compound wakes persister cells at 25 μM; however, the mechanism has not been determined. Also, the brominated furanone (Z)-4-bromo-5-(bromomethylene)-3-methylfuran-2(5H)-one (**Fig. 2**) wakes *P. aeruginosa* planktonic and biofilm persister cells (Pan et al. 2012) and mucoid *P. aeruginosa* (Pan et al. 2013).

Success has also been achieved by preventing persister cell formation by attenuating quorum sensing. By screening 284,256 compounds for interference with *pqsA* expression and by using structure-activity relationships, a family of compounds was identified including the benzimidazole derivative M64 (**Fig. 2**) that block the PqsR quorum sensing/virulence system of *P. aeruginosa* (Starkey et al. 2014). Using M64, persister cell concentrations were reduced up to 100 fold and the virulence of *P. aeruginosa* was reduced in several

murine model systems (Starkey et al. 2014).

Perspectives. It is reasonable that some percentage of the bacterial cell population should obtain a resting state to avoid various stresses since cells spend the bulk of their existence in a stressed state. Hence, part of the success of bacteria involves having the ability to respond to stress in an elegantly-regulated manner to reduce growth. So sometimes survival of the fittest means having the ability to grow slowly (or not grow at all), until the stress ceases, when growth may resume. Therefore, it is becoming increasing clear that perhaps all bacteria and Archaea have the ability to adopt a dormant state.

Given this prevalence of the dormant state, a new paradigm for evaluating antimicrobials is required: evaluating new pharmaceuticals for their efficacy for cells in the resting state. Luckily, some new techniques for battling persisters have been found in the past few years, as outlined above. However, the idea that killing planktonic cells to combat acute infections may in fact generate persisters and complicate chronic infections has not been embraced completely. For example, it has been argued that activating toxins (by deactivating antitoxins) is a possible method to fight infections (Chan et al. 2015). However, as is the case for many antimicrobials that are effective in killing growing cells, this often results in generating persister cells, which are non-trivial to treat. Perhaps the best approach then is to utilize a combination of drugs (at least one for growing cells and one for persisters) as was done by combining rifampicin with ADEP4 (Conlon et al. 2013) and by combining cefoperazone and doxycycline with daptomycin (Feng et al. 2015).

Given the plethora of new methods arising for combatting persister cells, we can be sanguine about our chances to eliminate their infections. However, we should remain critical and avoid complacency. For example, these novel methods to combat persister cells should be evaluated for their range of their applicability, including testing their efficacy with VBNC cells. Furthermore, the new treatment methods should be evaluated in terms of how readily resistance occurs. The field should strive to avoid the complacency seen for some groups developing anti-virulence compounds which have argued resistance is not important (Allen et al. 2014; Gerdt and Blackwell 2014), even in the face of mounting evidence that resistance to anti-virulence compounds is common (García-Contreras et al. 2013a; García-Contreras et al. 2013b; García-Contreras et al. 2015b; Maeda et al. 2012) and spreads (García-Contreras et al. 2015a).

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Figure Legends

Fig. 1 Generation of *E. coli* persister cells through the activation of toxins MqsR and YafQ of the MqsR/MqsA (Wang et al. 2011) and YafQ/DinJ (Hu et al. 2012) TA systems. Under non-stress conditions, antitoxin MqsA represses transcription of *rpoS* via binding to a palindromic region, represses transcription of the gene encoding biofilm regulator CsgD (CsgD positively controls curli and cellulose production) by binding to a palindromic region, and inhibits toxin MqsR by direct binding. Oxidative stress leads to degradation of antitoxin MqsA by protease Lon which leads to activation of the stationary-phase sigma factor RpoS and mRNA degradation by MqsR. Most cells combat the stress via activation of RpoS (RpoS positively controls catalase production) while some cells have too much mRNA degradation and become dormant due to a lack of protein production. Similarly, under non-stress conditions, antitoxin DinJ represses *rpoS* mRNA translation indirectly by repressing transcription of *cspE* (cold-shock protein CspE positively controls translation of *rpoS* mRNA) and inhibits toxin YafQ by direct binding. Antibiotic stress leads to degradation of antitoxin DinJ by protease Lon which leads to activation of RpoS and mRNA degradation by YafQ.

Fig. 2 Compounds that affect persister cells grouped by mode of action.

Fig. 3 Indole signaling in persister cells (Hu et al. 2015; Kwan et al. 2015c). Schematic showing how phosphodiesterase DosP and toxin YafQ both increase persistence by reducing indole concentrations. Activation of DosP reduces cAMP concentrations which prevents transcription of *tnaA*; *tnaA* encodes tryptophanase which produces indole from tryptophan. Stress activates Lon protease which degrades antitoxin DinJ; activated toxin YafQ degrades TnaA mRNA preventing indole production.

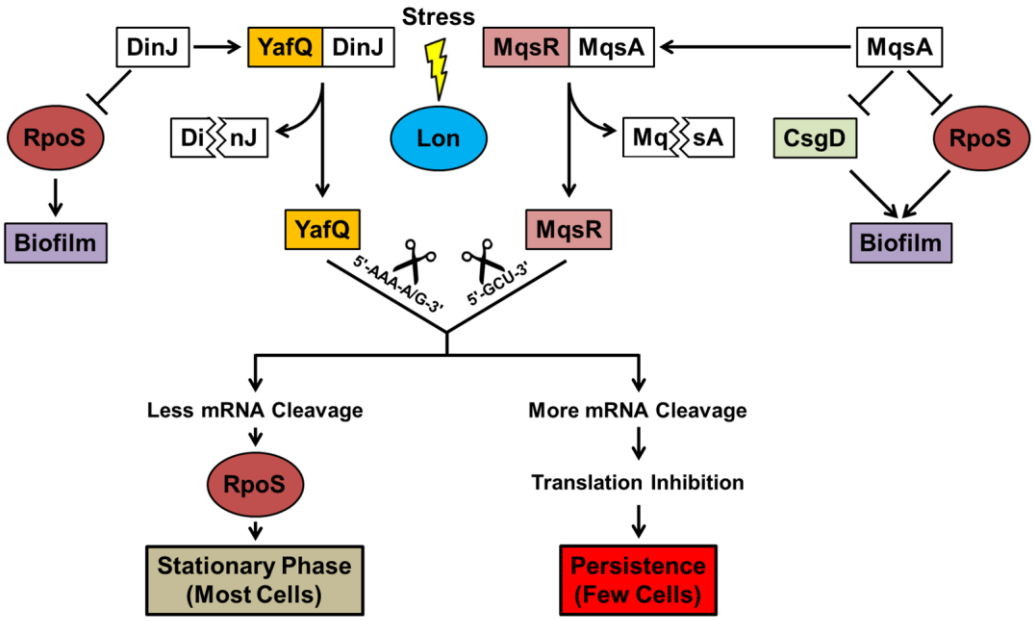

Figure 1

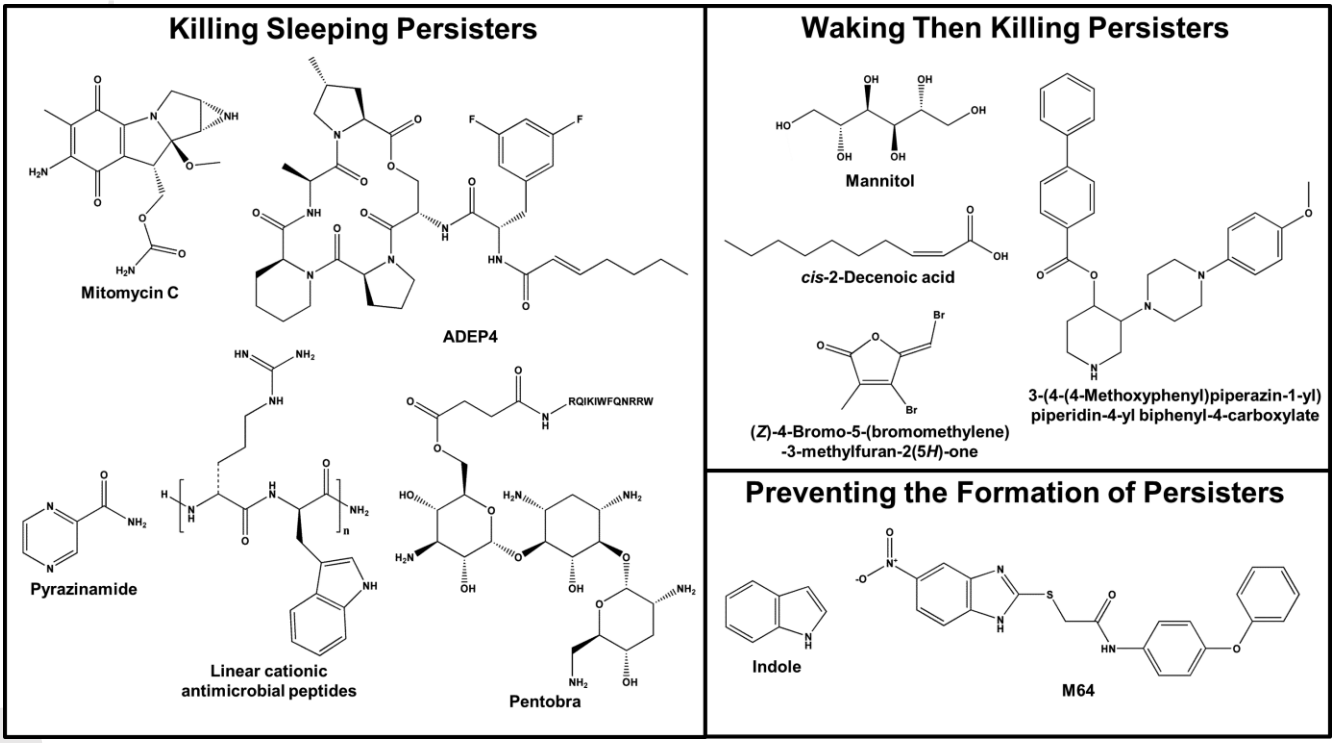

Figure 2

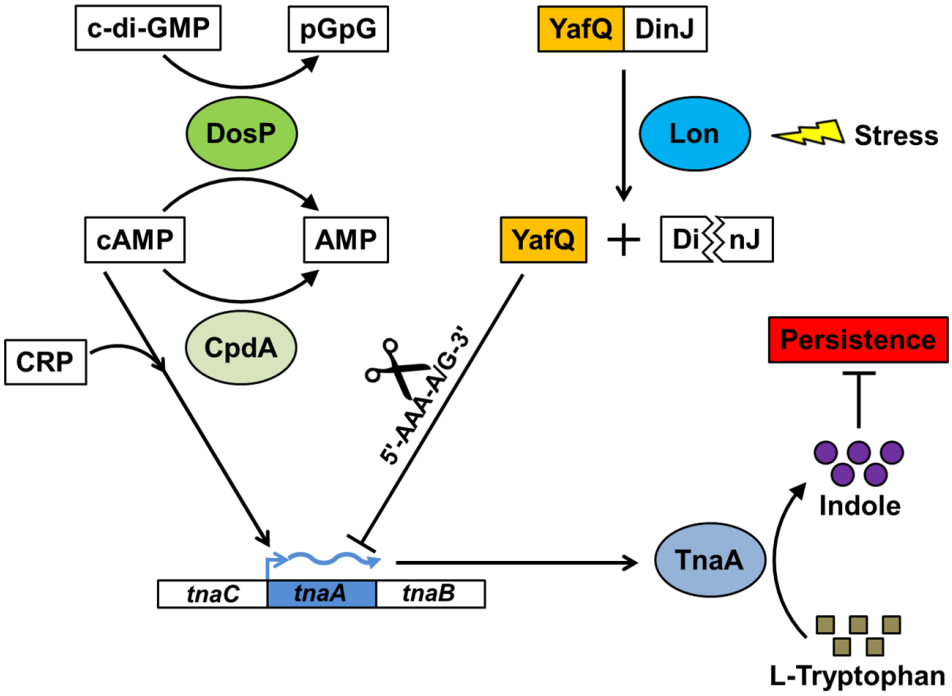

Figure 3