Persister Cells in Biofilm Associated Infections

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1 Introduction

Persisters were first identified by Joseph Bigger in 1944 (Bigger 1944). Bigger was the first to show that penicillin is a bactericidal antibiotic. He also found that addition of a lethal dose of penicillin to a population of *Staphylococcus aureus*, always resulted in a subpopulation of survivors. These surviving colonies could be regrown and the resulting population could also be lysed efficiently with penicillin to yield a subpopulation of survivors, or persister cells (Bigger 1944).

Persisters are dormant, phenotypic variants of regular cells, capable of surviving in the presence of high levels of bactericidal antibiotics. They can be pre-existing in a population, or their formation can be induced by environmental conditions (Balaban et al. 2004; Dorr et al. 2010). The relative abundance of persister cells in a population is dependent on the growth phase, with a particularly high number being observed in the stationary phase (Keren et al. 2004; Conlon et al. 2013). Persister cells often display multi-drug tolerance, meaning that an individual persister can survive for prolonged periods in the presence of a variety of antibiotics (Conlon et al. 2013). Persister formation can actually be induced by exposure to antibiotics of various classes (Dorr et al. 2010; Johnson and Levin 2013).

Persister cells have been identified in vivo and have been directly implicated in disease (Conlon et al. 2013; Helaine et al. 2014; Mulcahy et al. 2010). They are proposed to be the underlying reason for the tolerance demonstrated by bacterial biofilms. Targeting these dormant persister cells with novel antibiotics will allow us to efficiently treat chronic biofilm infections and eradicate bacterial populations (Conlon et al. 2013).

2 Mechanisms of Persister Formation

The *hipA* gene was the first gene demonstrated to have an impact on persister formation. Moyed and co-authors found that a particular allele of this gene, termed *hipA7*, led to at least a 100-fold increase in persister cells formation. In other words, antibiotic treatment of a population of *E. coli* harboring the *hipA7* gene, resulted in 100–1,000 times more survivors than an identical treatment of a wild-type population with a different *hipA* allele (Falla and Chopra 1998). Importantly, the minimal inhibitory concentration (MIC) of the antibiotics examined in this study, were identical for the wild-type and *hipA7* strains. A change in MIC suggests the development of resistance to an antibiotic, not apparent in a high persister (*hip*) phenotype.

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The *hipA* gene is the toxin portion of a toxin-antitoxin locus, *hipBA* (Falla and Chopra 1998). HipA is neutralized by its antitoxin HipB. It has been demonstrated that under cellular stress, the Lon protease degrades HipB allowing HipA to exert a toxic effect on the cell (Hansen et al. 2012; Tsilibaris et al. 2006). The toxicity of HipA is due to its kinase ability. It phosphorylates the glutamyl tRNA synthetase resulting in the accumulation of uncharged tRNA leading to activation of the stringent response, a shutdown of protein synthesis and subsequent persister formation (Germain et al. 2013).

The stringent response is activated under amino acid starvation (Durfee et al. 2008). Stalling of ribosomes due to accumulation of uncharged tRNA leads to activation of RelA which synthesize the alarmone (p)ppGpp (Durfee et al. 2008), and has a global impact on transcription, leading to downregulation of protein synthesis machinery and an induction of other genes including those involved in amino acid biosynthesis (Zhou and Jin 1998).

The stringent response has been heavily implicated in bacterial persister formation (Maisonneuve et al. 2013; Kaspy et al. 2013; Nguyen et al. 2011). Gerdes and co-authors have put forward a model whereby persister formation is governed by activation of the stringent response leading to antitoxin degradation by the Lon protease allowing free toxins to mediate persister formation (Germain et al. 2013).

Stochastic switching to slow growth results in persister cell formation and is governed by (p)ppGpp (Maisonneuve et al. 2013). Accumulation of ppGpp leads to the activation of the Lon protease (Balaban et al. 2004; Maisonneuve et al. 2011).

Degradation of the antitoxins is primarily mediated by the Lon protease in *E. coli* (Helaine et al. 2014; Hayes and Van Melderen 2011). The Clp protease has been shown to be responsible for antitoxin degradation in *S. aureus*, which lacks a Lon homologue (Donegan et al. 2010). Degradation of the antitoxin and subsequent freeing of the toxin can occur in response to specific environmental stimuli, leading to persister formation.

TA genes were first identified as addiction modules that play a role in plasmid maintenance (Ogura and Hiraga 1983). The antitoxin is far less stable than the toxin and is degraded by cellular proteases (Ogura and Hiraga 1983). TAs were subsequently discovered on bacterial chromosomes. Many studies have implicated chromosomal TAs to have a role in persistence which will be discussed in this section (Dorr et al. 2010; Falla and Chopra 1998; Hansen et al. 2012; Maisonneuve et al. 2011; Kim and Wood 2010; Moyed and Bertrand 1983).

There are 11 TA mRNA interferases in *E. coli* K12. Their involvement in persister cell formation was first suspected as overexpression of the toxin portion of the TA module led to cell shutdown and persister formation in vitro (Kim and Wood 2010; Donegan and Cheung 2009). They achieve this feat by cleavage of mRNA. Further investigation of the role of TAs in persister formation found that deletion of individual TAs did not lead to a measurable decrease in persister formation and it was hypothesized that redundancy between the TA modules may explain this discrepancy (Spoering et al. 2006).

A study that deleted 10 of these TA modules, in series, confirmed this to be the case (Maisonneuve et al. 2011). Deletion of 5 TAs led to a significant decrease in persister cells and the effect was heightened for each subsequent mutation, with the final Δ10 strain displaying a 100-fold reduction in persister formation. Furthermore the TA deletions were repeated in reverse order and, again, the phenotype was evident after five deletions and most pronounced after deletion of all 10 TA modules (Maisonneuve et al. 2011).

It should be mentioned that it is entirely possible that individual TA loci play an important role in persisters under certain specific environments or in certain strains. The fact that each TA locus in *E. coli* is unique and differentially regulated certainly suggests this (Gerdes et al. 2005). Norton et al. reported that mutation of the TA module PasTI had no phenotype when deleted in *E. coli* lab strain MG1655 but displayed a 100-fold reduction in persisters when deleted in a clinical isolate CFT073 (Norton and Mulvey 2012).

Dorr et al. showed that another toxin, *tisB* which is induced by the SOS-response could specifically induce persisters in response to fluoroquinolones (Durfee et al. 2008). They showed that ciprofloxacin induced the SOS system leading to increased persister formation and that this increase could be attributed to induction of the TisB toxin. TisB exerts its toxic effects by targeting the membrane causing a decrease in proton motive force which, at the right levels, can induce persister cell formation (Germain et al. 2013; Durfee et al. 2008). A *tisB* mutant also displayed a decrease in persisters but only to high levels of ciprofloxacin. The authors concluded that *tisB* likely mediates survival during extreme SOS stress when shutting down the cell, rather than attempting repair is more beneficial to survival.

3 Other Genes Implicated in Persister Cell Formation

A variety of studies and screens have identified a multitude of genes implicated in the persister phenotype. One such study identified the gene, *glpD*, involved in glycerol metabolism as having a significant impact on persister cell formation (Spoering et al. 2006). Interestingly, overexpression of *glpD* also induced persistence to ofloxacin and ampicillin suggesting that these persisters are multi-drug tolerant. Deletion of the *glpD* gene in a wild type background reduced levels of persisters to ciprofloxacin in stationary phase.

Various aspects of metabolism have been implicated in persister cell formation in numerous other studies (Bernier et al. 2013; Allison et al. 2011; Amato et al. 2014). Bernier et al. identified numerous amino acid auxotrophs that displayed an increase in persister cell formation (Bernier et al. 2013). Girgis et al. showed that mutation of methionyl tRNA synthase resulted in a 10,000-fold increase in persisters (Girgis et al. 2012). They also found that mutations in transketolase A (*tktA*) and glycerol-3-phosphate (G3P) dehydrogenase (*glpD*) increase persistence through metabolic flux alterations that increase intracellular levels of the growth-inhibitory metabolite methylglyoxal (Girgis et al. 2012). Another high-throughput screen in *Pseudomonas aeruginosa*, using a 5,000 gene knockout library, identified numerous genes involved in metabolism that had a significant impact on persister cell numbers (De Groote et al. 2009a). Another study in *Streptococcus mutants* identified persister genes associated with phosphate metabolism, phospholipid synthesis, purine metabolism, folate biosynthesis and energy metabolism (Leung and Levesque 2012). Persister levels increase as nutrients becoming limiting in the stationary phase (Keren et al. 2004). (p)ppGpp accumulation results in an increase in persister cell formation (Maisonneuve et al. 2013; Kaspy et al. 2013). Addition of sugars enables killing of persisters with an aminoglycoside (Allison et al. 2011). Together these findings demonstrate the central role metabolism plays in persister formation. This is important from the perspective of biofilm as these environments may be low in nutrients.

Another screen for persister genes utilized the Keio collection (Baba et al. 2006), an ordered deletion library of all 3,985 non-essential genes in *Escherichia coli*. A number of hits were confirmed and most of these were global regulators with relatively minor effects on persister levels (Hansen et al. 2008).

Similarly, studies of persisters in the pathogenic organism, *P. aeruginosa* identified a number of global regulators that altered persister levels although the precise mechanism by which they control persister formation remains unclear (Moker et al. 2010; Murakami et al. 2005; Viducic et al. 2006; De Groote et al. 2009b).

In general, although many genes in a variety of pathways appear to be involved in persister formation, they all lead to a common phenotype by achieving a level of dormancy that protects them from antibiotic treatment (Fig. 1.1).

4 Persisters and Biofilm

Bacterial biofilms have been implicated in an array of human diseases. A biofilm can protect bacterial cells from the host immune system, serving as a protective barrier against complement

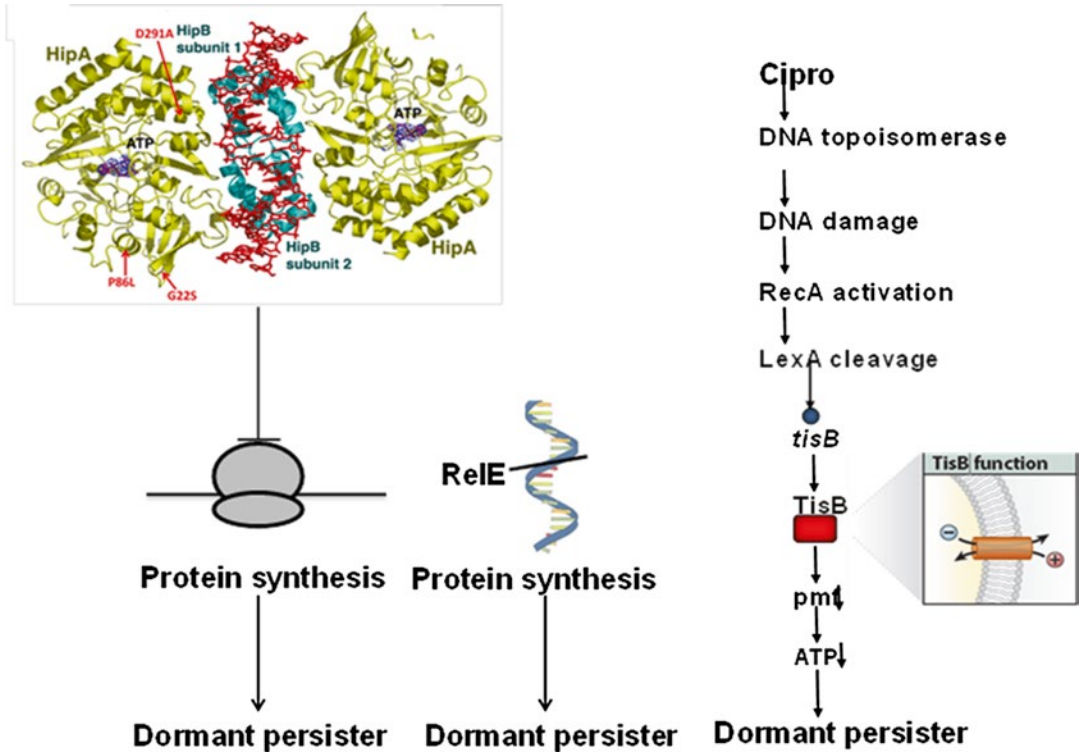

Fig. 1.1 Redundant pathways of persister formation

immunity and phagocytosis. The antibiotic tolerance of a microbial biofilm has also long been recognized and thought to be central to the difficulty in treating biofilm associated diseases (Costerton et al. 1987).

Biofilm associated infections such as a cystic fibrosis lung infection, infective endocarditis, wound infections and infections of indwelling devices are notoriously recalcitrant to antibiotic treatment, often leading to prolonged treatment regimens or extreme measures such as removal and replacement of an infected device or debridement of an infected wound (Ammons 2010).

It was hypothesized that biofilm protected indwelling cells from antibiotic penetration (Gristina et al. 1987). Upon detailed examination of this hypothesis, it was shown that antibiotics penetrate biofilm quite well (Vrany et al. 1997; Anderl et al. 2000; Darouiche et al. 1994; Jefferson et al. 2005). The tolerance of the biofilm was finally explained by the presence of persisters within the biofilm population (Lewis 2001). A bactericidal antibiotic will kill the majority of cells in a biofilm but persisters will survive. These persisters are shielded from the immune system by the biofilm matrix. Once antibiotic treatment is ceased, these persisters can revert to a growing state and repopulate the biofilm causing a relapse of infection (Fig. 1.2).

Persister cells are more abundant in the stationary phase of growth (Keren et al. 2004), presumably due to nutrient deprivation inducing cellular shutdown. Cells within a biofilm may be in a nutrient deprived state and thus the biofilm lifestyle may serve to induce persister cell formation. Indeed, *Staphylococcus aureus* biofilm populations tend to demonstrate far higher proportions of persisters than an exponential phase planktonic population (Conlon et al. 2013).

One recent study looked at what was known about persister formation in planktonic *E. coli*

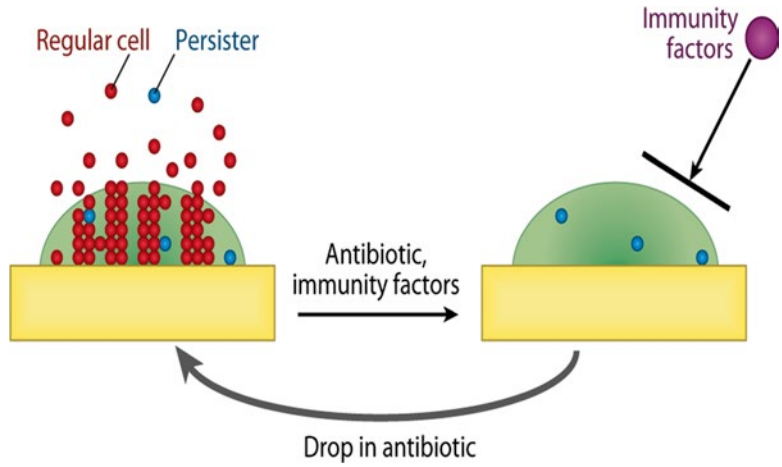

Fig. 1.2 A model of a relapsing biofilm infection

and applied it to the biofilm environment. What they found was that ppGpp was central to persister formation in the *E. coli* biofilm and persister cell formation was governed by nutrient transitions, again implicating metabolism as a central component of persister cell formation (Amato and Brynildsen 2014). The carbon source was altered and this was shown to impact persister levels in the biofilm through RelA and ppGpp (Amato and Brynildsen 2014). Interestingly, this ppGpp mediated persister formation did not implicate TA modules and was therefore independent of the proposed model involving (p)ppGpp accumulation, followed by Lon mediated degradation of antitoxins, which was previously discussed.

Another study, again using *E. coli* biofilm, conducted a screen to find genes involved in biofilm tolerance to the fluoroquinolone, ofloxacin (Bernier et al. 2013). Amino acid auxotrophs displayed higher levels of tolerance and this was biofilm specific. Again starvation plays a central role in the antibiotic tolerance phenotype. They also found that the SOS response played a central role in ofloxacin tolerance in biofilm associated persisters (Bernier et al. 2013). Interestingly, the group reported that induction of fluoroquinolones tolerance observed in biofilms compared to planktonic cells was independent of any of the TA mRNA interferases or TAs previously shown to be induced by the SOS response.

P. aeruginosa is an important opportunistic human pathogen. It can cause incurable biofilm associated infections in cystic fibrosis (CF) patients. Mulcahy et al. examined persister cell formation in longitudinal isolates from a number of cystic fibrosis patients and found that high-persister (hip) mutants emerged in 10 out of 14 cases studied (Mulcahy et al. 2010). Repeated exposures of the bacterial population to antibiotics selected for hip mutants over the course of disease progression. This directly implicates persisters in *P. aeruginosa* biofilm infections of the cystic fibrosis lung and the recalcitrance of this infection to antimicrobial treatment.

A similar investigation was performed with cancer patients with oral *Candida albicans* infection (Lafleur et al. 2010). *C. albicans* produces oral biofilms resulting in the disease known as oral thrush. Longitudinal isolates were studied from patients treated daily with chlorhexidine. The patients were divided into two groups, transient carriers and long-term carriers. Hip mutants were only identified in patients with long-term carriage again implicating persisters in this biofilm associated disease and drug tolerance (Lafleur et al. 2010).

A recent study involving a novel anti-persister antibiotic treatment further substantiates the role of persister cells in biofilm tolerance. The ability to kill a persister directly translates to the ability to target and kill the biofilm (Conlon et al. 2013).

5 Killing Persister Cells and Treating Biofilm Infection

Antimicrobial tolerance of the biofilm can be explained by the presence of dormant persister cells. Killing these persister cells would therefore lead to eradication of the biofilm. The problem with conventional antibiotics is that they rely on the corruption of active processes in the cell. Aminoglycosides corrupt protein synthesis leading to toxin peptide production (Davis 1987), B-lactams corrupt peptidoglycan biosynthesis leading to cell lysis (Tipper and Strominger 1965) and fluoroquinolones corrupt gyrase leading to double-strand breaks (Drlica and Zhao 1997). If the cell is dormant and these mechanisms are down-regulated or completely inactive, then the antibiotic does not kill and the persister cell survives. Within the biofilm, many such cells exist as energy may be limiting.

A mechanism to kill persisters within a biofilm was proposed by Allison et al. based on metabolite stimuli (Allison et al. 2011). Tolerance to aminoglycosides may be primarily due to low membrane potential in the persister cells. Aminoglycosides require a proton motive force for uptake. Adding specific sugars to the culture induced the uptake of gentamicin via generation of a proton motive force resulting in death of the persister cell. Importantly, they were able to show that this metabolite enabled eradication was not confined to *E. coli* and worked equally well against *S. aureus*. Addition of mannitol (*E. coli*) or fructose (*S. aureus*) to the treatment regimen allowed eradication of a biofilm in a mouse catheter infection model (Allison et al. 2011). More recently it has been demonstrated that L-arginine also facilitates biofilm eradication by gentamicin by affecting the pH. Again, the efficacy of treatment was demonstrated in a mouse catheter model of infection (Lebeaux et al. 2014).

It has also been shown that long term aminoglycoside treatment alone is capable of eradicating *P. aeruginosa* stationary phase cultures, implying a sufficient proton motive force is maintained in all cells while protein synthesis also remains active (Keren et al. 2012). This prolonged aminoglycoside treatment could inform improved dosing regimens in the clinic to deal with *P. aeruginosa* biofilm infections.

A persister specific compound capable of killing a persister without effecting regular cells was reported by Kim et al. in (2011). They demonstrated that this compound, termed C10, could eradicate a population of *E. coli* in combination with norfloxacin although the mechanism of action remains unknown.

Another approach to persister killing and biofilm eradication relied on the activation and corruption of a mechanism in the bacterial cell without the requirement for energy. The acyldepsipeptides are a family of antibiotics that bind the ClpP protease (Kirstein et al. 2009).

Proteolysis is an important aspect of protein homeostasis and quality control (Michel et al. 2006). It is a tightly regulated process involving the cooperation of ClpP, its associated unfoldases, ClpA, ClpX or ClpC and requires ATP (Michel et al. 2006). Under normal circumstances, a protein can be targeted for degradation, for example by an *ssrA* tag (Farrell et al. 2005). The unfoldases will unfold the protein at the expense of ATP and feed it into the catalytic chamber of ClpP where it is degraded (Farrell et al. 2005). ADEP4 is a synthetic acyldepsipeptides antibiotic that binds the ClpP protease, opening its proteolytic core and results in dysregulation of proteolysis (Brotz-Oesterhelt et al. 2005). This proteolysis no longer requires ATP, which may be at extremely low levels in persister cells (Dorr et al. 2010). A stationary phase population of *S. aureus* cells behaves like a persister population, meaning that it is completely tolerant to antibiotics (Conlon et al. 2013). Incubation of stationary phase *S. aureus* cells with ADEP4 for 24 h resulted in mass protein degradation (Falla and Chopra 1998). Over 400 proteins were significantly degraded by ADEP4 treatment, many of which were essential proteins. This coincided with death of the persister population. There was a caveat to ADEP4 however. The ClpP target is not essential so resistance occurs at high frequency. However, mutation of *clpP* resulted in an increased susceptibility to conventional antibiotics, rifampicin, linezolid and ciprofloxacin.

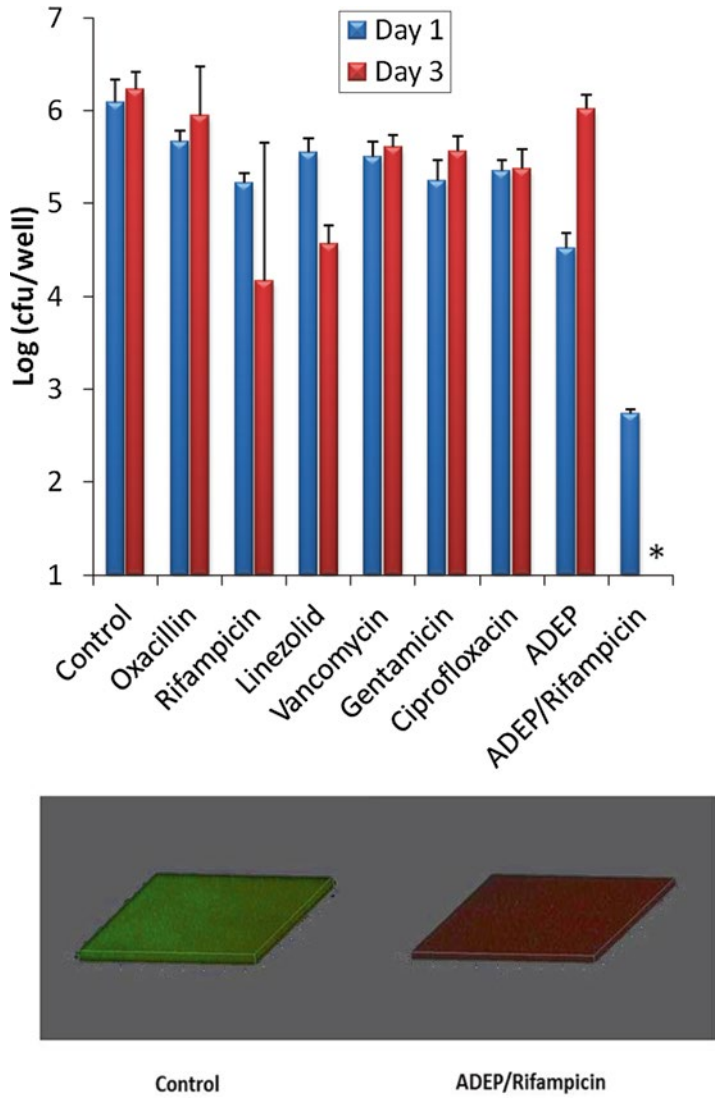

Fig. 1.3 Eradication of biofilm by ADEP4/rifampicin

Combining ADEP4 with rifampicin resulted in complete eradication of the population to the limit of detection in a variety of experiments (Conlon et al. 2013). Importantly, a *S. aureus* biofilm was also eradicated by an ADEP4/rifampicin combination (Fig. 1.3). Finally a biofilm infection was established in a mouse thigh and this too could be eradicated by the ADEP combination (Conlon et al. 2013). To date, antibiotic discovery has focused on how to kill fast growing cells while these recent studies provide much needed insight into how to eradicate a non-growing persister population.

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