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Persister Cells Generation and their Role in Recalcitrance of Biofilms Towards Antibiotics (Part 2)

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Summary

All pathogenic bacteria produce a small fraction of dormant cells that are highly tolerant to antibiotics called „perister cells" or „persisters". Unlike resistant mutants, perister cells do not proliferate in the presence of an antibiotics and this phenotypic tolerance is not correlated with any genetic alterations or modifications. The presence of these isogenic subpopulations of tolerant cells is a form of insurance strategy at the population level and is considered to be the most appriopriate theory to explain the biofilm recalcitrance toward antibiotics. Several number of factors and mechanisms are belived to be involved in generation of persister cells that appear through a phenotypic switch: dormancy, toxin-anti-toxin modules, nutrient limitation or stringent response.

Key words

persister cells; bacterial biofilm; antibiotic resistance; SOS response

Introduction

Despite the passage of time, acute bacterial infections, life-threatening diseases caused by microbial pathogens such as *Yersinia pestis* or *Vibrio cholerae* are still weak point of the mankind. Initially, due to discovery of antibiotics, vaccines and hygiene rules application, a significant reduction of lethal incidents related to bacterial infections was observed [1,2]. However, the golden age of antibiotic did not solve such challenges as occurrence of antibiotic-resistant microbes together with still rising level of chronic bacterial infections difficult to eradicate [3].

At the same time the progressive research conducted by environmental microbiologists confirmed prevalence of bacterial biofilm conglomerates in all types of natural niches and ecosystems [4]. Soon clear became the fact, that bacterial biofilms display specific biological properties in comparison to their planktonic counterparts [5]. First data pointing out the direct correlation between persistent infections and bacterial biofilm development came from J.W., Costerton and N., Hoiby during their research of *Pseudomonas areuginosa* colonization model on the lungs of CF patients [6]. Since then, a significant role of bacterial biofilms in the pathophysiology of tissue related infections has been widely confirmed and proved.

Whilst the planktonic bacteria can be easily eradicated by diverse antimicrobial drugs, a subset of biofilm bacteria highly tolerant to antibiotics survives the treatment and becomes a cause of infection recurrence [7]. The ability of bacterial biofilms to withstand harmful bactericidal antibiotic activity, even when these bacteria are susceptible to such antimicrobial agents is called “recalcitrance of biofilm bacteria towards antibiotics” [3,8]. Among many suggested reasons potentially able to explain the above mentioned recalcitrance phenomenon, the presence of an isogenic subpopulation of “persister cells” is now considered as the most important one [9].

Persister cells generation

Although there are many attractive hypotheses for biofilm-induced recalcitrance towards antibiotics, they cannot fully explain the phenomenon of biofilm recalcitrance. The best example came from fluoroquinolones – a class of antibiotics that are able to kill non-dividing bacterial cells due to lax diffusion through biofilm matrix, but still not capable to fully eradicate bacterial biofilms [10].

The inherence of stochastically formed dormant persisters in microbial populations has been known since the beginning of the antibiotic era. The very first data describing persistence phenomenon came in 1944 when Joseph Bigger studies revealed presence of persister cell population, survived as dormant, non-dividing cells, completely different from classical and well known antibiotic resistance mechanisms [11, 12, 13]. Bigger described the phenomenon when addition of a lethal amount of penicillin into exponentially growing population of *Staphylococcus aureus* often failed and resulted in survivor colonies. These subpopulations were fully capable of regrowing once the antibiotic level dropped. It is now well known that all pathogenic bacteria produce a small fraction of dormant cells that are highly tolerant to antibiotics called "persister cells" or "persisters" [9,14].

Recently, it has been found, that persister cells are responsible for the high tolerance of bacterial biofilms to antibiotics [15]. In a variety of bacterial species, the level of persister cells increased proportionally with the density of the culture [11], reaching 1% in stationary phase or in a biofilm of *Pseudomonas aeruginosa* [16], *Escherichia coli*, and *Staphylococcus aureus*.

Persisters are a group of generated cells being phenotypic variants of wild type cells, characterized by the surviving abilities in the presence of lethal levels of bactericidal antibiotics. Persistence occurs in subpopulations of slow or non-dividing bacteria, whereas drug indifference concerns the entire population. It is very important to distinguish that bacterial persistence is distinct from antibiotic resistance. Unlike resistant mutants, persister cells do not proliferate in the presence of the antibiotic and this phenotypic tolerance is not correlated with any genetic alterations or modifications. Once the antibiotic is removed, persisters resume growth and give rise to a population that displays an original non-persister antibiotic tolerance profile and produce small proportion (1%

of population) of persister cells. This observation proved that conversely to resistance, persistence is a non-inherited phenomenon. Moreover, it was observed that persisters can arise in response to multiple environmental factors including oxidative stress, presence of antibiotics, SOS response or limitation of nutrient components like carbon.

The presence of these isogenic subpopulation of tolerant cells is a form of insurance strategy at the population level and nowadays it is considered to be the most appropriate theory to explain the biofilm recalcitrance towards antibiotic [3]. Both *in vitro* and *in vivo* studies clearly showed the presence of persister cells in biofilm formed by Gram-negative bacteria [17, 18]. The capacity of a biofilm to limit the access of the immune system agents, together with the ability of persisters to sustain an antibiotic activity account for the recalcitrance of infections *in vivo*. Clinical image in the case of catheter-related bloodstream infections (CRBSI), showed that even after 14-day treatment with significant concentrations of antimicrobial agents, over 20% of infections relapse, mainly because survival abilities of persister cells reside inside the biofilm [3]. When bacterial community experiences intense stress, persister cells may survive, thereby providing survival of the whole community. Moreover, it is possible that persisters are fully capable to escape antibiotic-induced programmed cell death (PCD) [19].

Genes and mechanisms affecting persistence and tolerance towards antibiotics

There are many pathways that may potentially lead to persisters formation. As these dormant cells are present prior to the antibiotics occurrence, they are believed to preside as the result of phenotypic switch [20]. Several mechanisms are believed to be involved in generation of persister cells such as dormancy, toxin-antitoxin modules, nutrient limitation or SOS response. Several genes strongly affecting the level of persistence have been described and screened for the first time by Moyed and Bertrand [21]. Some environmental factors, stochastic gene expression or some passive and active mechanisms are potentially related to the presence of persister cells in bacterial populations. To date, screening of *Escherichia coli* mutant libraries has released no single mutants completely lacking persisters [22, 23]. Under particular conditions toxins from chromosomally encoded toxin–antitoxin (TA) modules have important

roles in the physiology of bacterial cells including functions during the biofilm formation together with multidrug resistance [24,25,26]. Originally TA genes were identified on plasmids where they proclaim a maintenance mechanism. Usually the toxin is a protein that regulates a crucial cellular function such as translation or replication, and forms a non-active complex with the antitoxin. TA systems are also frequently found on bacterial chromosomes, but their role is still unclear and unknown [11]. Despite the fact that molecular nature of TA evolves from RNA molecules to protein, TA modules in general consist of usually stable toxin which disrupts an essential cellular process and unstable, degradable antitoxin being remedy for the effect of the toxin activity, and most often encoded in an operon, resulting in co-translation and co-transcription of both toxin and antitoxin [27]. Toxin is a component that is responsible for inhibiting crucial cellular functions like replication or translation [28]. Degradation-prone antitoxin through formation of idle complex, antagonizes the effect of the toxin compound. Moreover, toxins seem to be attractive effectors of the switch to the persister mode as they stop growth and reduce the activity of the antibiotic target as well [29]. Toxin-antitoxin systems are remarked as a genetic basis in the transformation process of persisters from normal cells and have further been approved by mutagenesis studies [30, 31]. TA modules are widely spread in bacterial genome, at least 37 putative TA systems have been described in genome of *Escherichia coli* and around 65 in *Mycobacterium tuberculosis* H37Rv [32]. TA systems are involved in persister formation in *Escherichia coli*. Moreover, the examination of TA systems in *Mycobacterium* was proved to increase the abilities of entering the persistence state, and resulting in multidrug resistance [33].

Deletion of such operons as hipA, tisB or mqsR has a dramatic effect on the persistence level [34]. The first locus identified to have an impact on persister formation was *hip* ("higher persister"). Overexpression of HipA is highly toxic and halts cell division and is considered as locus that carries a toxin/antitoxin module [3]. According to Chaignon *et al.* HipA was initially the locus to phosphorylate translation factor EF-Tu, directly being a cause of persistence due to cell stasis [35]. Recent data point out that most likely variant is that HipA inhibits glutamyl-tRNA Snthetase (GltX) via phosphorylation and triggers the synthesis of ppGpp [3, 15]. The *hipA7* mutant is a result of a double mutation and has alike effects

as overexpression of HipA [36]. In this particular *hipA* mutant the level of persister is increased 1000-fold compared to the wild-type, with simultaneously increased degree of tolerance to such groups of antibiotics as aminoglycosides, β-lactams and fluoroquinolones [37]. Deletion of *hipB* is lethal for bacterial cell, because of strong toxicity effect of HipA, which may suggest that HipB is the native repressor of the operon. On the other hand, the deletion of hip locus has absolutely no impact on frequency of persister cells during the exponential phase of growth. Keren *et al*., by using a *hipA7 E. coli* mutant have confirmed that overexpression of *relE* lead directly to growth setback with simultaneous increase in the level of persisters cells [11]. Deletion of the *hipBA locus* has no impact on persister formation in a growing culture but leads to a decrease of persisters level in the stationary phase bacterial culture [3, 38]. Interestingly, deletion of other TA modules seemed to have no influence on persisters level in stationary microbial culture, which gave beginning to the redundancy hypothesis. Further experiments conducted by Maisonneuve et al., confirmed redundancy by showing that single mutation of 10 TA modules had completely no impact on persister occurrence, while combination of mutations significantly increased susceptibility towards ciprofloxacin and ampicillin [39]. Afterwards, the same group proved that *E. coli* cells with low ribosomal activity are enriched in persisters. The gene expression studies confirmed overexpression of *dinJ*, *yefM* and *yoeB* (known TA systems) but moreover also part of the *ygiUT* operon takes after a TA module. Important observation was that overexpression phenomenon of *ygiU* directly caused inhibition of growth together with increasing the tolerance level towards such antibiotics as cefotaxime and ofloxacin [3, 40].

Another well-characterized TA system important to persistence is MqsR/Mqs [41]. MqsR is an RNase toxin, which cleaves almost all mRNA [42]. Overproduction of MqsR was proven to significantly increase persistence (Kwan, B.W., et al., 2013) while the deletion of *mqsR* resulted in decreased persistence phenomenon, which was the very first data showing that the absence of a toxin affects persistence. Another similar observation was made with TisB/IstR-1; a TA system where the toxin, TisB, decreases ATP levels, and induces the *tisB* transcription via the SOS response increasing persistence [30]. Currently, a novel TA module, type V, has been correlated with persistence in *Escherichia coli* cells, composed of

GhoS anti-toxin and GhoT toxin module. GhoS conceals toxicity of GhoT via peculiar cleavage of *ghoT* mRNA and prevents the synthesis [43].

The term SOS response, known also as the DNA damage response, refers to repair process of the DNA in bacterial cells [44]. The SOS genetic network gathers diverse molecular mechanisms activated by genomic DNA damage caused by such conditions as oxidative radicals, ultraviolet radiation (UV) or mutagens (including quinolone antibiotics). This set of co-regulated genes is widespread in microorganisms and promotes survival of bacterial cells by repairing damage of the DNA. In gram-negative pathogen *E. coli* the SOS system consists of more than 40 genes characterized by different functions depending from the DNA damage type, including halt of cell division, translesion DNA replication, excision repair of nucleotides or homologous recombination [45]. The SOS system is regulated by the LexA repressor protein which downregulates the expression of other SOS genes, includes *lexA* itself and thereby controls the whole SOS network. Following ssDNA or dsDNA damage, the RecA nucleoprotein promoters assemble into filaments along stretches of ssDNA near arrested replication forks. The activated RecA together with the LexA repressor activates autocleavage of the LexA, what results in relieving repression of the SOS system. Inactivation of the LexA repressor leads to synthesis of SulA protein, which by binding to the protein responsible for division ring formation (FtsZ) completely halts cell division. Once the DNA-damaging exposure period ends, the SulA accumulates and completely inhibits bacterial cell division. On the other hand, upon DNA repair protein SulA is degraded by the Lon protease, maintaining continuity of cell division. Within the SOS regulon are also genes responsible for nucleotide-excision repair (*uvrABC*), genes required for recombination (*recA*) and also genes responsible for encoding DNA polymerases (*dinA*, *umuDC*, *dinB*) [46].

In 2004, scientists have established a correlation between the SOS network and tolerance [47]. The authors demonstrated that inactivation of penicillin-binding protein 3, caused by β-lactams-induced SOS in pathogenic gram-negative *Escherichia coli* via DpiBA system. The above mentioned phenomenon transitorily stops division of bacterial cells and enables survival upon other lethal antibiotic exposure. In 2013 Bernier *et al*., proved that nutrition limitation together with the SOS response can induce high biofilm specific tolerance toward antibiotic ofloxacin [48].

SOS-dependent biofilm specific ofloxacin tolerance is independent of toxin-antitoxin systems induced by the SOS response and previously associated with bacterial persistence. Consistently, we observed that SOS dependent ofloxacin tolerance increases with biofilm age. Recently more attention is paid to how starvation and SOS response could induce biofilm tolerance to ofloxacin [48]. Bernier *et al*., by performing experiments on modified strain of *E. coli*, characterized by strong abilities to form biofilms with higher tolerance upon antibiotics, have identified amino acid auxotrophs with strong tolerance towards antibiotics while starvation. Another significant observation was that *recA* together with other SOS genes were extremely induced in mature biofilms in comparison with planktonic cells [49].

The genetic heterogeneity of bacterial biofilms, which may arise through mutations, local nutrient limitations or stochastic gene expression, could be the milestone on the road to drug indifference. The importance of starvation and nutrient limitation seemed to be confirmed in the late stationary phase of growth when the level of persisters increases to finally reach the maximum [11]. Conversely, when bacterial culture is constantly kept at exponential phase, where constant dilution and medium renewal occur, persister cells disappear.

Other significantly important locus affecting cell survival is *relA*. In situation when nutrients become limited for growth, *Escherichia coli* cells switch their gene expression program from supporting growth to allowing for prolonged survival in stationary phase. Slow rate of growth lead to activation of RelA-dependent synthesis of ppGpp, which is responsible for inhibition of anabolic processes in microbial cells [50]. In most of bacterial species, a crucial point of this switch is the accumulation of the alarmones guanosine 5′,3′ bispyrophosphate (ppGpp) [51]. This ppGpp--mediated stringent response induced in nutrient limitation conditions for many years was postulated to have major role in phenotypic switch of persister cells. Finally the overexpression of ppGpp in *Escherichia coli* was described not only to increase level of antibiotic tolerance, but also to halt phospholipids and peptidoglycan synthesis ipso facto showing a link between antibiotic tolerance, the stringent response and starvation [3, 52].

Concluding notes

Biofilm recalcitrance phenomenon towards antibiotics and antiseptics is one of the main reasons responsible for most of the difficulties in the treatment of biofilm-related infections. Despite the fact, that major advances have been made in the characterization of factors associated with this problematic biofilm property, still little is known about nature of persisters. Recognition of the precise role played by persister cells and identification of molecular mechanisms involved in the persisters formation could be the key to developing a new promising antibiofilm strategies, thus novel approaches in treatment of chronic biofilm-based infections.

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