Biocatalysis and Agricultural Biotechnology 22 (2019) 101401

Contents lists available at ScienceDirect

Biocatalysis and Agricultural Biotechnology

journal homepage: http://www.elsevier.com/locate/bab

Persister cell development among Enterobacteriaceae, Pseudomonadaceae, Mycobacteriaceae and Staphylococcaceae biotypes: A review

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1. Introduction

1.1. Persisters

Treatment of infectious diseases is often long and requires patients to take drugs even after they have seemingly recovered. This is because of a phenomenon called persistence, which allows small fractions of the bacterial population to survive treatment despite being genetically susceptible (Martinecz and AbelzurWiesch, 2018). Studies have attributed this treatment failure to the presence of a small, transiently multidrug-tolerant subpopulation of cells, called persister cells. Wood et al. (2013) had defined that bacterial cells may escape the effects of antibiotics without undergoing genetic change; these cells are known as persisters. Fisher et al. (2017) had elaborated that, persister cells are subpopulation of transiently antibiotic-tolerant bacterial cells that are often slow-growing or growth-arrested and are able to resume growth after a lethal stress. These special cells survive antibiotic treatments by reducing their metabolism; i.e., rather than by mounting an active response to the antibiotic stress, such as activating efflux pumps and rather than undergoing mutation, they survive antibiotics by becoming dormant (Kim and Wood, 2016).

And more over, after the initial rapid elimination of bacteria, the rate of elimination slows down by following a biphasic killing pattern (Lewis, 2010). Hence, persister cells have been identified in every major pathogen, contribute to the antibiotic tolerance observed in biofilms, and are responsible for the recalcitrant nature of chronic infections (Defraine et al., 2018).

A modified method of TD (Tolerance detection) test (Gefen et al., 2016) for easy detection of bacterial tolerance and persistence in clinical isolates using conventional 'Disc diffusion' method (Bauer et al., 1966) was carried out in the laboratory of Medical Microbiology, Dept. of Biotechnology and Bioinformatics, Sambalpur University, India. Briefly, the strain BMS2 was availed from dept. of Microbiology, Veer Surendra Sai Institute of Science and Research, Burla, India. The appropriate diagnostic bacteriological tools (Pattnaik, 2017) like colony morphology, Biotyping tests, Biofilm assay (Congo Red and Spectrophotometric assay) and more over 16S rDNA sequencings were adopted for the purpose of characterization and identification. There was development of Black coloured colonies on the Congo Red agar plates (Fig. 1) indicating the presence of biofilm substantiated by spectrophotometric (absorbance taken at 570 nm, λ) analysis (Kirmusauglu, 2017). This is to mention that clinically relevant multi drug resistant bacteria with biofilm production are the potential candidates to produce persisters (Pang et al., 2019). Strong biofilm formation is an important prerequisite for persister development (Singh et al., 2017).

Gentamicin (drug of choice) impregnated discs (30mcg) had been placed on the Nutrient agar (NA) agar plates seeded with O/N (10^3 CFU/ml) cultures of BMS2 and incubated at 37 °C for a period of 14 h. After incubation period, it was observed that there was growth of a small population of colonies on the vicinity of zone of inhibition (Fig. 2). From this observation it was inferred that a sub population of persisters arose in low levels after antibiotic treatment and developed as secondary population maintaining bi-phasic growth curve pattern as suggested by Orman et al. (2016). The killing of Gentamicin could have followed a biphasic pattern—rapid death of the bulk of the population followed by a diminishing killing rate of a more tolerant subpopulation (Keren et al., 2011). Hence, the bacterial population developed in vicinity of zones of inhibition was considered as the persisters.

This small number of phenotypic variants, defined as 'persisters', are refractory to antibiotics and survive treatment. The mechanisms behind this phenomenon remain largely unknown in spite of recent advances, in great part because of the difficulty in isolating the very small fraction of the population that is in this state at any given time (Cañas-Duarte et al., 2014). Moreover, the procedure for persister cell isolation relies on activation of the SOS response together with stringent response (Dorr et al., 2010).

With this preliminary observation on the development of persisters of BMS2, this review work was initiated with an aim to understanding the biology of persisters of various biotype groups (Enterobacteriaceae, Pseudomonadaceae, Staphylococcaceae and Mycobacteriaceae)

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https://doi.org/10.1016/j.bcab.2019.101401
Received 3 April 2019; Received in revised form 6 October 2019; Accepted 25 October 2019
Available online 4 November 2019
1878-8181/

Fig. 1. Appearance of Black colonies on the Congo Red agar plates indicate development of biofilm. The merged colonies of BMS2 have produced a thick biofilm. The Red colour of Congo Red agar is clearly visible in the plate where the colonies have not grown. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

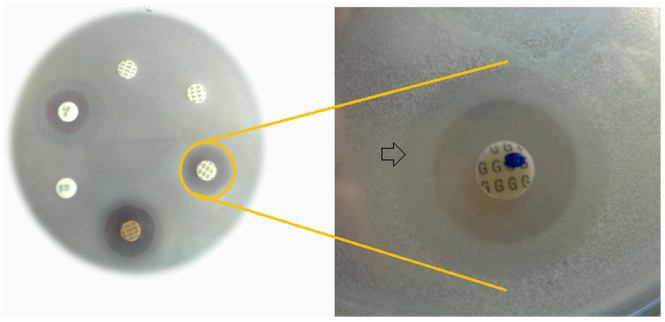

Fig. 2. The growth of a subpopulation of cells in the vicinity of zone of inhibition formed around a Gentamicin impregnated disc tested against a strain of *Staphylococcus aureus* (BMS 2).

which are notorious and causal organisms of chronic persistent infections.

1.2. Phenotype of persisters

Persister cells constitute a subpopulation of dormant cells within a microbial population which are genetically identical but phenotypically different from regular cells (Willenborg et al., 2014). Persistence is linked to pre-existing heterogeneity in bacterial populations because phenotypic switching occurred between normally growing cells and persister cells having reduced growth rates. When a population of genetically identical bacterial cells is exposed to a sufficiently strong antibiotic treatment, most of the population is killed. The death of the majority of the population takes place as a fast exponential decay characterized by a single parameter, the killing rate. After a few hours, the initial purely exponential decay of the killing curve changes to a more complex, slowly decreasing function. By the time the antibiotic is removed, a small fraction of the cells still survives. These cells have not genetically acquired antibiotic resistance: They re grow anew population that is as sensitive to the antibiotic (Balaban et al., 2004). It was suggested that they are transiently refractory to killing, without having acquired resistance through genetic modification (Keren et al., 2004). It might be expected that non-genetic inheritance would be involved in at least some aspects of the persister cell phenomenon (Day, 2016). This "bet-hedging strategy" is said to be a survival strategy under fluctuating environment conditions (Petchiappan and Chatterji, 2017). Hence, a bacterial population follows more than one way to make persisters with establishment of plurality (Allison et al., 2011; Muthuramalingam et al., 2016) and the concept was well established by mathematical derivations made by Ogle et al. (2017).

The Phenotypic plurality is embodied in the biofilm paradigm and genotypic plurality is embodied in the concepts of the supra-genome and the distributed genome hypothesis (Ehrlich et al., 2005). Interestingly, the dormant cells in one of these studies appear to be narrower than normally growing cells, suggesting that stationary-phase cellular dwarfing (Orunno et al., 2011) are antibiotic-resistant phenotypes (Miyaue et al., 2018). As an adaptive trait, persistence is heterogeneous and emerges via multiple mechanisms (Cabral et al., 2018). Although persistence mechanisms are conserved in terms of the gene function and pathways involved among different bacterial species, they may vary in gene homology and relative importance of a given pathway (Cui et al., 2016). Further, there are differences in the mech-

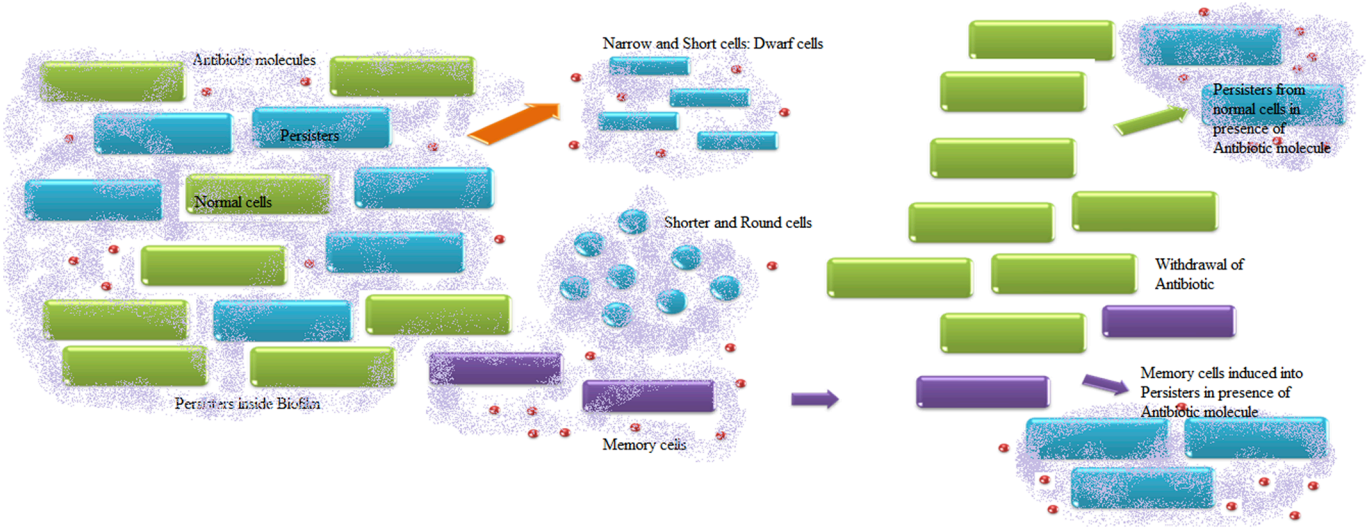

Fig. 3. A model showing a pictorial view of heterogenous resuscitation of persisters after Antibiotic removal. The persisters being inside a biofilm survive and attain three types.

anisms of persister cell formation in Gram-negative and Gram-positive bacteria. It has been reported by Kim and Wood (2018) that persisters have low levels of antibiotic-corrupting proteins and that their resuscitation is heterogeneous which was evidenced by (i) immediate division (Michael et al., 2010), (ii) immediate elongation followed by division (Barrett et al., 2019), (iii) immediate elongation but no division (Bos et al., 2015), (iv) delayed elongation/division (Yu et al., 2019), and (v) no growth of cells which are defined as VBNC (Viable but non-culturable forms, Ayrapetyan et al., 2015). The Persister cells typically form long polynucleoid filaments (elongated forms) and reach maximum SOS induction after removal of ofloxacin. Eventually, cell division resumes, giving rise to a new population (Goormaghtigh and Van Melderen and De Bast, 2009). Figs. 4 and 5 are hypothetical pictorial representation of putative morphotypes and events of persister cells after removal of antibioticsagainst Gram negative and Gram positive bacteria respectively.

The induction of elongated forms of a MDR (Multi Drug Resistant) and biofilm producing *Klebsiella pneumoniae* strain (BMKl-2) in presence of Amoxyllin was evidenced by an *in vitro* study, which was carried out in the laboratory of Medical Microbiology, Dept. of Biotechnology and Bioinformatics, Sambalpur University, India. BMKl-2 (a biosample isolated strain, taken from a patient with chronic urinary tract infection) was subjected to TD test (exposed with Amoxyllin) as described above, and the test concentration tolerant cells were observed under microscope (at 1000X). The cells were found to be both elongated and normal sized cells (Fig. 6). This was inferred that Mycobacterial cellular variations in growth and division increase heterogeneity in cell length, possibly contributed to cell-to-cell variation in host and antibiotic stress tolerance (Vijay et al., 2017).

In this context, Khlebodarova and Likhoshvai (2018) proposed a concept of 'population drift' in the space of multiple neutrally coupled mutations leading "neutrally coupled co-evolution" (NCCE), as a result of which there is appearance of cells capable of carrying out a single cell cycle in two or more alternative ways due to coupled transcription-translation system. But Chib et al. (2018) could imagine alternative models in which cells under stress preferentially partition functional components to one daughter, thus enhancing survival rates. While, Gerdes and Semsey (2016) proposed that persisters do have higher TolC levels than the drug-sensitive subpopulation.

1.3. Genomics of persisters

Lewis (2012) has reported that the persisters are developed due to the mutations in *hip A* or *hip B* gene present in the *HipAB* locus. But distinct from drug-resistant mutants with genetic modification, persisters do not replicate in the presence of antibiotics. However, once the drugs are removed, they resume growth and form a population equally susceptible to the antibiotics as the parental population. Persisters accumulate fewer antibiotics as a direct result of increased efflux rate with higher expression of efflux-associated genes particularly high expression of *tolC*, which is critical to promote persister formation. The Persisters combine active efflux and passive dormancy to survive antibiotic attack (Pu et al., 2016). It is also reported that *oxyR*, *dnaK*, *sucB*, *relA*, *rpoS*, *clpB*, *mqsR* and *recA* were prominent persister genes involved in persistence to multiple antibiotics (Wu et al.,

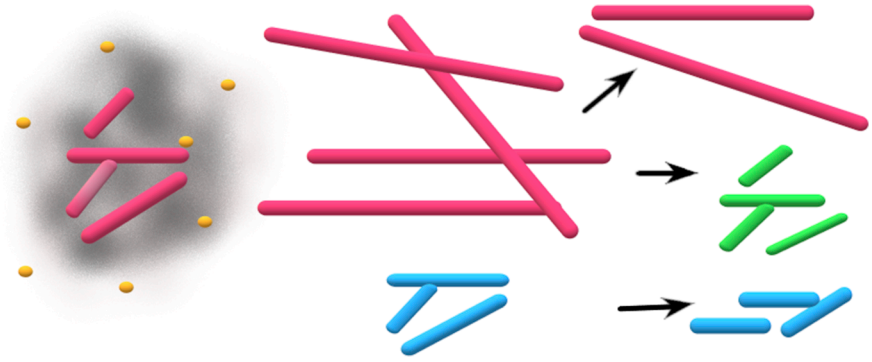

Fig. 4. Three types of typical Rod shaped cells formed inside the biofilm on exposure to antibiotics. Type I Persisters elongate but not killed by antibiotics (marked in Red). Type II Persisters elongate but killed by antibiotics (Marked in Green); Type III Normal Cells do not elongate but killed by the action of antibiotics (Marked Blue). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

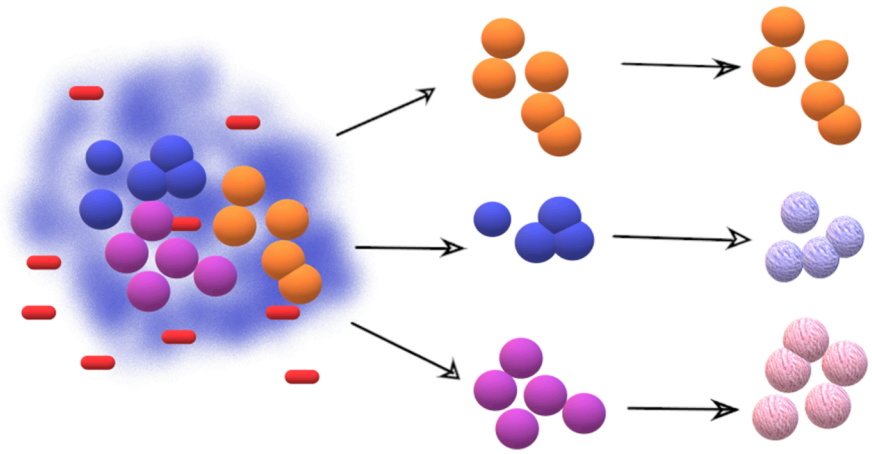

Fig. 5. Three types of typical spherical bacterial cells are formed in side biofilm on exposure to antibiotics. Type I Persisters are not killed by antibiotics (marked in Orange spherical cells); Type II Persisters are killed by antibiotics (Marked as fuzzy Blue cells), Type III Normal cells killed by antibiotics (Marked as fuzzy purple cells). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

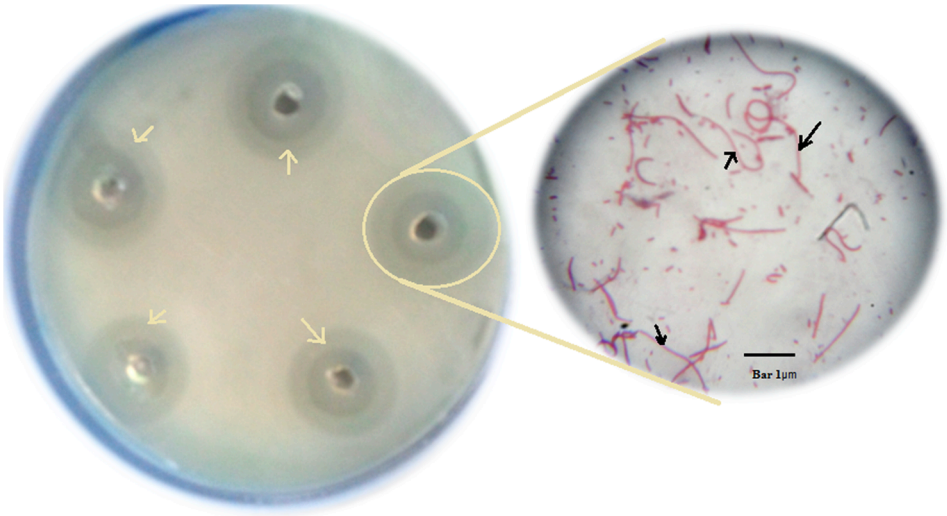

Fig. 6. Left panel showing a secondary ring of colonies around the well impregnated with Amoxyllin.the vicinity of zones are resuscitated with Persisters of a test strain of *Klebsiella pneumoniae* (BMKl-2). The Right panel is showing the Gram stained elongated cells (indicated by arrows) and normal cells.

2015). Further, Transcriptional analysis revealed that genes involved in guanosine tetraphosphate synthesis are upregulated in persisters, which represses transcription and DNA replication and leads to ofloxacin tolerance. Lactate dehydrogenase and several ATP-binding cassette transporters were upregulated in persisters to adapt to anaerobic metabolism (Matsumoto et al., 2018). The 16S rRNA promoter *rrnB* P1 was proposed to be a persister reporter and an indicator of toxin activation regulated by ppGpp (Shan et al., 2017). Fig. 7 is a hypothetical model showing the co localisation of hipBA promoters for persister formation in *Escherichia coli*, *Shigella sonnie* and *Klebsiella pneumoniae*, which are members of Enterobacteriaceae biotype group.

1.4. Proteomics of persistes

In addition, environmental conditions (nutrient availability, oxygen concentration, cell density, and sub-MIC antibiotic levels) regulate expression of biofilm adhesins and the type of biofilm matrix produced, which influences the rate at which cells in the biofilm enter the persister state and, accordingly, the antibiotic tolerance of the biofilm (Waters et al., 2016). Besides, PhoU protein is required for persister formation in bacteria (Zhang and Li, 2010). PhoU, whose expression is regulated by environmental changes like nutrient availability and age of culture, is a global negative regulator beyond its role in phosphate metabolism and facilitates persister formation by the suppression of many important cellular metabolic processes (Li and Zhang, 2007). This is pertinent to mention that the Phosphate (Pho) regulon is a global regulatory mechanism involved in bacterial Pi (inorganic Phosphate) management that was first characterized in *Escherichia coli* (Santos-Beneit, 2015).

1.5. Metabolomics of persisters

Maisonneuve and Gerdes (2014) have defined the persisters as dormant cells having a low metabolic activity. This is pertinent to mention that antibiotics are target oriented molecules. The molecular targets of a bacterial cell may be proteins, Enzymes, or Nucleic acids which are synthesized in actively growing cells. Hence, the antibiotics cannot make their way to corrupt the essential or active targets of bacterial cells of dormant Persisters, which have entered a state of 'freezing'. As a result of which, there is no further target molecule synthesis and no antibiotic targeting events among persisters, which can avail the drug tolerance phenomenon and increased the number of bacteria with a long lag time (Vulin et al., 2018). In addition, once cell division begins, the growth rate is that of exponential cells. Critically, the greater the ribosome content, the faster the persister cells resuscitate. Therefore, there is acquaintance of 'Coherent diversity' which is evolved due to differential physical association of parental DNA strands. One of the parental strands of DNA can be physically associated with proteins appropriate for a survival strategy whilst the other strand can be physically associated with proteins appropriate for

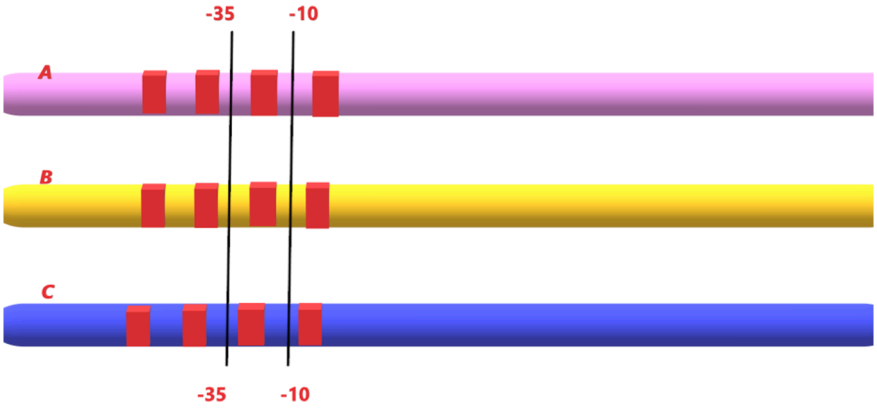

Fig. 7. Co localisation of *hip* genes of *Escherichia coli, Shigella sonnie and Klebsiella pneumoniae*. Schematic showing the -35 -10 boxes of hipBA promoters as binding sites respectively. ATG are the transcription start sites.

a growth strategy, so allowing division to generate daughters with different, coherent phenotypes (Nana et al., 2018). Computer aided simulations predicted (Bandyopadhyay et al., 2018) that cell lineage correlations and the sensitivity of growth to changes in toxin levels coincide in a critical regime. Further it was added that the dormant phenotype characterized by down-regulation of energy producing (ATP decrease) and biosynthetic factors (Lewis, 2005).

1.6. Biotypes and persiser development mechanism

The survival mechanism adopted by multi drug resistant genus *Escherichia* (Ramírez-Castillo et al.,2018; Odonkor and Addo, 2018), a member of Enterobacteriaceae is Gram-negative, non–spore-forming, facultative anaerobe that ferment glucose and other sugars, reduce nitrate to nitrite, and produce catalase, do not produce oxidase and are referred as Enterics (Donnenberg, 2015) was reviewed and inferences were drawn.

1.6.1. Development of persister in strains of Escherichia coli (Enterbacteriaceaebiotype)

Escherichia coli persisters profile indicated about downregulated biosynthetic pathways, consistent with their dormant nature, and over expression of toxin/antitoxin (TA) modules (Lewis, 2008; Kedzierska and Hayes, 2016). While, Van Melderen (2009) had suggested TA (Toxin and Antitoxin) loci are primarily plasmid borne operons that promote plasmid inheritance by the expression of a stable toxin, which is counteracted by an unstable antitoxin. This TA system enables cells to escape from antibiotics and the main cause of formation of persister cells. Therefore, in presence of antibacterial agents, bacteria respond by activating intracellular 'toxin' proteins, which arrest cell growth and induce formation of persister cells. When, antibacterial agents are removed, persisters can re-grow by synthesizing 'antitoxin' proteins that sequester toxin proteins. A multitude of different protein-based antitoxin species are known to be actively degraded by a limited number of shared proteolytic pathways, strongly suggesting interaction via competition between antitoxins for degradation machinery (Ogle et al., 2017). This view was well supported by Cho et al. (2017) that MazE antitoxin sequesters the activity of MazF toxin. Fenga et al. (2013) had also added that HipBA, a two-gene operon, is one of many chromosomally encoded toxin and antitoxin modules in *Escherichia coli* and the HipA7 allelic variant was the first validated high-persistence mutant. Further they have suggested a stochastic model to generate bistability and/or Multistability (Jayaraman, 2008) of the HipBA system, via the reciprocal coupling of free HipA to the cellular growth rate. Germaina et al. (2015) had defended that Stochastic variation of [(p)ppGpp] in single cells induced TA-encoded mRNases via a pathway involving polyphosphate and Lon protease. Polyphosphate activated Lon degrade all known type II antitoxins of *E. coli*, thus the activated mRNases induced persistence and multidrug tolerance. These dormant cells form stochastically, but also in response to environmental cues, by various pathways that are usually controlled by the second messenger (p)ppGpp (Harms et al., 2016). The mechanism of ciprofloxacin-induced persister formation was found, in *Escherichia coli*, to be partially due to the expression of the TisB toxin, which is involved in the decrease of ATP levels and the stop of the proton motive force (Mina and Marques, 2016). While, Renbarger et al. (2017) had reported that PhoU is a 'Persistence Switch'involved in Persister formation and tolerance to multiple antibiotics and stresses in *Escherichia coli*, an interesting note was given by Miyaue et al. (2018) about presence of a long-retention effect, or "memory effect" (Fig. 3) in the persister cell state of *E. coli* cells. Kussell et al. (2005) derived a mathematical model and proposed that the optimal rate of switching between normal and persister cells in *Escherichia coli* is found to depend strongly on the frequency of environmental changes and only weakly on the selective pressures of any given environment.

1.6.2. Development of persister in Klebsiella pneumoniae (Enterobacteriaceae biotype)

The toxin-antitoxin (TA) system has also been reported to be involved in the formation of drug-tolerant persister cells *K. pneumoniae* cells (Yi-Qing et al., 2016). Li et al. (2018) had provided evidence that persister formation is a growth phase-dependent and Type II persisters dominate the persister subpopulation during the entire exponential phase of *Klebsiella pneumoniae*. Carvalho et al. (2018) had given a plausible explanation on the effect of the switching dynamics between susceptible and persister cells on the capacity of biofilms to grow, survive and recover from antibiotic shocks by testing three switching strategies: (1) constant switches, (2) substrate-dependent switches and (3) antibiotic-dependent switches. Keaseya et al. (2019) have emphasized about role of proteomic features engaging persisters to grow. They have explained that drug-specific changes to proteomes included proteins for receptor-mediated membrane transport and sugar utilization, central metabolism, and capsule production, while mechanisms common to both antibiotics included elevated scavenging of reactive oxygen species and turnover of misfolded proteins. Resistance to combined antibiotics presented integrated adjustments to protein levels as well as unique drug-specific proteomic features, which was demonstrated in a strain of *Klebsiella pneumoniae* susceptibility, which involved global remodeling of the bacterial proteome to counter the effects of antibiotics and stabilize growth.

1.6.3. Development of persister in Salmonella typhi serovar typhi (Enterobacteriaceaebiotype)

Drescher et al. (2019) found persisters and unstable SCVs as phenotypic variants of *Salmonella enterica* that were able to survive ciprofloxacin exposure. TacT toxin of *Salmonella enterica* is known to induce a persister state in macrophages through the acetylation of aminoacyl-tRNAs (VanDrisse et al., 2017). Silva-Herzog et al., (2015) had suggested that of the six TA loci conserved across *Salmonella enterica* subspecies that cause systemic infections in mammals and birds, exogenous expression of *vapC* or *sehA* resulted in complete growth arrest. In addition, ectopic expression of three RelE family member toxins enhanced tolerance to ciprofloxacin (*relE*), hydrogen peroxide (*relE-3*) or low pH (*relE-5*). Slattery et al., (2013) had revealed that the shpAB locus (*Salmonella* high persistence) imparted a 3- to 4-order-of-magnitude increase in survival after ampicillin exposure throughout its growth phase and protected the population against exposure to multiple antibiotics. Genetic characterization revealed that shpAB is a newly discovered toxin-antitoxin (TA) module. The high-persistence phenotype was attributed to a nonsense mutation in the 3′ end of the shpB gene encoding an antitoxin protein.

1.6.4. Development of persister in Mycobacterium tuberculosis (Mycobacteriaceae biotype)

The biology of persisters of *Mycobacterium tuberculosis* causing latent infection in host tissues is largely unknown (Velayati et al., 2016; Torrey et al., 2016). The *Mycobacterium tuberculosis* phosphate-specific transport (Pst) system controls gene expression in response to phosphate availability by inhibiting the activation of the SenX3-RegX3 two-component system under phosphate-rich conditions, but the mechanism of communication between these systems is unknown (Namugenyi et al., 2017). But Wei et al. (2019) had proposed that the *Mycobacterium tuberculosis* CRISPR-associated Cas1 involves persistence and tolerance to anti-Tubercular drugs. Srinivas et al. (2018) had determined that the pre-existing persisters are a heterogeneous mix of 44 vapC30, mazF, and relA/spoT overexpressing cells. Deep et al. (2018) presented a plausible model for VapC11-tRNA (toxin-target) interactions and explained that in stress induced response, the

anti-toxin molecules are degraded by cellular proteases. The resulting free toxins inhibit translation by degrading either mRNA or rRNA or tRNA, thereby reprogramming cells to reduce cell growth to facilitate cell survival in the presence of antibiotic-or host induced stress. Unlike members of the γ- and β-proteobacteria lineages, which encode two functionally divergent RSH homologs (RelA and SpoT), *Mtb* encodes a single bifunctional RSH enzyme, Rel_{Mtb} (Dahl et al., 2003; Sala et al., 2014; Singh et al., 2010), which is conserved in all *Mycobacterium* species (Dutta et al., 2019). Although Korch et al. (2015) had reported that*Mycobacterium tuberculosis*possesses three Rel TA modules (Rel_{Mtb}): $RelBE_{Mtb}$, $RelFG_{Mtb}$ and $RelJK_{Mtb}$ (Rv1246c-Rv1247c, Rv2865-Rv2866, and Rv3357-Rv3358, respectively), which inhibit mycobacterial growth when the toxin genes: *relE, relG, relK*) are expressed independently of the antitoxin genes: *relB, relF, relJ* but substantial evidences regarding these T/A modules for persister formation is lacking in literature. Increased cell size and cell-to-cell variation in cell length were associated with bacteria in sputum and infected macrophages. A study provided by Shi and Zhang (2010) had validated that PhoU is involved in persistence not only in *E. coli* but also in *M. tuberculosis*. Zhang et al. (2012) had reviewed and mentioned that there are 56 upregulated persister-specific genes, including many toxin-antitoxin (TA) modules, universal stress protein (UspA), SigF, anti-sigma factor RsbW, and DnaE2, with 5 genes being common to the different persister models, including Acr2 α-crystallin heat shock protein, GntR transcriptional regulator family protein (Rv1152), PdhA pyruvate dehydrogenase component, Rv3290c (Lat) encoding an L-lysine-epsilon-aminotransferase, and Rv2517c encoding a hypothetical protein.

1.6.5. Development of persister in Pseudomonas aeruginosa (Pseudomonadaceae biotype)

Survival mechanism opted by cells of *Pseudomonas aeruginosa* was observed to be associated with *hip* mutants. Mulcahy et al. (2010) had proposed that high-persister mutants (hip mutants) of *Pseudomonas aeruginosa* produce elevated levels of drug tolerant cells. Wang and Wood (2011) suggested that nutritional environment determines the role, that quorum sensing plays in *Pseudomonas aeruginosa* biofilm formation.

1.6.6. Development of persister in Staphylococcus aureus/epidermidis (Staphylococcaceae biotype)

Staphylococcus genera also resist action of antibiotics making some population of persister cells. Yang et al. (2015) had investigated and confirmed that there are at least three subpopulations within a *S. epidermidis* biofilm: normal cells, dormant cells, and TBK cells. Biofilms comprise of more TBK cells and dormant cells than their log-planktonic counterparts (Fig. 5) in a test strain of *Staphylococcus epidermidis*. Shapiro et al. (2011) had demonstrated the presence of persister cells at high concentrations in stationary planktonic cultures and in biofilms of *S. epidermidis* RP62a. Persister cell numbers in *S. epidermidis* biofilms are quite low at first but within 24 h they become high. The persister number increases as the cell density increases in planktonic cultures and biofilms. The pre-exposure of bacteria to salt stress caused a 1–2.5 order of magnitude increase in persister formation in the bacterial population after antibiotic exposure, depending on the type and concentration of the antibiotic used (Kubistova et al., 2018).

1.7. Environment and cues for persister development

Regularly growing bacteria differentiate into persister cells stochastically at a basal rate, but this phenotypic conversion can also be induced by environmental cues indicative of imminent threats for the bacteria. Size and composition of the persister subpopulation in bacterial communities are largely controlled by stress signaling pathways, such as the general stress response or the SOS response, in conjunction with the second messenger (p)ppGpp that is almost always involved in persister formation (Harms et al., 2016). Granta et al. (2012) had found that persisters were surviving in the environment with drop in dissolved oxygen (DO) saturation (20%) in the face of bactericidal antibiotics. Zhang et al. (2012) had also made a similar statement that Tubercle bacilli (Mtb) reside in different micro environmental conditions that include high oxygen (in cavities) or low oxygen (in host macrophages or in granulomatous lesions) content, nutrient starvation, oxidative stress, and acidic pH, all of which affect their metabolic statuses. Such varied conditions constituted the basis for producing heterogeneous bacterial populations, including non-replicating persisters and growing bacteria with different capacities for persister formation. Kubistova et al. (2018) had investigated and proposed that salt or oxidative stress could play a role in the formation of *S. aureus* persisters outside the host's intracellular interface. While, Portillo et al. (2019) suggested that *sigB* and *sigE* were over-expressed in early adaptation to low oxygen concentration independently of the carbon source.

1.8. Methods for studying persisters and its development

In past, Keren et al. (2004) had developed a general method for isolating persisters through lysis of regular cells by ampicillin and also established a gene expression profile of persisters containing toxin-antitoxin (TA) modules and other genes that could block important cellular functions such as translation. But Orman et al., (2016) focussed on the metabolic activities of natively generated *Escherichia coli* persisters in presence of aminoglycosides. They had performed LIVE/DEAD fluorescent indicator cell lysis staining, metabolic aminoglycoside potentiation assay, and more over persister measurements assay. Canas and Duarte (2014) were able to develop a novel method for persister isolation, based on a combination of alkaline and enzymatic lysis that targets the cell membrane. Their protocol was to isolate persister cells of Type I and Type II by rapidly killing normally growing cells using a mixture of lytic solutions. Further, isolation was done of only Type I persisters by lysing cells of normally growing cells and type II persisters. Rowe et al. (2016) took interest and enumerated the persisters by adding lethal concentration of a bactericidal antibiotic to a population of cells. The bulk of the population died rapidly and the surviving persister fraction could be enumerated at various time intervals by removing an aliquot, washing with 1% NaCl, and plating serial dilutions for colony counting. The surviving persister fraction emerged in a clear biphasic curve in response to the antibiotic. The time period where the persister fraction remains constant was referred to as the “persister plateau”. Henry and Brynildsen (2016) had developed Persister-FACSeq, which was a method that uses fluorescence-activated cell sorting, antibiotic tolerance assays and next generation sequencing to interrogate persister physiology and its heterogeneity. As a proof-of-concept, they had used Persister-FACSeq on a library of reporters to study gene expression distributions in non-growing *Escherichia coli,* and found that persistence to ofloxacin was inversely correlated with the capacity of non-growing cells to synthesize protein. Grassi et al. (2017) had isolated Persister cells (PCs) of *Pseudomonas aeruginosa* and *Staphylococcus aureus* at high efficiency through membrane-perturbing antimicrobial peptides (AMPs) and two clinically used peptide-based antibiotics, (colistin and daptomycin). It was reported that exposure of stationary-phase cultures to optimized concentrations of the uncoupling agent cyanide *m*-chlorophenylhydrazone (CCCP) was able to generate at high efficiency PCs exhibiting an antibiotic-tolerant phenotype toward different classes of antibiotics, which were sorted by flow cytometry. While Windels et al. (2019) had presented a novel and highly effective persister isolation method involving cephalexin, an antibiotic that induces extensive filamentation of susceptible cells. They could separate

the antibiotic-tolerant cells by size after a short cephalexin treatment, and designated the isolated cells as 'genuine persisters' using microfluidic device. Choudhary et al. (2019) had explored the CRISPRi-based genetic repression for better understanding the effect of DNA gyrase depletion on Mtb physiology and response to anti-TB drugs. It was reported that, suppression of DNA gyrase drastically affects intra- and extracellular growth of Mtb. Interestingly, gyrase depletion in Mtb leads to activation of RecA/LexA-mediated SOS response and drug tolerance via induction of persister subpopulation. Chemical inhibition of RecA in gyrase-depleted bacteria results in reversion of persister phenotype and better killing by antibiotics.

1.9. Biofilm formation ability and persistence

Biofilms are surface-attached groups of microbial cells encased in an extracellular matrix that are significantly less susceptible to antimicrobial agents than non-adherent, planktonic cells (Hall and Mah, 2017). Cells in biofilm can produce persister cells which do not grow or die in the presence of antibiotics. Stress conditions in the host induce persister cells and influence biofilm formation by *Staphylococcus epidermidis* (Fraiha et al., 2019). Roberts and Stewart (2005) through their mathematical model, had hypothesized that the persister state is a hypothetical, highly protected state adopted by a small fraction of the cells in a biofilm. Persisters were assumed to be generated at a fixed rate, independent of the presence of substrate or antimicrobial agent. Cells were assumed to revert from the persister state when exposed to the growth substrate and incapable of growth. *Staphylococcus aureus* biofilms are embedded in an extracellular matrix composed of self-produced extracellular polysaccharides, DNA, and proteins or host-derived matrices such as fibrin, prompting speculation that limited drug diffusion into biofilms contributes to tolerance (Waters et al., 2016) and leads to poor antibiotic penetration, slow growth, adaptive stress responses (George and Halami, 2019) that have a marked influence on cellular physiology, and contribute to the occurrence of persister cells (Acker and Coenye, 2016). Chemical signaling communication, a phenomenon called quorum sensing (QS) has also active role for persister formation inside a biofilm (Maisonneuve and Gerdes, 2014).

2. Prevention and killing persisters

Eradication of tolerant persisters is a serious challenge. But studies are in progress to challenge the persisters. Pearl et al. (2008) had recommended lytic phages infecting bacterial persisters of *E. coli* that could lyse them when growth is resumed, suggesting that phage therapy could be a promising strategy to eradicate persisters. But, Kim et al. (2011) had shown that 3-[4-(4-methoxyphenyl)piperazin-1-yl] piperidin-4-yl biphenyl-4-carboxylate (C10), screened out of a chemical library, selectively kills bacterial persisters that tolerate antibiotic treatment but does not affect normal antibiotic-sensitive cells. C10 led persisters to antibiotic-induced cell death by causing reversion of persisters to antibiotic-sensitive cells. Antibiotic killing of *E. coli*, *P. aeruginosa* and *M. tuberculosis*in the presence of oxygen compared with an oxygen-limited environment had been suggested by Granta et al. (2012), which could be more effective. A combination of specifically persister-targeted approaches, such as catching them when active and susceptible, either by stimulating them to "wake up" or by intermittent drug dosing, kill Mycobacterial persisters (Zhang et al., 2012). Certain sugars were shown to induce a proton motive force, which in turn led to increased uptake of aminoglycosides and thereby bacterial killing (Helaine and Kugelberg, 2014). Mitomycin (Kwan et al., 2015) is a pro-drug which is converted into a reactive compound forming adducts with DNA upon entering the cell. Prolonged treatment with aminoglycosides that cause mistranslation leading to misfolded peptides can sterilize a stationary culture of *Pseudomonas aeruginosa*. In addition, rifampin, an inhibitor of RNA polymerase, was suggested because it "kills" by preventing persister resuscitation. Alumasa et al. (2017) had added that Ribosome Rescue Inhibitors can kill actively growing and non replicating persister *Mycobacterium tuberculosis* cells. While Chung and Ko (2019) had shown that combination of colistin and amikacin antibiotics would be effective at eradicating persister cells. In addition, the killing effect on persister cells was different with respect to the order of antibiotic treatment because, the mechanism of persister cell formation by colistin is different from that by other antibiotics.

3. Conclusion

From this comparative review report on the survival mechanism adopted by the members of *Escherichia coli, Salmonella typhi* and *Klebsiella pneumoniae* (Enterobacteriaceae, Biotype group), *Pseudomonas aeruginosa* (Pseudomonadaceae), *Mycobacterium tuberculosis* (Mycobacteriaceae) and *Staphylococcus aureus* (Staphylococcaceae), it was observed that all the members are following the global survival mechanism. But it is important to note that all the members undergo a 'Freezing' effect' (FE) in the presence of antibiotics. The said FE is referred to as hanging of enzymatic system required for the growth and multiplication of bacterial strains for a certain period of time. While analysing the biochemical properties of the said bacterial strains (under study), it is found that all the four bacterial strains are of different biotypes. Considering IMVIC test, *E. coli* is + +-- biotype, *Kl. pneumoniae* is --+ + biotype, *P. aeruginosa* is ---+biotype and *S. aureus* is -+ + + biotype. Likewise, these bacteria execute differential biochemical approaches in the reduction of Nitrate to Nitrite reduction, Urea hydrolysis, Coagulase, Oxidase production, Carbohydrate fermentation etc. But all the four Biotypes opt a master mechanism for stalling of their metabolic reactions and in most cases are facilitated by biofilm production. Hence, from this snapshot of a research review work carried out for this communication, it was understood that that AA metabolism is a critical mediator of persistence, and as one would expect, the stringent response, a major metabolic regulatory system controlled by ppGpp (Amato et al., 2014). Mok et al. (2015) investigated the role of seven global transcriptional regulators (ArcA, Cra, cyclic AMP [cAMP] receptor protein [CRP], DksA, FNR, Lrp, and RpoS) on persister metabolism.

The modulation of TCA cycle activity appears as a hallmark in persister metabolism. This regulation must be precisely controlled to avoid ROS formation with potentially destructive implications for persister cells. Multiple lines of evidence suggest that the metabolism of persisters can be tuned to alter their susceptibility toward antibiotics or to trigger programmed cell-death-like processes. In a number of cases, this is achieved simply by supplementing selected carbohydrates (Prax and Bertram, 2014). In addition, target inactivity and/or global cellular dormancy comprise mechanisms such as reduced cellular energy, halted DNA replication and blocked translation supporting persister formation (Wilmaerts et al., 2019).

Acknowledgement

This review and experimental studies are part of a Ph. D thesis registered under Sambalpur University.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.bcab.2019.101401.

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