ISOLATION AND PROTEOMIC CHARACTERIZATION OF *ESCHERICHIA COLI* PERSISTERS

By

AMRAPALI GHOSH

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ISOLATION AND PROTEOMIC

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PERSISTERS

Thesis Approved:

Dr. Kevin Wilson

Thesis Adviser

Dr. Jose Soulages

Committee Member

Dr. Andrew Mort

Committee Member

Dr. Erika Lutter

Committee Member

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Abstract: Bacteria are known to adapt in various unfavorable situations. The term 'persister' has emerged to signify their ability to survive under physiologically adverse environments. Persisters can even tolerate multiple lethal antibiotic treatments. They are of major clinical importance due to their involvement in many fatal chronic bacterial infections such as tuberculosis, lung infection associated with cystic fibrosis, urinary tract infection, and many more. Physiological dormancy is the main basis of their survival in antibiotic stress or host induced environmental stress. Persisters serve as a 'biological memory' and are capable of resuming their growth to repopulate when the favourable conditions are restored. Only little do we know about the diverse cascade of events taking place in persisters. Eradication of chronic infection requires elimination of persisters using a suitable anti-persister drug. It is imperative to analyze their physiology thoroughly for a better insight and to explore effective drug-targets. Naturally occurring persisters are present at a very low frequency in any bacterial population and also they lack specific biomarker to selectively fish them out. To isolate native persisters from a heterogeneous population, we first needed to develop a highly specific and quantitative method that selectively identifies and separates them. Using *E. coli* as a model, we developed an innovative and sophisticated flow cytometry method to isolate native persisters from planktonic liquid cultures. Taking advantage of their non-dividing nature, we exploited their inability to dilute fluorescence and thereby based on the pattern of fluorescent intensity they were quantitatively detected and sorted through flow cytometry. We also performed microscopic analysis to address the 'fluorescent dilution' ability of normal dividing cells. Whole proteomic analysis using Mass spectrometry of the sorted persisters revealed an involvement of tryptophanase enzyme as a key regulator of persister physiology. Further we performed microscopic and flow cytometric analysis to demonstrate the crucial role of tryptophanase in bacterial persistence. Thereby tryptophanase can be proposed as an attractive drug-target to develop effective anti-persister drug. Overall, our successful attempt to isolate and analyze persisters in a modern and more authentic manner paid off by reflecting a unique aspect of their physiology.

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CHAPTER I

INTRODUCTION

Bacteria are known to adapt in a diverse range of unfavorable circumstances such as pH stress, oxidative stress, antibiotic stress etc. [1, 42]. The term 'persister' has emerged to indicate the impressive capability of a many bacteria to endure any physiologically adverse environment and survive. Persisters are medically relevant as the underlying causative agents of relapsing fatal chronic infections such as tuberculosis, lung infections associated with cystic fibrosis, urinary tract infections, recurrent typhoid fever and healthcare-associated infections [4, 5, 40]. The remarkable potential of a small sub-fraction of pathogenic bacteria population to tolerate and survive bactericidal antibiotic stress and resume their growth to replicate and persist in host cells, impedes the permanent eradication of such recalcitrant infections [4].

The presence of persisters is characterized by the biphasic killing kinetics of bacterial population upon exposure to a lethal dose of bactericidal antibiotic. The vast majority of the rapidly growing sensitive cells are killed during the first rapid exponential decay whereas the dormant or slow-growing persisters evade the antibiotic-mediated killing [2, 3]. Later on, when antibiotic stress subsides, the surviving persisters can resume their growth to re-inoculate the infection and give rise to the original population with identical susceptibility to the lethal drug [4]. This noticeably establishes persistence as a phenotypic trait of a subset of bacteria in any existing genetically uniform bacterial population [6, 7]. Persisters utilize physiological quiescence

to elude multiple rounds of antibiotic treatment, host induced defensive mechanisms or any other catastrophe and eventually give rise to prolonged untreatable chronic infections if they are pathogenic in nature [5-12]. The delayed growth rate compensates for an evolutionary advantage in the bacterial community where the persisters obtain an upper hand [13]. Thereby persisters serve as a 'biological memory' or 'seed bank' of any bacterial community and prevent complete eradication of the population upon exposure to any physiologically challenging environment [12].

These features of the persisters have been actively tested and observed by infecting different animal models with pathogenic *E. coli*, *Samonella* or *Mycobacterium tuberculosis* [14-19]. Persisters are clearly different from antibiotic resistant cells which display a genetically inherited ability to grow in the presence of lethal antibiotics. However persisters are known to act as catalysts to trigger the emergence of genetically resistant cells leading to complete antibiotic treatment failure [20]. Therefore comprehensive understanding of detailed physiology of the persisters is pivotal to innovating novel effective therapeutic remedies to combat such deadly chronic infections [1, 2]. While physiological dormancy leading to slow growth or no growth are necessary persisters, they are not absolutely sufficient to shape persistence [8, 11, 21]. The level of (p)ppGpp and complex interplay between a variety of toxin-antitoxin molecules help these specialized persisters to cope up with antibiotic induced adversities [5, 7, 8, 11]. This special advantage of persisters is favoured by phenotypic variation accomplished either stochastically or in response to environmental cues [11, 22-26]. The controversy regarding the mechanism of generation of persisters remains largely unsettled still [41]. However, many pioneering scientists involved in research with persisters have accumulated a number of strong evidences based on genetic, microscopic and flow cytometric data which firmly support the stochastic pre-existing dormant nature of persisters [4-10, 12]. The phenotypic variance that aids the antibiotic tolerance is a cumulative outcome of a varied transcriptome and proteome [42]. Therefore it is vital to acquire knowledge of the transcriptome and proteome of native persisters to broaden our

understanding about the intricate regulatory phenomena taking place at the genomic, transcriptional or translational level in order to come up with novel drug targets which would help to eliminate persisters and advance medical science [3, 27, 28, 31].

Multiple technical hurdles to isolate native persisters have impeded our understanding of persister physiology [29, 30]. Naturally occurring persisters arise in a very low frequency (10^{-5} to 10^{-6} in exponentially growing *in vitro* culture) [6]. Specific biomarker(s) which could have been useful to fish out persisters are not known. The transient nature of persisters and their similarities to viable-but-non-culturable (VNBCs) cells makes it difficult to isolate and analyze native persisters eventually leading to a major hindrance to alleviate the burdens of recurrent infections [11, 27, 30, 32-34]. Traditionally, several model persisters had been designed by over-expressing several toxin proteins and were studied extensively to gain knowledge about the cascades of events taking place in persisters. But ultimately, these models have been questioned on the grounds of insufficient authenticity and inability to sufficiently reflect the natural persisters [27]. So the persister field has gradually shifted towards the application of more high-end and sophisticated techniques such as FACS (Fluorescence-Activated Cell Sorting), advanced microscopy, micro-fluidic device to overcome these technical obstructions and develop methods to address and analyze naturally-occurring persisters.

Lewis and his research group took a step forward and employed a green fluorescence reporter protein (GFP) fused to a ribosomal RNA promoter (*rrn BP1*) and monitored its in bacterial population [10]. This study took an attempt to segregate the persisters based on the low expression of the reporter protein (which implies suppression of rRNA synthesis and consequently protein synthesis) in a subgroup of cells. He could justify a 20-fold enrichment of the persisters in the low fluorescent reporter expressing cells which signified quite advancement in the process of native persister purification [10].

Brynildsen and research group initiated their investigation to recognize a detailed insight of how metabolic activities control and contributes to persistence. They also enlightened on native persister purification from *in vitro E. coli* culture. His lab carried out multiple works demonstrating a combination of FACS, NGS (Next Generation Sequencing) and complex mathematical models as a powerful tool to identify and quantify native persisters based on phenotypic distributions [11, 27, 30, 34, 35]. Brynildsen assigned a transcriptional fluorescent reporter to analyze hierarchical clustering of gene expression patterns. Combining these data with FACS and several complex mathematical analyses, he came to the conclusion that persistence to Ofloxacin (a bactericidal drug, routinely prescribed to the patients with chronic infection) is inversely proportional to the promoter activity [27]. He has taken initiatives to identify the unique aspects of the transcriptome and metabolome profile of the native persisters. Even though minimal metabolic activities and translation takes place, but suppression of major and essential cellular functions including protein synthesis leading to overall cellular dormancy has been assigned as the main trademark of persisters.

Helaine and research group addressed the dynamics of intracellular replication of pathogen *Salmonella enterica* (causes water or food-borne chronic typhoid fever) with the help of a translational fluorescent reporter [17, 36, 37]. She exploited overall translational dormancy and arrested cellular growth to mark the native persisters when the infected bacteria population replicate inside the macrophages. A sophisticated plasmid-based fluorescent reporter system was assigned to address the overall heterogeneity of replicating pathogens in macrophages. This study employed immuno-fluorescence microscopy and FACS and clearly illustrated a distinct subgroup consisting of very slow-growing and dormant-like bacteria within the infectious population. They could be spotted by their inability to dilute the fluorescent marker as a consequence of their non-replicating nature [36]. She also predicted that this small fraction of non-replicating dormant-like bacteria to be the reservoir of native persisters [17, 36, 37]. Later she applied this finetuned dual

fluorescent reporter system to *Mycobacterium tuberculosis* and demonstrated the heterogenic replication dynamics in this pathogen population both *in vivo* and *in vitro* [38]. Also, this work marked the enrichment of non-replicating native persisters upon macrophage uptake by testing the bactericidal drug tolerance phenotype. In addition to antibiotic stress, host induced stress also selects for the dormant-like persister phenotype [38].

Inspired by these approaches, we set out to identify, sort and analyze true persisters in our own unique way. In common laboratory practice, an *in vitro* growing bacterial culture is typically treated with a supra-lethal dose of any lethal antibiotic to isolate persisters. In reality this process frequently selects for a heterogeneous population surviving antibiotic stress which is not exclusively composed of native persisters. We first needed to develop a highly specific and quantitative method that selectively recognizes and separates native persisters from such heterogeneous population surviving the antibiotic stress. Followed by this, we aimed to focus on the analysis of whole proteome profile of the native persisters which was still largely unknown. Our main target was to address the comparative overview of the proteomic profiles of the native persisters and other antibiotic surviving cells. That way we anticipated to obtain a new insight regarding any physiological marker(s) which might be up or down regulated exclusively in persisters. This could potentially serve as a useful identifier for native persisters. Most importantly such marker might be a novel drug target to accelerate the therapeutic treatment to abolish relapsing life threatening diseases.

Keeping this big picture in mind, we embarked on the project, choosing *E. coli* as a model system, because of the previous studies on *E. coli* persisters [6, 10, 43-48] including our own lab [49, 50]. We used a specialized *E. coli* strain to overcome some anticipated technical difficulties. This strain chromosomally expresses HipA7 toxin leading to increased yields of persisters without affecting the cellular physiology or natural growth of the strain [43, 44, 51]. We integrated several versatile technologies to come up with an innovative method for isolating

native *E. coli* persisters. Our approach combined two layers of purification. The first layer was typical lysis of the sensitive population by bactericidal drug cefoxitin which selects for a heterogeneous population surviving drug treatment. This was followed by selective sorting of native persisters from such heterogeneous population using flow cytometry. The latter step needed a careful selection of fluorescent marker.

We obtained a plasmid-based dual fluorescent marker which was calibrated by Helaine and her research group, but in a different bacterial system [36]. We implemented this fluorescent marker to verify our central hypothesis and to recognize and segregate native *E. coli* persisters. We had a very straightforward fundamental hypothesis. We hypothesized that the naturally occurring persisters are dormant-like non-replicating cells whereas the normal cells would grow and divide spontaneously. The dual fluorescent marker plasmid could be employed to distinguish normal and persister cells. Growth and cell division of normal cells would dilute the fluorescence while native persisters would be prominent by keeping the fluorescence undiluted as a result of their dormant-like nature leading to no active cell division. We aimed to utilize fluorescent microscopy to demonstrate this fluorescent dilution event.

Treatment of such culture having various colour-based distribution of fluorescently labeled cells, with bactericidal drug cefoxitin in a supra-lethal dose would evidently select for antibiotic surviving cells. We could justify our hypothesis if we could show a significant enrichment of our hypothesized native persisters in the heterogeneous antibiotic stress surviving population. Quantitative flow cytometry would be best fitted to address this. Flow cytometeric analysis could demonstrate both the heterogenic nature of the antibiotic surviving population and enrichment of our hypothesized native persisters with undiluted fluorescence in the antibiotic stress surviving population. Most importantly, selective segregation and sorting of these native persisters was feasible through fluorescence-based flow cytometric sorting technique. After successful sorting, our next plan was to perform the major ground-breaking experiment with the

help of mass spectrometry to uncover the whole proteome of the native persisters and compare that to other antibiotic surviving cells.

1.1 Research Objectives

We hypothesized that this study would identify some novel physiological markers that are unique to native persisters. Accordingly we embarked on experiments to accomplish several objectives:

1. Identify the fluorescent dilution event which clearly distinguishes the normal cells from the native persisters.
2. Address the heterogenic nature of the antibiotic stress surviving population selected by lethal antibiotic treatment of *in vitro* growing bacterial culture.
3. Address the enrichment of hypothesized native persisters in antibiotic surviving population.
4. Sorting and analysis of the whole proteome data of the native persisters and compare that with the proteomic profile of antibiotic stress surviving cells.

The overall purpose of this project was to fill in some major gaps of knowledge regarding persisters. We integrated multiple sophisticated technologies to address our objectives. Our method of isolating native persisters can be adapted to apply in any type of bacteria that can be transformed with the plasmid-based dual fluorescent marker. I was excited to take over the project in the hope of contributing towards the progress of persister research field and open up new avenues to design effective anti-persister drug.

1.2 Thesis Organization

Chapter 2 focuses on a literature review most relevant to this thesis research. Chapter 3 elaborates on scientific methods and materials used to accomplish the research objectives of this thesis. Chapter 4 covers the outcomes of the experiments and explains their contextual significance. Chapter 5 connects the major outcomes of this thesis with the broader picture in the persister field and further discusses the significance of the results. This chapter also outlines the future directions of this research.

CHAPTER II

REVIEW OF LITERATURE

Persister cells are drawing major attention since last few decades, as they are known to be the causative agents for recurrent chronic infections [4, 5, 40]. Persisters are named so to symbolize their remarkable capacity to persist in any catastrophe and emerge as survivors [62]. That way they save the bacterial community from eradication due to adverse environmental conditions and serve as 'seed banks' keeping a provision for the entire population to recoup [63]. This has stymied successful medical treatment of many fatal chronic infections such as tuberculosis, lung infection associated with cystic fibrosis and also a number other relapsing infections such as recurrent typhoid fever, urinary tract infection etc [4, 5, 40]. These specialized persisters have an innate ability to tolerate multiple bactericidal antibiotics which makes it harder to find suitable ways to eradicate recurrent infections. Persisters are frequently confused with antibiotic-resistant cells. But resistant cells have genetically inherent capability to grow in the presence of antibiotic while persisters arise in a low frequency in a genetically uniform population of bacteria and dodge antibiotic mediated killing by manifesting phenotypic variance [20]. Limited metabolism and slow growth or dormancy leading to physiological quiescence aids persisters to evade antibiotic-mediated killing. Most importantly, the impressive ability of persisters to revert back after antibiotic stress subsides and repopulate giving rise to the parent population with identical susceptibility holds them responsible for recurrent noxious infections

[4]. Thus a comprehensive knowledge about persister physiology is needed before identifying an appropriate drug target to design anti-persister drug.

Joseph Bigger first noticed the presence of persisters in 1944 in penicillin treated staphylococcal population and classified them as non-dividing phase of bacteria [55]. Later the pivotal role of such bacteria remerged in the context of chronic infection and the importance of studying these specialized persisters was demonstrated [56]. Since then, persisters have grabbed major attention and have been studied extensively. However, the mechanism of persister formation is still unresolved. A group of scientists advocate for pre-existing stochastic persister formation whereas others consider that the persistence state is induced by environmental cues.

Balaban and colleagues had developed methods to study *E. coli* persisters at the single cell level with the help of micro-fluidics [6]. Their experiments demonstrated persistence as a phenotypic switch. Persisters represent a small sub-fraction of cells having innate phenotypic variance and pre-exist as a part of genetically homogeneous bacterial population. This pre-existing phenotypic heterogeneity in a genetically identical bacterial community clearly serves as a reservoir to prevent the population from weathering away and aid adaptation in unpleasant environmental conditions [6].

Due to very low frequency of occurrence of native persisters in any bacterial population, it had always been tricky to isolate them to sufficient purity from the regular cells. Lewis and his research group endeavored to address this problem by presenting a method based on flow cytometry and fluorescence microscopy to identify, sort and isolate persisters. This method relies on the hypothesis that persisters are dormant and possess a very low level of translational activity supporting the pre-existing dormant nature of persisters. They chose the promoter of a rRNA whose activity corroborates with the growth rate of *E. coli* and monitored the level of GFP expression as a reporter which was fused with rRNA coding gene. They sorted the cells which

were dimly fluorescent assuming that they were native persisters due to very low level of translational activity. These cells emerged as tolerant to bactericidal antibiotics which support their assumption. Using microarray analysis they showed a distinct transcriptome which is significantly different from either exponential or stationary phase cells, having the toxin-antitoxin modules (TA modules) extensively expressed in such dormant native persisters. They speculated major involvement of these modules in persistence [10].

Lennon and Jones described persistence and dormancy from the perspective of ecological implications by elaborating multiple metagenomics data [12]. Persisters and dormancy state had been elaborated from the perspective of both pre-existing spontaneous and induced responsive manner. Pre-existing inherent dormant state of a tiny fraction of bacterial population helps the entire community from extinction upon environmental fluctuation. This natural pre-existing dormancy helps the persisters to respond quickly to environmental perturbation without investing energy to regulate cellular machinery to switch physiological state in order to avoid hostile condition mediated damage. A complex interplay of toxin-antitoxin modules were again held responsible for phenotypic variance within the genetically consistent population giving persisters a benefit from the evolutionary and biodiversity standpoint [12].

Further progress in the field revealed the importance of phenotypic bi-stability that enables the co-existence of persisters and regular cells in the same population. This phenotypic differentiation of persisters is brought about by stochastic amplification of noise which is manifested as the expression of toxins (from the TA modules of chromosome) beyond a threshold level in a certain minor fraction of a population. This, in turn, allows very slow growth rate, dormancy and limited translational and metabolic activities which cumulatively exert a positive feedback effect to tolerate environmental fluctuations facilitating long term evolutionary implications [3]. In this context, Moyed had provided an example of *in vitro* evolution by exposing *E. coli* culture under cyclic administration of bactericidal drug which eventually

resulted in significantly raised level of persisters which were indeed mutants having increased antibiotic tolerance as they expressed a mutated version of toxin protein HipA having reduced and weaker interaction with its cognate antitoxin acting as an inhibitor of the toxin [52, 53]. Later, *in vivo* evolution was also addressed in a clinical study done with cystic fibrosis patient subjected under extensive antibiotic treatment where mutant *P. aeruginosa* were isolated from the patient's lung [54]. This could establish the value of persistence in bacterial evolution, but the redundancy of TA modules in different bacteria such as *E .coli*, *M. tuberculosis* remained unexplained. Possibly the multiplicity of such modules might contribute towards the robustness of persistence [3].

Alarmones such as second messenger (p)ppGpp (guanosine tetra or penta phosphate) and TA modules are crucial for dormancy and multidrug tolerance in persisters [63, 67-70]. There are intricate interactive cascades of (p)ppGpp and multiple toxins, antitoxins which manifest phenotypic variance in persisters. TA modules are diverse in nature and mostly abundant in bacteria, but also found in eukaryotes and archea [60]. Each TA locus consists of two consecutive closely linked genes in an operon located in bacterial chromosome where one gene codes for labile antitoxin and the other codes for stable toxin molecule. In *E. coli*, TA modules are broadly classified into five different types depending on the mode of interaction between toxin and its cognate antitoxin [60, 62, 63]. So far type I and type II TA modules has been found to have major involvement in persistence (Fig. 2.1). Antitoxin interferes with toxin functionality and inhibits them in normal cells. Antitoxins belonging to type I TA module inhibit its cognate toxin at the RNA level by the expression of small antisense RNAs which bind to its cognate toxin mRNAs and generate double stranded RNA molecules. This, in turn, triggers RNA interference process mediating complete degradation of these double stranded RNA complexes. Type II toxins are inhibited by direct binding of the antitoxin proteins to its cognate toxin proteins [60, 62, 63].

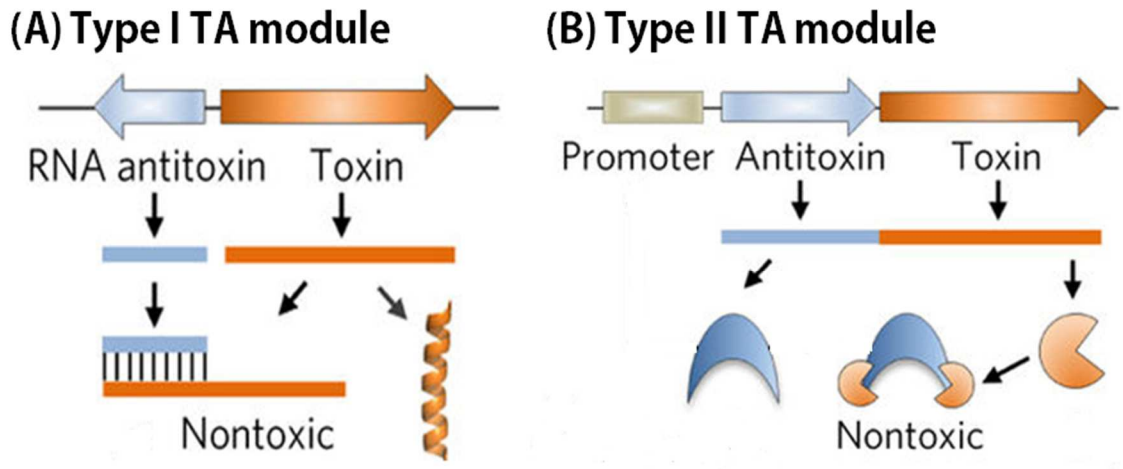


Figure 2.1: Type I and type II TA modules [63]. **(A)** Type I TA module generates antitoxin RNA which is antisense to the cognate toxin mRNA. Binding of antitoxin RNA to cognate toxin mRNA inhibits type I toxins at the mRNA level. **(B)** Type II antitoxin proteins are able to form complexes with cognate toxins and block the functionality of type II toxins.

Activated type I and type II toxins interrupt the functionalities of different cellular targets which ultimately lead to dormancy (Fig. 2.2) [60, 62, 63]. Type I toxins are membrane associated proteins exerting effect by forming small pores in the cell membranes which dissipate the proton motive force and thereby ATP synthesis is interrupted. ATP is required as crucial energy source for diverse essential cellular processes like DNA replication, transcription, translation and many other synthetic pathways. Type I toxins HokB and TisB are found to play vital roles in persistence [25, 67]. But the activation mechanism of both toxins differs. SOS response pathway (one of the stringent response pathways) activates TisB toxin. Damaged ssDNA activates RecA protein (component of SOS signalling pathway) which induces auto-cleavage of LexA protein (positive regulator of transcription of istR RNA which is antisense to TisB mRNA) and thus it relieves the inhibition from TisB toxin coding RNA [25, 62]. HokB toxin however is known to get activated by collective action of (p)ppGpp and Obg (a small highly conserved GTPase) [62, 73]. Activated type II toxins on the other hand are mostly ribonucleases and carry out cleavage of mRNAs in either ribosome dependent or independent manner. This suppresses global cellular

protein synthesis. So far eleven type II toxins have been discovered in *E. coli* and 10 of them are ribonucleasaes.

In addition to this, type II toxins also contribute towards elevating the (p)ppGpp level through intricately regulated cascades. *hipA* (high persister A) is the first gene discovered to be involved in persistence and it is the only exception in the category of type II toxins which has a different mode of action when activated [8, 45, 52]. HipA is a Ser/Thr kinase which executes ATP-dependent phosphorylation of glutamyl tRNA synthetase (GluX) at S239 position (part of a conserved ATP-binding domain) inhibiting its ability to perform aminoacylation of $tRNA^{Glu}$ with free glutamic acid [71]. This generates uncharged $tRNA^{Glu}$ which still can enter the ribosomal A-site. However ribosome gets stalled and translation come to a halt as a consequence. Furthermore, recruitment of deacylated tRNA in ribosome triggers structural changes in the ribosome which is recognized by RelA [71].

RelA is a (p)ppGpp synthetase. RelA can directly interact with uncharged tRNA bound to stalled ribosome which in turn helps to activate RelA. Activated RelA synthesizes ppGpp or pppGpp from either GDP and ATP or GTP and ATP [67]. (p)ppGpp can also be synthesized by SpoT, a bifunctional enzyme which either synthesizes or degrades (p)ppGpp depending on two different conformations [67, 69]. Conformational change is regulated by binding of a GTPase protein ObgE which is involved in 16S rRNA processing step of ribosomal assembly. When ObgE is bound to SpoT it performs (p)ppGpp hydrolysing activity whereas dissociation of ObgE triggers conformational changes when SpoT starts synthesizing (p)ppGpp [67]. Usually nutritional deprivation such as lack of certain amino acids, fatty acids or phosphate causes dissociation of ObgE from SpoT. The detailed regulation and exact mechanism are not yet clearly understood.

In this context it is required to mention that HipA7 is a mutant version of HipA protein which is frequently isolated from uropathogenic *E. coli* found in patients suffering from chronic urinary tract infection [48, 72]. HipA7 has two mutations (G22S and D291A), that reduces its affinity towards cognate antitoxin HipB and also it is nontoxic to cell growth. Thus HipA7 toxin protein greatly increases (100-1000 fold) the frequency of persister formation in *E. coli* cell populations without affecting its growth or physiology [48, 59].

Under normal circumstances, balance of phosphate level in bacterial cells is maintained by the synchronized action of two enzymes, Polyphosphate hydrolase (PPX) and Polyphosphate kinase (PPK) [7]. (p)ppGpp acts as competitive inhibitor of PPX. Thus, when (p)ppGpp level is elevated beyond a threshold, it binds and inhibits PPX which causes accumulation of high concentration of polyphosphate (PolyP) in the cell as PPK keeps producing polyphosphates [7, 8, 57]. Accumulated polyphosphate binds and stimulates the activation of ATP-dependent Serine protease Lon. Lon protease has a number of cellular targets including the labile type II antitoxins. Thus degradation of antitoxin relieves the inhibitions from the type II toxin and activated toxins work towards entailing cellular dormancy. In addition, type II HipA toxin activation triggers (p)ppGpp synthesis, the signal gets amplified and the cycle continues. Moreover, apart from its major role in toxin activation, elevated (p)ppGpp itself can competitively inhibit many translational GTPases [64, 65], and also it can interact with beta subunit of RNA polymerase to block its functionality [66]. These events comprehensively contribute towards persister phenotype.

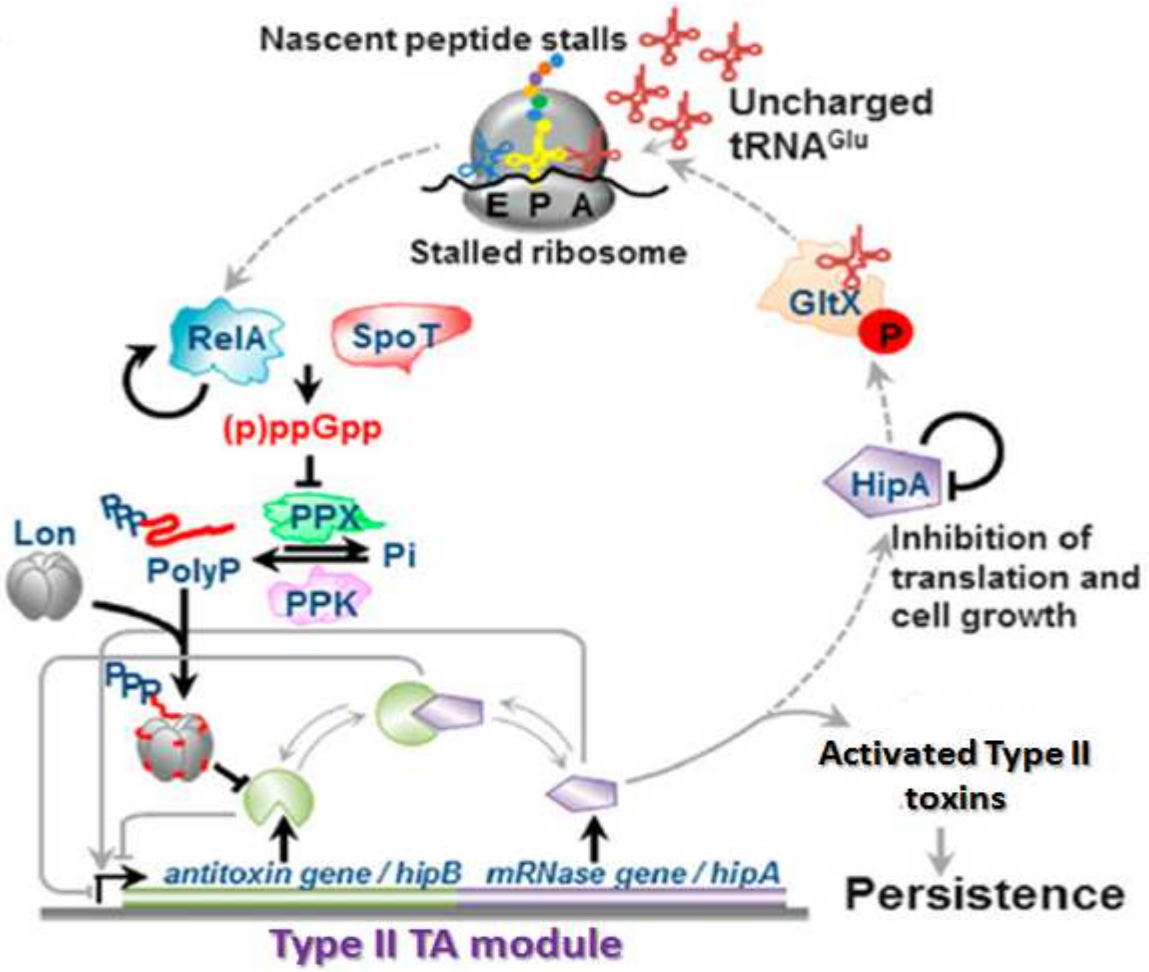


Figure 2.2: Molecular model integrating TA loci, HipA and (p)ppGpp contributing to persistence [8]. Free HipA kinase phosphorylates GltX and inhibits formation of Glu-tRNA^{Glu} and causes accumulation of uncharged tRNA^{Glu} which can enter the ribosomal A-site stalling the ribosome and inhibiting translation. Such stalled ribosome with uncharged tRNA activates (p)ppGpp synthetase RelA. RelA along with SpoT starts synthesizing (p)ppGpp. Elevated (p)ppGpp competitively inhibit PPX disrupting cellular phosphate balance. Increased PolyP binds and activates protease Lon which in turn degrades 11 type II antitoxins including HipB. This leads to activation of type II toxins (including HipA) which causes inhibition of translation, cell growth and brings about persistence. There's a feed-forward loop driven by HipA which should ideally lock the cells into a state with elevated (p)ppGpp. However such state has never been observed experimentally. This is possibly because of the ability of activated HipA toxin to inhibit itself by intermolecular phosphorylation (indicated by a solid line that points back to HipA) [96]. Solid arrow emerging from RelA and points back to itself represents a positive feedback loop where elevated (p)ppGpp causes more (p)ppGpp synthesis. Such positive feedback loop is responsible for stochastic variation of (p)ppGpp at the single cell level. Broken arrows indicate the signalling pathway driving the feed-forward loop that would lead to continuous activation of HipA and type II toxin ribonucleasaes and lock the cells with a state with high level of (p)ppGpp. Solid arrows represent the signalling pathways leading to persistence. GltX=Glutamate tRNA synthetase, PPX=Poly phosphate hydrolase and PolyP=Poly phosphate.

The central question here is that, from which point does the cycle begin in persisters? Gerdes and his colleagues showed the importance of 10 toxin mRNA endonucleases and Lon proteases in persisters [57]. By successive deletion of 10 type II TA modules in *E. coli*, progressive reduction in the persistence level demonstrated the harmonic effect of these toxins towards persistence phenotype. The importance of Lon proteases in persisters was established by the drastically reduced persistence level in strains lacking the *lon* gene. *E. coli* mutants overproducing Lon showed highly increased persistence level whereas the same *E. coli* mutants lacking 10 mRNA endonucleases showed largely reduced persistence level when exposed to either ampicillin or ciprofloxacin. This established type II antitoxins as substrates of Lon proteases and the importance of both in the context of persistence [57].

A couple of years later, Gerdes and research group threw light on more insights of the mechanistic details of persistence in the context of (p)ppGpp, toxin-antitoxin and stochastic pre-existing dormancy [7]. Using fluorescence time lapse microscopy and microfluidics they unveiled that the level of (p)ppGpp stochastically becomes elevated in a small fraction of cells in exponentially growing *E. coli* culture even in the absence of any kind of external stimuli [7]. These cells get phenotypically differentiated and show very slow growth and dormancy which aids their survival when subjected to bactericidal antibiotic mediated killing. Thus stochastic variation of this small signalling molecule brings about non-inherited persistence even in the absence of adverse environment. In addition, by testing a series of mutants, they established the hierarchy of (p)ppGpp mediated regulatory signalling cascade which is sequentially transmitted through inorganic polyphosphate, Lon proteases and toxin-antitoxins [7].

A few years later, Gerdes and colleagues proved convincingly at the single level that HipA activation and high level of (p)ppGpp in the absence of 10 toxin endoribonucleases is able to induce slow growth and dormancy but not multidrug tolerance or persistence [8].

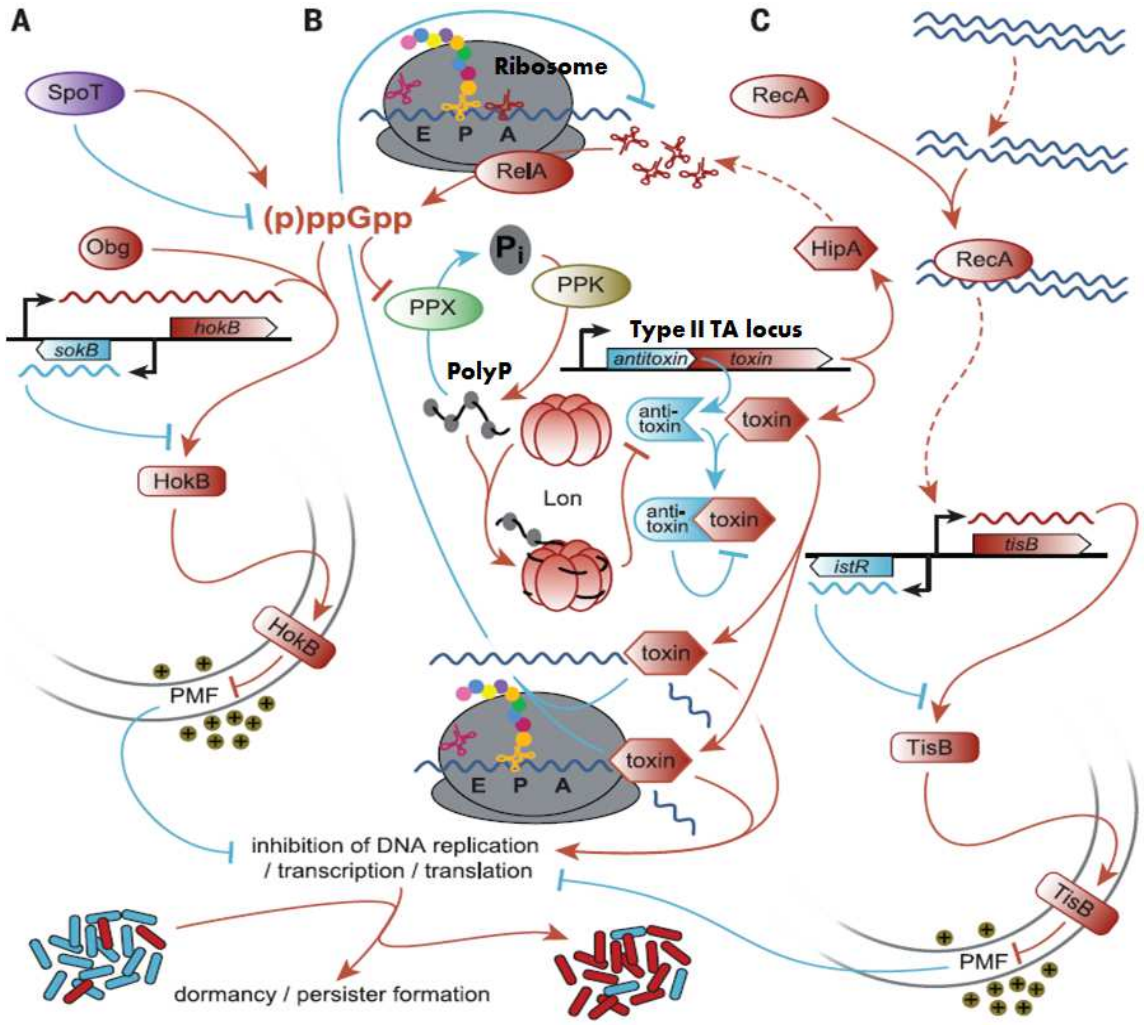


Figure 2.3: Interconnected regulatory pathways integrating (p)ppGpp and TA modules in persister formation [62]. Three central cascades in *E. coli* K-12 have been presented schematically in details. These pathways lead to interruptions of different essential cellular processes which ultimately bring about persistence. **(A)** Obg/HokB pathway and **(B)** Polyphosphate/Lon/mRNA interferase pathway which directly depend on the elevated (p)ppGpp level. Also **(C)** TisB pathway is activated by strong induction of SOS response pathway. Type I toxins TisB and HokB being small membrane-associated proteins dissipate proton motive force which interferes with cellular ATP production, ultimately inhibiting DNA replication, transcription and translation. Ten type II mRNA endonucleases cleave mRNA and interrupt ribosomal translation. HipA interestingly exerts both positive (by promoting RelA mediated (p)ppGpp formation) and negative (by contributing to lower the overall mRNA level which include toxin mRNAs also) effects on the process. Blue lines signify inhibition, red lines signify activation. E, P, and A indicate ribosomal exit site, peptidyl transferase centre, and aminoacyl tRNA binding sites or decoding centre, respectively. Pi=Inorganic phosphate, PPK=PolyPhosphate Kinase, PPX=PolyPhosphate hydrolase, PolyP=PolyPhosphate PMF=Proton Motive Force.

Consistent with their previous models, a new insight about the indispensability of the 10 type II toxins in persister physiology was added to our knowledge.

Wood and colleagues had carried out a work which elaborates the importance of overall decreasing environmental fitness of the cells towards formation of persisters [24]. They engineered type II toxin protein MsqR by two amino acid replacement that increases the stability and toxicity of the toxin and thereby overall increased fraction of *E. coli* persisters was obtained which was easier to isolate and analyze. They revealed that the MsqR variant contributed towards increased persistence by suppressing acid resistance, osmotic resistance and multidrug resistance genes. These findings collectively led to the postulation that overall unresponsiveness and dormancy of the cells to external stimuli is the key to persistence. Further study proved that master regulator of stress response RpoS coding gene deleted mutant and several other mutants having deleted genes which are under the control of RpoS, showed such vividly increased persistence [24].

Lewis and his colleagues provided mechanistic understanding of persister induction in responsive fashion [25]. Using *E. coli* as a model they demonstrated the central role of SOS stringent response pathway and type I TisB toxin in persister induction. Severe DNA damage in *E. coli* causes activation of SOS response and *tisAB* is one of the TA loci, being induced upon activation of SOS response pathway. They showed that *tisAB* knockout strain of *E. coli* showed dramatically decreased level of persisters when exposed to bactericidal drug ciprofloxacin which mediates its lethal effect by causing extensive DNA damage in target bacteria. Also exposing *E. coli* to ciprofloxacin in a step-wise manner induced persister formation which was dependent on *tisAB* locus and also cells producing TisB toxin exhibited multidrug tolerance. Mild overproduction of TisB toxin induces persister level which is consistent with their model [25].

Lewis and research group established oxidative stress as a way of persister induction [23]. The effect of oxidative stress was important to testify as macrophages from hosts' immune system use ROS (Reactive Oxygen Species) or RNS (Reactive Nitrogen Species) mediated oxidative stress to destroy the pathogens during chronic infection. Such oxidative stress is known to induce SoxRS and OxyR regulons in bacteria. A number of genes are in control of these regulons which help to reduce the oxidants and also trigger the repair system genes to get activated. They exploited external viologenic agent paraquat to test the effect of oxidative stress on persister formation using *E .coli*. Paraquat induces transfer of electrons from NADPH or NADH to oxygen promoting super oxide radical formation which causes oxidative stress. The number of cells tolerating fluoroquinolone antibiotic-mediated stress rose drastically upon pre-incubation of the cells with paraquat, whereas this did not influence other categories of bactericidal drugs like kanamycin or amplicillin-mediated killing. So the effect of paraquat was limited to fluoroquinolone class of bactericidal drugs only. However paraquat pre-treated cells surviving fluoroquinolone mediated lethal challenge, showed highly enhanced tolerance to multiple drugs of diverse classes. Further analysis revealed that increased persister level was achieved by paraquat induced enhanced expression of one of the SoxRS regulon genes coding for multidrug resistant efflux pump AcrAB-TolC. This efflux pump presumably flushed out fluoroquinolones from the cells which contributed to drug tolerance and thereby enhanced fraction of persister formation. Most importantly, a mutant unable to manifest SOS response clearly illustrated that pre-incubation with paraquat followed by exposure to fluoroquinolone is not enough to induce increased persister formation when SOS response pathway is not activated. This study elucidated the vital role of SOS response pathway in inductive persister formation [23].

Contemporarily, Ramakrishnan and group took an initiative to test the effect of an efflux pump inhibitor drug verapamil on persistence [15]. At first, they utilized *Mycobacterium*

marinum infected zebrafish larvae as an *in vivo* model to study the response of host upon *mycobacterium* infection and persistence. They interestingly found out that the drug tolerant population surface soon after the macrophages get infected and the replicating population become enriched in non-replicating physiologically quiescent persisters. Persisters become more enriched with time and get disseminated by tuberculous granuloma formation. Granulomas are complex immunological structures which occur at a developed stage of tuberculosis representing local infection lesions where the bacteria reside either within macrophages or extracellularly forming necrotic cores. Using cultured human macrophages infected with *Mycobacterium tuberculosis* mutants they proved that the multidrug tolerant efflux pumps are central to mediate persistence of chronic tubercular infection. Followed by this they employed efflux pump inhibitor drug verapamil to test the level of persister formation when cultured macrophages get infected with wild type *Mycobacterium tuberculosis* pathogens. This drug reduced the tolerance level as expected. Thereby it is capable of shortening the period of anti-tubercular treatment. However to eradicate the pathogens permanently requires more comprehensive understanding of *in vivo* lifestyle and the physiology of these non-growing quiescent persisters [15].

The recurrence of chronic human urinary tract infection is caused by uropathogens such as *E. coli*, *Staphylococcus saprophyticus*. The common notion was that overall metabolic dormancy which holds the pathogenic persisters unresponsive to lethal drug is the basis of drug tolerance. Reid and group had provided experimental evidences which demonstrated a different aspect of drug tolerance mechanism [26]. They employed different antibiotics of various classes like ciprofloxacin, gentamicin and ampicillin to analyse the effect in drug tolerance of uropathogenic population when different cellular targets are aimed to interrupt DNA replication, transcription, translation or cell-wall biosynthesis. The level of persister formation differed as results of differential responses manifested against different drugs and also drug tolerance level varied between two different species of bacteria as well. Moreover, type I (arising from

exponentially growing bacterial culture) and type II (arising from stationary phase of bacterial culture) persisters arising from each bacterial population showed varying susceptibility to each drug. Furthermore, their experimental results clearly shows that SOS-deficient strains were sensitized by ciprofloxacin attack which indicates the importance of DNA gyrase activity in persisters even though they are assumed to be overall metabolically dormant. Also the inevitable role of SOS response mediated repair pathways for drug tolerance was established. They also revealed that pre-exposure of different bacterial culture with sub-inhibitory concentration of specific lethal antibiotic helps to enhance the tolerance for that particular drug and thereby manifests significantly increased level of persister formation. This was possible by selective shut down of the particular drug target(s) if the organism had a history of exposure to that drug. These findings led to the conclusion that global cellular dormancy is not sufficient for persistence, rather selective inactivation of cognate cellular targets contributes towards drug tolerance ability in a drug-specific inductive manner. Uropathogenic *E. coli* isolated from patients with recurrent urinary tract infection, disclosed the fact these pathogen populations were highly enriched in persisters. The authors speculated that the low-dose suppressive antibiotic treatment and prophylactic antibiotic administration which are widespread regimens of medical therapy should be taken care of in order to abolish severe chronic infections [26].

Heinemann and group demonstrated persister formation in the light of conditional bet-hedging upon nutrient-shift [22]. Their data revealed that an isogenic population of *E. coli* diversifies into two phenotypic dissimilar subpopulations when the carbon source is shifted from glucose to various gluconeogenic substrates. The abundance of intracellular metabolites commonly addressed as metabolic flux serves crucial underlying role in accomplishing such molecular mechanism. A cell is usually able to smoothly transit to gluconeogenesis from glycolysis only if it achieves a gluconeogenic flux above a certain threshold. Cells growing extremely fast under glycolytic condition fail to accumulate sufficient initial gluconeogenic flux

and hence become bound to enter dormant state disregarding the new gluconeogenic carbon sources being offered. Such non-growing sub-population adapts to nutrient fluctuation and also becomes tolerant to various other stresses such as lethal antibiotic treatment. They are able to revert back once glycolytic conditions are restored. In contrast, the cells which were not growing very fast are able to acquire adequate gluconeogenic flux to switch to gluconeogenesis-based metabolism strategy by up-taking and utilizing the gluconeogenic substrates. Thus this explains population level adaptation of *E. coli* in response to changes in nutrient environment [22].

McKinney and colleagues showed the overall dynamic nature of persisters using *Mycobacterium smagmatis* as a model [61]. Using time-lapse fluorescence microscopy and micro-fluidics, they uncovered the fact that *M. smagmatis* persisted by dividing in the presence of lethal anti-tubercular drug isoniazid (frequently prescribed to patients with TB). Also they showed that catalase persoxidase KatG which activates isoniazid was actively expressed in these bacteria and was produced stochastically via a range of varying pulsing. The pulses of production of KatG were inversely proportional to the cell survival rate. Authors speculated that a possible adaptation in such lethal stress could be achieved by gradual selection of cells with infrequent KatG pulses under cyclic administration isoniazid. Such cells could emerge as persisters [61].

In this perspective, Brynildsen and research group proved that persisters cannot be stereotyped by mere dormancy and metabolic inactivity [11]. There are far more complexities associated to this unique phenotype. Utilizing a combination of Fluorescent Activated Cell Sorting (FACS), fluorescent marker for cell division and metabolic activity and persistence assay they showed that persisters even arise from growing cells even though the proportion is less than those arising from non-growing cells. This led them to conclude that dormancy and metabolic inactivity only increases the likelihood of a cell to achieve persistence but not sufficient. In addition, they showed that persisters reflect a wide spread variety of metabolic profile which might be an obstacle to produce any specific drug target. However, more detailed transcriptome

and proteome level understanding of their physiology would be useful to design some broad-spectrum anti-persister drug [11].

It's vital to realize that both stochastic and inductive phenotypic differentiation come with a biological cost [28]. Pre-existing stochastic nature of persister formation pre-adapts the population to survive lethal stress while the population pays the cost by reduced fecundity. In contrast, the responsive switching of phenotype of a fraction of sub-population to become persisters has energetic cost to regulate the cellular machineries to sense the environmental stimuli and manifest response. However, pre-existing spontaneous phenotypic switching has long term evolutionary repercussions. This stochastic pre-existing concept can be viewed as an evolutionary 'bet-hedging' strategy which is embraced by diverse organisms when they suffer from reduced fitness in typical conditions whereas enhanced fitness can be achieved under stressful conditions. Mathematical models suggest that stochastic switching is favored when the environmental stresses are infrequent whereas responsive switching is effective in rapidly fluctuating environments [28].

CHAPTER III

METHODOLOGY

3.1 Bacterial strains and plasmid

E. coli strains MG1655 and MG1655-hipA7 were obtained from Prof. Thomas Hill (University of North Dakota. *E. coli* strain GL69 (MG1655 *tnaA–sfgfp : : frt*) was obtained from Prof. Kevin D. Young (University of Arkansas Medical School) [76]. Plasmid pDiGi was obtained from Prof. Sophie Helaine (Imperial College London, U.K) via Addgene [36]. Information about the plasmid and strains is described in Table 3.1.

Table 3.1: Strains and plasmid

Strains	**Features**	**Reference**
MG1655	*E. coli* K-12, Sex: F-, Chromosomal Markers: λ^-, *rph-1* (CGSC # 6300)	Guyer et al. (1981) CSHSQB 45:135. [97]
MG1655-hipA7	HipA7 (having two mutations G22S and D291A) toxin coding gene. Parent strain: MG1655.	Korch et al. (2003) Mol. Microbiol. 50:1199-1213. [48]
GL69	A SF-GFP (Super Folding-Green Fluorescent Protein) tagged Trypotophanase is encoded by the chromosome. Parent strain: MG1655.	Li & Young, (2015) BMC Microbiology 15:14. [76]
Plasmid	**Features**	**Reference**
pDiGi	A vector containing DsRed (excitation maxima 560nm and emission maxima 587nm) encoding gene under arabinose inducible promoter pBAD, and GFP (excitation maxima 488nm and emission maxima 509nm) encoding gene under IPTG (Isopropyl β-D-1-thiogalactopyranoside) inducible promoter pLac, Amp^R (Ampicillin resistant).	Helaine et al. (2010), PNAS, 107(8): p. 3746–3751. [36]

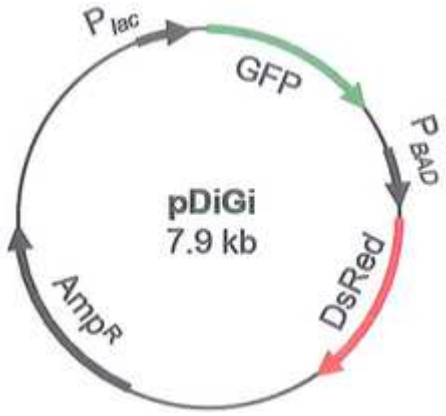


Figure 3.1: Plasmid map of pDiGi [Ref: Addgene]. This plasmid is 7.9 kb long and contains ampicillin resistant gene (Amp^R) as a selection marker. It has GFP coding gene cloned under pLac promoter which can be induced by IPTG (0.5mM) and DsRed coding gene cloned under pBAD promoter which can be induced by arabinose (0.2%). GFP=Green Fluorescent Protein, IPTG=Isopropyl β-D-1-thiogalactopyranoside.

3.2 Transformation

Materials:

1. LB (Luria Bertani) broth powder (Fisher BioReagents, Cat # BP1426-2)
2. LB agar powder (Fisher BioReagents, Cat # BP1423-500)
3. Sterile water
4. Sterile 10% glycerol and 50% glycerol
5. Filter steriled 25 mg/ml ampicillin stock solution (in water)

Instruments:

1. Autoclave (ADV-PB, 250˚F heat, 15 psi pressure, liquid cycle = 30 mins)
2. Incubator (New Brunswick Scientific, US patent # 3002895)
3. Electroporation apparatus (Gene Pulser, Bio-Rad)
4. Spectrophotometer (HP, diode array, 845A)
5. Centrifuge (Beckman, Product # J-6M/E)

Method:

Plasmid pDiGi was transferred into MG1655-hipA7 strain by electroporation. To make competent cells for transformation, MG1655-hipA7 cells were grown in 250ml LB broth until log-phase (OD_{600} ~ 0.6) at 250 rpm at 37˚C. These cells were chilled at 4˚C for 15 minutes and centrifuged at 4200 rpm in 4˚C for 10 min. The supernatant was discarded and the remaining pellet was resuspended in 250 ml ice-cold sterile water. These cells were centrifuged and washed with 250 ml ice-cold water again (total three times). After washing, the volume of the pellet was estimated and equal volume of sterile ice-cold 10% glycerol was added and resuspended. Fifty µl aliquots of these cells (competent MG1655-hipA7) were frozen into a 1.5 ml microcentrifuge tubes and stored at -80˚C. For transformation, the electroporation apparatus was set to the voltage of 1.5 KV, the capacitance of 250 µFD, and the resistance of 200 Ohms. After pulsing 50 µl of the competent MG1655-hipA7 cells with 0.5µl (1µg) plasmid pDiGi, the transformed cells (MG1655-hipA7/pDiGi) were immediately mixed with 1ml fresh sterile LB, followed by the incubation at 250 rpm and 37˚C for 1hr. Hundred µl of the cells were spread onto LB-agar plates with 50 µg/ml ampicillin and were incubated at 37˚C overnight. On the following day, one of the colonies was streaked onto LB-agar plates containing ampicillin (50µg/ml) and incubated again. On the next day, one of the colonies from the streak was chosen and inoculated into 5ml LB with the 50 µg/ml ampicillin at 37˚C overnight. From the overnight cells, 1ml of the cells was removed, mixed with 500 µl of 50% glycerol and stored at -80˚C.

3.3 Fluorescent proteins (GFP and DsRed) expression and growth conditions

Materials:

1. LB (Luria Bertani) broth powder (Fisher BioReagents, Cat # BP1426-2)
2. LB agar powder (Fisher BioReagents, Cat # BP1423-500)
3. Sterile water
4. Filter sterilized 10% arabinose stock solution (in water)

5. Filter sterilized 1M IPTG (Isopropyl β-D-1-thiogalactopyranoside) stock solution (in water)
6. Filter sterilized 172 mg/ml cefoxitin stock solution (in water)
7. Filter sterilized 25 mg/ml ampicillin stock solution (in water)

Instruments:

1. Autoclave (ADV-PB, 250˚F heat, 15 psi pressure, liquid cycle = 30 mins)
2. Incubator (New Brunswick Scientific, US patent # 3002895)
3. Spectrophotometer (HP, diode array, 845A)

Method:

Overnight culture of MG1655-hipA7/pDiGi was grown in 5ml LB containing 50μg/ml ampicillin, 0.2% arabinose (inducer of DsRed) and 0.5mM IPTG (inducer of GFP). On the next day the overnight culture was diluted (1:500) in two sets of large cultures (500 ml each). The first set being, fresh LB + 0.2% arabinose and the second set being, fresh LB + 0.5mM IPTG to allow the fluorescent dilution of either GFP or DsRed respectively. In both the cases, the cultures were allowed to grow at 37˚C under shaking condition (250 rpm) until mid-log phase (OD_{600}~0.8), when antibiotic cefoxitin (16 μg/ml) was added. To visualize the effect of cefoxitin on cell growth, OD_{600} was measured every 30 min. Fresh sterile LB media was used as a blank for all spectrophotometric readings. Samples were collected before and after 3 hrs of antibiotic treatment for microscopic and flow cytometric analysis. One control set was grown in parallel without any of the fluorescent protein inducers either in overnight or large scale culture.

3.4 Cell growth in minimal media (MOPS Glc)

Materials:

1. MOPS Glc minimal media (Product # M2106, Tenkova)
2. Sterile water
3. Filter sterilized 172 mg/ml cefoxitin stock solution (in water)
4. Filter sterilized 500 mM indole stock solution (in methanol)

Instruments:

1. Autoclave (ADV-PB, 250˚F heat, 15 psi pressure, liquid cycle = 30 mins)
2. Incubator (New Brunswick Scientific, US patent # 3002895)
3. Spectrophotometer (HP, diode array, 845A)

Method:

All the experiments involving strain GL69 were done using MOPS Glc minimal media. GL69 strain was inoculated in 5 ml MOPS Glc media from its glycerol stock and was allowed to grow at 37˚C for 24 hrs with shaking (250 rpm). This culture was diluted (1:10000) in 250 ml fresh sterile MOPS Glc media and was allowed to grow at 37˚C with shaking (250 rpm) for 12 hrs until the OD_{600} reading reached 0.062. At this OD_{600}, 250 µl of indole (Stock solution concentration=500 mM) was added to the culture to achieve a final concentration of 0.5 mM and the culture was allowed to grow at 37˚C with shaking (250 rpm) until the OD_{600} reached 0.534. At this OD_{600}, cefoxitin (256µg/ml) was added to the culture (to check the effect of cefoxitin on tryptophanase expression) and was incubated under the same conditions. Samples were collected before cefoxitin addition (at the OD_{600} = 0.534), 3 hrs after cefoxitin addition and 10 hrs after cefoxitin addition for microscopic and flow cytometric analysis. Fresh sterile MOPS Glc + indole was used as a blank for spectrophotometric readings.

3.5 Light microscopy

3.5.1 Visualization of fluorescent dilution

Materials:

1. Sterile 1X Phosphate-Buffered Saline or PBS (pH=7.4)
2. 1% sterile LB-agarose solution
3. Clean glass slides (75mm x 25mm, Fisher Scientific, Cat # 12-544-4)
4. Clean coverslips (25mm x 25mm, Fisher Scientific, Cat # 12-540C)

Instruments:

1. Epi-fluorescence microscope (XYL-146Y, Amscope)
2. Phase contrast lens (PCT200-INF, Amscope)
3. Digital camera (MU1000-CK, Amscope).
4. Centrifuge (Eppendorf, Product # 5415 C)
5. Autoclave (ADV-PB, 250˚F heat, 15 psi pressure, liquid cycle = 30 mins)

Method:

For microscopy, cell samples collected at different time points were harvested by centrifugation at 10K rpm for 5 mins at 4˚C and the supernatant was discarded. The cell pellets were resuspended in appropriate volume (10-20 µl) of 1X PBS. A thin plastic sheet (cut according the dimension of a slide) with a hole in the middle was placed on a clean glass slide. Molten 1% LB-agarose of appropriate volume (~200 µl) was added in the hole. A second slide was placed on top of the first slide immediately to trap the LB-agarose in between two slides which would shape a thin layer of it. The slides were placed in 4˚C immediately after placing the second slide and kept there for at least 1 hr to allow the solidification of the LB-agarose. The slides were taken out at room temperature after 1 hr and the top slide was carefully separated from the bottom slide keeping the plastic sheet and thin LB-agarose layer intact. Following this, the plastic sheet was carefully pulled out in such a manner that would only leave the thin LB-agarose layer behind on

the glass slide. Wet mounting was done by trapping a small volume (~5ul) of the resuspended cells between this thin layer of 1% LB-agarose on glass slide and a coverslip. Samples were visualized using an epi-fluorescence microscope with phase contrast lens and pictures were taken using a digital camera.

3.5.2 Visualization of cells stained with FM 5-95 dye

Materials:

1. Sterile 1X Phosphate-Buffered Saline or PBS (pH=7.4)
2. 1% sterile LB-agarose solution
3. Clean glass slides (75mm x 25mm, Fisher Scientific, Cat # 12-544-4)
4. Clean coverslips (25mm x 25mm, Fisher Scientific, Cat # 12-540C)
5. FM 5-95 dye (FM ® Lipophilic Styryl Dyes, Molecular Probes, Invitrogen, Cat# T23360)
6. Dimethyl Sulfoxide or DMSO (Fisher Scientific, Cat # BDH1115-1LP)

Instruments:

1. Epi-fluorescence microscope (XYL-146Y, Amscope)
2. Phase contrast lens (PCT200-INF, Amscope)
3. Digital camera (MU1000-CK, Amscope).
4. Centrifuge (Eppendorf, Product # 5415 C)
5. Autoclave (ADV-PB, 250˚F heat, 15 psi pressure, liquid cycle = 30 mins)

Method:

Cells were stained with FM 5-95 dyes, using a modified version of the manufacturers' protocol. 100 µl of DMSO was added to 1 mg lyophilized dye and mixed well to achieve 10 mg/ml stock solution concentration which was kept at -20˚C wrapped with aluminium foil. 1 µl from this stock solution was taken to mix with 199 µl of sterile 1X PBS in a tube to achieve 50 µg/ml working stock concentration and the tube was kept at 4˚C, wrapped with aluminium foil. Cells grown in

liquid MOPS Glc media were harvested (1.5 ml) at different time points and were washed (10K rpm, 5 min, 4˚C) to resuspend the pellets in 20 µl sterile 1X PBS and kept chilled at 4˚C. 5 µl of this cell suspension was mixed with 10 µl of chilled dye of 50 µg/ml concentration and was incubated for 10 mins at 4˚C in dark. Wet mounting and visualization were done as quickly as possible after incubation. Wet mounting was done by trapping 8 µl mixtures of cell suspension and dye sandwiched between a thin layer of 1% LB-agarose on glass slide and coverslip. Samples were visualized using an epi-fluorescence microscope with phase contrast lens and pictures were taken using a camera.

3.6 Flow cytometry

3.6.1 Fluorescent proteins expressing bacterial sample analysis

Materials:

1. LB (Luria Bertani) broth powder (Fisher BioReagents, Cat # BP1426-2)
2. Sterile water
3. Sterile 1X Phosphate-Buffered Saline or PBS (pH=7.4)
4. Sterile flow cytometer tubes (BD Falcon, Product #352235)

Instrument:

BD FACSAria II (BD Biosciences) with the help of technical assistant Marie Montelongo in the lab of Prof. Jerry Ritchey in Centre for Veterinary Health Sciences, OSU. This instrument had 4 different lasers associated with it and they are red laser (640 nm), blue laser (488 nm), yellow-green laser (561 nm) and violet laser (405 nm).

Method:

BD FACSAria II flow cytometer was used to quantify the cells (MG1655-hipA7/pDiGi) expressing both GFP and DsRed before and after lethal antibiotic addition (cefoxitin). Same strain without any expression of any of the fluorescent proteins was used as control in flow

cytometer to get rid of the internal auto-fluorescence. All the flow cytometer functions were operated by BD FACSDiva software. All the samples were analyzed right after thawing. Sterile flow cytometer tube was filled with 500 µl to 1000 µl of the half diluted (with sterile LB) sample under laminar hood. Flow cytometer tube was loaded in the tube holder in the flow cytometer work station. The machine was allowed to perform its analysis by sucking up the liquid sample through a narrow capillary tube to pass it through a thin nozzle and analyze individual cells. The software was set to analyse 10,000 events per sample and project the data in terms of FSC (Forward SCatter), SSC (Side SCatter) and the color filters (Yellow-Green filter, Red laser filter) depending on the colour of the fluorescent protein(s) being expressed by the cell sample. The sample chamber was kept chilled (4˚C) by circulating chilled water through a tube while analysing and also sorting each sample. The flow rate was kept medium (35-45 µl/min) while cell sorting which ensured optimal purity of the sorted samples. The sorted cells were collected in sterile flow cytometric tubes and each cell flew out as a tiny droplet being suspended in sterile 1X PBS. Same protocol was followed while analysing the GL69 strain which expresses a chromosomal sf-GFP tagged trytophanase.

3.6.2 SYTOX Blue Viability analysis

Materials:

1. SYTOX Blue Dead Cell Stain (Invitrogen, Molecular probes, Cat # S34857).
2. Sterile 1X Phosphate-Buffered Saline or PBS (pH=7.4)
3. Sterile flow cytometer tubes (BD Falcon, Product #352235)

Instruments:

1. Centrifuge (Eppendorf, Product # 5415 C)
2. BD FACSAria II (BD Biosciences)
3. Autoclave (ADV-PB, 250˚F heat, 15 psi pressure, liquid cycle = 30 mins)

Method:

For dead cell staining, I used SYTOX Blue Dead Cell Stain and followed the manufacturers' instructions. Cells grown in liquid LB were harvested (1 ml) at different time points and were washed (10K rpm, 5 min, 4˚C) to resuspend the pellets in 1 ml sterile 1X PBS. The cell concentrations were adjusted to be from $1x10^5$ to $5x10^7$/ml by diluting in sterile 1X PBS. 1 ml from this diluted cells were transferred in a flow cytometry tube. The vial containing the SYTOX Blue stain (Stock concentration 1mM provided) was removed from the freezer (being protected from light) to allow the contents to equilibrate to room temperature. 1 µl of SYTOX Blue stain was added to each tube and was mixed well where the final concentration of dye was 1 µM. The flow cytometry tubes were incubated for 5 min in dark at room temperature followed by analysis with 440/40 nm bandpass filter of the BD FACSAria II apparatus.

3.7 Preparation of cell extracts for proteomics

Materials:

1. Sterile water
2. Liquid nitrogen

Instruments:

1. Centrifuge (Eppendorf, Product # 5415 C)
2. Sonicator (Fisher Scientific Model 120 Sonic Dismembrator)
3. Probe (Fisher Scientific, diameter 1/8 inches)
4. Speed vacuum concentrator (Thermo Fisher Scientific)

Method:

We developed a cell lysis protocol for the FACS sorted cells to maximize the protein yield in order to derive the whole proteome data. This lysis procedure combines freeze-thaw and probe-sonication. First, we centrifuged the cells at 10K rpm, for 10 minutes at 4˚C, followed by careful

removal of supernatant and resuspension of the pellet in 300 µl autoclaved sterile water in a sterile microcentrifuge tube. Then the samples were subjected to three consecutive freeze-thaws in liquid nitrogen and water. Following this, probe-sonication was done using a sonicator. The probe (diameter 1/8 inches) was submerged halfway through the liquid samples in the microcentrifuge tubes. The sonication was done at Amplitude=20%, Pulse=1 sec on 5 sec off, Time=1 min while placing the sample tubes in ice. The sonicated samples were frozen at -80˚C immediately. Next day, the frozen samples were placed in the speed vac (in the core facility of Biochemistry and Molecular Biology) and the water was evaporated for 2 hours at 25˚C, leaving behind a very fine hardly visible concentrated pellet at the bottom of each microcentrifuge tube.

3.8 Mass spectrometry

Materials:

1. Buffered Urea Reducing Solution or BURS (pH=7)
2. 100 mM Tris-2carboxyethyl phosphorine or TCEP solution (pH=7)
3. 100 mM Iodoacidamide or IAA
4. 8X Tris buffer (pH=8.5)
5. 0.25 µg/µl trypsin stock solution (Promega SeqGrade)
6. 100% Trifluoro acetic acid or TFA
7. HPLC-grade pure water (B&J Brand)
8. 75 µm x 40 cm fused silica column
9. 3-µm Magic C18 AQ beads

Instruments:

1. Orbitrap Fusion mass spectrometer (Thermo Fisher Scientific) by Janet Rogers at a core facility under the direction of Dr. Steve Hartson at Oklahoma State University.
2. Nanospray Flex ion source (Thermo Fisher Scientific)
3. NanoLC chromatography (Eksigent Technologies)

Method:

For concentrated pellets of cell lysates, pellets were dissolved in neutral solvents to produce a solution compatible to liquid chromatography. Then proteins were digested by trypsin into peptide fragments. Next liquid chromatography-based initial separation of the mixtures of multiple peptide fragments helps the following mass spectrometric analysis. Mass Spectrometer has three major components, an ionizer, a mass analyzer and a detector. Ionizer has an ion source creating ions from the peptide fragments. These ions are then subjected to electric or magnetic field. The mass analyzer analyses them by the amount of their deflection. Deflection of peptide ions in any magnetic or electric field depends on the mass-to-charge ratio of individual peptide ion. Mass analyzer coordinates with the detector to report the data. Detector finally consolidates computer-based results in terms of relative abundances of each ion as a function of mass-to-charge ratio.

Cell extracts (see previous section) were submitted for Mass Spectrometry in the form of concentrated pellets. Fresh BURS was prepared with 8M Urea, 100mM Tris-buffer (pH 8.5). Reducing agent 100 mM TCEP solution was prepared and 25 µl 100 mM TCEP solution was mixed with 500 µl buffered urea. The pellets were dissolved in 50 µl BURS for 30 min using vortexing and mixing thoroughly (dissolving by heat or even hand friction is prohibited). 5 µl of 100 mM alkylating agent IAA per 50 µl reaction was added followed by incubation in the dark at room temperature for 20 min. The reaction was diluted by mixing 57.5 µl of sample with 25 µl 8X Tris buffer and 117.5 µl water. Next 3.2 µl of 0.25 µg/µl trypsin stock (4 µg/ml final concentration) was added to it and the digestion was allowed to take place at 37˚C in a dark place overnight. The next day, 2 µl of 100% TFA was added and the peptide extracts are now ready for analysis. Peptide extracts were analyzed on an Orbitrap Fusion mass spectrometer coupled to a Nanospray Flex ion source and a NanoLC chromatography system. Peptides were analyzed by trapping on a 5 cm pre-column in a vented column configuration, followed by analytical separation on a 75 µm x 40 cm fused silica column packed in house with 3-µm Magic C18 AQ

beads, and terminating with stainless-steel emitter needle. Peptides were eluted using a 0-40% Acetonitrile/0.1% formic acid gradient performed over 60 min at a flow rate of 250 nL/min.

During elution, samples were analyzed using Top Speed methodology. Each scan cycle included one full-range FT-MS scan (nominal resolution of 120,000 FWHM, 300 to 1500 m/z) and as many data-dependent MS/MS scans as possible during the 3-second cycle period. Ions were isolated for MS/MS using the quadrupole (1.6 m/z width), fragmented by CID, and fragment masses determined using the ion trap sector. MS/MS settings used a trigger threshold of 10,000 counts, monoisotopic precursor selection (MIPS), and rejection of parent ions that had unassigned charge states, were previously identified as contaminants on blank gradient runs, or that had been previously selected for MS/MS.

The mass spectra from the samples were searched against a 4,141 proteins of *E. coli* MG1655 database (NCBI; ftp.ncbi.nlm.nih.gov) including 115 protein genes of laboratory contaminants (www.thegpm.rog/cRAP/index.html). MaxQuant software (version 1.4.1.2, Max Planck institute) was used to quantify the amount of proteins in terms of peptide intensities. The derived data files were analysed using a statistical software Perseus (version 5.8.3.1). The generic matrix containing the data of protein intensities were first uploaded in a blank Perseus file. The intensity values of the proteins of all samples were first transformed to $\log_2$ ('Basic' button in toolbar has the 'Transform' option in it) for better handling of data. Protein intensity columns were sorted to arrange them in ascending order. For searching any particular protein or proteins of interest the 'Filter row' button in the toolbar is useful. It opens up a small window were the desired words for searching the protein name can be entered. Choosing 'Keep matching column' option in the same window would generate a new filtered matrix according to the search. Scatter plot can be generated using the scatter plot icon in the tool bar. The intensities of proteins of 'yellow' sample in x-axis and 'red' sample in y-axis were plotted to generate the scatter plot. The parameters for each of the axes of the scatter plot can be chosen by pulling down the arrows in

the corresponding small dialogue box that appears. The scatter plot can be directly saved in .pdf format using the ‘save image’ icon.

CHAPTER IV

RESULTS AND DISCUSSIONS

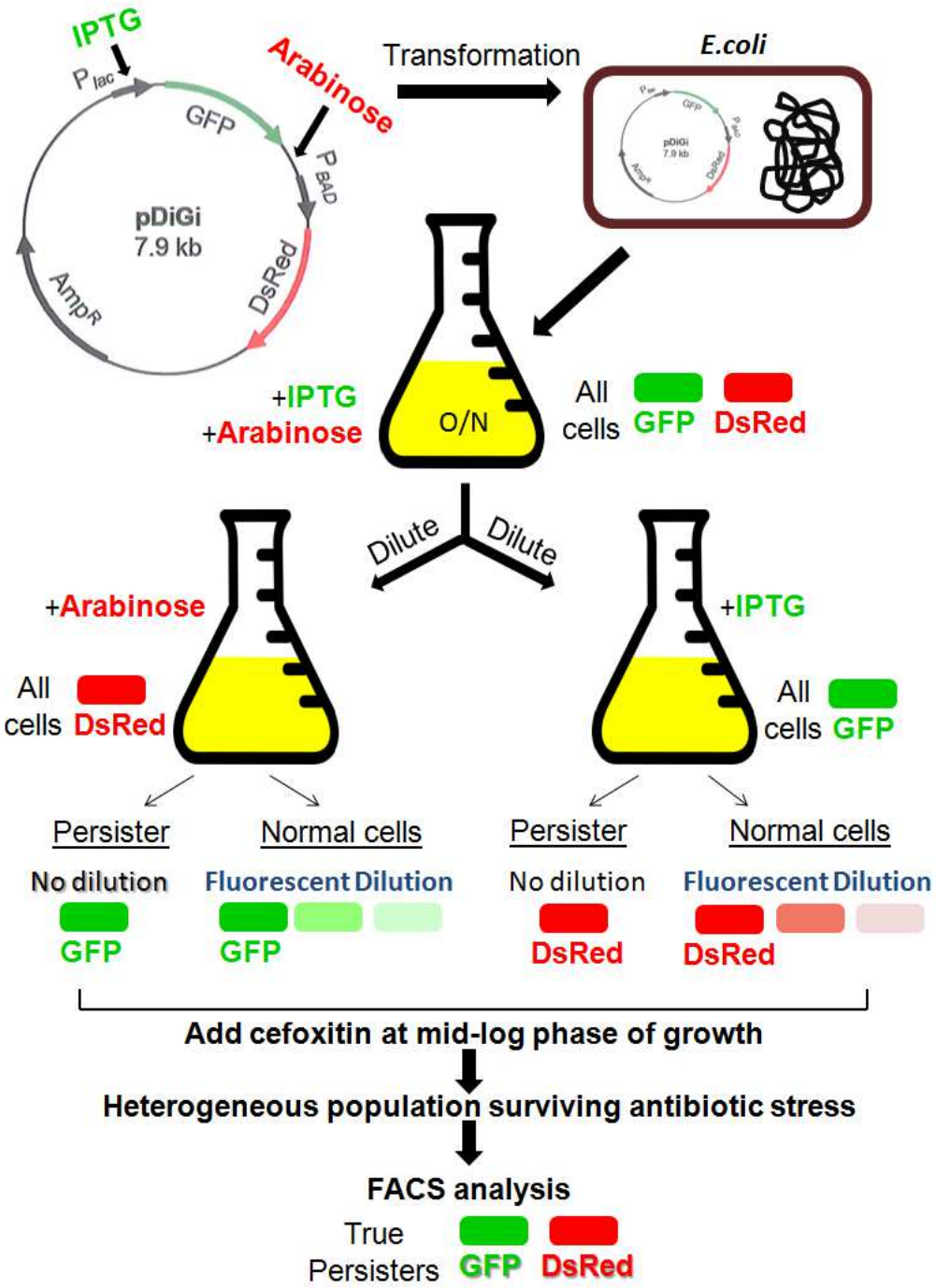
4.1 Cell growth and harvest of true persisters from a heterogeneous population

Isolation of native persisters has always been difficult because of their low frequency and lack of specific biomarker. We developed an innovative strategy to isolate authentic persisters. This method was developed based on the hypothesis that persisters, being dormant, refrain from active cell division unlike the normal cells which actively grow and multiply.

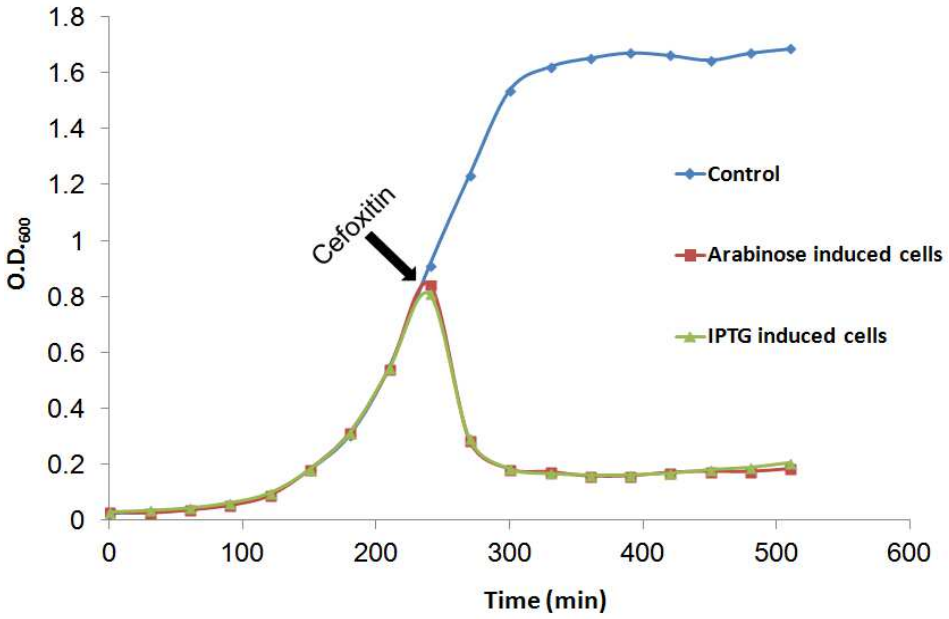
Figure 4.1A sketches out our overall experimental strategy to isolate *E. coli* persisters. Persisters are rare in any bacterial population (10^{-5} to 10^{-6} in exponentially growing *in vitro* culture) [6]. HipA is the first toxin protein discovered to be associated with persistence. HipA7 is a mutant version of HipA protein having two mutations (G22S and D291A) which reduces its affinity towards cognate antitoxin HipB. Also HipA7 protein is nontoxic to cell growth [48]. As a result, HipA7 expression greatly increases (100-1000 fold) the frequency of persister formation in *E. coli* cell populations [45, 46, 48]. First we needed to construct a new *E. coli* strain that co-expresses HipA7 toxin and fluorescent proteins. We obtained *E. coli* MG1655-hipA7 strain which has *hipA7* gene inserted in its chromosomal DNA. This strain expresses HipA7 toxin protein, instead of HipA. This was a technical tactic to improve the overall persister frequency. We also obtained pDiGi plasmid (Fig. 4.1 A) [36] as a dual fluorescent marker. This plasmid has a GFP (Green Fluorescent Protein) coding gene cloned under a pLac promoter which can be induced by

IPTG and a DsRed (Discosoma Red) fluorescent protein coding gene cloned under a pBAD promoter which can be induced by arabinose.

(A)



(B)



<u>Figure 4.1</u>: Cell growth and harvest of true persisters from a heterogeneous population: (A) pDiGi is a 7.9 Kb plasmid having GFP and DsRed encoding genes cloned under pLac and pBAD respectively which are induced by IPTG (0.5mM) and Ara (0.2%) respectively [36]. pDiGi was transformed in *E. coli* MG1655-hipA7 strain and was selected with LB+Amp media. Overnight culture of this new strain (MG1655-hipA7/pDiGi) was grown in LB+Amp (50µg/ml)+IPTG (0.5mM)+Ara (0.2%) which allowed the cells to express both GFP and DsRed. This overnight culture was diluted either in fresh LB+IPTG (0.5mM) or in fresh LB+Ara (0.2%) and was allowed to grow until mid-log phase (OD_{600}~0.8). In case of the former, the normal cells progressively diluted their red fluorescence while keeping their green fluorescence intense as a result of the presence of only IPTG in the media. On the other hand, in the latter, the normal cells progressively diluted their green fluorescence while keeping their red fluorescence intense as a result of the presence of only Ara in the media. Interestingly, in both the cases, persisters due to their non-diving dormant nature kept both the fluorescence intense. Both sets of cultures were treated with a lethal dose (16 µg/ml) of the bactericidal drug cefoxitin at the mid log phase which selects for a heterogeneous population surviving antibiotic stress. FACS analysis of such population was expected to easily identify and sort true persisters as they would distinctively appear intense in terms of both red and green fluorescence. **(B) Cell growth curves.** Three cultures of MG1655-hipA7/pDiGi were grown at 37˚C, 250 rpm in LB. A control culture (blue line) was grown without addition of cefoxitin at any time point. This culture came from an uninduced overnight culture which was freshly diluted in LB lacking any of the inducers for fluorescent proteins. The other two cultures came from overnight cultures having both IPTG (0.5mM) and Ara (0.2%) which induces GFP and DsRed respectively. The induced overnight culture was diluted either in LB+Ara (0.2%) and was grown until mid-log phase (~210 min), when cefoxitin (16µg/ml) was added (maroon line), or in LB+IPTG (0.5mM) and was grown until mid-log phase (~210 min), when cefoxitin (16µg/ml) was added (green line). OD_{600} readings measured in every 30 min were plotted against time to generate these curves. IPTG=Isopropyl β-D-1-thiogalactopyranoside, Ara=Arabinose, GFP=Green Fluorescence Protein, DsRed=Discosoma Red, Amp=Ampicillin.

Isolation of native persisters has always been difficult because of their low frequency and lack of specific biomarker. We developed an innovative strategy to isolate authentic persisters. This method was developed based on the hypothesis that persisters being dormant refrain from active cell division unlike the normal cells which actively grow and multiply.

Figure 4.1A sketches out our overall experimental strategy to isolate *E. coli* persisters. Persisters are rare in any bacterial population (10^{-5} to 10^{-6} in exponentially growing *in vitro* culture) [6]. HipA is the first toxin protein discovered to be associated with persistence. HipA7 is a mutant version of HipA protein having two mutations (G22S and D291A) which reduces its affinity towards cognate antitoxin HipB. Also HipA7 protein is nontoxic to cell growth [48]. As a result, HipA7 expression greatly increases (100-1000 fold) the frequency of persister formation in *E. coli* cell populations [45, 46, 48]. First we needed to construct a new *E. coli* strain that co-expresses HipA7 toxin and fluorescent proteins. We obtained *E. coli* MG1655-hipA7 strain which has *hipA7* gene inserted in its chromosomal DNA. This strain expresses HipA7 toxin protein, instead of HipA. This was a technical tactic to improve the overall persister frequency. We also obtained pDiGi plasmid (Fig. 4.1 A) [36] as a dual fluorescent marker. This plasmid has GFP (Green Fluorescent Protein) coding gene cloned under pLac promoter which can be induced by IPTG and DsRed (Discosoma Red) fluorescent protein coding gene cloned under pBAD promoter which can be induced by arabinose. The feasibility of simultaneous expression and detection of GFP and DsRed in *E. coli* was shown by Stefan Jakobs and his researcher group [74]. Plasmid pDiGi has ampicillin resistant gene (Amp^R) as selection marker which helps to maintain it in the host bacterium. Thus, we constructed a new strain by transforming pDiGi into MG1655-hipA7 strain and selected for the new strain (MG1655-hipA7/pDiGi) in LB + ampicillin media.

We adopted a method for isolating pure *E. coli* persisters which is based on a combination of two layers of purification where the first level was typical lysis of the sensitive population by bactericidal drug cefoxitin. Our chosen dual fluorescent marker plasmid pDiGi had amplicillin resistant gene as a selective marker which codes for beta lactamase enzyme. Beta

lactamase confers resistance to any beta lactam group antibiotic including ampicillin by cleaving the beta lactam ring. Being transformed with pDiGi, our new *E. coli* strain MG1655-hipA7/pDiGi became resistant to ampicillin. However cefoxitin is resistant to beta lactamase mediated hydrolysis because of the presence of a methoxy group at the 7-alpha position of its beta lactam ring [78, 79, 80]. This is why we chose cefoxitin to treat the bacterial culture at its supra-lethal dose (16μg/ml) to exert its killing effect [78]. Cefoxitin is a broad spectrum bactericidal drug frequently prescribed to treat a variety of bacterial infections including many recurrent infections such as urinary tract infection. It is a second generation cephalosporin drug which interferes with bacterial cell wall biosynthesis by binding and inhibiting transpeptidase or Penicillin Binding Protein ultimately leading to cell lysis [78, 79, 80]. Followed by cefoxitin mediated lysis, our second layer of purification was fluorescence intensity-based flow cytometric sorting of true persisters from the heterogeneous population surviving cefoxitin stress. This combinatory strategy greatly enhances the overall level of purity while isolating the persisters.

As shown in Fig. 4.1 A, the MG1655-hipA7/pDiGi strain was grown overnight in LB in the presence of both the inducers so as to allow the cells to express both the fluorescent proteins simultaneously. These cells were diluted (1:500) using fresh LB media in two different sets each having only one of the inducers added in the media. Both sets were allowed to grow at 37˚C in shaking condition until mid log phase (OD_{600}~0.8). This approach allows the cells to start off expressing both the fluorescent proteins. In case of the set LB+arabinose (Fig.4.1 A), the normal cells dilute green fluorescence as a mark of their active cell division in the absence of GFP inducer IPTG. However, these normal cells keep the red fluorescence intense as the media contains arabinose. On the other hand, the second set being LB+IPTG (Fig. 4.1 A) allows the normal cells to dilute the red fluorescence while they actively grow and divide in the absence of DsRed inducer. Here these normal cells keep the green fluorescence intact as the media contains GFP inducer IPTG. It was vital to allow both the sets to grow till OD_{600}~0.8 so as to permit

sufficient rounds of cell divisions in the exponential phase. Here we allowed the provision of four complete rounds of cell divisions in the exponential phase which ensures that the normal cells would dilute the fluorescence adequately (an event called 'Fluorescent Dilution'). These phenomena of 'Fluorescent Dilution' could be potentially visualized by microscopy to establish the base of our hypothesis (See next section). Interestingly, in both the cases the persisters would retain both red and green fluorescence intensity intact throughout as a result of their dormant nature. However, persisters are rare, so treating both the sets with bactericidal drug cefoxitin at the mid log phase aids the lysis of the vast majority of the sensitive cells while enriching for persisters. Thus presumably true persisters could readily be recognized and sorted from the heterogenous population surviving antibiotic stress with the help of multicolour flow cytometric analysis. We refer our hypothesized true persisters as 'yellow' cells for convenience.

Growth patterns of both sets of cultures (Set l=LB+IPTG, Set 2=LB+arabinose) were closely monitored throughout by measuring OD_{600} every 30 min and were plotted against time to generate the respective curves (Fig. 4.1 B, green line and maroon line for Set 1 and Set 2 respectively). The blue line (Fig. 4.1 B) represents the growth curve of a control culture which came from an uninduced (absence of both inducers) overnight culture of MG1655-hipA7/pDiGi and was diluted (1:500) in fresh LB without any inducers. This culture was allowed to grow in parallel at 37˚C with shaking, but without interrupting its growth by antibiotic addition. OD_{600} readings were taken until late stationary phase for this control culture and were plotted against time which generated a typical sigmoid growth pattern. We can clearly observe that the OD_{600} readings of two experimental cultures (Fig. 4.1 B, green and maroon lines) were superimposed on those of the control culture (Fig. 4.1 B, blue line) until cefoxitin was added to the former two sets to lyse the sensitive cells. Thus this control culture was crucial to understand that adding either IPTG or arabinose in LB did not exert any effect on the growth of this strain. For both green and maroon curves, we observed a sharp drop in absorbance at 600 nm after cefoxitin addition. This

indicates lysis of cefoxitin-sensitive population (Fig. 4.1 B). However the OD_{600} readings got stalled at around 0.2 and did not decrease afterwards forming a plateau (Fig. 4.1 B, green and maroon line). These plateau patterns signify existence of some residual cells surviving cefoxitin mediated killing.

To strengthen our claim we performed a quick CFU (Colony Forming Unit) assay which showed that CFU/ml was $167x10^6$ at the mid-log phase (OD_{600}~0.8) before addition of cefoxitin and went down to 10^6 CFU/ml after two hours (OD_{600}~0.2) of cefoxitin addition. So these stalled values of OD_{600} forming such plateau shapes (Fig. 4.1 B, green and maroon lines) were clearly indicative of some residual viable cells which managed to sustain the cefoxitin stress. Such populations are presumably enriched in true persisters ('yellow' cells). This accomplishes the first level of purification of persisters. We harvested cells before cefoxitin addition (mid log phase) and 3 hrs after cefoxitin addition from both the sets (LB+IPTG and LB+arabinose) for flow cytometric and microscopic analysis (see next sections of results).

4.2 Microscopic visualization of various samples and addressing 'Fluorescent Dilution'

Our primary aim was to address the 'Fluorescent Dilution' event. We wanted to carefully observe a diverse set of cell samples to understand their fluorescent protein expression pattern and morphology. Fig. 4.2 represents phase contrast and fluorescence microscopic images of MG1655-hipA7/pDiGi strain taken under various growth phases and treatments.

We can clearly visualize that there is no fluorescent protein expression in the uninduced overnight (Fig. 4.2, 1st panel) and uninduced actively growing mid-log phase (Fig. 4.2, 3rd panel) cells. Almost all of the cells in the induced (with IPTG & arabinose) overnight culture as expected expressed both red and green fluorescent proteins (Fig.4.2, 2nd panel). But a comparative view of the DIC (Differential Interference Microscopy) bright field of the overnight induced (Fig. 4.2, 2nd panel) and uninduced (Fig. 4.2, 1st panel) cells shows that induced overnight cells are relatively longer. As Hell and group demonstrated, GFP expressing cells are always a little longer than DsRed expressing cells [74]. So here the overnight cells in the induced culture are probably longer because of the expression of the fluorescent proteins, notably GFP.

When GFP and DsRed induced overnight cells were freshly diluted in LB + IPTG and were allowed to grow until mid-log phase, the culture showed diluted red fluorescence as expected. Much fewer red fluorescent cells were found compared to a much higher number of cells having green fluorescence (Fig. 4.2, 4th panel). Cefoxitin treatment of these IPTG induced actively growing mid-log phase cells selects for a heterogeneous population (Fig. 4.2, 5th panel) surviving cefoxitin stress. A close comparative view of the red and green filter images of this population showed that the majority of cells expressing both red and green fluorescent proteins with a few cells expressing only GFP, but not DsRed for example, one long green cell at the top right hand corner and another at the bottom (Fig. 4.2, 5th panel, green filter). These are probably some normal sensitive cells which managed to escape the antibiotic mediated killing and thereby

contributing towards the heterogeneity of the antibiotic-tolerant population. In addition, careful observation of the DIC bright field of cefoxitin-surviving population (Fig. 4.2, 5th panel) reveals the fact that there is cell debris present in the field. This makes it obvious that cefoxitin was active by lysing the vast majority of the sensitive normal cells of the population. Also there is an overall variance in the cell-length (Fig. 4.2, 5th panel).

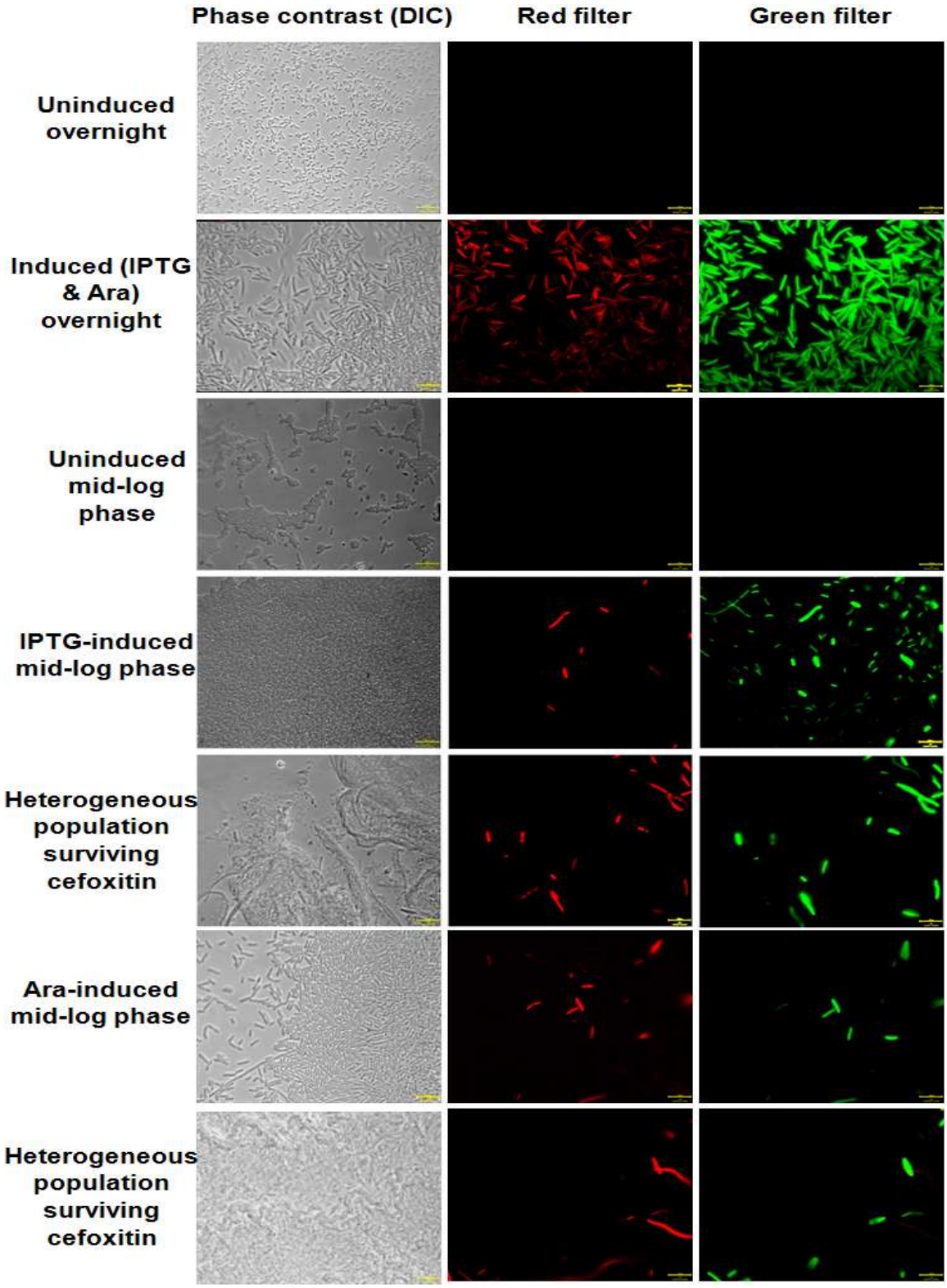


Figure 4.2: Visualization of cells (MG1655-hipA7/pDiGi) under microscope. Left panels: cells imaged by phase-contrast lens, Middle panels: same field under red filter, Right panels: same field under green filter. The yellow bar represents 10µm. IPTG= Isopropyl β-D-1-thiogalactopyranoside, Ara=Arabinose.

On the other hand, GFP and DsRed expressing overnight culture was also diluted in LB+arabinose and was allowed to grow till mid-log phase when they were analyzed microscopically to address the 'Fluorescent Dilution' (Fig. 4.2, 6th panel). As expected, fewer green fluorescent cells were found compared to a larger number of cells having red fluorescence in this sample. Cefoxitin treatment of these arabinose induced actively growing mid-log phase cells selects for a heterogeneous population (Fig. 4.2, 7th panel). As we can see in the phase contrast DIC image (Fig. 4.2, 7th panel), the field is full of debris which demonstrates cefoxitin activity. Like before, this heterogeneous population also showed majority of the cells expressing both red and green fluorescent proteins with a few exceptional cells expressing only DsRed, but not GFP for example, two long red cells at the middle of the red filter field (Fig. 4.2, 7th panel). These are probably some normal sensitive cells which managed to escape the antibiotic treatment and contribute towards the heterogeneity of the population surviving cefoxitin treatment. However, our focus was on the true persisters ('yellow' cells) which retained undiluted intense red and green fluorescence. Overall the microscopic images convincingly represented both red and green fluorescent dilution events. Also, the images visually addressed the enrichment of the 'yellow' cells when the actively growing mid-log phase cultures were subjected to lethal antibiotic stress.

4.3 Flow cytometry revealed significant enrichment of persisters upon lethal drug exposure:

Flow cytometry is a powerful technique to analyse the distribution of cells in a population addressing the heterogeneity and also can sort individual cells based on fluorescent stain or internally expressed fluorescent proteins. Multicolor flow cytometry primarily distinguishes cells on the basis of size and internal complexity using two parameters FSC and SSC. FSC (Forward SCatter) is a measure of cell size and SSC (Side SCatter) is a measure of internal complexity. Optimization of this complex machine at the beginning is critical to make sure that the stream coming out of the nozzle has only one cell coming along the steam-line at a time. This is called hydrodynamic focusing. This way each cell is analysed thoroughly in terms of size, internal complexity and fluorescent colour which is followed by data acquisition.

We took the advantage of this technique to further test our hypothesis. We wanted to figure out the proportion of the 'yellow' cells before and after antibiotic addition to address the question of their enrichment. We were expecting considerable enrichment of 'yellow' cells in the antibiotic tolerant population representing them as the dominant survivor in antibiotic stress. It would provide strong evidence in accord to our hypothesis of the 'yellow' cells being the true persisters. We were also expecting overall heterogeneity in the population surviving antibiotic stress and were aiming to selectively sort out the 'yellow' cells from this heterogeneous population to analyse them further. Each dot in every gate (Fig. 4.3 and 4.4) represents a cell. Fig. 4.3 A represents arabinose induced actively growing mid log phase sample and Fig. 4.3 B represents the population that survived antibiotic stress when the arabinose induced mid log phase culture was treated with cefoxitin. The first gates (data plot of FSC vs. SSC) of Fig. 4.3A and 4.3B represent the distribution of 10,000 cells in terms of their size and complexity. From the FSC vs. SSC plots of 3A and 3B, it is clear that the population is quite scattered throughout in both the cases, but mostly concentrated along a fairly large triangular smear towards the left corner for 3A and along a fairly wide vertical smear towards the left for 3B. So overall, majority

of the cells in both the population were small in terms forward scatter or size. However a widely varied side scatter or internal complexity was observed for the antibiotic surviving population whereas the mid log phase culture had cells with relatively less varied internal complexity. This reflects the more heterogeneous nature of the cells of antibiotic surviving population. Next gate is to choose the singlets from the population in terms of comparable SSC-Height (SSC-H) and SSC-Area (SSC-A) and leave behind the doublets which can mislead in terms of fluorescent intensities of the cells (Fig. 4.3 A and B). The final gate (Intensity of GFP vs. Intensity of DsRed) is most informative that represent the singlet distribution according to cells those fluoresce exclusively as red ('only red'), exclusively as green ('only green') or both red and green ('Yellow') for both actively growing mid-log phase cells (Fig. 4.3 A) and the antibiotic surviving population (Fig. 4.3 B). The bars were set (at 10^3) using control uninduced mid-log phase cells (grown in parallel under same experimental conditions) to nullify the usual auto-fluorescence of bacteria (Fig. 4.3 A and B). Any cell located towards the right side or up across the bar would be considered either green or red fluorescent cell respectively.

We aimed to address the question of enrichment of our hypothesized persisters after antibiotic mediated cell lysis. Thus we were interested in the quantitative comparison of the 'yellow' cells before and after antibiotic addition. There were 13% 'yellow' cells in the actively growing mid-log phase culture whereas the percentage goes way above to 70% in the population surviving cefoxitin (Fig. 4.3 A and B). Firstly, 'yellow' cells constitute a major portion of the antibiotic surviving population and we can clearly observe a significant enrichment (~5.4 fold) of 'yellow' cells after cefoxitin addition. This enrichment advocates the fact that the 'yellow' cells emerged as the dominant surviving population upon antibiotic stress. This goes in accord with our hypothesis that 'yellow' cells represent true persisters.

We sorted significant numbers of 'yellow' (1267925) and 'only red' (905214) cells using BD FACSAria II and collected the cells in autoclaved nanopure water to perform Mass

Spectrometric analysis to obtain the whole proteome data and compare them with the aim of identifying unique proteins associated solely with true persister physiology so as to distinguish them from other cells in the heterogeneous antibiotic surviving population. Cell sorting is a very sophisticated, tedious and expensive technique. So we had to choose very carefully to sort the significant number of cells from the absolutely necessary sets of samples to perform relevant comparative whole proteome study.

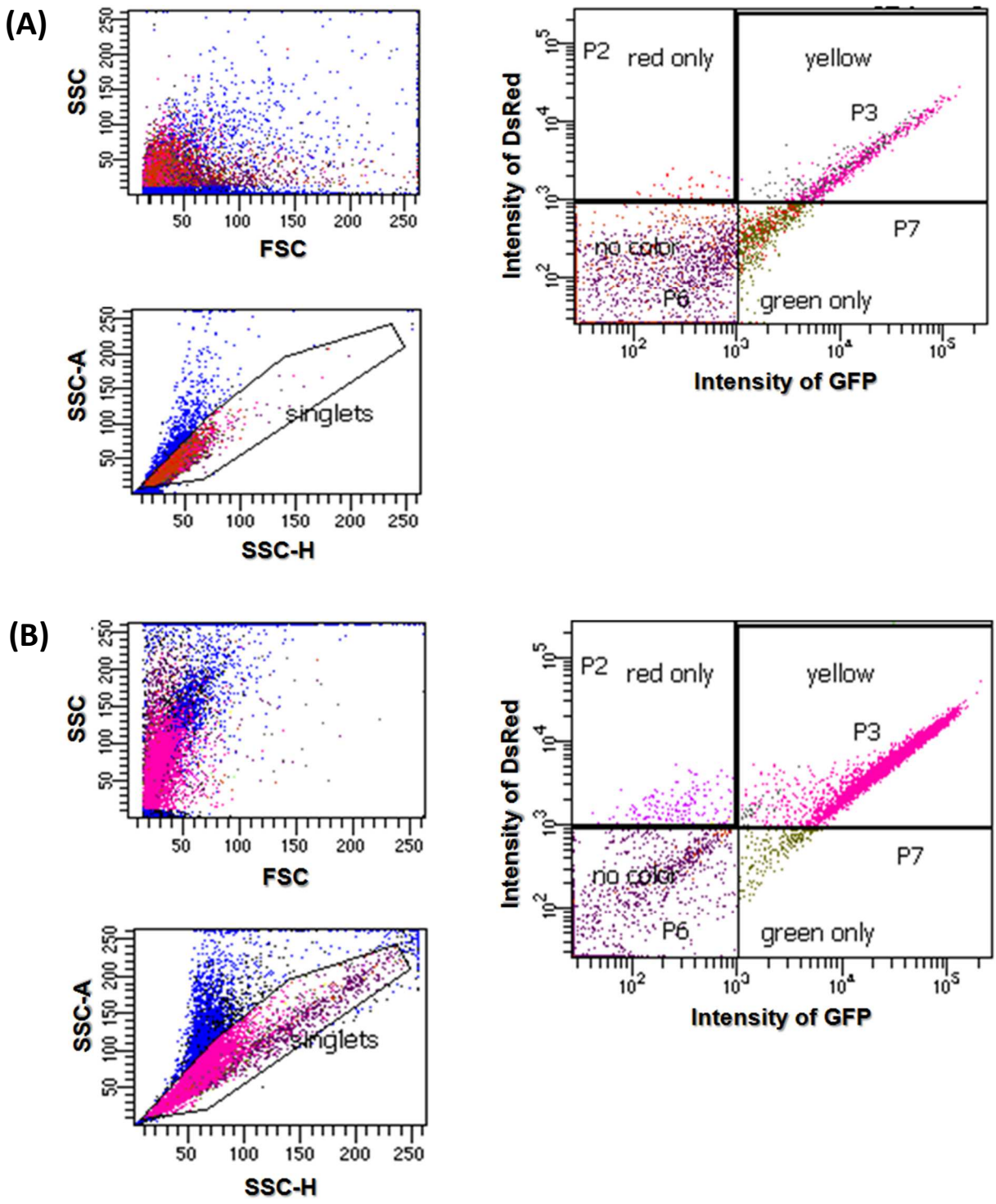


Figure 4.3: Distribution of arabinose induced cells in terms of GFP & DsRed expression before and after cefoxitin addition. (A) Distribution of actively growing mid-log phase culture. We observed that 13% of the singlets expressed both GFP & DsRed ('Yellow'). **(B) Distribution of heterogeneous population surviving antibiotic.** We observed that 70% of the singlets expressed both GFP & DsRed ('yellow'). This suggests significant (5.4 fold) enrichment of 'yellow' cells upon cefoxitin treatment.

To strengthen our hypothesis further, it was important to make sure that the enrichment of 'yellow' cells takes place independent of the inducer being added in the media. So we wanted to figure out whether the 'yellow' cells are getting enriched upon antibiotic exposure of IPTG induced mid-log phase cells. Thus flow cytometry-based quantitative distribution was also evaluated for the IPTG induced mid log phase sample (Fig. 4.4 A) and the antibiotic surviving population selected from it upon cefoxitin addition (Fig. 4.4 B).

As mentioned before, the first gates of each of Fig. 4.4A and B also represent the distribution of 10,000 cells in terms of their size and complexity. From the first FSC vs. SSC plots of Fig. 4.4A and B, it is clear that both the population are quite scattered throughout, but mostly concentrated along a vertical line towards the left. Majority of the cells in both of the population were small in size but had a varying degree of internal complexity. But the IPTG induced mid log phase culture was less scattered (Fig. 4.4A) compared to the antibiotic surviving population (Fig. 4.4B) which reflects the more heterogenic nature of the latter. Likewise, the next gates (Fig. 4.4A and B) were to screen for the singlets from these population in terms of comparable SSC-Height (SSC-H) and SSC-Area (SSC-A) and leave out the doublets which can mislead in terms of fluorescent intensities of the cells. The final gate (Intensity of GFP vs. Intensity of DsRed) is most informative that represent the singlet distribution according to cells those fluoresce exclusively as red ('only red'), exclusively as green ('only green') or both red and green ('Yellow') for both the IPTG induced actively growing mid-log phase cells (Fig. 4.4 A) and antibiotic surviving population (Fig. 4.4 B). The bars were set (between 10^2 and 10^3) using control uninduced mid-log phase cells (grown in parallel under same experimental conditions) to nullify the usual auto-fluorescence of bacteria (Fig. 4.4 A and B). We were interested in the quantitative comparison of the 'yellow' cells before and after antibiotic addition. There were 3% 'yellow' cells in the actively growing mid-log phase culture whereas the percentage was raised to 21% in the cefoxitin surviving population (Fig. 4.4 A and B). So we can clearly observe

significant enrichment (7 fold) of ‘yellow’ cells after cefoxitin addition. This enrichment supports our hypothesis that ‘yellow’ cells represent true persisters.

So overall, significant enrichments of ‘yellow’ cells were observed in antibiotic surviving heterogeneous population derived from both IPTG and arabinose induced mid log phase cells. Thus, these two sets of FACS data (Fig. 4.3 and 4.4) imply that irrespective of the inducer present in the media and the colour of fluorescence we are allowing to get diluted as an indicator of cell division, we would end up selecting for a heterogeneous antibiotic population upon lethal drug treatment. And such heterogeneous population is greatly enriched in our hypothesized true persisters. These experiments had lead us one more step closer to our hypothesis and intrigued us to perform the next experiment to look for the comparative whole proteome data of the sorted antibiotic surviving ‘yellow’ and ‘only red’ cells derived from the arabinose induced mid log phase cells

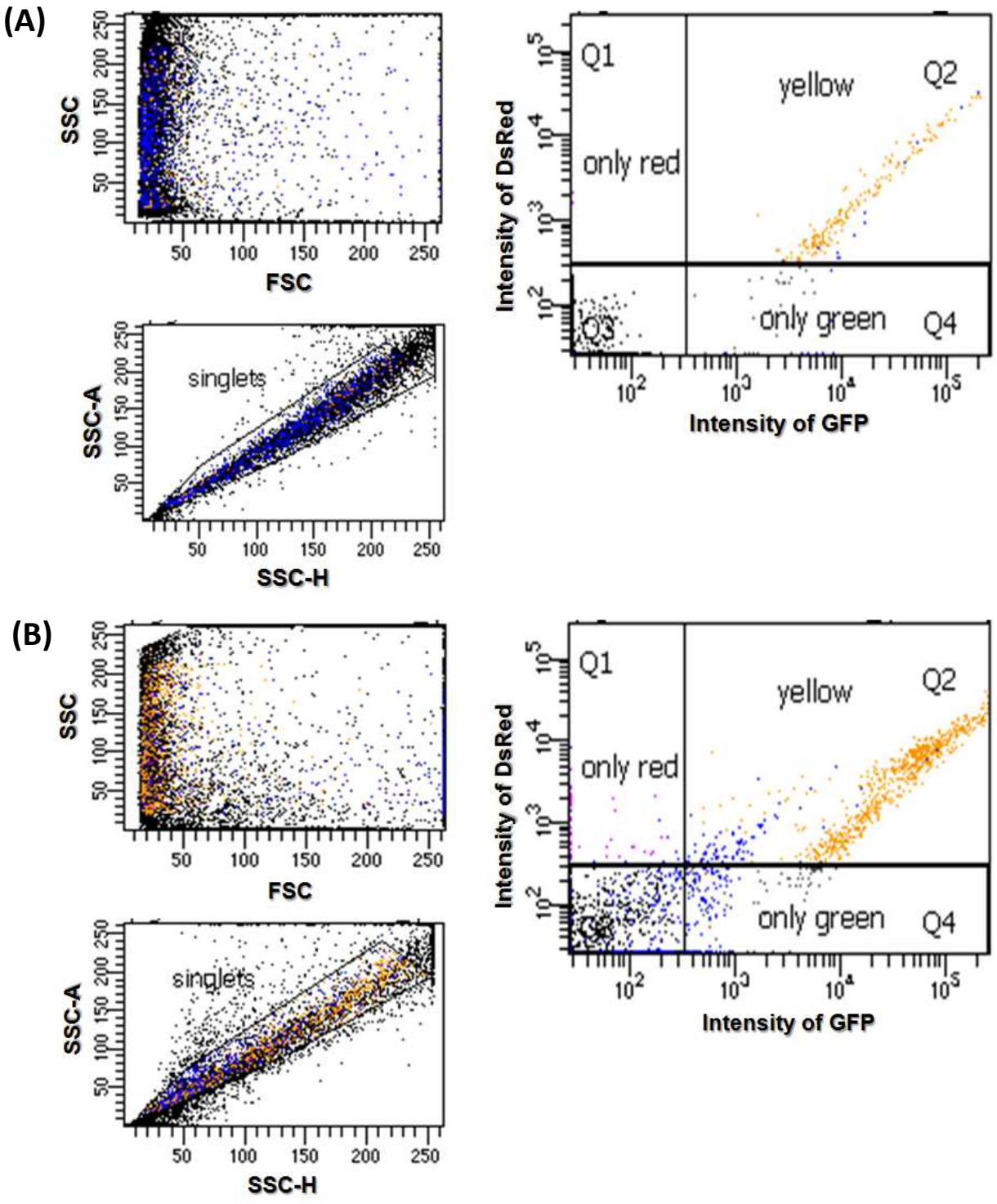


Figure 4.4: Distribution of IPTG induced cells in terms of GFP & DsRed expression before and after cefoxitin addition. (A) Distribution of actively growing mid-log phase culture. We observed that 3% of the singlets expressed both GFP & DsRed ('yellow'). **(B) Distribution of heterogeneous population surviving antibiotic.** We observed that 21% of the singlets expressed both GFP & DsRed ('yellow'). This suggests significant (5.4 fold) enrichment of 'yellow' cells upon cefoxitin treatment.

4.4 Viability test with true persisters showed that vast majority of the cells are alive:

Before analyzing the whole proteome of the 'yellow' cells, we aimed to check their viability. This was to make sure that we were analyzing the population having majority of the viable cells. We used SYTOX Blue dead cell stain for viability test. It is a flow cytometry equipped fluorescent stain and it's entry inside the cell depends on the integrity of the cell membrane. It can easily penetrate the cells having compromised cell membranes and can bind to the nucleic acid giving bright blue fluorescence when excited with 405 nm violet laser light. So the cells that fluoresces blue upon excitation represent dead cells where as cells that are non-blue are the live cells as the dye cannot enter these live cells having intact cell membrane.

Fig. 4.5 A depicts the positive control done with the dye to confirm that it works reliably. The plot SSC vs. Intensity of SYTOX blue of the left panel of Fig. 4.5 A represents the distribution of uninduced unstained mid log phase cells and the bar was set before 10^3 to nullify the blue auto-fluorescence of the strain. Any cell positioned at the right hand side of the bar would be considered fluorescing blue after staining. Dye was added to the same set of cells in the same tube after heating the cells at 75˚C for 45 min in a dry bath which ensures that the cells are dead. Thus we expected majority of the cells to fluoresce blue after being stained with SYTOX blue. Flow cytometric analysis was done after incubating the heated cells in dark with SYTOX blue for 5 min and the data was represented in the second plot of SSC vs. Intensity of SYTOX blue (Fig. 4.5 A, right panel)). We can clearly observe a shift of the cell cloud in terms of blue fluorescence where 99% of the cells are now fluorescing blue or in other words, are indicated as dead. So as per this positive control, the dye worked perfectly. Next, we tested the viability of the 'yellow' cells and the 'only red' cells after staining with SYTOX blue dye (Fig 4.5 B). We can clearly observe two separate populations under blue filter in case of both types of cells. For 'yellow' cells, only 0.7% of the population fluoresced blue and are clearly separated forming a different cloud at the right side of the bar (Fig. 4.5 B, left panel). So it was obvious that almost all

the cells in our hypothesized persisters were viable. This made the ground of our hypothesis even stronger. For the 'only red' cells, two separate clouds were observed under blue filter and only 5% of the population fluoresced blue (Fig. 4.5 B, right panel) indicating their death. Here also majority of the cells were viable.

Hence in short, both types of cells surviving antibiotic stress were viable and are worth comparison in terms of whole proteome data to investigate if there are any unique proteins exclusive to the true persister physiology. These proteins might serve as valuable markers for distinctively identify the true persisters.

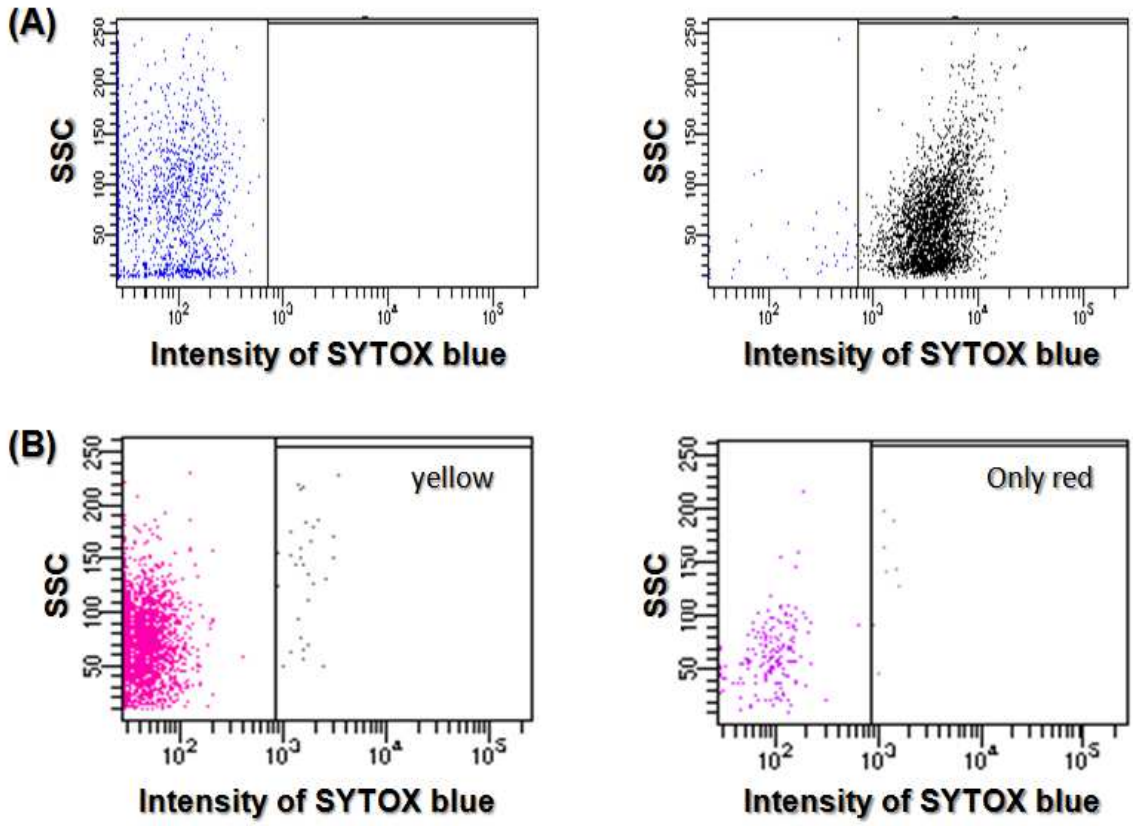


Figure 4.5: Viability test with SYTOX Blue dead cell stain. (A) Positive control with actively growing mid-log phase cells. Data depicts a clear shift of the cell cloud after heating the cells at 75˚C for 45 min which ensures the death of the cells. SYTOX blue staining showed confirmed 99% of the cells as dead. **(B) Viability test with arabinose induced population surviving antibiotic.** Data depicts 0.7% of the 'yellow' cells and 5% of the 'only red' cells as dead upon SYTOX blue staining. This confirms majority of the cells surviving lethal antibiotic are viable.

4.5 Whole proteome data revealed tryptophanase as a unique marker being highly over-expressed in true persisters:

It was important to uncover the whole proteome profile of true persisters to gain an authentic insight regarding key physiological regulators. It is known that approximately $155x10^{-15}$ grams of protein can be extracted upon lysis of one *E. coli* cell [58]. So we aimed to sort at least a million cells to get necessary and sufficient amount of protein to be detected through Mass Spectrometry. We sorted 1267925 'yellow' cells and 905214 'only red' cells from the heterogeneous population surviving antibiotic. We lysed them carefully using a MS compatible protocol that combines freeze-thaw and probe-sonication to obtain maximum possible yield. The precipitated protein pellets were analyzed using Mass Spectrometry for very sensitive detection as we had very limiting number of cells sorted and thereby limiting amount of extracted protein.

We were able to detect 403 proteins from 'only red' cells and 741 proteins from 'yellow' cells. First, the numerical values of intensities or abundances of the proteins in each sample were transformed to $\log_2$ scale in order to tackle the numbers more conveniently. Then we aimed to compare the abundances of proteins detected in these two samples to figure out if there were any protein(s) in the 'yellow' sample which shows significant difference in abundance compared to 'only red' cells. It is important to mention here that the 'only red' and 'yellow' samples are internally controlled as they were part of a single population grown in the same culture flask under same conditions and survived cefoxitin stress. They were sorted and lyzed using the same protocol as well. Thus if any difference in the protein abundance is manifested between these two samples, that would help us to distinctively identify the true persisters within the heterogeneous population those survived antibiotic stress.

The protein abundance values of these two samples were plotted in a Scatter plot to find out if there is any correlation between them. We plotted 'yellow' sample in x-axis and 'only red'

sample in y-axis and generated the Scatter plot (Fig. 4.6). Each point in the plot represents the abundance of a particular protein in both the samples. A more or less diagonal trend of distribution of the protein abundance values were observed, except for an outlier which was way off the diagonal. Every point which was included in the diagonal trend of distribution of the values represents no significant variance in terms of abundance in two different samples. But the single point which was way off the diagonal caught our attention (marked by the red circle in Fig. 4.6). It was disclosed from the protein name database in the perseus file that this particular point represents the abundance value of enzyme tryptophanase. This data was reproduced in the technical replicates as well. Tryptophanase had more than 2000 fold higher abundance in the true persisters compared to the 'only red' sample. Highly over-expressed tryptophanase seemed to serve as a potential innate and exclusive feature of true persisters as opposed to other cells surviving antibiotic. We were excited by this remarkable finding and came across a paper by Collins and colleagues that correlates tryptophanase, indole signalling and persistence [9].

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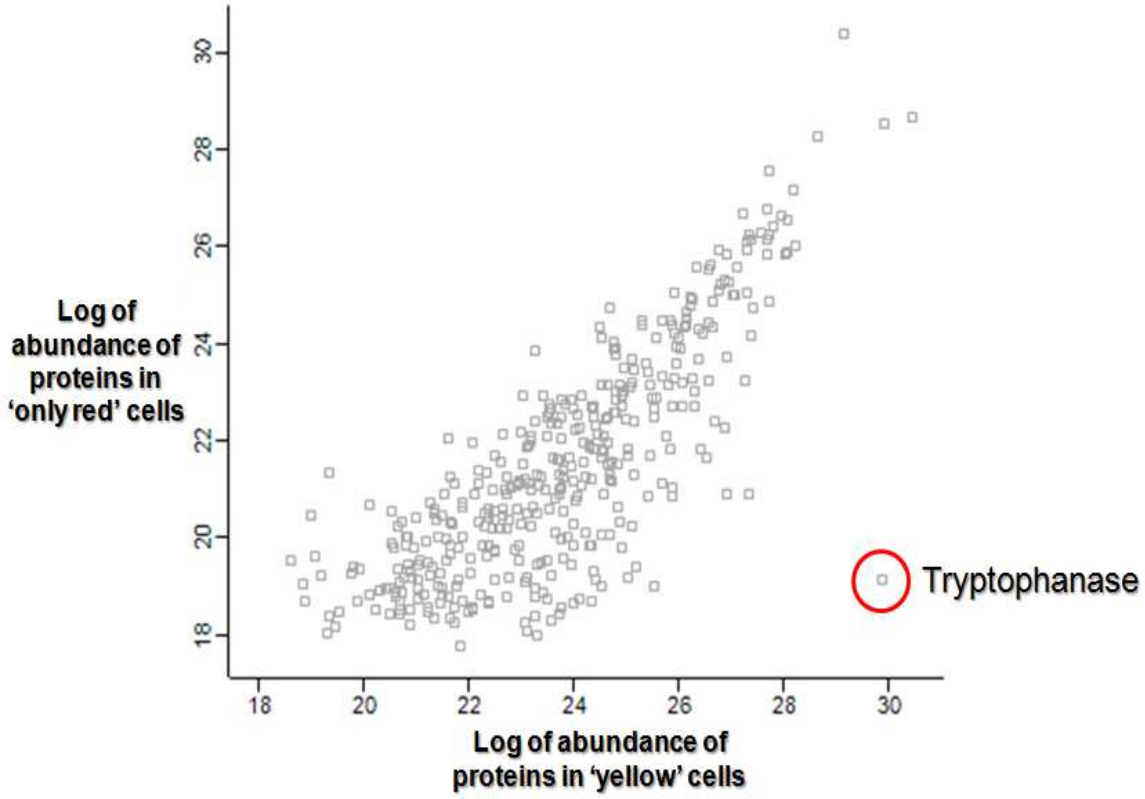

Figure 4.6: Scatter plot to represent a correlation between the whole proteome data of 'yellow' and 'only red' samples. The data clearly revealed approximately 2000 fold more abundance of tryptophanase enzyme in the 'yellow' cells compared to the internally controlled 'only red' cells.

4.6 Microscopic visualization revealed that tryptophanase over-expressing cells survived lethal antibiotic stress:

Based on the outcome of the whole proteome data, we hypothesized that tryptophanase is one of the key players that regulate persister physiology. To validate our hypothesis, our initial aim was to take a microscopy-based qualitative and visual approach to gain some fundamental idea about the role of tryptophanase in persistence. Collins and research group showed that indole induces persistence in *E. coli* [9]. Indole is a small nonpolar signalling molecule. It is produced as one of the products when enzyme tryptophanase lyse amino acid tryptophan (L-tryptophan + H_2O → indole + pyruvate + NH_3). A vital experiment was performed using microfluidics, time-lapse microscopy and an indole-responsive fluorescent reporter strain of *E. coli* strain which demonstrated the crucial role of tryptophanase in persistence [9]. Physiological concentration of indole was added to the media just before the cells started entering the log phase and this caused stochastic activation of the promoter of tryptophanase operon in few cells which showed fluorescence. Lethal antibiotic was added to the culture at mid-log phase. Interestingly, the cells survived the antibiotic mediated lysis were mostly fluorescent or in other words, had activated tryptophanase expressing promoter [9].

Taking inspiration from this work, we obtained an *E. coli* strain GL69 [75, 77] which has a sf-gfp (super folding-green fluorescence protein) gene fused with the tryptophanase coding gene *tnaA* under the native promoter in the chromosome. This strain was a useful and authentic tool to directly visualize tryptophanase over-expression under microscope in terms of green fluorescence. We chose minimal media (MOPS Glc) instead of complex media LB to assure the manifestation of sole effect of added indole on the cells. So, according to our hypothesis we expected a fraction of actively growing mid log phase cells to have green fluorescence. These cells were likely to have a protective effect against antibiotic mediated lysis. We aimed to treat the culture with bactericidal drug cefoxitin in order to test this. Significant enrichment of the cells

over-expressing tryptophanase was anticipated upon antibiotic addition. The cell growth kinetics of *E. coli* in MOPS Glc minimal media is very slow (doubling time ~2 hr) compared to rich and complex media LB (doubling time ~30 min). The MIC (Minimal Inhibitory Concentration) of cefoxitin for GL69 in MOPS was 128µg/ml. As typically done for LB, we chose to treat the cells with 2X of MIC to perform the persister assay. GL69 overnight culture (in MOPS Glc) was diluted to 1:10000 fold in fresh MOPS Glc and was allowed to grow till just before the entrance to log phase (OD_{600}=0.062) and indole was added to the media at this point. This helps the cells to exponentially grow and multiply throughout the log phase under the influence of indole which would help to manifest the effect of indole more evidently.

Microscopic visualization revealed that green fluorescent spherical foci (as small green dots) are visible in a small fraction of mid log phase cells (Fig. 4.7 A) as expected. Indole induced tryptophanase over-expression heterogeneously in the *E. coli* population. We chose to stain the cells with a membrane staining dye (FM 5-95) to precisely observe the foci in the cells. FM 5-95 is a lipophilic styryl dye that inserts itself in the cell membrane. This helps to stain and visualize the clear border of each cell as a red hallo. We took the pictures in DIC, green filter and red filter and overlapped the red and green filter images to visualize the green foci in the red bordered cells. Careful observation revealed that the foci tend to have a location near the cell membrane in most of the cells in mid log phase (Fig. 4.7 A). This was possibly because tryptophanase serves some function near the cell membrane that we are not aware of. But no specific position (polar or centred) of the tryptophanase foci in terms of cell length could be conferred and the size of the foci also varied but they all were uniformly spherical in shape.

Next we added lethal dose 256 µg/ml cefoxitin to lyse the sensitive cells. Typically for *E. coli* grown in LB, we wait until 3 hrs after antibiotic addition (lethal dose) to allow the antibiotic to work optically lysing the sensitive cells. Likewise we collected the sample 3 hrs after antibiotic addition and visualized under microscope (Fig. 4.7 B). It was clear that even if many of cells were

lysed and debris were visible, still we could see almost same number of visible green foci as we did before antibiotic addition, so it's obvious that majority of the cells having over-expressed tryptophanase tend to survive the cefoxitin mediated cell lysis. Also, like the previous sample, the foci were spherical shaped, closely associated with the cell membrane, but the position of the foci in terms of cell length was random. The foci also varied in size.

From the bright field (Fig. 4.7 B), it was clear that many cells were not lysed yet and visibly less amount of cell debris were present than are typically expected. It might be due the slower cell lysis kinetics in minimal media compared to LB. So to let the antibiotic work optimally, we decided to collect another set of sample for microscopy after 10 hrs from cefoxitin addition (Fig. 4.7 C).

This sample had maximum proportion of cell debris which indicates optimal activity of the antibiotic. Also, in the green filter (Fig. 4.7 C), it was observed that the proportion of green foci was consistent with the previous two samples which ensured even more enrichment of the cells having over-expressed tryptophanase as the sensitive cells were extensively lysed over the period of 10 long hours of antibiotic exposure. Also the size of the foci varied but they all were spherical. However it was not feasible to visualize this sample after staining with FM 5-95 dye, because of the high proportion of cell debris present in the sample. The debris are mostly composed of lysed tiny fragments of cell membrane which are nonpolar in nature and are stained bright red with FM 5-95. Even though the membranes of the remaining cells are also stained red, but the high proportion of stained debris tend to form red clustered cloudy view under the red filter overshadowing the remaining intact cells. Thus it was not possible to produce a microscopic view of the 10 hrs sample stained with FM 5-95. Therefore we could only accomplish the DIC and unstained green filter images of this sample (Fig. 4.7 C) which nonetheless clearly demonstrate more enrichment of the cells having green foci compared to the 3 hrs sample (Fig. 4.7 B). Also it was previously demonstrated the TnaA-GFP foci disappear upon cell lysis [76].

Thus it was evident that the presence of foci was clearly indicative of the presence of unlysed intact cells.

Overall, according to the depiction of qualitative microscopic data, there was a clear gradual enrichment of the cells having over-expressed tryptophanase over the time after lethal antibiotic addition. So the cells having over-expressed tryptophanase proved to be the eventual survivor under the lethal antibiotic stress. To validate the conclusion in a quantitative approach, we aimed to perform the flow cytometric analysis with the same samples which could reveal the actual proportion of cells having green fluorescence over the time (Fig. 4.8).

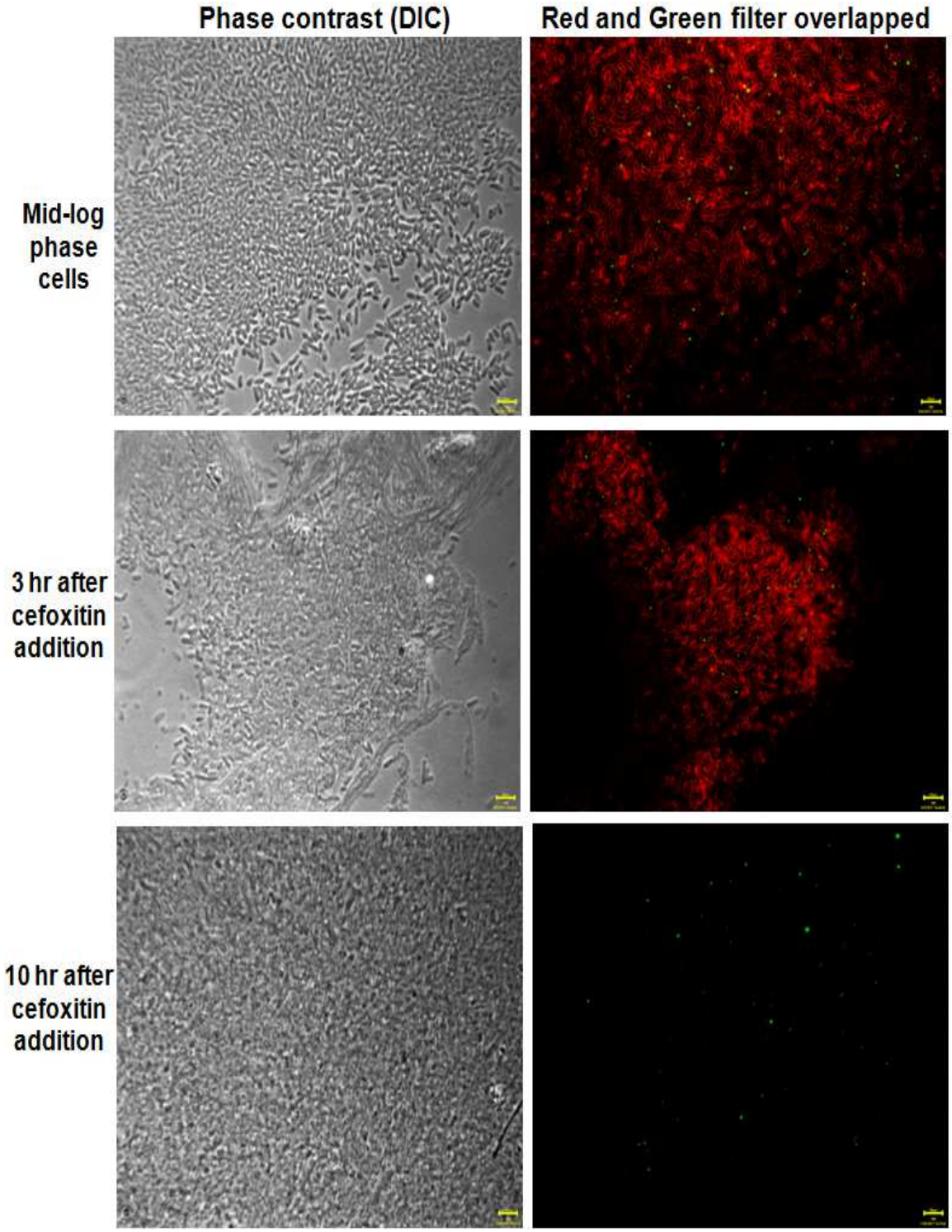

Figure 4.7: Visualization of cells (GL69) under microscope with FM 5-95 dye. Left panels: cells imaged by phase-contrast lens. Right panels: same field was visualized under green filter and red filter separately and the images were superimposed for the first two samples. The sample collected after 10 hrs of cefoxitin addition was not stained with FM 5-95 because of the technical difficulties. The yellow bars represent 5μm.

4.7 Flow cytometry demonstrated the pivotal role of tryptophanase in persister physiology:

Based on the striking finding from the whole proteome data (Fig. 4.6) we hypothesized that tryptophanase possibly serves as one of the major regulators of persister physiology. We first addressed the hypothesis by microscopic data (Fig. 4.7). And finally, we performed flow cytometric analysis (Fig. 4.8) with the same set of samples using the same strain (GL69) to complement the microscopic data in a quantitative approach.

We analyzed three samples namely, actively growing mid log phase cells (Fig. 4.8 A), 3 hr after cefoxitin treatment (Fig. 4.8 B) and 10 hr after cefoxitin treatment (Fig. 4.8 C). The cell growth kinetics of GL69 was very slow in MOPS Glc media and even the cell lysis kinetics seemed slower from the microscope observation. So it was obvious that we would get gradually increased proportion of cell debris in the flow cytometric analysis as cefoxitin works by lysing the cells. As per microscopic observation, there was no visibly increased cell debris after 10 hrs from the time of antibiotic addition. So using GL69 we analysed these three comparable samples (Fig. 4.8, A, B, C) to quantitatively estimate the proportion of cells having green fluorescence. As per our hypothesis we expected majority of the cells in the surviving population to over-expressed tryptophanase. We expected enrichment of cells having green fluorescence as we move from mid log phase cells (before adding cefoxitin) to 3 hrs after cefoxitin addition to 10 hrs after cefoxitin addition. This progressive enrichment of cells having green fluorescence would clearly demonstrate the vital role of tryptophanase in persister physiology to aid the survival ability under the stress of bactericidal antibiotic.

First it was made sure that the debris were left out while the expected ‘live cells’ were analyzed. The debris are very tiny and have very less FSC and SSC values and are usually identified as being concentrated at the left hand side corner at the bottom of the FSC vs. SSC

plots. Therefore, for each sample, we selected all the cells from a particular hexagonal area (Fig. 4.8) from the FSC vs. SSC plot which assures the exclusion of debris.

It can be observed that the actively growing mid log phase cells (Fig. 4.8 A) have almost no debris whereas the proportion of debris gradually gets increased as we move from the cell sample collected after 3 hrs of cefoxitin addition (Fig. 4.8 B) to the sample collected after 10 hrs of antibiotic addition (Fig. 4.8 C). The last sample had maximum proportion of cell debris as visible in the plot (Fig. 4.8 C) which was expected. The SSC vs. Intensity of GFP plots (Fig. 4.8 A, B, C) were necessary to perform the quantitative analysis of the cells in terms fluorescence intensity. The bar was set using the mid log phase cells of control wild type (MG1655) strain that do not express GFP tagged tryptophanase and was grown under the same conditions and same media (MOPS Glc + indole). Here we can visualize clear gradual shifting of the cell cloud towards the right side of the bar as we look through the mid log phase cells (Fig. 4.8 A), the cells after 3 hrs of antibiotic addition (Fig. 4.8 B) and the cells after 10 hrs of antibiotic addition (Fig. 4.8 C). Mid log phase cells have a small fraction of cells (4%) expressing green fluorescence (Fig. 4.8 A). These might represent the potential persisters. Sample collected after 3 hrs of cefoxitin addition, have a clear jump in the fraction of the cells having green fluorescence from 4% to 73%. So at this time point, majority of the cells surviving the cefoxitin mediated lysis, have over-expressed tryptophanase. This makes us consider the importance of this enzyme in persister physiology. Finally, 91% cells of the sample collected after 10 hrs of cefoxitin addition had green fluorescence. So almost all of the cells survived through 10 hrs of cefoxitin stress, have over-expressed tryptophanase.

Overall, these quantitative data evidently reveal gradual enrichment of the cells having over expressed tryptophanase. This huge enrichment (~23 fold) makes it obvious that the majority of cells surviving the stress exerted by the lethal drug have over expressed tryptophanase. So

tryptophanase clearly has some pivotal role that aids the surviving ability of the cells under stress and hence it is one of the key regulators of persister physiology.

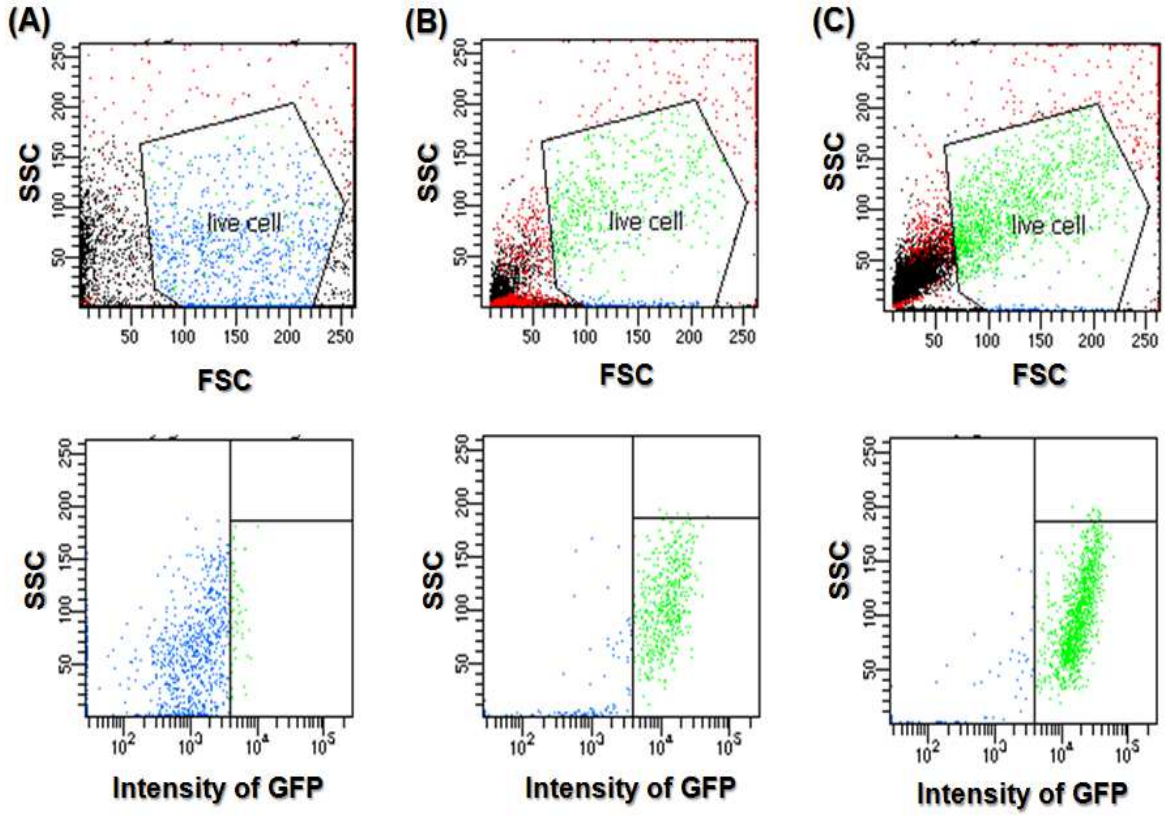

Figure 4.8: **Quantitative distribution of cells (GL69) in terms of green fluorescence. (A) Actively growing mid log phase cells.** Only 4% of the cells had green fluorescence. The sample had almost no debris. **(B) 3 hrs after cefoxitin addition.** The percentage of cells having green fluorescence raised up to 73%. The cell sample had some debris concentrated at the left hand side corner at the bottom of the FSC vs. SSC plot. **(C) 10 hrs after cefoxitin addition.** The percentage went even higher where 91% of the cells are now having green fluorescence. This sample had maximum proportion of debris concentrated at the left hand side corner at the bottom of the FSC vs. SSC plot. Cells from a best possible chosen area of each of the FSC vs. SSC plot was selected to assure the exclusion of debris.

CHAPTER V

CONCLUSION

Elimination of persisters is vital to completely eradicate chronic bacterial infections. Designing an effective anti-persister drug requires deep knowledge about persister physiology. Isolating persisters in sufficient purity has been a hurdle in the way to gain better understanding of their physiology. In this Thesis, I demonstrated a method to selectively isolate authentic persisters and characterized their proteome by Mass Spectrometry. My finding provides insight into the physiology of true persisters. The most remarkable revelation was the presence of tryptophanase in great abundance only in true persisters but not in other cells surviving antibiotic stress *in vitro*.

Tryptophanase (also called tryptophan-indole lyase) is a pyridoxal phosphate dependent homotetrameric enzyme which breaks down L-tryptophan into three products: pyruvate, indole and ammonia [98]. Tryptophanase is encoded by *tnaA* gene which is part of *tnaCAB* operon in *E. coli* [99]. The regulation of this operon is very complex. *tnaC* is a leader sequence which controls the expression of *tnaA* and *tnaB* via translational attenuation in response to the abundance of tryptophan [99, 100]. TnaB is a low affinity tryptophan permease which is associated with the transportation of tryptophan across plasma membrane for catabolism [100]. Expression of tryptophanase can also be controlled by catabolite repression at the transcriptional level even though it is not the major regulatory mechanism [101]. Bacterial tryptophanase helps to utilize tryptophan as source of carbon and nitrogen for growth. Apart from that, tryptophanase

exerts major multidimensional effect on bacterial physiology via metabolite indole production [91].

Indole is a small nonpolar molecule which plays a crucial role in bacterial physiology as both extracellular [90] and intracellular [88, 89] signalling molecule. Interestingly indole can modulate bacterial behaviour and response to a number of phenomena. They include persister formation [9] which is of our utmost interest and also biofilm formation [83], acid tolerance [86], antibiotic resistance [85], virulence [84], motility [83], inter-kingdom signalling [87]. These complicated broad-ranging phenomena might be influential to each other which are not known in details.

Collins and group demonstrated how addition of physiological concentration of indole in the media pre-adapted a subset of cells in *E. coli* culture to tolerate lethal antibiotic stress [9]. They showed that one hour of indole treatment of exponential phase culture of *E. coli* increased (by at least an order of magnitude) their survival rate when subjected to three different bactericidal drugs. They also provided a direct observation of indole induced persistence with the help of microfluidics and time-lapse microscopy which is consistent with the previous observation. They utilized an indole-responsive transcriptional fluorescent reporter strain of *E. coli* which is designed to report transcriptional activation of the promoter of tnaCAB. This result clearly showed that allowing the cells to divide in the presence of indole stochastically induced fluorescence in a small sub-fraction of cells. Ampicillin mediated extensive lysis of such population proved that cells with indole-responsive fluorescence are mostly tolerant to antibiotic stress. Their results illustrated that the heterogeneously arising indole response manifests a protective effect in a sub-fraction of bacterial population and also this effect is proved be generalized against multiple lethal drugs. To validate their claim further they showed that indole treatment of ΔtnaA strain of *E. coli* showed significantly decreased persister formation. Thus their work established that indole ultimately induces activation of tryptophanase leading to increased

intracellular indole concentration which activates indole mediated signalling cascades to enhance antibiotic tolerance or persistence. Further investigation of genome-wide transcriptional response to indole in *E. coli* revealed significant up-regulation of genes of phage shock and OxyR pathway. Also *E. coli* strains with deleted phage shock or OxyR pathway genes led to substantial decrease in persister level [9]. However the details of how such convoluted and broad-ranging indole signalling works remains yet to be explored.

Our findings clearly establish tryptophanase as a major regulator of persister physiology. This suggests that tryptophanase can be established as an attractive therapeutic target to design effective anti-persister drug. Our proteome data showing dramatic up-regulation of tryptophanase exclusively in persisters was consistent with the literature [9]. Using a GFP-labelled TnaA translational reporter strain of *E. coli* we wanted to learn if indole is causing up-regulation of tryptophanase at the protein level and most importantly if tryptophanase over-expressing cells are able to survive lethal antibiotic mediated lysis demonstrating the vital role of this enzyme in persister physiology. It was known that tryptophanase is localized in polar focus in bacterial cell (one focus per cell) and such polar focus is distinct from inclusion body [77]. The enzyme remains functional in the focus. But the purpose of such localization remains elusive [76, 77]. My investigation revealed formation of stochastic over-expression of tryptophanase in the form of green spherical foci in a sub-fraction of mid-log phase culture as an effect of indole. Eventually these cells with green foci sustained extensive cell lysis. The flow cytometric data quantitatively complemented the microscopic data showing a large enrichment (~23 fold) of tryptophanase over-expressing cells upon antibiotic mediated lysis.

We speculate that over-expressed tryptophanase contributes towards increasing intracellular indole concentration which ultimately activates indole signalling cascades leading to antibiotic tolerance. Unfortunately indole signalling pathway and its regulation still remain mysterious. It was shown that indole contributes to persistence through activation of phage shock

and OxyR pathways [9]. Also OxyR, the central regulator of bacterial oxidative stress response was identified as one of the major virulence factors of uropathogenic *E. coli* responsible for chronic urinary and extra-urinary infections [92]. Inactivation of *oxyR* gene leads to increased sensitivity of mycobacterium towards anti-tubercular drug isoniazid [93]. Phage-Shock-Protein response pathway is conserved in different bacteria and is known to associate with extra-cytoplasmic stress response leading to overall reduction of the energy status of the cell [94]. *E. coli psp* mutants have been found to have defects in biofilm formation and trouble in maintenance of proton motive force [94]. *psp* mutants in *Salmonella enterica* (causing chronic typhoid fever) leads to reduction in virulence [95]. However how indole does actually activate phage shock and OxyR pathways and how the interactive components of these complex pathways lead to antibiotic tolerance are unexplained.

This study was limited to only one antibiotic cefoxitin. Persisters by definition should be tolerant to multiple lethal antibiotics. This requires further investigation with different bactericidal drugs. We don't know what happens to the tryptophanase foci over time upon persister resuscitation. We can openly speculate that possibly tryptophanase focus would slowly disappear over the time upon persister recovery as a result of down-regulation of the enzyme. However the possible underlying mechanism of such intricate regulation in response to environmental fluctuations is yet to be explored. In addition, we are also unaware of the transcriptome profile and internal cell morphology of the native persisters.

5.1 Future Research Directions:

It was previously reported that the internal morphology of MazF–modelled *E. coli* persisters and also *P. aeruginusa* persisters differs substantially from normal cell morphology [49, 81]. The changed internal morphologies of MazF-modelled persisters were indicative of disrupted transcription-translation coupling. We were able to isolate authentic persisters based on multicolor flow cytometric analysis and without toxin over-expression. Thus it would be interesting to investigate the internal morphology of such persisters. The whole proteome data of these persisters unveiled the importance of tryptophanase as a valuable marker of their physiology. Revealing the transcriptome profile of persisters is likely to draw our attention to other novel vital physiological regulators. We can measure the intracellular indole concentration of persisters to and compare that with normal cells to confirm the claim of indole playing a major role in persister as a signalling molecule. It is also possible to testify the level of persister formation with several mutants of indole signalling targets or using inhibitors such targets. We also don't know about the potential role of tryptophanase in persister regrowth. Therefore it would be exciting to perform a time-course experiment with fluorescent reporter strain to track the tryptophanase fluorescence over the time during revival of persisters. A synthetic analogue of tryptophan named L-bishomotryptophan has been reported to be a highly efficient inhibitor of *E. coli* tryptophanase having no observed inhibitory effect towards Trp synthase [82]. Given the fact that tryptophanase could potentially serve as a valuable drug target, it would be interesting to explore the level of persister formation upon exposure to such inhibitors. Most importantly it would be worth trying out our approach of isolating persisters using different bacteria such as several other pathogens like *Pseudomonas aeruginosa*, *Salmonella enterica* causing chronic infections and different bactericidal drugs.

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APPENDICES

Table A1. Type I and type II toxin and antitoxin molecules.

	Toxin	Antitoxin	Feature and function of the toxin		Reference
Type I	HokB	SokB	Membrane protein.	Interferes with ATP synthesis.	[8, 62]
	TisB	IstR			
Type II	ChpB	ChpS	Ribosome dependent endoribonuclease.	Inhibits translation.	
	MazF	MazE			
	MsqR	MsqA			
	YoeB	YefM			
	HicA	HicB			
	HipA	HipB	Serine Kinase		
	YafN	YafO	Ribosome independent endoribonulease.		
	HigB	HigA			
	RelE	RelB			
	YafQ	DinJ			
	YhaV	PrlF			

VITA

Amrapali Ghosh

Candidate for the Degree of

Master of Science

Thesis: AMRAPALI GHOSH

Major Field: Biochemistry and Molecular Biology

Biographical:

Education:

Completed the requirements for the Master of Science in Biochemistry and Molecular Biology major at Oklahoma State University, Stillwater, Oklahoma in December, 2017.

Completed the requirements for the Master of Science in Biochemistry major at University of Calcutta, Kolkata, India in 2013.

Completed the requirements for the Bachelor of Science in Microbiology (Hons.) major at St. Xavier's College, Kolkata, India in 2011.

Experience:

Graduate Research Assistant, Oklahoma State University, Stillwater, Oklahoma (2015-2017).

Graduate Teaching Assistant, Oklahoma State University, Stillwater, Oklahoma (2017).

Visiting Research Assistant, University of Calcutta, Dept. of Biochemistry, Kolkata, India (2014).