Master's Thesis

Surviving Bacterial Predation: A Comparative Study Between Predation Persistence and Antibiotic Persistence

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Approved by

Advisor
Robert J. Mitchell

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07/10/2018

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Abstract

Many infectious diseases may one day become uncontrollable. Thus, antibiotic-resistant bacteria have become a serious issue. And as the failing of antibiotics becoming a big problem, the needs for finding new antimicrobial agents are increasing. For an alternative of antibiotics, predatory bacteria are a promising approach since *Bdellovibrio bacteriovorus* is an obligate predator of other Gram-negative bacteria. However, even after predation by *B. bacteriovorus*, a small sub-population of prey cells remains a characteristic that is similar to bacterial persisters. And in a long-term predation in the continuous system was conducted to characterize these remaining sub-population of prey. One interesting finding from long-term predation is phenotypic changes of prey. So-called small colony variants (SCVs). The bacterial persisters are a small sub-population of the entire culture that exists within a dormant state and, although they lack any genetic mutations or modifications, they survive longer during exposures to antibiotics due to this temporal state. As these persister cells survive in the presence of different antibiotics, their existence is considered important as they may cause chronic or recurrent infections.

In this study, I characterized both persister cells induced by a pre-treatment with antibiotics, *i.e*., antibiotic persisters (AP), and the sub-population of prey cells which survive predation by *B. bacteriovorus* HD100, *i.e.*, the predation persisters (PP) cells. I found AP cells were also resistant to *B. bacteriovorus* predation and, conversely, the PP cells were better at surviving antibiotic treatments. The characteristics of these cultures were studied further to find common features for the two persister populations, by comparing the expression level of persister related genes in SCVs using transcriptome analysis.

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Abbreviation

E. coli : *Escherichia coli*

DNA : Deoxyribonucleic acid

RNA : Ribonucleic acid

TA : Toxin, Antitoxin

BALOs : Bdellovibrio and like organisms

USA : United States of America

SCVs : Small colony variants

ppGpp : Guanosine tetraphosphate

pppGpp: Guanosine pentaphosphate

RPM : Revolution per minute

PFU : Plaque forming unit

CFU : Colony forming unit

LB : Luria-Bertani broth

DNB : Residual cell

OD : Optical density

w.t. : wild-type

SDS : Sodium Dodecyl Sulfate

R cell : Residual cell

Chapter 1 Introduction

1.1 Predatory bacteria, *Bdellovibrio bacteriovorus*

In microworld, there are bacteria which can prey on the other bacteria as the predator exists in the animal world. The predatory bacteria, *Bdellovibrio* is a genus of Gram-negative, obligate aerobic bacteria. The unique property of this predatory bacteria is that it could predate on the other gram-negative bacteria. There are different predatory bacteria which has the different property of predation, however, *Bdellovibrio bacteriovorus* which was found accidentally by Stolp and Petzold in 1962 is the most well studied predatory bacteria. And it is highly motile and found easily in nature such as soil, lakes, and sewage. [1-6]

While predation of *Bdellovibrio bacteriovorus* HD100 on other gram-negative bacteria, it can degrade genome and protein of prey cell. That could block horizontal gene transfer by inhibiting the prey gene transfer to the surroundings. Furthermore, it has a broad range of prey. [6] Because of this unique property of predatory bacteria, it is a promising approach for alternatives to antibiotics.

1.1.1 The life cycle of *Bdellovibrio bacteriovorus*

The life cycle of *Bdellovibrio bacteriovorus* (Fig1.1.) has two different phases called “attack phase” and “growth phase”. [8] During the “attack phase”, BALOs are freely swimming fast using their flagella and seeking a prey around. Once they find the prey, *B.bacteriovorus* can attach to the outer membrane and peptidoglycan layer of prey cell. And it remains reversibly attached to the prey for short recognition period via the pole opposite the flagellum. After the recognition period, it becomes irreversibly attached. Finally, *B.bacteriovorus* enters the periplasmic space of prey making the small hole prior to “growth phase” starts. When the *Bdellovibrio* enters the periplasmic space of prey cell, it seals the membrane hole and transforms the host cell into a spherical morphology. This complex is called a bdelloplast. Then the *Bdellovibrio* consumes and digests the cytoplasm of prey and elongate inside of the host cell and break down the host cell molecule via a different type of hydrolytic enzymes. After the prey nutrients are exhausted, the progeny of *Bdellovibrio* become motile and lyse the host cell and are released from the prey cell. The whole cycle of predation takes three to four hours. [5,6,8,]

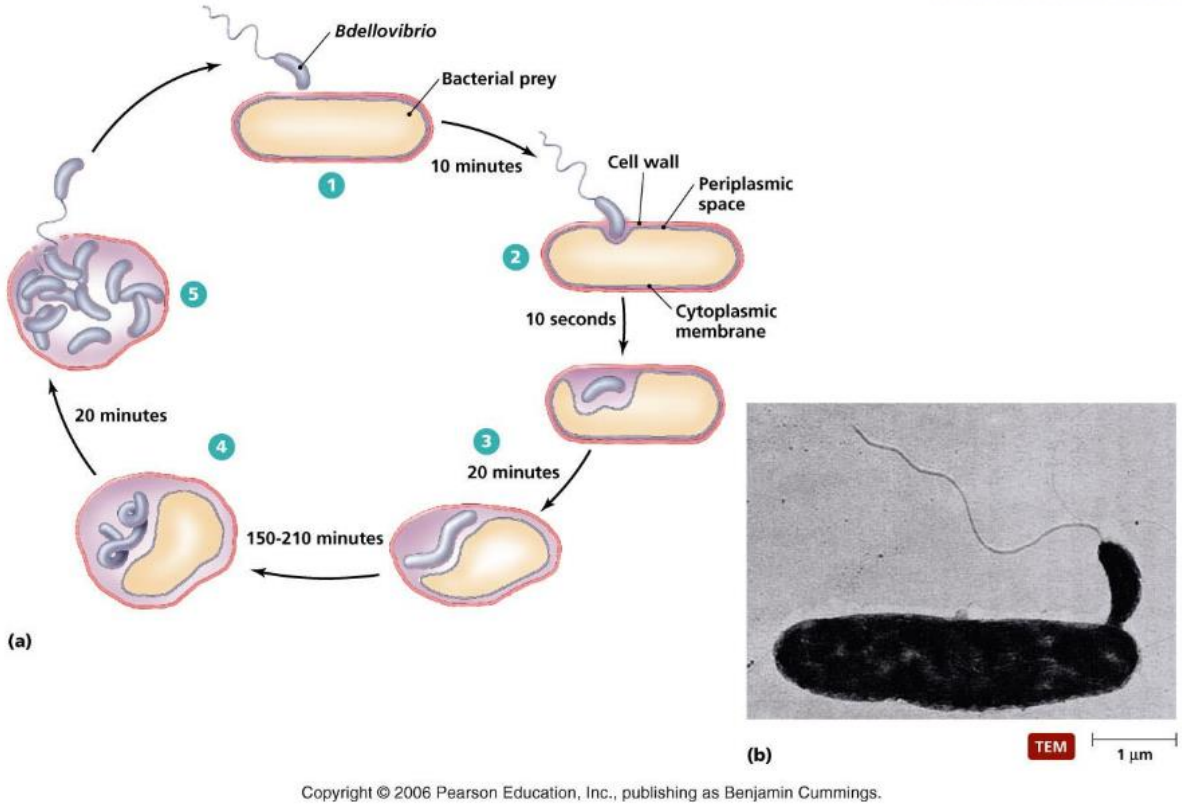


Figure 1.1. The life cycle of predatory bacteria, *Bdellovibrio bacteriovorus*.

1.1.2 Residual prey cell after predation

As an alternative to antibiotics, predatory bacteria are a promising approach since *Bdellovibrio bacteriovorus* is an obligate predator of other Gram-negative bacteria. However, even after predation by *B. bacteriovorus*, a small sub-population of prey cells remains. The predation of *Bdellovibrio bacteriovorus* leads the multiplication of the predator and the lysis, but it does not result in the eradication of the prey. (Fig1.2.) [10] The small sub-population of prey cell which remained after predation regards as resistant cell towards predation by *Bdellovibrio bacteriovorus.* This tolerance does not show any genetic mutation and it disappears after removal of the predator.

The mechanism of interaction between prey and predator is not elucidated. However, in the past, this phenomenon was explained by the low chance for a predator to find a prey around as predation occur. [11,12] However, the result of a study from Edouard Jurkevitch explained that the co-existence of prey and predator is possible because plastic phenotype of prey populations allow exhibit "reversible resistance" to a predator. [10] This experiment showed that prey population influence to make them have reversible resistance to BALOs upon exposure to the predatory bacteria. [10] In that study, *Bdellovibrio bacteriovorus* SNE were used as predatory bacteria and prey was Erwinia carotovora ssp. carotovora (Ecc) which can be predated by the predatory bacteria but not totally eliminated. The lysate obtained from overnight cultures with prey and predator were used at two different final concentration. (10^6-10^7 and 10^8-10^9 pfu ml^{-1}) Therefore, it contains different level of residual prey cell from the predation carried along. As the lysate containing a low concentration of residual cell were added to fresh prey cell, the prey population decreased more in the suspension with a low concentration of predators. However, the predation was more efficient with a higher concentration of predatory bacteria when the 0.45 μm filtered predatory cell lysate added to the fresh prey cell. And the naïve prey cell formed bdelloplast rapidly whereas the residual cell took longer and less attachment with predator upon exposure to predatory bacteria. (Fig 1.2.) However, this phenomenon was disappeared after the residual cell were overnight grown in medium without predators. Therefore, increased resistance to predation was not a stable chracteristic but appeared to be transient since the residual cell becomes sensitive to predation as naïve prey cell. This transient property of resistant phenotype exhibit that it is not a mutational event but a phenotypic plastic response. In conclusion, it is shown that the non-eradication of the residual prey population in BALO-prey interaction does not stem from the incapacity of the predator to encounter prey cells at low concentration, as suggested previously.

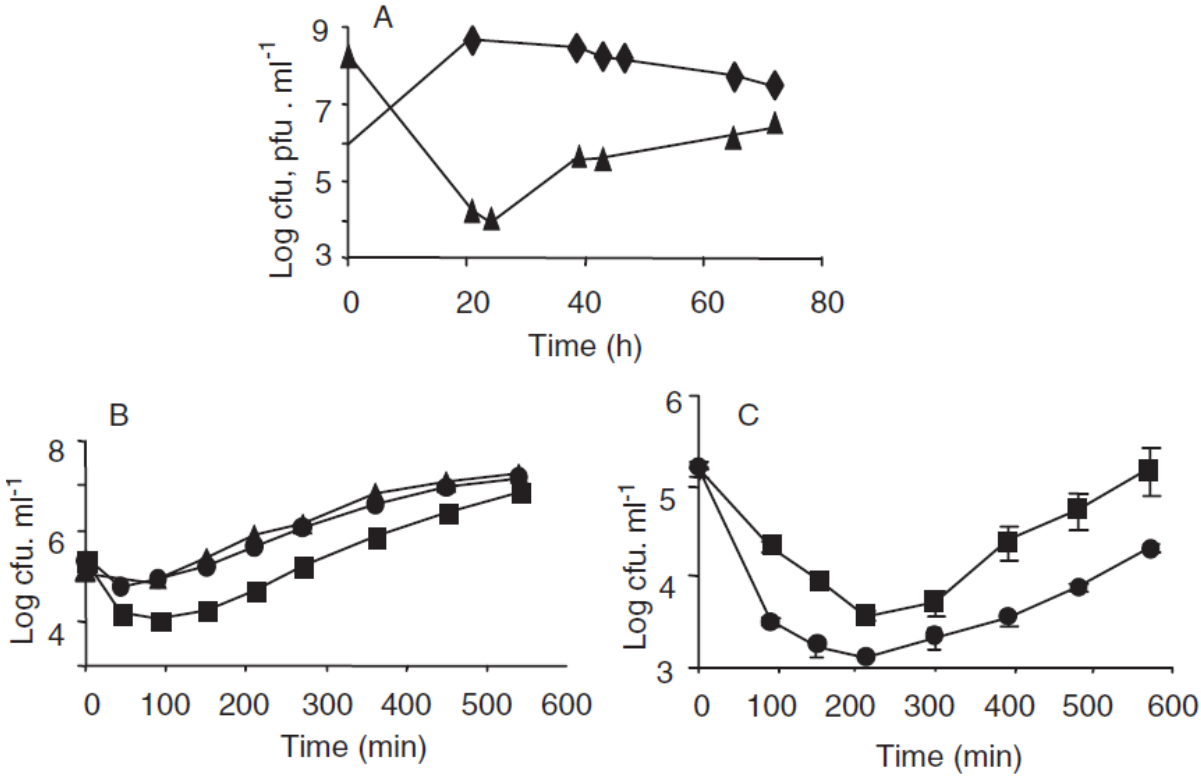


Figure 1.2. Residual prey cell after predation. (Y Shemesh, E Jurkevitch - Environmental Microbiology, 2004)

B. bacteriovorus SNE predation on an *Erwinia carotovora* ssp. *Carotovora* (Ecc) prey. A. Lysate were obtained by growing the predator from a plaque with an Ecc prey: SNE (filled diamonds), Ecc (filled triangles). A residual prey population were always remained after predation. B. Predation of fresh Ecc prey cells at a different concentration of predator containing residual prey cell. (high -filled circles /low -filled squares) No predator added (filled triangles). C. Predation of fresh prey cells with different concentration of 0.45-um-filtered predatory SNE cells. (high -filled circle /low -filled squares) Suspensions were adjusted to contain similar levels of residual prey.

1.1.3 Small colony variants (SCVs)

Members in my lab, performed predation experiments in the continuous cultures to see the prey and predator interaction in long term. (Fig 1.3. (A),(B)) And found that survived population from predation is not defined by the availability of nutrients and that this population is not completely resistant to predation.

One interesting finding from the chemostat experiments was the development of small colony variants (SCVs) (Fig 1.3. (C)) which shows phenotypic changes when *E. coli* MG1655 pUCDK was used as the prey. To explain the phenotypic differences seen with the SCVs, therefore, the genomes from three independent SCV isolates were sequenced. And no clear mutations were found that account for these phenotypic differences. If the residual prey population was resistant to predation, it follows the *B. bacteriovorus* HD100 numbers within the chemostat should decrease due to a wash-out effect. As the predatory populations remain steady, however, this shows the prey is not completely resistant. (Fig 1.4. (A)) When these SCVs were grown, they were grown slower from early exponential phase and not grown more than OD_{600} around 1.5 while wild-type entered stationary phase around 2.5. (Fig 1.4. (B))

Moreover, predation experiments found the SCV cultures grown from stock were just as susceptible to predation as the wild-type *E. coli* MG1655 cultures. These results fit with the accepted concept of “plastic resistance” amongst bacterial prey as describe previously. Briefly, this resistance does not stem from a genetic mutation but rather a phenotypic variation that makes a sub-population temporarily resistant.

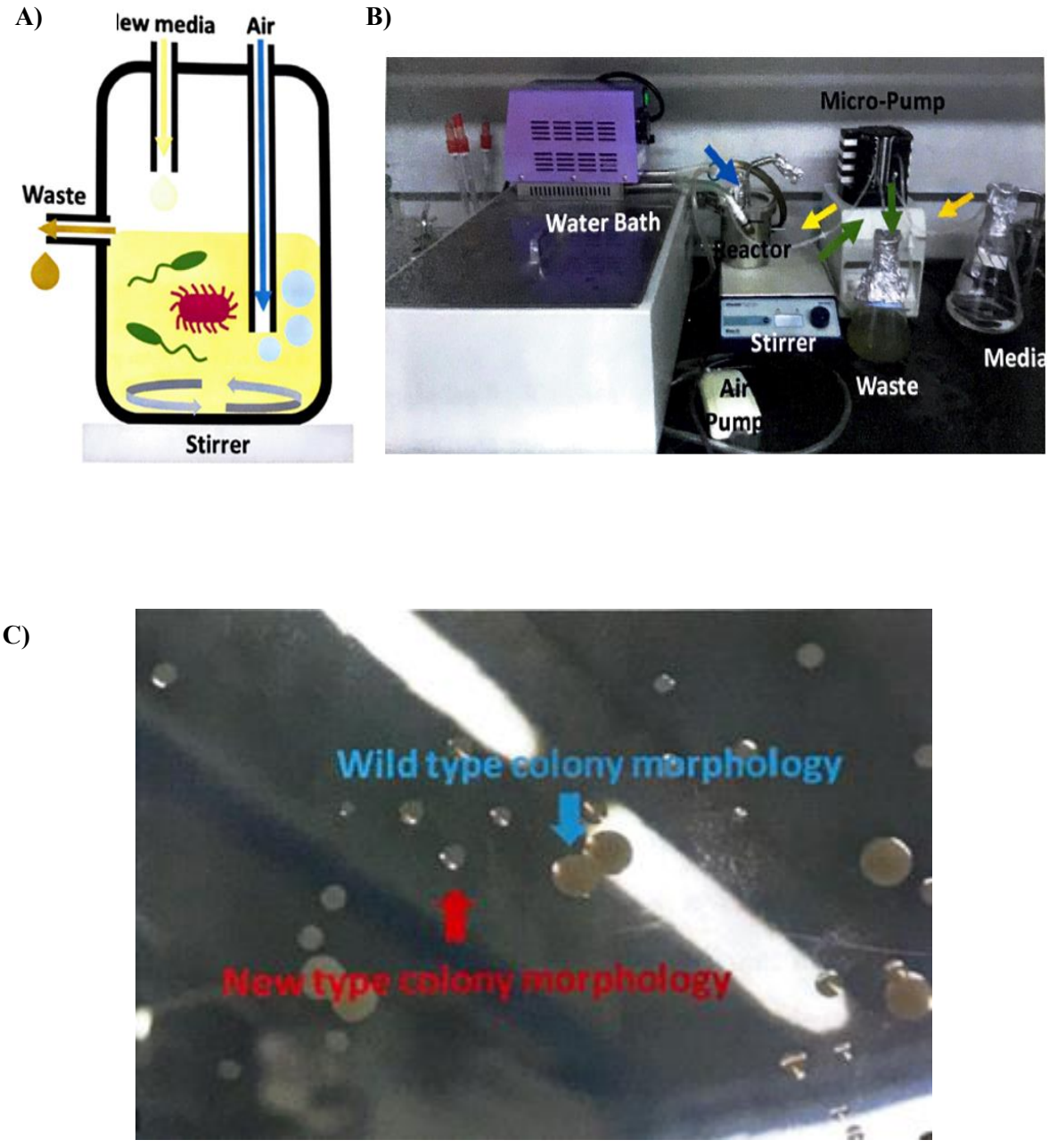
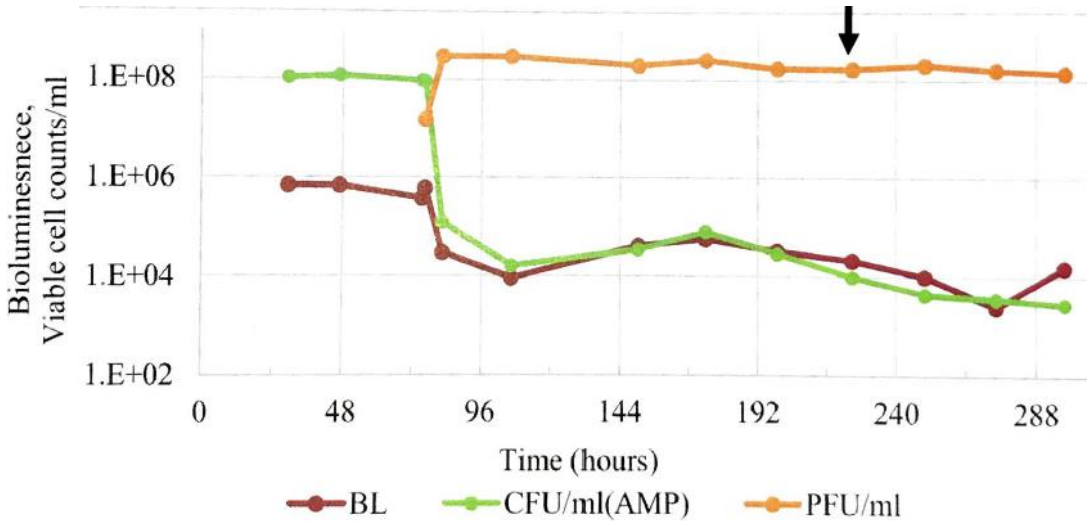


Figure 1.3. Development of SCVs in continuous system (A) Schematic view inside the reactor. (B) Overview of actual system. Media flowed into the reactor through yellow arrow direction while waste flowed out through green arrow. Air pumped into the reactor through blue arrow. (C) Small colony variants (SCVs) from long-term predation exposure in continuous culture system.

A)



B)

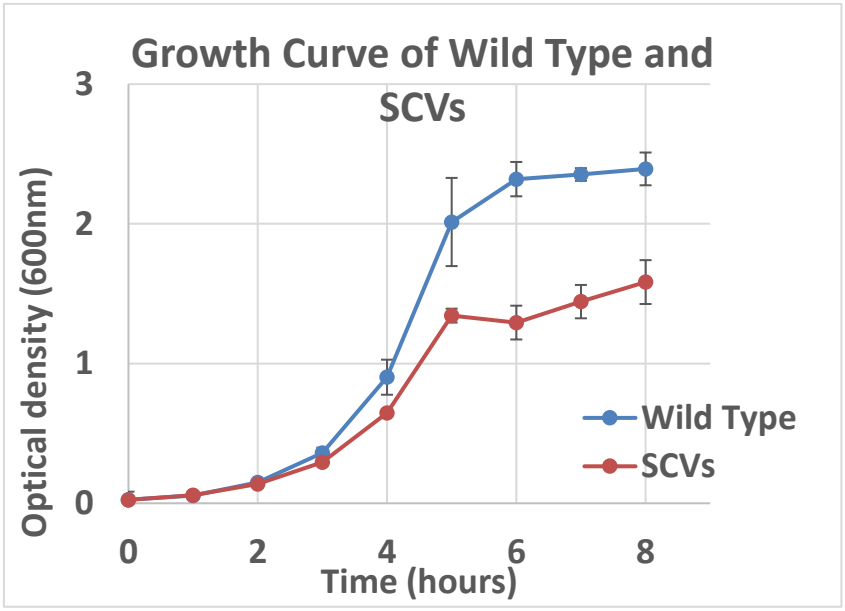


Figure 1.4. Development of SCVs of *E. coli* MG1655 pUCDK

(A) Long-term exposure *E.coli* MG1655 pUCDK *to B. bacteriovorus* HD100 in continuous system

(B) Growth curve for each colony type, wild type and SCVs.

1.2 Persister cell & Dormancy

As a modern biological therapeutic skill to treat pathogens have been improved, the strategies of bacteria to fight against antibiotics also have been developed. Since the rate of antibiotic taker is on the increase, the infection of super bacteria by the abuse of these antibiotics and the resulting death toll from superbug are gradually raising. Therefore, the importance of understanding their strategy against antimicrobial agents has been increasing. For a bacterial cell, one of the well-known ways to survive under treatment of antimicrobial agents is that having resistance towards antimicrobial agents through genetic mutations. However, being persister cell is also a way to survive under the effects of antibiotics. This phenomenon was first found by Joseph Bigger. He found that there were survived cell when he tried to sterilize the flask cultures of *Staphylococcus aureus* using penicillin. The penicillin could not kill the cell completely until longer treatments were used. And he named that as "persister" first. [34]

Bacterial persisters are a small sub-population of the entire culture (typically 10^{-4} to 10^{-6} of the bacterial population) that exist within a dormant state and, although they lack any genetic mutations or modifications they survive longer during exposures to antibiotics due to this temporal state. (Fig 1.5.) As these persister cells are able to survive in the presence of different antibiotics, their existence is considered important as they may cause chronic or recurrent infections. [12,14,15,27, 34] Unlike a resistant cell, the persister cells can persist temporarily under the stress such as antibiotic treatments without undergoing genetic changes. Therefore, this persister cell can be spontaneously switched back to the state which has the susceptibility towards antibiotics again in the absence of stress. The property of persister is nonheritable since it is not gained from genetic mutations whereas resistance is inherited ability of microorganisms. [43]

The persister cell is in dormant status or slow-growing which has very low metabolic activity. Under stress environment, persister cell does not proliferate and stay in dormancy. The mechanism of persister cell formation is not completely established. However, this dormancy status helps the cell to have tolerance towards antibiotics since many of antibiotics target on the actively growing bacteria by disrupting protein synthesis. [25, 27, 30, 34] Therefore, the persister cell which is not dividing would not be affected by antibiotics. The study of the mechanisms underlying bacterial persistence is important for the development of new antimicrobial agents which can reduce persistence.

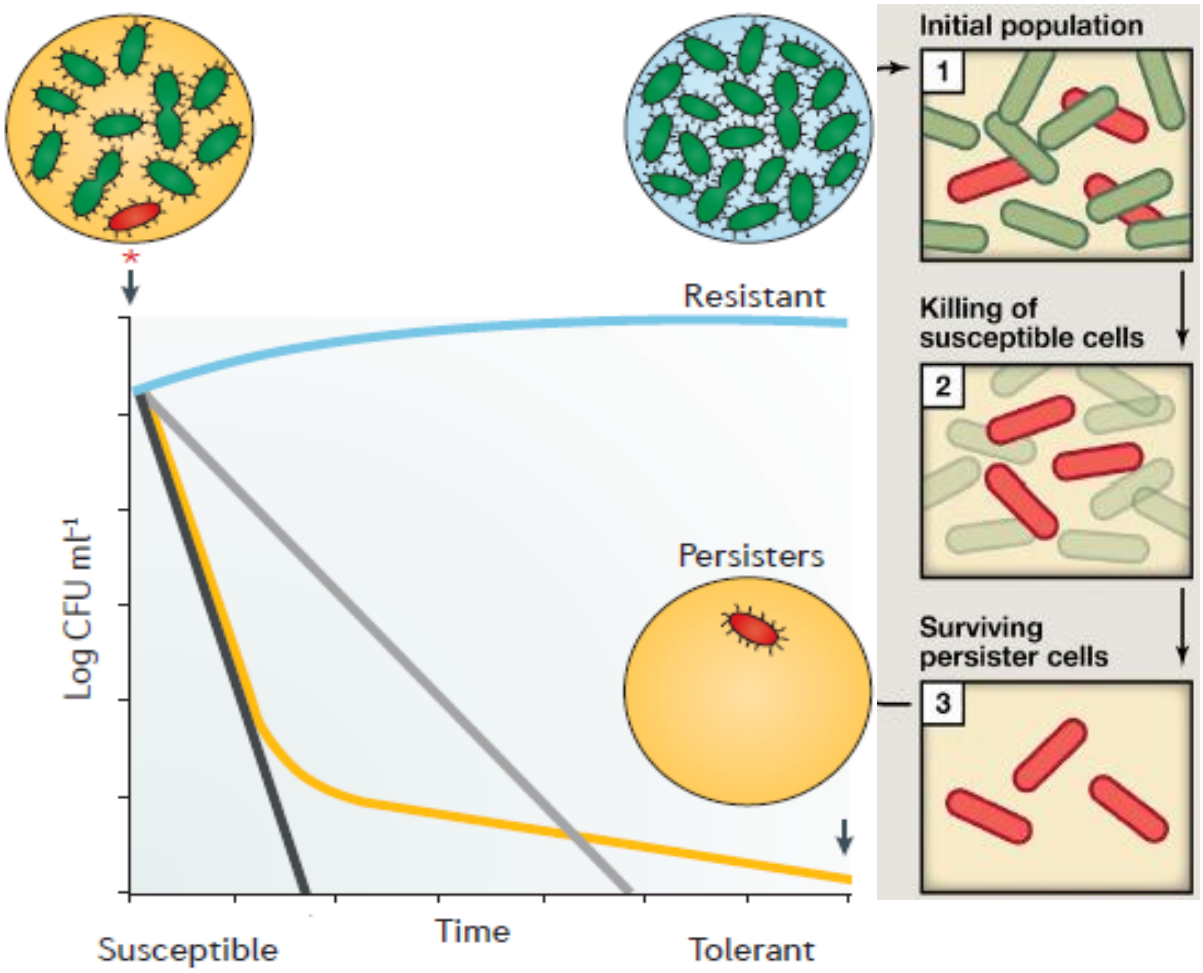


Figure 1. 5. Antibiotic killing kinetics of resistant, tolerant and persister cells. (RA Fisher, B Gollan, S Helaine - Nature Reviews Microbiology, 2017)

1.2.1 Toxin-antitoxin system in persister cell formation

For living organisms, it is important to regulate the cell death and cell growth in certain conditions. It is also essential for the bacterial cell to survive under stress conditions by regulating cell growth and cell death. Typically, toxin-antitoxin systems act on crucial cellular processes such as translation, replication, cytoskeleton formation, membrane integrity and cell wall biosynthesis. Toxin-antitoxin system is prevalent in the bacterial genome. Through this toxin-antitoxin system, the bacterial cell can adapt to the stress by slowing down cell growth or inhibiting cell growth. The toxin-antitoxin systems are consist of the two gene set which encodes the stable toxin protein and unstable antitoxin. The toxin could disrupt the essential mechanism for the cellular process including DNA replication, mRNA stability, protein synthesis, cell-wall biosynthesis and the antitoxin mediates the effects of the toxin. There are different types of toxin-antitoxin systems and these are classified with the way of neutralization of toxin by antitoxin. Type I antitoxins are antisense sRNAs which inhibit toxin translation. And it is type III if the antitoxin inhibits toxin activity by binding the toxin protein. Type II TA systems are well studied than the other TA systems, and both toxin and antitoxin are a protein which forms a protein-protein complex to neutralize the toxin. Type IV antitoxins are a protein which prevents the toxin from binding its target not inhibiting the toxin directly, and type V antitoxins are proteins that cleave the toxin mRNA specifically. [11, 15, 17, 23]

As mentioned above, the mechanism of persister cell formation is not completely elucidated. However, the Toxin-antitoxin systems are considered as it is involved in the persister cell formation for inducing a dormancy status. Toxin-antitoxin systems were first considered as a genetic basis for the formation of persister cell from normal cells as they induce a state of dormancy via overproduction of toxin which led to increase in persistence. In 2006, DNA microarrays performed on carefully isolated dormant cells using a green fluorescent protein (GFP) reporter downstream from a ribosomal promoter, metabolically inactive cells were isolated via fluorescence-activated cell sorting(FACS) based on diminished fluorescence. (Figure 1.7., Fig1.8.) [39] Theses dormant cells showed tolerance to ofloxacin compared to normal growing cell and showed TA related genes with differential transcription included dinJ, yoeB, yefM, yafQ,dinJ, relE, and maze compare to the non-persister cell. Only two TA pairs (type II MqsR/MqsA and type I TisB/IstR-1) are directly related to persistence by their deletion. On the other hand, overproduction almost any TA system-related genes increase persistence. [11,15,20,34]

As mentioned above, the suggested mechanisms by which Toxin-Antitoxin systems results in persistence are related to dormancy. For the type II MqsR/MqsA TA system, toxin MqsR cleaves most of the transcripts in the cell so that the toxin leads the cell to dormant status by diminishing translation. For the type I TisB/IstR-1 system, the toxin makes cell to dormancy status by decreasing the proton motive force and ATP levels. Corroborating the importance of TA systems for persister cell formation, Lon protease has been shown to be necessary for persister cell formation as the Lon protease degrades

the antitoxin in type II TA systems. [20,21,23-25]

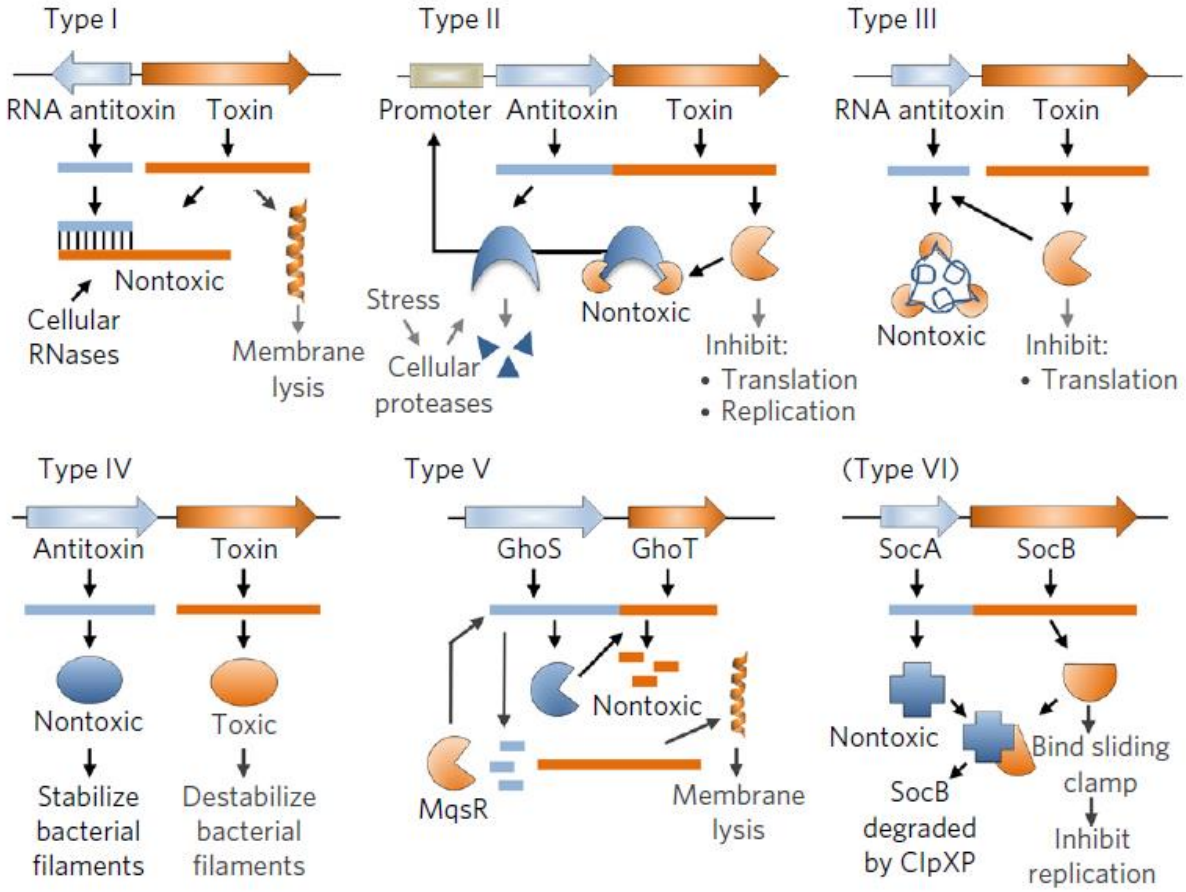


Figure 1.6. Toxin-antitoxin systems (R Page, W Peti - Nature chemical biology, 2016)

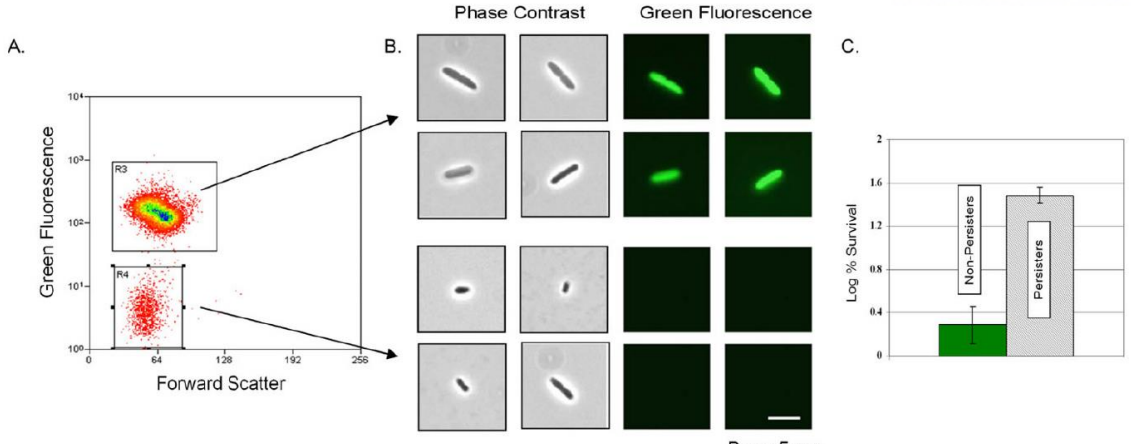


Figure 1.7. Isolation of persister cells (D Shah, Z Zhang, AB Khodursky – BMC, 2006)
E. coli ASV cells containing reporter cassette were grown in LB medium to mid exponential phase at 37°C (A) The different level of fluorescence was detected using forward light-scatter. (B) The sorted persister cell and non-persister cell were visualized by epifluorescent microscopy. (C) Sorted populations were treated with ofloxacin (5μg/ml) for three hours.

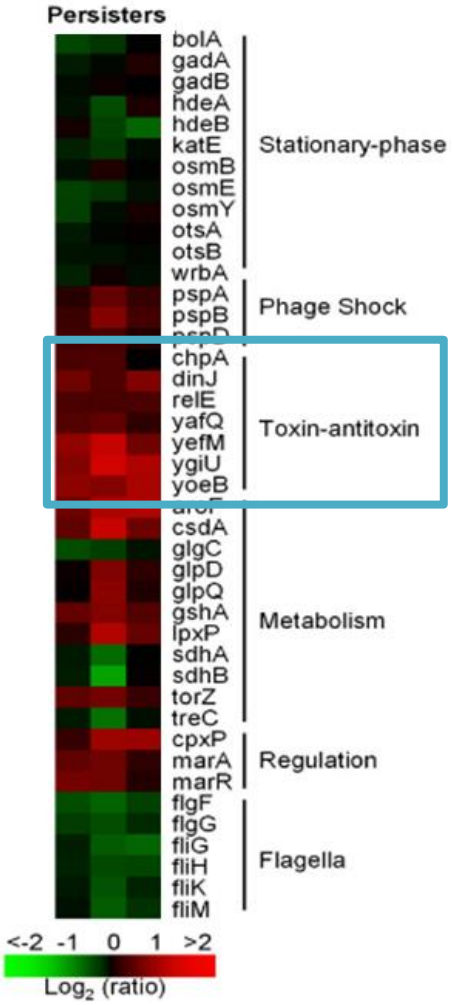


Figure 1.8. Gene expression profile of FACS isolated *E. coli* persisters. (D Shah, Z Zhang, AB Khodursky – BMC, 2006)
Heat map comparison of representative genes differentially expressed in persisters compared to non-persisters.

1.2.2 (p)ppGpp and persistence

Both ppGpp (guanosine tetraphosphate) and pppGpp (guanosine pentaphosphate) are important bacterial signaling molecules that are involved in the stringent response. The stringent response is a stress response of bacteria which react to amino-acid starvation, fatty acid limitation, iron limitation, heat shock and other stress conditions. Through stringent response, cellular metabolism can be reprogrammed from rapid to slow growth by regulating the expression of genes. (Durfee et al., 2008; Traxler et al., 2008) And in the stringent response, (p)ppGpp can regulate transcription of many other genes (up to 1/3 of all genes in the cell) which are involved in stress response and inhibit RNA synthesis. This makes the cell to avert resources away from division and growth to survive until conditions improve. The synthesis and degradation of (p)ppGpp have been most extensively characterized in the model system *E. coli*. Accumulation of (p)ppGpp promotes via RelA which is pppGpp synthase under stress condition. And SpoT is responsible for degradation of (p)ppGpp when the amino acid balanced is restored in the cell.

As described above, it is found that TA systems are one of notable elements in models of persistence, and (p)ppGpp is also regulated persister formation by stochastically switching to a high level through the connection with TA activity. [19,20,21,25] As the relA is activated stochastically or under stress conditions, it synthesizes ppGpp. And the accumulation of ppGpp inhibits the enzyme called exopolyphosphatase (Ppx) which degrades the polyphosphate. When the level of polyphosphate goes up in the cytoplasm of bacteria, it activates the Lon protease. Lon protease can degrade all known type II antitoxin in *E. coli*. Since the antitoxin which can neutralize the toxin effects is degraded, the toxin is free to act on its target RNA. This confers persistence. And one experiment showed that (p)ppGpp is involved in inducing persister cell through deletion of relA and spoT exhibit extremely low level of persister cells in exponentially growing cultures. And ppGpp level is positively correlated with the level of persistence accordingly. (Fig 1.9) [11, 23, 24,25]

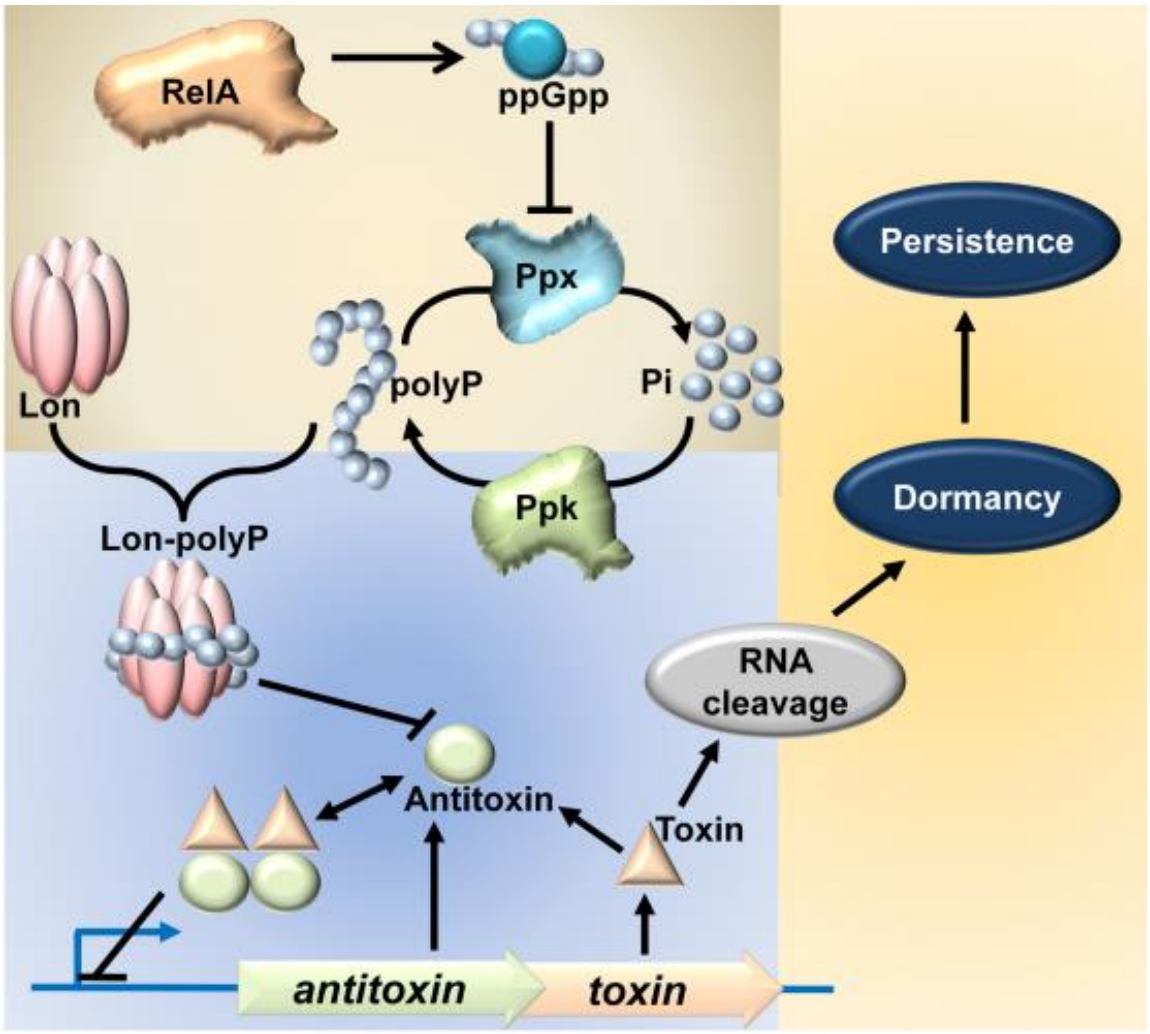


Fig 1.9. Model linking stringent response, TAS, and persistence (Maisonneuve et al., 2011, 2013)

Chapter 2 Experimental Method & Materials

2.1 Bacterial strain

The following Bacterial strains are used in this study. *Escherichia coli* str. MG1655, *Escherichia coli* BW25113 (Keio collections), *Acinetobacter baumannii* clinical isolation, *Klebsiella pneumoniae* clinical isolation, *Escherichia coli* MG1655 pUCDK which were obtained by transformation of pUCDK plasmid DNA into *E. coli* MG 1655 were used for bioluminescence test. *Bdellovibrio bacteriovorus* HD100 (predatory bacteria) was purchased from the German Collection of Microorganisms and Cell Cultures (DSMZ). All Strains were stored in 25% glycerol stock at -80°C. Strains of need except for *Bdellovibrio bacteriovorus* HD100 were streaked on Luria-Bertani broth agar (1.7% micro agar) plates (BD Difco™, USA) which contains proper antibiotics for each plasmid transformed to the bacteria and grown overnight at 37°C. The bacteria with pUCDK plasmid was grown with 100μg/ml of ampicillin. From the agar plates, a single colony was taken by the disposable tip and inoculated into 15ml of LB medium with shaking at 250rpm (liquid culture) at 37°C in the shaking incubator.

2.2 Used media

LB

All bacterial strain in this study except *Bdellovibrio bacteriovorus* HD100, grown in Luria-Bertani (LB) medium. It is composed of tryptone 10.0g, yeast extract 5.0g, and sodium chloride 10.0g per liter. The pH of media is 7.0±0.2. For the CFU, the LB agar plates were used with the agar concentration of 1.7%. All bacterial strain in this study except *Bdellovibrio bacteriovorus* HD100, grown in Luria-Bertani (LB) medium with shaking at 250 rpm (liquid culture) at 37 °C.

HEPES

Bdellovibrio bacteriovorus HD100 was grown in HEPES buffer media mixed with prey, N-(2-Hydroxymethyl) piperazin-N`-(2-ethanesulfonic acid) from Sigma Aldrich. Molecular weight is 238.30g/mol. BALO salt (2mM $MgCl_2$ and 2mM $CaCl_2$) is mixed with the 25mM of HEPES buffer as final concentration. And the pH was adjusted to 7.2.

DNB

DNB is an abbreviation for 'Diluted Nutrient Broth'. The final pH is 6.8±0.2. DNB media was used for dilution before doing the CFU (colony forming unit).

2.3 The culture of predatory bacteria, *Bdellovibrio bacteriovorus* HD100

The predatory bacterium *B. bacteriovorus* was grown routinely in HEPES buffer with 2mM of CaCl2 and 2mM of $MgCl_2$ salts added *Escherichia coli* MG1655 pUCDK as the prey. The prey,

Escherichia coli MG1655 pUCDK, was grown overnight in 15ml of LB broth at 37°C in the shaking incubator. And it was centrifuged down (2,000×g, 15 minutes) and adjusted the Optical Density to 1.0 of liquid cultured *Escherichia coli* MG1655 pUCDK with HEPES buffer. Add 1/100 of 0.45 μm (Merck Millipore) filtered grown *Bdellovibrio bacteriovorus* HD100

2.4 Induce persister cell formation by pre-treatment of antibiotics

I followed protocol from the previous research Dr. Thomas K. Wood in 2013 to induce a high population of persister cell using antibiotics pretreatment. An overnight culture of *E. coli* MG1655 was diluted 1:100 in 20ml of fresh LB broth and grown to mid-exponential phase (turbidity of 0.7-0.8 at OD_{600}). When it reaches the optical density (OD_{600}) of 0.7-0.8 treat antibiotics (rifampin or tetracycline 100μg/ml, 50μg/ml for each antibiotic) and incubate for 1 hour with shaking at 250rpm at 37°C in the shaking incubator. Wash the persister cell to remove the remaining antibiotic 1 time by centrifugation (2,000×g, 15 minutes) with HEPES buffer. Adjust the turbidity 1.0 (OD_{600}). The number of the viable cells measured with CFU at this time point was set as an initial number of induced persister cell in the further experiments. Control cell without pretreatment of antibiotics and resistant cell with pretreatment of antibiotics were prepared as the same way.

2.5 Development of rifampin-resistant cell

E. coli MG 1655 WT resistant cell to rifampin was developed as a control of persister cell to check whether the remaining antibiotic affects to BALOs predation. A single colony of *E. coli* MG 1655 WT was inoculated to 15ml of LB and overnight cultured at 37°C in the shaking incubator. Spread 100μl of the cultured cell on LB plate containing rifampin(100μg/ml) and incubate at 37°C incubator. Take the single colony from the rifampin LB plate and inoculate into fresh LB media containing rifampin. And incubated in shaking incubator at 37°C overnight. After 24h the 1ml of the sample was taken and inoculated to fresh 9ml of LB media with rifampin added. Repeat those process 3 times more and the survived cell from the culture with rifampin was stored in 25% glycerol stock at -80°C.

2.6 Persister cell predation by *Bdellovibrio bacteriovorus* HD100

Induced *E. coli* MG1655 persister cell with the pretreatment of antibiotics for 1h (rifampin or tetracycline 100μg/ml, 50μg/ml for each antibiotic) were prepared as turbidity 1.0 with HEPES buffer. At the same time control cell and resistant cell were prepared as the same way. Added 1/100 of overnight cultured *Bdellovibiro bacteriovorus* HD100 which were filtered with 0.45μm syringe filter to 10ml of each persister prey and control prey. After 18h and 24h from the point where predatory bacteria was added, OD and CFU were measured.

2.7 Antibiotic susceptibility test of induced persister cell

After pretreatment of antibiotics, cultures were centrifuged down and resuspended with HEPES to remove the remaining antibiotics and then adjusted to the turbidity of 1.0 at OD_{600}. Then 1ml of an aliquot of persister cells (turbidity of 1.0 at OD_{600}) from each sample were centrifuge down (13,000×g, 1 min). Each aliquot of samples was resuspended with LB containing antibiotics (ciprofloxacin 5 μg/ml). Incubate the samples at 37°C in the incubator. And the samples were taken after 3hours and 24hours to measure the cell viability based on the CFU. Colonies were counted after incubation at 37°C overnight. Experiments were conducted at least six independent cultures.

2.8 SDS toxicity test

The bioluminescent strain of *E. coli* MG1655 pUCDK was used to measure the prey population. The detergent which was used in this study is SDS (Sodium Dodecyl Sulfate). To determine the appropriate concentration of detergent to halt the predation without killing prey cell, the toxicity test for prey and BALOs were conducted. First, the different concentration of detergent was prepared in 96 well plates by dilution prior to adding the prey cell only. (The highest concentration was 8%) After that BALOs were added together with prey cell. (with BALOs, the highest concentration was 0.02% since the BALOs were more sensitive to detergent than its prey) The MOI for predator and prey were around 16. The bioluminescent were measured (GloMax® 20/20 Luminometer, Promega, USA) for 4h at 30°C. CFU and PFU were performed with the concentration of detergent around where BALOs shows predation. Each prey sample turbidity was adjusted with DNB media for BALOs predation.

2.9 Isolate residual prey cell using 0.1 % SDS

The prey, *E. coli* MG1655, was overnight cultured in the LB media at 37°C and adjusted turbidity of 1.0 at OD_{600} prey with HEPES buffer. Overnight grown *Bdellovibrio bacteriovorus* is filtered by 45μm syringe filter (Merck Millipore LTD., USA) to remove the prey cell and added as 1/100 diluted to 100ml of the prey of turbidity 1.0 at OD_{600}. Incubate the prey and BALOs at 30°C for over-night. Treat the chosen concentration 0.1% of Sodium Dodecyl Sulfate(SDS) to prey and predator mixture for 1 hour at 30°C in shaking incubator. After treatment of SDS for 1h, wash out the SDS from the prey by centrifuge down (2,000×g, 15 minutes) and resuspend with HEPES buffer one time.

2.10 Antibiotic susceptibility test of the residual cell from predation

The isolated residual prey cells were further tested for antibiotic susceptibility test. The total cell pallet after washing SDS was resuspended with 1ml of HEPES and 100μl aliquots were resuspend

with 900μl LB contained ciprofloxacin (5 μg/ml). LB which is not containing antibiotics were used as a control. Incubate it at 30°C in the incubator for 3h. After 3h the CFU was checked.

Chapter 3 Results

3.1 Antibiotic persister is resistant to predation by *Bdellovibrio bacteriovorus* HD100

To compare the characteristic between antibiotic persister and predation persister, first I checked whether the antibiotic persister has resistance towards predation like residual cell after predation. For predation test, inducing a high population of persister cell is important for further study. The protocol used in this study to induce a high level of persister cell is followed from Dr. Thomas K. Wood paper. In this paper, rifampin was used to induce persister cell to disrupt protein synthesis that mimics the effects of toxins such as MqsR and TisB. I tested whether a rifampin-pretreated cell (rifampin100 μg/ml) shows slow loss of viability that is characteristic of persister cells compare to control cell with the prolonged exposure (6h) of ciprofloxacin (5 μg/ml). *E. coli* MG 1655 strain was used mainly in this study for inducing persister cell. Ciprofloxacin was used to isolate persister cells since this antibiotic is effective at killing cells in all phases of growth. Therefore, I confirmed that I can induce the "true" persisters. (Figure3.1.1.)

Using the protocol mentioned above to test the property of persister cells, I induced a high level of persister cells and see if predation by *B. bacteriovorus* HD100 occurs with persister cell as prey. After inducing the persister cells the preparation of prey cell was same with a normal predation we use for the daily culture of predatory bacteria. The control cell is one that without pretreatment of rifampin. And rifampin-resistant cell which was mutationally gained the resistance against rifampin was used in case of the remaining antibiotic after washing effects the predation by *B. bacteriovorus* HD100. The turbidity and CFU were measured after 18h and 24h from predation starting point. (Figure3.1.2.) As shown in the Figure3.1.2., the predation of control cell and resistant cell occurred well, however, when the persister cells are used as prey it showed that the persister cell has resistance toward *B. bacteriovorus* HD100 predation. The other bacterial strain *A. baumannii*, *K. pneumoniae* and *E. coli* BW 25113 which were often used as prey were induced as persister cell. Persister predation with different bacterial strains, also showed that the predation was blocked. (Figure 3.1.3.) Furthermore, another antibiotics, tetracycline which is translator inhibitor were also able to generate persister cell which shows resistant to predation.

From the following result, it is found that the antibiotic persisters were resistant to B. bacteriovorus predation.

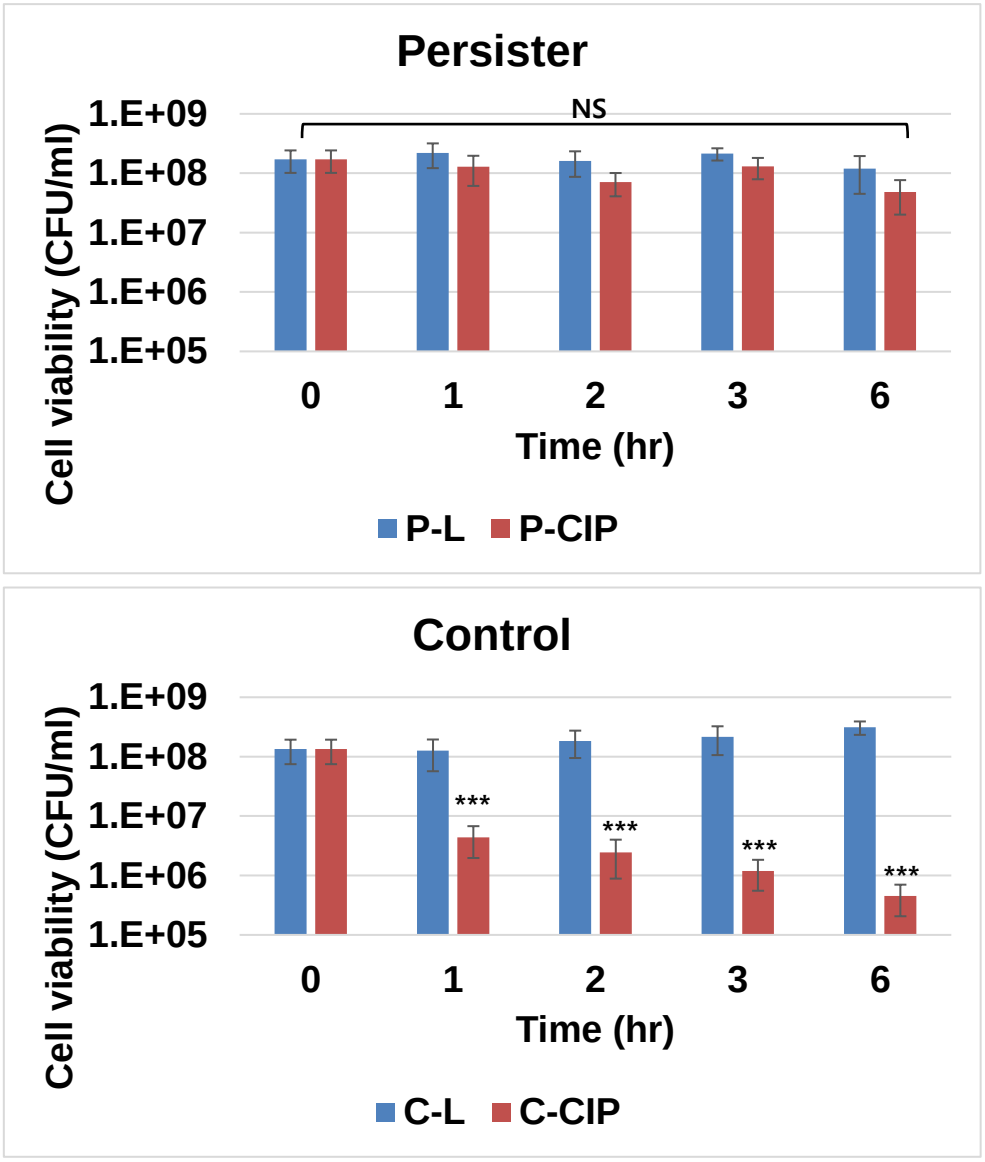
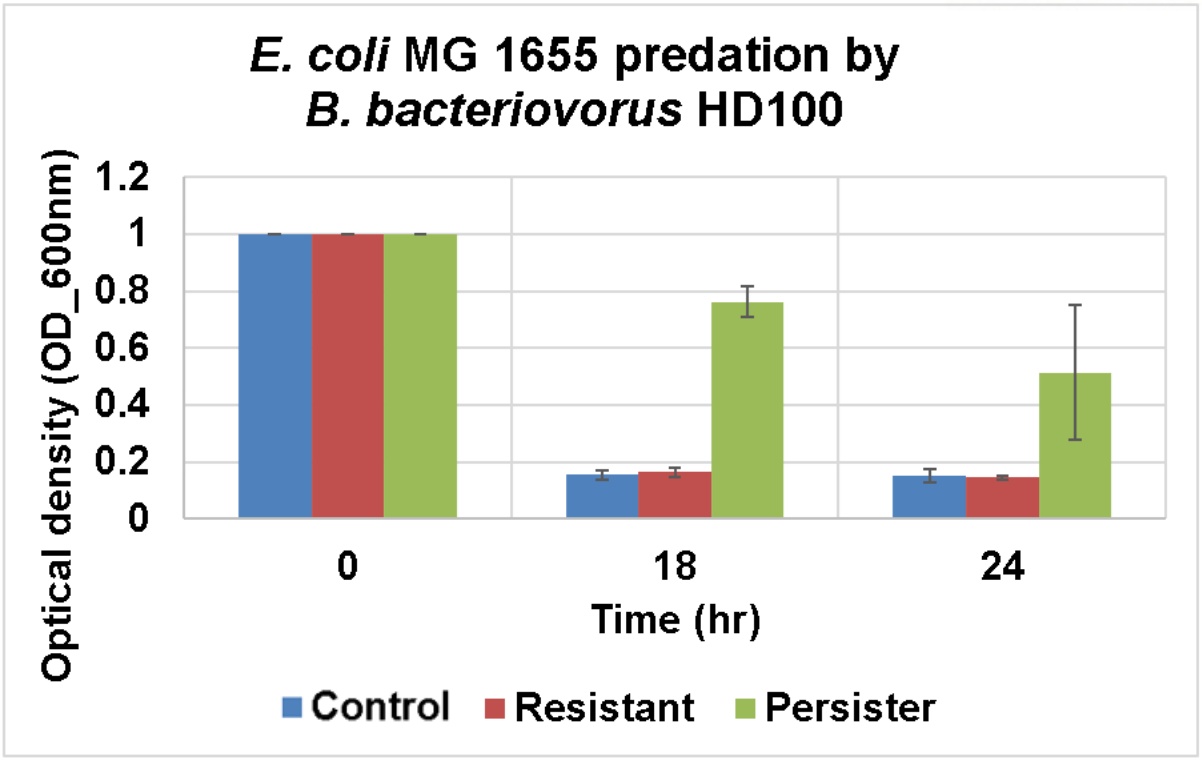


Figure 3.1.1 Cell viability of rifampin-induced persister under prolonged antibiotic exposure

Cell viability under prolonged antibiotic (ciprofloxacin 5 μg/ml) exposure of rifampin-induced (100 μg/ml, 1h) persisters. Antibiotics were pretreated to mid-exponential phase cultures of *E. coli* MG 1655(turbidity of OD_{600} 0.8-1.0) prior to antibiotic (ciprofloxacin 5 μg/ml) exposure for 6h.
(P – Persister / C – Control / L – LB / CIP – Ciprofloxacin treated)



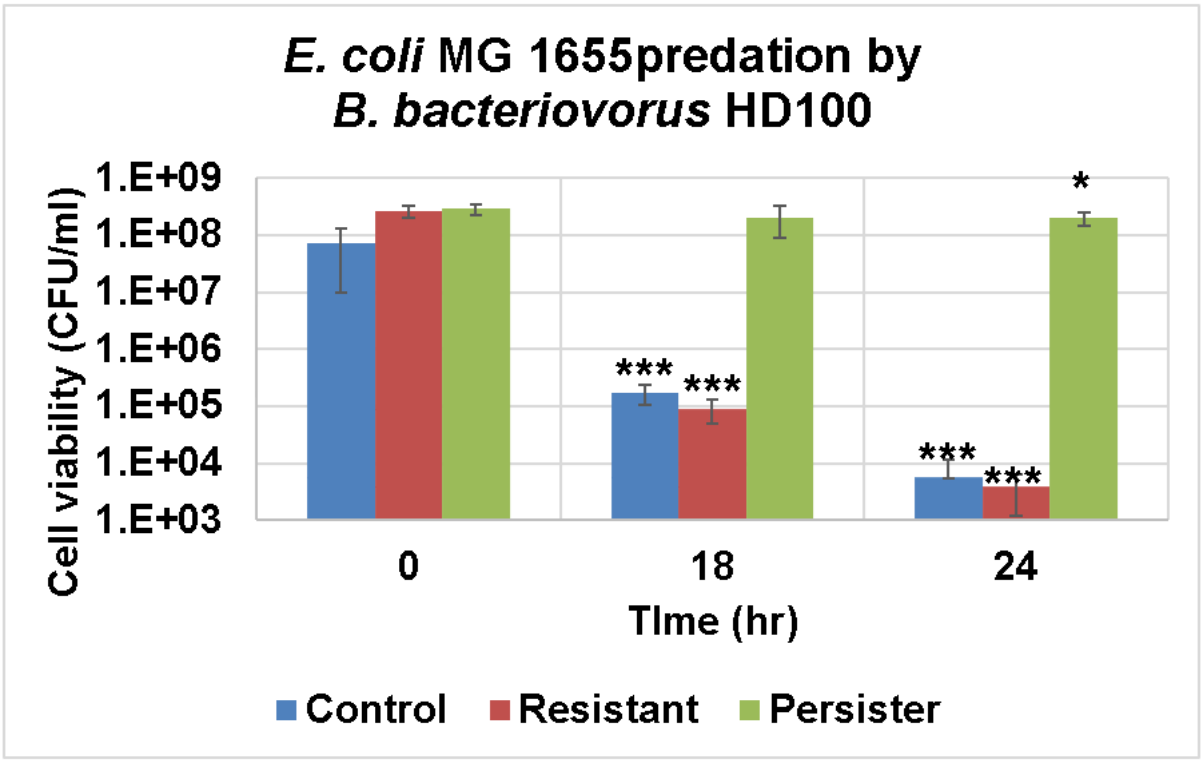


Figure 3.1.2 The predation of persister, resistant cell and control cell by *Bdellovibrio bacteriovorus* HD100

OD and CFU was measured at 18hr and 24hr after predatory bacteria were added.

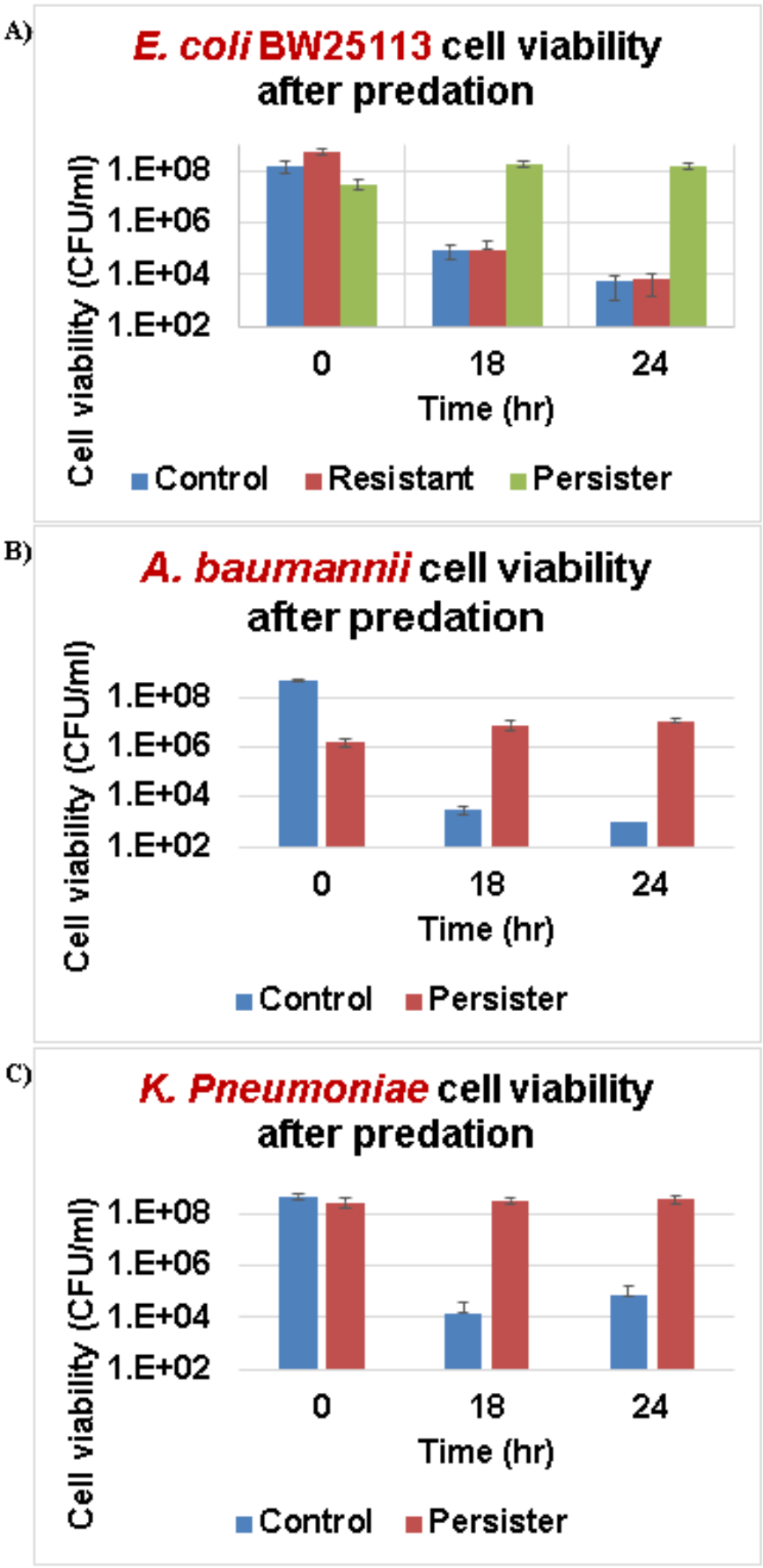
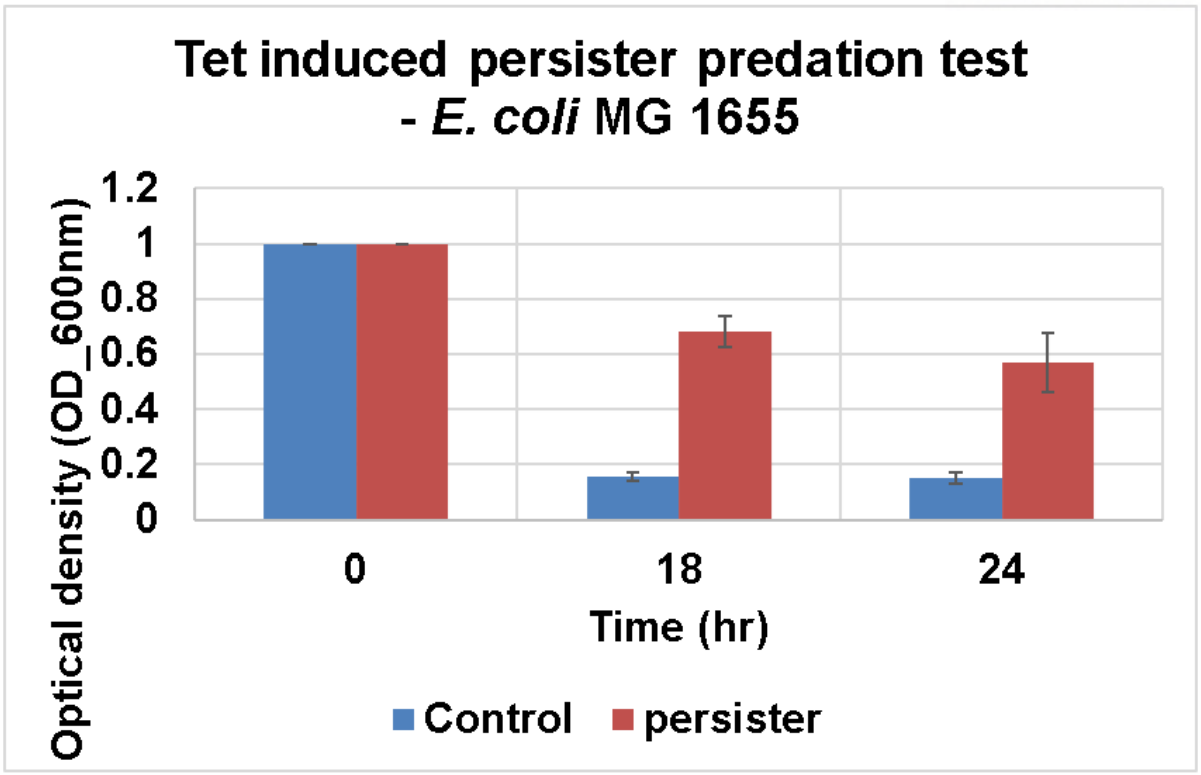


Figure 3.1.3 predation of persister by *B. bacteriovorus* HD100

Different strain were induced as persister cell for predation test



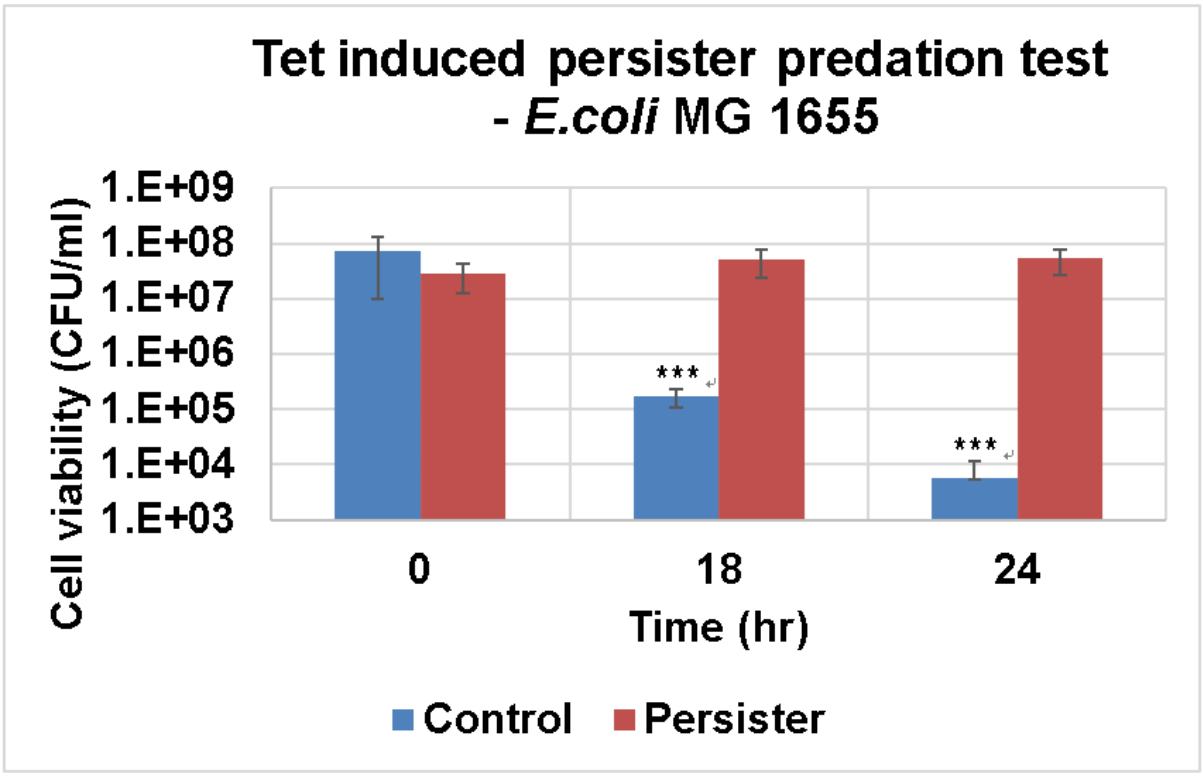


Figure 3.1.4 Predation of persister which induced with pretreatment of tetracycline

Tetracycline(50μg/ml) pretreatment for 1h was used to induce persister cells.

3.2 Predation persister show resistant to antibiotics

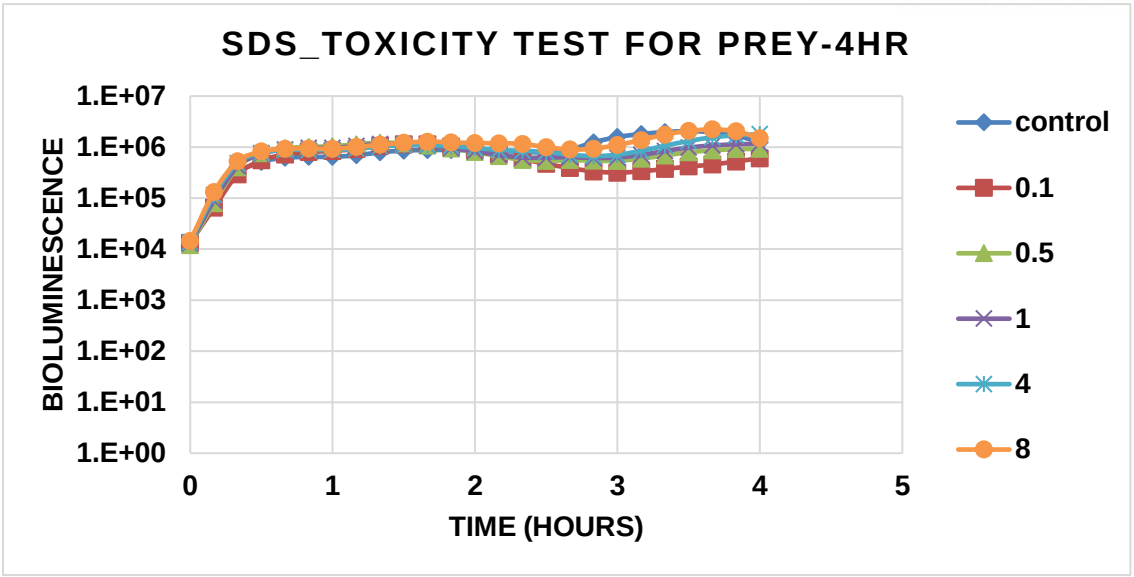
Secondly, an experiment performed to examine whether the predation persister has a similar property to antibiotic persister such as tolerance to antibiotics treatments. In this experiment, it is important to isolate the residual cell after predation for testing antibiotics tolerance. Centrifugate the overnight cultured mixture of prey and predatory bacteria to isolate the remaining prey bacteria and concentrate 100-fold. However, there was no significant difference in viable cell number of prey between before and after centrifugation. It could be explained as reattacking of predatory bacteria occurred during centrifugation. Hence, we needed to develop a way to stop predation without harming the remaining prey. And an idea was obtained from the detergent study which tests the different sensitivity of prey and predatory bacteria against detergent treatment.

In the detergent experiment, I figured out that the detergents are much more effective against to predatory bacteria than prey at the same concentration. Sodium Dodecyl Sulfate (SDS) is one of detergent which tested in the detergent study. The fitness of B. bacteriovorus HD100 was determined with predation activity through bioluminescence of prey. When the detergent was added to prey only, prey bioluminescence still did not decrease at a concentration of 8% of SDS (weight/volume). (Fig3.2.1.(A)) However, when predatory bacteria were added, bioluminescence of prey did not reduce from 0.01% of SDS or higher which means predation did not occur. (Fig3.2.1.(B)) And confirmed with CFU and PFU. (Fig3.2.1.(C)) We chose 0.1% of SDS which is much higher from the concentration where effects predatory bacteria but not prey. Before using 0.1% of SDS to isolate residual prey cell, we tested whether SDS has any effect on inducing persister formation at a given concentration. Predation of SDS pretreated prey and unpretreated prey as a control were tested since persister cells show resistance to predation by *B. bacteriovorus* HD100. (Fig3.2.2.)

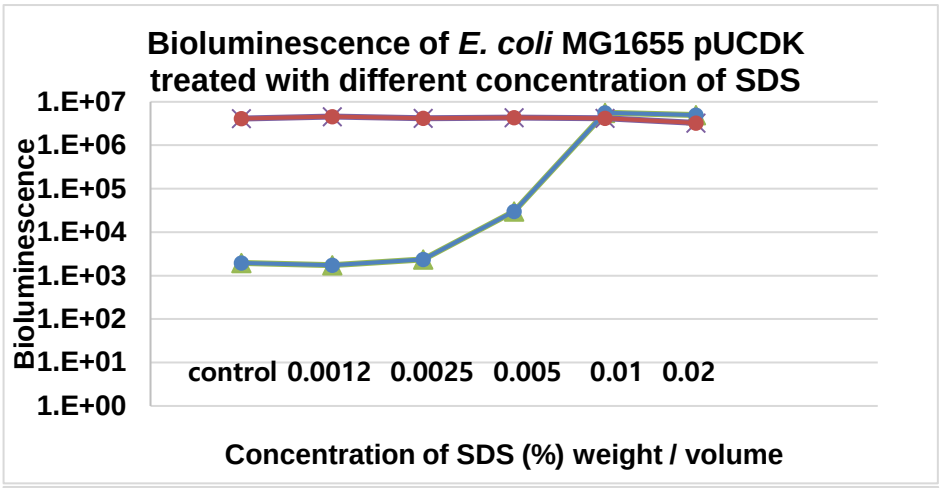
After isolating residual prey cell after predation, the ciprofloxacin was treated for 3h to check the susceptibility of the residual cell. And the residual cell from predation shows higher tolerance against antibiotic treatment compared to the control cell. As a result, the predation persister also shows the property of antibiotic persister. (Fig 3.2.3.)

From these studies, I found out that the antibiotic persister cells were also resistant to *B. bacteriovorus* predation and, conversely, the predation persister cells were better at surviving antibiotic treatments.

A)



B)



C)

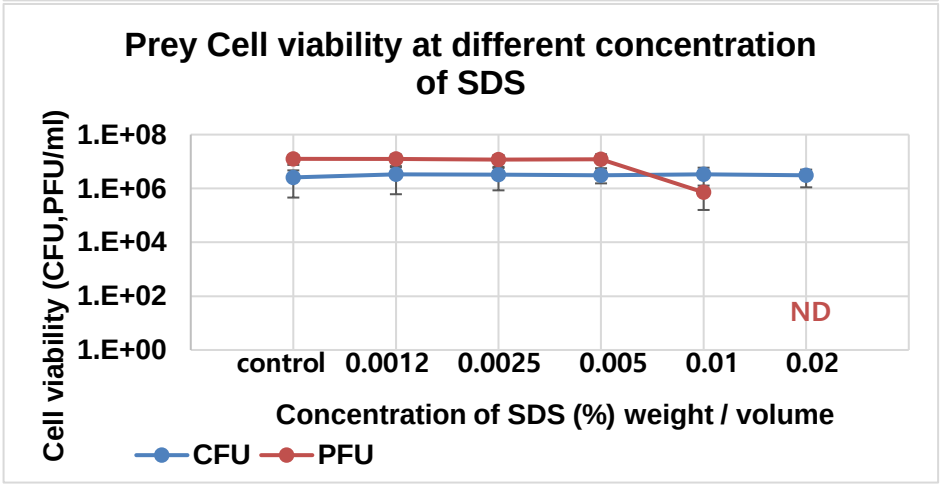


Figure 3.2.1. Different sensitivity of prey and predatory bacteria against SDS treatment (A)Bioluminescence of *E. coli* MG1655 pUCDK for 4h with SDS treatment (the highest concentration is 8%) (B) Bioluminesence of *E. coli* MG 1655 pUCDK and *B. bacteriovorus* HD100 (the highest concentration is 0.02%)(C) CFU and PFU after exposure SDS for 4h

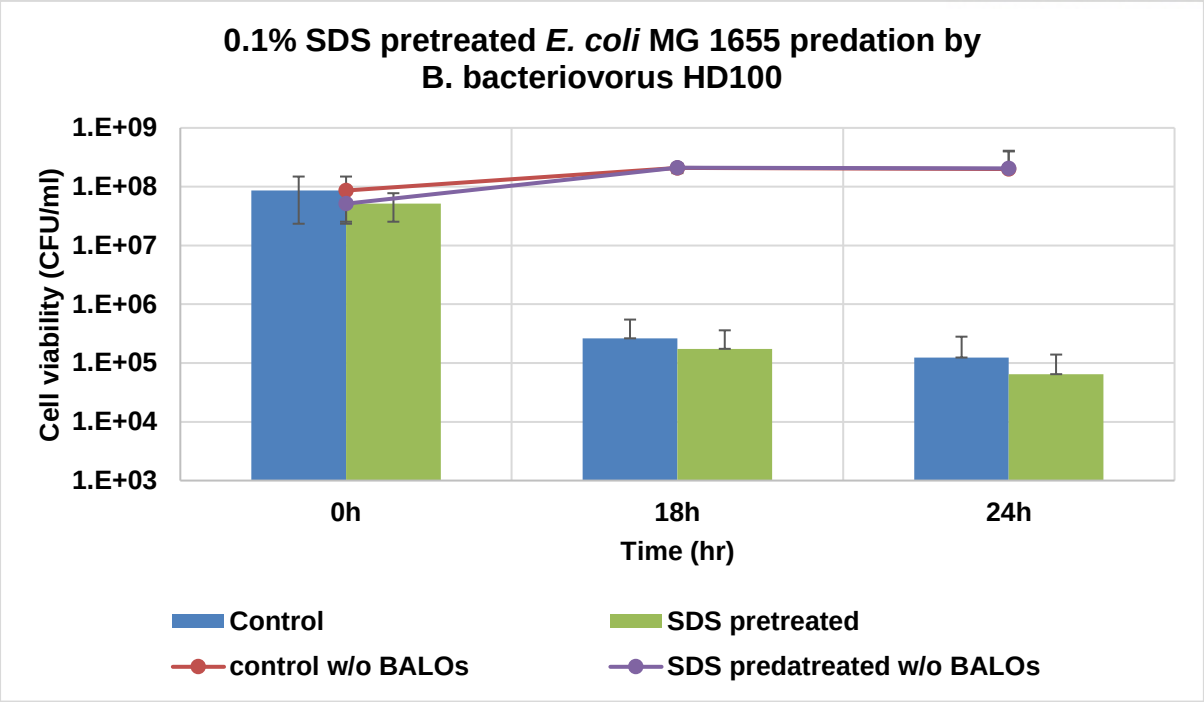


Figure 3.2.2 Predation of SDS pretreated prey cell by *B. bacteriovorus* HD100

Pretreatment of 0.1% SDS to *E. coli* MG1655 for 1h does not show any effect to predation

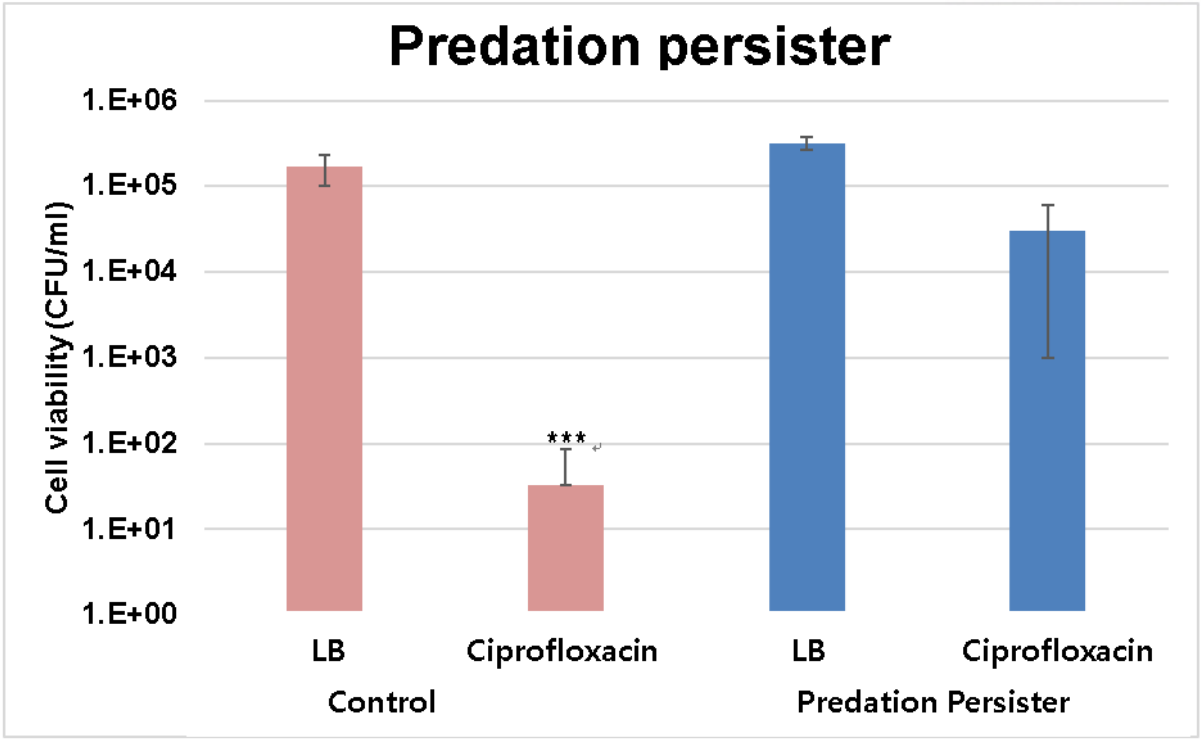


Figure 3.2.3 Cell viability of predation persister after 3h antibiotic treatment

Treatment of ciprofloxacin 5 μg/ml were prolonged for 3h after isolating the remaining prey cell, *E. coli* MG 1655, from predation.

3.3 Transcriptomic analysis of antibiotic persister and SCVs

Lastly, I analyzed the transcriptome data of SCVs looking specifically for persister related genes to compare the gene expression patterns between antibiotic persister and SCVs which has resistance to predation. In the antibiotic persister, the toxin-antitoxin systems are considered as a genetic basis for the formation of persister cell since the TA system can induce the dormant status which gives the antibiotic tolerance. Therefore, I wondered where the predation resistance of SCVs comes from and whether the predation resistance obtained from the dormant status such as antibiotics persisters. The paper from Kim Lewis, they isolated pre-exist persister of the population in dormant status using an *E. coli* strain which expresses degradable GFP from a ribosomal promoter that is only active under conditions of rapid growth was used to isolate non-growing dormant cell. Fluorescence-activated cell sorting (FACS) isolated cell were used for genome-wide expression profiling. (Fig 1.7.) And compare to the non-persister cell, 45 genes showed a 2-fold increase in expression at least. (Fig 1.8.) And among the overexpressed genes in the transcriptome of persister, the genes which contribute to the dormant state were elements of TA modules such as dinJ, yoeB, ydfM. [39,41] Even though, multiple pathways were involved for persister formation including TA modules, SOS stress response, oxidative stress, slow metabolism, however, dormancy through TA modules can be explained for the multidrug tolerance. Thus, I looked at some specific genes which were related to TA systems in the transcriptome data of SCVs. With the transcriptome data, I looked through the transcriptome data of SCVs especially for TA modules related genes which are shown in the figure 3.4.1. And I could see that some of TA system-related genes were upregulated compared to the WT however, it was not significant. (<2-fold) [39] From this data, the given temporal resistance to predation were not completely due to the dormancy drove by the TA modules.

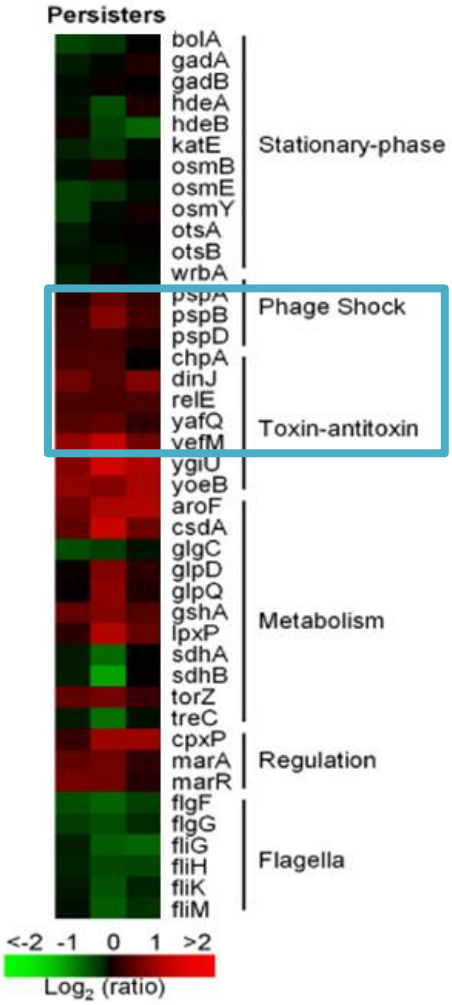


Figure 1.8. Gene expression profile of FACS isolated *E. coli* persisters. (D Shah, Z Zhang, AB Khodursky – BMC, 2006)

Heat map comparison of representative genes differentially expressed in persisters compared to non-persisters.

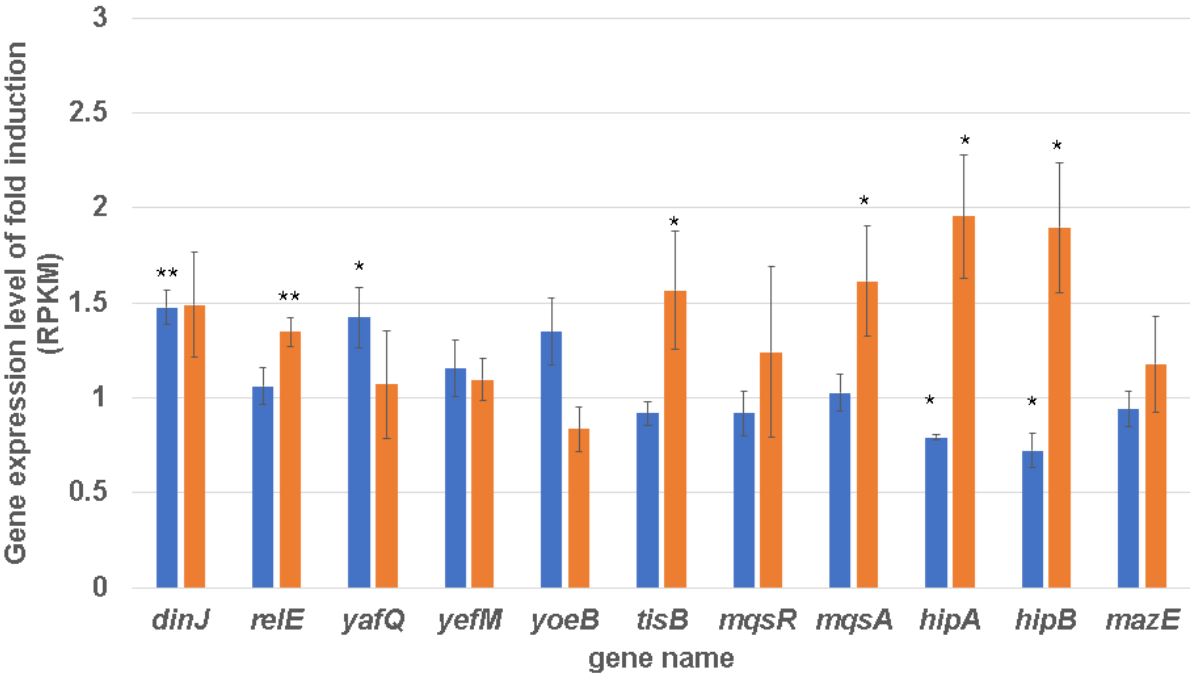


Figure 3.4.1 transcriptome analysis of SCVs focused on the antibiotic persister related genes

Chapter 4 Conclusion

This study is needed for understanding the prey and predator interaction for better application of the predatory bacteria as living antibiotics in the future. Also, as the failing of antibiotics due to the antibiotic-resistant pathogens, understanding bacterial strategy for surviving under stress is important. And one strategy for the bacterial cell is “bet-hedging strategy” known as bacterial persisters.

In this study, the characterization of antibiotics persister and predation persister were performed which started from the curiosity of any possibility that they are related. From the experiments, I figured out that they overlapped in a way that they share the property of each other. The antibiotics persister showed the resistance toward predation and the predation persister showed antibiotics tolerance. And to study where this persistence comes from, the transcriptome data were compared. Through my study, I have demonstrated that although they were showing similar characteristics, it seems that the factors what gives the persistence were not the same.

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