Starvation induced physiological changes in *Vibrio cholerae*

By
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A thesis submitted to the
Board of Studies in Life Sciences

In partial fulfillment of requirements
for the Degree of

DOCTOR OF PHILOSOPHY
of
HOMI BHABHA NATIONAL INSTITUTE

June 2020

Homi Bhabha National Institute

Report of Ph.D. Viva-Voce

Board of Studies in LIFE Sciences

A. General Details:

1. **Name of the Constituent Institution:** Bhabha Atomic Research Center, Mumbai

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3. **Enrolment Number:** LIFE01201404007
4. **Date of Enrolment in HBNI:** 01.08.2014
5. **Date of Submission of Thesis:** 07.02.2020
6. **Title of the Thesis:** Starvation induced physiological changes in *Vibrio cholerae*

7. **Number of Doctoral Committee Meetings held with respective dates:**

Review Period	Date	Review Period	Date
1. Aug 2015 – Aug 2017	25.08.2020	2. Aug 2016 – Feb 2017	23.02.2017
3. Feb 2017 – Feb 2018	31.1.2018	4. Feb 2018- Dec 2018	22.01.2019
5. Pre-synopsis	14.11.2019		

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Recommendations of the Examiner 2 (Thesis Evaluation) (i) accepted, (ii) ~~accepted after revisions~~, or (iii) ~~rejected~~:

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1. **Date of Viva Voce Examination:** 29.06.2020

2. **Name and affiliation of External Examiner:** Dr. Anirban Banerjee, Assistant Professor, Department of Biosciences and Bioengineering, IIT Mumbai.

3. **Whether there were other experts / faculty/students present?** Please enclose a soft copy of attendance sheet indicating participation in person/over video as per proforma given below at (5) Attached.
4. **Recommendations for the award of the Ph.D. degree: Recommended / ~~Not Recommended~~**
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The present work explains the starvation biology and persister cell formation in *V. cholerae*. Antibiotics and starvation both can induce the persister cell population. However, both populations differ in their stress tolerance and reversion back to the normal population. The starved *V. cholerae* cells showed reduced genome ploidy. The ploidy depends on the nutrient availability, and genome content is controlled by DNase activity. The survival under carbon, nitrogen, and phosphate limitation was also studied. The *V. cholerae* showed acute sensitivity to phosphate limitation. One of the primary causes of cell death was active metabolism and protein synthesis in phosphate-limited cells. Irregular metabolism under phosphate starvation can lead to the accumulation of NADH, membrane damage, and cell death. Cells exposed to protein synthesis inhibitors did not form persisters. Starvation, anaerobic conditions, and inhibition of ATP synthesis also induced persisters, but not when protein synthesis was inhibited. Glucose changed antibiotic sensitivity in a growth phase-dependent manner. The stationary phase cells, which show higher antibiotic tolerance, could be sensitized to ciprofloxacin and ampicillin by glucose supplementation.

The work presented in the thesis is comprehensive and led to 4 publications and multiple conferences, including one international conference on persister cells. This work also opened new avenues in the area of persister cells and starvation biology of *V. cholerae*.

5. Attendance at Viva Voce (Doctoral Committee, External Examiner, others):

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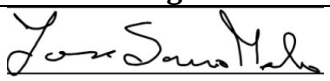
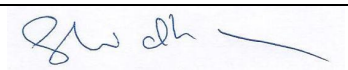
1.	**Chairman**	Dr. J. S Melo	
2.	**Convener** (Guide)	Dr. Shashidhar. R	
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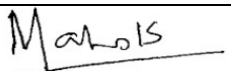
6	**Member**	Dr. Mahesh Subramanian	
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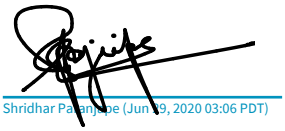


Shridhar Suresh Paranjape

DECLARATION

I, hereby declare that the investigation presented in the thesis has been carried out by me. The work is original and has not been submitted earlier as a whole or in part for a degree/diploma at this or any other Institution / University.

Shridhar Suresh Paranjape

List of Publications arising from the thesis

Journal

1. "The ploidy of *Vibrio cholerae* is variable and is influenced by growth phase and nutrient levels", Paranjape, S.S., Shashidhar, R., *FEMS Microbiol. Lett*, **2017**, 364
2. "Inhibition of protein synthesis eradicates persister cells of *V. cholerae*", Paranjape, S.S., Shashidhar, R., *3 Biotech*, **2019,** 9:380
3. "Comparison of Starvation-Induced Persister Cells with Antibiotic-Induced Persister Cells", Paranjape, S.S., Shashidhar, R., *Current Microbiology*, **2019,** 1495–1502
4. "Glucose Sensitizes the Growing and Persistent Population of *Vibrio cholerae* to Antibiotics", Paranjape, S.S., Shashidhar, R., *Archives of Microbiology*, **2019**

Conferences

1. Paranjape, S.S., Shashidhar, R., **2018.** Altered physiological conditions lead to antibiotic persistence in *Vibrio cholerae*. EMBO Symposium on Bacterial persistence and antimicrobial therapy**.** University of Basel, Switzerland.
2. Paranjape, S.S., Shashidhar, R., **2018.** Simple sugars can increase the antibiotic susceptibility of Gram-negative pathogens. Life Science Symposium, Mumbai, India.
3. Paranjape, S.S., Shashidhar, R., **2016.** The *Vibrio cholerae* reduces its DNA during starvation - survival tactics of food-borne pathogen. International Conference on Current trends in Biotechnology" (ICCB16)**.** Vellore, India

Others

Conference publication

1. Paranjape, S.S., Shashidhar, R., **2019.** Carbon, Nitrogen and Phosphorous assimilation in *V. cholerae* – a case of imperfect nutrient sensing. Access Microbiology. 1- 1A.

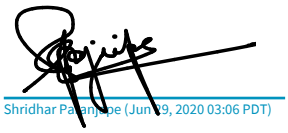
GenBank submission

Quinolone resistance determining region (QRDR) of ciprofloxacin persister cells of *Vibrio cholerae* – 3 locus.

1. *gyrA* (MN458507)
2. *gyrB* (MN458508)
3. *parC* (MN458509).

Manuscript under preparation

1. Phosphate limitation leads to futile metabolic cycles and cell death in *Vibrio cholerae.*



Shridhar Suresh Paranjape

DEDICATIONS

This thesis is dedicated to my son, who reminds me of what it takes to be a caring father and a responsible man

ACKNOWLEDGMENTS

A research advisor is the most important person you meet in your life who can shape not only as a scientist but also as a mature human being. I learned (and will continue to learn) a great deal from my Guru Dr. Shashidhar. R. He managed to take out time for his students despite his busy schedules. His continued support and mentoring helped me sail through the ups and downs of professional and personal life. I thank him with sincere gratitude. The first paper in Ph.D. life is always special. If not for the critical observations of my supervisor, the paper would not have seen the days light.

At the outset, I would like to express my appreciation to Dr. J. S. Melo, chairman of my doctoral committee, my committee members Dr. Hema Rajaram, and Dr. Mahesh Subramanian for their positive criticism and support throughout my tenure. The committee was cordial and mentored me for five long years, keeping me focused on making my dream a reality.

Associate Director Dr. Venugopalan and both, the former Head of Division Padmashri Dr. Sharad P. Kale and the current head Dr. S. K. Ghosh and have been very supportive throughout my Ph.D. life.

I would like to appreciate my department's "peers," Dr. Sahyog Jamdar, Dr. Bibhuti Misra, Dr. Sachin Hazare, and Dr. Vandan Nagar, who always would help me even on short notice. I happen to be on talking terms with almost all the scientific staff in the division and their concern towards me, my work and family has made my stay in the department warm.

I want to thank my global "peers," starting with Dr. Kim Lewis (Northeastern University college of science, Boston, USA), Dr. Nathalie Questembert-Balaban

(Professor of Physics, The Hebrew University of Jerusalem), and Professor Dan Andersson (Uppsala Antibiotic Center, Sweden). They all helped me develop a deeper understanding of bacteria and their survival mechanisms.

My admirations go to my lab colleagues Sagar Vehale, Dr. Aniruddha Vaidya, Dr. Sandeep Newase, Kirti Sawant, Lipika Pansare-Godambe, Indu Pant, and Lokesh Mishra. Thanks to my friends and batchmates from training school days, Shikha Pachauri, Megha Sodani, Pooja Negi-Rao, Priya, Avi Raizada, Raj Bhadur Singh and Rohit Sharma. The support staff in the FTD office TC&TSC office, the lab technicians (Kalpana Bagade, Vaishali Mahale, and Shabbir Alam) have made me feel homely in the department.

Thanks to the reviewers of my accepted and rejected papers for spending their time in reading my work and giving me useful comments. Special thanks to the Homi Bhabha National Institute for the International Travel Grant, 2018. A special thanks to countless bacteria especially *Vibrio cholerae*, who lived and died for my thesis. Not to mention my appreciation towards my bike and my computer who never failed me and kept me and my results intact.

Thanks to my parents in and outside the law for their support.

It appears customary to thank the spouse in the end, and I didn't want to break the norm. Akshata has been part of everything I have been doing and not doing. This thesis is as much hers as is mine.

CONTENTS

SUMMARY

The present work attempts to explore the starvation biology and antibiotic persistence of *V. cholerae,* from a physiological perspective.

Abiotic stress leads to the formation of persister cell sub-populations that can tolerate variety of stresses. In the present study, two such sub-populations, one triggered by nutrient limitation (E-cells) and another by exposure to a lethal concentration of antibiotics (P-cells) were isolated and characterized. Both the sub-populations differ concerning morphology, temperature tolerance, oxidative stress tolerance, and recovery pattern. The two subpopulations were found to be fundamentally distinct, and each subpopulation might offer survival advantages during antibiotic exposure or nutrient limitation. The starvation induced persisters were further characterized for changes in macro molecular content during starvation survival. The present work established that *V. cholerae* is a (mero-) oligoploid or polyploid bacteria. The ploidy levels per cell were found to be growth-phase regulated. In addition to the growth phase, an external parameter such as nutrient level influences the ploidy. The activity of the DNase enzyme increased during starvation that decreased the ploidy. The ploidy was restored to the pre-starvation levels with nutrient supplementation. Additionally, the survival under carbon, nitrogen, and phosphate limitation was also studied. The *V. cholerae* showed acute sensitivity to phosphate limitation. One of the primary causes of cell death was active metabolism and protein synthesis in phosphate limited cells. Irregular metabolism under phosphate starvation can lead to the accumulation of NADH, membrane damage, and cell death. The *V. cholerae* might never have experienced complete phosphate limitation, due to its sizeable intracellular phosphate and the abundance of phosphate in its natural habitat estuarine water.

Apart from starvation, antibiotics also induce persister cell formation. Cells, when exposed to protein synthesis inhibitors (kanamycin, chloramphenicol, tetracycline, erythromycin, and gentamicin), did not form persisters. Starvation, anaerobic conditions, and inhibition of ATP synthesis also induced persisters, but not when protein synthesis is inhibited. Interestingly, glucose changed the antibiotic sensitivity in a growth phase-dependent manner. The stationary phase cells, which show higher antibiotic tolerance, could be sensitized to ciprofloxacin and ampicillin by glucose supplementation. Furthermore, the study emphasizes on the role of protein synthesis for persister formation, persister maintenance, and dormancy maintenance in the *V. cholerae*.

Findings presented in the thesis are not a means to an end; instead, are windows of opportunity and fascinating research in areas of antibiotic tolerance associated with starvation, starvation recovery, pathogen survival in the famine conditions, and fundamental metabolic pathways involved in central metabolism.

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1 INTRODUCTION AND REVIEW OF LITERATURE

1.1 Starvation biology in bacteria

1.1.1 Fast and famine, with a rare feast

Bacteria often experience acute and prolonged nutrient limitation. Bacteria have evolved various metabolic pathways to survive the long duration of starvation[1]. For example, the carbon content in the marine system can be as low as 1 mg mL^{-1} to 0.5 mg mL^{-1} compared to the 10 mg mL^{-1} used in typical bacterial growth media[2]. In nature, bacteria can experience two different cases of starvation. One is a nutrient limitation, and another is energy limitation. Nutrient limitation limits the growth of an organism because of the non-availability of one of the few essential nutrients. The most common limiting nutrient in terrestrial and aquatic ecosystems are nitrogen, phosphorus, and iron. The energy limitation arises because of an overall reduction in a total flux of available energy. Most of the time, microbes can be simultaneously energy and nutrient-starved, since many compounds act as both energy and nutrient sources[3]. The physiological state resulting from an insufficient amount of nutrient and energy can be termed as starvation-survival[1]. Therefore, the "starvation" refers to both nutrient and energy limitation unless otherwise mentioned[1].

The study of bacterial starvation begun as early as 1950 with soil and water microorganisms. Isolation of bacteria from the mid-Pacific continental shelf, which represents material laid down millions of years ago, sparked a new wave of studies in microbiology. Biologists and environmentalists led by Richard Y Morita, Kjellberg, Lipmann, and Rita Colwell from 1950 to 1980s explored the mechanisms to survive under low energy conditions. The starvation-survival provides a convenient mechanism to tolerate and survive under low energy conditions. Bacteria survive under prolonged starvation, by entering a state of low metabolic activity, which rarely

divides[3]. It only takes one cell per environment for the survival of a species, which can divide and repopulate the entire colony once the conditions become favorable. One of the oldest and longest studies of starvation survival was carried in the 1960s on *Pseudomonas syringae* subsp. *syringae*. The organism survived in distilled water for 24 years, yet retained its antigenic properties[1]. About 3 x 10^{29} cells reside in the deep biosphere, yet they have access to only 1% of the photosynthetically fixed carbon on the surface of the earth. These bacteria are not yet taxonomically characterized; their physiology and biochemistry still unknown [3].

1.1.2 The process of starvation

The starvation process begins as soon as the organism ceases its uptake of nutrients. Thus, depending on the time spent under starvation, it can be divided as short, medium, and long-term starvation-survival. The severity of starvation increases with the time spent under starvation[1]. Several authors have documented the morphological and physiological changes and adaptations of microbial cells to starvation[1,4–8]. The changes in cell size, cell shape, cell motility, and cell adhesion are some commonly observed responses to starvation.

1.1.3 Cell size and volume

The typical response of most bacterial species to starvation is the reduction in cell size and cell volume. The shrinking of individual cells or cell division without growth (fragmentation) can reduce the cell size and volume[3]. The metabolically active bacteria from freshwater ponds have sizes ranging from 1.6 to 2.4 µm, whereas the bacteria from marine water are as small as 0.4 µm. Such ultra-micro bacteria in the marine environment appear as cocci, vibrio, bacilli, horseshoe, and sigmoid forms. Within limits, the cell size decreases with the extent of starvation. During long-term

starvation, cells would probably reach their minimum size[1]. The changes in cell size and shape under starvation varies drastically among members of different species. Some species respond to starvation by a reduction in cell size and change in cell shape from rod to spherical. This type of response is called copiotrophic starvation response (CSR). Examples include *Vibrio cholerae* and marine *Vibrio* Species Ant-300, both are present initially as vibrio, but as starvation proceeds, they turn to a spherical shape. Another characteristic response to starvation is the oligotrophic starvation response (OSR). In this condition, the microbes are adapted and can grow in oligotrophic conditions. The cells under this category are usually spherical irrespective of energy levels and undergo minimal changes in cell size during starvation. Examples include *Arthrobacter* sp. and certain chemolithoautotrophs[3].

1.1.4 Changes in the cell membrane

The composition of the cell membrane changes upon induction of starvation. For example, the *Vibrio cholerae* changes its lipid content dramatically within seven days of starvation. The lipids, preferably Poly-hydroxy butyrate (PHB) is used extensively as an energy reserve to be utilized during starvation[9]. In another marine *Vibrio* species called Ant-300, the cell surface becomes rigid, often resisting the cell degradation and lysis. The scavenging ability of the bacteria also increases in the oligotrophic environment, and this may reflect the changes associated with bacterial membrane[1].

1.1.5 Cell motility

Motility provides access to better sources of nutrition and energy, niche compartmentalization, and colonization. Motility propelled by the flagella movement comes with a significant energy cost to the cell. The energy-driven process is often slowed down or stopped to survive starvation. The starvation-induced loss of motility

is shown in *Photobacterium angustum* strain S14, α-proteobacterium *Sinorhizobium meliloti*, *Pseudomonas* strain, and hyper-thermophiles archaeon *Methanocaldococcus jannaschii*. Interestingly, the *Vibrio* sp. Ant-300 was shown to be motile only under starvation. Thus, the bacterial response to starvation can be an increase or decrease in motility and chemotaxis. The motility is restored within hours of nutrient upshift[10,11].

1.1.6 Cell adhesion

Bacteria can be free-living or attached living depending on environmental conditions. The cells present in the biofilms can turn to free-living if enough nutrients are available. Many microbes growing on the surface are motile, and many free-living microbes are non-motile oligotrophs with minimal energy expenditure. The increase in cell adhesion during starvation is documented in *E. coli*, *Shewanella oneidensis, Vibrio sp. strain DW1*, and *Pseudomonas* sp., but there are exceptions too. *Klebsiella pneumoniae,* for example, reduces the adhesion under starvation. The increase or decrease in adhesion under starvation depends on the habitat type, energy substrates, and broader evolutionary mechanisms[3].

1.1.7 Changes in cell macromolecular composition

The cell composition changes profoundly during starvation. The changes in lipid composition and adjustments in macromolecular contents are commonly observed during starvation-survival[1,3,5]. The composition changes are species-dependent with profound differences between copiotrophic starvation response (CSR) and oligotrophic starvation response (OSR)[3]. The *V. cholerae*, an extreme example of CSR, reduces the lipid and carbohydrate content by 99.8% and 88.7% within a week of starvation, respectively. The protein and DNA content also decreases by 75 %, and RNA decreased by over 20 % over 30 days of starvation[7]. Similar observations are

also made in *Nocardia corallinae* and *Salmonella typhimurium.* The reduction in lipid content correlated with the loss of cell viability and membrane integrity. The OSR strains also show a reduction in carbohydrate content, protein, and RNA. However, unlike CSR, the DNA content in OSR does not change. Examples include *A. globiformis, A. nicotianae, Brevibacterium linens, Corynebacterium fascians, Mycobacterium rhodochrous, N. roseum*[3].

1.2 Adaptation during starvation-survival

Every cell maintains energy reserves in the form of adenylate energy charge and ATP. Adequate energy utilization depends on growth temperature, redox, and pressure. Because of the niche-specific perturbations, the populations, are never at the theoretical carrying capacities[3]. The cell under such low energy levels can enter different states as a surviving mechanism to long-term starvation.

1.2.1 Spore formation

Starvation triggers the formation of spores in several soil bacteria. Spores are metabolically not active and can survive long-term starvation and other abiotic stresses. Spores can germinate once favorable conditions return and populate the colony. Spores are usually formed by gram-positive bacteria such as *Bacillus subtilis*. Other persistent surviving mechanisms include the formation of a cyst, myxospores, and akinetes[12]. The gram-negative organisms do not form spores. They show other starvation survival strategies such as VBNC formation, GASP phenotype, ultra-microcells, and rugose colony formation.

1.2.2 Growth Advantage in Stationary Phase (GASP) phenotype

GASP is the ability of long-term starved cultures to take over the young cells (cells that just entered the stationary phase). During long-term starvation, mutants with

higher fitness than the parental strains arise in the population. These mutations confer selective advantages during starvation-survival. This shows that even under extreme starvation, cell physiology is dynamic, and mutants continue to arise in higher frequencies. To date, four types of GASP mutants are identified, of which RpoS and Lrp mutants are well characterised[13].

1.2.3 Viable But Non-culturable (VBNC) phenotype

During prolonged starvation, certain bacterial species can lose cultivability but retain the viability. Such cells are unable to form colonies on nutrient agar plates but can be revived back, and this phenomenon is called VBNC[13]. This phenomenon is a survival strategy that forms an essential reservoir of pathogens in the environment. The VBNC have low metabolic activity yet can be cultured upon resuscitation. The VBNC has been associated with recurrent infections in humans and other animals. The pathogens can escape the antimicrobials because of low metabolic activity. The important pathogens which can enter VBNC state include *Campylobacter* spp., *E. coli* (including the pathogenic EHEC strains), *Helicobacter pylori*, *Listeria monocytogenes, M. tuberculosis, Pseudomonas aeruginosa*, several *Salmonella* and, *Shigella* spp. and numerous pathogenic *Vibrio* species such as *V. cholerae* and *V. vulnificus*[14]. Long-term starvation induces a characteristic "persister" behavior among bacterial species, where the cells enter a state of suspended animation and gain tolerance to antibiotics[15]. The persister and VBNC states, both of which are tolerant of antibiotics appear to be a part of the "dormancy continuum'[16].

1.3 The physiological and genetic basis of starvation-survival

The primary goal of cells under starvation is to preserve the energy and biosynthetic precursors[17]. A basal level of energy called "maintenance energy" is maintained in the

form of proton motive force (PMF) during starvation-survival. PMF is crucial for the substrate transport across the cell membrane in all bacteria for motility in flagellated bacteria and ATP synthesis, where substrate-level phosphorylation is not enough. The transition from a dormant state to a growth state is an energy-consuming process, and the spore-forming bacteria will, therefore, be a disadvantage. Long-term starvation appears to be the most appropriate strategy to survive millions of years as compared to the spore formation[18].

Two global regulators, RpoS and (p)ppGpp, well-characterized in *E. coli*, regulate the transition of bacteria from growth to starvation physiology. Together these two regulators bring about Stringent response. The RpoS is a sigma factor induced by stress such as heat and osmotic stress and also by nutrient limitation. The RpoS favors the expression of stress-adaptive genes with non-consensus promoters. The (p)ppGpp or Guanosine Penta phosphate is a stringent response alarmone which is synthesized in response to carbon and amino acid limitation. It has pleiotropic effects on many cellular processes[19]. The alarmone (p)ppGpp activates proteases, to increase the amino acid pool in the cell. (p)ppGpp inhibits the intracellular exopolyphosphatase activity, leading to a significant increase in the accumulation of polyphosphate. It is also involved in regulating glycogen synthesis by repressing the regulator CsrA. Along with a co-regulator DksA, the (p)ppGpp strongly represses rRNA and gene expression of ribosomal proteins. The DksA and (p)ppGpp are required during the transition from growth to low nutrient conditions. However, both DksA and (p)ppGpp offer no help during long-term starvation. The RpoS mutation confers Growth Advantage in Stationary Phase (GASP) phenotype, which is better at tolerating long-term starvation.

These findings suggest that a delicate balance exists between the growth arrest and surviving a prolonged non-growing state[17].

1.4 Antibiotic tolerance of starved cells

The antibiotics often do not eliminate bacterial population, leaving behind a small number of surviving cells called persister cells[20]. They can tolerate and survive in the presence of antibiotics and repopulate the colony once the antibiotics are withdrawn. The persister cells are not genetic mutants and are not resistant to antibiotics. The persisters can arise randomly in a population or as a response to environmental stimuli such as starvation[21]. Successful starvation survival requires long periods of cell growth arrest[1]. The nutrient limitation acts as a signal to arrest the cell division. The cells enter a dormant state and can survive the long-term starvation. The starved cells, often gain transient tolerance to lethal doses of antibiotics[22]. Cells that can tolerate high doses of antibiotics arise due to one or several environmental triggers such as starvation are called Type-I persister cells[23]. Whether all types of starvation conditions result in persister induction remains to be seen. Other than starvation, biofilm formation, quorum sensing, exposure to the host immune system, and exposure to antibiotics can trigger Type-I persister formation[24,25].

The role of stringent response in the development of persistence is studied by several authors[15,26–31]. The protein RelA triggers the alarmone (p)ppGpp in response to amino acid starvation. Another protein SpoT can trigger the (p)ppGpp in response to iron, phosphorus, or fatty acids starvation. Stringent response downregulates the ribosome activity, initiation of DNA replication, and can also take part in DNA repair by promoting RNA polymerase backtracking[24]. Toxin: Antitoxin (TA) modules are

studied as a possible mechanism for cell growth arrest and persister cell formation. The earliest known incidence of the T: A system in persistence is *hipA7* mutation. The mutation in the *hipA* allele causes an increase in persister formation in *E. coli*[26]. Though a link exists between the stringent response, persistence, and TA systems, the persistence being a transient physiological state is more likely triggered by multiple molecular events[24].

1.5 Starvation biology of *Vibrios*

The genus *Vibrio* comprises gram-negative, comma-shaped bacteria, highly motile, oxidase-positive, non-spore forming, and facultative anaerobes belonging to the class γ-proteobacteria. The species of *Vibrio* are pathologically important. The *Vibrios* can infect and colonize a wide range of hosts, ranging from zooplankton, crustaceans, fish, and humans. The three important species of significance to human health include *V. cholerae*, which causes cholera, *V. vulnificus*, and *V. parahaemolyticus*, both of which cause acute enteritis[32].

Vibrio species are generally used as the model organism for studies on the starvation-induced response in non-differentiating bacteria[1–3]. The bacteria can survive under carbon-limited waters and in the nutrient-rich colon of a human host, thus cycling between feast and famine[1]. The stress tolerance and long-term starvation resistance of *Vibrio* make them convenient model organisms to study a) cryptic growth b) formation of ultra-micro cell c) development of a heterogeneous population with starvation e) physiological changes in starvation and f) genetic and proteomic analysis[4,8,33]. *Vibrio* species are widely present in estuarine water, deep-sea waters, kelp beds, gastrointestinal microflora of fish and invertebrates, and in humans. The *Vibrio* exists as free-living beings or as biofilms in association with zooplankton and fishes. The

ubiquitous presence of *Vibrio* suggests that they are well adapted to survive in starvation conditions[1].

1.5.1 Starvation-induced proteins

The starvation induced (Sti) proteins are synthesized in the first hours of starvation. The addition of inhibitors during initiation of starvation significantly affects the long-term survival. Hence these proteins appear to be essential for a successful long-term starvation-survival. The majority of Sti proteins impart starvation-induced cross-protection to abiotic stresses such as heat, osmotic stress, and oxidation. The regulation of Sti proteins is carried out by global regulators such as RpoS. The 2-D analysis of proteins during one h of carbon starvation has led to the conclusion that response to starvation is an ordered array of events, together they allow for long-term starvation survival. Even though Sti proteins are produced, severe starvation can cause oxidation of specific polypeptides, which increase the risk of oxidative damage. The proteins related to the peptide chain elongation, protein folding, stress resistance, and carbon metabolism become oxidized, which increases the risk of aberrant protein production. The starvation of amino acids results in uncharged tRNA, which reduces translation accuracy. This would cause selective degradation of a subset of proteins in the starved cells[34].

1.5.2 Multi-nutrient starvation in *Vibrios*

The majority of work on multi-nutrient starvation in *Vibrio* is carried out in the model organism *Vibrio* sp. S14 (*Photobacterium angustum*). The *Vibrio* sp. S14 was found to survive better under carbon starvation than the nitrogen or phosphorus starvation. When the cells were simultaneously starved for all the three nutrients (carbon, nitrogen, and phosphate), the survival kinetics was like carbon starvation survival. The

phosphorous starvation did not induce transient stress resistance, unlike carbon starvation. The cells could not survive the long-term phosphorus starvation, and no viable colonies could be observed within a few days of phosphate starvation. Starvation of carbon and nitrogen results in the accumulation of ppGpp. The accumulation of ppGpp is essential for starvation survival, especially during the first hours of starvation. The low phosphorus is detected by PhoR, which activates PhoB, the primary phosphate regulator. The formation of long chains of inorganic phosphates has been observed in most of the organisms during phosphate starvation. These inorganic phosphate polymers are essential for adaptation to stress and survival. A similar response was observed for nitrogen starvation, where the cells showed reduced survival during long-term starvation. The limitation of nitrogen does not induce a stable population during long-term starvation, and the population declines continuously[34].

1.6 Starvation biology of *V. cholerae*

The *V. cholerae* is well adapted for nutrient-rich human intestine and nutrient scarce estuarine and marine water[32]. The *V. cholerae* is well studied for its ability to enter VBNC state under cold and nutrient-stress[14]. Apart from forming VBNC, the bacteria can survive the long-term starvation and retain viability and motility. Some basic studies on *V. cholerae* shed light on the morphological changes and cell composition changes during starvation. Though most of the work in starvation survival is carried out using *Photobacterium angustum* (a distant relative of *Vibrio* species)[33,35,36], no significant studies are reported from *V. cholerae*.

1.6.1 Starvation induced morphological changes in *V. cholerae*

In *V. cholerae,* the detailed work on morphological changes is reported by Baker et al[7]. The significant changes under long-term starvation include, a) coccoid cell shape with over 90% reduction in cell volume after 30 days of starvation, b) increase in cell number because of reduction division, c) loss of small granule and three-layered integrity (outer membrane, peptidoglycan, and inner membrane), d) compression to nuclear region to center of the cell with dense cytoplasm and e) formation of extended or convoluted structures from cell wall. Upon nutrient up-shift, the cell shape changed from coccoid to vibrioid within two hours, and cells began to divide within five hours. Maintenance of viability during starvation and quick response to nutrient upshift show the efficient survival mechanisms during long-term starvation.

1.6.2 Changes in the cellular composition of *V. cholerae*

The cellular composition of *V. cholerae* changes drastically upon nutrient downshift. Among the macromolecules, the carbohydrates and total lipids decreased respectively by 88.7% and 99.8% within seven days of starvation. The poly-β-hydroxybutyrate (PHB) also decreased during starvation, suggesting that along with carbohydrates and lipids, the PHB is also used as an energy resource during starvation. The RNA decreases by less than 2% during the first 14 days of starvation and by 20% after 30 days. The decrease in RNA was not as severe as protein and carbohydrates, presumably due to stable ribosomes. The DNA and protein, however, decrease by over 80% after 30 days of starvation. The initial reduction in DNA per cell is attributed to the reduction division, which takes place as soon as starvation begins. Even after the reduction division has stopped, the DNA continues to decline during starvation-survival[37]. Whether the reduction in DNA represents a reduction in extra DNA copies

or configuration changes in molecules or some unknown process is still unfamiliar. Interestingly, the patterns of reduction in DNA, RNA, and proteins do not resemble the pattern observed in another *Vibrio* strain ANT-300[9].

1.6.3 Extracellular polysaccharides and biofilms

During long-term starvation, bacteria increase surface adherence and form biofilms, as detailed in section 1.1.6. The *V. cholerae* successfully forms biofilms on a variety of surfaces, which is critical for its ability to cause pandemic disease. Biofilms can protect from harsh chemicals, antibiotics, and abiotic stress. The formation of biofilm is directly related to the ability of persistence in the environment. Elaborate and 3-dimensional biofilms formed by both pathogenic biotypes of *V. cholerae* O1 El Tor and O139. The formation of biofilm is enhanced in the presence of chitin. The chitin is the polymer found on crustaceans, implicating that the *V. cholerae* has evolved and well adapted to survive on the crustacean surface. The formation of biofilm depends on the expression of exopolysaccharide (EPS). Biofilm enables *V. cholerae* to survive in association with chitinaceous surfaces, the mucilaginous sheath of algae, and copepods[9]. The EPS is also produced in response to nutrient starvation.

1.6.4 The rise in antibiotic resistance

Antibiotics are widely used to treat microbial infections. The antibiotics, depending on the mechanism of action, can either inhibit the growth of microbe (static) or can kill the microbes (cidal). The widespread use of antibiotics has led to the emergence of antibiotic resistance in most of the human pathogens, including *V. cholerae*. The antibiotic-resistant bacteria can survive and grow in the presence of antibiotics, whereas the susceptible ones are eliminated. The development of antibiotic resistance in *V. cholerae* usually occurs because of the excessive use of antibiotics in aquaculture.

The antibiotics are often added in a large amount, leaching into nearby soil and water bodies. The microbes with natural resistance to such antibiotics get selected and can spread resistance among other susceptible microbes.

The cholera is not a systemic disease[38]. Hence, the use of antibiotics is not the sole treatment regime for cholera patients. However, cholera patients are prescribed antibiotics to reduce the duration and frequency of infection. Antibiotics help to reduce the severity of the infection and to maintain hydration. The most widely used antibiotics during cholera are tetracycline and quinolones[39]. Along with doxycycline, erythromycin has also yielded promising results, especially in children. However, the emergence of numerous strains with tetracycline resistance has reduced its usage. The antibiotic resistance in *V. cholerae* was first reported in Tanzania, 1977, and later, it was reported in multiple cholera outbreaks across the globe[40]. The World Health Organization (WHO) does not recommend the general use of antibiotics to curtail the rise of antibiotic resistance[40].

V. cholerae can become drug-resistant by exporting the antibiotics using efflux pumps, mutations, and transferable genetic resistance. The efflux pumps help to pump out antibiotics, dyes, and detergents irrespective of their chemical structure. The efflux pumps drive energy either by transmembrane H^+ or Na^+ ions. One of the noted efflux pumps in *V. cholerae* is the ATP-driven VcaM pump. It can confer resistance to several antibiotics, including tetracycline, norfloxacin, ciprofloxacin, and doxorubicin. The random mutations confer resistance to various antibiotics by acting as an antibiotic inhibitor, or target modulator. The mutations in *GyrA*, for example, reduces the binding to ciprofloxacin to DNA gyrase, thus conferring resistance. The genes of antibiotic

resistance can also be acquired by horizontal gene transfer mediated by plasmids (conjugation) or mobile genetic elements (SXT).

The seminal paper by Balaban and co-workers[41] has established the link between antibiotic resistance and tolerance. The development of antibiotic resistance precedes the enhanced tolerance to antibiotics. The tolerance is distinct from antibiotic resistance. When a bacterium is said to be tolerant of an antibiotic, it reduces its metabolic rate and does not divide in the presence of the antibiotic. Once the antibiotics are withdrawn, the cells again divide and repopulate the colony. The term antibiotic persistence is often loosely used as an alternative to tolerance. Although there are some differences between antibiotic persistence and tolerance, both are a part of the dormancy continuum[42]. Hence detection of antibiotic tolerance or persistence can reasonably indicate the developing antibiotic resistance. The higher the tolerance to antibiotics, the higher are the chances of resistance.

1.7 Scope of the present work

The response to starvation is a combined effort by several genes, pathways, and protein-protein interactions, which are best studied at a holistic or physiological level. A bacterial cell has evolved over millions of years to suit the ever-changing environment. In doing so, it has also gained several habitats and niche-specific genes which enhance the survival of the population.

The studies of starvation can either take a reductionist molecular approach or a holistic physiological approach. The reductionist approach aims to cut down the problem into several small pieces, like genes and proteins. The reductionist approach builds on the role played by individual genes and proteins and extrapolates to the observed

phenomenon. The reductionist approach has led us to discover such central regulators and their pathways. The holistic approach relies upon the famous saying by Aristotle, "The whole is more than the sum of its parts." The reductionist and physiological approaches are interdependent and complementary to each other[43], which have led to the discovery of several key molecules involved in starvation survival including ppGpp, relA, spot, and rpoS.

The theme of the present work is to develop an understanding of how the *V. cholerae* survives starvation and antibiotics with minimal loss of viability. The thesis work was carried out with three primary objectives:

1. Studies on the physiological state of starved cells.
2. Studies on starvation (carbon, nitrogen, and phosphate) induced persister/dormant cells and their response to antibiotics and other stresses.
3. Expression studies of important genes involved in starvation survival and recovery.

The present work, as discussed ahead, represents a tiny step towards understanding the physiology of long-term starvation in *V. cholerae*.

2 MATERIALS AND METHODS

2.1 Materials

2.1.1 Plasticware and glassware

Disposable polypropylene micro-centrifuge tubes (1.5 mL and 2 mL), PCR tubes (0.2 mL or 0.5 mL), micropipette tips (10µL – 1.0 mL), plastic Petri dishes, polypropylene oak ridge tubes, and GSA bottles were obtained from Tarson (India) or Axygen (USA). Conical flasks, glass beakers, and measuring cylinders were purchased from Borosil (India). All these plastic wares and glasswares were sterilized by autoclaving. Cryovials (2 mL) and low-temperature storage boxes were procured from Laxbro (India) and Axygen (USA).

2.1.2 Chemicals and media ingredients

Dehydrated culture media components were purchased from Himedia (India). Fine chemicals were purchased either from Sigma-Aldrich-Merck (Germany). Inorganic and organic salts, antibiotics and organic solvents of analytical and molecular grade were purchased either from Sigma-Aldrich- Merck (Germany) or Loba Chemie Pvt Ltd (India).

2.1.3 Enzymes and other molecular biology reagents

PCR reagents were from New England Biolabs or Thermo Fisher Scientific (USA). The DNA molecular weight standards were obtained from Himedia (India) or Promega (USA). PCR purification kit, Gel extraction kit, plasmid isolation kit, and bacterial genomic DNA isolation kit were from Qiagen (Germany) or Sigma-Aldrich-Merck (Germany).

2.1.4 Stock Solutions of different chemicals

Tris - HCl solution: To prepare 1 M solution, 121.14 g of Trizma base was dissolved in 800 mL of distilled water, and pH was adjusted using 1 N HCl. Volume was made to 1 L. The solution was autoclaved and stored at room temperature.

Potassium phosphate monobasic solution (KH_2PO_4): To prepare 0.75 M solution, 102 g of KH_2PO_4 was dissolved in 800 mL of distilled water, and volume was made to 1 L and solution was autoclaved and stored at room temperature.

NaH_2PO_4 solution: To prepare 1 M solution, 119.98 g of NaH_2PO_4 was dissolved in 800 mL of distilled water and volume made to 1 L. The solution was autoclaved and stored at room temperature.

Na_2HPO_4 solution: To prepare 1 M solution, 141.96 g of Na_2HPO_4 was dissolved in 800 mL of distilled water and volume made to 1 L. The solution was autoclaved and stored at room temperature.

Phosphate buffered saline: NaCl 8 g, KCl 0.2 g, Na_2HPO_4 1.44 g, and KH_2PO_4 0.2 g were dissolved in 800 mL of distilled water. The pH was adjusted to 7.4 with HCl and volume made to 1 L. The solution was autoclaved and stored at room temperature.

Sodium chloride (NaCl) solution: To prepare a 5 M solution, 292 g of sodium chloride was dissolved in 700 mL of distilled water and volume made to 1 L.

Saline: To prepare saline, 0.85 g of NaCl was dissolved in 100 mL of distilled water. The solution was autoclaved and stored at room temperature.

NaOH solution: To prepare 10 N solution, 4g of NaOH pellets were dissolved in 10 mL autoclaved ultrapure Milli-Q® water, stored at room temperature.

Magnesium sulfate ($MgSO_4$) solution: To prepare 1 M solution, 12.03 g of $MgSO_4$ was dissolved in 70 mL of distilled water and volume made to 100 mL. The solution was autoclaved and stored at room temperature.

Calcium chloride ($CaCl_2$) solution: To prepare 1 M solution, 14.7 g of $CaCl_2$ dihydrate was dissolved in 70 mL of distilled water and volume made to 100 mL. The solution was autoclaved and stored at 4 ˚C.

EDTA solution: To prepare a 0.5 M solution, 186.1 g of disodium EDTA.$2H_2O$ was dissolved in 800 mL of distilled water and pH adjusted to 8.0 using NaOH pellets. The final volume was made to 1 L, autoclaved, and stored at room temperature.

Glycerol solution: To prepare a 50% solution, 50 mL of glycerol was mixed with equal volumes of distilled water. The solution was autoclaved and stored at room temperature.

IPTG solution: To prepare 1 M solution, 2.38 g of IPTG (isopropyl-beta-D-thiogalactopyranoside) powder was dissolved in 7 mL of autoclaved distilled water and volume made to 10 mL. The solution was filter sterilized and aliquots of 1 mL stored at -20ºC.

Lysozyme: 1 g of lyophilized lysozyme powder was dissolved in 10 mL of 10mM Tris-HCl (pH 8.0) and stored at -20ºC. This will make a 100 mg mL^{-1} solution.

2.1.5 Reagents for agarose gel electrophoresis

TBE: To prepare 10X TBE, 108 g Tris base, 55 g boric acid (borate) and 40 mL 0.5 M EDTA (pH = 8.0) were suspended in distilled water, and the volume was made up to 1 L.

TAE (Tris-acetate EDTA) buffer: To prepare 50X TAE, 242 g of Tris base was dissolved in 700 mL of distilled water, and 57.1 mL of glacial acetic acid was added to

it. Additionally, 100 mL of 0.5M EDTA, pH 8.0 was added, and pH was adjusted to 8. Buffer was autoclaved and stored at room temperature.

DNA loading dye: Bromophenol blue or xylene cyanol (25mg) and 4g of sucrose were dissolved in autoclaved distilled water, and volume was made to 10 mL. The dye was autoclaved and aliquoted before storing it at -20 ºC. The prepared dye solution is 6X concentrated.

Ethidium bromide solution: Ethidium bromide (EtBr) (100 mg) was dissolved in 10 mL of autoclaved ultrapure MQ water, aliquoted, and stored at room temperature. Thus, the prepared solution is 10 mg mL^{-1} concentrated.

2.2 Antibiotic stock solution

All the antibiotics were prepared, as described in Table 2.1. They were dissolved in respective solvents, filter sterilized, and stored in -20 ˚C.

Table. 2.1: List of antibiotic stock solutions

Antibiotics	**Stock solution (mg mL^{-1})**	**Solvent**
Ampicillin	100	water
Carbenicillin	100	water
Kanamycin	25	water
Chloramphenicol	25	Absolute ethanol
Gentamycin	100	water
Tetracycline	10	water
Rifampicin	100	Dimethylsulfoxide (DMSO)
Erythromycin	50	Absolute ethanol

2.3 Methods

2.3.1 Strain, media, and growth conditions.

The model organism *Vibrio cholerae* El Tor O1 N 16961was used throughout this study. The strain was confirmed by Next Generation Sequencing (NGS). All other cultures used in the study are tabulated in Table 2.2.

Table. 2.2: Bacterial cultures used in the present work

Organism	Collection source
***Vibrio cholerae* El Tor O1 N16961**	Food Isolate, FTD[a]
***Vibrio vulnificus* YJ016**	National Cheng-Kung University[b]
Vibrio harveyi	Environmental, FTD[a]
Vibrio parahaemolyticus	Food Isolate, FTD[a]
***Escherichia coli* MG1665**	MTCC1655[c]
***Escherichia coli* DH5α**	MTCC 1652
***Salmonella enteritica enteritica* Typhimurium LT2**	MTCC98[c]
Aeromonas hydrophilla A331	CECT839[d]
Cronobacter sakazakii	Food Isolate, FTD[a]

[a] FTD- Food Technology Division, Bhabha Atomic Research Centre, Mumbai, India.

[b] *A kind gift from Dr. Lien I Hor*

[c] *Microbial type culture collection, Chandigarh, India.*

[d] *Spanish type culture collection, Spain.*

All cultures were grown in Tryptone Soya broth (TSA) or Luria–Bertani broth (Himedia, India) adjusted to pH of 7.5 at 37°C with shaking at 150 rpm, unless otherwise mentioned. The stationary phase culture was diluted 1:1000 by autoclaved LB to start the primary culture. Every new experiment was done by reviving the glycerol stocks maintained at -80 °C. After reviving the stocks on TSA plates, the colonies were

re-streaked on selective media before initiating experiments. All other media used in the experiment are detailed in Annexure. The media used for persister assay were filter sterilized. Autoclaving causes degradation of media components and unpredictably alters the composition. This contributes to the inconsistency of results.

Aeration has a strong influence on bacterial physiology. Always maintain uniform aeration conditions in all experiments related to persister assay. Besides, other factors such as strain's genotype, growth medium, and temperature, fold dilution of initial inoculum, the growth phase of both the test culture and inoculum source can influence the outcome.

Serial dilutions are made in 96-well microtiter plates. While using a multichannel pipette for making serial dilutions, 10X dilutions are recommended. All the wells are filled with 90 µL of LB. Samples of 10 µL are transferred from wells of row A to row B, and then from row B to row C, so on till we get the desired level of dilution.

2.3.2 Determination of cell viability.

The cell viability was determined by the serial dilution method. 100 µL of culture was diluted in 900 µL of Artificial Sea Water (ASW) or saline. The culture was further serially diluted to get countable colonies on the plate. From each dilution tube, 100 µL of the solution was spread plated on TSA plate. Alternatively, 10 µL of the sample was dropped on plates. After the culture drops have dried up, the plates are kept inverted in the incubator at 37 °C for 24 h. In a single plate, 5 – 6 individual drops can be made circularly. Overcrowding the drops may result in the overlapping of colonies from adjacent dilutions. Colony Forming Units [CFU] mL^{-1} was determined using the following equation:

$$Colony\ forming\ units\ (CFU)\ per\ mL = \frac{Number\ of\ colonies\ on\ the\ plate}{Volume\ added\ on\ the\ plate} \times 100$$

2.3.3 Preparation of glycerol stock of cultures.

A single colony of pure culture was streaked on the TSA plate and incubated for 24 h at 37 °C. Around 2 – 5 mL of Glycerol: skim milk in 1:1 ratio was added on the plates and using the sterile glass spreader colonies were suspended in the solution. The suspended cells were then distributed in cryovials and stored at -80 °C till further use. Multiple vials were prepared and used only once per experiment.

2.3.4 Selective media for *V. cholerae*

Thiosulfate-citrate-bile salts-sucrose agar or TCBS agar is the selective media for *V. cholerae*. The colonies grew in the TSA plate where streaked heavily onto TCBS plate and incubated at 37 °C for 24- 48 h. The formation of golden color colonies in the green media background confirms the isolation of *V. cholerae*.

2.3.5 Polymerase Chain Reaction (PCR) for gene identification

The primers for the gene of interest are designed using the IDT primer quest tool (www.idtdna.com/site). The PCR recipe is detailed in Annexure. The annealing temperature for each gene pair is tabulated in Table 2.2. The agarose gel was prepared by dissolving 0.8% agarose, Genei, MERCK (Germany) by boiling in 0.5X TBE until the solution is clear. After the solution cools down to 50 °C - 60 °C, Ethidium bromide (Honeywell Fluka™, Switzerland) was added (3 µL per 40 mL agarose gel). The gel was cast, and after it solidified, 5 µL of PCR samples were mixed with a drop of loading dye and was loaded in wells. 4 µL of 100bp DNA ladder (Himedia, India) was also loaded along with the samples. The electrophoresis was carried out under 75 V, 50 mA

current till the dye front reaches at least 3/4th of gel slab. DNA bands were visualized on UV illuminator.

2.3.6 RNA isolation and cDNA preparation

Total RNA was extracted from the cell samples using the RNeasy mini kit (QIAGEN, US) as per the manufacturer's protocol. The quality and quantity of isolated RNA were measured using a photometer (Eppendorf, Germany). Pure RNA has an OD_{260}/OD_{280} ratio of ~2.0. An OD_{600}=1 at 260 nm for RNA molecules corresponds to 40 ng uL^{-1} of RNA. The samples are usually diluted to 1:50 or 1:100 depending on concentration. The following formula is used to determine the concentration of RNA in the isolated sample.

$$RNA\ concentration\ (ng\ per\ \mu L) = OD\ at\ 260nm\ * 40\ ng\ per\ \mu L * dilution\ factor$$

About 100 ng of the RNA was reverse transcribed using a DyNAmo cDNA synthesis kit (Finnzymes, Espoo, Finland) as per manufacturer's protocol. The expression profile of the cDNA products was determined using the real-time quantitative PCR (qPCR) and analyzed using the Rotor-Gene Q Series Software 2.1.0 (Build 9) analysis template. The gene-specific primers (Table S4) were designed using the integrated DNA technologies Primer Quest software (www.idtdna.com/site). The qPCR recipe and thermal cycle are given in Annexure.

2.3.7 Nutrient limitation

The overnight grown cultures in LB were centrifuged at 8000 g for 2 min. The cell pellet was washed thrice in respective starvation media. Later the starvation inoculum was prepared to achieve 10^7 or 10^8 CFU mL^{-1}, as mentioned in the results. The nutrient-limited culture was incubated at 37 °C. At regular intervals, the cell number was

determined by the serial dilution method. To induce carbon (C), nitrogen (N) and phosphate (P) limitation, 1 % of overnight grown culture were inoculated in ASW and allowed to incubate at 37 °C. The multi-nutrient starvation was induced by removing one or more components from minimal media. The detailed composition of all the limited nutrient media is given in Annexure.

2.3.8 Growth curve analysis

A single colony from the TSA plate was inoculated in LB broth and incubated as described in section 2.2.1. At regular intervals, a small aliquot of culture was removed, serially diluted, and plated on TSA plates. A graph of cell number on the X-axis and time on the Y-axis was plotted. The doubling time and growth rate are calculated using the following equations.

$$Doubling\ time\ (G) = \frac{t}{3.3 \log\left({Nt}/{No}\right)}$$

G = no. of generations

Nt = population at time t

No = initial population at time 0

t = time

$$Specific\ growth\ rate\ (k) = slope\ of\ the\ growth\ curve\ graph.$$

2.3.9 Minimal inhibitory concentration (MIC) determination

The MIC values of the antibiotics were determined using the conventional microdilution method in Muller Hinton broth (MHB). The exponential culture (OD_{600}=0.4) was grown in LB was used for MIC determination. To each well of a 96-well microtiter plate, Corning (US), 100 µL of MHB (2X concentration) was dispensed.

To wells of column 1, were dispensed with antibiotics of 2x concentration and mixed well by pipetting. From the column 1, 100 µL of the sample was added to column 2, from column 2 to 3, and so on until column 10. From lane 10, remove 100 µL of sample and discard. Starting from column 1 to column 11, to all corresponding wells, 10^4-10^5 CFU mL^{-1} (1 µL of exponential culture) were inoculated. The column 12 was not inoculated, and it would serve as a negative control. Column 11 would be a positive control that had not received antibiotics. The plate was incubated at 37 °C for 16 h. The lowest concentration of antibiotics that inhibited the visible growth of bacteria was interpreted as Minimal Inhibitory Concentration (MIC). All the assays were repeated twice in triplicates. The antibiotic concentration was adjusted to yield a broad range of concentration to test inhibitory concentrations. The range of concentrations was narrowed down to arrive at the exact MIC of antibiotics.

Table. 2.3: The minimum inhibitory concentration (MIC) of antibiotics used in the experiments is tabulated. Organism: *Vibrio cholerae*. Units: (µg mL^{-1})

Antibiotics	Cation adjusted Muller Hinton broth	Luria-Bertani broth
Ampicillin	4	4-5
Ciprofloxacin	0.1	0.1-0.125
Kanamycin	8	10
Erythromycin	8	10
Gentamicin	2	2
Chloramphenicol	8	6-8
Tetracycline	0.25	0.3-0.5
CCCP	4.5	4.2-5.0

2.3.10 Persister cell isolation: time-dependent kill method

The persister assay was done using time-dependent killing kinetics.[44]

Day 1: An isolated bacterial colony was picked using a sterile loop and inoculated in 15 mL of culture medium in a 100 mL flask. We used three independent overnight cultures for each strain and treatment of interest. The culture was incubated for 16 ± 1 h at 37 °C with shaking (150 rpm).

Day 2: The overnight grown cultures were diluted 1:100 in 100 mL flasks and incubated on a shaker (150 rpm) at 37 °C (corresponding to around $3*10^7$ CFU mL^{-1}). When cultures reached OD_{600}=0.4, cells were exposed to the 10-fold MIC of antibiotics and incubated further. At regular intervals, typically 0, 1, 2, 4, 6, 8, and 24 h, 1 mL of the sample was centrifuged. The cell pellet was suspended in 1 mL of fresh ASW, serially diluted, and 10 µL of the cell suspension was spotted on TSA plates. The plates were further incubated at 37°C for 16 – 24 h until colonies appeared.

Day 3: Count the cells on plates and determined CFU according to using the formula given in section 2.3.2. A graph of CFU mL^{-1} v/s time was plotted. The plateau region in the graph where the rate of killing is minimal indicates the formation of persister fraction. The persister fraction was determined using the formula:

$$Persister\ fraction\ (\%) = \left(\frac{Nt}{No}\right) \times 100$$

N_t = CFU mL^{-1} after antibiotic exposure in the plateau region.

N_o = CFU mL^{-1} before antibiotic exposure.

2.3.11 Persister recovery

The exponential phase cells were exposed to the tenfold MIC of ciprofloxacin and allowed to form persisters. After a stable persister population was formed, the cells were washed in LB and inoculated in LB media without ciprofloxacin. The culture was incubated at 37 °C on 150 rpm shaker for 24 h. Meanwhile, the growth curve was plotted, and the growth rate and lag time were measured. Persisters were also inoculated

in LB with antibiotics and incubated. The MIC of ciprofloxacin was calculated for the regrown population. Next, using tenfold MIC concentration, persisters were allowed to form in the new population, and persister frequency was measured. The persister frequency of the original population and the regrown population was compared.

2.3.12 Detection of Viable But Non-Culturable cells (VBNC)

We carried out the resuscitation studies to determine whether the persister population harbor viable but non-culturable (VBNC) subtypes, using the protocol described by Mishra[45]. Briefly, the persisters were resuscitated in ASW by releasing the stress (antibiotic) at 25 °C, for 72 h at room temperature. After 72 h of resuscitation, the cells were serially diluted and plated on LB agar plates without antibiotics. Viable cell count (CFU mL^{-1}) was determined following plating on TSA agar. If VBNC is present among the persisters, the cell count after resuscitation would be more than cells count immediately after antibiotic treatment. The two-tailed unpaired t-test was carried out to determine the statistical significance of the data (cut-off $p = 0.05$).

2.3.13 Live/Dead Viability assay

The viability of kanamycin-treated cells was determined using the LIVE/DEAD Bac light™ viability kit (Molecular Probes, Invitrogen, USA), as per manufacturer's protocol. Briefly, the Propidium iodide (PI) and Syto9 dyes were mixed in a 1:1 ratio and incubated in the dark for 15 min. After the incubation, the live/dead cell count was taken using Nikon Eclipse Ni fluorescent microscope (Nikon Instruments Inc. Japan). A xenon lamp of 100 W was used to deliver light to FITC and TRITC filters. The images were captured individually under each filter and then merged using Nikon NIS elements software. The viability of kanamycin-treated cells was checked at different time points

(0, 1, 2 and 4 h after kanamycin exposure), and images were taken. All experiments were carried out in triplicates.

2.3.14 Quinolone resistance-determining region (QRDR) sequencing

The mutations in quinolone resistance-determining region (QRDR) locus of *gyrA, gyrB,* and *parC* genes was screened using the protocol as details by Kim[21]. Briefly, the PCR amplification of the QRDR region was carried out using primers listed by Kim et al. (2010). The PCR amplicons were purified using Exo Pro Star™ S PCR and Sequence Reaction Clean-up Kit (GE Healthcare, US) and sequenced using the Sanger sequencing method. The sequences were compared with the genome sequence of *V. cholerae* O1 strain N16961, available at NCBI Gen Bank.

2.3.15 Glucose supplementation and antibiotic susceptibility

V. cholerae was cultured in LB at 37 °C under shaking condition, it was supplemented with glucose in different growth phases and measured the antibiotic susceptibility. Glucose concentrations were adjusted to deliver 60 mM carbon.

Condition 1: Growth phase—lag: glucose was added to media at the time of culture inoculation. The overnight grown culture of *V. cholerae* was inoculated in a sterile LB flask. Glucose (10 mM) was immediately added to the media, and the culture was incubated at 37 °C, 150 rpm shaking condition. The control was also maintained without glucose supplementation, and after three h of incubation, the antibiotics were added at tenfold MIC concentration and incubated for six h further. At the end of the antibiotic treatment, the viability was enumerated by serial dilution and plating on LB plates.

Condition 2: Growth phase—log: glucose was added to the cultures in the exponential phase. The cultures were inoculated in LB, and the glucose was supplemented to the media Glucose (+) after 3 h of incubation. Control was maintained without glucose

supplementation Glucose (−). Immediately after glucose addition, cultures were challenged with antibiotics (tenfold MIC) for 6 h. At the end of the antibiotic challenge, the survivors were enumerated by plating.

Condition 3: Growth phase—stationary: glucose added in the non-dividing state. The overnight grown stationary phase culture was supplemented with 10 mM glucose. The antibiotics were added to the culture immediately and treated for 6 h Glucose (+). Control was maintained without glucose addition Glucose (−), and the percentage of survival was determined after six hours of antibiotics challenge.

Condition 4: Persister stage: the glucose was added to the persister cells were isolated using methods as described by Keren *et al*[46]. Briefly, the exponentially growing culture of *V. cholerae* in LB was challenged with tenfold MIC of ciprofloxacin and ampicillin individually. The culture was incubated at 37 °C, and the survivors were enumerated at regular time intervals. The persister cells obtained by ciprofloxacin challenge were termed as P-ciprofloxacin; the persister cells obtained by ampicillin challenge were termed as P-ampicillin. Glucose (10 mM) was added to the persister population, and changes in the cell number were determined after 2 h. The cultures were always maintained under antibiotic stress to retain the persister physiology.

2.3.16 Stress tolerance

Cultures were incubated in LB at 45 °C for 180 min. The cell number was enumerated at regular intervals by serial dilution method. To induce oxidative stress, the P-cells and E-cells were incubated with 5 mM Hydrogen peroxide for 180 min. The cell number was enumerated at regular intervals by serial dilution method. To study the radiation sensitivity, the persister sub-populations were washed and suspended in saline (10^8 CFU mL^{-1}). Care was taken to maintain the antibiotic concentration in the media containing

persister sub-populations. Cells were irradiated on ice in the dose range of 0.1–1 kGy using a Co^{60} source (Gamma Cell 5000, BRIT, Mumbai, India) at a dose rate of 7 kGy h^{-1}. After irradiation, 100 µL of different dilutions were plated on LA agar plates, and CFUs were enumerated after 24 h of incubation at 37 °C, and the decimal reduction dose (D_{10}), the dose which kills 90 % of the cells, was determined from the response curve.

2.3.17 Catalase activity

The spectrophotometric procedure described by Beers and Sizer was followed[47].

Sample: The cell-free extract containing 10 µg of protein

Buffer: 50mM potassium- phosphate buffer (pH 7)

Reagent: H_2O_2 solution - 0.75 mL of 30 % H_2O_2 in 100 mL of potassium- phosphate buffer

Assay: Sample containing 10 µg of protein was diluted up to 2 mL with a buffer which was transferred to a quartz cuvette, and 1 mL of freshly prepared H_2O_2 solution was added. Catalase activity was measured by following the decrease in absorbance (240 nm) caused by the disappearance of H_2O_2 in the potassium-phosphate buffer. One unit is defined as the amount of enzyme which decomposes 1 µmol of H_2O_2 per minute.

$$Catalase\ activity\ (U\ /mg) = \frac{\Delta As - \Delta Ao * 3\ ml}{0.0436 * 2\ ml}$$

0.0436: Milli molar extinction coefficient of hydrogen peroxide at 240 nm (cm^2 μmol^{-1})

Δ As: decrease in absorbance calculated per min using the linear portion of the curve of the sample

ΔA_0 : decrease in absorbance calculated per min using the linear portion of the curve of blank

2.3.18 Recovery from nutrient and antibiotic stress

E-cells were supplemented with LB and cultured at 37 °C for 24 h. P-cells were washed in LB to remove the antibiotics and inoculated in LB and cultured at 37 °C for 24 h. The recovery studies were carried out only after normalizing the cell number among the sub-populations (10^5 CFU mL^{-1}).

2.3.19 Quantification of DNA

The DNA of samples was isolated from cultured cells using Bacteraemia DNA Isolation kit, Biostic (Mo Bio Laboratories, US) using the manufacturer's protocol. The quality and integrity of the eluted DNA were verified on 1% agarose gel electrophoresis. Spectra readings of the isolated DNA were recorded using a Nanodrop photometer, and DNA per cell was calculated.

2.3.20 Cell disruption for ploidy analysis

The cells from the appropriate growth condition were harvested by centrifugation. The cell number was determined using a Neubauer cell counter. The cells were lysed using 4mg mL^{-1} lysozyme in Tris buffer (pH 7.2). The lysate was plated to measure lysis efficiency. After the lysis, the cell debris was removed by centrifugation (8000 g, 5 min). 0.1 mL of the supernatant was used as the cytoplasmic extract for further analysis. The integrity of genomic DNA was checked using agarose electrophoresis.

2.3.21 Ploidy determination using qPCR method

The ploidy was determined by a real-time quantitative PCR method with the following modifications[48]. Fragment of 900bp region of *V. cholerae recA* gene was amplified using standard PCR from the genomic DNA of *V. cholerae*. The fragments were

purified by preparative agarose gel electrophoresis and the GenElute^{tm} PCR clean-up kit (Sigma-Aldrich, US). The PCR amplified DNA mass concentrations were determined photometrically, and the concentrations of DNA molecules were calculated using the molecular weights computed with 'oligocalc' (www.basic.northwestern.edu/biotools). A dilution series was generated for each standard fragment and used for qPCR analysis with the dilution series. The qPCR was carried out by following the MIQE guidelines. The genome copy number per cell was calculated using the cell number present at the time of cell lysis. Three independent replicates were performed for each sample.

2.3.22 Ploidy determination using the spectroscopic method

Ploidy was also determined using the spectroscopic method. The DNA was isolated using the Bacteraemia DNA Isolation kit, Biostic (Mo Bio Laboratories, US). The isolated DNA was verified by 1% agarose gel electrophoresis. The spectrophotometric readings (260 nm) of the isolated DNA were recorded. The cell number and absorption at 260 nm were used to calculate the ploidy per cell using the following parameters[49]: an absorption value of 1 at OD_{600} equals a DNA concentration of 50 µg mL^{-1}, the mean molecular mass of one base pair is 660 g mol^{-1}. The genome size of *V. cholerae* is 4.34 Mbp[50].

2.3.23 DNase activity measurement.

The activity of DNase was monitored using the Agarose gel method (Gerceker *et al*., 2009). Different aliquots of whole cells were harvested from different growth conditions. The calf thymus DNA (500 µg mL^{-1}) was added to the cell aliquots, and the cells were incubated at 37°C for various time intervals. The degradation of DNA was checked using 0.9% agarose gel electrophoresis.

2.3.24 NADH measurement

To make a standard assay, pure NADH was sourced from Sigma-Aldrich, Merck, Germany. NADH/NAD was prepared in Tris-EDTA (10mM Tris, 1mM EDTA) to stock concentration of 1 mg mL^{-1}, pH 8. Serially diluted (1:2) standard solution was prepared using 100 µL of total volume per well. The solution was directly measured fluorescence reader with excitation at 340 nm and emission at 440 nm. Only NADH absorbs at 340 nm. A standard graph was prepared with absorbance and concentration of NADH. Cells were washed in 1x PBS and centrifuged. To the washed pellets, 300 µL of 0.2 M NaOH (for NADH extraction) and 300 µL of 0.2 M HCl (for NAD extraction) were added. The samples were heated for 55°C for 10 min. This was followed by the addition of 300 µL of 0.1 M HCl (for NADH extraction) and 300 µL of 0.1 M NaOH (for NAD extraction). Centrifuge the samples at 12000 g for 5 min. The supernatant was collected, stored at -20°C and used for NADH assay. The sample cell lysate, as prepared above, was measured at 340/440 nm. We used two types of blank to neutralize background absorption. One blank contained only media and no cells. And another black solution contained dormant cells that were starved for several days (to contain very low metabolic activity, hence, very low NADH in the background). The black absorbance was subtracted from sample absorbance, and the concentration of NADH was calculated.

2.3.25 Propidium Iodide uptake measurement

The membrane damage in P-limited cells was measured and compared with the dormant cells (carbon, nitrogen, and phosphate starved cells). The membrane-permeable Propidium Iodide dye (PI). Only dead cells would take up the dye and fluoresce red since the dye is permeable only if the cell membrane is damaged. Cells samples were

washed in 1x PBS, and 1.3 µg mL^{-1} of PI dye was added. Cells were treated for 20 min in the dark at room temperature. At the end of treatment, sample fluorescence was measured with excitation at 544nm and emission at 620nm. A graph of sample v/s PI uptake was plotted.

2.3.26 CreC protein analysis

The CreC protein sequence of *E. coli* (WP_097745917.1) was used as a query sequence, and protein BLAST was performed against all the species used in the experiment (see sections). The BLAST results were analyzed, and proteins with the highest hit were selected as a subject sequence for further analysis. The evolutionary history was inferred using the Neighbour-Joining method. The optimal tree with the sum of branch length = 5.15821847 is shown. The tree is drawn to scale, with branch lengths in the same units as those of the evolutionary distances used to infer the phylogenetic tree. The evolutionary distances were computed using the Poisson correction method and are in the units of the number of amino acid substitutions per site. This analysis involved 8 amino acid sequences. All ambiguous positions were removed for each sequence pair (pairwise deletion option). There was a total of 516 positions in the final dataset. Evolutionary analyses were conducted in MEGA X[51].

2.3.27 Statistical Analyses

Statistical analyses were performed using the software GraphPad Prism 7.0 software. Also, Student's t-test or ANOVA (Analysis of variance) was used for the statistical significance of data. P-values obtained at 95 % confidence intervals are shown as (*) for <0.05, (**) for <0.01 and (***) for <0.001. The ploidy number as obtained from qPCR and the spectroscopic method was analyzed using the Bland-Altman plot for the test of agreement (Altman *et al*., 1986). The correlation between the reduction in DNA

and ploidy during starvation was also determined using regression – correlation analysis. The Student's t-test was carried out to determine the significant results at $p < 0.05$.

3 RESULTS AND DISCUSSION

3.1 Isolation and characterization of persister phenotypes

The phenotypic variations brought about by non-genetic mechanisms are challenging to study owing to the stochastic nature of physiological states[52]. Such variations often influence the propensity of persisters cells to form during stress conditions[53]. One of the most studied phenotypic variations is the paradox of antibiotic-induced persistence[26,54]. The antibiotic persisters can survive lethal doses of antibiotics with characteristic bi-phasic kill curve[46]. Apart from antibiotics, several other stresses, such as nutrient limitation, acid stress, phages, and metal toxicity, can prompt the persister formation[52]. Starvation-triggered persistence and antibiotic-triggered persistence poses a compelling question. Do they represent a common subpopulation of cells, or are they different sub-populations? Do they represent phenotypic heterogeneous sub-populations? To clarify these questions, we isolated the persisters from *V. cholerae* population and studied the inter-relatedness of environmental persistence and antibiotic persistence.

The present chapter explains the persister phenotypes in *V. cholerae.* Bacteria were subjected to two types of abiotic stress, one nutrient limitation, and another was a lethal dose of antibiotics. The effect of these abiotic factors on persister frequency was monitored, and persister populations were characterized. The findings of the present chapter have resulted in the following publication.

*Paranjape, S. S. & Shashidhar, R. Comparison of Starvation-Induced Persister Cells with Antibiotic-Induced Persister Cells. Curr Microbiol **76**, 1495–1502 (2019).*[55]

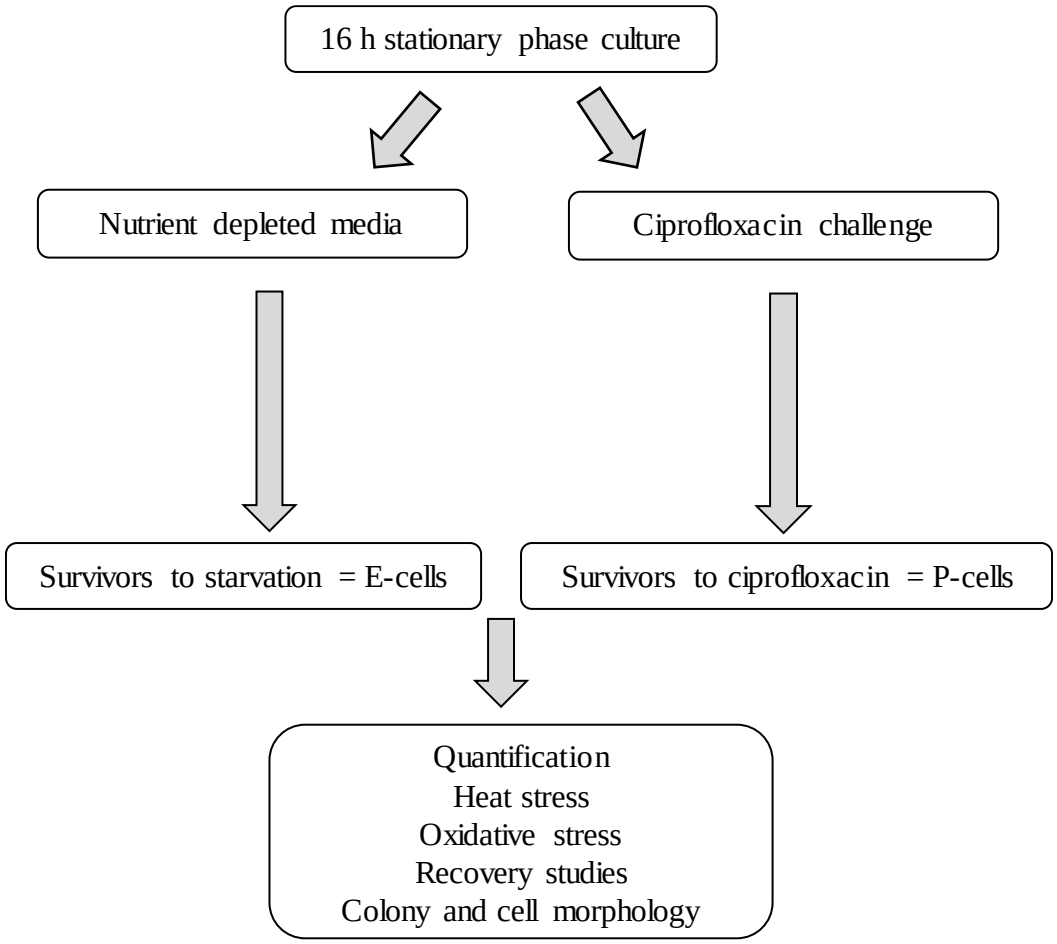

Figure. 3.1. Workflow to isolate the persister subpopulations. The stationary culture of V. cholerae was challenged with either starvation inoculum or inhibitory concentrations of antibiotics. The survivor populations were isolated and considered as two separate subpopulations. The subpopulation isolated from starvation are called environmental persisters (E-cells). The subpopulation isolated from antibiotic stress are called antibiotic persisters (P-cells). Both the subpopulations were analysed for stress tolerance and colony morphology.

In the present study, two sub-populations from 16 h old stationary phase cells of *V. cholerae* were isolated, as depicted below (**Figure. 3.1**).

E-cells: The stationary cells were exposed to simultaneous carbon, nitrogen, and phosphate limitations, and cell number was monitored. The cell number reduced by

50% in the initial 4 - 6 h of starvation; after that, it formed a stable population of 10^8 Colony Forming Unit (CFU) mL^{-1} (**Figure. 3.2**).

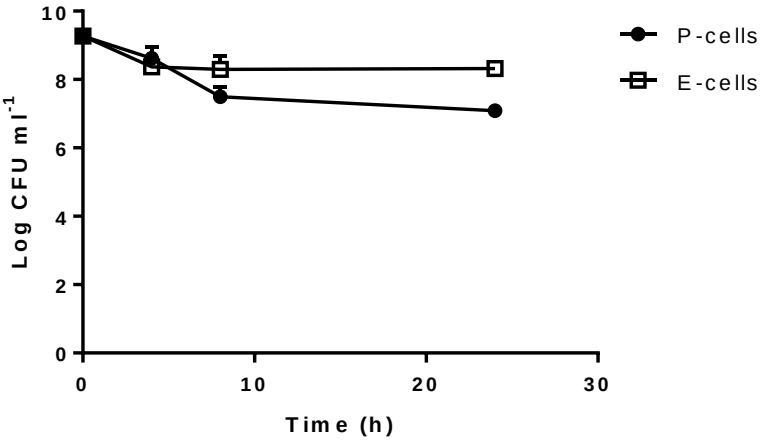

Figure. 3.2. Time dependent killing to isolate the subpopulations from stationary phase cells. The squares represent the cells which survived the starvation (Environmental persister cells, E-cells). The filled circle represents the persister cells which survived the antibiotics (Antibiotic persister cells, P-cells).The graph represents the average of three biological replicates with S.D.

The cells of the plateau region in **Figure 3.2** where population size does not reduce further indicates the formation of the persister population. Accordingly, the cells of 24 h starved culture was considered as starvation-triggered persister cells and are denoted as E-cells.

P-cells: Another subpopulation was isolated by treating the stationary cells with an antibiotic. The choice of antibiotic was ciprofloxacin, because of its efficiency to kill both growing as well as non-growing bacteria. Cells were exposed to 10-fold Minimum Inhibitory Concentration (MIC) of ciprofloxacin. Antibiotic addition resulted in a rapid reduction in cell number for the initial 3 – 4 h; after that, a stable population of 10^7 CFU mL^{-1} was formed (**Figure. 3.2**). The cells that survived the high dose of antibiotics are termed as antibiotic-triggered persister cells, denoted as P-cells.

Antibiotic exposure often results in the rise of antibiotic-resistant mutants. The resistant mutants have a significant increase in MIC value and can grow in the presence of antibiotics. Accordingly, we isolated P-cells and inoculated into LB without antibiotics. The MIC value of the recovered population had not changed (**Table. 3.1**).

Table. 3.1. The MIC values of cultures before and after treatment with ciprofloxacin.

Culture	Before ciprofloxacin treatment ($\mu g\ mL^{-1}$)	After ciprofloxacin treatment ($\mu g\ mL^{-1}$)
V. cholerae cells	0.1	0.1

The recovered population was challenged with 10-fold MIC of ciprofloxacin, and kill kinetics was determined. The cells were as sensitive as the previous population, and no change in the kill kinetics was observed (**Figure 3.3**); these results indicate that the presence of resistant clone among the P-cells is unlikely.

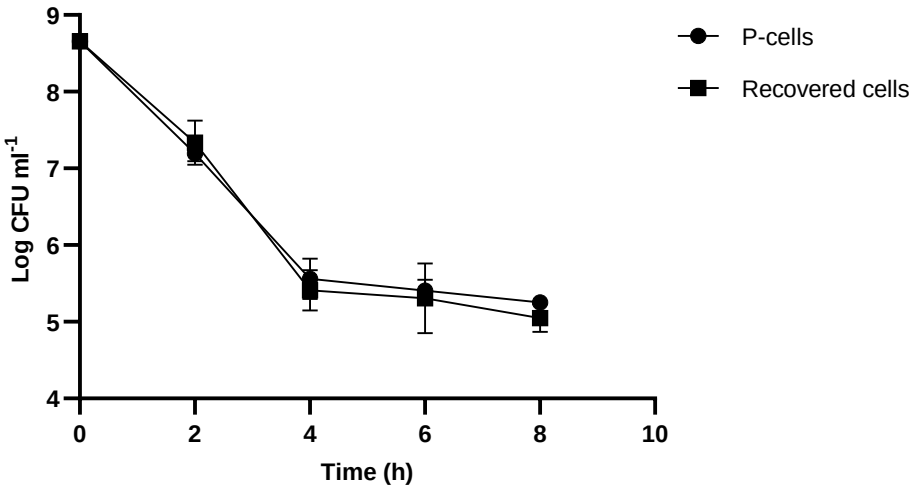

Figure. 3.3. Antibiotic susceptibility of recovered P-cells. The cells were recovered in LB and exposed to 10-fold MIC ciprofloxacin. The kill kinetics was prepared with Log cell number on X-axis and Time on the Y-axis. The kill kinetics of original P-cell population was taken as control and was compared with the kill kinetics of recovered cells. The difference between the kill kinetics was measured using one-tailed t-test with cut off p= 0.05. The results plotted above represent the mean on three biological replicates with S.D.

Earlier reports have suggested that antibiotic tolerant populations often contain cells in Viable But Non-culturable (VBNC) state[16]. In the present study, we performed the VBNC resuscitation assay to determine whether the P-cell population contains VBNC. Post antibiotic exposure, we recovered the surviving P-cells in Artificial Sea Water (ASW) and incubated it for 3 days at room temperature. There was no significant increase in the P-cell population after the overnight resuscitation (**Table. 3.2**). VBNC formation in the antibiotic-triggered population may require more stringent conditions[56,57]. Therefore, it is unlikely that the isolated P-cells to contain the VBNC population.

Table. 3.2. Test for VBNC formation. The cells were resuscitated post-antibiotic challenge, and cell number was determined. The cell number after resuscitation has not increased, and the cell number is like one obtained immediately after the antibiotic challenge.

Antibiotic treatment Time (h)	P-cells immediately after treatment	P-cells after resuscitation	Unpaired t-test of significance
0	9.27 ± 0.155	9.41 ± 0.154	Not significant
4	8.6 ± 0.32	8.72 ± 0.24	Not significant
8	7.5 ± 0.302	7.78 ± 0.294	Not significant
24	7.08 ± 0.177	7.67 ± 0.168	Not significant

The average of three biological triplicates with S.D is given in the table (n=3). The difference between the cell number before and after resuscitation was determined using unpaired t-test, p=0.05.

Both P-cell and E-cell sub-populations comprise 1% and 10% of stationary phase cells, respectively, which qualifies them as sub-populations. Both the sub-populations share common characteristics such as no increase in cell number and tolerance to antibiotics (as discussed in chapter 3.3 under section 3.3.1 and 3.3.7). The E-cells and P-cells may form either as a response to stress or owing to stochastic physiological effects. The dynamic physiological and morphological changes contribute to the formation of E-

cells during nutrient depletion[9,58], and the P-cells can occur as a small population in a growing culture and need not form in response to stress[26,46]. The formation of both subpopulations may exhibit overlapping molecular events, such as TA modules, SOS response, growth arrest, and reduced PMF[2,22,56,59,60].

3.1.1 Characterization of E-cells and P-cell subpopulations

Morphology

We documented the cell size and colony characteristics of both the sub-populations. The E-cells undergo several changes in cell wall composition, cell size and volume, motility, and macro-molecular content (**Figure. 3.4**). E-cells were smaller (1.18 ± 0.27µm), and the P-cells were longer (14.5± 4.37µm) compared to non-stressed growing cells (2.56± 0.13µm). P-cells were a long filamentous chain of smaller cells; however, the number of cells in each chain was varying. The colony characteristics of both P-cell and E-cell subpopulations were documented after incubating the plates at 37°C for 16h. E-cells formed smaller colonies of 1 – 2 mm diameter, and P-cells formed regular colonies of size 2 – 3 mm in diameter. We could not find any detailed report on changes in cellular morphology and macro-molecular content of persister cells. However, as one study points out, the elongated P-cells become spherical with ageing[16]. One earlier work has shown the spontaneous appearance of rugose colonies upon long-term nutrient starvation[61]. However, in the present work, neither E-cells nor P-cells showed rugose morphology.

3.1.2 Antibiotic persisters are better at tolerating stress

Heat stress: The heat stress reduced the P-cells and E-cells population in a biphasic manner (**Figure. 3.5 A**). In 60 minutes of heat stress, 99% of the P-cell population, and over 99.99% of the E-cells population were eliminated. The E-cells cell number

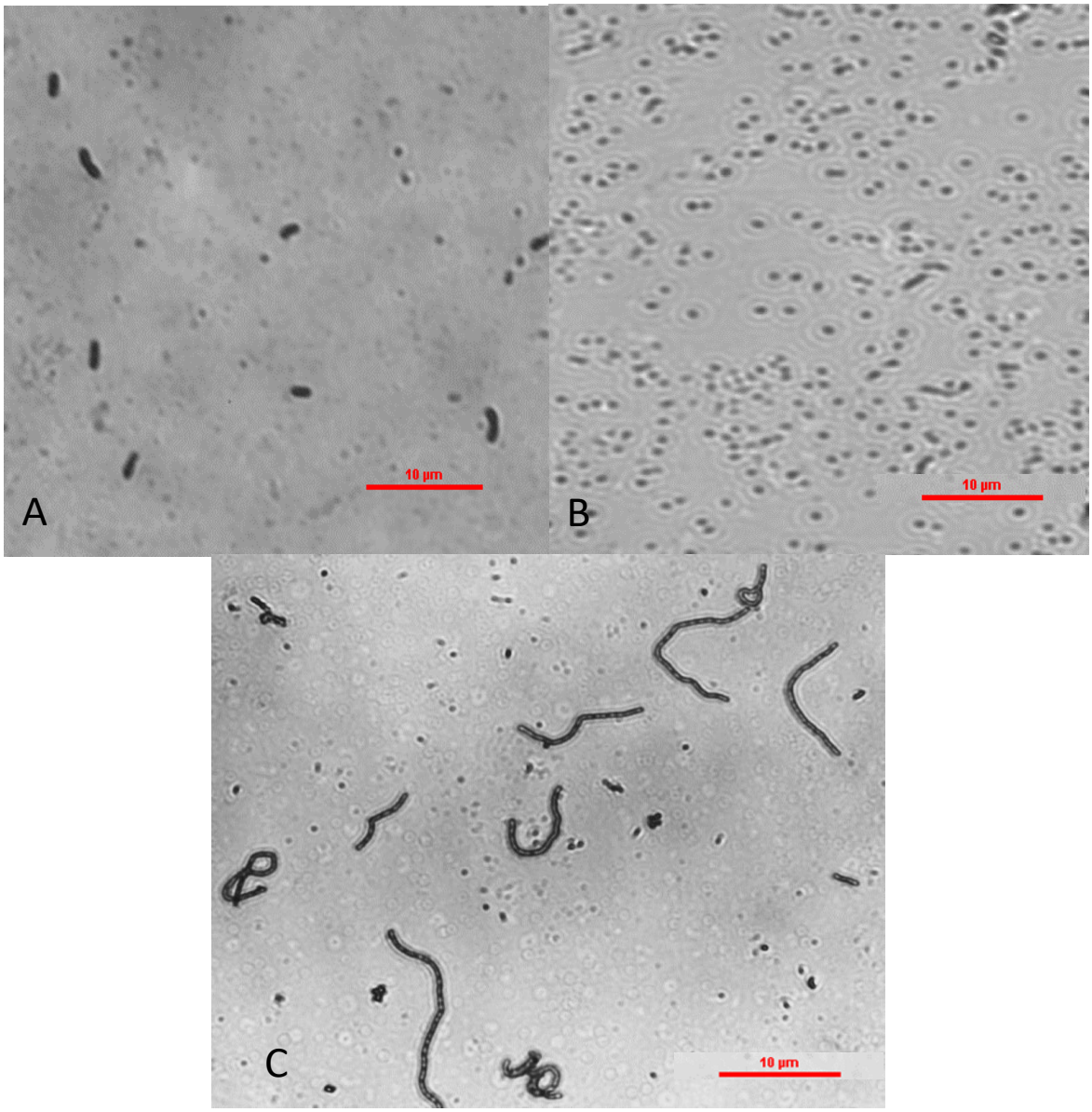

Figure. 3.4. The microscopic images of persister subpopulations. A) Non-stressed control growing population of V. cholerae. B) E-cells obtained after starvation for 24 h C) P-cells obtained 4 h after exposure to 10-fold MIC ciprofloxacin Scale bar: 10 µm. The image was taken in Nikon Ni-U microscope and analysed through NIS-Elements.

reduction was rapid when compared to both P-cells and non-stressed population.

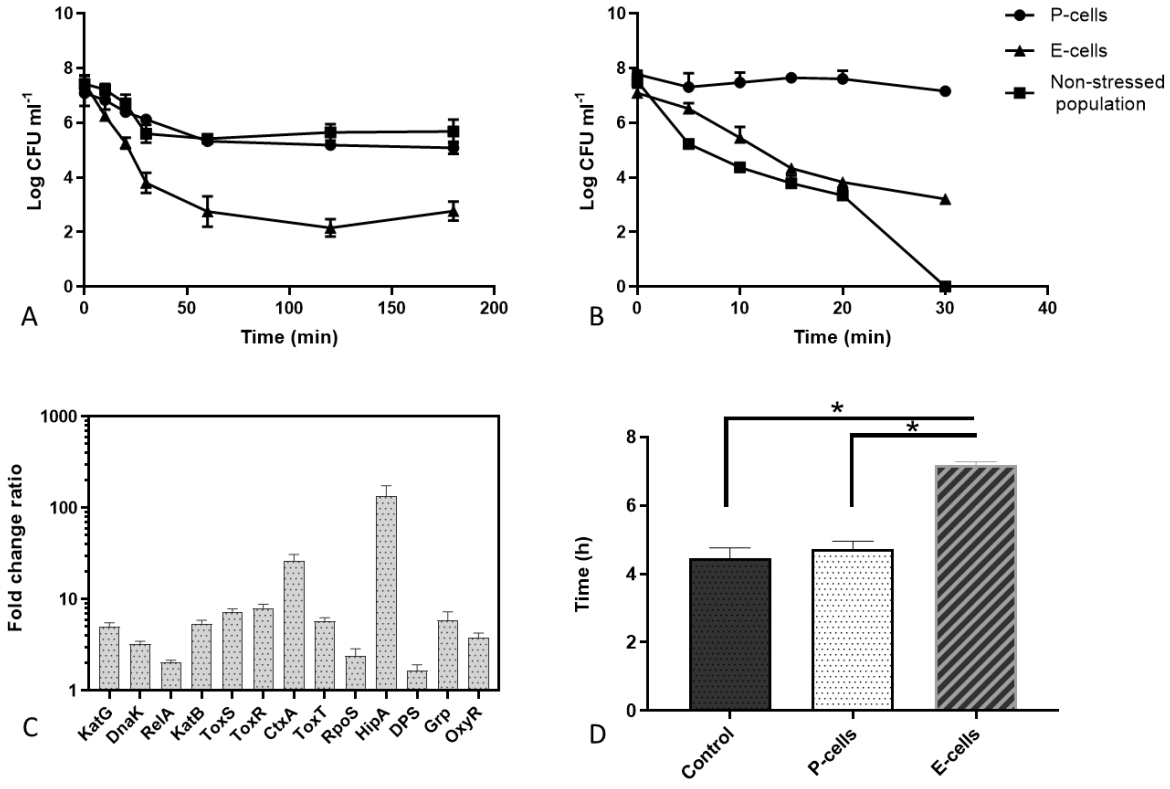

Figure. 3.5. Abiotic stress tolerance of persister subpopulations. A) The heat stress response of E-cells and P-cells at 45 °C for 180 min. B) The oxidative stress response of E-cells and P-cells at 5mM H_2O_2 for 180 min. C) The quantitative PCR analysis of important gene involved in oxidative stress and TA system. The gene expression of P-cells was compared with that of E-cells. 16s rRNA and recA were used as house-keeping genes. X-axis indicates the fold change in expression of mRNA in P-cells with respect to mRNA in E-cells. D) The lag time measurement during recovery from stress. The lag time of subpopulations were compared with that of the growing non-stressed population. The number of cells in all the test samples was maintained ~ 10^5 CFU ml^{-1}. The bar graph represents the mean with SD, *p ≤ 0.05 (n=3).

However, after the initial killing, the survivors of both the sub-populations could tolerate the heat stress for the remaining course of the experiment, and we did not observe the further reduction in cell number. The heat stress tolerance pattern of the non-stressed control population was like P-cells.

Oxidative stress: In 30 minutes of oxidative stress, 99.9% of the E-cells were killed; however, the P-cells could tolerate oxidative stress with no loss of viability (**Figure. 3.5 B**). In comparison, the non-stressed population did not survive oxidative stress beyond 30-minute. We measured the catalase enzyme activity in both the sub-populations. The P-cells had higher catalase activity (13.08 U mg^{-1}) than the E-cells (6.84 U mg^{-1}), and this difference was significant at p = 0.05. The catalase activity of the non-stressed population was less than the sub-populations (4.68 U mg^{-1}). The high catalase activity in P-cells may help tolerate oxidative stress.

Radiation stress: Both the sub-populations were exposed to γ-radiation. The D_{10} value of E-cell and P-cell was 59 Gy and 43 Gy, respectively (**Figure. 3.6**).

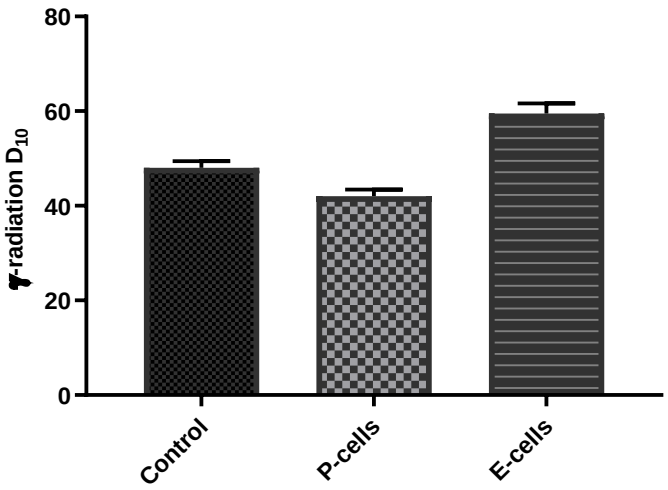

Figure. 3.6. The gamma radiation sensitivity of persister subpopulations. The values in bar represent the average D_{10} values in Gy with S.D (n=3). The difference between D_{10} values were tested for significance using Student's t-test at p=0.05 and was found to be not significant.

In comparison, the D_{10} of exponentially growing cells of *V. cholerae* was 48 Gy. The D_{10} values of the sub-populations and the control population were not significant at p = 0.05 (Student's t-test).

Long-term starvation in *V. cholerae* results in the reduction in cell volume, reduction division, loss of inclusion bodies, loss of membrane integrity, compressed nuclear volume, and formation of convoluted structures[1]. The starved cells of copiotrophs such as *E. coli*, *P. putida*, and *Vibrio* sp. strain S14 survived the heat stress better than the non-starved counterparts[35]. Contrary to the above reports, the E-cells (*V. cholerae* El Tor O1 strain) were more sensitive to heat and oxidative stress. This may be because of the difference in the extent of starvation and medium used to impart nutrient depletion. Heat stress kills bacteria by destabilizing the lipid bilayer allowing small molecules to escape which change the electrical conductivity of the cell leading to the loss of viability[62].

The morphological changes such as membrane modifications may sensitize the cells to heat stress and oxidative stress. P-cells arise from a well-nourished growing population and resemble dormant cells with low metabolic activity[63]. The antibiotic persister cells because of being in a "healthier nutrient state" could survive the heat stress, although with considerable loss of viability. The radiation resistance depends on the genetic and physiological makeup of the cell[64]. Results from the present study suggest that both the sub-populations are sensitive to gamma radiation. Though the P-cells are tolerant of other stresses, they are sensitive to DNA damaging stresses. The morphological changes in E-cells and high antioxidant activity of P-cells could not protect them from the DNA damage. The starved bacterial cells are more tolerant of non-ionizing radiation such as UV radiation[65]. But the high energy ionizing radiation can still reduce the dormant population of *V. cholerae* with high potency. When compared to both the sub-populations the non-stressed cells were sensitive to oxidative stress. The higher antioxidants in E-cells and P-cells could protect them from oxidative challenge.

3.1.3 Antibiotic persisters show enhanced catalase activity and *hipA* expression

E-cells and P-cells both represent a dormant cell condition under abiotic stress. Both may likely have common gene expression patterns. The expression of relevant genes involved in persister cell formation and toxin production of E-cells and P-cells was studied using RT-PCR. The list of primers is given in Annexure. The gene expression of E-cells was taken as control, and relative to that, the fold change in the gene expression of P-cell was determined (**Figure. 3.5 C**). The genes involved in stress tolerance, such as *dnaK* (+3.25-fold), *dps* (+1.65-fold), and *grp* (+5.89-fold) were up-regulated in the P-cells as compared to E-cells. The cholera toxin gene *ctxA* (+ 26-fold), its transcription activators, such as *toxS* (+ 7.2-fold) and *toxR* (+ 7.9-fold) were also up-regulated in the P-cells. The antioxidant gene cascade involving *katG* (+5.01-fold), *katB* (+5.45-fold), *oxyR* (+3.8-fold) were also up-regulated in P-cells. One of the TA systems implicated in persistence, *hipA* was up-regulated by 134-fold in the P-cells.

The goal of the present experiment was to compare the persister subpopulation among themselves. The genes that were measured belong with stress-tolerance, cholera toxin expression, and antioxidant enzymes. These genes are expected to show higher expression during stress, such as nutrient limitation or antibiotic exposure. Besides, the non-stressed cells are actively growing, overall gene expression is higher in non-stressed cells as compared to any cell population under stress[66]. Hence, the gene expression of subpopulation was compared to identify minor changes in gene expression. A comparison between non-stressed and persister cells would lead to the underestimation of the expression of essential genes involved in persister cell physiology.

The gene expression data of 16s rRNA and rpoS was used as the internal control for normalization of gene expression (house-keeping genes). The 16s rRNA is a widely used house-keeping gene that has stable expression over a wide range of physiological conditions[67]. The recA was chosen because of its exceptional stability during starvation survival[68]. Besides, both the house-keeping genes were validated for stable expression during persister formation and starvation survival.

Bactericidal antibiotics and oxidative agents can induce reactive oxygen species (ROS) formation by stimulating the respiration and depleting NADH formation[69]. The stimulated respiration would increase the electron transport resulting in increased levels of superoxide and other ROS; such free radicals may damage DNA, lipids, and proteins that may result in cell death[70]. Cells can tolerate oxidative stress by synthesizing antioxidant enzymes such as catalase and peroxidase to scavenge the ROS and other free radicals[69]. Besides catalase-peroxidase genes, the P-cells also showed increased expression of the genes involved in cholera toxin and general stress tolerance. The antibiotics kill bacterial cells by increasing oxidative stress[71]. The cell responds by producing a high level of antioxidant enzymes to counter the damage, which appears to be true for the present study. Specific environmental stresses, such as starvation and antibiotics, can induce the expression of toxin-antitoxin (TA) modules[72]. The *hipA* gene was over-expressed in P-cells, but not in the E-cells, showing that the hipBA toxin-antitoxin system is not involved in the starvation-triggered persistence in *V. cholerae*. The nutrient-deprived cells down-regulate the rate of protein synthesis (by about 20%) and speed up ribosome hibernation to reduce the overall metabolism and gene expression[2]. The P-cells are also in the lower metabolic state, but there can be gradients

in metabolic activity[73]. Even though both the populations could tolerate a high dose of ciprofloxacin, only one of them appears to depend on the *hipA* TA system.

3.1.4 Recovery studies

The growth kinetics of sub-populations during recovery (Nutrient up-shift) was studied (**Figure. 3.5 D**). The E-cells and P-cells recovered from dormancy after six h and four h of lag time, respectively. The lag time of P-cell was comparable with that of control the non-stressed population (**Figure. 3.7**). The rest of the kinetic parameters such as growth rate and yield of cells remained the same in both the sub-populations. The starved cells recovered from dormancy with an increase in lag phase[35]. The starved cells of *Vibrio* sp. S14 keeps an excess of protein-synthesizing machinery, including stable RNA and ribosome[35]. However, the delayed cell division initiation and the continuous degradation of endogenous material might cause an increase in lag time[35]. Persisters arise because of a stochastic reduction in metabolism[56]. Nevertheless, as

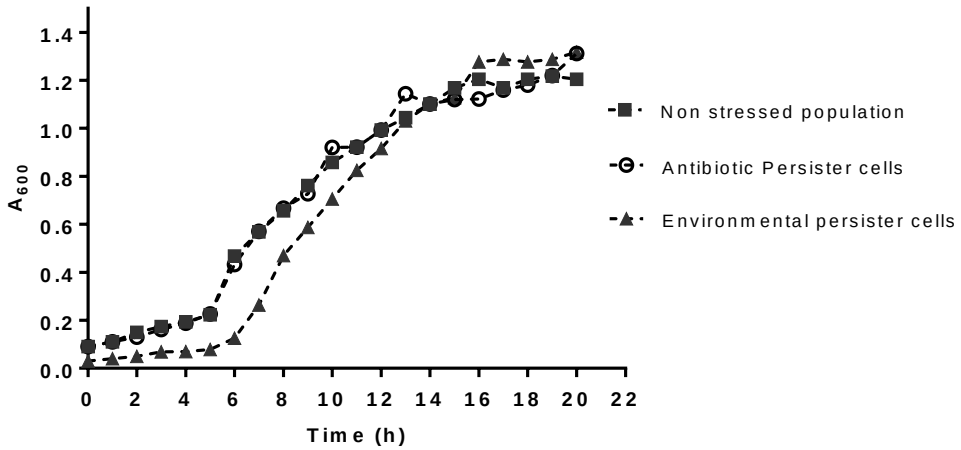

Figure. 3.7. The recovery kinetics of persister subpopulations. Both E-cell and P-cell were inoculated in LB and allowed to grow. The non-stressed population was taken as control. The values in the graph represent the mean of three biological experiments with S.D.

reported here, the P-cells are elongated filaments which can quickly divide into multiple small cells and regrow, with no extension in lag time.

The single-cell assays have established the heterogeneous nature of clonal populations[74]. However, the open question is the biological importance of observed cellular heterogeneity[74]. The phenotype of a large population is often the phenotype expressed by most of the cells. A small minority of cells away from the mean physiology of the population may have distinct phenotypes. Phenotypic variants can arise as a bet-hedging strategy to cope with a fluctuating stressful environment[52,74]. Starvation and antibiotics represent two different environmental triggers that can restrict bacterial growth. In both the conditions, only the persister survives and can regrow when the opportunity arises.

Table 3.3. The comparative chart of persister subpopulations.

	Nutrient-triggered persister cells (E-cells)	**Antibiotic-triggered persister cells (P-cells)**
Heat stress	++	+++++
Oxidative stress	+++	+++++++
Radiation stress	D_{10} - 59	D_{10} - 43
Recovery from stress	Extended lag	No lag extension
Cell Morphology	Small (1.18 ± 0.27µm)	Elongated (14.5 ± 4.37µm)
Colony	Small (1 – 2 mm)	Regular (2 – 3 mm)
Growth arrest	Yes	Yes
Antibiotic tolerant	Yes	Yes

Note: The '+' sign indicates the tolerance

The non-growing population could be triggered to produce at least two subpopulations, the P-cell and E-cell persister subpopulation. Both the sub-populations differ from each

other regarding cell and colony morphology. The sub-populations are not genetic mutants and do not harbor VBNC phenotypes. Both the sub-populations differ in abiotic stress tolerance and recovery, yet they share common features such as antibiotic tolerance and growth arrest. The antibiotic-triggered persisters change colony morphology but show no lag extension during recovery. The two persister sub-populations may give fitness advantage during adverse environmental conditions. Molecular mechanisms and physiological changes that lead to the formation of two subpopulations are challenging to determine, and these studies warrant a need for a better selection method for separating the subpopulation based on unique molecular markers.

Further studies can shed more light on the decrease in cell diameter of filamentous persister cells. Both subpopulations are dormant cell types, and hence stringent responses may be involved in the initiation and maintenance of persister phenotypes. The results presented here extend the possibility of the presence of physiologically distinct subpopulations in any given microbial culture. Some of these subpopulations may have overlapping phenotypes, making them difficult to isolate and characterize. Further, this study also makes a stronger argument for the study of single-cell bacterial physiology.

3.1.5 Chapter summary

The phenotypic heterogeneity in a large population arises because of variation in microenvironments and stochastic gene expressions. In the current chapter, we isolated two types of persistent sub-populations of *V. cholerae*. One of the sub-populations was triggered by nutrient limitation (E-cells) and another by exposure to a lethal concentration of antibiotics (P-cells). We characterized the E-cells and P-cells for stress

tolerance, colony morphology, and toxin gene expression. Both the sub-populations differ concerning morphology, temperature tolerance, and oxidative stress tolerance. The E-cells were smaller than the P-cells, and their colonies appeared tiny (1-2 mm). The E-cells were more sensitive to heat and oxidative stress as compared to P-cells. The up-regulated genes of P-cells included the genes of antioxidant enzymes (>5 fold), cholera toxin (>26 fold), and toxin: antitoxin protein *hipA* (>100 fold). Upon supplementing nutrients, the E-cell recovered after a lag time of 6 h. However, such a slow recovery was not observed during P-cell recovery, suggesting that P-cell physiology is more comparable to healthy cells than E-cells. This is the original comparative study on the two distinct persister sub-populations of *V. cholerae*.

3.2 Starvation induced persisters

In the present section, the starvation-induced persisters were further studied. *V. cholerae* experiences nutrient limitations during prolonged survival in seawater between the epidemic. After the cholera infection, a large number of the bacteria is shed in the stool; from there, it makes its way in drainage water and later into fresh or marine water[75]. While it travels, it experiences uneven nutrient distribution. In fresh or estuarine water, the bacteria form biofilm on the crustacean and mollusks for survival[9]. During long term starvation in *Vibrio*, cellular macromolecules such as carbohydrates, β-hydroxybutyrate, proteins, RNA, and DNA per cell reduced drastically, up to 90 % of the original amount[5]. Members of the *Vibrio* genus poorly survive the carbon, nitrogen, and phosphate starvation, and stringency is less efficiently activated than other species of *Enterobacteriales*[33,35]. Most of the study in multi-nutrient starvation and associated cellular changes in *Vibrio* is carried out using *Vibrio* sp. S14 as model organism[8]. However, the S14 strain now classified as *Photobacterium angustum*, is not in the *Vibrio* genus and is photogenically distinct from *V. cholerae*[76,9].

The section deals with the multi-nutrient starvation profile of *V. cholerae* and determines why the DNA decreases during starvation. Further, the section explores how the individual nutrient starvation (carbon, nitrogen, and phosphate) impacts the survival of *V. cholerae*, with emphasis on phosphate limitation. Present work is derived from the following publication:

Paranjape S, Shashidhar R. The ploidy of Vibrio cholerae is variable and is influenced by growth phase and nutrient levels, FEMS Microbiology Letters, Volume 364, Issue 19, October 2017, fnx190, https://doi.org/10.1093/femsle/fnx190 [77]

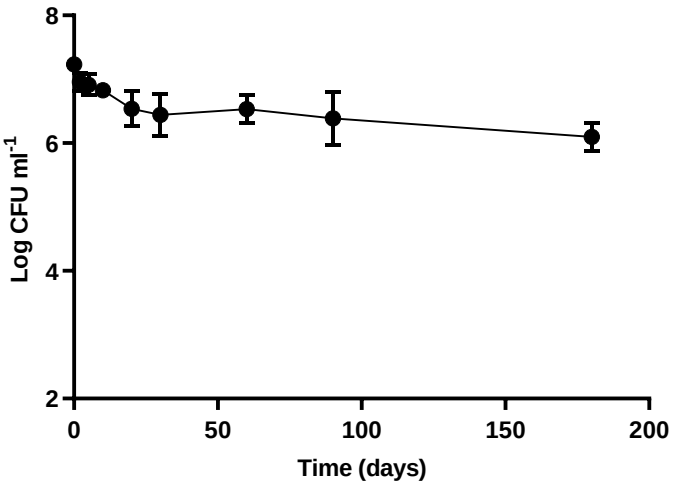

Figure 3.8. Survival under multi-nutrient starvation. V. cholerae was exposed to simultaneous carbon, nitrogen and phosphate starvation. Cell number was monitored over 180 days. The Y-axis denotes cell number in log scale and X-axis is time in days. The data represents the average of three biological replicates with standard deviation (n=3).

V. cholerae survives the long terms starvation in ASW (carbon, nitrogen, and phosphate limitation) for over 6 months (**Figure. 3.8**). Only a 10% reduction in cell number was observed. We tested whether cells had gone into the VBNC phenotype and found that no VBNC was formed. During such long-term starvation earlier, researchers have reported a reduction in DNA per cell. We investigated the causes of the reduction in DNA per cell in *V. cholerae*.

3.2.1 *V. cholerae* is a polyploid organism

V. cholerae is a polyploid with genome copy number reaching 71 ± 5.6 per cell during the 8 h exponential phase (**Figure. 3.9**). The cell maintained its polyploidy condition even after 24 h of incubation in LB. The ploidy of *Escherichia coli* was determined as

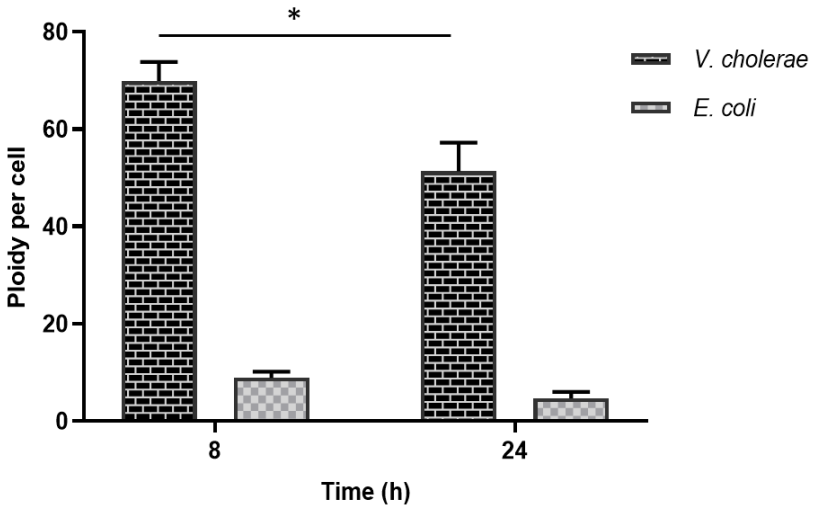

Figure. 3.9. Determination of the ploidy of V. cholerae during the laboratory growth condition. The ploidy of V. cholerae was determined during the exponential phase and in the stationary phase using qPCR-based method. The ploidy of E. coli was also determined as a positive control. The (*) indicates the level of significance at p<0.05. The mean of three biological replicates with standard deviation is plotted (n=3).

a positive control. During the exponential phase, the *E. coli* ploidy number was eight, and it agreed with the earlier reports[48]. The ploidy calculated from the spectrophotometric method and the qPCR method was compared in Bland-Altman plot [78] and were found to be in excellent agreement (**Figure. 3.10**). The p-value of the t-test was <0.05, indicated that there is no significant difference between the ploidy values obtained from the spectrophotometer and the qPCR. All further experiments were carried out using the qPCR method.

3.2.2 The ploidy number depends on the growth phase and nutrient levels

The ploidy during different stages of the *V. cholerae* growth curve was determined (**Figure. 3.11**). The ploidy increased steadily from the lag phase to the log phase. The highest ploidy (71 ± 2.5) was observed after 8 h of growth. During the prolonged stationary phase (48 h) ploidy number decreased to 20 ± 1.4 per cell. The ploidy was also determined under the limited nutrient condition in ASW.

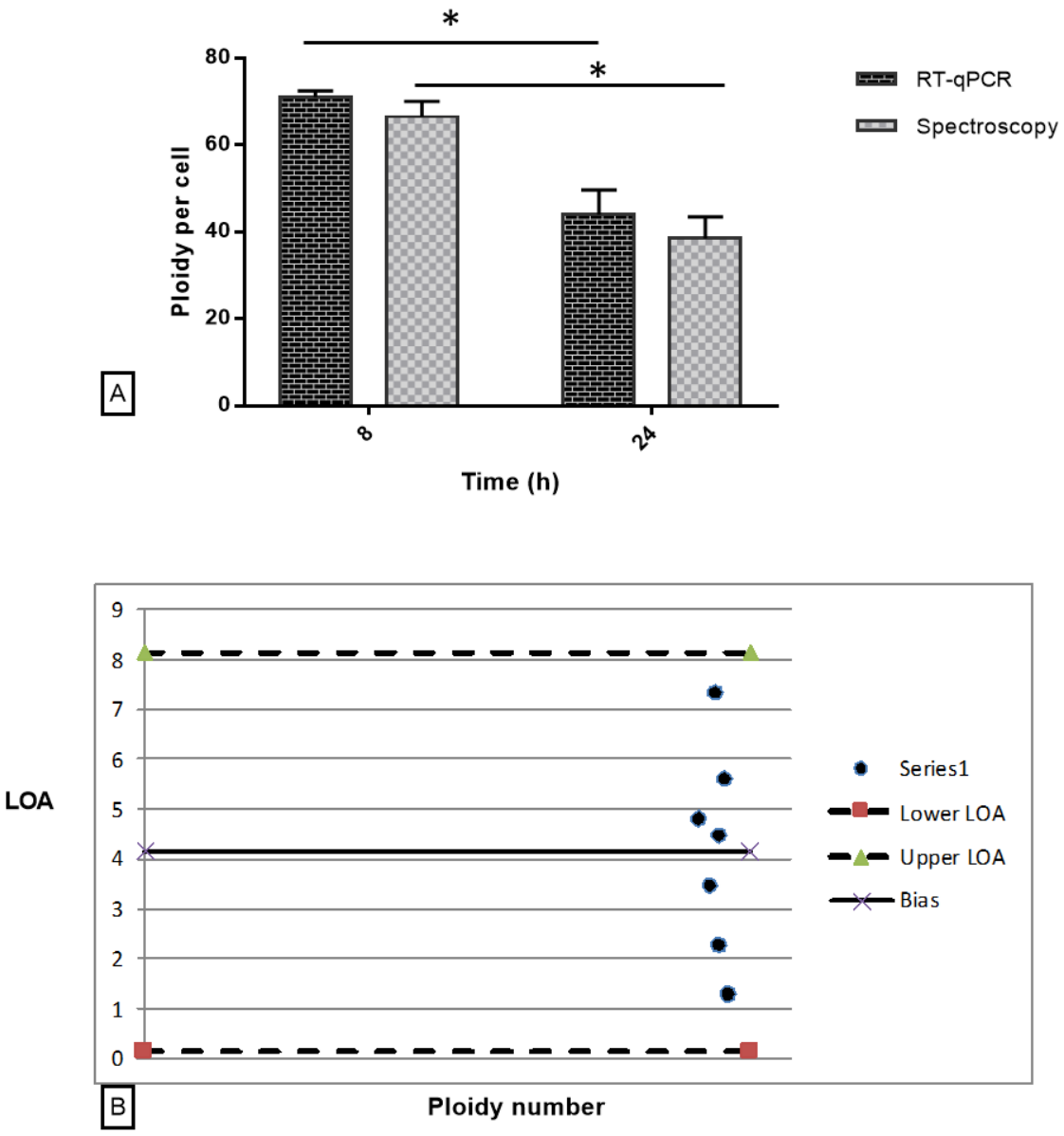

Figure. 3.10. ***The ploidy of V. cholerae as determined by qPCR and by spectroscopy. A) The ploidy was determined during exponential growth phase (8h) and during the stationary phase (24 h). The experiments were carried out in triplicates. B) The Bland-Altman plot of agreement. Here the ploidy values from both the methods are plotted. The area between the Upper LOA and lower LOA indicates the zone of agreement. The values are calculated with the bias of the experiment. These values which fall within the zone of agreement are said to be correlated and in agreement. The p values were calculated from Student's t-test for both the methods and by conventional criteria, this difference is not statistically significant. The (*) indicates the level of significance at p<0.05 between 8h and 24h.***

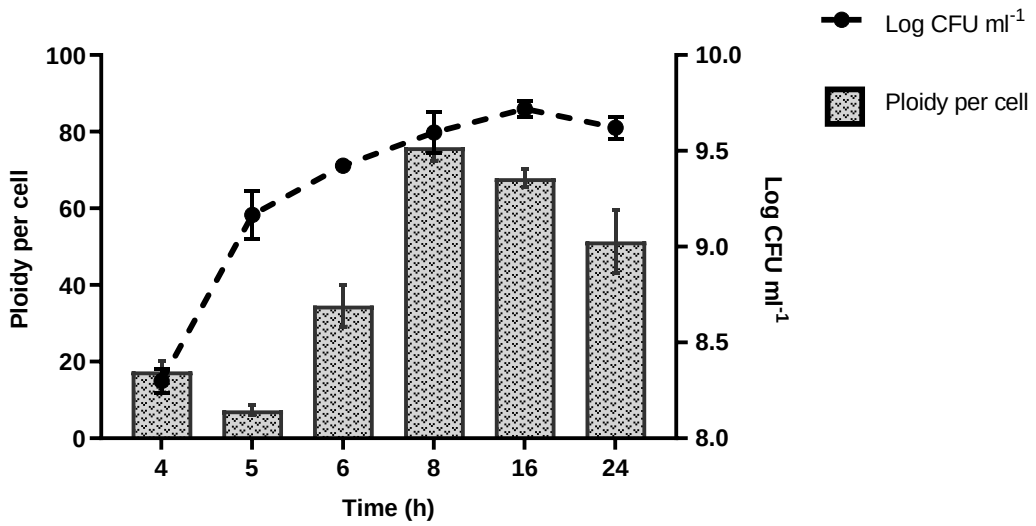

Figure. 3.11. The changes in ploidy as a function of growth curve. The dashed line indicates the growth curve of V. cholerae during laboratory growth conditions. The Solid bars represent the ploidy per cell. The ploidy per cell was determined using qPCR method and has been validated with biological and technical triplicates (n=3).

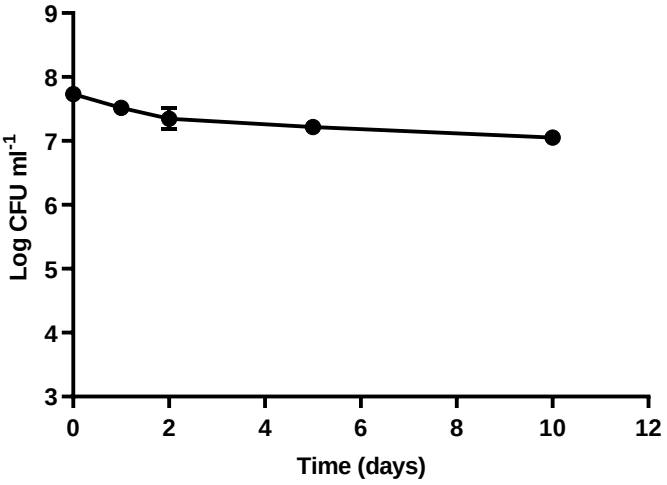

Figure. 3.12. The survival of V. cholerae in ASW. The survival was tracked till 1o days into ASW. Average of three biological triplicates with S.D is shown above.

The cell number reduction in ASW was negligible during the ten days of starvation (**Figure. 3.12**). However, the ploidy of the starved cells was considerably reduced (**Figure. 3.13**). The starved cells reduced their ploidy number to 15.7 ± 4.9 per cell within two hours of starvation. Further, the reduction in ploidy progressed gradually,

and by 24 h, the ploidy number reached 6.46 ± 1.3 per cell. The ploidy was 2.34 ± 1.4 per cell, even after ten days of starvation.

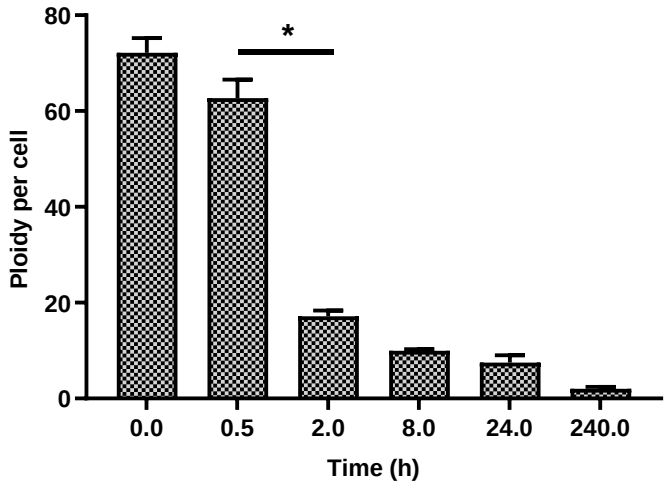

Figure. 3.13. The reduction of ploidy levels during starvation. The ploidy was plotted against the time of incubation in low nutrient media. The ploidy was calculated up to 10 days of starvation. The significant difference in ploidy ($p < 0.05$) is indicated by the * mark. The reading was carried out in triplicate samples.

Several species of the phylum Proteobacteria are oligo or polyploid, such as *Azotobacter vinelandii*, three species of *Neisseria, Buchnera* species, and two species of *Desulfovibrio*. Another member of the proteobacteria, *Neisseria gonorrhoeae*, is a diploid with two genomes before and four genomes after replication. This ploidy depends on growth rate and optimal conditions [49]. Hence, we postulated that the reduction in DNA is due to the reduction in extra DNA copies or ploidy. The ploidy of *V. cholerae* has not been reported earlier, and this is the first report on the ploidy of *V. cholerae*.

The ploidy of *V. cholerae* varied with the growth stages as well as under the nutrient stress. Cells in the lag and the stationary phase divide slowly, in comparison to the log phase. The decrease in cell division rate, in turn, downregulates the DNA replication rate. Both lag and stationary phases have reduced metabolic rate and consequently

reduced DNA replication and cell division. Therefore, in lag- and stationary-phase cells, a reduced ploidy number was observed. However, the optimum cell division conditions in the log phase lead to high DNA synthesis and cell division. Hence, log-phase cells accumulate higher DNA copies and thus become polyploids. These observations draw strength from Amir *et al*[79], where the connection between cell volume, cell division, and DNA replication has been extensively reviewed. Among the 11 species of the proteobacteria, only three are truly monoploid[48]. The *E. coli* is a monoploid during prolonged growth but becomes a mero-oligoploid during fast growth. Mero-oligoploids are essentially haploid organisms with partially duplicated chromosomes. Multiple origins of replication are fired up to fasten the DNA replication rate resulting in a partially replicated chromosome. The ploidy is influenced by growth rates, as seen in *Desulfovibrio*, which has 17 genome copies during rapid growth in batch cultures and 9 genomes per cell in chemostat cultures[80].

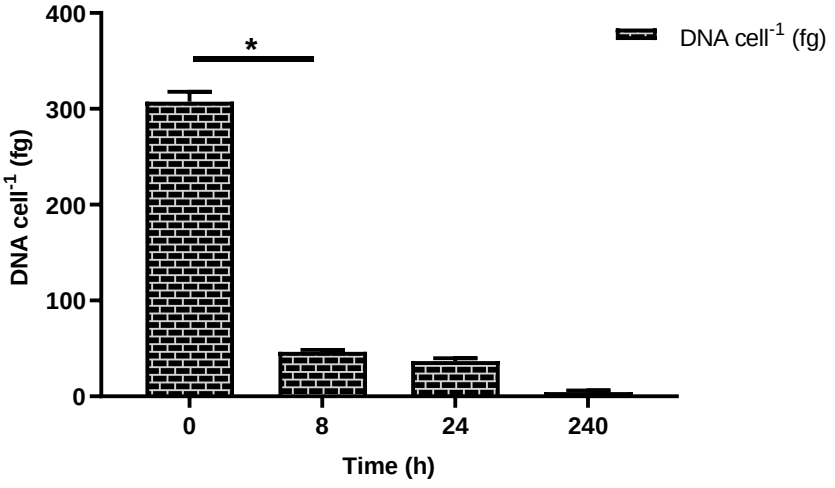

Figure. 3.14. Reduction in DNA per cell during starvation. The DNA isolated from starved cells was quantified by spectroscopic method at 260nm. The data represents the mean of three biological triplicates with standard deviation. The significant difference in DNA per cell ($p < 0.05$) is indicated by the (*) mark.

3.2.3 DNA per cell reduces during starvation.

The reduction in DNA content per cell was monitored in the starvation regime. The reduction in DNA content per cell was rapid during the first 8 h of starvation in ASW; however, the cell number reduction was minimal (**Figure. 3.14**). The initial DNA content per cell was 314.7 ± 18.9 fg per cell, which was reduced to < 70 ± 3.5 fg per cell within 8 h. After this, a gradual reduction of DNA per cell was observed throughout the starvation phase. On the 10^{th} day of starvation, DNA was reduced to 10.89 ± 1.5 fg per cell, corresponding to a 97% reduction from initial DNA content. The reduction of DNA was biphasic with an initial rapid fall in DNA per cell, followed by a gradual but continuous decline over the entire period of starvation. This pattern of DNA reduction during starvation correlates with that of the ploidy with r = 0.995 (**Figure. 3.15**).

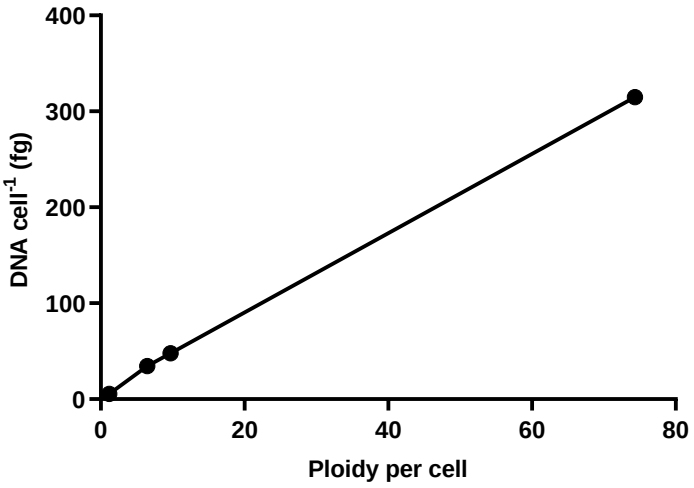

Figure. 3.15. The correlation between the reduction in DNA and corresponding ploidy reduction during starvation. The DNA (fg per cell) are plotted against the ploidy per cell. The regression coefficient was, r = 0.995.

Hood *et al*. had observed the rise in cell number during initial hours of starvation, and they had concluded that the DNA per cell reduction is due to this increase in cell number[81]. However, in this study, we did not observe any increase in cell numbers

during starvation. The cell number declined by 10% of the initial population soon after induction of starvation; after that, the cell number remained constant through the 10 days of starvation. The observed rise in cell number by earlier authors may be due to the carryover effect of nutrients from culture inoculum or stored polyphosphates or PHB within the cell that might have provided essential nutrients for survival under starvation. Morita and Novitsky have suggested several possibilities for the reduction in DNA, such as loss of DNA due to the leaky cell membrane or reduction in extra DNA copies or degradation of DNA due to stress or changes in the configuration of nucleotide, or some unknown mechanism[82].

3.2.4 Effect of nutrients supplementation on ploidy number

The ASW does not contain any phosphate sources. Hence, phosphate supplementation was postulated to slow down DNA reduction. However, the ploidy levels did not increase in the presence of the phosphate (**Figure. 3.16**), and the cells did not show any

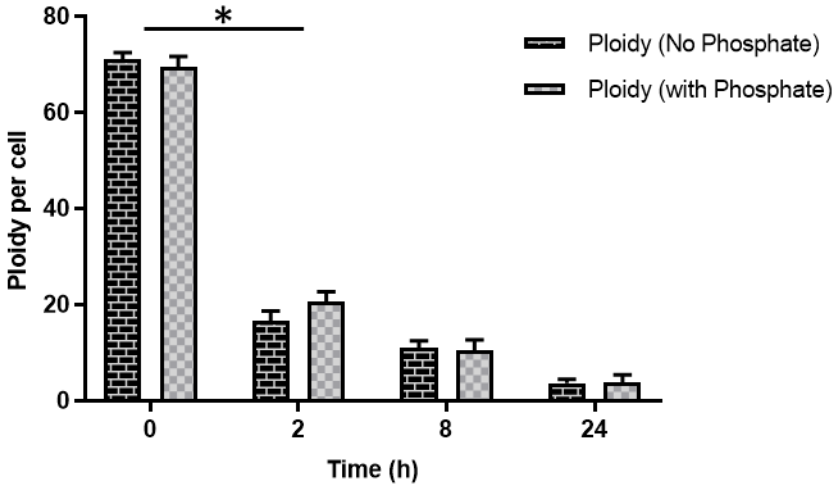

Figure. 3.16. Effect of phosphate supplement on ploidy. The reduction in ploidy with or without phosphate supplement was determined. The ASW was supplemented with 10mM K_2HPO_4 (Light column) and ploidy levels were monitored. The ploidy from non-supplemented ASW (dark column) was used as positive control. In both the conditions the ploidy reduced significantly 2h post nutrient limitation(* $p<0.05$). The mean of three biological triplicates with the standard deviation are provided.

changes in survivability in the presence or the absence of the phosphate. The starved cells were tested for their ability to recover the ploidy number, during the nutrient upshift. The starved cell inoculum was supplemented with 1X LB broth. The availability of all essential nutrients prompted the cells to grow exponentially to maximum population density, though, with an extended lag period. The cells recovered their ploidy quickly during the nutrient upshift and reached maximum ploidy of 72 per cell during the exponential phase (**Figure. 3.17**).

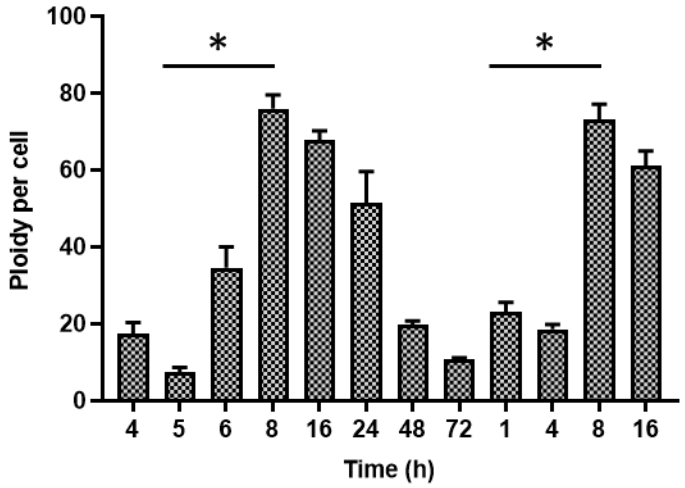

Figure. 3.17. The recovery of ploidy during nutrient up-shift. The initial phase (A) represents the rise in ploidy during the exponential growth. The next phase (B) represents the decline in ploidy during the late stationary and the starvation period, where the ploidy goes below 10 per cell. The final phase (C) represents the recovery of ploidy, as the starved cells resume growth upon nutrient up-shift. The (*) indicates the significant difference between ploidy of late exponential phase and early exponential phase at p<0.05.

3.2.5 DNase activity increases during starvation

The cells from exponentially growing culture and the cells from 2 h starved culture was compared for their DNase activity. The starved cells showed increased DNA degradation than that of fresh cells (**Figure. 3.18**). The DNA degradation increased, with as the starvation duration was increased, indicated by the extended smear formation on the agarose gel. The addition of EDTA inhibited the degradation of DNA,

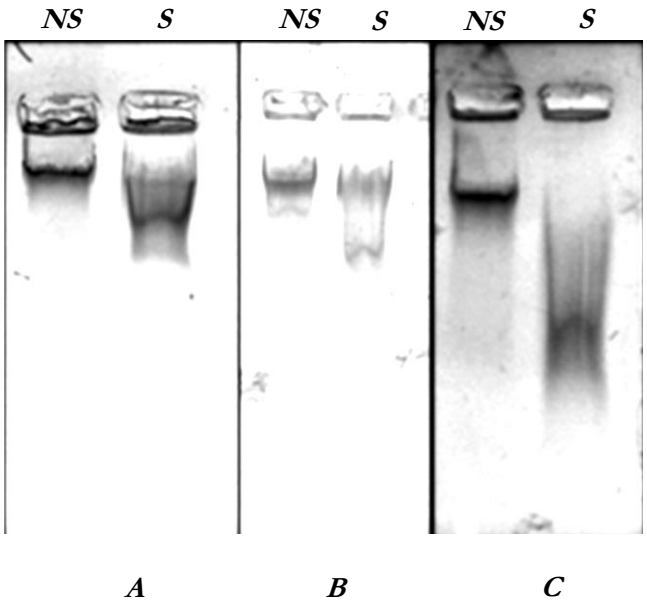

Figure. 3.18. The DNase activity of starved cells. The starved and non-starved cells were tested for their DNase activity. The DNase activity was measured at 30 min (A), 60 min (B) and at 2 h (C) of starvation. The lanes are represented as NS (Non-starved) and S (Starved). The extent of smear indicates the DNase activity.

suggesting the role of DNase activity. Mutation studies to inhibit the DNase enzymes and their impact on ploidy levels can give more clarity on the role of DNase in ploidy reduction.

3.2.6 DNase gene expression increases during starvation

The expression profile of DNase genes of 2 h starved cells in ASW was compared with that of actively growing cells (OD_{600} = 0.5) (**Figure. 3.19**). The list of DNase primers is given in Annexure. The DNase enzymes such as extracellular deoxyribonuclease (VC0470), endonuclease III (VC1011) and 5' deoxyribonucleotidase (VC1978) were upregulated by 1.88-fold, 1.7-fold, and 1.95-fold during starved conditions, respectively. The exodeoxyribonuclease V (VC2319) was downregulated by 0.75-fold during starvation. The fold change of 1.5 and above was considered as significant. The starved cell-extracellular deoxyribonuclease, the endonuclease III, and the

5'deoxyribonucleotidase had more than 1.5-fold change increased expression compared to the non-starved cells. The enzymes extracellular deoxyribonuclease is actively produced, and it is found in the exterior cell wall of the cell, whereas the 5'deoxyribonucleotidase is in the periplasmic space. Both these enzymes might be involved in the degradation of external DNA. More study is needed to understand the activity of DNase enzyme and the role of each enzyme in reducing the ploidy.

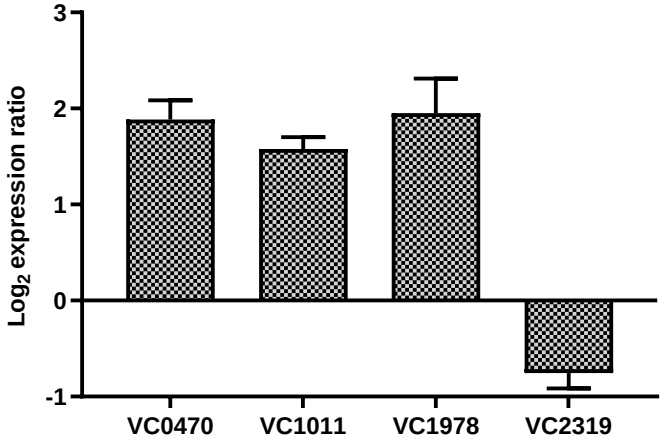

Figure. 3.19. Relative expression ratio plot. The expression of DNase genes was checked during starvation and compared with the levels of gene expression during non-starved phase. The expression profile includes the four important genes of DNase such as the extracellular deoxyribonuclease (VC0470), endonuclease III (VC1011) 5' Deoxyribonucleotidase (VC1978) and the exodeoxyribonuclease V (VC2319). The experiment was carried out using triplicates samples as per MIQE guidelines.

DNA is the only macromolecule that has large deposits of C, N, and P aggregates. DNA can be a good source of macronutrients. Earlier studies have concluded that in *Haloferax volcanii* and *Synechocystis* sp. PCC 6803 nutrient availability determines ploidy level, and that genomic DNA is used as a phosphate storage polymer[49,83]. Recent studies have raised the possibility of the evolution of DNA as a nutrient storage molecule because of its rich phosphate content[83]. We speculated that the DNA would serve as a phosphate store in *V. cholerae*. However, we did not observe any difference

in ploidy in the presence or the absence of phosphate in the media (**Figure. 3.16**). The availability of phosphate in media did not affect the ploidy during growth and starvation. Evolution might not have preferred the DNA as phosphate storage molecule in *V. cholerae* as it thrives in mineral-rich estuarine waters[32,84]. The higher ploidy may offer the advantage of being able to support more cell division process. Under adverse conditions, the increase in DNase activity may contribute to the recycling of the macromolecules. Further work is needed to explore the fate of the degraded DNA and its associated pathways.

3.2.7 Limitation of carbon, nitrogen, and phosphate

In the previous sections (Chapter 3.1 section 3.1.1; Chapter 3.2 section 3.2.1 – section 3.2.6), the impact of carbon, nitrogen, and phosphate starvation on survival was studied in detail. In the present section, the impact of individual nutrient limitation is explored. The limitation of carbon (glucose) resulted in a rapid decline in cell numbers over five days (**Figure 3.20 A**). After that, the cell number declined gradually to form a stable population number of 10^4 cells mL^{-1}. The survivor cell colonies were identical to the non-starved control population.

Most of the bacteria are tolerant of carbon limitation[33,36,85]. Bacterial cells have a large number of glycogen stores to be used during carbon limitation[85]. Apart from that, lipids are also used up as energy source if glycogen stores are insufficient. In *Vibrio sp*. S14, researchers have shown that during short term carbon limitation, cell number reduction is marginal. Ultra-microcell formation has been observed in response to carbon and multi-nutrient limitation [36].

Contrary to what has been observed in another *Vibrio sp*., our study showed that long term carbon starvation could lead to the formation of a low number of persisters[36].

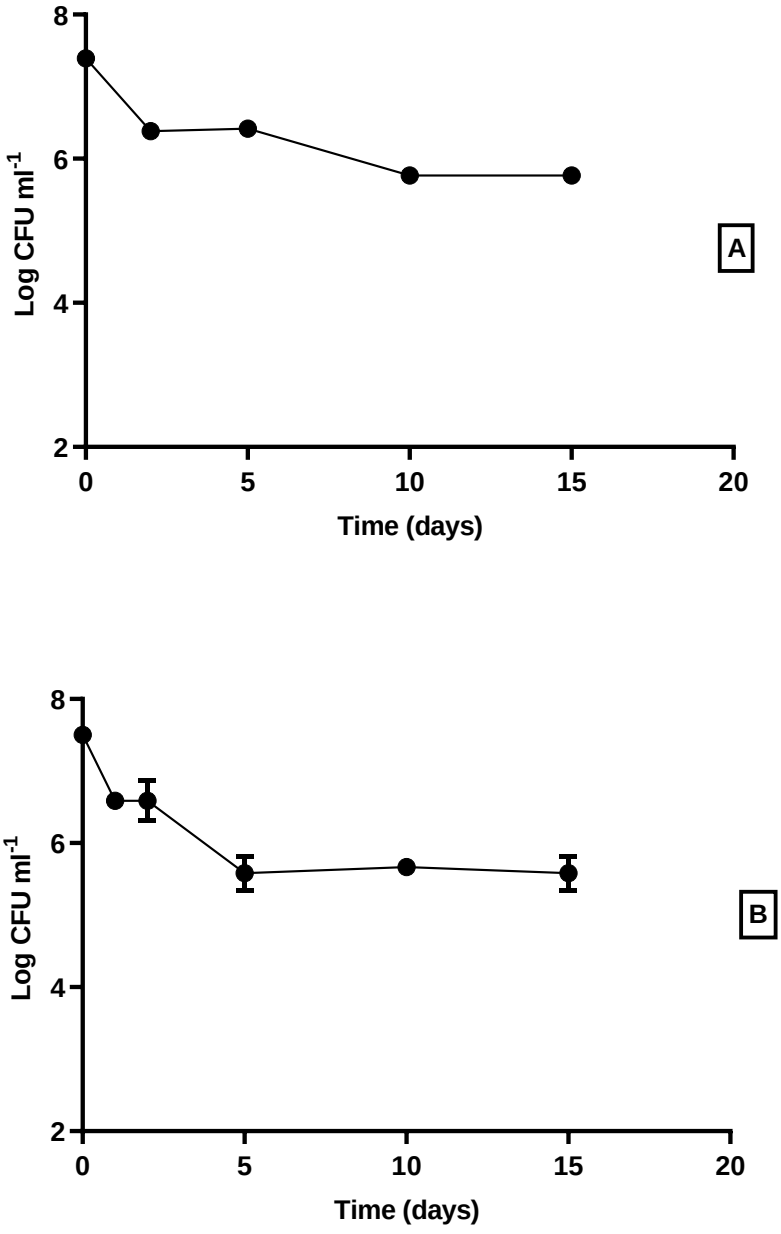

Figure. 3.20. Limitation of individual nutrients. A. Carbon limitation. Cells were inoculated in carbon limited cells and cell number was observed for 15 days. B. Nitrogen limitation. Y-axis denoted cell number in log scale and X axis denoted time in days. The data represents average of three biological replicates with standard deviation (n=3).

Further, we observed that the limitation of nitrogen was more lethal than carbon starvation (**Figure. 3.20 B**).

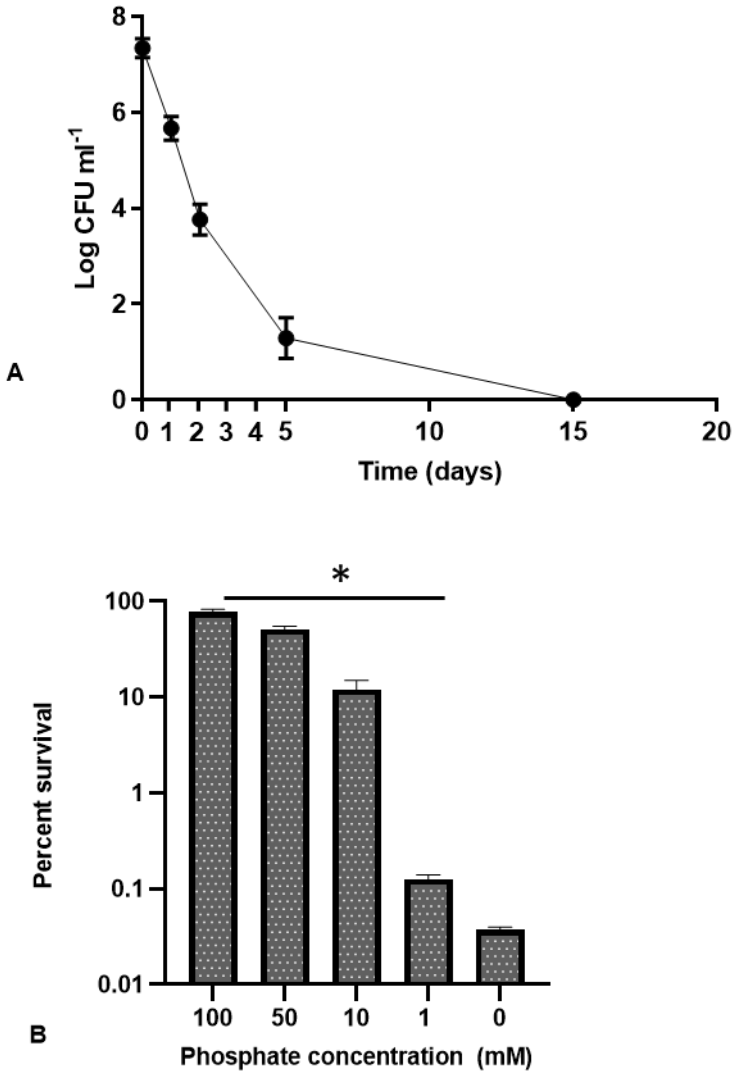

Figure. 3.21. Limitation of Phosphate and survival of V. cholerae. A) The reduction of cell number during phosphate starvation. The cell number reached below detectable levels after 15 days of P- limitation. (Lowest detection limit is 10^1 CFU ml^{-1}). The X axis denotes time in days and Y axis is log cell number. Average of three biological triplicates with S.D is depicted above (n=3). B). Phosphate limit as a function of survival. Lowest amount of phosphate required for survival was measured. Y axis indicates percentage survival after 48 h of starvation. The (*) indicates the level of significance at p<0.05.

The nitrogen limitation resulted in a rapid decline in cell numbers. After 5 days of continued cell death, a stable persister population tolerant of nitrogen starvation was formed. *Vibrio sp.* is sensitive to nitrogen starvation and shows an acute decline in population size[9,86]. Interestingly, the phosphate starvation profile of *V. cholerae* was

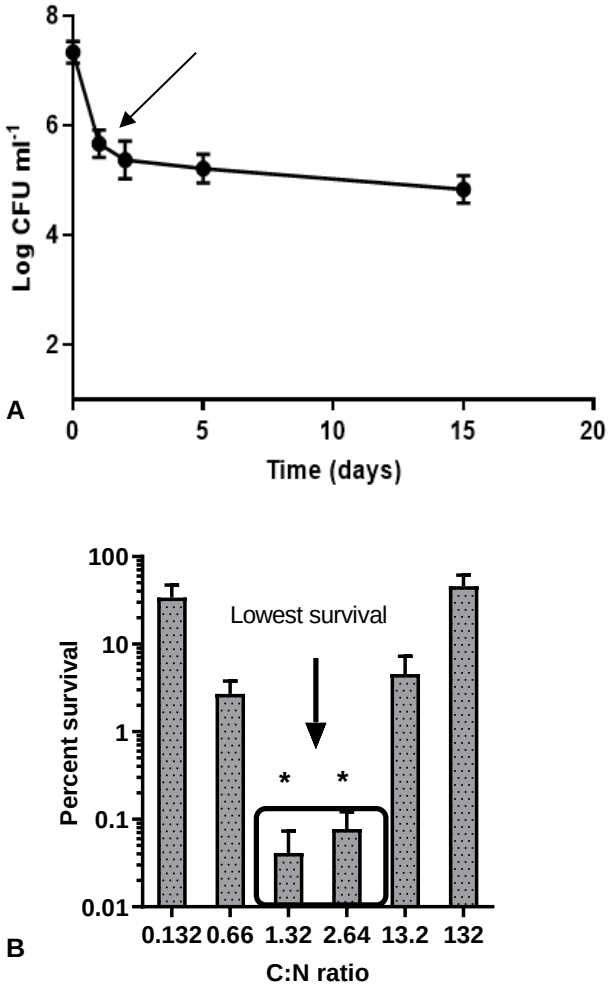

Figure. 3.22: A). Phosphate supplementation reduces the cell death. The cells were starved for phosphate for 24 h (indicated by an arrow) and then were supplemented with 10mM NaH_2PO_4. The rate of cell death decreased, and stable population was observed till the end of experiment on day 15. The data represents the average of three biological replicates with standard deviation. B) Influence of changes to C: N ratio on survival under P-limitation. D). Activation of stringent response by pre-treatment with sub MIC chloramphenicol. The pre-treated cells were exposed to P-limitation and survival was monitored after 48h. All values represent average of three biological triplicates (n=3), *p<0.001.

significantly different from carbon and nitrogen starvations. During P-limitation 3-$\log_{10}$ reduction in cell number was observed within 48 h. After 15 days of starvation, there were no survivors (**Figure. 3.21 A**). The lack of survivors or persisters to phosphate limitation in *V. cholerae* led us to examine possible causes for this acute sensitivity. The most plausible explanations of cell death in phosphate starvation include pH, osmolarity, membrane damage, failure to sense phosphate limitation, failure to induce a stringent response, and low ATP level. The initial two causes, the pH and osmolarity, were ruled out by adjustment to our starvation media composition[87]. The remaining probable cause of cell death, including stringency and low ATP conditions, were further investigated. First, the response of cells to fluctuations in phosphate content in the medium was determined. The phosphate concentration was decreased from 100 mM and 0 mM, without changing carbon and nitrogen concentrations (**Figure. 3.21 B**). Survival was critically affected below 10 mM phosphate concentration. Interestingly, upon external phosphate addition, the rate of cell death decreased, and cell survival improved. (**Figure. 3.22 A**).

3.2.8 C: N ratio and effect on survival

The ratio of carbon: nitrogen (C: N) in the medium was altered, and its effect on survival under P-limited condition was monitored. The C: N ratio of 1.32:1 resulted in a maximum loss of viability under phosphate limitation (**Figure. 3.22 B**). The C: N ratio of 1.32:1 is the optimal ratio present in the minimal media where bacteria show rapid cell division[88]. In optimum C: N ratio, the metabolism is not hindered or slowed down, even under P-limitation. The evolution might have shaped the bacterium to recognize only the C: N ratio regardless of phosphate availability; It may also be a possibility that the bacterium may not have metabolic checkpoints that would halt the metabolism upon

sensing P-limitation. The cells which are in simultaneous carbon, nitrogen, and phosphate limitation show limited metabolic activity and are considered starved cells.

3.2.9 Amino acids as common sources of carbon and nitrogen

Carbon and nitrogen can enter the metabolism independently, with different uptake rates and participate via different pathways. Metabolizable amino acids were used as a single source of carbon and nitrogen. This eases the study of P-limited cells. P-limited media was prepared using either metabolizable (glutamate) or non-metabolizable (arginine) amino acids. In the presence of metabolizable amino acid, 3-log cells lost viability (**Figure. 3.23**), in 48 h of phosphate starvation. However, we did not observe significant cell death in the cells supplemented with non-metabolizable amino acids. The amino acids are directly entering the central carbon metabolic pathways via

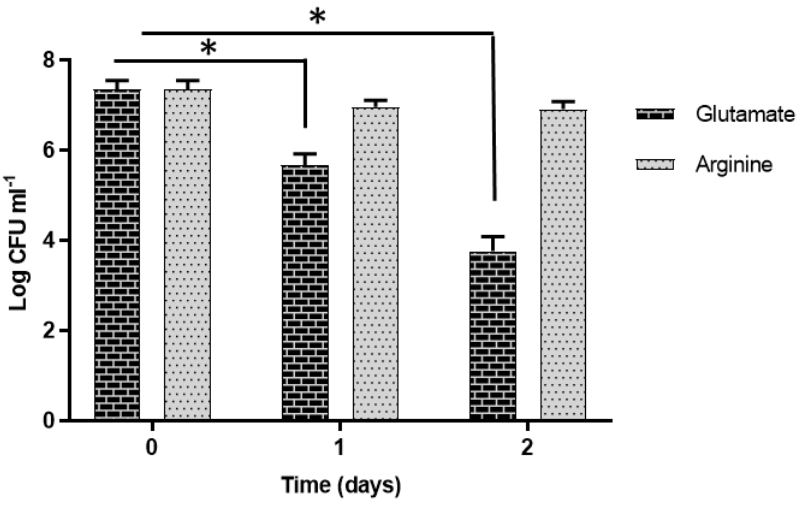

Figure. 3.23. Survival in the presence of amino acids as common source of carbon and nitrogen. The glutamate is used as the metabolizable amino acid and Arginine is the non-metabolizable amino acid. Both the conditions were phosphate limited. Cell survival was monitored for two days. The (*) indicates the level of significance at p<0.05. The graph represents the average of three biological triplicates with S.D.

TCA cycle. Therefore, the circumstantial evidence suggests the active metabolism is taking place in the cell despite the absence of phosphate.

The above argument suggests that metabolism is continued under P-limitation, which may be the cause of cell death. If metabolism could be slowed down or stopped, the cell survival may increase, even under P-limitation. Therefore, cell metabolism was altered by incubating P-limited cells at suboptimal temperatures. P-limited cells that were cultured at 37 °C, showed maximum cell death compared to the cells at sub optimal temperatures such as 15 °C and 40 °C. Only $1\log_{10}$ CFU lost viability at 15 °C compared to $4\log_{10}$ at 37 °C (**Figure. 3.24**). Interestingly, the cells incubated at 25 °C showed intermediate tolerance, more than 37 °C but less than 15°C. The growth rate of *V. cholerae* is highest at 37 °C (optimal growth conditions), and reduces if temperature is reduced below 30 °C. The temperature is effective way to slow down the rate of cell division and thereby reduce the metabolic activity[89].

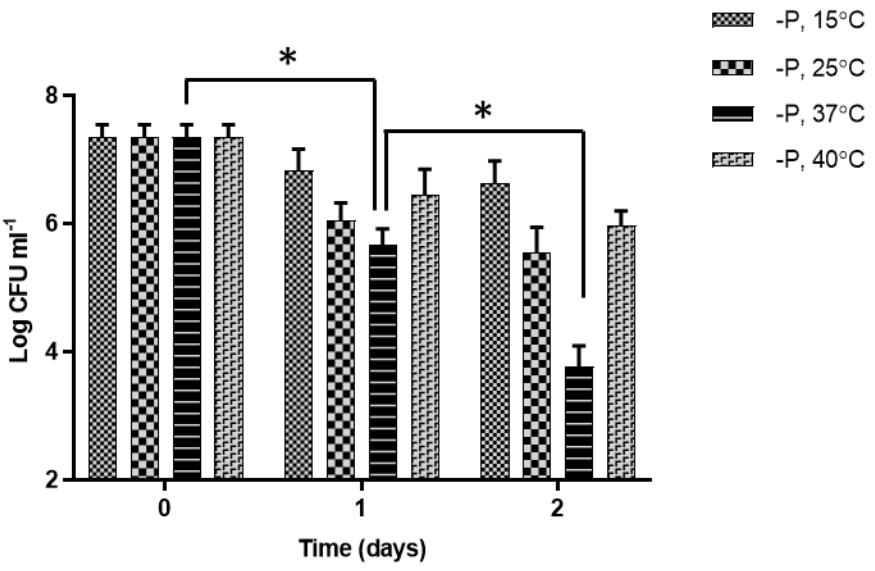

Figure. 3.24. The survival of P-limited cells at different growth temperature. The cells were starved for phosphate and incubated at different temperature and survival was monitored for 2 days. The (*) indicates the level of significance at p<0.05. The data represents the average of three biological replicates with S.D.

These results showed that P-limited cells were metabolically active, and that reduction in metabolic activity could increase cell survival. Cellular ATP levels are one of the crucial indicators of the metabolic state of the cell[90]. The P-limited cells carry out robust metabolism, which may be reflected in the ATP levels. ATP levels in P-limited cells were less than 0.5 mM, which was less than the exponentially growing cells (more than 10-15-fold). The dormant cells simultaneously starved for carbon, nitrogen, and phosphate also showed less than 0.5 mM ATP (**Figure. 3.25**). The ATP levels in P-limited cells were as low as dormant cells even as the metabolism was active (one-tailed t-test not significant). Why were cells low on ATP, even though metabolism was robust in P-limited cells?

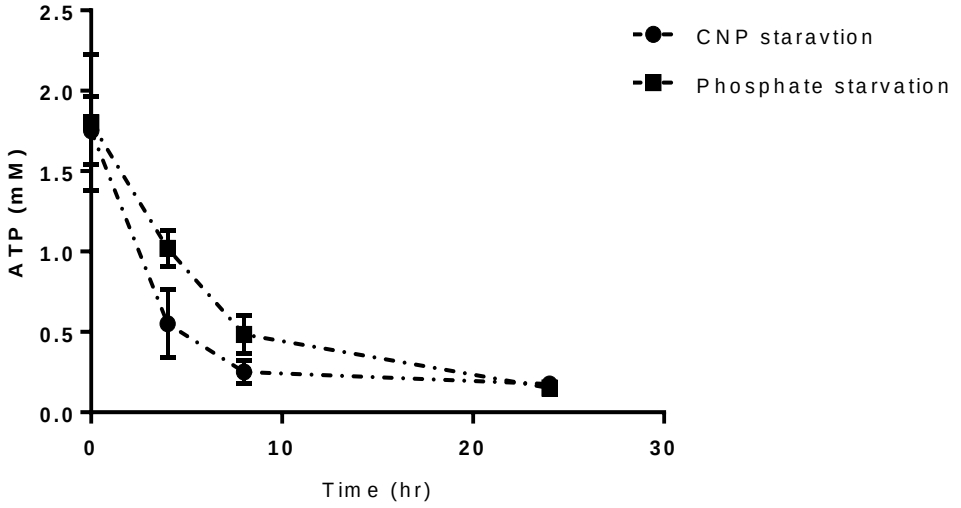

Figure. 3.25. ATP measurement in starved cells. Cells were starved for 24 h and ATP was measured at specific time points. The Cell number was taken into consideration while measuring ATP. CNP starvation means the cells starved for carbon, nitrogen and phosphate. The data represents the average of two biological replicates with S.D. The one tailed t-test was carried out at p-value 0.05.

3.2.10 Activation of the stringent response

So far it was clear that P-limited cells have continued metabolism. The reduction in metabolism and ribosome activity upon nutrient limitation is the hallmark of stringent response. Previous data suggest that *V. cholerae* lacks an efficient stringent response to multi-nutrient starvation[33,36]. Stringent response influences the cellular metabolic rate and survival[91]. Hence, it was hypothesized that, activation of stringent response may increase the survival under P-limitation. The exponentially growing cells were pre-treated with the sub MIC concentration of chloramphenicol before inoculating into P-limited media[92]. The pre-treated cells survived the P-limitation for two days with only a 10% loss of viability (**Figure 3.26**), whereas the untreated cells did not survive the P-limitation.

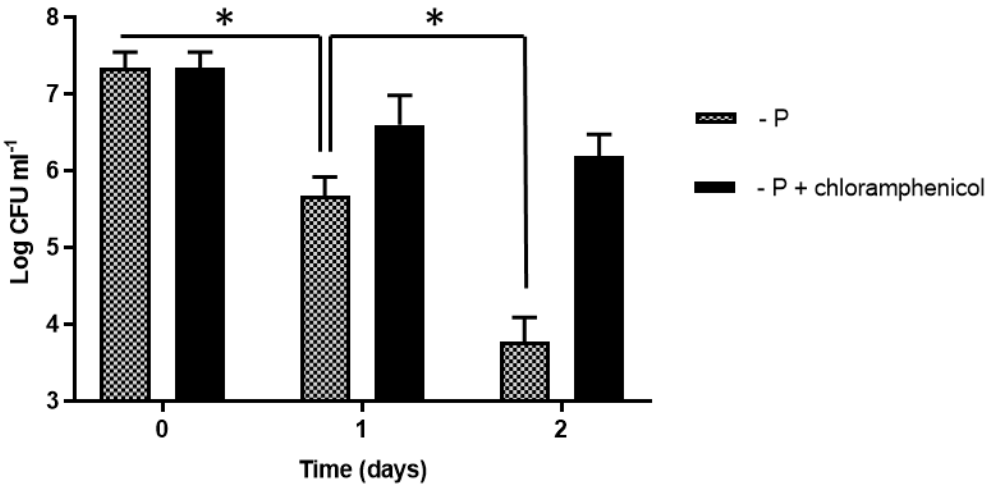

Figure. 3.26. Activation of stringent response and survival under P-limitation. The cells were pre-treated with chloramphenicol and subsequently challenged with P-limitation. The survival was monitored for 2 days. The (*) indicates the level of significance at $p<0.05$. The graph represents the average of three biological triplicates with S.D.

Exposing cells to the sub-lethal concentration of chloramphenicol inhibits ribosome activity and leads to the induction of stringent response. Once stringency is activated,

cells attain metabolic dormancy and can tolerate the phosphate limitation[19]. Therefore, P-limited cells may be less efficient in stringent response and that may lead to cell death. To strengthen the hypothesis, we explored the downstream effects of inefficient stringent response in the presence of carbon and nitrogen.

3.2.11 Inhibitors fail to rescue the phosphate starved cells

The findings from the above experiments were that the stringent response is not activated in P-limited cells; that prompts high metabolic activity. To maintain the metabolic activity, ATP was used up and hence the low ATP in P-limited cells (**Figure. 3.25**). However, the dormant cells in ASW also have low ATP, but they do not lose viability, like P-limited cells. There can be two possibilities, one may be that the cell is unable to produce enough ATP, or another could be a high turnover of ATP due to increased metabolism; under both the conditions, the ATP levels would be very low. Uncoupling the ATP synthesis from metabolism would reveal where it affects survival under P-limitation. The ATP uncoupler Carbonyl cyanide m-chlorophenyl hydrazine (CCCP), was used to reduce the ATP synthesis. The hypothesis was that if P-limited cells do not produce ATP, then the addition of uncoupler would not have any effect on survival. If the ATP is actively produced, then in the presence of the uncoupler, the ATP levels in P-limited cells would go down, and cells enter dormancy. This may increase survival under P-limitation. However, the P-limited cells exposed to 1x MIC of CCCP continued to lose viability (**Figure. 3.27**). Another inhibitor hydroxyurea was used to see if cells are trying to replicate and a failed replication event leading to cell death. However, the presence of hydroxyurea (1 X MIC) could not improve the survival under P-limitation. The cells under P-starvation may not be dying due to unsuccessful cell division. Ciprofloxacin is the inhibitor of DNA gyrase. The cells were insensitive to the

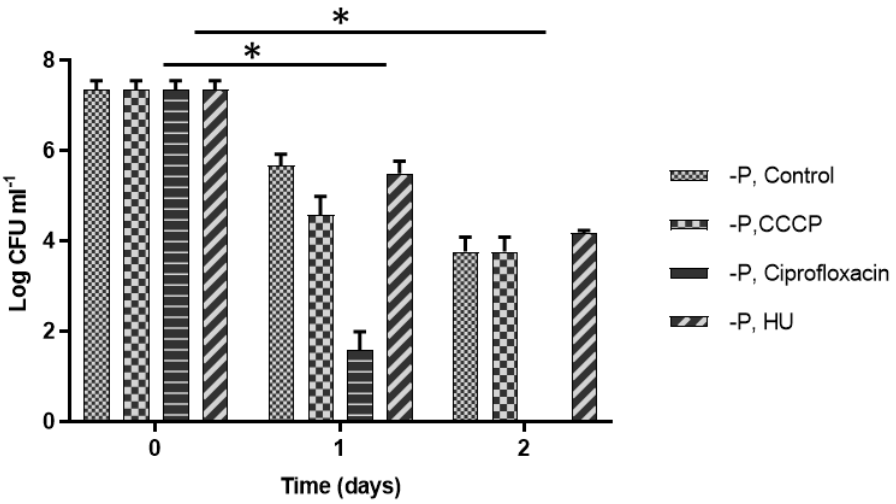

Figure. 3.27. The effect of metabolic inhibitors on survival under P-limitation. The P-limited cells were treated with CCCP, an inhibitor of ATP synthesis, or Ciprofloxacin or Hydroxyurea. The untreated P-limited cells were the control samples. The (*) indicates the level of significance at p<0.05. The data represents the average of two biological replicates with S.D.

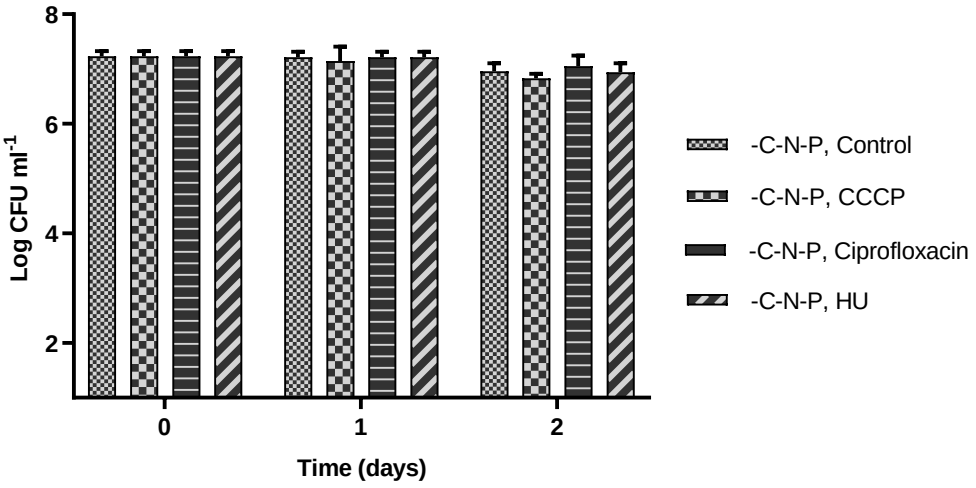

Figure. 3.28. The effect of metabolic inhibitors on survival under multi-nutrient starvation. Cultures were simultaneously starved for carbon, nitrogen and phosphate and survival was monitored. The starved cells were exposed to metabolic inhibitors for 2 days and their survival was plotted. The untreated starved cells were the control samples. The difference in survival was not significant at $p \leq 0.05$. The data represents the average of two biological replicates with S.D.

presence of ciprofloxacin, implying that either DNA replication or transcription are continued even under P-limited conditions. Thus, it appears that the cells under P-limitation are not producing net ATP, have not shut down electron transport chain, have active DNA replication or transcription but no cell division.

In comparison, dormant cells simultaneously starved for C, N and P tolerated the CCCP treatment and showed no change in cell number (**Figure. 3.28**). That means the P-limited cells are metabolically active with no net ATP synthesis. We also treated P-limited and dormant cells with 1X MIC ciprofloxacin (DNA gyrase inhibitor) and 1X MIC of Hydroxyurea (cell division inhibitor). The inhibitors could not improve the survival of P-limited cells (**Figure. 3.27)**. In comparison, the inhibitors had no effect on dormant cells, which did not show significant changes in cell number (**Figure 3.28**). The phosphate limited cells were metabolically active and continued to die in the presence of inhibitors. If in the presence of inhibitors, P-limited cells had reduced their metabolism, more cells would have survived the starvation. However, the metabolism is turbulent or erratic, and the targets of inhibitors might not be active or functional. Such a situation would leave the cells non-responsive to the metabolic inhibitors.

3.2.12 Phosphate starvation survival among other members of γ-proteobacteria.

Apart from *V. cholerae*, other members of γ-proteobacteria were also subjected to P-limitation and their survival patterns were compared with *V. cholerae* (Figure. 3.29). The marine bacterium *V. vulnificus* showed acute sensitivity to P-limitation. Emerging pathogen *Aeromonas hydrophila* and *V. harvey* fared better, with 1% of the population surviving even after 48 h into P-limitation. The common enteric bacteria *E. coli* and *S.* Typhimurium fared much better than other members for P-limitation; more than 10%

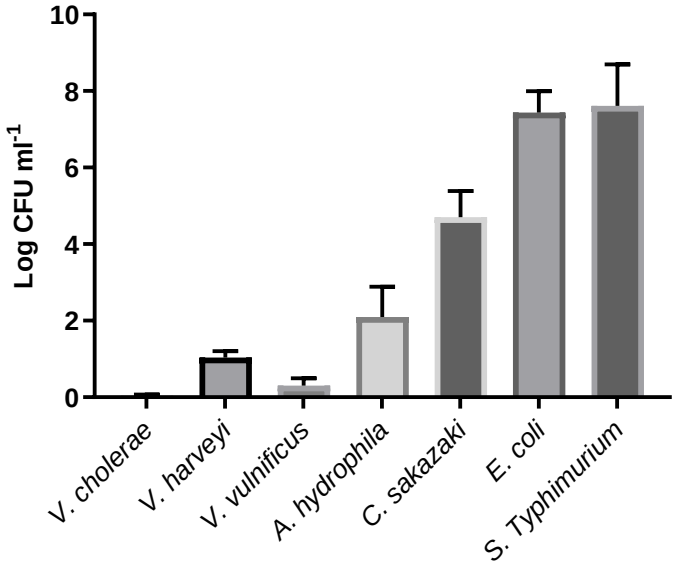

Figure. 3.29. P-limitation profile among other members of γ-proteobacteria. Survival was measured for 2 days under P-limitation (carbon and nitrogen were supplied). Average of three biological triplicates with S.D is given in the graph.

of these bacterial populations survived after 48 h into P-limitation. Persisters to P-limitation were also formed in *C. sakazaki*. From the survival pattern, it appeared that closely related members share a similar tolerance pattern. *V. cholerae* and *V. vulnificus* are closely related and can show similar acute sensitivity. Hence, we retrieved the phosphate regulon sequence of *V. cholerae* and compared it with the other species tested here.

One of the striking differences was in the two-component system CreC/CerB. The CreC is an activator of phosphate regulon[93]. Using the protein sequence of CreC, we constructed a phylogenetic tree. We observed a correlation between percent conserved creC sequence and survival under P-limitation (**Figure. 3.30)**. A simple correlation cannot explain causation. Further detailed investigation on creC mutants is essential to validate whether the correlation is causal.

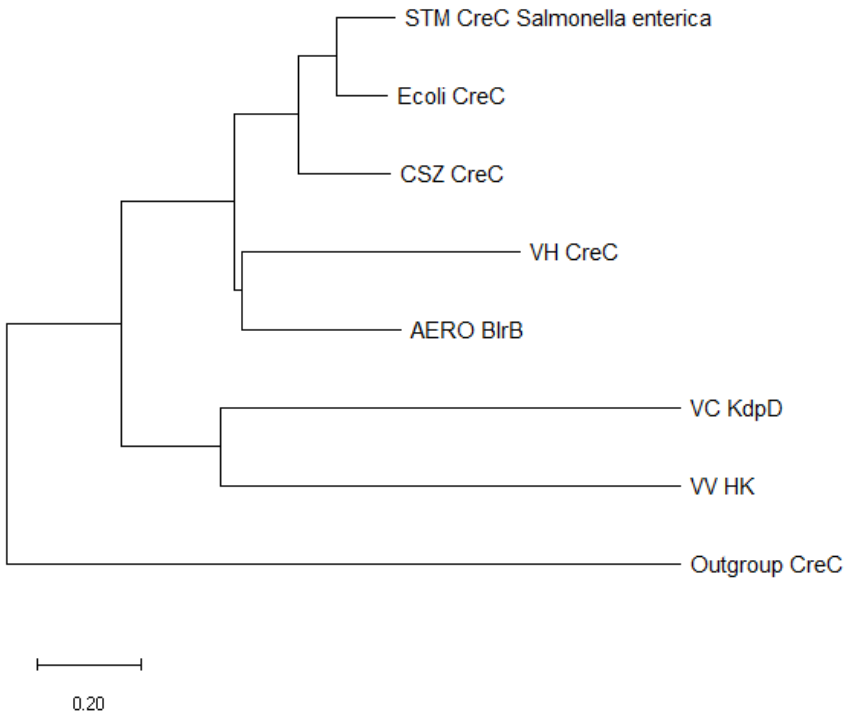

Figure. 3.30. Phylogeny analysis of bacterial species according to CreC protein sequence. STM - Salmonella enteritica typhimurium; E. coli- E. coli; CSZ-C. sakazaki; VH-V. harveyi; AERO-A. hydrophila; VC-V. cholerae; VV- V. vulnificus; outgroup-Mycobacterium. The evolutionary history was inferred using the Neighbour-Joining method[15]. The optimal tree with the sum of branch length = 5.15821847 is shown. The tree is drawn to scale, with branch lengths in the same units as those of the evolutionary distances used to infer the phylogenetic tree. The evolutionary distances were computed using the Poisson correction method[16] and are in the units of the number of amino acid substitutions per site. This analysis involved 8 amino acid sequences. All ambiguous positions were removed for each sequence pair (pairwise deletion option). There was a total of 516 positions in the final dataset. Evolutionary analyses were conducted in MEGA X[17].

The presence of carbon and nitrogen sources in media triggers cellular uptake of nutrients and represses gluconeogenesis. Cells carry out ATP demanding anabolic reactions by utilization of polyphosphate deposits. At the onset of P-limitation, *V. cholerae* may carry out healthy metabolism using large phosphate deposits, which are 100-fold more than *E. coli*[94]. Because of rapid metabolism, the phosphate deposits deplete and reduces the flux of phosphates for ATP synthesis. Enzymes of central

carbon metabolism and other cellular pathways use phosphate to regulate the enzyme activity[95]. Because of the presence of carbon and nitrogen, P-limited cells do not activate a stringent response, which prompts continued nutrient uptake, anabolism, and a further reduction in ATP. In pursuit of ATP, cells attempt futile nutrient cycling by catabolism. Such a scenario would result in simultaneous activation of Anaplerotic reactions that would increase the accumulation of intermediate metabolites and reducing agents in the cell.

3.2.13 NADH levels and membrane damage

The P-limited cells are actively metabolizing without net ATP synthesis. The continued passaging of nutrients would generate reducing agents like NADH and $FADH_2$. Lack of ATP indicates that some or all the steps of the electron transport chain are not functioning. This means the NADH and $FADH_2$ would not be oxidized, resulting in a

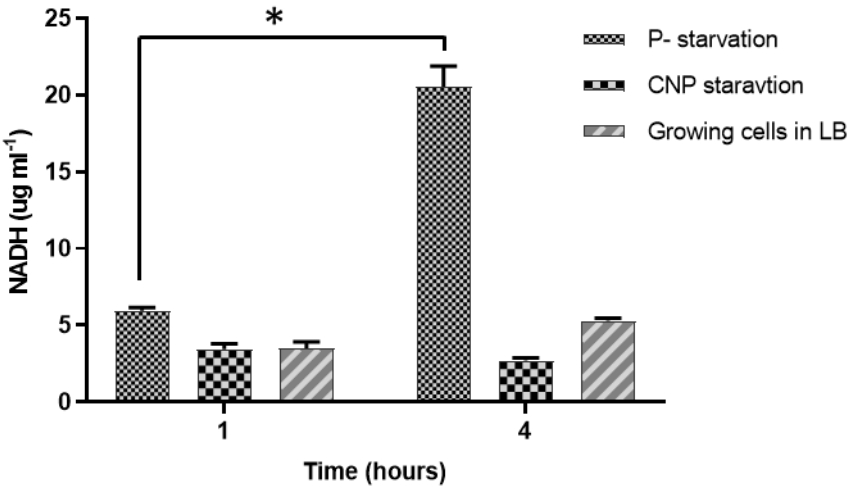

Figure. 3.31. NADH levels during starvation survival. NADH levels were measured by fluorescent method. X axis represents the NADH concentration (µg ml^{-1}) and Y axis is duration of starvation. P-limited cells, carbon, nitrogen and phosphate limited dormant cells and growing cells in LB were takes as three independent samples and NADH was measured at 1 h and 4 h time points. The (*) indicates the level of significance at $p<0.05$. Average of three biological triplicates with S.D is given in the graph.

build-up of reducing agents in the cell. The NADH level in the cell was measured using in P-limited cells and was compared to that of the carbon, nitrogen, and phosphate starved dormant cells. As soon as the P-starvation sets in, the intracellular NADH concentration reduced in the dormant population. However, the P-limited cells maintained a high NADH level even after 4 h into P-limitation (**Figure. 3.31**). Higher NADH accumulation is toxic to cells[96]. NADH acts as a reducing agent that can damage membranes and other cellular organelles[96].

The build-up of reducing agents means an imbalance in the redox state of the cell[97]. The immediate effect of redox imbalance is on the cell membrane because the redox state of the cell determines the membrane potential and membrane permeability. The increase in PI uptake indicates cell death; the dormant and VBNC cells do not take up the dye. The PI uptake was 10-fold higher in P-limited cells compared to the dormant cells after four h of starvation (**Figure. 3.32**).

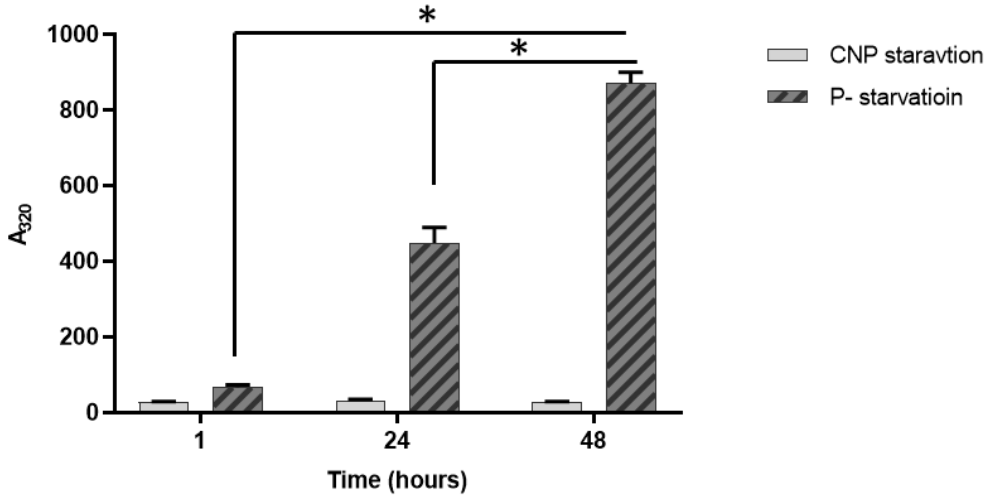

Figure. 3.32. Propidium uptake (PI) up take is measured by fluorescent method (Ex:544nm. Emm:620nm). X axis denote the A_{320} corresponding to PI uptake by P-limited cells and carbon, nitrogen and phosphate limited dormant cells were determined at 1 h and 4 h into starvation. The (*) indicates the level of significance at p<0.05. Average of three biological triplicates with S.D is given in the graph.

The P-limited cells have increased membrane damage resulting in loss of cell viability. The unstable and intermediate metabolites accumulate, leading to the rise of oxidative stress and membrane damage. Starved cells of *E. coli* can get nutrients from dead cells and maintain a small population size[98]. In P-limited *V. cholerae*, the acquisition of nutrients from dead material may increase only worsen the situation. An incomplete link may exist between phosphate store and stringent response activation in *V. cholerae*.

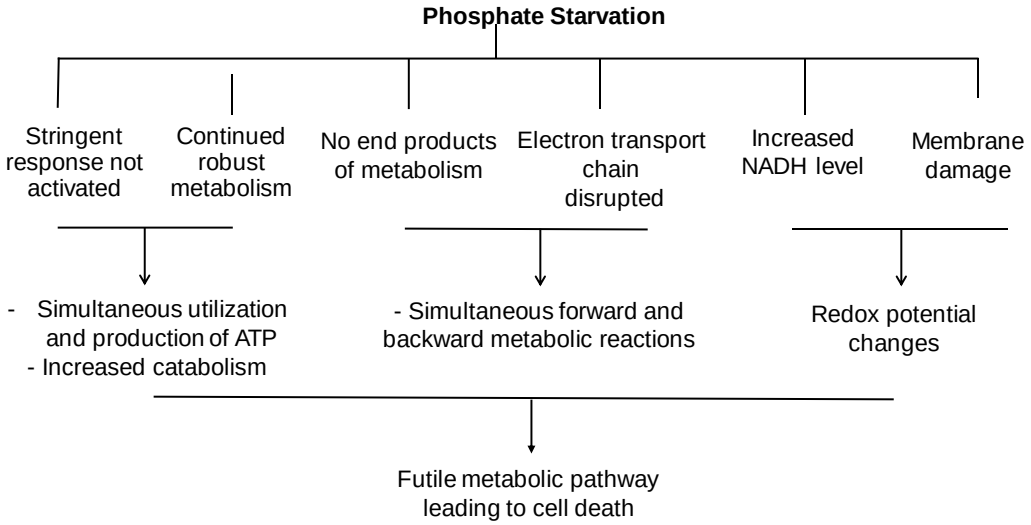

Figure. 3.33. A representative image of futile cycling of metabolites. The futile cycling involves increased nutrient uptake, reduced stringency, increased ATP demand, reduced ATP synthesis, and failure to recognize acute shortage of ATP. The final effects are change in redox state and membrane damage and imminent cell death.

In the present work, we explored the acute sensitivity of *V. cholerae* to phosphate limitation. One of the primary causes of cell death is active metabolism and protein synthesis in P-limited cells. Irregular metabolism because of P-limitation can lead to the accumulation of NADH, membrane damage, and cell death. *V. cholerae* may not have evolved to tolerate complete phosphate limitation because of large intracellular

phosphate store and availability of abundant phosphates in estuarine water. This work suggests that *V. cholerae* has poor phosphate starvation management (**Figure. 3.33**).

3.2.14 Chapter summary

In the present chapter, the survival of *V. cholerae* was studied under multi-nutrient and individual nutrient starvation. The ploidy of *V. cholerae* reduces under multi-nutrient starvation. The *V. cholerae* was found to be (mero-) oligoploid or polyploid. The ploidy levels per cell were found to be growth phase regulated with the highest ploidy recorded in the early stationary phase. In addition to the growth phase, an external parameter such as nutrient level influences the ploidy, i.e., ploidy reduces rapidly at the onset of starvation. The activity of the DNase enzyme increased during starvation that decreased the ploidy. The ploidy was restored to the pre-starvation levels with nutrient supplementation.

Multi-nutrient starvation profiles are emerging as an essential feature of human pathogen studies[1,8]. These investigations provide insight into how pathogens survive during long-term dormancy and famine between outbreaks. In the present work, we measured the metabolism in phosphate-starved cells of *V. cholerae* using metabolism inhibitors and under sub-optimal growth conditions. We further examined the Pho regulon of *V. cholerae* and other representatives of γ-proteobacteria. The conclusions presented here show that *E. coli* and other representatives of γ-proteobacteria slow down their metabolism as soon as they experienced phosphate deprivation. However, the *V. cholerae* cannot slow down the metabolism, leading to a futile turnover of metabolites. The futile nutrient cycling contributes to the accumulation of NADH, no net ATP synthesis, and loss of cell survival.

3.3 Antibiotic induced persisters

This chapter studies the antibiotic-induced persisters concerning antibiotics and growth conditions. The practice of using antibiotics in aquaculture has increased the risk of antibiotic resistance among the *Vibrio* species of clinical and commercial importance[99,100]. A recent study on *V. cholerae* O139 strain revealed the difference in antibiotic resistance among planktonic and biofilm cultures[101]. However, not much information is available on the antibiotic persistence phenomenon in *V. cholerae*.

Persistence being a physiological manifestation is influenced by media components and growth conditions. Accordingly, in the present chapter, we examined the ability of *V. cholerae* to form persister cells in laboratory growth conditions. The fraction of persisters was determined in different media and the presence of inhibitors of respiration. We also carried out studies to understand the role of glucose in modulating the antibiotic susceptibility of *V. cholerae*.

The chapter is subdivided into two sections. Section A explores the effect of antibiotics on persister formation, and Section B investigates the effect of glucose on antibiotic susceptibility and persister formation. The findings presented here are reported in the following two publications:

1. Paranjape, S.S., Shashidhar, R. 2019. Inhibition of protein synthesis eradicates the persister cells of V. cholerae. 3 Biotech. (https://doi.org/10.1007/s1320501919160)[102]

2. Paranjape, S.S., Shashidhar, R., 2019. Glucose Sensitizes the Growing and Persistent Population of Vibrio cholerae to Antibiotics. Archives of Microbiology. Springer (https://doi.org/10.1007/s00203-019-01751-8)[103]

3.3.1 Effect of different antibiotics on persister frequency of *V. cholerae*

We exposed the growing culture of *V. cholerae* to the 10-fold MIC of antibiotics (ciprofloxacin, ampicillin, and kanamycin) (**Table. 2.3 Chapter 2**). The viable cell number declined rapidly during the initial 2 -3 h of antibiotic challenge (**Figure. 3.34**).

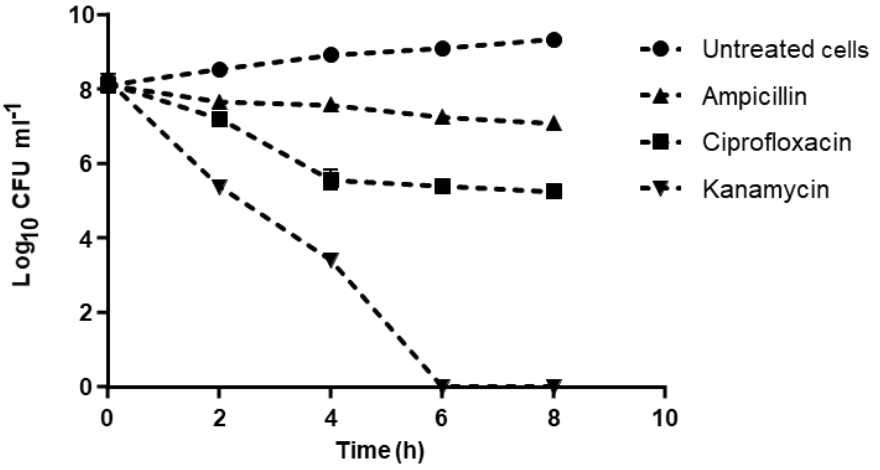

Figure.3.34. The effect of different antibiotics on persister frequency of exponential phase cultures. The cells were subjected to lethal dose of antibiotics and cell number was enumerated at regular interval. The graph above shows the biphasic kill curve. The formation of plateau region indicates the persister formation. Results shown are the average of three biological triplicates with standard deviation (n=3). The reduction in cell number 4 h post-antibiotic exposure was significant at p = 0.05 (Two-tailed t-test), when compared with the cell number at the beginning of the experiment T0.

There onwards, the rate of cell death decreased and formed a plateau region with a stable population size. This biphasic kill curve is the hallmark of persister formation[46]. Exposure to antibiotics often results in a rise of antibiotic-resistant mutants. A series of experiments were performed to determine if the persister population had any resistant mutants. The persisters were recovered in LB without antibiotics and again exposed to a lethal dose of antibiotics (**Figure.3.35**). The MIC values and persister frequencies did not change before and after the antibiotic treatments (**Table. 3.4**).

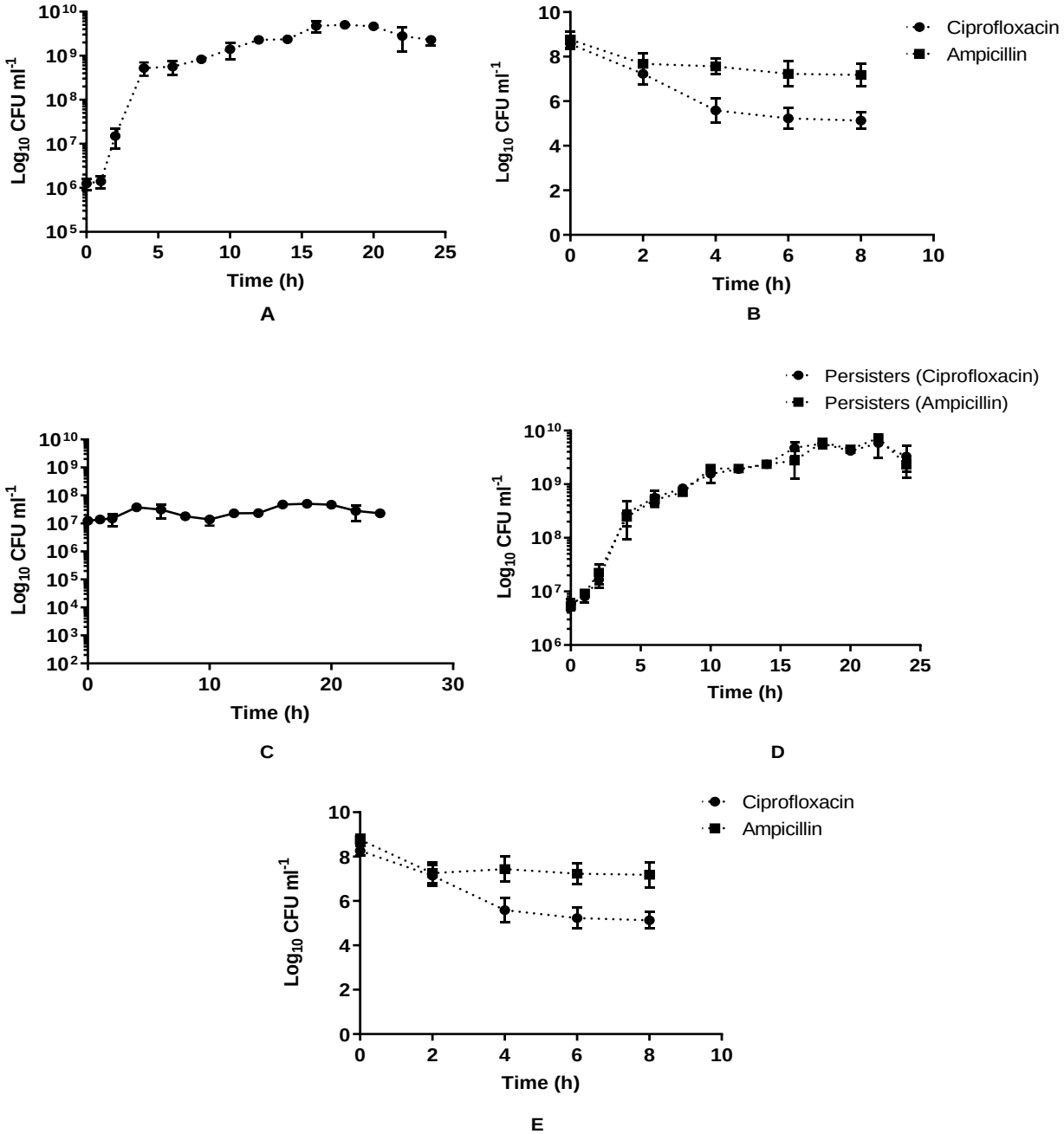

Figure 3.35. Persister cells are not genetic mutants. A) The cells were cultured overnight in LB and incubated at 37°C for 24 h. B) 10-fold MIC of antibiotics were added to the overnight grown culture from A. After 6 h of antibiotic addition the cells from B were inoculated into fresh LB with (C) or without (D) antibiotics. In the presence of the antibiotics the survivors did not grow (C) and in the absence of antibiotics, survivors regained growth and formed regular colonies(D). The regrown population was again exposed to antibiotics and persisters were isolated. The persister frequency before (B) and after (E) antibiotic exposure. All the tests are carried out in triplicates and average of the three readings with standard deviation is plotted against the time.

Table. 3.4. The persister frequency and MIC values of V. cholerae before and after exposure to ciprofloxacin. The average of three biological replicates is tabulated. The Student's t-test was carried out and the significance of the data was measured at p = 0.05. The MIC was calculated in Cation adjusted Muller Hinton broth. No significant difference in MIC and persister frequency was observed (P < 0.05).

	MIC (μg mL^{-1})		**Persister frequency (%)**	
	Before exposure	**After exposure**	**Before exposure**	**After exposure**
Ciprofloxacin	0.1	0.1	0.1 ± 0.059	0.2 ± 0.015
Ampicillin	4	4	7.19 ± 0.415	6.89 ± 0.538

Further, we also sequenced the quinolone resistance determining region (QRDR) of ciprofloxacin persister cells. No mutations were observed in the QRDR locus of the genes *gyrA* (MN458507), *gyrB* (MN458508), or *parC* (MN458509). The persister cells obtained in the present work were exposed only once to the lethal concentration of ciprofloxacin, and no further selection was made on the survivors. Hence the antibiotic tolerance due to mutation is highly unlikely in the persister cell population. However, there is always a possibility that isolation of ciprofloxacin-resistant *V. cholerae* from the environment as reported from India[104]. Thus, we ruled out the possibility of resistant mutants among the persister formation.

3.3.2 Persister formation is a density-dependent phenomenon

Density-dependent persister formation was studied. Cultures of *V. cholerae* in different stages of growth were exposed to a lethal dose of antibiotics, and persister fraction was measured. The persister fraction at the start of the experiment (T_0) represents the carry forward persisters from stationary phase inoculum. Fresh nutrients and the absence of antibiotics induce active metabolism and cell division in persister cells, which makes

them sensitive to antibiotics; this is indicated by the dip in the lag phase. Persister fraction increased as the cells advanced to the log phase and continued to increase until the culture entered the stationary phase (**Figure. 3.36**). Interestingly, the stationary phase cells of *V. cholerae* were sensitive to ampicillin and ciprofloxacin and could form only 10% of persisters (**Figure. 3.36**).

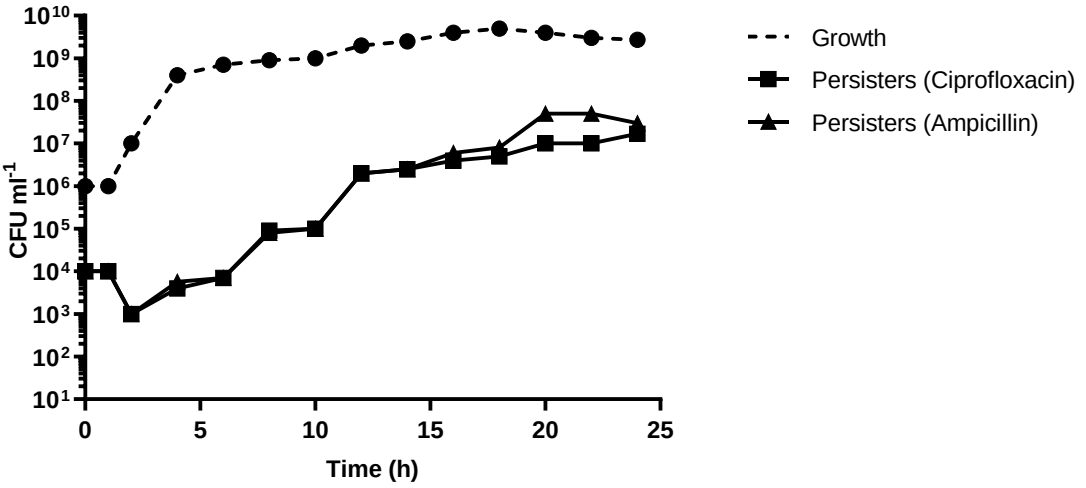

Figure. 3.36. Growth phase dependent persister formation. The cells were subjected to lethal dose of antibiotics and time dependent kill curve was plotted from lag phase to stationary phase. The "growth" indicates the normal growth of the cells without antibiotics. The persisters formed under ciprofloxacin and ampicillin exposure are calculated at regular intervals and was plotted against the time. Results shown are the average of three biological triplicates with S.D.

In contrast to *V. cholerae*, the stationary cells of *E. coli* were more tolerant to both ciprofloxacin and ampicillin[26,46]. Stationary cells of *B. burgdorferi* were sensitive to antibiotics, which supports our report[105]. Stationary phase cells are generally more tolerant of ampicillin since they are not in the active dividing stage. It may be possible that stationary phase cells of *V. cholerae* continue to grow and die in a steady state. Another possibility is that the cell wall of *V. cholerae* continues to remodel in the stationary phase making cells sensitive to β-lactams[102].

3.3.3 Effect of growth media on persister formation

Various growth media used for bacterial cultivation have distinct effects on growth kinetics and survival. Persistence is a physiological phenomenon that changes with the growth stage of the cell. Hence, we hypothesized that media compositions might have a significant influence on the persister frequency. *V. cholerae* was cultured to exponential phase in different growth media, and the persisters were enumerated (**Table. 3.5**).

Table. 3.5. The persister frequency of V. cholerae changes with the type of media. All the tests were carried out with three biological triplicates (n=3) One-way ANOVA is performed to determine the significant variations in persister formation among different media. The persister frequency in different media was compared with that of LB. The difference between BPW and APW was also found to be significant at p = 0.05.

Media type	Persister fraction (%)
LB	0.1 ± 0.032
TSB	10 ± 1.5
APW	0.001 ± 0.0007
BPW	15 ± 2.6

The cells cultured in Buffered peptone water (BPW) formed the highest persister fraction (15 ± 2.6), and the lowest was in Alkaline peptone water (APW) with 0.001% persisters. We could isolate 0.1% and 10% persisters in regular culture media used in the Luria-Bertani (LB) and Tryptone soya broth (TSB), respectively. The differences in persister frequency were significant at p = 0.05. BPW is the most common media used during the isolation and maintenance of *V. cholerae* from clinical and environmental samples. Antibiotic persistence often precedes the rise of genetic resistance to

antibiotics[41]. Future studies on antibiotic persistence and resistance may also consider the influence of media and its compositions on cellular physiology.

3.3.4 Protein synthesis is essential for persister formation

V. cholerae failed to form persist when exposed to the 10-fold MIC of kanamycin (**Figure. 3.34**), an inhibitor of protein synthesis. In order to validate the finding, *V. cholerae* was exposed to four more antibiotics that would also inhibit protein synthesis (chloramphenicol, erythromycin, tetracycline, and gentamicin) (**Table. 3.6**).

Table. 3.6. The percent persister fraction (%) of V. cholerae when challenged with protein synthesis inhibitors. Percent persister frequency was calculated after 6 h of antibiotic addition. The average of three biological replicates is tabulated.

Antibiotics	**Exponential cells**	**Stationary cells**	**Preformed persister cells to ciprofloxacin**	**Preformed persister cells to ampicillin**
Kanamycin	0	0	0	0
Chloramphenicol	0	0	0	0
Erythromycin	0	0	0	0
Gentamycin	0	0	0	0
Tetracycline	0	0	0	0

Both the exponential and stationary cultures of *V. cholerae* were sensitive to inhibition of protein synthesis and could not form persisters. It appears that the formation of persisters in *V. cholerae*, depends on the active protein synthesis. Furthermore, we exposed the exponential and stationary phase cells of *E. coli* to the 10-fold MIC of all the antibiotics used in the study. Persister population was observed after exposure to all protein synthesis inhibiting antibiotics. The percent persister fraction ranged from 0.1 to 1 for exponential cells and from 1 – 10 for stationary cells (**Table. 3.7**).

Table. 3.7. The percent persister fraction (%) of E. coli when challenged with protein synthesis inhibitors. Percent persister frequency was calculated after six h of antibiotic addition. The average of three biological replicates is tabulated. 1-way Multiple comparison ANOVA was carried out. The significance of the data was measured at p =0.05. There was no significant difference between the exponential cells and pre-formed persisters to ciprofloxacin and ampicillin.

Antibiotics	**Exponential cells (%)**	**Stationary cells (%)**	**Preformed persister cells to ciprofloxacin (%)**	**Preformed persister cells to ampicillin (%)**
Kanamycin	0.1 ± 0.019	1 ± 0.22	0.1 ± 0.02	0.1 ± 0.031
Chloramphenicol	1.0 ± 0.43	10 ± 3.25	1.0 ± 0.17	1.0 ± 0.52
Erythromycin	1.0 ± 0.27	10 ± 1.87	1.0 ± 0.36	1.0 ± 0.16
Gentamicin	0.1 ± 0.038	1 ± 0.64	0.1 ± 0.013	0.1 ± 0.049

3.3.5 The inhibition of protein synthesis does not form the VBNC population.

Earlier work has shown that persister cells and viable but non-culturable cells (VBNC) can co-exist together. Cells under VBNC do not grow in agar plates giving a false impression of loss of viability. Only after resuscitation, the VBNC can form colonies on agar plates. We performed a VBNC assay to determine if the kanamycin treated cells enter the VBNC state. An increase in the colonies on agar plates after resuscitation indicates the presence of VBNC in the persister population. However, in our study, we did not observe changes in cell numbers after resuscitation (**Table. 3.8**). Although dormant, VBNC maintains the cell membrane integrity. We performed the LIVE/DEAD test to determine if kanamycin treated cells enter the VBNC state. The LIVE/DEAD analysis revealed that with an increase in the kanamycin exposure time, more cells took up Propidium Iodide (PI) dye and fluoresced red (**Figure. 3.37**). The 0 h and one h (**Figure. 3.37 A and B**) images have a higher proportion of green fluorescent cells. After 4h of kanamycin treatment (**Figure. 3.37 D**) majority of cells

fluoresce red color. The cells which fluoresce red are dead since they take up PI dye and cells that fluoresce green are alive because their membrane is impermeable to PI dye. These studies indicated that kanamycin exposure killed the cells rather than inducing VBNC.

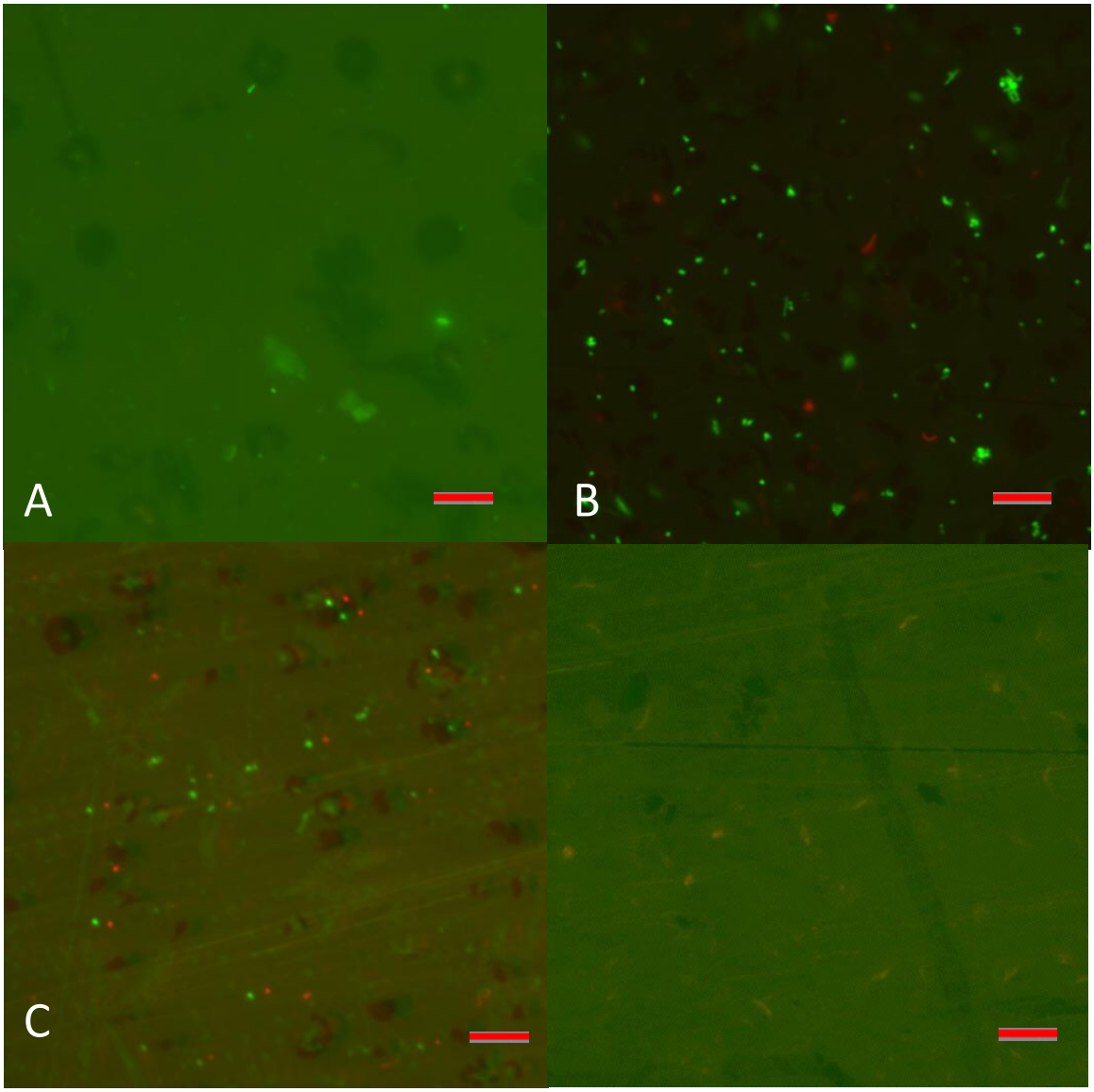

Figure. 3.37. Determination of viability of V. cholerae upon exposure to 10-fold MIC kanamycin using LIVE/DEAD Baclight Viability kit under fluorescent microscope. Panel A shows the cells before exposure to antibiotic. Panel B shows the cells after 1 h, Panel C 2 h, Panel D 4 h after kanamycin exposure. Bar represents 10 µm length.

Table. 3.8. Test for VBNC formation. The cells were resuscitated post-antibiotic challenge, and cell number was determined. The average of three biological triplicates with S.D is given in the table (N=3). Two-tailed t-test was carried out with a cut-off p-value at 0.05.

Antibiotics	**Persisters immediately after 6 h of treatment (%)**	**Persisters after 72 h resuscitation (%)**	**Significance**
Ampicillin	7.14 ± 0.263	7.12 ± 0.41	Not significant
Ciprofloxacin	0.1± 0.05	0.1 ± 0.04	Not significant
Kanamycin	0	0	0

3.3.6 Protein synthesis is essential for persister maintenance

As we continued the study, we questioned whether the protein synthesis is essential for maintaining the persistence physiology in *V cholerae*. We challenged the growing culture with 10-fold MIC of ciprofloxacin and isolated the persister population. These pre-formed persisters were then challenged with 10-fold MIC of kanamycin and at viability was monitored with time. (**Table. 3.6**). The pre-formed persisters were killed by kanamycin, and after 6 h of antibiotic challenge, no viable cells were recovered. Active protein synthesis appears to be involved in the persister maintenance in *V. cholerae*. To find out how the pre-formed persisters of *E. coli* behave, we challenged them with 10-fold MIC of proteins synthesis inhibitors. The pre-formed persisters of *E. coli* survived the antibiotics with no loss of viability (**Table. 3.7**). The 1-way multiple comparisons ANOVA revealed that the difference in survival of pre-formed persisters and exponential cells was not significant at $p < 0.05$. The mechanism of persister formation and maintenance is different in *V. cholerae* and *E. coli*.

3.3.7 Protein synthesis inhibition eradicates starved persister cells

Nutrient limitation in *V. cholerae* imparts a characteristic persister cell formation[106]. As already mentioned, cells under acute starvation attain a dormant state where metabolism is inhibited[1], and for this purpose, we obtained the starved cells of *V. cholerae* by culturing the cells in Artificial Sea Water (ASW) for 24 h. The starved cells were subsequently challenged with 10-fold MIC of antibiotics to determine the persister fraction. Nutrient limited cultures survived ciprofloxacin and ampicillin exposure with no significant loss of viability (**Table. 3.9**).

Table. 3.9: The percent persister frequency (%) in the starved population. Percent persister frequency of V. cholerae is tabulated post 24 h of starvation.

Antibiotics	**Persister fraction (%)**		
	Aerobic	**Anaerobic**	**CCCP**
Ampicillin	1 ± 0.42	10 ± 2.63	10± 3.59
Ciprofloxacin	0.1 ± 0.039	10	10± 1.85
kanamycin	0	0	0

However, within 6 h of exposure to kanamycin, all the starved cells were eliminated without leaving any survivors. The starved cell, though metabolically dormant, maintain active protein synthesis. The inhibition of protein synthesis may be critical for starvation survival or continuation of dormancy in *V. cholerae*.

3.3.8 Protein synthesis is essential for survival and resurrection of persister cells

The respiration was inhibited by culturing the cells in an anaerobic condition or by pre-treatment with a sub-MIC level of Carbonyl cyanide m-chlorophenyl hydrazone (CCCP) and subsequently exposed to antibiotics. Both ampicillin and ciprofloxacin treatment resulted in 10% of persisters under the anaerobic condition as well as in the

presence of CCCP. However, V. *cholerae* was sensitive to kanamycin, and no persisters were formed (**Table. 3.10**).

Table. 3.10. The percent persister frequency (%) of metabolically dormant V. cholerae. The cells were challenged with all three antibiotics and resulting survival was tabulated. The persister frequency was determined by exposure to different antibiotics, in the presence of CCCP or under anaerobic conditions. The average of three biological replicates with standard deviation is tabulated.

Antibiotics	**Persister fraction (%)**
Ampicillin	98 ± 1.2
Ciprofloxacin	95 ± 3.7
Kanamycin	0

During long-term stress tolerance, the cells lower the metabolic rate and enter a dormant state. The characteristics of dormancy include a reduction in ATP levels, a low rate of cell division, and changes in cell morphology. Those cells are tolerant of a variety of stresses such as temperature, oxidative stress, and antibiotics. The inhibition of respiration and metabolism could increase the persister formation in *E. coli* and *S. aureus.* Between the cholera epidemics, *V. cholerae* stays in a low nutrient condition of estuarine water for several months.

The low energy state could result in the formation of dormant cells, which may show high drug-tolerance. However, the metabolically dormant cells of *V. cholerae* show sensitivity to inhibitors of protein synthesis. The inhibition of ATP synthesis in *E. coli* and *S. aureus* can turn the entire population into persisters[63,90]. The majority of the antibiotic targets require ATP to function, and a reduction in ATP will lead to decreased activity of antibiotic targets, resulting in increased drug tolerance[90]. However, it cannot explain why the reduced metabolism could not save *V. cholerae* from inhibitors of

protein synthesis. It is very interesting to understand the extreme dependence on proteins synthesis to survive both starvation and antibiotics (**Figure 3.37**).

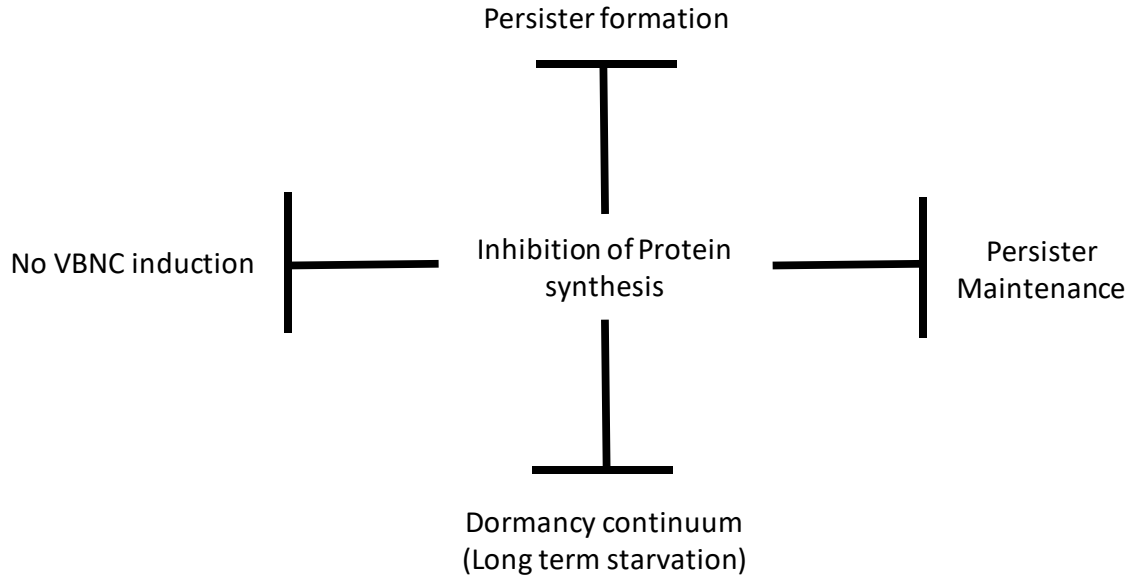

Figure. 3.38. The protein synthesis appears to be critical for persister formation, maintenance and long-term starvation survival, at least in V. cholerae. This is the most important finding of the present chapter. Protein synthesis inhibition in any level from transcription to translation elongation can inhibit persister formation and maintenance. This can be effective strategy to reduce the risk of persister formation during vibrio infections.

3.3.9 Effect of glucose on persister frequency of *V. cholerae*

Earlier reports on *E. coli* have shown that sugars can sensitize the viable cells to β-lactams while another group has elaborated on the role of proton motive force (PMF) in sugar-induced potentiation of aminoglycoside on persister population. Sugar could also increase the effectiveness of antibiotics against resistant clones of *E. tarda*. Hence in the present study, the effect of glucose on antibiotic persisters of *V. cholerae* was determined. The results of previous section A, subsection 3.3.3, showed that persister frequency changed with the type of media (LB, TSB, APW, or BPW). The significant difference between the media is a carbon source. Hence the study was continued by supplementing LB with glucose and measured the antibiotic susceptibility. First, the effective glucose concentration was determined, which would provide significant changes to antibiotic susceptibility. The stationary phase culture of *V. cholerae* was

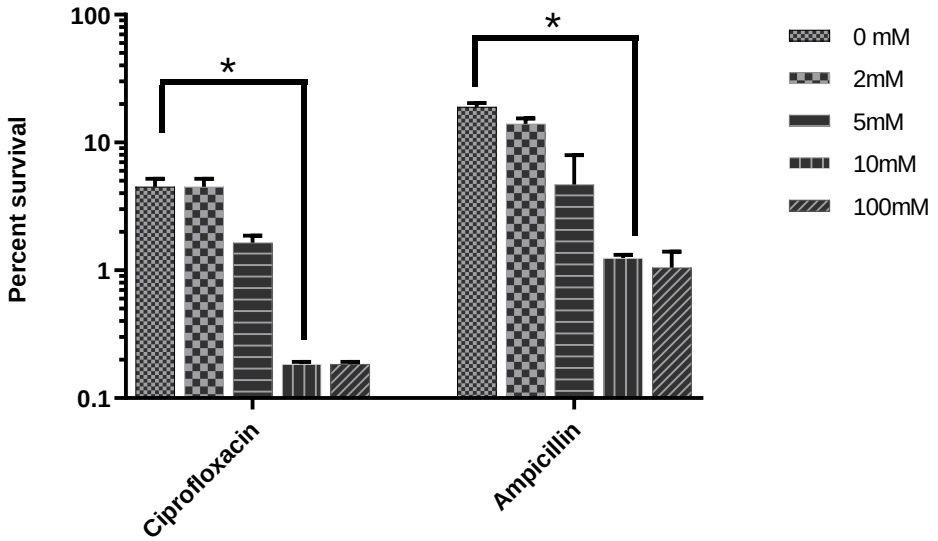

Figure 3.39. The correlation between glucose concentration and antibiotic susceptibility. The stationary phase cells were given glucose supplements for 2 h and later exposed to antibiotics ciprofloxacin and ampicillin. The glucose concentration was increased from 0 to 100 mM and corresponding changes in antibiotic susceptibility was determined. The percent survival [(N/N0) *100] was determined from the serial dilution method. The results indicate the mean of three biological triplicates (n=3) with standard deviation. (*p < 0.05).

supplemented with different concentrations of glucose. The stationary phase cells were supplemented with different concentrations of glucose and subsequently exposed to antibiotics. As the concentration of glucose increased from 0 to 10 mM, the antibiotic susceptibility also increased (**Figure. 3.39**) to ciprofloxacin and ampicillin. However, beyond 10 mM, further increased in glucose could not change antibiotic susceptibility. Glucose at 10 mM concentration was found to be most effective in sensitizing *V. cholerae* to antibiotics.

The study of the effect of glucose on antibiotic susceptibility was undertaken with the following experimental design (**Figure. 3.40**). Cultures in four different conditions were treated with antibiotics, and persister fraction was determined.

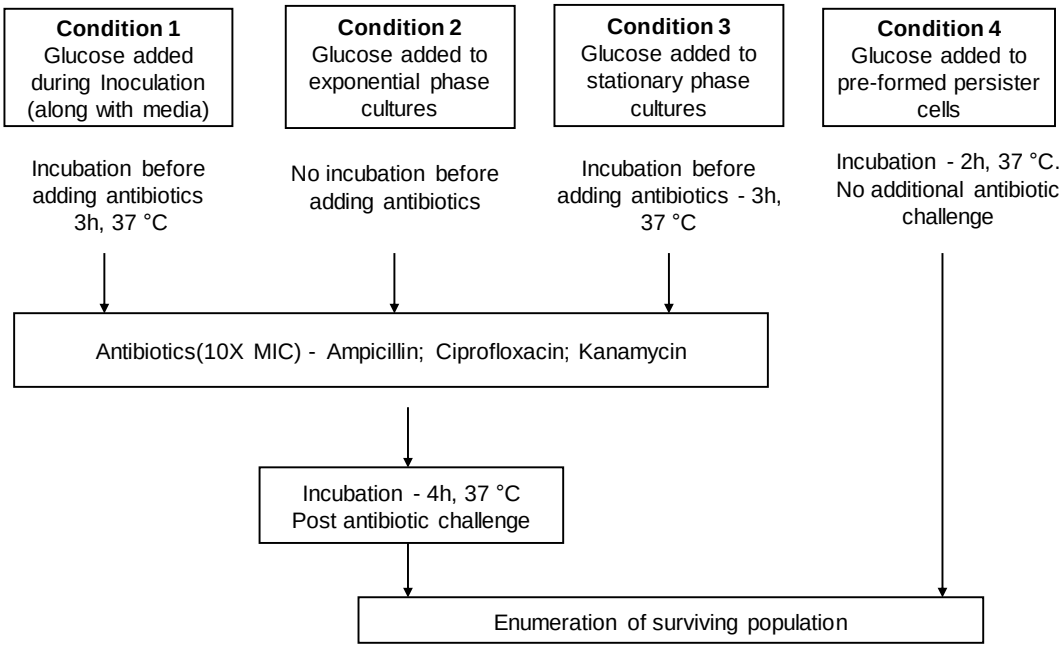

Figure 3.40. Experimental design to study the effect of glucose on antibiotic susceptibility. Four different samples were tested using glucose and three different antibiotics (ampicillin, ciprofloxacin and kanamycin). The cell number was enumerated by standard serial dilution method.

3.3.10 Condition 1: glucose added at the time of inoculation

The *V. cholerae* grown in a media without glucose (Glucose (-)) was treated with ciprofloxacin (10 X MIC), and after the treatment, less than 0.1 % survived. Interestingly, *V. cholerae* grown in the media with glucose (Glucose (+)) had 10-fold more survivors to ciprofloxacin (**Figure. 3.41**), and this difference was statistically significant (P<0.05). However, both the cultures, Glucose (+), and Glucose (-) did not show a significant difference in susceptibility when challenged with ampicillin and kanamycin.

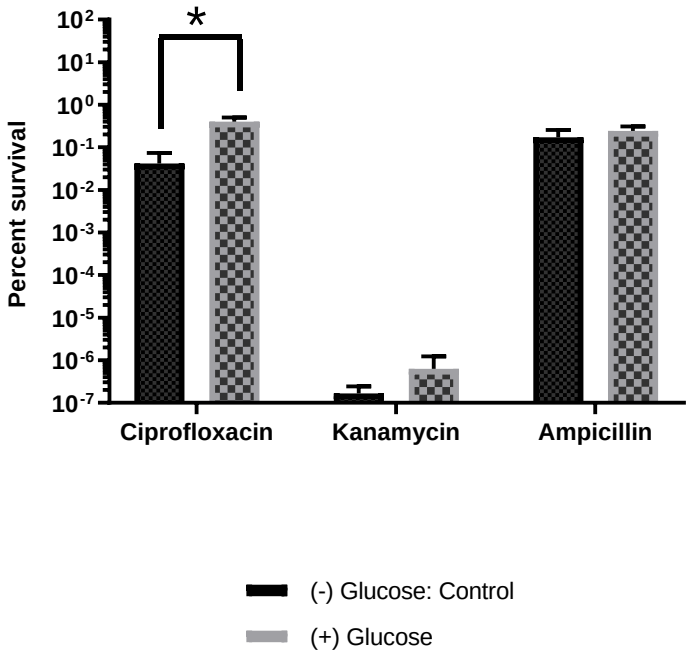

Figure. 3.41. Condition-1. Cultures were grown either in the presence or in the absence of glucose in the media, and as they reach exponential phase, antibiotics were added. After 4h into antibiotics, the cultures were isolated and cell number was determined. The above graph represents the persister population that survived the antibiotics. The control population refers to the cultures growing in LB without additional glucose. The (+) glucose population represents the cultures growing in LB supplemented with 10mM glucose. The (*) indicates the level of significance at p<0.05. The data represents the average of three biological triplicates with S.D.

In condition 1, the cells were cultured in the presence of glucose. The Glucose (+) culture was more tolerant to ciprofloxacin than Glucose (-) culture, but not as tolerant as stationary phase cells. Both cultures were actively dividing and hence showed similar sensitivity to ampicillin. Since this study focuses on *V. cholerae*, it is important to say that it is sensitive to aminoglycosides, and glucose did not rescue cells from the lethality of antibiotic effect.

The apparent increase in tolerance to ciprofloxacin in condition one was further investigated. The growth curve showed that in the presence of glucose, the growth rate had increased from 0.15 h^{-1} to 0.19 h^{-1} ($p = 0.0299$). This result suggests that in the presence of glucose, the cells would divide faster and advance in the growth phase. Therefore, once the glucose is added, the cells would be in the late log phase or the early stationary phase, and we know that stationary cells are resistant to antibiotics. The growth difference between Glucose (+) and Glucose (-) conditions was nullified by sampling cells at same growth phase ($OD_{600} = 0.4$); we observed no significant difference in susceptibility to antibiotics (**Figure. 3.42**)

The major carbon source in LB media is metabolizable amino acids. When glucose is added to the media, cells would prefer to use glucose over amino acids for ATP generation and growth. *V. cholerae* El Tor strains produce acetoin when grown in glucose-rich condition[107]. Acetoin is a neutral fermentation product that does not inhibit bacterial growth. This can be another cause of increased growth rate when cultured with glucose. When the antibiotic was added at a specific time point, the Glucose (+) cultures, due to the faster growth rate, had already advanced to the next growth phase.

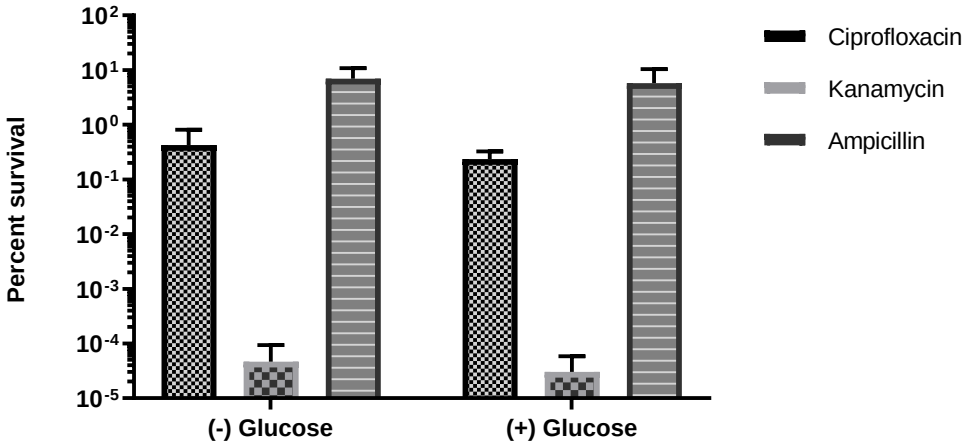

Figure. 3.42. Survival of culture after the synchronizing the growth phase at OD_{600} = 0.4. The difference in survival in the presence or in the absence of glucose was not significant at $p \leq 0.05$.

However, when both Glucose (+) and Glucose (-) were synchronized for growth, the effect of glucose on antibiotic susceptibility was nullified. The physiological age might be the reason for the observed changes in antibiotic susceptibility. The addition of glucose in the media at the time of inoculation can result in significant changes in growth kinetics, and the parameters must be carefully considered while determining the antibiotic susceptibility of growing cultures.

3.3.11 Condition 2: glucose added in exponential phase

The glucose and antibiotic were added together to the actively growing culture in media, and here both the cultures Glucose (+) and Glucose (-) had 0.1% survivors to both ciprofloxacin and ampicillin (**Figure. 3.43**). The glucose addition failed to change the antibiotic susceptibility of an exponentially growing population, irrespective of the mode of action of antibiotics (ciprofloxacin, ampicillin, and kanamycin).

In condition 2, the exponentially growing cultures received glucose and antibiotic simultaneously. The glucose could not sensitize the exponentially growing culture of *V.*

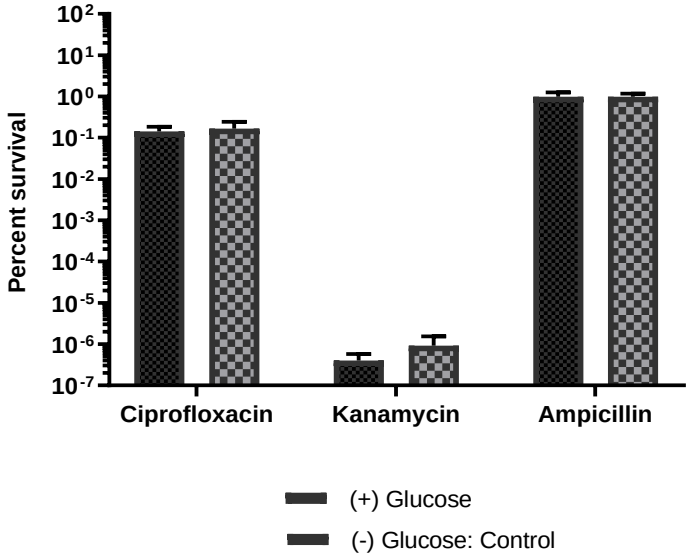

Figure. 3.43. Condition 2. Glucose was supplemented to the cultures which had already reached exponential phase. Along with glucose, cultures were also exposed to 10-fold MIC of antibiotics. The graph represents the persister population that survived the 4 h of antibiotics treatment. The control population refers to the cultures exposed to antibiotics without glucose. The (+) glucose population represents the cultures exposed to antibiotics in the presence of 10mM glucose. The difference in survival in the presence or in the absence of glucose was not significant at $p \leq 0.05$. The data represents the average of three biological triplicates with S.D.

cholerae to the antibiotics, so our results with *V. cholerae*, contradict a previous study on *E. coli*[108] in which the authors have shown that glucose can sensitize the exponentially growing culture to carbenicillin but increase the tolerance to gentamicin.

3.3.12 Condition 3: glucose supplemented in stationary phase

The stationary phase cells were more tolerant of antibiotics than the actively growing population (**Figure. 3.44**). The Glucose (+) cultures were over 10-fold more susceptibility to ciprofloxacin, ampicillin and kanamycin, in comparison to Glucose (-) cultures ($p < 0.05$). The stationary cultures were supplemented with non-metabolizable sugars such as sorbitol and sucralose. No change in antibiotic susceptibility was observed in the presence of non-metabolizable sugars (**Figure. 3.45)**.

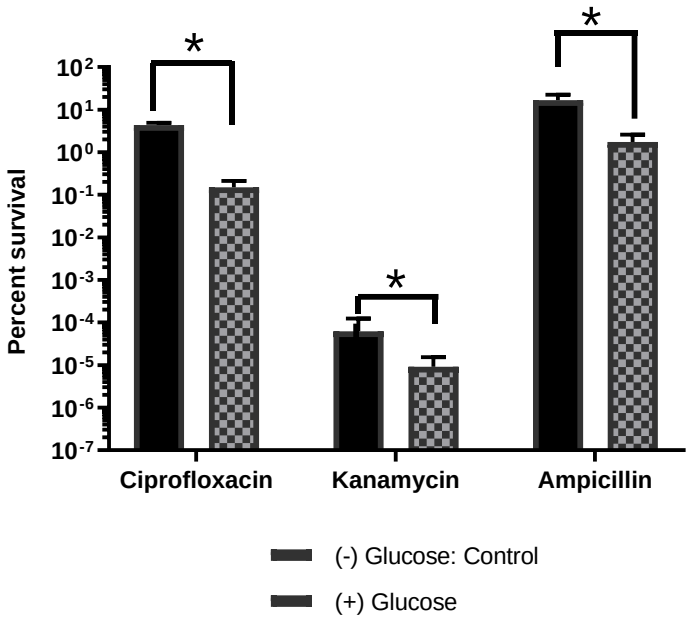

Figure. 3.44. Condition 3. Glucose was supplemented to stationary phase cultures. The cultures were then challenged with antibiotics for 4 h and cell number was determined. The graph represents the persister population that survived the 4 h of antibiotics treatment. The control population refers to the cultures exposed to antibiotics without glucose. The (+) glucose population represents the cultures exposed to antibiotics in the presence of 10mM glucose. The (*) indicates the level of significance at p<0.05. The data represents the average of three biological triplicates with S.D.

In the condition-3, stationary phase cells were challenged with antibiotics after the addition of glucose. The stationary phase is characterized by starvation of metabolizable carbon sources[109], and those cells are better at tolerating antibiotics than the exponential cells, which is in agreement with the earlier reports[26]. Interestingly, the glucose supplementation reduced the antibiotic tolerance almost to the level of late exponential phase cells. Similar results were obtained in *E. coli* by Gutierrez [110], where the authors have observed a rise in susceptibility to ciprofloxacin once the stationary cells are provided with glucose. The authors have also pointed out that along with glucose, oxygen is also required to sensitize the stationary cells[110]. The glucose-induced

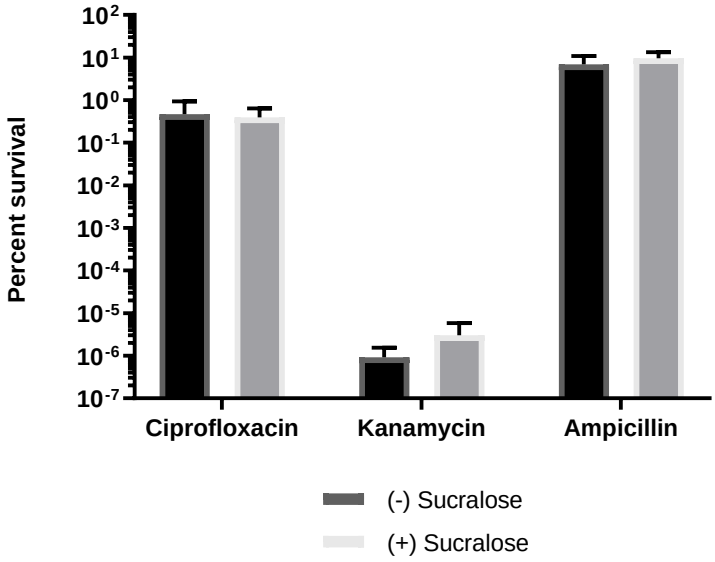

Figure. 3.45. The effect of non-metabolizable sugar Sucralose on the antibiotic susceptibility of V. cholerae. The sucralose was supplemented during inoculation and antibiotic was challenged in the exponential phase. The survival was measured 4 h post antibiotic treatment. Values in all bar graph depict the mean ± SD. The difference in survival in the presence or in the absence of sucralose was not significant at $p \leq 0.05$.

the sensitivity of the stationary population to daptomycin and beta-lactams is also documented in *S. aureus* and in *E. coli*, respectively[108,111]. The replenishment of glucose triggers the metabolism and consequently sensitizes the cells[112,113]. The rise in metabolism can increase the respiration and production of ATP[63], which results in fastening the antibiotic uptake, thereby sensitizing cells to antibiotics[114]. In contrast, in *S. aureus*, the glucose-mediated sensitivity to antibiotic daptomycin was not due to the generation of proton motive force (PMF)[111].

3.3.13 Condition 4: glucose was added to the persister cells.

The persister cells obtained by ciprofloxacin challenge were termed as P-ciprofloxacin; the persister cells obtained by ampicillin challenge were termed as P-ampicillin. The 10-fold antibiotic concentration was maintained in the medium throughout the

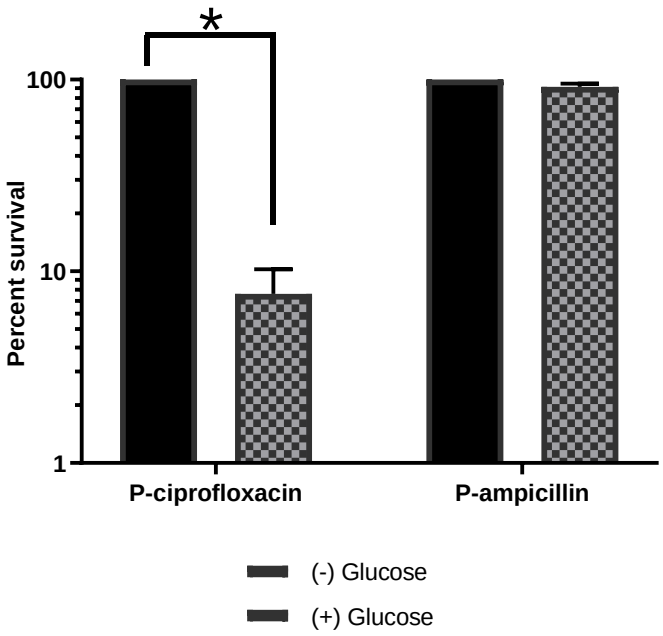

Figure. 3.46. Effect of glucose on preformed persister cells. Persister cells were obtained by treating either with ciprofloxacin (P-ciprofloxacin) or ampicillin (P-ampicillin). The persisters were then supplemented with glucose and incubated for 2 h and cell number was determined. The control population refers to the pre-formed persisters exposed to antibiotics without glucose, they are considered as 100% survivors. The (+) glucose population represents the pre-formed persisters exposed to antibiotics in the presence of 10mM glucose. Above graph represents the pre-formed persisters that survived the 4 h of antibiotics treatment in relation to the control population. The (*) indicates the level of significance at p<0.05. The data represents the average of three biological triplicates with S.D.

experiment to keep a stable persister population. After the addition of glucose to this stable persister population, the P-ciprofloxacin population reduced by 10-fold (**Figure. 3.46**), but the P-ampicillin, however, did not show significant cell number reduction upon glucose supplementation.

In condition 4, the persister cells of *V. cholerae* were obtained in the presence of glucose using ciprofloxacin and ampicillin to which they were tolerant. The persister cells are now appreciated as an important factor that precedes the spread of antibiotic resistance[115], and they represent a transient physiological state, with reduced

metabolism and cell division[116]. The presence of glucose can trigger the cells to exit the persister state and initiate active metabolism, which made them sensitive to ciprofloxacin (this study) and aminoglycosides[112,113,117]. However, due to the continued presence of ampicillin, the persister cells cannot be triggered to initiate cell division.

In comparison, the stationary phase cells reduce their metabolism significantly due to the non-availability of carbon sources[109]. The glucose addition can quickly increase the metabolism and initiate cell division in stationary cells. Metabolites can sensitize the persister cells of *E. coli* to aminoglycosides and quinolones; *S. aureus* to aminoglycosides; *M. tuberculosis* to isoniazid and *P. aeruginosa* to aminoglycosides[113] and *V. cholerae* to ofloxacin, aminoglycosides and β-lactams (**Figure. 3.44**).

3.3.14 Role of respiration in the glucose-mediated rise in antibiotic susceptibility

The stationary phase cells were pre-treated with 1 X MIC of CCCP, to inhibit the ATP synthesis, by uncoupling the electron transport chain. This pre-treatment appears to have protected the cells from deleterious effects of both ciprofloxacin and ampicillin, and glucose showed no effect on the antibiotic susceptibility (**Figure. 3.47**). Upon inhibition of ATP synthesis, the subsequent addition of glucose could not increase antibiotic susceptibility.

Cholera patients are given the high volume of Oral Rehydration Solution (ORS) containing a large volume of glucose to prevent dehydration[118]. In the presence of glucose, the dormant cells of other opportunistic pathogens may show higher sensitivity to antibiotics, which would help decrease the intestinal bacterial load during cholera infections. As we found in the present study, the growth rate of *V. cholerae* increased in the presence of glucose, but the antibiotic susceptibility did not change (Conditions

2). Glucose can effectively increase the antibiotic susceptibility of non-dividing cells of the stationary phase or persister state (Condition 3 and 4).

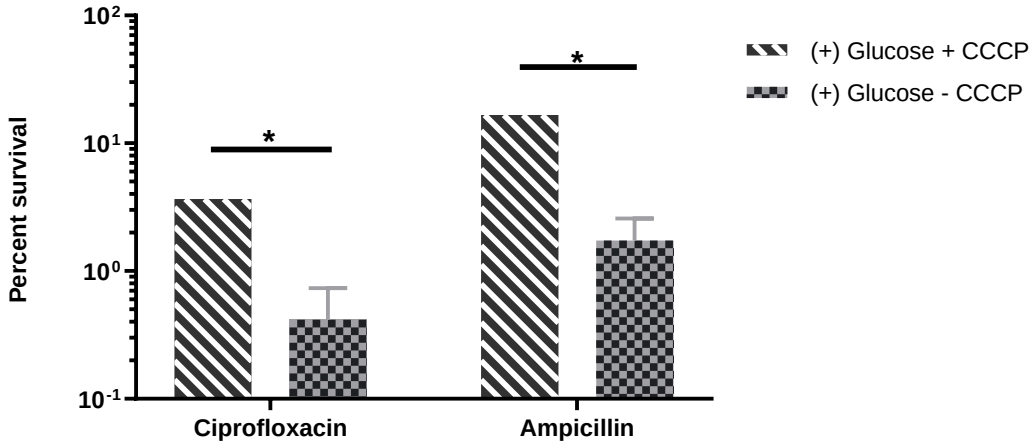

Figure. 3.47. Effect of CCCP pre-treatment on the effect of glucose on antibiotic susceptibility. The (+) CCCP indicates that the cultures grown in the presence of glucose were pre-treated with CCCP and then exposed to antibiotics. The (-) CCCP indicates that the cultures grown in the presence of glucose were directly exposed to antibiotics without CCCP pre-treatment. Values in all bar graphs depict the mean ± SEM with significance reported as p-values in comparison with untreated control (-CCCP): *p ≤ 0.01, (n = 3).

However, the correlation between the beneficial effects of glucose on growth and deleterious effects on antibiotic susceptibility needs further attention. The minimum effective concentration of glucose was found to be 10 mM. Remarkably, the average blood glucose is varying between 4 mM – 6 mM in the fasting condition, and above 10 mM, is termed as hyperglycemia. The effect of glucose on antibiotic susceptibility is maximum at the boundary of normal and diabetic blood glucose levels. However, at this point, we cannot comment on antibiotic effectiveness in diabetic and non-diabetic patients without clinical data.

3.3.15 Chapter summary

Persister formation is a complex physiological phenomenon. The current work describes the effect of a different class of antibiotics on *V. cholerae* and *E. coli* on persister cell formation. The second important component that would influence persister formation is the growth condition. Persister frequency fluctuates with the bacterial growth phase with minimal persisters in the log phase, reaching a peak in the stationary phase. The frequency of ciprofloxacin induced persisters of *V. cholerae* would fluctuate with the bacteriological media used for the growth of the cells. When challenged with protein synthesis inhibitors (kanamycin, chloramphenicol, tetracycline, erythromycin, and gentamicin), *V. cholerae* did not form persisters. Persister recovery assay, LIVE/DEAD analysis, and QRDR sequence analysis showed that the persister population neither included resistant mutants nor VBNC population. Starvation, anaerobic conditions, and inhibition of ATP synthesis also induced persisters, but not when protein synthesis is inhibited.

In the present study, we observed contrasting differences in persister formation in *E. coli* and *V. cholerae* when exposed to protein synthesis inhibitors. When exposed to kanamycin, *E. coli* can form persister cells in both exponential and stationary phases. However, *V. cholerae* was eliminated in both the growth phases. The mechanism of persister formation in *E. coli* has been investigated by several studies[26,90,112]. The main contributors to persister formation in *E. coli* are Toxin: Antitoxin (TA) modules, stringency, and starvation responses[24,28]. In *E. coli* during dormancy, the ribosome content decreases, thus reducing the targets for protein synthesis inhibitors[112]. However, in *V. cholerae*, during dormancy, the ribosome machinery is actively maintained[119], and therefore cells show higher sensitivity to inhibitors of protein synthesis. The persister

mechanisms in *V. cholerae* are not well characterized, and not much of information is available about the role of stringent responses in persister formation in *V. cholerae*. Notably, even with the deletion of 10 TA pairs, the persister frequency in *E. coli* remains unchanged. *V. cholerae* has several putative TA modules, but how these impacts the persister formation is still not known.

Metabolites can alter cell physiology and hence, can change the antibiotic susceptibility. Glucose could change the antibiotic susceptibility in a growth phase-dependent fashion; however, the antibiotic susceptibility of exponentially growing cells was not influenced due to the presence of glucose. Stationary phase cells that show higher antibiotic tolerance could be sensitized to ciprofloxacin and ampicillin by glucose supplementation (tenfold sensitive). The glucose increases the respiration, which in turn increases the metabolism and cell division rate. We propose that persistence being a physiological phenomenon is affected by the growth rate, growth medium, growth phase, type of antibiotics, and metabolites.

4 REFERENCES

1. *Starvation in Bacteria*. (Springer US, 1993).

2. Navarro Llorens, J. M., Tormo, A. & Martínez-García, E. Stationary phase in gram-negative bacteria. *FEMS Microbiol. Rev.* **34**, 476–495 (2010).

3. Lever, M. A. *et al.* Life under extreme energy limitation: a synthesis of laboratory- and field-based investigations. *FEMS Microbiol. Rev.* **39**, 688–728 (2015).

4. Nyström, T., Albertson, N. H., Flärdh, K. & Kjeileberg, S. Physiological and molecular adaptation to starvation and recovery from starvation by the marine Vibrio sp. S14. *FEMS Microbiol. Lett.* **74**, 129–140 (1990).

5. Morita, R. Y. Starvation-Survival of Heterotrophs in the Marine Environment. in *Advances in Microbial Ecology: Volume 6* (ed. Marshall, K. C.) 171–198 (Springer US, 1982).

6. Morita, R. Y. Bioavailability of energy and its relationship to growth and starvation survival in nature. *Can. J. Microbiol.* **34**, 436–441 (1988).

7. Baker, R. M., Singleton, F. L. & Hood, M. A. Effects of nutrient deprivation on Vibrio cholerae. *Appl. Environ. Microbiol.* **46**, 930–940 (1983).

8. Srinivasan, S. & Kjelleberg, S. Cycles of famine and feast: the starvation and outgrowth strategies of a marineVibrio. *J. Biosci.* **23**, 501–511 (1998).

9. Wai, S. N., Mizunoe, Y. & Yoshida, S. How Vibrio cholerae survive during starvation. *FEMS Microbiol. Lett.* **180**, 123–131 (1999).

10. Stretton, S., Danon, S. J., Kjelleberg, S. & Goodman, A. E. Changes in cell morphology and motility in the marine Vibrio sp. strain S14 during conditions of starvation and recovery. *FEMS Microbiol. Lett.* **146**, 23–29 (1997).

11. Watson, S. P., Clements, M. O. & Foster, S. J. Characterization of the Starvation-Survival Response of Staphylococcus aureus. *J. Bacteriol.* **180**, 1750–1758 (1998).

12. Sporulation and Cell Differentiation. *Biol. Prokaryotes* (1998) doi:10.1002/9781444313314.ch25.

13. Finkel, S. E. Long-term survival during stationary phase: evolution and the GASP phenotype. *Nat. Rev. Microbiol.* **4**, 113 (2006).

14. Oliver, J. D. Recent findings on the viable but nonculturable state in pathogenic bacteria. *FEMS Microbiol. Rev.* **34**, 415–425 (2010).

15. Wood, T. K., Knabel, S. J. & Kwan, B. W. Bacterial Persister Cell Formation and Dormancy. *Appl. Environ. Microbiol.* **79**, 7116–7121 (2013).

16. Kim, J.-S., Chowdhury, N., Yamasaki, R. & Wood, T. K. Viable but non-culturable and persistence describe the same bacterial stress state. *Environ. Microbiol.* **20**, 2038–2048 (2018).

17. Bergkessel, M., Basta, D. W. & Newman, D. K. The physiology of growth arrest: uniting molecular and environmental microbiology. *Nat. Rev. Microbiol.* **14**, 549–562 (2016).

18. Hoehler, T. M. & Jørgensen, B. B. Microbial life under extreme energy limitation. *Nat. Rev. Microbiol.* **11**, 83 (2013).

19. Pal, R. R., Das, B., Dasgupta, S. & Bhadra, R. K. Genetic components of stringent response in Vibrio cholerae. *Indian J. Med. Res.* **133**, 212–217 (2011).

20. Fauvart, M., De Groote, V. N. & Michiels, J. Role of persister cells in chronic infections: clinical relevance and perspectives on anti-persister therapies. *J. Med. Microbiol.* **60**, 699–709 (2011).

21. Lewis, K. Persister cells. *Annu. Rev. Microbiol.* **64**, 357–372 (2010).

22. Nguyen, D. *et al.* Active starvation responses mediate antibiotic tolerance in biofilms and nutrient-limited bacteria. *Science* **334**, 982–986 (2011).

23. Balaban, N. Persistence: mechanisms for triggering and enhancing phenotypic variability. *Genet. Syst. Biol.* **21**, 768–775 (2011).

24. Van den Bergh, B., Fauvart, M. & Michiels, J. Formation, physiology, ecology, evolution and clinical importance of bacterial persisters. *FEMS Microbiol. Rev.* **41**, 219–251 (2017).

25. Harms, A., Maisonneuve, E. & Gerdes, K. Mechanisms of bacterial persistence during stress and antibiotic exposure. *Science* **354**, aaf4268 (2016).

26. Balaban, N. Q., Merrin, J., Chait, R., Kowalik, L. & Leibler, S. Bacterial persistence as a phenotypic switch. *Science* **305**, 1622–1625 (2004).

27. Radzikowski, J. L., Schramke, H. & Heinemann, M. Bacterial persistence from a system-level perspective. *Curr. Opin. Biotechnol.* **46**, 98–105 (2017).

28. Kint, C. I., Verstraeten, N., Fauvart, M. & Michiels, J. New-found fundamentals of bacterial persistence. *Trends Microbiol.* **20**, 577–585 (2012).

29. Kaldalu, N., Hauryliuk, V. & Tenson, T. Persisters—as elusive as ever. *Appl. Microbiol. Biotechnol.* **100**, 6545–6553 (2016).

30. Harms, A., Brodersen, D. E., Mitarai, N. & Gerdes, K. Toxins, Targets, and Triggers: An Overview of Toxin-Antitoxin Biology. *Mol. Cell* **0**, (2018).

31. Ramisetty, B. C. M., Ghosh, D., Roy Chowdhury, M. & Santhosh, R. S. What Is the Link between Stringent Response, Endoribonuclease Encoding Type II Toxin–Antitoxin Systems and Persistence? *Front. Microbiol.* **7**, (2016).

32. Colwell, R. R. & Spira, W. M. The Ecology of Vibrio cholerae. in *Cholera* (eds. Barua, D. & Greenough, W. B.) 107–127 (Springer US, 1992).

33. Nyström, T., Olsson, R. M. & Kjelleberg, S. Survival, stress resistance, and alterations in protein expression in the marine vibrio sp. strain S14 during starvation for different individual nutrients. *Appl Env. Microbiol* **58**, 55–65 (1992).

34. *The Biology of Vibrios*. (American Society of Microbiology, 2006).

35. Nyström, T., Albertson, N. H., Flärdh, K. & Kjelleberg, S. Physiological and molecular adaptation to starvation and recovery from starvation by the marine Vibrio sp. S14. *FEMS Microbiol. Ecol.* **7**, 129–140 (1990).

36. Nyström, T., Flärdh, K. & Kjelleberg, S. Responses to multiple-nutrient starvation in marine Vibrio sp. strain CCUG 15956. *J. Bacteriol.* **172**, 7085–7097 (1990).

37. M A Hood, D. C. W. & J B Guckert, F. D. Effect of nutrient deprivation on lipid, carbohydrate, DNA, RNA, and protein levels in Vibrio cholerae.

38. Colwell, R. R. Global Climate and Infectious Disease: The Cholera Paradigm*. *Science* **274**, 2025–2031 (1996).

39. Dengo-Baloi, L. C. *et al.* Antibiotics resistance in El Tor Vibrio cholerae 01 isolated during cholera outbreaks in Mozambique from 2012 to 2015. *PLOS ONE* **12**, e0181496 (2017).

40. WHO | Cholera. *WHO* http://www.who.int/csr/don/archive/disease/cholera/en/.

41. Levin-Reisman, I. *et al.* Antibiotic tolerance facilitates the evolution of resistance. *Science* **355**, 826–830 (2017).

42. Ayrapetyan, M., Williams, T. C. & Oliver, J. D. Bridging the gap between viable but non-culturable and antibiotic persistent bacteria. *Trends Microbiol.* **23**, 7–13 (2015).

43. Fang, F. C. & Casadevall, A. Reductionistic and holistic science. *Infect. Immun.* **79**, 1401–1404 (2011).

44. Goormaghtigh, F. & Van Melderen, L. Optimized Method for Measuring Persistence in Escherichia coli with Improved Reproducibility. in *Bacterial Persistence: Methods and Protocols* (eds. Michiels, J. & Fauvart, M.) 43–52 (Springer, 2016). doi:10.1007/978-1-4939-2854-5_4.

45. Mishra, A., Taneja, N. & Sharma, M. Viability kinetics, induction, resuscitation and quantitative real-time polymerase chain reaction analyses of viable but nonculturable Vibrio cholerae O1 in freshwater microcosm. *J. Appl. Microbiol.* **112**, 945–953 (2012).

46. Keren, I., Kaldalu, N., Spoering, A., Wang, Y. & Lewis, K. Persister cells and tolerance to antimicrobials. *FEMS Microbiol. Lett.* **230**, 13–18 (2004).

47. Beers, R. F. & Sizer, I. W. A Spectrophotometric Method for Measuring the Breakdown of Hydrogen Peroxide by Catalase. *J. Biol. Chem.* **195**, 133–140 (1952).

48. Pecoraro, V., Zerulla, K., Lange, C. & Soppa, J. Quantification of Ploidy in Proteobacteria Revealed the Existence of Monoploid, (Mero-)Oligoploid and Polyploid Species. *PLOS ONE* **6**, e16392 (2011).

49. Zerulla, K., Ludt, K. & Soppa, J. The ploidy level of Synechocystis sp. PCC 6803 is highly variable and is influenced by growth phase and by chemical and physical external parameters. *Microbiology,* **162**, 730–739 (2016).

50. Heidelberg, J. F. *et al.* DNA sequence of both chromosomes of the cholera pathogen Vibrio cholerae. *Nature* **406**, 477–483 (2000).

51. Kumar, S., Stecher, G., Li, M., Knyaz, C. & Tamura, K. MEGA X: Molecular Evolutionary Genetics Analysis across Computing Platforms. *Mol. Biol. Evol.* **35**, 1547–1549 (2018).

52. Gefen, O. & Balaban, N. Q. The importance of being persistent: heterogeneity of bacterial populations under antibiotic stress. *FEMS Microbiol. Rev.* **33**, 704–717 (2009).

53. Jõers, A. & Tenson, T. Growth resumption from stationary phase reveals memory in Escherichia coli cultures. *Sci. Rep.* **6**, 24055 (2016).

54. Balaban, N. Q. *et al.* Definitions and guidelines for research on antibiotic persistence. *Nat. Rev. Microbiol.* 1 (2019) doi:10.1038/s41579-019-0196-3.

55. Paranjape, S. S. & Shashidhar, R. Comparison of Starvation-Induced Persister Cells with Antibiotic-Induced Persister Cells. *Curr. Microbiol.* **76**, 1495–1502 (2019).

56. Cabral, D. J., Wurster, J. I. & Belenky, P. Antibiotic Persistence as a Metabolic Adaptation: Stress, Metabolism, the Host, and New Directions. *Pharmaceuticals* **11**, 14 (2018).

57. Oliver, J. D. Recent findings on the viable but nonculturable state in pathogenic bacteria. *FEMS Microbiol. Rev.* **34**, 415–425 (2010).

58. Ostling, J., Holmquist, L. & Kjelleberg, S. Global analysis of the carbon starvation response of a marine Vibrio species with disruptions in genes homologous to relA and spoT. *J. Bacteriol.* **178**, 4901–4908 (1996).

59. Chubukov, V. & Sauer, U. Environmental Dependence of Stationary-Phase Metabolism in Bacillus subtilis and Escherichia coli. *Appl Env. Microbiol* **80**, 2901–2909 (2014).

60. Wang, J. D. & Levin, P. A. Metabolism, cell growth and the bacterial cell cycle. *Nat. Rev. Microbiol.* **7**, 822–827 (2009).

61. Mizunoe, Y., Wai, S. N., Takade, A. & Yoshida, S.-I. Isolation and Characterization of Rugose Form of Vibrio cholerae O139 Strain MO10. *Infect. Immun.* **67**, 958–963 (1999).

62. Ebrahimi, A., Csonka, L. N. & Alam, M. A. Analyzing Thermal Stability of Cell Membrane of Salmonella Using Time-Multiplexed Impedance Sensing. *Biophys. J.* **114**, 609–618 (2018).

63. Conlon, B. P. *et al.* Persister formation in *Staphylococcus* aureus is associated with ATP depletion. *Nat. Microbiol.* **1**, 16051 (2016).

64. Sukhi, S. S., Shashidhar, R., Kumar, S. A. & Bandekar, J. R. Radiation resistance of Deinococcus radiodurans R1 with respect to growth phase. *FEMS Microbiol. Lett.* **297**, 49–53 (2009).

65. Häder, D.-P., Helbling, E., Williamson, C. & Worrest, R. Effects of UV radiation on aquatic ecosystems and interactions with climate change. *Photochem. Photobiol. Sci.* **10**, 242–260 (2011).

66. Nagar, V., Bandekar, J. R. & Shashidhar, R. Expression of virulence and stress response genes in Aeromonas hydrophila under various stress conditions. *J. Basic Microbiol.* **56**, 1132–1137 (2016).

67. Sun, J. *et al.* Comparative analysis of the survival and gene expression of pathogenic strains Vibrio harveyi after starvation. *FEMS Microbiol. Lett.* **363**, (2016).

68. Gomes, A. É. I. *et al.* Selection and validation of reference genes for gene expression studies in Klebsiella pneumoniae using Reverse Transcription Quantitative real-time PCR. *Sci. Rep.* **8**, 9001 (2018).

69. Grant, S. S. & Hung, D. T. Persistent bacterial infections, antibiotic tolerance, and the oxidative stress response. *Virulence* **4**, 273–283 (2013).

70. Redza-Dutordoir, M. & Averill-Bates, D. A. Activation of apoptosis signalling pathways by reactive oxygen species. *Biochim. Biophys. Acta BBA - Mol. Cell Res.* **1863**, 2977–2992 (2016).

71. Wu, Y., Vulić, M., Keren, I. & Lewis, K. Role of Oxidative Stress in Persister Tolerance. *Antimicrob. Agents Chemother.* **56**, 4922–4926 (2012).

72. Kim, J.-S. & Wood, T. K. Tolerant, Growing Cells from Nutrient Shifts Are Not Persister Cells. *mBio* **8**, e00354–17 (2017).

73. Ackermann, M. A functional perspective on phenotypic heterogeneity in microorganisms. *Nat. Rev. Microbiol.* **13**, 497 (2015).

74. Altschuler, S. J. & Wu, L. F. Cellular Heterogeneity: Do Differences Make a Difference? *Cell* **141**, 559–563 (2010).

75. Lutz, C., Erken, M., Noorian, P., Sun, S. & McDougald, D. Environmental reservoirs and mechanisms of persistence of Vibrio cholerae. *Front. Microbiol.* **4**, 375 (2013).

76. Thompson, C. C. *et al.* Genomic taxonomy of vibrios. *BMC Evol. Biol.* **9**, 258 (2009).

77. Paranjape, S. S. & Shashidhar, R. The ploidy of Vibrio cholerae is variable and is influenced by growth phase and nutrient levels. *FEMS Microbiol. Lett.* **364**, (2017).

78. Myles, P. S. & Cui, J. I. Using the Bland–Altman method to measure agreement with repeated measures. *BJA Br. J. Anaesth.* **99**, 309–311 (2007).

79. Amir, A., Männik, J., Woldringh, C. L. & Zaritsky, A. Editorial: The Bacterial Cell: Coupling between Growth, Nucleoid Replication, Cell Division, and Shape Volume 2. *Front. Microbiol.* **10**, 2056 (2019).

80. Griese, M., Lange, C. & Soppa, J. Ploidy in cyanobacteria. *FEMS Microbiol. Lett.* **323**, 124–131 (2011).

81. Hood, M. A., Guckert, J. B., White, D. C. & Deck, F. Effect of nutrient deprivation on lipid, carbohydrate, DNA, RNA, and protein levels in Vibrio cholerae. *Appl. Environ. Microbiol.* **52**, 788–793 (1986).

82. Novitsky, J. A. & Morita, R. Y. Survival of a Psychrophilic Marine Vibrio Under Long-Term Nutrient Starvation 1. *Appl. Environ. Microbiol.* **33**, 635–641 (1977).

83. Zerulla, K. *et al.* DNA as a Phosphate Storage Polymer and the Alternative Advantages of Polyploidy for Growth or Survival. *PLOS ONE* **9**, e94819 (2014).

84. Ali, A., Rashid, H. & K R Karaolis, D. High-Frequency Rugose Exopolysaccharide Production by Vibrio cholerae. *Appl. Environ. Microbiol.* **68**, 5773–8 (2002).

85. Iyer, S., Le, D., Park, B. R. & Kim, M. Distinct mechanisms coordinate transcription and translation under carbon and nitrogen starvation in Escherichia coli. *Nat. Microbiol.* **3**, 741–748 (2018).

86. Nyström, T., Flärdh, K. & Kjelleberg, S. Responses to multiple-nutrient starvation in marine Vibrio sp. strain CCUG 15956. *J. Bacteriol.* **172**, 7085–7097 (1990).

87. M9 Minimal Medium (with Tris replacing phosphate). *Team_HKUST-Rice_2015_M9_Minimal_Medium_(Tris)* http://2015.igem.org/wiki/images/2/2d/Team_HKUST-Rice_2015_M9_Minimal_Medium_(Tris).pdf.

88. M9 minimal medium (standard). *Cold Spring Harb. Protoc.* **2010**, pdb.rec12295 (2010).

89. Martinez, R. M., Megli, C. J. & Taylor, R. K. Growth and Laboratory Maintenance of Vibrio cholerae. *Curr. Protoc. Microbiol.* **0 6**, Unit-6A.1 (2010).

90. Shan, Y. *et al.* ATP-Dependent Persister Formation in Escherichia coli. *mBio* **8**, e02267–16 (2017).

91. Flärdh, K., Axberg, T., Albertson, N. H. & Kjelleberg, S. Stringent control during carbon starvation of marine Vibrio sp. strain S14: molecular cloning, nucleotide sequence, and deletion of the relA gene. *J. Bacteriol.* **176**, 5949–5957 (1994).

92. Kwan, B. W., Valenta, J. A., Benedik, M. J. & Wood, T. K. Arrested protein synthesis increases persister-like cell formation. *Antimicrob. Agents Chemother.* **57**, 1468–1473 (2013).

93. Godoy, M. S., Nikel, P. I., Gomez, J. G. C. & Pettinari, M. J. The CreC Regulator of Escherichia coli, a New Target for Metabolic Manipulations. *Appl. Environ. Microbiol.* **82**, 244–254 (2016).

94. Kim, K.-S., Rao, N. N., Fraley, C. D. & Kornberg, A. Inorganic polyphosphate is essential for long-term survival and virulence factors in Shigella and Salmonella spp. *Proc. Natl. Acad. Sci. U. S. A.* **99**, 7675–7680 (2002).

95. Chubukov, V., Gerosa, L., Kochanowski, K. & Sauer, U. Coordination of microbial metabolism. *Nat. Rev. Microbiol.* **12**, 327–340 (2014).

96. Ying, W. NAD+/NADH and NADP+/NADPH in Cellular Functions and Cell Death: Regulation and Biological Consequences. *Antioxid. Redox Signal.* **10**, 179–206 (2007).

97. Degli Esposti, M. Inhibitors of NADH–ubiquinone reductase: an overview. *Biochim. Biophys. Acta BBA - Bioenerg.* **1364**, 222–235 (1998).

98. Takano, S., Pawlowska, B. J., Gudelj, I., Yomo, T. & Tsuru, S. Density-Dependent Recycling Promotes the Long-Term Survival of Bacterial Populations during Periods of Starvation. *mBio* **8**, e02336-16 (2017).

99. Cabello, F. C. Heavy use of prophylactic antibiotics in aquaculture: a growing problem for human and animal health and for the environment. *Environ. Microbiol.* **8**, 1137–1144 (2006).

100. Tang, H.-J. *et al.* In Vitro and In Vivo Activities of Newer Fluoroquinolones against Vibrio vulnificus. *Antimicrob. Agents Chemother.* **46**, 3580–3584 (2002).

101. Gupta, P. *et al.* Increased antibiotic resistance exhibited by the biofilm of Vibrio cholerae O139. *J. Antimicrob. Chemother.* **73**, 1841–1847 (2018).

102. Paranjape, S. S. & Shashidhar, R. Inhibition of protein synthesis eradicates persister cells of V. cholerae. *3 Biotech* **9**, 380 (2019).

103. Paranjape, S. S. & Shashidhar, R. Glucose sensitizes the stationary and persistent population of Vibrio cholerae to ciprofloxacin. *Arch. Microbiol.* (2019) doi:10.1007/s00203-019-01751-8.

104. Baranwal, S., Dey, K., Ramamurthy, T., Nair, G. B. & Kundu, M. Role of Active Efflux in Association with Target Gene Mutations in Fluoroquinolone Resistance in Clinical Isolates of Vibrio cholerae. *Antimicrob. Agents Chemother.* **46**, 2676–2678 (2002).

105. Sharma, B., Brown, A. V., Matluck, N. E., Hu, L. T. & Lewis, K. Borrelia burgdorferi, the causative agent of Lyme disease, forms drug-tolerant persister cells. *Antimicrob. Agents Chemother.* AAC.00864-15 (2015) doi:10.1128/AAC.00864-15.

106. Jubair, M., Jr, J. G. M. & Ali, A. Survival of Vibrio cholerae in Nutrient-Poor Environments Is Associated with a Novel "Persister" Phenotype. *PLOS ONE* **7**, e45187 (2012).

107. Pradhan, S., Baidya, A. K., Ghosh, A., Paul, K. & Chowdhury, R. The El Tor Biotype of Vibrio cholerae Exhibits a Growth Advantage in the Stationary Phase in Mixed Cultures with the Classical Biotype. *J. Bacteriol.* **192**, 955–963 (2010).

108. Thorsing, M., Bentin, T., Givskov, M., Tolker-Nielsen, T. & Goltermann, L. The bactericidal activity of β-lactam antibiotics is increased by metabolizable sugar species. *Microbiology* **161**, 1999–2007 (2015).

109. Sezonov, G., Joseleau-Petit, D. & D'Ari, R. Escherichia coli Physiology in Luria-Bertani Broth. *J. Bacteriol.* **189**, 8746–8749 (2007).

110. Gutierrez, A. *et al.* Understanding and Sensitizing Density-Dependent Persistence to Quinolone Antibiotics. *Mol. Cell* **68**, 1147–1154.e3 (2017).

111. Prax, M., Mechler, L., Weidenmaier, C. & Bertram, R. Glucose Augments Killing Efficiency of Daptomycin Challenged Staphylococcus aureus Persisters. *PLOS ONE* **11**, e0150907 (2016).

112. Allison, K. R., Brynildsen, M. P. & Collins, J. J. Metabolite-enabled eradication of bacterial persisters by aminoglycosides. *Nature* **473**, 216–220 (2011).

113. Meylan, S. *et al.* Carbon Sources Tune Antibiotic Susceptibility in Pseudomonas aeruginosa via Tricarboxylic Acid Cycle Control. *Cell Chem. Biol.* **24**, 195–206 (2017).

114. Ye, J. *et al.* Identification and efficacy of glycine, serine and threonine metabolism in potentiating kanamycin-mediated killing of Edwardsiella piscicida. *J. Proteomics* **183**, 34–44 (2018).

115. Fridman, O., Goldberg, A., Ronin, I., Shoresh, N. & Balaban, N. Q. Optimization of lag time underlies antibiotic tolerance in evolved bacterial populations. *Nature* **513**, 418–421 (2014).

116. Amato, S. M. & Brynildsen, M. P. Nutrient Transitions Are a Source of Persisters in Escherichia coli Biofilms. *PLOS ONE* **9**, e93110 (2014).

117. Su, Y. *et al.* Pyruvate cycle increases aminoglycoside efficacy and provides respiratory energy in bacteria. *Proc. Natl. Acad. Sci.* **115**, E1578–E1587 (2018).

118. Oh, Y. T. *et al.* Selective and Efficient Elimination of Vibrio cholerae with a Chemical Modulator that Targets Glucose Metabolism. *Front. Cell. Infect. Microbiol.* **6**, (2016).

119. Flärdh, K. & Kjelleberg, S. Glucose upshift of carbon-starved marine Vibrio sp. strain S14 causes amino acid starvation and induction of the stringent response. *J. Bacteriol.* **176**, 5897–5903 (1994).

Annexure

Media Constituents

Composition of Artificial Sea Water (ASW)

Constituents Per 1 litre (1 X)	Weight in grams
NaCl	24.7
KCl	0.67
$CaCl_2$. H_20	1.36
$MgCl_2$. $6H_20$	4.66
$MgSO_4.H_20$	6.29
$NaHCO_3$	0.18
Deionized H_2O	to 1 litre

[a] Filter-sterilize and store at 4°C.

Composition of M9 salts

Constituents per 1 Litre (1 X)	Weight in grams
$Na_2HPO_4•7H_2O$	64
KH_2PO_4	15
NaCl	2.5
NH_4Cl	5.0
Deionized H_2O	to 1 litre

Divide the salt solution into 200-ml aliquots and sterilize by autoclaving for 15 minutes at 15 psi (1.05 kg/cm 2) on liquid cycle.

Composition of M9 minimal media

Reagent	Amount to add (per 100 mL)
M9 salts (5X)	20 mL
Glucose (20%; Sigma-Aldrich)[a]	2 mL
$MgSO_4$ (1 M; Sigma-Aldrich)[b]	200 µL
$CaCl_2$ (1 M; Sigma-Aldrich)[b]	10 µL
Deionized H_2O	78 mL

[a] Filter-sterilize and store at 4°C.

Summary and Future perspectives

In the present work, an attempt has been made to understand starvation biology from physiological perspectives. The aim of the current work was to understand physiological changes associated with long term starvation survival and antibiotic tolerance.

In **chapter 3.1**, two types of persister cells were isolated and characterized. One of the sub-populations was triggered by nutrient limitation (E-cells) and another by exposure to a lethal concentration of antibiotics (P-cells). Both the sub-populations differ concerning morphology, temperature tolerance, oxidative stress tolerance, and recovery pattern. Several genes of P-cells were up-regulated, including the genes of antioxidant enzymes, cholera toxin and toxin: antitoxin protein *hipA*. The two subpopulations were found to be fundamentally distinct, and each subpopulation might offer survival advantages during antibiotic exposure or nutrient limitation.

Chapter **3.2** describes the starvation-induced persisters in more detail. The present work established that *V. cholerae* was found to be (mero-) oligoploid or polyploid. The ploidy levels per cell were found to be growth-phase regulated. In addition to the growth phase, an external parameter such as nutrient level, influences the ploidy. The activity of the DNase enzyme increased during starvation that decreased the ploidy. The ploidy was restored to the pre-starvation levels with nutrient supplementation.

Additionally, the survival under carbon, nitrogen, and phosphate limitation was also studied. The *V. cholerae* showed acute sensitivity to phosphate limitation. One of the primary causes of cell death was active metabolism and protein synthesis in P-limited cells. Irregular metabolism because of P-limitation can lead to the accumulation of NADH, membrane damage, and cell death. The *V. cholerae* has 100- fold more phosphate stores compared to *E. coli*. The *V.*

cholerae might never have experienced complete phosphate limitation, due to its sizeable intracellular phosphate and the abundance of phosphate in its natural habitat estuarine water.

The **chapter 3.3**, compiles the detailed studies on antibiotic-induced persisters of *V. cholerae*. The frequency of ciprofloxacin-induced persisters of *V. cholerae* would vary with the bacteriological media used for the growth of the cells. Cells, when exposed to protein synthesis inhibitors (kanamycin, chloramphenicol, tetracycline, erythromycin, and gentamicin), did not form persisters. Persister recovery assay, LIVE/DEAD analysis, and Quinolone Resistance Determining Region (QRDR) sequence analysis showed that the persister population neither included resistant mutants nor Viable But Non-Culturable (VBNC) population. Starvation, anaerobic conditions, and inhibition of ATP synthesis also induced persisters, but not when protein synthesis is inhibited.

Further, the effect of glucose on antibiotic susceptibility of *V. cholerae* was also studied. Glucose could change antibiotic sensitivity in a growth phase-dependent manner. The stationary phase cells, which show higher antibiotic tolerance, could be sensitized to ciprofloxacin and ampicillin by glucose supplementation. The glucose increases the respiration, which in turn increases the metabolism and cell division rate. In general, bacterial susceptibility can be increased by combining antibiotics with glucose. The chapter concludes that persistence depends on the media components and growth phase. Furthermore, the chapter emphasizes the role of protein synthesis for persister formation, persister maintenance, and dormancy maintenance in the *V. cholerae*.

Future perspectives

The results presented in the thesis extend the possibility of the presence of physiologically distinct sub-populations in any given microbial culture. This can inspire more work towards isolating such sub-populations in pure form. The finding that *V. cholerae* is sensitive for

protein synthesis inhibitors invites more research to design effective drugs to treat *Vibrio* infections. Changes in ploidy levels also raise questions about the role of ploidy during starvation survival. It would be fascinating to know if a cell can use self-genetic material as a source of nutrients in crisis. One interesting observation of the study was the acute sensitivity of the *V. cholerae* towards phosphate limitation. The results, as reported here, show a possible link between failed stringency activation and continued metabolism as a cause of cell death. However, more research is needed to establish a clear connection between the rate of metabolism in phosphate starved cells and stringent responses. More research should explore direct pieces of evidence for the futile cycling of nutrients and the effect of cell physiology.

Findings presented in the thesis are not a means to an end; instead, are windows of opportunity and fascinating research in areas of antibiotic tolerance associated with starvation, starvation recovery, pathogen survival in the famine conditions, and some fundamental metabolic pathways involved in central metabolism.

FEMS Microbiology Letters, 364, 2017, fnx190

doi: 10.1093/femsle/fnx190
Advance Access Publication Date: 7 September 2017
Research Letter

RESEARCH LETTER – Physiology & Biochemistry

The ploidy of *Vibrio cholerae* is variable and is influenced by growth phase and nutrient levels

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One sentence summary: Reduction of ploidy in *Vibrio cholera* due to starvation.
Editor: Hermann Heipieper

ABSTRACT

The ploidy of *Vibrio cholerae* was quantified under different growth conditions. The *V. cholerae* was found to be (mero-) oligoploid or polyploid. The ploidy levels per cell were found to be growth phase regulated. The ploidy is highest during the early stationary phase (56–72 per cell) and lowest in the long-term starved state. In addition to growth phase, an external parameter such as nutrient level influences the ploidy, i.e. ploidy reduces rapidly at the onset of the starvation. The reduction is significant with P-value < 0.05 within 2 h of starvation. Even after prolonged starvation of 10 days, the ploidy number remained above 2 per cell. Failure to obtain a monoploid *V. cholerae* indicates that during starvation the genome is not distributed equally to daughter cells. The activity of DNase enzyme increased during starvation that decreased the ploidy. The ploidy was restored to the pre-starvation levels with nutrient supplementation.

Keywords: *Vibrio cholerae*; starvation; ploidy; DNase; endonuclease; ASW

INTRODUCTION

The non-dividing prokaryotes are considered to be monoploid with a single copy of their chromosome. However, some species are known to be polyploid, e.g. *Deinococcus radiodurans*, *Azotobacter vinellandii* and the cyanobacterium *Anabaena cylindrica*. In recent years, it has been shown that the majority of species of prokaryotes are oligoploid or polyploid (Pecoraro *et al*. 2011). The polyploidy offers many advantages to the bacteria including low mutation rate, resistance against double-strand breaks, gene redundancy, global regulation of gene dosage, survival over geological times, large cell size and storage of phosphate (Zerulla *et al*. 2014; Zerulla, Ludt and Soppa 2016). Recently, many species of proteobacteria except *Neisseria lactamica* and *Escherichia coli* are found to be polyploid. Polyploidy appears to be a common condition with monoploid being an exception.

Vibrio cholerae is a gram-negative, motile, autochthonous organism and a human pathogen causing cholera (Colwell and Spira 1992; Ali *et al*. 2015). It survives in sea water for prolonged period wherein it experiences starvation stress. During starvation, the reduction in the macromolecular content of the cell such as carbohydrates, β-hydroxybutarate, proteins and several fatty acids is documented (Morita and Novitsky 1977). The reduction in the DNA content of the starved cell has also been reported in several studies (Hood *et al*. 1986; Morita and Novitsky 1977). The DNA per cell reduced up to 90% of the original amount. DNA reduction during starvation could be due to the reduction of additional DNA copies or loss of DNA due to cell envelope stress, or changes in the configuration of nucleotide or some unknown mechanism (Morita and Novitsky 1977; Moyer and Morita 1989; Wai, Mizunoe and Yoshida 1999; Baumler *et al*. 2008).

Received: 15 June 2017; **Accepted:** 6 September 2017

The *V. cholerae* is a member of the phylum Proteobacteria, and many members of this phylum are polyploids. Therefore, there is a possibility that *V. cholerae* could also be a polyploid organism. The *V. cholerae* is known to survive prolonged starvation conditions. Hence, it could accumulate higher ploidy to survive the long-term starvation. The reduction in ploidy during starvation can explain the accompanying loss of DNA per cell. This study was carried out to understand the ploidy number variation in *V. cholerae*. A rapid and refined technique of ploidy determination using qPCR was carried out. The changes in ploidy during growth condition were measured. The ploidy number was also determined under reduced nutrient condition. The results of this study would help in understanding the starvation biology of *V. cholerae*.

MATERIALS AND METHODS

Starvation inoculum

The *Vibrio cholerae* O1 El Tor lab isolate was used in the study. The cells were cultured regularly in Luria-Bertani broth (HiMedia, Mumbai, India) supplemented with 1% NaCl at 37°C (Colwell 1996). To induce nutrient-limited conditions, the cells were inoculated in artificial sea water (ASW) and allowed to incubate at 28°C (Colwell 1996). At regular intervals, the starved cells were harvested, cell number was enumerated and ploidy was determined. The cell number was determined by plating appropriate dilutions at particular intervals. The cell number was also determined by Neubauer cell counter. For phosphate supplement studies, the cells were cultured in ASW supplemented with 10mM K_2HPO_4.

Quantification of DNA

The DNA was isolated from cultured cells using Bacteremia DNA Isolation kit, Biostic (MoBio Laboratories, Carlsbad, CA, USA) at different time intervals. The quality and integrity of the eluted DNA were verified on 1% agarose gel electrophoresis. Spectra readings of the isolated DNA were recorded using a Nanodrop photometer, and DNA per cell was calculated.

Cell disruption for ploidy analysis

The cells from appropriate growth condition were harvested by centrifugation. The cell number was determined using Neubauer cell counter. The cells were lysed using 4mg mL^{-1} lysozyme in Tris buffer (pH 7.2). The lysate was plated to measure the lysis efficiency. After the lysis, the cell debris was removed by centrifugation (8000 × *g*, 5 min). 0.1 mL of the supernatant was used as the cytoplasmic extract for further analysis. The integrity of genomic DNA was checked using agarose electrophoresis.

Ploidy determination using qPCR method

The ploidy was determined by real-time quantitative PCR (RT-qPCR) method (Pecoraro *et al*. 2011) with following modifications. Fragment of 900 bp region of *V. cholerae recA* gene was amplified using standard PCR from genomic DNA of *V. cholerae* (Table S1, Supporting Information). The fragments were purified by preparative agarose gel electrophoresis and the GenElute PCR clean-up kit (Sigma-Aldrich, USA). The PCR amplified DNA mass concentrations were determined photometrically, and the concentrations of DNA molecules were calculated using the molecular weights computed with 'oligocalc' (www.basic.northwestern.edu/biotools). A dilution series was generated for each standard fragment and used for qPCR analysis with the dilution series. The qPCR was carried out by following the MIQE guidelines. The genome copy number per cell was calculated using the cell number present at the time of cell lysis. Three independent biologic replicates were performed for each sample.

Ploidy determination using spectroscopic method

Ploidy was also determined using the spectroscopic method. The DNA was isolated using Bacteremia DNA Isolation kit, Biostic. The isolated DNA was verified by 1% agarose gel electrophoresis. The spectrophotometric readings (260 nm) of the isolated DNA were recorded. The cell number and absorption at 260 nm were used to calculate the ploidy per cell using the following parameters (Zerulla, Ludt and Soppa 2016): an absorption value of 1 at OD_{600} equals a DNA concentration of 50 μg mL^{-1}, the mean molecular mass of 1 bp is 660 g mol^{-1}. The genome size of *V. cholerae* is 4.34 Mbp (Heidelberg *et al*. 2000).

DNase activity measurement

The activity of DNase was monitored using agarose gel method (Gerceker *et al*. 2009). Different aliquots of whole cells were harvested from different growth conditions. 500 μg mL^{-1} calf thymus DNA was added to the cell aliquots, and the cells were incubated at 37°C for various time intervals. The degradation of DNA was checked using 0.9% agarose gel electrophoresis.

RNA isolation and cDNA preparation

Total RNA was extracted from cultures using the RNeasy mini kit (Qiagen, Valencia, CA, USA) as per manufacturer's protocol. Samples were stored at −80°C until used. RNA quantity and quality was measured using a photometer (Eppendorf, Hamburg, Germany). About 1 μg of the RNA was reverse transcribed using a DyNAmocDNA synthesis kit (Finnzymes, Espoo, Finland). The expression profile of the cDNA products was determined using the RT-qPCR and analyzed using the Rotor-Gene Q Series Software 2.1.0 (Build 9) analysis template. The gene-specific primers for DNase enzymes (Table S2, Supporting Information) were designed using integrated DNA technologies Primer Quest software (www.idtdna.com/site).

Statistical analysis

All the tests were carried out in triplicates with appropriate biological replicates. The mean and standard deviation of the replicates are provided wherever required. The ploidy number as obtained from qPCR and spectroscopic method were analysed using Bland–Altman plot for the test of agreement (Altman and Bland 1986). The correlation between reduction in DNA and ploidy during starvation was also determined using regression—correlation analysis. The Student's t-test was carried out to determine the significant results at $P < 0.05$.

RESULTS

Vibrio cholerae is a polyploid with genome copy number reaching 71 ± 5.62 per cell during exponential phase (Fig. 1). The ploidy in the overnight grown culture was found to be 62 ± 3.53 per cell. The cell maintained its polyploidy condition even after 48h of

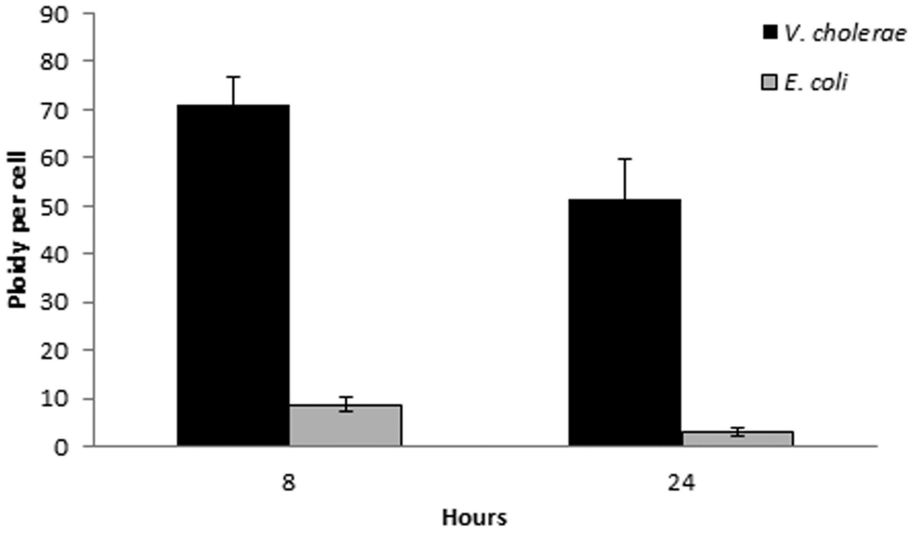

Figure 1. Determination of the ploidy of *V. cholerae* during the laboratory growth condition. The ploidy of *V. cholerae* was determined during the exponential phase and also in the stationary phase using qPCR based method. The ploidy of *E. coli* was also determined as a positive control. All the experiments were carried out in triplicates.

incubation in LB. The ploidy of *E. coli* was determined as a positive control. During the exponential phase, the *E. coli* ploidy number was 8, and is in agreement with the earlier reports (Pecoraro *et al.* 2011). The ploidy calculated from the spectrophotometric method and the qPCR method was in excellent agreement (Fig. S1) as shown by the Bland–Altman plot (Altman and Bland 1986). The *P*-value of t-test was < 0.05, indicated that there is no significant difference between the ploidy values obtained from the spectrophotometer and the qPCR. All further experiments were carried out using the qPCR method.

The ploidy number depends on growth phase and nutrient levels

The ploidy during different stages of *V. cholerae* growth curve was determined (Fig. 2). The ploidy increased steadily from lag phase to log phase. The highest ploidy (71 ± 2.56) was observed after 8 h of the growth. During the prolonged stationary phase (48 h) ploidy number decreased to 20 ± 1.42 per cell.

The ploidy was also determined under the limited nutrient condition in ASW. The cell number reduction in ASW was negligible during the 10 days of starvation (Fig. S2, Supporting Information). But the ploidy of the starved cells was considerably reduced (Fig. 3). The starved cells reduced their ploidy number to 15.7 ± 4.86 per cell within 2 h of starvation. Further, the reduction in ploidy progressed gradually, and by 24 h, the ploidy number reached 6.46 ± 1.28 per cell. The ploidy was 2.34 ± 1.38 per cell even after 10 days of starvation.

DNA per cell reduces during starvation

The reduction in DNA content per cell was monitored in starvation regime (Fig. S3, Supporting Information). The reduction in DNA content per cell was rapid during the first 8 h of starvation in ASW; however, the cell number reduction was minimal (Fig. S2, Supporting Information). The initial DNA content per cell was 314.76 ± 18.92 fg per cell, which was reduced to $<70 \pm 3.46$ fg per cell within 8 h. After this, a gradual reduction of DNA per cell was observed throughout the starvation phase. On 10th day of starvation, DNA was reduced to 10.89 ± 1.5 fg per cell corresponding to 97% reduction from initial DNA content. The reduction of DNA was biphasic with initial rapid fall in DNA per cell followed by gradual but the continuous decline over the entire period of starvation (Fig. S3, Supporting Information). This pattern of DNA reduction during starvation correlates with that of the ploidy with $r = 0.995$. (Fig. S4, Supporting Information)

Effect of phosphate supplementation on ploidy number

The ASW does not contain any phosphate sources. Hence, phosphate supplementation was postulated to slow down the DNA reduction. However, the ploidy levels did not increase in the presence of the phosphate (Fig. S5, Supporting Information) and also the cells did not show any changes in survivability in the presence or in the absence of the phosphate.

The ploidy recovery during nutrient upshift

The starved cells were tested for their ability to recover the ploidy number, during the nutrient upshift. The starved cell inoculum was supplemented with 1X LB broth. The availability of all essential nutrients prompted the cells to grow exponentially to maximum population density though, with an extended lag period. The cells recovered their ploidy quickly during the nutrient upshift and reached maximum ploidy of 72 per cell during the exponential phase (Fig. S6, Supporting Information).

Increase in DNase activity during starvation

The cells from exponentially growing culture and the cells from 2 h starved culture were compared for their DNase activity. The starved cells showed increased DNA degradation than that of fresh cells (Fig. 4). The DNA degradation increased, with as the starvation duration was increased, indicated by the extended smear formation on the agarose gel. The addition of EDTA

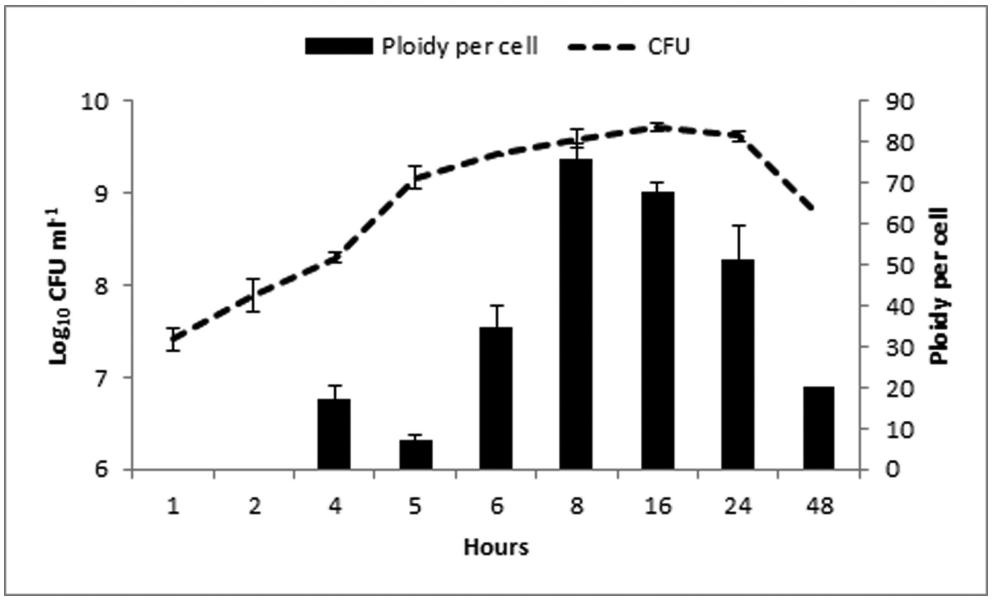

Figure 2. The changes in ploidy as a function of growth curve. The dashed line indicates the growth curve of *V. cholerae* during laboratory growth conditions. The solid bars represent the ploidy per cell. The ploidy per cell was determined using qPCR method and has been validated with biological and technical triplicates.

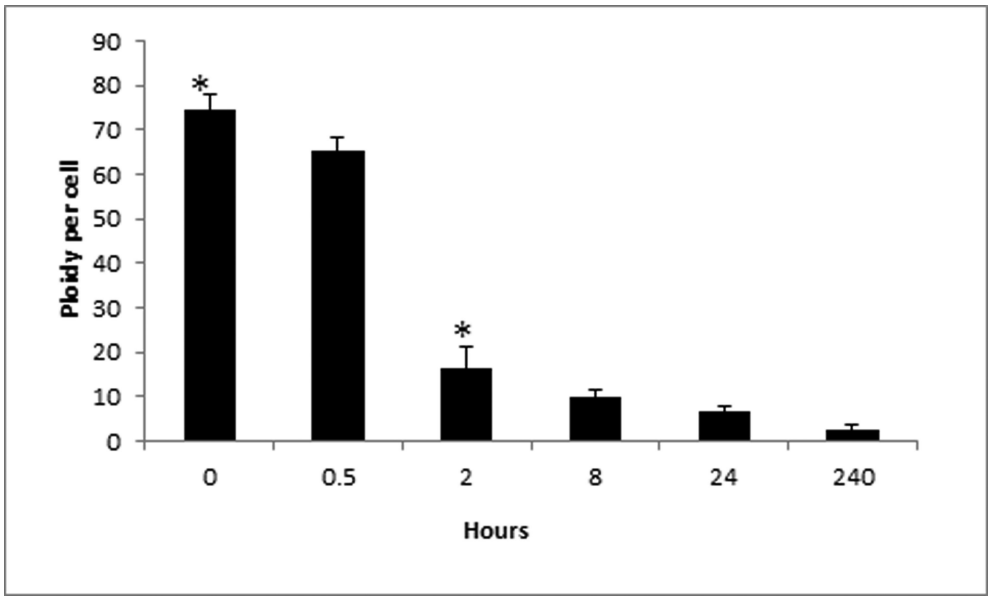

Figure 3. The reduction of ploidy levels during starvation. The ploidy was plotted against the time of incubation in low nutrient media. The ploidy was calculated up to 10 days of starvation. The significant difference in ploidy ($P < 0.05$) is indicated by the *mark. The reading was carried out in triplicate samples.

inhibited the degradation of DNA suggesting the role of DNase activity.

Expression analysis of DNase genes during starvation

The expression profile of DNase genes of 2 h starved cells in ASW was compared with that of actively growing cells (OD_{600} = 0.5; Fig. 5). The DNase enzymes such as extracellular deoxyribonuclease (VC0470), endonuclease III (VC1011) and 5′ deoxyribonucleotidase (VC1978) were upregulated by 1.88-fold, 1.7-fold and 1.95-fold during starved conditions, respectively. The exodeoxyribonuclease V (VC2319) was downregulated by 0.75-fold during starvation. The fold change of 1.5 and above was considered as significant.

DISCUSSION

Several species of the phylum proteobacteria are oligo or polyploid, such as *Azotobacter vinelandii*, three species of *Neisseria*, *Buchnera* species and two species of *Desulfovibrio*. Another member of the proteobacteria, *Neisseria gonorrhoeae* is a diploid with two genomes before and four genomes after replication. This ploidy depends on growth rate and optimal conditions (Zerulla, Ludt and Soppa 2016). Hence, we hypothesized that the reduction in DNA is due to the reduction in extra DNA copies or

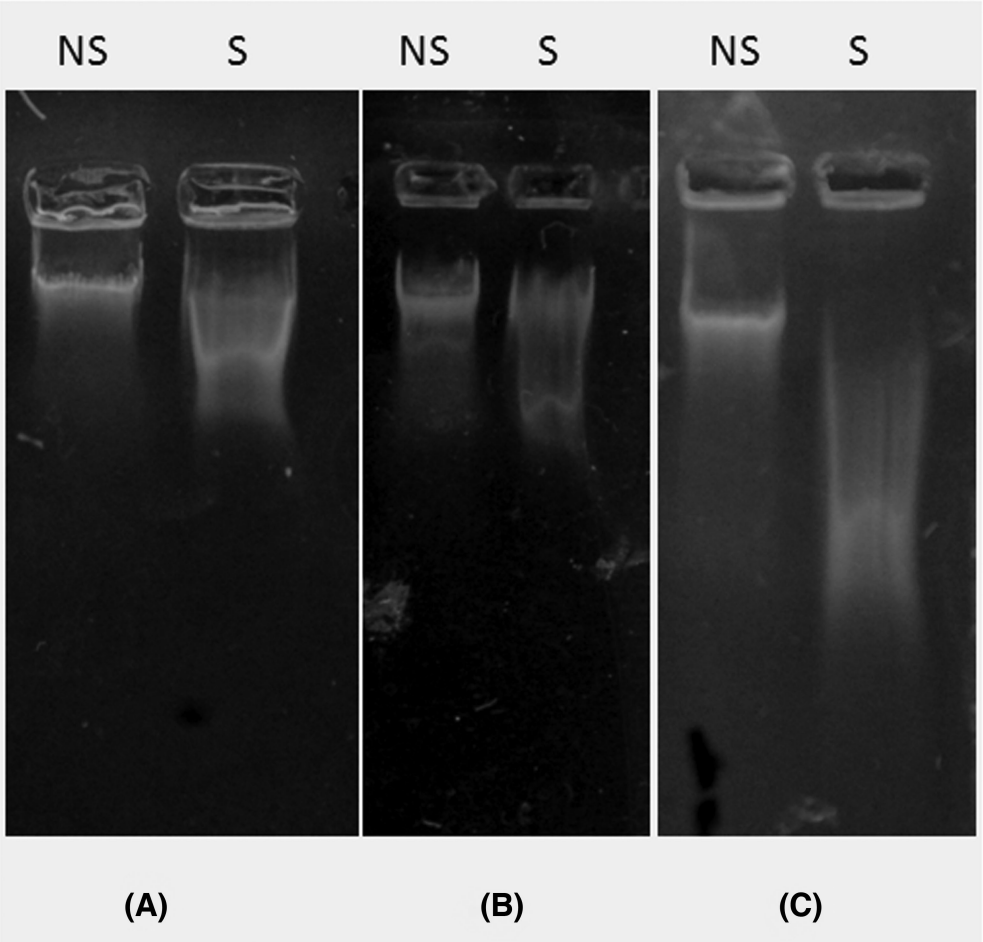

Figure 4. The DNase activity of starved cells. The starved and non-starved cells were tested for their DNase activity. The DNase activity was measured at 30 min (**A**), 60 min (**B**) and at 2 h (**C**) of starvation. The lanes are represented as NS (non-starved) and S (starved). The extent of smear indicated the DNase activity.

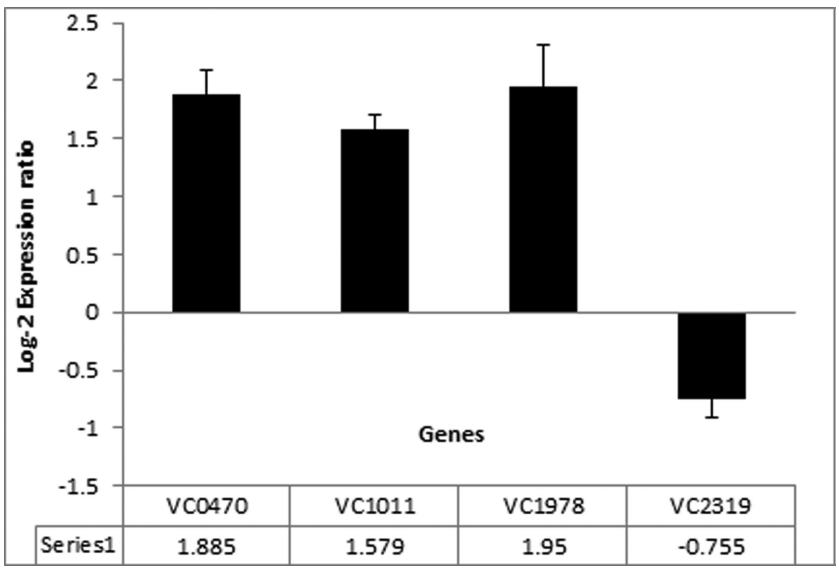

Figure 5. Relative expression ratio plot. The expression of DNase genes was checked during starvation and compared with the levels of gene expression during non-starved phase. The expression profile includes the four important genes of DNase such as the extracellular deoxyribonuclease (VC0470), endonuclease III (VC1011) 5′ deoxyribonucleotidase (VC1978) and the exodeoxyribonuclease V (VC2319). The experiment was carried out using triplicates samples as per MIQE guidelines. The significant change in gene expression is indicated by the *mark.

ploidy. The ploidy of *Vibrio cholerae* has not been reported earlier, and this is the first report on ploidy of *V. cholerae*. The ploidy of *V. cholerae* varied with the growth stages as well as under the nutrient stress. Cells in the lag and the stationary phase divide slowly, in comparison to the log phase. The decrease in cell division rate, in turn, downregulates the DNA replication rate (Griese, Lange and Soppa 2011). Both lag and stationary phase have reduced metabolic rate and consequently reduced DNA replication and cell division. Therefore, in lag- and stationary-phase cells, reduced ploidy number was observed. However, the optimum cell division conditions in log phase lead to high DNA synthesis and cell division. Hence, log phase cells accumulate higher DNA copies and thus become polyploids. These observations draw strength from Zaritsky, Woldringh and Mannik (2016), where the connection between cell volume, cell division and DNA replication has been extensively reviewed.

Among the 11 species of the proteobacteria, only three are truly monoploid (Pecoraro *et al*. 2011). The *Escherichia coli* is a monoploid during prolonged growth but becomes a merooligoploid during fast growth. The ploidy is influenced by growth rates, as seen in *Desulfovibrio*, which has 17 genome copies during rapid growth in batch cultures and 9 genomes per cell in chemostat cultures (Griese, Lange and Soppa 2011). The ploidy is also influenced by media type, for example in *A. vinelandii*, the ploidy at late exponential phase are well above 40 in rich growth medium but not in synthetic medium. In several species, the growth phase also influences the ploidy level (Zerulla, Ludt and Soppa 2016). The ploidy was found to be higher in exponential phase than the stationary phase. Similarly, in our study, the *V. cholerae* showed the difference in ploidy during the growth phase (Fig. 2). When the cells are grown under optimal conditions, the generation time becomes less than the replication/segregation time, leading to the re-initiation of replication before the previous replication round had been terminated (Griese, Lange and Soppa 2011), which leads to increased ploidy in exponential growth phase. When the batch culture reaches stationary phase, the growth rate drops and the cell number becomes stable. However, the ploidy of *V. cholerae* was maintained near 20 even after 48 h. This observation suggests that *V. cholerae* has a mechanism to support high ploidy number.

When the *V. cholerae* was inoculated in ASW, the loss of cell viability is marginal (<10% of initial inoculum). ASW has no carbon, nitrogen and phosphate sources and yet the bacteria survived and maintained the constant cell number. More than 90% of the DNA per cell is lost within 24 h of starvation (Fig. S3, Supporting Information). The reduction in DNA per cell was higher during initial hours of starvation. After that, the gradual reduction in DNA per cell continued over the entire course of starvation. The fall in DNA is biphasic and significant (Fig. S3, Supporting Information). Hood *et al*. (1986) had observed the rise in cell number during initial hours of starvation, and they had concluded that the DNA per cell reduction is due to this increase in cell number. However, in this study, we did not observe any increase in cell number during starvation (Fig. S2, Supporting Information). The cell number declined by 10% of the initial population soon after induction of starvation, after that the cell number remained constant through the 10 days of starvation. The observed rise in cell number by earlier authors (Hood *et al*. 1986) may be due to carryover effect of nutrients from culture inoculum or stored polyphosphates or PHB within the cell that might have provided with essential nutrients for survival under starvation. Morita and Novitsky (1977) have suggested several possibilities for the reduction in DNA such as loss of DNA due to the leaky cell membrane or reduction in extra DNA copies or degradation of DNA due to stress or changes in the configuration of nucleotide, or some unknown mechanism.

The DNase enzymes are responsible for the degradation of DNA. Hence, the DNase activity of the *V. cholerae* was tested before and after the starvation. The DNase activity increased with the induction of starvation (Fig. 4). The starved cells showed higher DNA degradation than the cells of exponential phase. The increase in gene expression of DNAse enzymes was evident in the qPCR data (Fig. 5). The starved cell extracellular deoxyribonuclease, the endonuclease III and the 5′ deoxyribonucleotidase had more than 1.5-fold change increased expression compared to the non-starved cells. The enzymes extracellular deoxyribonuclease is actively produced, and it is found in the exterior cell wall of the cell, whereas the 5′ deoxyribonucleotidase is located in the periplasmic space. Both these enzymes might be involved in degradation of external DNA. More study is needed to understand the activity of DNase enzyme and the particular role of each enzyme in reducing the ploidy.

The DNA is the only macromolecule that has large deposits of C, N and P aggregates. DNA can be a good source of macronutrients. The studies by Zerulla *et al*. (2014), Zerulla, Ludt and Soppa (2016) have concluded that in *Haloferax volcanii* and *Synechocystis* sp. PCC 6803 nutrient availability determines ploidy level, and that genomic DNA is used as a phosphate storage polymer. Recent studies have raised the possibility of evolution of DNA as storage molecule, because of its rich phosphate content (Zerulla *et al*. 2014). We speculated that the DNA would serve as phosphate store in *V. cholerae*. However, we did not observe any difference in ploidy in the presence or the absence of phosphate in the media (Fig. S4, Supporting Information). The availability of phosphate in media did not affect the ploidy during growth and starvation. Evolution might not have preferred the DNA as phosphate storage molecule in *V. cholerae* as it thrives in mineral rich estuarine waters (Colwell and Spira 1992; Ali *et al*. 2015).

The higher ploidy may offer an advantage of being able to support more cell division process. Under adverse conditions, the increase in DNase activity may contribute to the recycling of the macromolecules. Further work is needed to explore the fate of the degraded DNA and its associated pathways. The results presented here show that the ploidy in *V. cholerae* varies with its metabolic/nutrient state.

SUPPLEMENTARY DATA

Supplementary data are available at *FEMSLE* online.

Conflicts of interest. None declared.

REFERENCES

Ali M, Nelson AR, Lopez AL *et al*. Updated global burden of cholera in endemic countries. *PLoS Negl Trop Dis* 2015;**9**:e0003832. DOI: 10.1371/journal.pntd.0003832.

Altman DG, Bland JM. Statistical methods for assessing agreement between two methods of clinical measurement. *Lancet* 1986;**1**:307–10.

Baumler DJ, Hung KF, Jeong KC *et al*. Molybdate treatment and sulfate starvation decrease ATP and DNA levels in *Ferroplasma acidarmanus*. *Archaea* 2008;**2**:205–9.

Colwell RR, Spira WM. The ecology of *Vibrio cholerae*. In: Barua D, Greenough WB (eds). *Cholera*. III edn. New York: Plenum Medical Book Co., 1992, 107–27.

Colwell RR. Global climate and infectious disease: the cholera paradigm. *Science* 1996;**274**:2025–31.

Gerceker D, Karasartova D, Elyürek E *et al*. A new, simple, rapid test for detection of DNase activity of microorganisms: DNase tube test. *J Gen Appl Microbiol* 2009;**55**:291–4.

Griese M, Lange C, Soppa J. Ploidy in cyanobacteria. *FEMS Microbiol Lett* 2011;**323**:124–31. DOI: 10.1111/j.1574-6968.2011.02368.x.

Heidelberg JF, Eisen JA, Nelson WC *et al*. DNA sequence of both chromosomes of the cholera pathogen *Vibrio cholerae*. *Nature* 2000;**406**:477–83. DOI: 10.1038/35020000.

Hood MA, Guckert JB, White DC *et al*. Effect of nutrient deprivation on lipid, carbohydrate, DNA, RNA, and protein levels in *Vibrio cholerae*. *Appl Environ Microb* 1986;**52**:788–93.

Morita RY, Novitsky JA. Survival of a psychrophilic marine *Vibrio* under long-term nutrient starvation. *Appl Environ Microb* 1977;**33**:635–41.

Moyer CI, Morita RY. Effect of Growth rate and starvation-survival on cellular DNA, RNA, and protein of a psychrophilic marine bacterium. *Appl. Environ. Microb* 1989;**55**:2710–6.

Pecoraro V, Zerulla K, Lange C *et al*. Quantification of ploidy in Proteobacteria revealed the existence of monoploid, (mero-)oligoploid and polyploid species. *PLoS ONE* 2011;**6**:e16392. DOI: 10.1371/journal.pone.0016392.

Wai SN, Mizunoe Y, Yoshida S. How *Vibrio cholerae* survive during starvation. *FEMS Microbiol Lett* 1999;**180**:123–31.

Zaritsky A, Woldringh CL, Mannik J. The bacterial cell: coupling between growth, nucleoid replication, cell division and shape. *Front Microbiol* 2016;**7**:116

Zerulla K, Chimileski S, Nather D *et al*. DNA as a phosphate storage polymer and the alternative advantages of polyploidy for growth or survival. *PLoSONE* 2014;**9**:e94819. DOI: 10.1371/journal.pone.0094819.

Zerulla K, Ludt K, Soppa J. The ploidy level of *Synechocystis* sp. PCC 6803 is highly variable and is influenced by growth phase and by chemical and physical external parameters. *Microbiology* 2016;**162**:730–9. DOI: 10.1099/mic.0.000264.

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https://doi.org/10.1007/s13205-019-1916-0

SHORT REPORTS

Inhibition of protein synthesis eradicates persister cells of *V. cholerae*

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Received: 3 May 2019 / Accepted: 24 September 2019

Abstract

In the present work, we studied the antibiotic-induced persister formation in *Vibrio cholerae*. Persisters vary with the bacterial growth phase with minimum persisters in log phase and maximum in stationary phase. Only 10% of the stationary phase cells of *V. cholerae* were tolerant of ampicillin and ciprofloxacin. In comparison, more than 90% of the stationary phase cells of *E. coli* were tolerant of ampicillin and ciprofloxacin. Frequency of ciprofloxacin-induced persisters of *V. cholerae* would vary with the bacteriological media used for the growth of the cells. In tryptone soy broth (TSB) and in buffered peptone water (BPW), *V. cholerae* could form more than 10% persisters, whereas in Luria–Bertani broth (LB) and alkaline peptone water (APW) persister fraction was less than 1%. When exposed to protein synthesis inhibitors (kanamycin, chloramphenicol, tetracycline, erythromycin and gentamicin), *V. cholerae* did not form persisters. Persister recovery assay, LIVE/DEAD analysis and QRDR sequence analysis showed that persister population neither included resistant mutants nor VBNC population. Starvation, anaerobic conditions and inhibition of ATP synthesis also induced persisters, but not when protein synthesis is inhibited. These observations suggest that the protein synthesis is critical for persister formation, persister maintenance, and also for dormancy maintenance in *V. cholerae*. Contrary to these observations, *E. coli* can form persisters when protein synthesis is inhibited, suggesting fundamental mechanistic differences between the two species.

Keywords *Vibrio cholerae* · Persisters · Ciprofloxacin · Protein synthesis · Starvation · ATP synthesis

Introduction

The persistence is a transient phenotypic variation which imparts significant survival advantage under a lethal dose of antibiotics. Unlike antibiotic resistance, the persistence is not a heritable phenotype (Balaban et al. 2004). Data from several studies suggest that the physiological state of the cell significantly influences the persister formation (Kint et al. 2012; Amato et al. 2014). The physiology of a cell readily responds to fluctuations in the nutrient levels, the presence of competitors or growth inhibitors, changes in temperature and pH of the media (Magdanova and Golyasnaya 2013). Stochastic nature of persister formation shows that persister physiology can be one such physiological state favourable under a lethal dose of antibiotics.

V. cholerae is a food-borne bacterium, that survives in estuarine and brackish water and thrives by forming biofilm on shellfish and zooplanktons (Colwell and Spira 1992). This bacterium infects a wide range of hosts including humans and several marine animals such as fishes, crustaceans and molluscs. The regular use of antibiotics in aquaculture has risked the development of antibiotic resistance among the *Vibrio* species of clinical and economic importance (Tang et al. 2002; Cabello 2006), and besides that, several other pathogens are forming clinically significant multi-drug-tolerant persisters.

Recent study on *V. cholerae* O139 strain showed the difference in antibiotic resistance among planktonic and biofilm cultures (Gupta et al. 2018). The biofilm-associated cultures showed higher resistance to antibiotics and the surviving clones exhibited virulence and increased resistance. However, no much information is available on antibiotic persistence phenomenon in *V. cholerae*. Therefore, in this work, we investigated the ability of *V. cholerae* to

Electronic supplementary material The online version of this article (https://doi.org/10.1007/s13205-019-1916-0) contains supplementary material, which is available to authorized users.

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form persister cells in laboratory growth conditions where the persister fraction was determined in different media and in the presence of inhibitors of respiration. This paper also sheds light on the differences between *V. cholerae* (*Vibrionales*) and *E. coli* (*Enterobacteriales*) concerning persisters forming abilities.

Materials and methods

Strains, media and growth conditions

V. cholerae O1 El Tor N16961 strain was used throughout the experiment. Cation-adjusted Muller Hinton Broth (MHB), Tryptic soy broth (TSB), Luria–Bertani broth (LB), alkaline peptone water (APW) and buffered peptone water (BPW), all procured from Himedia (Mumbai, India) were used to culture the bacteria and study the persisters. The uncoupling agent carbonylcyanide-3-chlorophenylhydrazone (CCCP) and antibiotics (Table. S1) were purchased from Sigma-Aldrich Merck (Germany). Overnight cultures were prepared by streaking the glycerol stocks (− 80 °C) onto agar plates and culturing a single colony in the broth with aeration at 37 °C for 16–20 h. Starvation was induced by culturing cells in artificial sea water (ASW) for 24 h. ATP synthesis was inhibited by exposing the culture to sub-MIC of CCCP and subsequently exposed to antibiotics. To induce anaerobic environment, the culture was incubated in anaerobic chamber.

Minimal inhibitory concentration (MIC) determination

The MIC values of the antibiotics were determined using the conventional micro-dilution method in cation-adjusted Muller Hinton broth and in Luria–Bertani broth. Briefly, the exponential culture was back diluted 1:10 into fresh media. To the first lane in 96-well plate (Corning, US), 10 µl of culture and 1:10 diluted stock concentration of antibiotic were added. The volume of the wells was made up to 200 µl using MHB/LB. To each of the remaining wells, 100 µl of MHB/LB was added. The solution from the first lane was diluted twofold to proceeding lanes. The plates were incubated at 37 °C for 16 h. The lowest concentration of antibiotics that inhibited the visible growth was interpreted as Minimal Inhibitory Concentration (MIC). All the assays were repeated twice in triplicates. The MIC of all the antibiotics is summarized in Table. S1. We did not find significant difference between the MIC values in MHB and LB. Hence, all further studies were carried out in LB.

Antibiotic susceptibility assay

The overnight grown cultures were diluted 1:100 in culture flasks and incubated on a shaker (150 rpm) at 37 °C for 3 h. At this point, the cells were challenged with tenfold MIC of each antibiotic separately and cultured for another 8 h. At regular intervals, small aliquots of the sample were centrifuged. The cell pellet was diluted in LB, and 10 µl was spotted on to TSA plates. The plates were incubated at 37 °C for 16–24 h until colonies appeared. A graph of cell number v/s time was plotted to obtain the kill curve. The formation of biphasic kill curve indicates the formation of persister cells.

Persister recovery assay

The exponential phase cells were exposed to tenfold MIC of ciprofloxacin and allowed to form persisters. After stable persister population was formed, the cells were washed in LB and inoculated in LB media without ciprofloxacin. The culture was incubated at 37 °C on 150 rpm shaker for 24 h. Meanwhile, growth curve was plotted and growth rate and lag time was measured. Persisters were also inoculated in LB with antibiotics and incubated. The MIC of ciprofloxacin was calculated for the regrown population. Next, using tenfold MIC concentration, persisters were allowed to form in the new population and persister frequency was measured. The persister frequency of original population and regrown population was compared. Persister frequency = (no. of persisters/total no. of cells before antibiotic exposure) × 100.

We carried out the resuscitation studies to determine whether the persister population harbour viable but non-culturable (VBNC) subtypes, using protocol described by Mishra et al. (2012). Briefly, the persisters were resuscitated in ASW by releasing the stress (antibiotic) at 25 °C, for 72 h at room temperature. After 72 h of resuscitation, the cells were serially diluted and plated on LB agar plates without antibiotics. Culturability (CFU ml^{-1}) was determined following plating on TSA agar. If VBNC is present among the persisters, the cell count after resuscitation would be more than cells count immediately after antibiotic treatment. Two-tailed unpaired *t* test was carried out to determine the statistical significance of the data (cutoff $p = 0.05$).

Viability assay

The viability of kanamycin-treated cell was determined using LIVE/DEAD Baclight™ viability kit (Molecular probes, Invitrogen, USA), as per manufacturer's protocol. Briefly, the propidium iodide (PI) and Syto9 dyes were mixed in 1:1 ratio and incubated in dark for 15 min. After the incubation, the live/dead cell count was taken using Nikon

Eclipse Ni fluorescent microscope (Nikon Instruments Inc. Japan). A xenon lamp of 100 W was used to deliver light to FITC and TRITC filters. The images were captured individually under each filter and then merged using Nikon NIS elements software. The viability of kanamycin-treated cells was checked at different time points (0, 1, 2 and 4 h after kanamycin exposure) and images were taken. All experiments were carried out in triplicates.

Screening of QRDR mutations

The mutations in quinolone resistance-determining region (QRDR) locus of *gyrA, gyrB* and *parC* genes was screened using protocol as details by Kim et al. (2010). Briefly, the PCR amplification of QRDR region was carried out using primers listed by Kim et al. (2010). The PCR amplicons were purified using ExoProStar S PCR and Sequence Reaction Clean-up Kit (GE Healthcare, US) and sequenced using Sanger-sequencing method. The sequences were compared with the genome sequence of *V. cholerae* O1 stain N16961, available at NCBI Gen Bank.

Statistical analysis

All the tests were conducted with three independent biological replicates and the data are expressed as mean ± SD. The student's *t* test and one-way multiple comparison ANOVA was used to determine the statistical significance with a cut-off value of $p = 0.05$.

Results and discussion

V. cholerae forms drug-tolerant persister cells

The MIC values were calculated in cation-adjusted Muller Hinton broth as per CLSI guidelines. We also calculated the MIC in Luria–Bertani broth and found no significant differences between MIC values of MHB and LB (Table S1).

We exposed the growing culture of *V. cholerae* to tenfold MIC of antibiotics (ciprofloxacin, ampicillin and kanamycin) (Table S1) and observed a rapid decrease in viable cell count during the initial 2–3 h of antibiotic challenge (Fig. 1). There onwards, the rate of cell death decreased and formed a plateau region with stable population size. This biphasic kill curve is the hallmark of persister formation (Keren et al. 2004). Additionally, we performed the recovery studies and found that the survivors were not genetic mutants (Fig. S1). The MIC values and persister frequencies did not change before and after the antibiotic treatments (Table S3). We analysed the quinolone resistance-determining region (QRDR) of ciprofloxacin persister cells and observed no mutations in the QRDR locus of the genes *gyrA* (MN458507), *gyrB* (MN458508) or *parC* (MN458509). The persister cells obtained in the present work were exposed only once to the lethal concentration of ciprofloxacin and no further selection was done on the survivors. Hence, the antibiotic tolerance due to mutation is highly unlikely in persister cell population. However, there is always a possibility that isolation of ciprofloxacin-resistant *V. cholerae* from environment as AQ2 reported by Baranwal et al. (2002) from India.

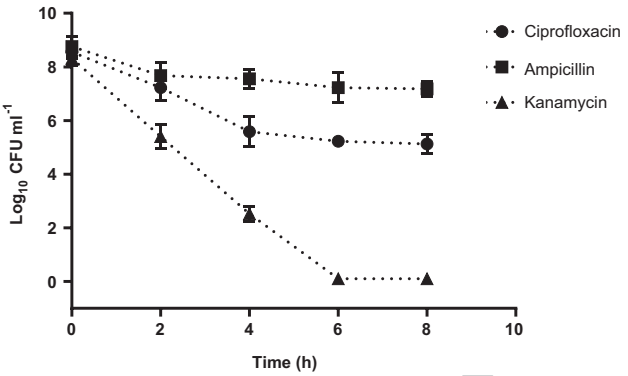

Fig. 1 The effect of different antibiotics on persister frequency of exponential phase cultures. The cells were subjected to lethal dose of antibiotics and cell number was enumerated at regular interval. The graph above shows the biphasic kill curve. The formation of plateau region indicates the persister formation. Results shown are the average of three biological triplicates with standard deviation ($n = 3$)

The resuscitation of cells post-antibiotic treatment did not increase the persister fraction. (Table S4). This showed that the kanamycin-treated cells did not form VBNC sub-population. The LIVE/DEAD analysis revealed that with increase in the kanamycin exposure time, more cells took up PI dye and fluoresce red (Fig. S3). The 0 h and 1 h (Fig. S3 A and B) images have higher proportion of green fluorescent cells. After 4 h of kanamycin treatment (Fig. S3 D), majority of cells fluoresce red colour. The cells which fluoresce red are dead since they take up PI dye and cells that fluoresce green are alive because their membrane are impermeable to PI AQ3 dye. These studies indicated that kanamycin exposure killed the cells rather than inducing VBNC. To determine the persister fraction with respect to growth phase, the cultures in different stages of growth were exposed to antibiotics and persister fraction was measured. After a dip in the lag phase, the persister fraction increased as the cells advanced to log phase, that continued to increase till the culture entered the stationary phase (Fig. S2). The stationary phase cells of *V. cholerae* were sensitive to ampicillin and ciprofloxacin and could form only 10% of persisters (Fig. S2).

The persister formation is a physiological phenomenon which depends on the metabolic state of the cell (Van den Bergh et al. 2017). In contrast to our observation, the stationary cells of *E. coli* were more tolerant to both ciprofloxacin and ampicillin (Keren et al. 2004). Similar to our results, the

stationary cells of *B. burgdorferi* were sensitive to antibiotics (Sharma et al. 2015). Stationary phase cells are generally more tolerant to ampicillin, since only dividing cells are sensitive to the antibiotic (Thorsing et al. 2015). One of the possibilities is that the *V. cholerae* stationary cells continue to grow and die in a steady state. Another possibility is that the cell wall of *V. cholerae* continues to remodel in stationary cells which might make the cells sensitive to β-lactam (Sharma et al. 2015).

Various growth media used in general microbiology laboratory have diverse effects on bacterial growth kinetics and survival. We hypothesized that since persisters depend on the growth stage of the cell, the media compositions may have considerable influence on the persister formation. *V. cholerae* was cultured to exponential phase in different growth media and the persisters were enumerated (Table. S5). The cells cultured in BPW formed highest persister fraction (15 ± 2.6) and the lowest was in APW with 0.001% persisters. We could isolate 0.1% and 10% persisters in regular culture media used in the LB and TSB, respectively. The differences in persister frequency were significant at $p = 0.05$. BPW is the most common media used during the isolation and maintenance of *V. cholerae* from clinical and environmental samples. Antibiotic persistence often precedes the rise of genetic resistance to antibiotics (Levin-Reisman et al. 2017). Future studies on antibiotic persistence and resistance may also consider the influence of media and its compositions on cellular physiology.

Protein synthesis is essential for persister formation

In the above results, we found that *V. cholerae* fails to form persisters when exposed to tenfold MIC of kanamycin (Fig. 1). To validate the finding, we exposed the growing culture of *V. cholerae* to four more antibiotics that inhibit the protein synthesis (chloramphenicol, erythromycin, tetracycline, and gentamicin) (Table 1). Both the exponential and stationary cultures of *V. cholerae* were sensitive to inhibition of protein synthesis and could not form persisters. It appears that the formation of persisters in *V. cholerae*, depends on the active protein synthesis. Furthermore, we exposed the exponential and stationary phase cells of *E. coli* to tenfold MIC of all the antibiotics used in the study. Persister population was observed after exposure to all protein synthesis inhibiting antibiotics. The percent persister fraction ranged from 0.1 to 1 for exponential cells and from 1 to 10 for stationary cells (Table. S6).

Protein synthesis is essential for persister maintenance

As we continued the study, we questioned whether the protein synthesis is essential for maintaining the persistence physiology in *V cholerae*. We challenged the growing culture with tenfold MIC of ciprofloxacin and isolated the persister population. These pre-formed persisters were then challenged with tenfold MIC of kanamycin and at viability, was monitored with time. (Table 1). The pre-formed persisters were killed by kanamycin and after 6 h of antibiotic challenge, no viable cells were recovered. Active protein synthesis appears to be involved in the persister maintenance in *V. cholerae*. To find out how the pre-formed persisters of *E. coli* behave, we challenged them with tenfold MIC of proteins synthesis inhibitors. The pre-formed persisters of *E. coli* survived the antibiotics with no loss of viability (Table. S6). The one-way multiple comparison ANOVA revealed that the difference in survival of pre-formed persisters and exponential cells was not significant at $p < 0.05$. The mechanism of persister formation and maintenance is different in *V. cholerae* and *E. coli*.

Protein synthesis inhibition eradicates starved "persister cells"

Nutrient limitation in *V. cholerae* imparts a characteristic "persister" cell formation (Jubair et al. 2012). As already mentioned, cells under acute starvation attain a dormant state where metabolism is inhibited (Kjelleberg 1993) and for this purpose, we obtained the starved cells of *V. cholerae* by culturing the cells in Artificial Sea Water (ASW) for 24 h. The starved cells were, subsequently challenged with tenfold MIC of antibiotics to determine the persister fraction.

Table 1 The percent persister fraction (%) when challenged with protein synthesis inhibitors

Antibiotics	Exponential cells	Stationary cells	Pre-formed persister cells to ciprofloxacin	Pre-formed persister cells to ampicillin
Kanamycin	0	0	0	0
Chloramphenicol	0	0	0	0
Erythromycin	0	0	0	0
Gentamycin	0	0	0	0
Tetracycline	0	0	0	0

Percent persister frequency was calculated after 6 h of antibiotic addition. The average of three biological replicates is tabulated

Nutrient limited cultures survived ciprofloxacin and ampicillin exposure with no significant loss of viability (Table. S7). However, we could not isolate any persisters after 6 h of exposure to kanamycin. The inhibition of protein synthesis may be critical for starvation survival or continuation of dormancy in *V. cholerae*.

Protein synthesis inhibition eradicates the metabolically dormant cells

Here, we studied the effect of protein synthesis inhibition in dormant cells. The respiration was inhibited by culturing the cells in an anaerobic condition or by pre-treatment with sub-MIC level of CCCP to inhibit the ATP synthesis and subsequently exposed to antibiotics. Ampicillin treatment resulted in 10% of persisters under reduced respiration (anaerobic and CCCP). The exposure to ciprofloxacin also resulted in 10% persisters in both anaerobic and CCCP treatment. However, under reduced respiration, the cells were susceptible to kanamycin (Table. S8).

It appears that if protein synthesis is inhibited, reduced respiration cannot rescue *V. cholerae*. During long-term stress tolerance, the cells lower the metabolic rate and enter a dormant state (Kjelleberg 1993). The characteristic of dormancy includes reduction in ATP levels, a low rate of cell division, and changes in cell morphology (Wai, Mizunoe, & Yoshida, 1999). Those cells are tolerant to a variety of stresses such as temperature, oxidative stress and antibiotics (Kaprelyants et al. 1993). The inhibition of respiration and metabolism could increase the persister formation in *E. coli* and *S. aureus* (Conlon et al. 2016; Shan et al. 2017). Between the cholera epidemics, *V. cholerae* stays in a low nutrient condition of estuarine water for several months. The low energy state could result in the formation of dormant cells which may show high drug tolerance. However, the metabolically inert cells of *V. cholerae* show sensitivity to inhibitors of protein synthesis. The inhibition of ATP synthesis in *E. coli* and in *S. aureus* can turn the entire population into persisters (Conlon et al. 2016; Shan et al. 2017). Majority of the antibiotic targets require ATP to function, and a reduction in ATP will lead to decreased activity of antibiotic targets, resulting in increased drug tolerance (Shan et al. 2017). But it cannot explain why the reduced metabolism could not save *V. cholerae* from inhibitors of protein synthesis. Notably, the persister levels are the same in the presence or in the absence of protein synthesis inhibitors in *E. coli* strain with deletions of 10 toxin:antitoxin (TA) modules (Shan et al. 2017). *V. cholerae* has several putative TA modules, but how these impact the persister formation is unknown.

In the present study, we observed contrasting differences in persister formation in *E. coli* and *V. cholerae* when exposed to protein synthesis inhibitors. When exposed to kanamycin, *E. coli* can form persister cells in both exponential and stationary phase. However, *V. cholerae* was eliminated in both the growth phases. The mechanism of persister formation in *E. coli* has been investigated by several studies (Balaban et al. 2004; Allison et al. 2011; Shan et al. 2017). Main contributors to persister formation in *E. coli* is toxin:antitoxin modules, stringency, ppGpp, RelA, Spot etc., (Kint et al. 2012; Van den Bergh et al. 2017). In *E. coli* during dormancy, the ribosome content decreases, thus reducing the targets for protein synthesis inhibitors (Allison et al. 2011). But in *V. cholerae*, during dormancy, the ribosome machinery is actively maintained (Flärdh et al. 1994), and, therefore, cells show higher sensitivity to inhibitors of protein synthesis. The persister mechanisms in *V. cholerae* is not well characterized and not much of information is available about the role of stringent responses in persister formation in *V. cholerae*. Further studies on TA modules and stringent response can throw more light on the persister mechanism in *V. cholerae*.

Conclusion

To conclude, *V. cholerae,* estuarine bacteria of human significance can form drug-tolerant persister cells. The persister fraction increases as the cells divide and reach the stationary phase and this fraction varies with the antibiotics and media type. However, *V. cholerae* fails to form persister cells upon exposure to antibiotics that inhibit the protein synthesis. The active protein synthesis appears essential for both persister formation and for persister maintenance in *V. cholerae*. Additionally, the nutrient limited cells and the cells with reduced metabolism were also sensitive to inhibitors of protein synthesis. Finally, these findings highlight the fundamental differences between the persister formation in *V. cholerae* and in *E. coli* and critical importance of protein synthesis in persisters of *V. cholerae*.

Acknowledgements We would like to acknowledge the Department of Atomic Energy, India for the financial support to carry out this research.

Funding The research work was supported by the Department of Atomic Energy, India.

Compliance with ethical standards

Conflict of interest The authors declare no conflict of interest.

Ethical approval No animal or human subjects were used in the experiment.

References

Allison KR, Brynildsen MP, Collins JJ (2011) Metabolite-enabled eradication of bacterial persisters by aminoglycosides. Nature 473(7346):216–220. https://doi.org/10.1038/nature10069

Amato SM, Fazen CH, Henry TC, Mok WWK, Orman MA, Sandvik EL, Brynildsen MP (2014) The role of metabolism in bacterial persistence. Front Microbiol. https://doi.org/10.3389/fmicb.2014.00070

Balaban NQ, Merrin J, Chait R, Kowalik L, Leibler S (2004) Bacterial persistence as a phenotypic switch. Science (New York, NY) 305(5690):1622–1625. https://doi.org/10.1126/science.1099390

Baranwal S, Dey K, Ramamurthy T, Nair GB, Kundu M (2002) Role of active efflux in association with target gene mutations in fluoroquinolone resistance in clinical isolates of *Vibrio cholerae*. Antimicrob Agents Chemother 46:2676–2678. https://doi.org/10.1128/AAC.46.8.2676-2678.2002

Cabello FC (2006) Heavy use of prophylactic antibiotics in aquaculture: a growing problem for human and animal health and for the environment. Environ Microbiol 8(7):1137–1144. https://doi.org/10.1111/j.1462-2920.2006.01054.x

Colwell RR, Spira WM (1992) The ecology of *Vibrio cholerae*. In: Barua D, Greenough WB (eds) Cholera. Springer, Boston, pp 107–127. https://doi.org/10.1007/978-1-4757-9688-9_6

Conlon BP, Rowe SE, Gandt AB, Nuxoll AS, Donegan NP, Zalis EA, Lewis K (2016) Persister formation in *Staphylococcus* aureus is associated with ATP depletion. Nat Microbiol 1(5):16051. https://doi.org/10.1038/nmicrobiol.2016.51

Flärdh K, Axberg T, Albertson NH, Kjelleberg S (1994) Stringent control during carbon starvation of marine *Vibrio* sp. strain S14: molecular cloning, nucleotide sequence, and deletion of the relA gene. J Bacteriol 176(19):5949–5957. https://doi.org/10.1128/jb.176.19.5949-5957.1994

Gupta P, Mankere B, Chekkoora Keloth S, Tuteja U, Pandey P, Chelvam KT (2018) Increased antibiotic resistance exhibited by the biofilm of *Vibrio cholerae* O139. J Antimicrob Chemother 73(7):1841–1847. https://doi.org/10.1093/jac/dky127

Jubair M Jr, Morris JG, Ali A (2012) Survival of *Vibrio cholerae* in nutrient-poor environments is associated with a novel "Persister" phenotype. PLoS One 7(9):e45187. https://doi.org/10.1371/journal.pone.0045187

Kaprelyants AS, Gottschal JC, Kell DB (1993) Dormancy in non-sporulating bacteria. FEMS Microbiol Lett 104(3–4):271–286. https://doi.org/10.1111/j.1574-6968.1993.tb05871.x

Keren I, Kaldalu N, Spoering A, Wang Y, Lewis K (2004) Persister cells and tolerance to antimicrobials. FEMS Microbiol Lett 230(1):13–18

Kim HB, Wang M, Ahmed S, Park CH, LaRocque RC, Faruque AS, Hooper DC (2010) Transferable quinolone resistance in *Vibrio cholerae*. Antimicrob Agents Chemother 54(2):799–803. https://doi.org/10.1128/AAC.01045-09

Kint CI, Verstraeten N, Fauvart M, Michiels J (2012) New-found fundamentals of bacterial persistence. Trends Microbiol 20(12):577–585. https://doi.org/10.1016/j.tim.2012.08.009

Kjelleberg S (eds) (1993) Starvation in bacteria, Springer. www.springer.com/in/book/9780306444302

Levin-Reisman I, Ronin I, Gefen O, Braniss I, Shoresh N, Balaban NQ (2017) Antibiotic tolerance facilitates the evolution of resistance. Science 355(6327):826–830. https://doi.org/10.1126/science.aaj2191

Magdanova LA, Golyasnaya NV (2013) Heterogeneity as an adaptive trait of microbial populations. Microbiology 82(1):1–10. https://doi.org/10.1134/S0026261713010074

Mishra A, Taneja N, Sharma M (2012) Viability kinetics, induction and resuscitation and quantitative real-time polymerase chain reaction analyses of viable but nonculturable *Vibrio cholerae* O1 in freshwater microcosm. J Appl Microbiol 112(5):945–953. https://doi.org/10.1111/j.1365-2672.2012.05255.x

Shan Y, Gandt AB, Rowe SE, Deisinger JP, Conlon BP, Lewis K (2017) ATP-dependent persister formation in *Escherichia coli*. MBio 8(1):e02267–16. https://doi.org/10.1128/mBio.02267-16

Sharma B, Brown AV, Matluck NE, Hu LT, Lewis K (2015) Borrelia burgdorferi, the causative agent of Lyme disease, forms drug-tolerant persister cells. Antimicrob Agents Chemother. https://doi.org/10.1128/AAC.00864-15

Tang H-J, Chang M-C, Ko W-C, Huang K-Y, Lee C-L, Chuang Y-C (2002) In vitro and in vivo activities of newer fluoroquinolones against *Vibrio vulnificus*. Antimicrob Agents Chemother 46(11):3580–3584. https://doi.org/10.1128/AAC.46.11.3580-3584.2002

Thorsing M, Bentin T, Givskov M, Tolker-Nielsen T, Goltermann L (2015) The bactericidal activity of β-lactam antibiotics is increased by metabolizable sugar species. Microbiology 161(10):1999–2007. https://doi.org/10.1099/mic.0.000152

Van den Bergh B, Fauvart M, Michiels J (2017) Formation, physiology, ecology, evolution and clinical importance of bacterial persisters. FEMS Microbiol Rev 41(3):219–251. https://doi.org/10.1093/femsre/fux001

Wai SN, Mizunoe Y, Yoshida S (1999) How *Vibrio cholerae* survive during starvation. FEMS Microbiol Lett 180(2):123–131. https://doi.org/10.1111/j.1574-6968.1999.tb08786.x

Archives of Microbiology
https://doi.org/10.1007/s00203-019-01751-8

ORIGINAL PAPER

Glucose sensitizes the stationary and persistent population of *Vibrio cholerae* to ciprofloxacin

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Received: 27 December 2018 / Revised: 13 May 2019 / Accepted: 16 October 2019

Abstract

The subject of analysis in this report was the antibiotic susceptibility of *V. cholerae* under glucose supplementation since the metabolites can significantly alter the antibiotic sensitivity of bacteria. Glucose could change the antibiotic susceptibility in a growth phase-dependent manner, however, the antibiotic susceptibility of exponentially growing cells was not affected in the presence of glucose. What has been shown is that the stationary phase cells which show higher antibiotic tolerance, could be sensitized to ciprofloxacin and ampicillin by glucose supplementation (tenfold sensitive). The glucose increases the respiration which in turn increases the metabolism and cell division rate. Furthermore, the addition of glucose could increase the susceptibility of persister cells to ciprofloxacin only. In general, the bacterial susceptibility can be increased by combining the antibiotics with glucose.

Keywords *Vibrio cholerae* · Glucose · *E. coli* · Persister cells · Ciprofloxacin

Introduction

Vibrio cholerae is an autochthonous estuarine bacterium and a causative organism of human disease cholera (Colwell and Spira 1992). Some of the symptoms that indicate the appearance of this bacterium include profuse vomiting and rice water stools leading to dehydration and death if not treated properly (Abubakar et al. 2018). Ever since 2007, the incidents of cholera across the globe are on the rise and often coincide with or resulting from natural disasters like flood or drought (Abubakar et al. 2018). In spite of extensive efforts by Governmental and non-Governmental agencies over the decades, cholera continues to be a major cause of epidemic disease and death in tropical countries. Though rehydration therapy is the mainstay and simplest treatment for cholera, oral antibiotics are given to dehydrated patients as soon as the vomiting stops (Hatchette and Farina 2011). However, *V. cholerae* is rapidly developing resistance to antibiotics used for the treatment of cholera (Kitaoka et al. 2011) and in addition to that formation of multi-drug tolerant persister population among several pathogens are gaining clinical significance (Defraine et al. 2018).

Earlier reports on *E. coli* have shown that the sugars can sensitize the growing cells to β-lactams (Thorsing et al 2015), while another group has elaborated on the role of Proton Motive Force (PMF) in sugar-induced potentiation of aminoglycoside on persister population (Allison et al. 2011). Sugar was also found to potentiate the antibiotic action against genetic resistant clones of *E. Tarda* (Peng et al. 2015). On the other hand, the *V. cholerae* is an important water-borne pathogen and has distinct physiology as compared to enteric pathogens, but unfortunately, there are no studies on modulation of antibiotic susceptibility of this pathogen unlike other enteric pathogens. Therefore, this study was initiated to understand the role of glucose in modulating the antibiotic susceptibility of *V. cholerae* focusing on growth phase.

Communicated by Djamel DRIDER.

Electronic supplementary material The online version of this article (https://doi.org/10.1007/s00203-019-01751-8) contains supplementary material, which is available to authorized users.

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Published online: 29 October 2019

Methods

Bacterial strains and growth conditions

All cultures were grown in Luria–Bertani (LB) broth adjusted to a starting pH of 7.5 at 37 °C with shaking, unless otherwise stated and for the purpose of this study, we have used *V. cholerae* El Tor O1 N 16961. The uncoupling agent carbonylcyanide-3-chlorophenylhydrazone (CCCP), antibiotics (Ciprofloxacin, Ampicillin and Kanamycin), and glucose were procured from Sigma-Aldrich, US and all fine chemicals are filter-sterilized separately and added as described in the results. Overnight cultures were prepared from glycerol stocks (− 80 °C) by streaking onto agar plates and culturing a single colony in the broth with aeration at 37 °C for 16–20 h. Additionally, the percentage of survival indicates the percentage of cells, which survive after the 4 h of antibiotic exposure. The formula used to calculate the percentage of survival is $= (N/N_0) \times 100$, where N is the CFU ml^{-1} after antibiotic challenge; N_0 is the CFU ml^{-1} of colonies before antibiotic challenge.

Glucose supplementation and antibiotic susceptibility

Vibrio cholerae was cultured in LB at 37 °C under shaking condition, it was supplemented with glucose in different growth phases and measured the antibiotic susceptibility. Glucose concentrations were adjusted to deliver 60 mM carbon. Refer Fig. 1 for the outline.

Condition 1: Growth phase—lag: glucose added at the time of inoculation

The overnight grown culture of *V. cholerae* was inoculated in a sterile LB flask. Glucose (10 mM) was immediately added to the media and the culture was incubated at 37 °C, 150 rpm shaking condition. The control was also maintained without glucose supplementation and after 3 h of incubation, the antibiotics were added at tenfold MIC concentration and incubated for 6 h further. At the end of the antibiotic treatment, the viability was enumerated by serial dilution and plating on LB plates.

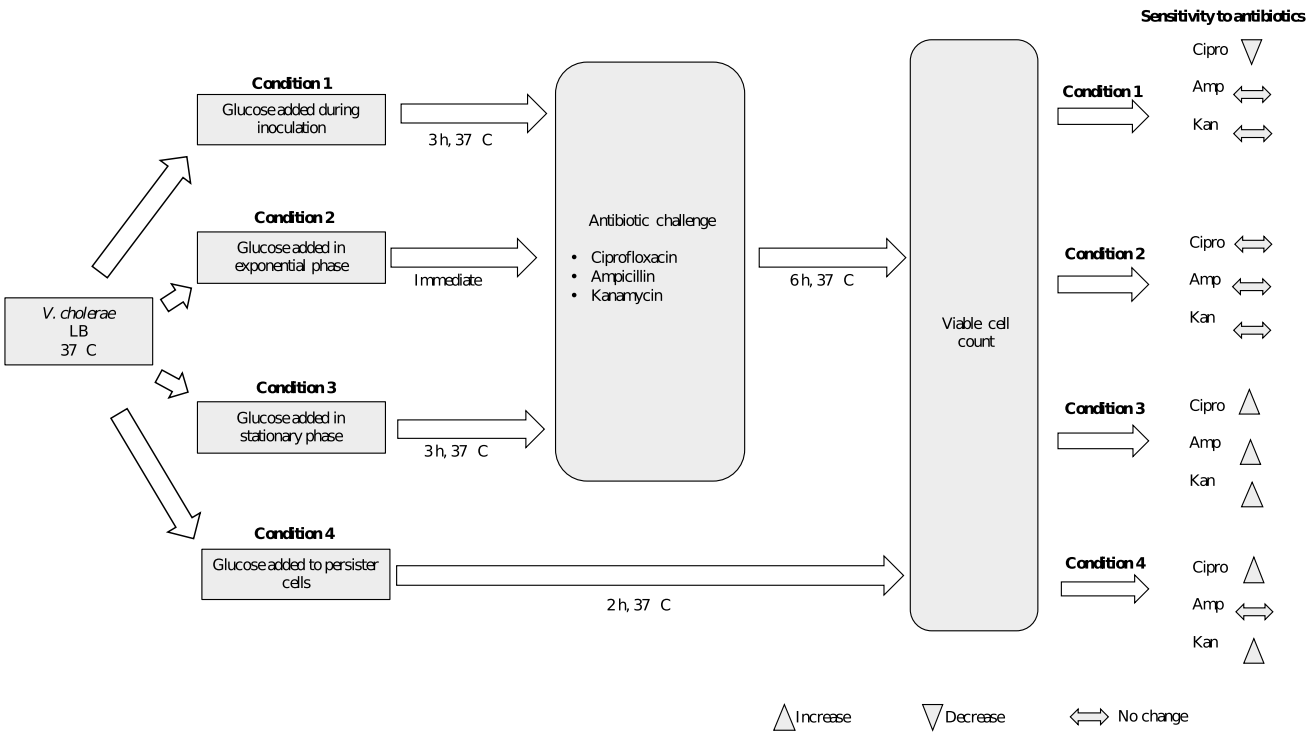

Fig. 1 The outline of growth dependent effect of glucose on antibiotic susceptibility of *V. cholerae*. The Luria–Bertani media was used throughout the study, with or without glucose supplementation. The susceptibility was determined by plating the survivor cells on LA plate post-antibiotic treatment. Ciprofloxacin (Cipro), ampicillin (Amp) and Kanamycin (Kan) antibiotics each with unique mechanism of action were used in the study

Condition 2: Growth phase—log: glucose added to the exponential phase

The cultures were inoculated in LB and the glucose was supplemented to the media (+ Glucose) after 3 h of incubation. A control was maintained without glucose supplementation (− Glucose). Immediately after glucose addition, cultures were challenged with antibiotics (tenfold MIC) for 6 h. At the end of antibiotic challenge, the survivors were enumerated by plating.

Condition 3: Growth phase—stationary: glucose added in the non-dividing state

The overnight grown stationary phase culture was supplemented with 10 mM glucose. The antibiotics were added to the culture immediately and treated for 6 h (+ Glucose). A control was maintained without glucose addition (− Glucose) and the percentage of survival was determined by enumerating the survivors after 6 h of antibiotics challenge.

Condition 4: Persister stage: the glucose was added to the persister cells

Persister cells were isolated using methods as described by Keren (2004). Briefly, the exponentially growing culture of *V. cholerae* in LB was challenged with tenfold MIC of ciprofloxacin and ampicillin (for MIC values, please refer Table S1), individually. The culture was incubated at 37 °C and the survivors were enumerated at regular time intervals. The persister cells obtained by ciprofloxacin challenge were termed as P-ciprofloxacin; the persister cells obtained by ampicillin challenge were termed as P-ampicillin. Glucose (10 mM) was added to the persister population and changes in the cell number were determined after 2 h. The cultures were always maintained under antibiotic stress to retain the persister physiology.

Minimal inhibitory concentration (MIC) determination

The MIC values of ciprofloxacin, kanamycin and ampicillin were determined using the conventional micro-dilution method (European Committee on Antimicrobial Susceptibility Testing, 2003). The MIC is the lowest antibiotic concentration required for inhibiting visible bacterial growth. Refer Table S1 for MIC values of antibiotics.

Statistical analysis

All the tests were conducted with three independent biological replicates and the data are expressed as mean ± SD ($n = 3$). The two-tailed t test was used to determine the statistical significance with a cutoff p value of ≤ 0.05.

Results

Condition 1: growth phase—lag: glucose added at the time of inoculation

The *V. cholerae* grown in a media without glucose (− Glucose) was treated with ciprofloxacin (10 × MIC) and after the treatment less than 0.1% survivors were observed. Interestingly, *V. cholerae* grown in the media with glucose (+ Glucose) had tenfold more survivors to ciprofloxacin (Fig. 2a) and this difference is statistically significant ($p < 0.05$). However, both the cultures (+ Glucose) and (− Glucose) did not show significant difference in susceptibility when challenged with ampicillin and kanamycin.

Condition 2: glucose added in exponential phase

The glucose and antibiotic were added together to the actively growing culture in media and here both the cultures (+ Glucose) and (− Glucose) had 0.1% survivors to both ciprofloxacin and ampicillin (Fig. 2b). The glucose addition failed to change the antibiotic susceptibility of exponentially growing population, irrespective of mode of action of antibiotics (ciprofloxacin, ampicillin, and kanamycin).

Condition 3: glucose added in the non-dividing state

The stationary phase cells were more tolerant to antibiotics than the actively growing population (Fig. 2c). The (+ Glucose) cultures were over tenfold more susceptible to ciprofloxacin and ampicillin and kanamycin, in comparison to (-Glucose) cultures ($p < 0.05$). The stationary cultures were supplemented with non-metabolizable sugars such as sorbitol and sucralose. No change in antibiotic susceptibility was observed in the presence of non-metabolizable sugars (Fig. S1).

Condition 4: Growth phase—persister stage: the glucose was added to the persister cells

The persister cells obtained by ciprofloxacin challenge were termed as P-ciprofloxacin; the persister cells obtained by ampicillin challenge were termed as P-ampicillin (Fig. 3). The tenfold antibiotic concentration was maintained in the

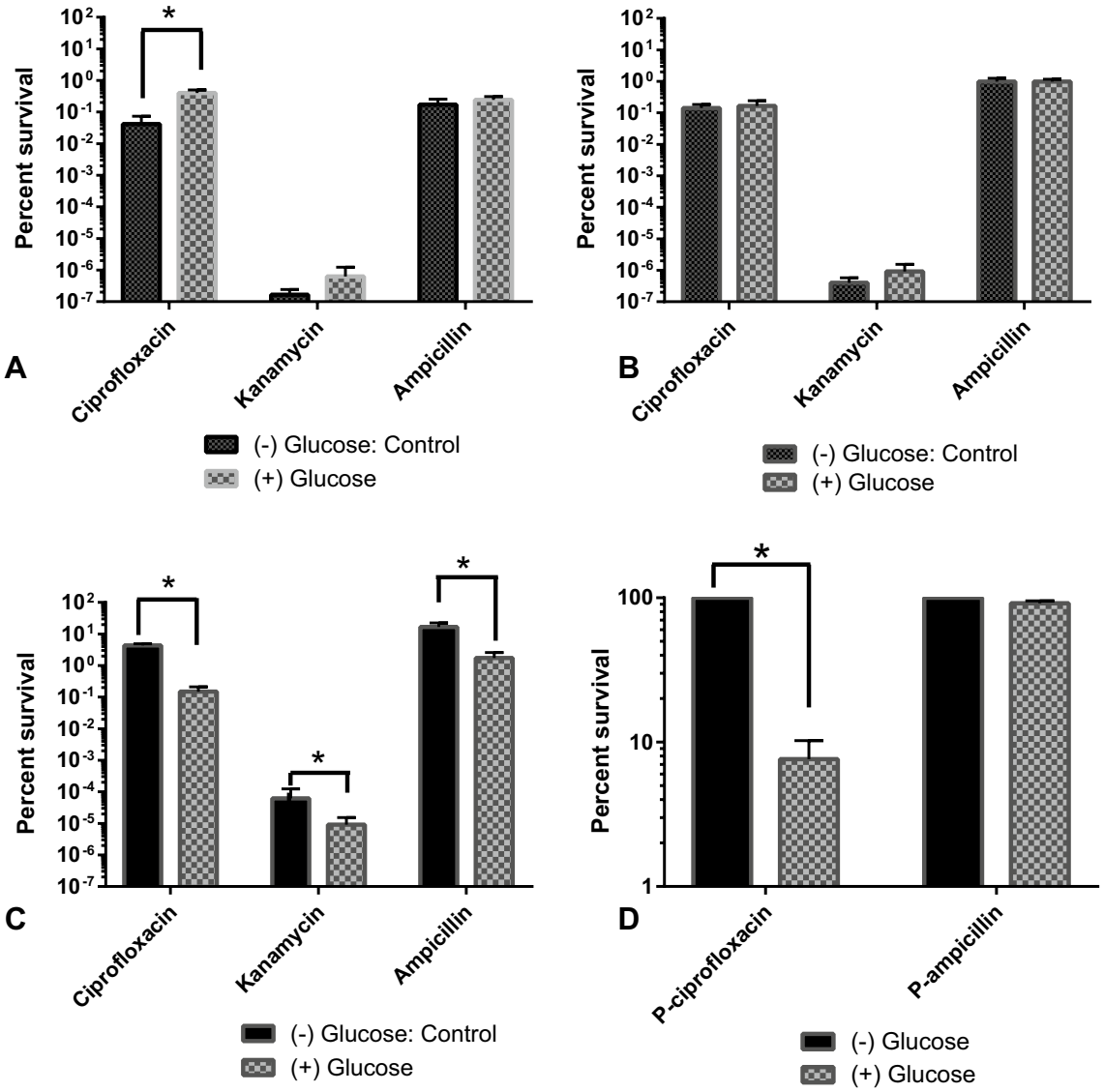

Fig. 2 The effect of glucose on antibiotic susceptibility. The (+) glucose cultures are depicted as black column and (−) glucose cultures as grey column. The (−) glucose is the control experiment. Percent survival is plotted after antibiotic treatment. **a** Condition 1: glucose was added to the culture at the time of inoculation. **b** Condition 2: glucose was added to the exponentially growing cells. **c** Condition 3: glucose was added to the stationary phase cells. **d** Condition 4: glucose was supplemented to persister population. P-ampicillin, P-ciprofloxacin refers to persister cells generated from ampicillin and ciprofloxacin treatment, respectively. Two-tailed *t* test was carried out to determine the statistical significance between (+) glucose and (−) glucose cultures ($n=3$) (*$p<0.05$)

medium throughout the experiment to keep stable persister population. After the addition of glucose to this stable persister population, P-ciprofloxacin population reduced by tenfold (Fig. 2d) ($p<0.05$), but the P-amp, however, did not show significant cell number reduction ($p>0.05$) upon glucose supplementation.

Growth kinetics

The apparent rise in antibiotic tolerance of + Glucose cultures in condition 1 was further investigated. Growth curve of + Glucose and − Glucose (Fig. S2) revealed that the growth rate of *V. cholerae* in LB increased from 0.15 h^{-1} to 0.19 h^{-1} in the presence of glucose ($p=0.0299$). This result suggests that in the presence of glucose, the cells would divide faster and advance to next growth phase. Therefore, once the glucose is added, the cells would be in the early stationary phase and stationary cells are resistant to antibiotics (as in condition 3). When the growth difference between + Glucose and − Glucose was nullified by sampling cells at same growth phase (A_{600}0.4), there was

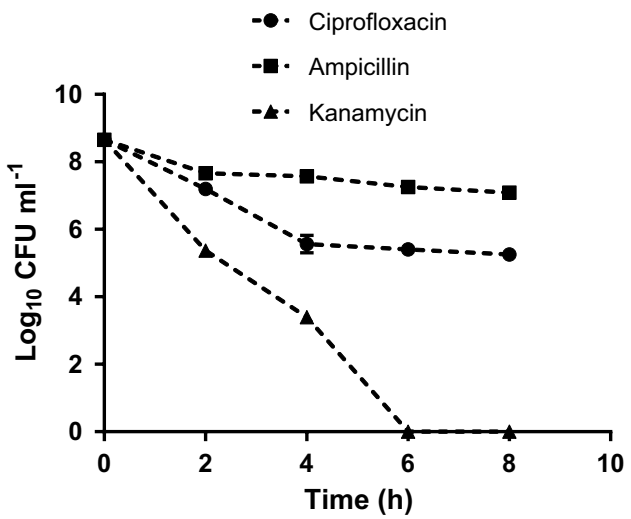

Fig. 3 Biphasic kill curve to obtain persister population. The stationary phase *V. cholerae* was challenged with tenfold MIC of ampicillin, ciprofloxacin and kanamycin (Table S1). The percent survival $[(N/N_0)\times 100]$ is determined and plotted against time (*h*). The average of three biological replicates are plated with mean and SEM ($n=3$)

no significant change in the sensitivity to antibiotics as observed in condition 1 (Fig. S3).

Dynamics of glucose concentration and antibiotic susceptibility

We changed the concentration of glucose and measured its effect on antibiotic susceptibility. The stationary phase cells were supplemented with different concentration of glucose and subsequently exposed to antibiotics (condition 3). As the concentration of glucose increased from 0 to 10 mM, the antibiotic susceptibility also increased (Fig. S4) for ciprofloxacin and ampicillin. However, beyond 10 mM, further increase in glucose could not change the antibiotic susceptibility. Glucose at 10 mM concentration was found to be the most effective in sensitizing the bacteria to antibiotics.

Inhibition of ATP production

The stationary phase cells were pretreated with 1 × MIC of CCCP, to inhibit the ATP synthesis, by uncoupling the electron transport chain. This pretreatment appears to have protected the cells from deleterious effects of both ciprofloxacin and ampicillin and glucose showed no effect on the antibiotic susceptibility (Fig. S5). Upon inhibition of ATP synthesis, subsequent addition of glucose could not increase the antibiotic susceptibility.

Discussion

The glucose induced changes in antibiotic susceptibility are documented in *E. coli*, *Pseudomonas*, and *S. aureus* (Meylan et al. 2017; Prax et al. 2016; Thorsing et al. 2015). Other than glucose, metabolites such as fructose, sucrose, maltose and amino acids such leucine, glycine, alanine can also alter the antibiotic susceptibility (Allison et al. 2011; Meylan et al. 2017; Prax et al. 2016; Thorsing et al. 2015; Ye et al. 2018). In the case of the *V. cholerae,* it inhabits the nutrient rich intestine, when inside the human body and once the patient is diagnosed with cholera, antibiotics are often prescribed to reduce any further chances of infection. However, we could not find any report on effects of sugar supplements on antibiotic susceptibility of *V. cholerae*. As mentioned at the beginning of this study, the effect of glucose on the antibiotic susceptibility of *V. cholerae* at different growth phases was investigated. To go a step further, this study also determined the kinetic effect of changes in glucose concentration on the antibiotic susceptibility. It was found that the 10 mM is the minimum effective glucose concentration at which the sensitivity of *V. cholerae* increases. Interestingly, the normal blood glucose varies between 4 and 6 mM in the fasting condition and when it is above 10 mM, it is termed as hyperglycemia (Hinson et al. 2010). Therefore, the conclusion from this would be that the effect of glucose on antibiotic susceptibility is at its maximum at the boundary of normal and diabetic blood glucose levels. However, at this point, we cannot comment on the antibiotic effectiveness in diabetic and normal patients without clinical data.

In condition 1, the cells were cultured in the presence of glucose. The (+ Glucose) culture was more tolerant to ciprofloxacin than (− Glucose) culture, but not as tolerant as stationary phase cells. Both of the cultures were actively dividing, and hence showed similar sensitivity to ampicillin. Since this study focuses on the *V. cholerae*, it is important to say that it is sensitive to aminoglycosides (unpublished data) and glucose did not rescue cells from lethality of antibiotic effect.

The apparent increase in tolerance to ciprofloxacin in condition 1 was further investigated. The growth of *V. cholerae*, which had increased in the presence of glucose, could be influenced by the media type and culture conditions (Martinez et al. 2010) (Fig. S2). When glucose is added to the media, the cells would prefer the glucose over amino acids predominantly present in LB for ATP generation. For that reason, the *V. cholerae* responded positively to the presence of glucose, by increasing the metabolism and cell division rate. *V. cholerae* El Tor strains, produce acetoin when grown in glucose-rich condition (Pradhan et al. 2010). Acetoin is a neutral fermentation end product

that does not inhibit bacterial growth. On the other hand, the growth of bacteria including classical strains of *V. cholerae* is often inhibited by the production of growth inhibiting substances, which lower the pH of the media (Oh et al. 2016). This can be another cause of increased growth rate when cultured with glucose.

When the antibiotic was added at specific time point, the (+ Glucose) cultures had already advanced to the next growth phase due to a faster division rate. However, when both the cultures (+ Glucose and − Glucose) were synchronized for growth, the effect of glucose on antibiotic susceptibility was nullified. The physiological age might be the reason for the observed changes in the antibiotic susceptibility. The addition of glucose in the media at the time of inoculation can result in significant changes in growth kinetics and the parameters must be carefully considered, while determining the antibiotic susceptibility of growing cultures.

In the condition 2, the cultures growing exponentially received glucose and antibiotic simultaneously. The glucose could not sensitize the exponentially growing culture of *V. cholerae* to the antibiotics, so our results with *V. cholerae* contradict a previous study on *E. coli* (Thorsing et al. 2015) in which the authors have shown that glucose can sensitize the exponentially growing culture to carbenicillin, but increase the tolerance to gentamicin.

In the condition 3, stationary phase cells were challenged with antibiotics after the addition of glucose. Stationary phase is characterized by starvation of metabolizable carbon sources (Sezonov et al. 2007) and those cells are better at tolerating antibiotics than the exponential cells (Fig. 2a–c), which is agreeing with the earlier reports (Balaban et al. 2004). Interestingly, the glucose supplementation reduced the antibiotic tolerance almost to the level of late exponential phase cells. Similar results were obtained in *E. coli* by Gutierrez et al. 2017, where the authors have observed a rise in susceptibility to ciprofloxacin once the stationary cells are provided with glucose. The authors have also pointed out that along with glucose, oxygen is also required to sensitize the stationary cells (Gutierrez et al. 2017). The glucose induced sensitivity of stationary population to daptomycin and beta-lactams is also documented in *S. aureus* and in *E. coli*, respectively (Prax et al. 2016; Thorsing et al. 2015). The replenishment of glucose triggers the metabolism and consequently sensitizes the cells (Allison et al. 2011; Meylan et al. 2017). The rise in metabolism can increase the respiration and production of ATP (Conlon et al. 2016), which results in fastening the antibiotic uptake thereby sensitizing cells to antibiotics (Ye et al. 2018). In contrast, in *S. aureus*, the glucose-mediated sensitivity to antibiotic daptomycin was not due to generation of proton motive force (PMF) (Prax et al. 2016).

In condition 4, the persister cells of *V. cholerae* were obtained in the presence of glucose using ciprofloxacin and ampicillin to which they were tolerant. The persister cells are now appreciated as an important factor which precedes the spread of antibiotic resistance (Fridman et al. 2014) and they represent a transient physiological state, with reduced metabolism and cell division (Amato and Brynildsen 2014). The presence of glucose can trigger the cells to exit the persister state and initiate active metabolism, which made them sensitive to ciprofloxacin (this study) and aminoglycosides (Allison et al. 2011; Meylan et al. 2017; Su et al. 2018). However, due to the continued presence of ampicillin, the persister cells cannot be triggered to initiate cell division. In comparison, the stationary phase cells reduce their metabolism significantly due to non-availability of carbon sources (Sezonov et al. 2007). The glucose addition can quickly increase the metabolism and initiate cell division in stationary cells. Metabolites can sensitize the persister cells of *E. coli* to aminoglycosides and quinolones; *S. aureus* to aminoglycosides; *M. tuberculosis* to isoniazid and *P. aeruginosa* to aminoglycosides (Meylan et al. 2017), and *V. cholerae* to ofloxacin.

Cholera patients are given high volume of Oral Rehydration Solution (ORS) containing a large volume of glucose to prevent dehydration (Oh et al. 2016). In the presence of glucose, the dormant cells of other opportunistic pathogens may show higher sensitivity to antibiotics, which would help decrease the intestinal bacterial load during cholera infections. As we found in the present study, growth rate of *V. cholerae* increased in the presence of glucose, but the antibiotic susceptibility did not change (condition 2). Glucose can effectively increase the antibiotic susceptibility of non-dividing cells of stationary phase or persister state (conditions 3 and 4). However, the correlation between the beneficial effects of glucose on growth and deleterious effects on antibiotic susceptibility needs further attention.

To conclude, this is the first detailed report on susceptibility of *V. cholerae* to antibiotics in different growth phases and under the glucose supplementation. What has been found is that the sensitivity of *V. cholerae* to ciprofloxacin could be significantly modified by the addition of glucose during inoculation. Unlike *E. coli*, glucose cannot alter the antibiotic susceptibility of the actively growing culture of *V. cholerae*. The glucose addition can trigger the metabolism in persister cells of *V. cholerae* by generating the PMF and could change their physiology to growth, thereby increasing the susceptibility to ciprofloxacin. The sugar-induced changes in the antibiotic susceptibility appear to be a general phenomenon across bacterial species, but with species–specific responses.

Acknowledgements We would like to acknowledge Department of atomic energy for their support.

Funding This work was funded by Department of atomic energy, India.

Compliance with ethical standards

Conflict of interest We declare no conflict of interest.

References

Abubakar A, Bwire G, Azman AS, Bouhenia M, Deng LL, Wamala JF et al (2018) Cholera epidemic in South Sudan and Uganda and need for international collaboration in cholera control. Emerg Infect Dis J 24(5):883. https://doi.org/10.3201/eid2405.171651

Allison KR, Brynildsen MP, Collins JJ (2011) Metabolite-enabled eradication of bacterial persisters by aminoglycosides. Nature 473(7346):216–220. https://doi.org/10.1038/nature10069

Amato SM, Brynildsen MP (2014) Nutrient transitions are a source of persisters in *Escherichia coli* biofilms. PLoS ONE 9(3):e93110. https://doi.org/10.1371/journal.pone.0093110

Balaban NQ, Merrin J, Chait R, Kowalik L, Leibler S (2004) Bacterial persistence as a phenotypic switch. Science (New York) 305(5690):1622–1625. https://doi.org/10.1126/science.1099390

Colwell RR, Spira WM (1992) The ecology of *Vibrio cholerae*. In: Barua D, Greenough WB (eds) Cholera, Springer US, Boston, pp 107–127. http://dx.doi.org/10.1007/978-1-4757-9688-9_6

Conlon BP, Rowe SE, Gandt AB, Nuxoll AS, Donegan NP, Zalis EA et al (2016) Persister formation in *Staphylococcus aureus* is associated with ATP depletion. Nat Microbiol 1(5):16051. https://doi.org/10.1038/nmicrobiol.2016.51

Defraine V, Fauvart M, Michiels J (2018) Fighting bacterial persistence: current and emerging anti-persister strategies and therapeutics. Drug Resist Updates 38:12–26. https://doi.org/10.1016/j.drup.2018.03.002

Fridman O, Goldberg A, Ronin I, Shoresh N, Balaban NQ (2014) Optimization of lag time underlies antibiotic tolerance in evolved bacterial populations. Nature 513(7518):418–421. https://doi.org/10.1038/nature13469

Gutierrez A, Jain S, Bhargava P, Hamblin M, Lobritz MA, Collins JJ (2017) Understanding and sensitizing density-dependent persistence to quinolone antibiotics. Mol Cell 68(6):1147–1154.e3. https://doi.org/10.1016/j.molcel.2017.11.012

Hatchette TF, Farina D (2011) Infectious diarrhea: when to test and when to treat. CMAJ 183(3):339–344. https://doi.org/10.1503/cmaj.091495

J Hinson, P Raven, S Chew (2010) Insulin and the regulation of plasma glucose. In: Hinson J, Raven P, Chew S (eds) The endocrine system, 2nd edn. Churchill Livingstone, London, pp 129–145. https://www.sciencedirect.com/science/article/pii/B9780702033728000112

Keren I, Kaldalu N, Spoering A, Wang Y, Lewis K (2004) Persister cells and tolerance to antimicrobials. FEMS Microbiol Lett 230(1):13–18

Kitaoka M, Miyata ST, Unterweger D, Pukatzki S (2011) Antibiotic resistance mechanisms of *Vibrio cholerae*. J Med Microbiol 60(4):397–407. https://doi.org/10.1099/jmm.0.023051-0

Martinez RM, Megli CJ, Taylor RK (2010) Growth and laboratory maintenance of *Vibrio cholerae*. Curr Protoc Microbiol 17:6A. https://doi.org/10.1002/9780471729259.mc06a01s17

Meylan S, Porter CBM, Yang JH, Belenky P, Gutierrez A, Lobritz MA et al (2017) Carbon sources tune antibiotic susceptibility in *Pseudomonas aeruginosa* via tricarboxylic acid cycle control. Cell Chem Biol 24(2):195–206. https://doi.org/10.1016/j.chembiol.2016.12.015

Oh YT, Kim HY, Kim EJ, Go J, Hwang W, Kim HR et al (2016) Selective and efficient elimination of *Vibrio cholerae* with a chemical modulator that targets glucose metabolism. Front Cell Infect Microbiol 6:156. https://doi.org/10.3389/fcimb.2016.00156

Peng B, Su Y, Li H, Han Y, Guo C, Tian Y, Peng X (2015) Exogenous alanine and/or glucose plus kanamycin kills antibiotic-resistant bacteria. Cell Metab 21(2):249–262. https://doi.org/10.1016/j.cmet.2015.01.008

Pradhan S, Baidya AK, Ghosh A, Paul K, Chowdhury R (2010) The el tor biotype of *Vibrio cholerae* exhibits a growth advantage in the stationary phase in mixed cultures with the classical biotype. J Bacteriol 192(4):955–963. https://doi.org/10.1128/JB.01180-09

Prax M, Mechler L, Weidenmaier C, Bertram R (2016) Glucose augments killing efficiency of daptomycin challenged *Staphylococcus aureus* persisters. PLoS ONE 11(3):e0150907. https://doi.org/10.1371/journal.pone.0150907

Sezonov G, Joseleau-Petit D, D'Ari R (2007) *Escherichia coli* physiology in luria-bertani broth. J Bacteriol 189(23):8746–8749. https://doi.org/10.1128/JB.01368-07

Su Y, Peng B, Li H, Cheng Z, Zhang T, Zhu J et al (2018) Pyruvate cycle increases aminoglycoside efficacy and provides respiratory energy in bacteria. Proc Natl Acad Sci 115(7):E1578–E1587. https://doi.org/10.1073/pnas.1714645115

Thorsing M, Bentin T, Givskov M, Tolker-Nielsen T, Goltermann L (2015) The bactericidal activity of β-lactam antibiotics is increased by metabolizable sugar species. Microbiology 161(10):1999–2007. https://doi.org/10.1099/mic.0.000152

Ye J, Lin X, Cheng Z, Su Y, Li W, Ali F et al (2018) Identification and efficacy of glycine, serine and threonine metabolism in potentiating kanamycin-mediated killing of *Edwardsiella piscicida*. J Proteom 183:34–44. https://doi.org/10.1016/j.jprot.2018.05.006

Current Microbiology
https://doi.org/10.1007/s00284-019-01777-7

Comparison of Starvation-Induced Persister Cells with Antibiotic-Induced Persister Cells

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Received: 14 January 2019 / Accepted: 17 September 2019

Abstract

The phenotypic heterogeneity in a large population arises because of fluctuation in microenvironments and stochastic gene expressions. In this report, we isolated two types of persistent sub-populations of *Vibrio cholerae*, one triggered by starvation and another by antibiotics. We characterised starvation-induced (E-cells) and antibiotic-induced (P-cell) persister cells for stress tolerance, colony morphology and toxin gene expressions. Both the sub-populations differ with respect to morphology, temperature tolerance and oxidative stress tolerance. The E-cells were smaller than the P-cells and formed tiny colonies (1–2 mm). The E-cells were more sensitive to heat and oxidative stress compared with P-cells. The up-regulated genes of P-cells include, genes of antioxidant enzymes (>5 fold), cholera toxin (>26 fold) and toxin: antitoxin protein *hipA* (>100 fold). Upon nutrient up-shift, the E-cells recovered after lag time of 6 h. However, such lag extension was not visible during P-cell recovery, suggesting that P-cell physiology is more akin to normal cells than E-cells. This is the first comparative report on the two different persister sub-populations of *V. cholerae*. The E-cells and P-cells are similar regarding antibiotic tolerance. However, the sub-populations differ significantly in stress tolerance and other phenotypes studied.

Introduction

The phenotypic variations generated by non-genetic mechanisms are difficult to study due to stochastic nature of physiological states [1]. Such variations often carry the memory of past events which influences the propensity of persister cells to form during stress conditions [2]. One of the most studied phenotypic variations is the phenomenon of antibiotic-induced persistence [3, 4]. The phenotypic variants survive lethal doses of antibiotics with characteristic bi-phasic kill curve [5]. Apart from antibiotics, several other stresses such as nutrient limitation, acid stress, phages and metal toxicity can trigger the persister formation [1].

Antibiotics rarely sterilise a given microbial culture, leading to antibiotic persister cell formation [5]. Persistence—a non-genetic phenomenon, is gaining clinical significance because of its role in chronic and relapse infections [6]. The persister cells have the higher propensity of formation in the stationary phase of laboratory cultures [7]. Another class of persister sub-populations arise during nutrient depletion. The pathogenic bacteria can scavenge the nutrients from the surrounding environment to build biomass and increase the cell number [8]. When the scavenging fails, starvation for carbon sources begins, the cells enter a dormant non-dividing phase [8], which exhibits increased stress tolerance and decreased cell division rate [9]. Environmental stress such as nutrient depletion, or any such hostile abiotic factors can arrest or slow down the cell division [10]. Nutrient starvation can trigger a variety of molecular events to reduce the overall metabolism and subsequent the energy demand [4, 11]. Several species of bacteria can remain viable under the starvation and attain a characteristic "persister" phenotype [12]. Several reports have shown that long-term starved cells also exhibit enhanced antibiotic tolerance as a population-wide phenotype [11–13].

The starvation-triggered persistence and antibiotic-triggered persistence poses an interesting question. Do they represent a common sub-population of cells or are they different

Electronic Supplementary Material The online version of this article (https://doi.org/10.1007/s00284-019-01777-7) contains supplementary material, which is available to authorized users.

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Published online: 25 September 2019

sub-populations? Do they represent phenotypic heterogeneous sub-populations? To answer these questions, we chose *Vibrio cholerae* as a model organism to study the inter-relatedness of environmental persistence and antibiotic persistence. *V. cholerae* is the causative organism of water-borne illness cholera [14]. It persists in the estuarine water between the outbreaks of the disease [15]. The starvation-triggered persister cells can form elaborate biofilms on abiotic and biotic surfaces and also in interaction with a multitude of organisms [16]. We could not get any report on biofilm formation by antibiotic-triggered persister cells. *V. cholerae* survives long-term starvation with no loss of viability [14] and in addition, exhibits enhanced antibiotic tolerance (unpublished data). There are many studies on the persister population and their molecular details [3, 5, 7]. However, the studies on the comparison of persister sub-populations isolated from different physiological conditions are lacking. In the present study, we compared the starvation-triggered and antibiotic-triggered persister sub-population regarding various abiotic stress conditions.

Methods

Strain and Sub-populations

Vibrio cholerae El Tor O1 N 16,961 was used in this study. Culture was grown in Luria–Bertani (LB) broth adjusted to a starting pH of 7.5 at 37 °C with shaking unless otherwise stated. Ciprofloxacin was obtained by Sigma-Aldrich, California, US added as described below. We used the stationary phase (16-h overnight grown culture) to isolate the persister sub-populations. 16-h old stationary phase cells were pelleted, suspended and inoculated in Artificial Sea Water (ASW). We starved the cells for 24–48 h to get the starvation-triggered persister sub-population selected under nutrient deficiency (E-cells). Another set of stationary phase cells were challenged with ciprofloxacin (tenfold MIC), and antibiotic-triggered persister cells (P-cells) were isolated as mentioned earlier [5]. The overnight grown cultures were diluted 1:100 in culture flasks and incubated on a shaker (150 rpm) at 37 °C for 16 h; at this point, the cells were challenged with a tenfold MIC of ciprofloxacin and cultured for another 3–6 h. At regular intervals, small aliquots of the sample were centrifuged. The cell pellet was diluted in a phosphate buffer, and 10 µl was spotted on to LB plates. The plates were incubated at 37 °C for 16–24 h until colonies appeared. A graph of cell number v/s time was plotted to get the antibiotic kill curve. The formation of biphasic kill curve shows the formation of persister cells. We took care to normalise the cell number difference among the persister sub-populations before challenging with different stresses. The antibiotic tolerances of both the sub-population were determined using antibiotic kill curve. Briefly, the sub-populations were isolated and treated with tenfold MIC ciprofloxacin. The cell number was periodically determined, and a graph of change in cell number with time was plotted.

Determination of MIC

The MIC values of ciprofloxacin were determined using the conventional micro-dilution method. The exponential culture was back diluted 1:10 into fresh LB. To the first lane in 96-well plate (Corning, US), 10 µl of culture and 1:10 diluted stock concentration of ciprofloxacin were added. The volume of the well in this lane was made up to 200 µl using LB. To each of the remaining wells, 100 µl of LB was added. Solution from the first lane was diluted twofold to proceeding lanes. The plates were incubated at 37 °C for 16 h. The lowest concentration of ciprofloxacin that inhibited the visible growth was interpreted as Minimal Inhibitory Concentration (MIC). All the assays were repeated twice in triplicates.

Stress Tolerance

The P-cell and E-cell sub-populations were collected as described above (3.1 strains and sub-populations). The cultures were incubated in LB at 45 °C for 180 min. The cell number was enumerated at regular intervals by serial dilution method. To induce oxidative stress, the P-cells and E-cells were incubated with 5 mM Hydrogen peroxide for 180 min. The cell number was enumerated at regular intervals by serial dilution method. To study the radiation sensitivity, the persister sub-populations were washed and suspended in saline (10^8 C.F.U ml^{-1}). Care was taken to maintain the antibiotic concentration in the media containing persister sub-populations. Cells were irradiated on ice in the dose range of 0.1–1 kGy using a 60Co source (Gamma Cell 5000, BRIT, Mumbai, India) at a dose rate of 7 kGy h^{-1}. After irradiation, 100 µl of different dilutions were plated on LA agar plates,and C.F.Us were enumerated after 24 h of incubation at 37 °C, and the decimal reduction dose (D_{10}), the dose which kills 90% of the cells, was determined from the response curve [17].

Catalase Activity

Cell-free extract of sub-population was used to determine the catalase activity using H_2O_2 degradation method [18].

Recovery from Stress

E-cells were supplemented with LB and cultured at 37 °C for 24 h. P-cells were washed in LB to remove the antibiotics and inoculated in LB and cultured at 37 °C for 24 h. We

carried out the recovery studies only after normalising the cell number among the sub-populations (10^5 C.F.U ml^{-1}).

RNA Isolation and cDNA Preparation

Total RNA was extracted from the E-cell and P-cell sub-populations using the RNeasy mini kit (QIAGEN, US) as per manufacturer's protocol. The quality and quantity of isolated RNA was measured using a photometer (Eppendorf, Germany). About 1 µg of the RNA was reverse transcribed using a DyNAmocDNA synthesis kit (Finnzymes, Espoo, Finland) as per manufacturer's protocol. The expression profile of the cDNA products was determined using the real-time quantitative PCR (RT-qPCR) and analysed using the Rotor-Gene Q Series Software 2.1.0 (Build 9) analysis template. The gene-specific primers (Table S4) were designed using the integrated DNA technologies Primer Quest software (www.idtdna.com/site).

Results and Discussions

In the present study, we isolated two sub-populations from stationary phase cells of *V. cholerae* (Fig. 1). Upon nutrient limitation cell number reduced with the time and after 24 h of starvation it formed a stable population of 10^8 C.F.U ml^{-1} (Fig. 2). We call the stable persister population triggered by starvation as E-cells. Another sub-population was isolated by treating the stationary cells with a tenfold MIC ciprofloxacin. After antibiotic addition, cell number declined and a stable persister population of 10^8 C.F.U ml^{-1} was formed after 4 h of exposure to ciprofloxacin (Fig. 2). We call these antibiotic-triggered persister cells as P-cells. We cultured the P-cells in LB without antibiotics and found that the MIC values had not changed in the re-grown population (Table S2). Again, we challenged the re-grown population with the tenfold MIC ciprofloxacin and got the characteristic biphasic kill curve. This confirmed the formation of persistent cells and not antibiotic resistant mutants. Both P-cell and E-cell sub-populations comprise 1% and 10% of stationary phase cells, respectively. Both the sub-populations did not undergo cell division (No increase in cell number) and exhibited antibiotic tolerance (Fig. S1). Post antibiotic exposure, we resuscitated the surviving P-cells in ASW and incubated it overnight at 37 °C. There was no significant increase in the P-cell population after the overnight resuscitation (Tables S2, S3). This showed that in the antibiotic treatment did not form VBNC sub-populations.

The E-cells and P-cells may form either as a response to stress or due to stochastic physiological effects. The active physiological and morphological changes lead to E-cells formation during nutrient depletion [19, 20]. In one study on antibiotic-induced persisters, those authors have shown

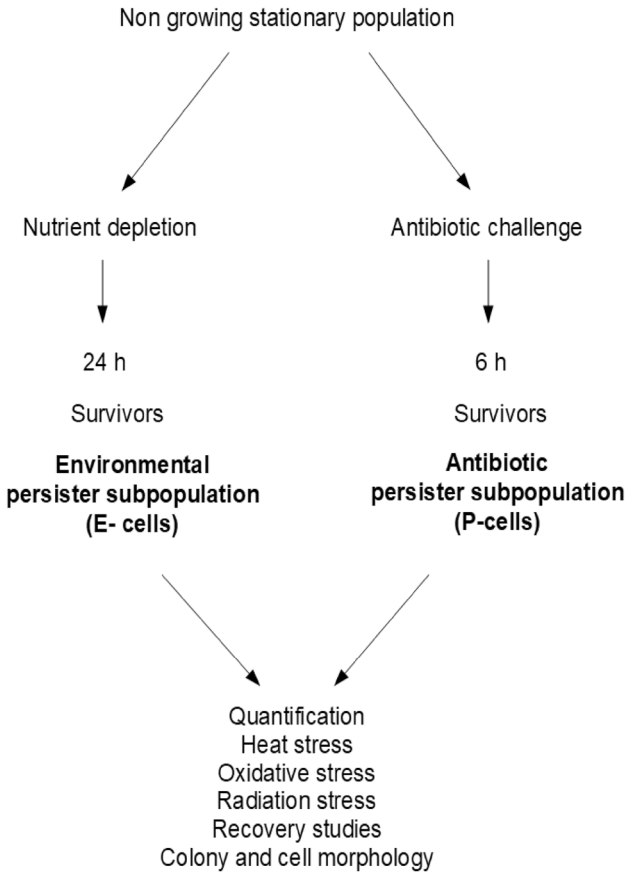

Fig. 1 The workflow used to isolate the persister sub-populations. The stationary culture of *V. cholerae* was challenged with either starvation or inhibitory concentrations of antibiotic ciprofloxacin. The survivor populations were isolated and considered as two separate sub-populations. The starvation-triggered persister cells are called Environmental persisters (E-cells). The sub-populations isolated from antibiotic stress are called Antibiotic-triggered persisters (P-cells). Both the sub-populations were analysed for stress tolerance and colony morphology

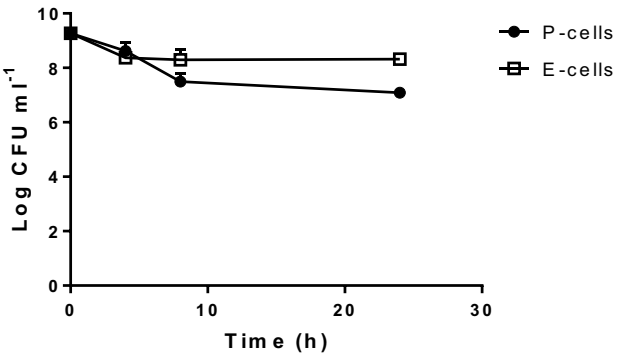

Fig. 2 Time-dependent killing to isolate the sub-populations from stationary phase cells. The squares represent the starvation-triggered persisters (E-cells) and the filled circle represents the antibiotic-triggered persisters (P-cells). The viability was obtained by serially diluting and plating the cells on LB agar plates and incubated overnight at 37 °C. The data represent the mean of three biological triplicates with standard deviation ($n = 3$)

that the persister cells exist as a small population in a growing culture and do not form in response to stress [3]. The formation of both the sub-populations may exhibit overlapping molecular events, such as TA modules, SOS response, growth arrest, and reduced PMF [7–11]. The P-cell and E-cell, respectively, make up less than 1% and 10% cells of non-dividing cells, which qualifies them as sub-populations. Formation of VBNC requires specific stress factors such as nutrient stress, cold stress or antibiotics [21, 22]. In the present case, we could not isolate VBNC population in antibiotic-treated cells. VBNC formation in antibiotic-triggered population may require more stringent conditions.

Morphology

We documented the cell size and colony characteristics of both the sub-populations (Fig. 3). E-cells were smaller (1.18 ± 0.27 µm) and the P-cells were longer (14.5 ± 4.37 µm) compared to non-stressed growing cells (2.56 ± 0.13 µm) (Table S1). P-cells were a filamentous long chain of smaller cells; however, the number of cells in each chain was varying. E-cells formed smaller colonies of 1–2 mm diameter and P-cells formed regular colonies of size 2–3 mm diameter with a few intermittent tiny (<1.5 mm) colonies.

The E-cells undergo several changes in cell wall composition, cell size and volume, motility and macro-molecular content [23]. We could not find any detailed report on changes in cellular morphology and macro-molecular content of persister cells. However, as one study points out, the elongated P-cells become spherical with ageing [22]. One earlier work has shown the spontaneous appearance of rugose colonies upon long-term nutrient starvation [24]. However, in the present work, neither E-cells nor P-cells showed rugose morphology.

Antibiotic Persisters are Better at Tolerating Stress

Heat stress: The heat stress reduced the P-cells and E-cells population in a bi-phasic manner (Fig. 4a). In 60 min of heat stress, 99% of the P-cell population and over 99.999% of the E-cells population declined. The E-cells cell number

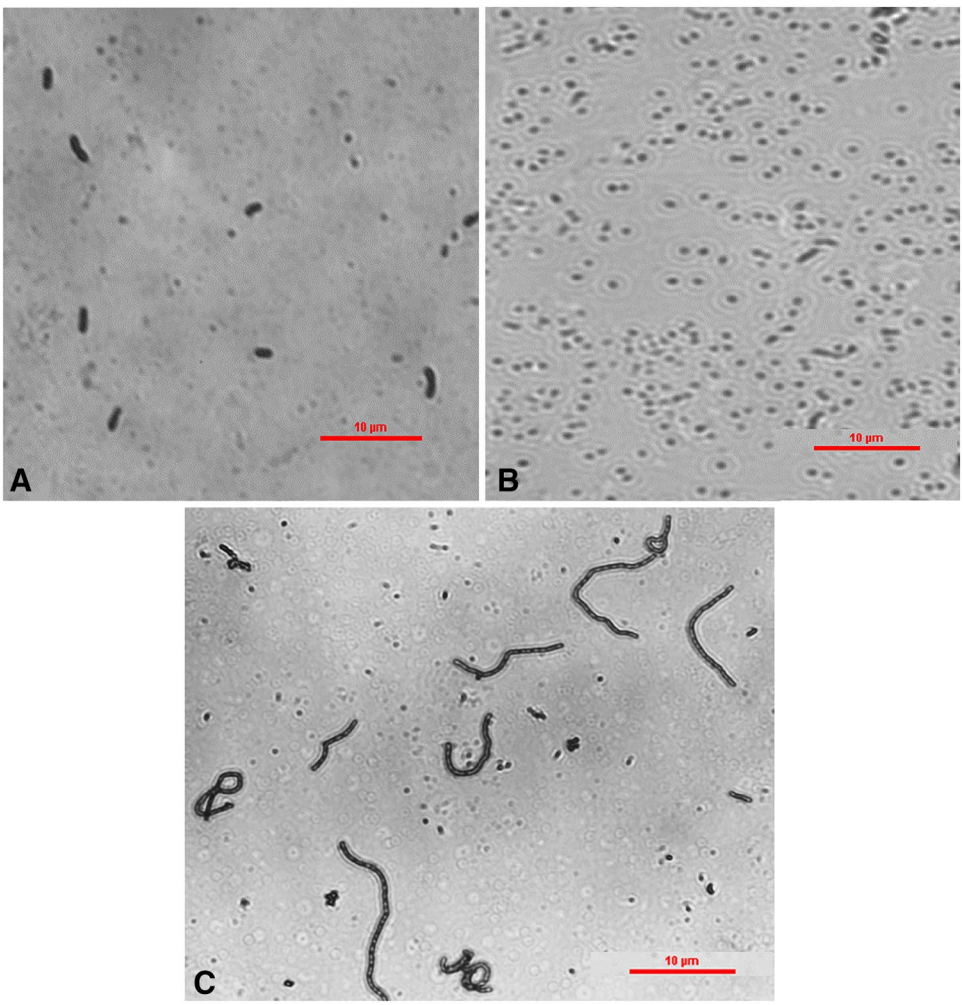

Fig. 3 The microscopic images of sub-populations. **a** Non-stressed control growing population of *V. cholerae*. **b** P-cells obtained after exposure to tenfold MIC ciprofloxacin **c** E-cells obtained after starvation for 24 h. Scale bar: 10 µm. The image was taken in Nikon Ni-U microscope and analysed through NIS-Elements

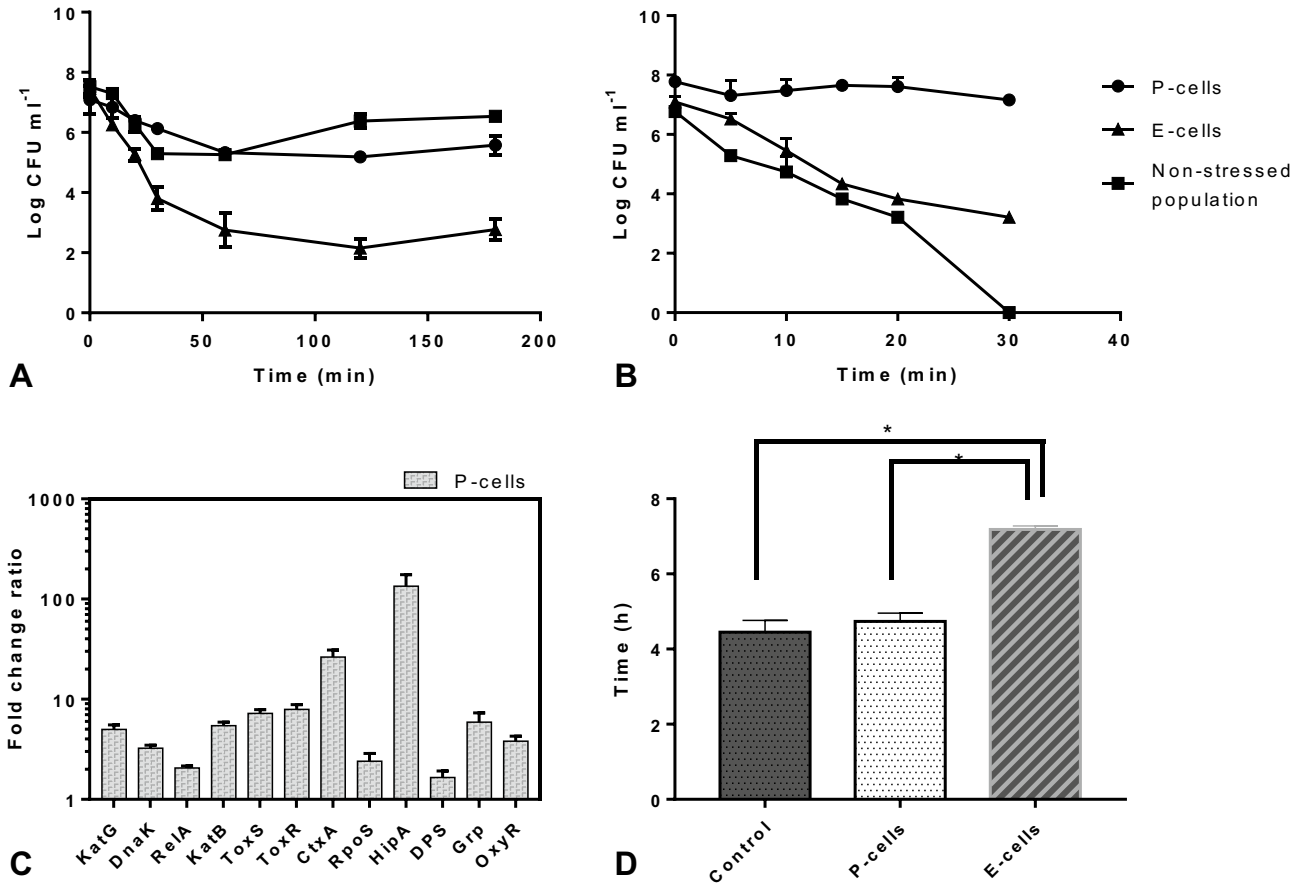

Fig. 4 The stress tolerance of E-cell and P-cell sub-populations. **a** The heat stress responses of sub-populations and non-stressed control population at 45 °C for 180 min. **b** The oxidative stress responses of E-cells and P-cells at 5 mM H_2O_2 for 180 min. **c** The quantitative PCR analysis of important genes involved in oxidative stress and TA system. The gene expression of P-cells was compared to that of E-cells. The X-axis indicates the fold change in expression of mRNA in P-cells with respect to mRNA in E-cells. **d** The lag-time measurement during recovery from stress. The lag time of sub-populations was compared to that of the growing non-stressed population. The number of cells in all the test samples was maintained at ~ 10^5 C.F.U ml^{-1}. Student's *t* test was used to determine the statistical difference in the lag time of samples. The bar graph represents the mean with SD, $*p \leq 0.05$ ($n = 3$)

reduction was rapid compared to those of both P-cells and non-stressed population. However, after the initial killing, the survivors of both the sub-populations could tolerate the heat stress for the remaining course of the experiment, and we did not observe the further reduction in the cell number. The heat stress tolerance pattern of non-stressed control population was similar to that of P-cells.

Oxidative stress: In 30 min of oxidative stress, 99.9% of the E-cells were killed; however, the P-cells could tolerate the oxidative stress with no loss of viability (Fig. 4b). In comparison, the non-stressed population did not survive oxidative stress beyond 30-minute. We measured the catalase enzyme activity in both the sub-populations. The P-cells had higher catalase activity (13.08 U mg^{-1}) than the E-cells (6.84 U mg^{-1}), and this difference was significant at $p = 0.05$. The catalase activity of non-stressed population was less than the sub-populations (4.68 U mg-1). The high catalase activity in P-cells may help tolerate oxidative stress.

Radiation stress: Both the sub-populations were exposed to γ-radiation. The D_{10} values of E-cell and P-cell were 59 Gy and 43 Gy, respectively (Fig. S2). In comparison, the D_{10} of exponentially growing cells of *V. cholerae* was 48 Gy. The D_{10} values of the sub-populations and the control population were not significant at $p = 0.05$ (Student's *t* test).

Long-term starvation in *V. cholerae* causes several changes to cell morphology such as the reduction in cell volume, reduction division, loss of inclusion bodies, loss of membrane integrity, compressed nuclear volume and formation of convoluted structures [23]. The starved cells of copiotrophs such as *E. coli*, *P. putida*, and *Vibrio* sp. strain S14 survived the heat stress better than the non-starved counterparts [25]. Contrary to the above reports, the E-cells (*V. cholerae* El Tor O1 strain) were more sensitive to heat and to oxidative stress. This may be because of the difference in the extent of starvation and medium used to impart nutrient depletion. Heat stress kills bacteria by destabilising

the lipid bilayer allowing small molecules to escape which change the electrical conductivity of the cell leading to the loss of viability [26]. The morphological changes such as membrane modifications may sensitise the cells to heat stress and oxidative stress. P-cells arise from a well-nourished growing population and resemble dormant cells with low metabolic activity [27]. The antibiotic persister cells because of being in "healthier nutrient state" could survive the heat stress although with considerable loss of viability. We could not find any detailed report on the cellular composition of antibiotic persister cells. The radiation resistance depends on genetic and physiological makeup of the cell [17]. Results from the present study suggest that both the sub-populations are sensitive to gamma radiation. Though the P-cells are tolerant of other stresses, they are sensitive to DNA damaging stresses. The morphological changes in E-cells and high antioxidant activity of P-cells could not protect them from the DNA damage. The starved bacterial cells are more tolerant of non-ionising radiation such as UV radiation [28]. However, the high-energy ionising radiation can still reduce the dormant population of *V. cholerae* with high potency. When compared to both the sub-populations the non-stressed cells were sensitive to oxidative stress. The higher antioxidants in E-cells and P-cells could protect them from oxidative challenge.

Antibiotic Persisters Show Enhanced Catalase and Toxin hipA Expression

E-cells and P-cells both represent a dormant cell condition under abiotic stress. The gene expression of few relevant genes involved in persister cell formation and toxin production of E-cells and P-cells was carried out using RT-PCR (Table S4). The gene expression of E-cells was taken as control and relative to that, the fold change in the gene expression of P-cell was determined. The genes involved stress tolerance such as *DnaK* (+3.25 fold), *DPS* (+1.65 fold) and *GRP* (+5.89 fold) were up-regulated in the P-cells as compared to E-cells (Fig. 4c). The cholera toxin gene *ctxA* (+26 fold), its transcription activators, such as *ToxS* (+7.2 fold) and *ToxR* (+7.9 fold) were also up-regulated in the P-cells. The antioxidant gene cascade involving *KatG* (+5.01 fold), *KatB* (+5.45 fold), *OxyR* (+3.8 fold) were also up-regulated in P-cells. One of the TA system implicated in persistence, *HipA* was up-regulated by 134-fold in the P-cells than the E-cells (Fig. 4c).

The significant up-regulation across all the genes was observed in P-cells. This gene expression comparison was with E-cells but not with non-stressed cells (non-persister). Because, the non-stressed cells are actively growing, overall gene expression is higher in non-stressed cells as compared to any cell population under stress [29]. Therefore, comparison between non-stressed and persister cell would lead to under estimation of gene expression of key proteins involved in persister cell physiology. Bactericidal antibiotics and oxidative agents can induce reactive oxygen species (ROS) formation by stimulating the respiration and depleting NADH formation [30]. The stimulated respiration would increase the electron transport resulting in increased levels of superoxide and other ROS. The free radicals would lead to hydroxyl radical formation that damage DNA, lipids and proteins resulting in cell death [31]. Cells can tolerate oxidative stress by synthesising antioxidant enzymes such as catalase and peroxidase to scavenge the ROS and other free radicals [30]. Besides catalase-peroxidase genes, the P-cells also showed increased expression of the genes involved in cholera toxin and general stress tolerance. The antibiotics kill bacterial cells by increasing oxidative stress [32]. The cell responds by producing a high level of antioxidant enzymes to counter the damage, which appears to be true for the present study. Certain environmental stresses, such as starvation and antibiotics can induce expression of toxin–antitoxin (TA) modules [33]. The *hipA* gene was over-expressed in P-cells, but not in the E-cells, showing that the hipBA toxin–antitoxin system is not involved in the starvation-triggered persistence in *V. cholerae*. The nutrient deprived cells down-regulate the rate of protein synthesis (by about 20%) and speed up ribosome hibernation to reduce the overall metabolism and gene expression [9]. The P-cells are also in the lower metabolic state, but there can be gradients in metabolic activity [16]. Even though both the populations could tolerate a high dose of ciprofloxacin, only one of them appears to depend on hipA TA system.

Recovery Studies

The growth kinetics of sub-populations during recovery (Nutrient up-shift) were determined (Fig. 4d; Fig. S3). The E-cells and P-cells recovered from dormancy after 6 h and 4 h of lag time, respectively (Fig. 4d). The lag time of P-cell was comparable with that of control non-stressed population. Rest of the kinetic parameters such as growth rate and yield of cells did not change in both the sub-populations.

The starved cells recover from dormancy with an increase in lag phase [25]. In this report, we found that E-cells recovered from starvation, with a significant extension in the lag period. The starved cells of *Vibrio* sp. S14 keeps an excess of protein synthesising machinery, including stable RNA and ribosome [25]. However, the delayed cell division initiation and the continuous degradation of endogenous material might cause an increase in lag time [25]. The P-cells recovered with no extension of the lag period in comparison with the control population. Persisters arise because of a stochastic reduction in metabolism [7]. However, as reported here, the P-cells are elongated filaments which can quickly

Table 1 The comparative chart of persister sub-populations

	Nutrient-triggered persister cells (E-cells)	Antibiotic-triggered persister cells (P-cells)
Heat stress	+ +	+ + + + +
Oxidative stress	+ + +	+ + + + + + +
Radiation stress	D_{10}59	D_{10}43
Recovery form stress	Extended lag	No lag extension
Cell morphology	Small (1.18 ± 0.27 µm)	Elongated (14.5 ± 4.37 µm)
Colony	Smaller (1–2 mm)	Regular (2–3 mm)
Growth arrest	Yes	Yes
Antibiotic tolerant	Yes	Yes

The '+' sign indicates the tolerance

divide into multiple small cells and regrow, with no extension in lag time.

The single cells assays have established the heterogeneous nature of clonal populations [34]. However, the open question is the biological importance of observed cellular heterogeneity [34]. Phenotype of a large population is often the phenotype expressed by most of the cells. A small minority of cells away from the mean physiology of the population may have distinct phenotypes. Phenotypic variants can arise as a bet-hedging strategy to cope with a fluctuating stressful environment [1, 34]. The starvation and antibiotics represent two different environmental trigger which can impose restrictions on bacterial growth. Under both the conditions, only the persister survive and can regrow when opportunity arise.

Conclusion

Comparison of two persister sub-populations led to the following conclusions (Table 1). The non-growing population could be triggered to produce at least two sub-populations, viz. the P-cell and E-cell persister sub-populations. Both the sub-populations differ from each other regarding cell and colony morphology. The sub-populations are not genetic mutants and do not harbour VBNC phenotypes. Both the sub-populations differ in abiotic stress tolerance and recovery, yet, they share common features such as antibiotic tolerance and growth arrest. The antibiotic-triggered persisters undergo changes in colony morphology but show no lag extension during recovery. The two persister sub-populations may give fitness advantage during adverse environmental conditions. Molecular mechanisms and physiological changes which lead to the formation of two sub-populations are difficult to determine, and these studies warrant a need of better selection method for separating the sub-populations based on certain unique molecular markers. Further studies can shed more light on the decrease in cell diameter of filamentous persister cells. Both sub-populations are dormant cell types, and hence, stringent responses may be involved in initiation and maintenance of persister phenotypes. The results presented here extend a possibility of the presence of physiologically distinct sub-populations in any given microbial culture. Some of these sub-populations may have overlapping phenotypes making them difficult to isolate and characterise. Further, this study also makes a stronger argument for the study of the single cell bacterial physiology.

Funding Funding was provided by Department of Atomic Energy, India.

References

1. Gefen O, Balaban NQ (2009) The importance of being persistent: heterogeneity of bacterial populations under antibiotic stress. FEMS Microbiol Rev 33:704–717
2. Jõers A, Tenson T (2016) Growth resumption from stationary phase reveals memory in *Escherichia coli* cultures. Sci Rep 6:24055
3. Balaban NQ, Merrin J, Chait R, Kowalik L, Leibler S (2004) Bacterial persistence as a phenotypic switch. Science 305:1622–1625
4. Balaban NQ et al (2019) Definitions and guidelines for research on antibiotic persistence. Nat Rev Microbiol 17:441–448
5. Keren I, Kaldalu N, Spoering A, Wang Y, Lewis K (2004) Persister cells and tolerance to antimicrobials. FEMS Microbiol Lett 230:13–18
6. Fauvart M, De Groote VN, Michiels J (2011) Role of persister cells in chronic infections: clinical relevance and perspectives on anti-persister therapies. J Med Microbiol 60:699–709
7. Cabral DJ, Wurster JI, Belenky P (2018) Antibiotic persistence as a metabolic adaptation: stress, metabolism, the host, and new directions. Pharmaceuticals 11:14
8. Chubukov V, Sauer U (2014) Environmental dependence of stationary-phase metabolism in *Bacillus subtilis* and *Escherichia coli*. Appl Environ Microbiol 80:2901–2909
9. Navarro Llorens JM, Tormo A, Martínez-García E (2010) Stationary phase in gram-negative bacteria. FEMS Microbiol Rev 34:476–495
10. Wang JD, Levin PA (2009) Metabolism, cell growth and the bacterial cell cycle. Nat Rev Microbiol 7:822–827
11. Nguyen D et al (2011) Active starvation responses mediate antibiotic tolerance in biofilms and nutrient-limited bacteria. Science 334:982–986
12. Jubair M, Jr JGM, Ali A (2012) Survival of *Vibrio cholerae* in nutrient-poor environments is associated with a novel 'persister' phenotype. PLoS ONE 7:e45187
13. Fung DKC, Chan EWC, Chin ML, Chan RCY (2010) Delineation of a bacterial starvation stress response network which can mediate antibiotic tolerance development. Antimicrob Agents Chemother 54:1082–1093

14. Colwell RR, Spira WM (1992) The ecology of Vibrio cholera. In: Barua D, Greenough WB (eds) Cholera. Springer, US, Boston, pp 107–127
15. Munro PM, Colwell RR (1996) Fate of *Vibrio cholerae* O1 in seawater microcosms. Water Res 30:47–50
16. Ackermann M (2015) A functional perspective on phenotypic heterogeneity in microorganisms. Nat Rev Microbiol 13:497
17. Sukhi SS, Shashidhar R, Kumar SA, Bandekar JR (2009) Radiation resistance of *Deinococcus radiodurans* R1 with respect to growth phase. FEMS Microbiol Lett 297:49–53
18. Catalase-Assay. https://www.worthington-biochem.com/CTL/assay.html
19. Ostling J, Holmquist L, Kjelleberg S (1996) Global analysis of the carbon starvation response of a marine *Vibrio* species with disruptions in genes homologous to *relA* and *spot*. J Bacteriol 178:4901–4908
20. Wai SN, Mizunoe Y, Yoshida S (1999) How *Vibrio cholerae* survive during starvation. FEMS Microbiol Lett 180:123–131
21. Oliver JD (2010) Recent findings on the viable but nonculturable state in pathogenic bacteria. FEMS Microbiol Rev 34:415–425
22. Kim J-S, Chowdhury N, Yamasaki R, Wood TK (2018) Viable but non-culturable and persistence describe the same bacterial stress state. Environ Microbiol 20:2038–2048
23. Kjelleberg S (ed) (1993) Starvation in bacteria. Springer, New York
24. Mizunoe Y, Wai SN, Takade A, Yoshida S-I (1999) Isolation and characterization of rugose form of *Vibrio cholerae* O139 strain MO10. Infect Immun 67:958–963
25. Nyström T, Albertson NH, Flärdh K, Kjelleberg S (1990) Physiological and molecular adaptation to starvation and recovery from starvation by the marine *Vibrio* sp S14. FEMS Microbiol Ecol 7:129–140
26. Ebrahimi A, Csonka LN, Alam MA (2018) Analyzing thermal stability of cell membrane of *salmonella* using time-multiplexed impedance sensing. Biophys J 114:609–618
27. Conlon BP et al (2016) Persister formation in *Staphylococcus aureus* is associated with ATP depletion. Nat Microbiol 1:16051
28. Häder D-P, Helbling E, Williamson C, Worrest R (2011) Effects of UV radiation on aquatic ecosystems and interactions with climate change. Photochem Photobiol Sci 10:242–260
29. Nagar V, Bandekar JR, Shashidhar R (2016) Expression of virulence and stress response genes in *Aeromonas hydrophila* under various stress conditions. J Basic Microbiol 56:1132–1137
30. Grant SS, Hung DT (2013) Persistent bacterial infections, antibiotic tolerance, and the oxidative stress response. Virulence 4:273–283
31. Redza-Dutordoir M, Averill-Bates DA (2016) Activation of apoptosis signalling pathways by reactive oxygen species. Biochem Biophys Acta 1863:2977–2992
32. Wu Y, Vulić M, Keren I, Lewis K (2012) Role of oxidative stress in persister tolerance. Antimicrob Agents Chemother 56:4922–4926
33. J.-S. Kim and T. K. Wood (2017) Tolerant, growing cells from nutrient shifts are not persister cells. mBio 8:e00354–17.
34. Altschuler SJ, Wu LF (2010) Cellular heterogeneity: do differences make a difference? Cell 141:559–563

[b] Autoclave and store at room temperature.

Composition of minimal media – carbon limited.

Reagent	Amount to add (for 100 mL)
M9 salts (5X)	20 mL
$MgSO_4$ (1 M; Sigma-Aldrich)	0.2 mL
$CaCl_2$ (1 M; Sigma-Aldrich)	0.01 mL
Deionized H_2O	78 mL

Composition of minimal media – Nitrogen limited

The M9 salts composition was modified to remove NH_4Cl. The Chloride ions reduction is made up by increasing the NaCl from 0.25% to 0.5%% (*V. cholerae* is tolerant up to 3% of NaCl concentration in media)

Constituents per 1 Litre (1 X)	**Weight in grams**
$Na_2HPO_4•7H_2O$	64
KH_2PO_4	15
NaCl	5
Deionized H_2O	to 1 litre

The above M9 salts were used to prepare M9 – Nitrogen minimal media, as given in Table.3

Composition of minimal media – Phosphate limited

The M9 salts composition was modified to remove phosphates. Tris-Cl was used as buffer to balance the pH of the media.

Constituents per 1 Litre (1 X)	**Weight in grams**
KCL	1.3
Tris-cl	0.5
NaCl	5
NH_4Cl	5.0
Deionized H_2O	to 1 litre

The above M9 salts were used to prepare M9 –Phosphate minimal media, as given in Table.3

The combinations of starvation media (- NC, - NP, - CP) are made by removing respective components from the M9 salt. Tris buffer was used to balance the pH.

PCR recipe (Using PCR master mix)

Reagents	**Volume (µL)**
PCR master mix (2X) [a]	10
Template DNA	1
Primers- Forward	1
Primers- Reverse	1
Nuclease free Water [a]	7

[a] *Thermo Fisher scientific, USA*

PCR recipe (Individual reagents)

Components	**25 µL reaction**	**Final Concentration**
10X Taq Reaction Buffer [a]	2.5	1X
10 mM dNTPs [b]	0.5	200 µM
10 µM Forward Primer	0.5	0.2 µM (0.05–1 µM)
10 µM Reverse Primer	0.5	0.2 µM (0.05–1 µM)
Template DNA	Variable	<1,000 ng
Taq DNA Polymerase [a]	0.125	1.25 units/50 µl PCR
Nuclease-free water [a]	to 25	

[a] *Thermo Fisher scientific, USA*

[b] *BRIT, India*

PCR thermal cycle

Cycle step	**Temperature (°C)**	**Time**	**Cycles**
Initial denaturation	95	30 min	1
• Denaturation	95	15-30 s	30
• Annealing	45 – 68 (depending on primer	15-30s	
• Extension	Tm)	1 minute per kb	
	72		
Final extension	72	5	1
Hold	4	∞	

qPCR – Reverse transcriptase PCR recipe (20 µL reaction mix)

Reagents	Volume (µL)
qPCR SYBR green master mix (2 X) [a]	10
cDNA	1
Primers- Forward	1
Primers- Reverse	1
Nuclease free Water [a]	7

[a] *Thermo Fisher scientific, USA*

qRT-PCR thermal cycle

Cycle step	Temperature (°C)	Time	Cycles
Initial denaturation	95	10 min	1
• Denaturation	95	15 s	40
• Annealing	45 – 68 (depending on primer	30 s	
• Extension	Tm)	15 s	
	72		
Final extension	72	10	1
Hold	4	∞	

Genes and Primers

Gene	NCBI gene ID	Primer sequence (5' to 3')
ctxA	VC1457	Forward: AAGCAGTCAGGTGGTCTT
		Reverse: TCCCGTCTGAGTTCCTCT
ToxS	VC0983	Forward:
		GGTAGACGTCACTTCCAATGT

		Reverse: CGCTTTCTGCACTATTGTCATC
ToxR	VC0820	Forward: CATCAGCCACTGTAGTGA
		Reverse: GGAAGTAAGACCGCTATC
hipA	VC0815	Forward: TAGCAGCCACGACTTACA
		Reverse: TTTCAGTTAGCTCGTGGT
oxyR	VC2636	Forward: CGGGAGCGAAAGATGATG
		Reverse: AAGGCTAGCTCAGGCAAT
DPS	VC0139	Forward: CAGGGATTGGTGGATGGT
		Reverse: CTCTTGCTCGCGGATGTA
KatG	VC1560	Forward: CGATGCGACAATCGAACA
		Reverse: CTTCCGGTGTGACCACAT
dnaK	VC0855	Forward: TACGGCGGAAGATAACCA
		Reverse: GGTTGATGCCCTCTAGGT
RelA	VC2710	Forward: GCAGATCCGTACCGAAGAT
		Reverse: GTGCCACCTCGTTCACTATT
KatB	VC1585	Forward: GAGAAGAAAGTCGGTACG
		Reverse: GGTCTTCCGATGGCTCAA
RpoS	VC0534	Forward: CGTTACAGCAACCGAGGA
		Reverse: CCATGTTGCGTAGGTAGA
GrpE	VC0854	Forward: AAGCCATGTCGATTCAAG
		Reverse: CACCATAGCAGGGCGTAA
RecA		Forward: CGG AGA GAG TAA TGG ACG AGA
		Reverse: AGG CAT TCG CTT TAC CTT GG
Extracellular deoxyribonuclease	VC0470	Forward: TGAGTGAGCAGTACGGTTTG
		Reverse: CTTTGGTCACGCACACATTC
Endonuclease III	VC1011	Forward: ACATCTTTCGCGTCTCCAATC
		Reverse: CAGTGGTGCACATCGAGTTTA

Exodeoxyribonuclease V	VC2319	Forward: AGGGCAGACTGAAAGTCTATT Reverse: GCTGCCTTGCGATTTATGTATG
5' Deoxyribonucleotidase	VC1978	Forward GGATATGCTCCCAGAAGAACTG Reverse ATCGCATCGGCTTGCTTA

_____END of THESIS_____