Quorum Regulated Resistance of *E. coli* against Environmental Bacteriophages

By

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A thesis submitted to the Department of Department of Mathematics and Natural Sciences in partial fulfillment of the requirements for the degree of
Master of Science in Biotechnology

Department of Mathematics and Natural Sciences
BRAC University
September 2019

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Ethics Statement

No human or animal model was used in this study.

Abstract

Predation of bacteria by their corresponding phages have a significant influence on the population structure of different communities of bacteria. *E. coli* infection has become a safety concern globally and they are causing a multitude of problems from food borne diseases to systemic infections. The *E. coli* cells interact with *E. coli* phages present in the aquatic systems and also in the GI system of humans and many mammalian species where they reside either during an infection or as a symbiotic or opportunistic microbe. As the *E. coli* cells survive phage predation in their habitats, there must be some underlying mechanisms which they resort to, to exist along with the phages present in the environment or evade infection. However, these techniques employed might hamper their ecological fitness and they may not be a permanent mutation and these cells might just revert back to their previous form when an environmental or chemical signal is sensed. In this experiment autoinducer molecules which are involved in bacterial quorum sensing were used to study phage bacterial interaction and it was found that growing *E. coli* cells in the presence of autoinducer AI-2 can significantly make them resistant to their corresponding phages than their parent strain. On the other hand, autoinducer CAI-1 seemed to have no effect on phage predation of *E. coli* bacterial species. Moreover, after culturing the *E. coli* bacterial cells in presence of exogenous autoinducer AI-2 there was a huge reduction in percentage of phage adsorption which might be due to downregulation of the receptors being used by the phage. All these results further prove the distinct effects of quorum sensing and will help scientists to formulate treatment strategies using Bacteriophages.

Keywords: Quorum Sensing, QS, Autoinducer, AI-2, CAI-1, *E.coli*, *V. cholerae*

Dedicated to my parents and siblings

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Md. Arafat Khan

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List of Acronyms

GI= Gastrointestinal

DPD= 4,5-dihydroxy-2,3-pentanedione

AHL= Acyl Homoserine Lactone

3OC6-HSL= N-3-oxohexanoyl-L-homoserine lactone

QS= Quorum Sensing

PQS= 2-Heptyl-3hydroxy-4(1H)-quinolone Pseudomonas quinolone signal

PAME= Hydroxyl-palmitic acid methyl ester

AI-1= Autoinducer 1

AI-2= Autoinducer 2

DPD= 4,5-dihydroxy-2,3-pentanedione

T3SS= Type III secretion system

HUS = Hemolytic uremic syndrome

CHAPTER 1

Introduction

1.1 Brief History of Quorum Sensing

Bacterial QS utilizes various types of extracellular chemical moieties which are produced by the bacteria and they accumulate to a certain level in the local environment that causes transcriptional regulation and activation of many bacterial genes (Papenfort & Bassler, 2016; Fuqua, Winans & Greenberg, 1994). QS was first detected in the 1960s and 1970s, when researchers showed luminescence in two marine bacteria (Nealson, Platt & Hastings, 1970) and in case of *Streptococcus pneumonia* it was genetic competence (Tomasz, 1965), all of which needed the production of some extracellular chemical molecules. This form of cell to cell communication was initially considered as chemical communication. Unfortunately, these earlier publications did not get widespread acceptance and were neglected for many years. In the 1980s several important discoveries occurred regarding QS. Firstly, the genes that regulated luminescence (lux) of *Vibrio fischeri* were identified which is a marine bacterium (Engebrecht & Nealson, 1983). These genes that are needed for control of luminescence by QS, the luxI and luxR (Engebrecht & Silverman, 1984) and how they control transcription of lux genes. Secondly, the QS signal in the bacterium *V. fischeri* was traced back to a molecule called 3OC6-HSL (Eberhard, et al., 1981). The luxI and luxR genes have two separate functions- luxI encodes for the protein named autoinducer synthase which must be present for the synthesis of 3OC6-HSL. On the other hand, luxR gene is responsible for coding of a transcriptional activator of lux genes that is responsive to 3OC6-HSL.

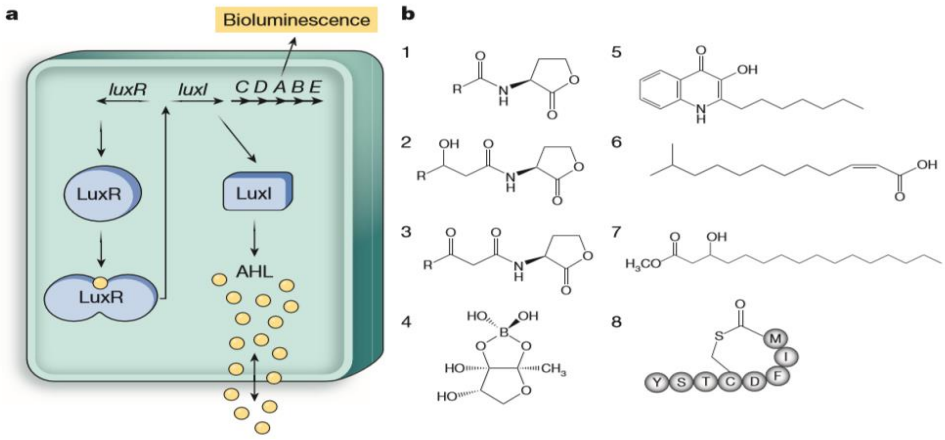

Figure 1: Canonical Quorum Sensing and the signals' chemical variety (Whiteley, Diggle & Greenberg, 2017).

a, *V. fischeri* Quorum sensing. LuxI synthesizes autoinducer molecule 3OC6-HSL (autoinducer AHL, denoted by yellow spheres), which interacts specially with the LuxR transcription regulator when it reaches concentrations of nanomolar levels. This contributes to the operon named luxICDABE and then bioluminescence is being expressed. **b**, Examplar of both gram-negative QS signals and gram-positive QS signals. (**1**) AHL. (**2**) 3-hydroxy-AHL. (**3**) N-(3-oxoacyl)-l-lactone (3-oxo-AHL) homoserine. R may be a 4–18 carbon fatty acyl band either with a carbon–carbon bond unsaturated in nature or not, the last carbon of the chain may have branching, and in case of some R groups may be acids of aromatic structure (cinnamic or p-coumaric acid). (**4**) AI-2(autoinduucer-2) from *V. harveyi*, the ester form of furanosyl borate. (**5**) PQS. (**6**) DSF and methyl dodecenoic acid (**7**) PAME. (**8**) *Staphylococcus aureus* autoinducing peptide 1 (AIP-1). In this tiny cyclic peptide quorum sensing signal, amino acids are shown by the balls which have letters inside them (Whiteley, Diggle & Greenberg, 2017).

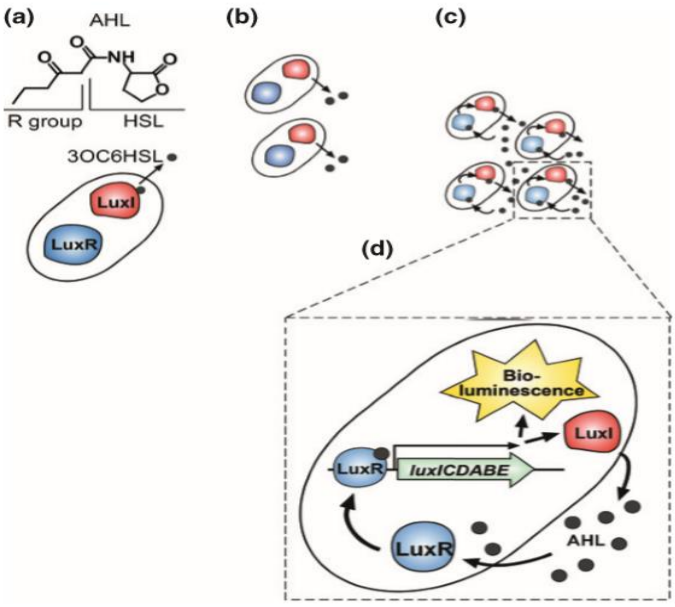

Figure 2: Quorum Sensing via a Positive Feedback Loop in *Vibrio fischeri* (Pereira, Thompson, & Xavier, 2013)

a) The autoinducer, 3OC6HSL, is produced by the enzyme LuxI, which diffuses from inside of the bacterial cell. **(b)** With the growth of bacteria, 3OC6HSL accumulates and it happens extracellularly. **(c)** At a specific cell density, critical concentration is achieved which further allows 3OC6HSL diffusion back within the cell and there it binds with LuxR, the associated receptor of this autoinducer and also stabilizes it. **(d)** The conformation shift that occurs after the generation of complex of ligand and receptor allows LuxR to steer transcription of lux operons, which encodes enzymes needed for the expression of luciferase enzyme and luxl. A positive feedback loop is created in this process: signal detection encourages more signal synthesis and further bioluminescence induction. The autoinducer 3OC6HSL is from AHL autoinducer class, in which it is a prominent member, composed of a prevalent homoserine lactone (HSL) ring combined with a R group which is specific to each species. In gram-negative bacterial species, AHLs are the primary Quorum sensing signaling molecules (Pereira, Thompson, & Xavier, 2013).

The general interest in Quorum sensing pathway stayed muted for another decade. In the 1990s due to advancements in DNA sequencing and sequence analysis, the homologous sequences of luxI and luxR genes were identified and it piqued the interest of some researchers. As a result, a multitude of new findings emerged and it was seen that many other bacteria used luxI-luxR-similar systems to control their antibiotics production, extracellular enzyme synthesis and also the genes used in conjugation (Fuqua, Winans & Greenberg, 1994). Interestingly, there were some common discoveries- luxI homologues always catalyzed the production of an AHL and the homologous molecules of luxR had affinity specifically for the AHL related to them. All these discoveries culminated to the concept of Quorum Sensing that we know today, that the AHL molecules that are diffusible in nature worked as a substitute for cell density and permitted a strain of bacteria to generate costly extracellular public materials only at that time when there was sufficient bacterial cells to profit from the materials generated (Schuster, Sexton, Diggle & Greenberg, 2013). Shortly afterwards, the signaling molecule for QS from *Streptococcus pneumoniae* (often called pheromone) was shown to be a tiny peptide (Havarstein, Coomaraswamy, & Morrison, 1995), and tiny cyclic peptide was shown to be used by Staphylococcus aureus which signals to trigger expression of genes to synthesize exotoxins (Ji, Beavis & Novick, 1995). An initial research has showed that *Vibrio harveyi,* which is a luminescent bacterium of marine environment, has the ability to recognize a signal produced by itself and also perceive a signal or multiple signals produced by other bacterial strains to cause production of light (Greenberg, Hastings, & Ulitzur, 1979). This phenomenon is regarded to be a sort of QS in which cells of many species in a blended microbial community, through a molecule called autoinducer-2 (AI-2) sense the overall bacterial population density (Chen, et al., 2002; Schauder, Shokat, Surette, & Bassler, 2001). Further studies have also defined QS-like structures in eukaryotic microbes (Candida and *Histoplasma* pathogenic fungi) (Hornby, et al., 2001; Sebghati, 2000) and lately in viruses (Erez et al., 2017), thus giving easily understandable examplars of convergent evolution. Few types of QS signals have a volatile nature, for example the DSF and PAME signals shown in Fig. 1b, and some evidence has been found that volatile signaling may take place in a local atmosphere (Groenhagen, 2013). Following the discovery of many of these structures, functional trials disclosed that QS mutants showed significantly decreased virulence for many plant and animal pathogens (Pearson, Feldman, Iglewski & Prince,2000; Pirhonen, Flego, Heikinheimo & Palva, 1993). The early link between

QS and pathogen virulence made the concept of aiming quorum sensing as a new strategy to treat infections caused by bacterial species exciting.

1.2 AI-2

Quorum sensing using AI-1 is known as signaling function between the same species (March and Bentley, 2004), where an inter-species signaling system being suggested for the autoinducer-2 (AI-2) system (Federle and Bassler, 2003). The gene for LuxS is responsible for encoding the enzyme AI-2 synthase which synthesizes AI-2 molecules from a 5-carbon precursor- DPD (Xavier et al., 2007). QS pathway is also utilized by several pathogenic bacterial species to control virulence factors; thus, interfering in the quorum sensing pathway is considered as a new way of target-specific strategy and an alternative to conventional antibiotics (Rasmussen and Givskov, 2006; Finch et al., 1998; Rasko and Sperandio, 2010). Theoretically, the cell density-dependent function of QS signaling systems is thought not to affect bacterial growth or viability when there is a lack of signal; therefore, inhibition of quorum sensing signals or quenching strategies are anticipated to prevent pathogen resistance (Otto, 2004).

QS Inhibitors (QSIs) were extensively researched for both applications in medical profession as well to ensure the safety of food on the basis of potential advantages in the fight against pathogens (Medellin-Peña et al., 2007; Hentzer et al., 2003; Park et al., 2014; Smith and Iglewski, 2003). According to newer reports, however, brought up questions about the prospective benefits of quorum sensing and thus implied a necessity to further explore the features of QSIs previously revealed. First of all, the anticipated anti-pathogenicity of Quorum sensing inhibitors may be debilitated. Bacteria can grow resistance against quorum sensing inhibitors by many ways-using several different QS systems, environmental circumstances, mutations or efflux systems (Defoirdt et al., 2010; Kalia et al., 2014). Second of all, interference in quorum sensing can have an impact on bacterial viability and it is completely different from the previous theory. According to concurrent studies conducted on quorum sensing which has revealed new roles of QS systems that have an effect on both regulation of bacterial physiology as well as collective signaling (Thompson et al., 2015; Van Kessel et al., 2015; Lee et al., 2013). In particular, experiments done on AI-2 QS participation under stressful conditions have shown important impacts on bacterial growth,

bacterial adaptation in different conditions, metabolism, colonization and survival in different adverse conditions (Sun et al., 2015; Christiaen et al., 2014; Lebeer et al., 2007, 2008;).

Many of the *E. coli* strains are involved in multiple types of infections such as traveler's diarrhea, urinary tract infection, cholecystitis, bacteremia, neonatal meningitis etc. and thus causing a global problem. Enterohemorrhagic *Escherichia coli* serotype O157:H7 (EHEC) causes food-borne disease and because of its widespread prevalence it has now become a global apprehension (Nguyen and Sperandio, 2012). Enterohemorrhagic *Escherichia coli* has the tendency to colonize in the epithelium of human colon in which place it produces Shiga-toxin (an endotoxin) that causes acute inflammation of the colon at the A/E lesions which are made by T3SS and this leads to development of HUS (hemolytic-uremic syndrome) (Pacheco and Sperandio, 2012; Garmendia et al., 2005). Enterohemorrhagic *Escherichia coli* (EHEC) bacterial strains further have a QS signaling method for LuxS / AI-2 to express its virulence factors (Sperandio et al., 1999). According to a recent study conducted by Park et al., using AI-2 quorum sensing *E. coli* strain 0157:H7 can increase their resistance to osmotic and gastrointestinal stress in vitro and thus contribute to their viability in gastrointestinal tract. These changes brought by QS can significantly reduce their chances of success in any kind of *E. coli* infection and that's why further studies need to be conducted on these bacterial strains and QS.

1.3 Finding of the Typical Communication System of Bacteria: How the Discovery of AI-2 Progressed

Foremost sign of inter-species-signaling among bacteria arose from Greenberg et al. (1979)'s study where *V. harveyi's* bioluminescent reaction was activated simply by the incorporation of the supernatant (from which cells were removed beforehand) from other bacterial species culture who produce auto-inducer molecules. These bacteria responded to something that the adjacent marine species being examined were producing. In 1993, further information were obtained after Bassler et al. (1993) observed in their study that mutant *V. harveyi* species which could not produce AHL stayed capable of activating genes which were quorum sensing dependent. For this reason, it was proposed that there was another quorum sensing methodology, for which the messenger molecule was named AI-2 (Bassler et al., 1994). Then an AHL-deficient mutated bacterial strain was made

that would only become bioluminescent when it was induced using the second autoinducer system using AI-2 signal molecule. This mutant strain was found to respond to culture supernatant fluids which were taken from bacterial strain completely different from it. This experiment proved that that the production of AI-2 was not limited to bacterial species of a solitary type (Bassler et al., 1997) and it was later reaffirmed by identifying the genetic segment that was accountable for the activity of AI-2 molecule. The gene was named LuxS and different homologues of this gene were found in several genome sequences (Surette et al., 1999).In 537 of the total 1402 bacterial genomes which were sequenced at that time homologues of LuxS gene could be identified. The identification of the operation of AI-2 in the environments outside bacterial cells of each species of luxS-containing bacteria investigated encouraged the concept that bacteria use autoinducer AI-2 to interact among different bacterial species (Surette et al., 1999; Bassler, 1999).

In 2001, understanding of biosynthesis of autoinducer AI-2 materialized. Genome sequence analysis has shown that the gene of LuxS from *Borrelia burgdorferi* was the third gene in an operon of 3 genes (Winzer et al., 2002b; Schauder et al., 2001). Two other genes present in the operon are pfs and metK and they code for enzymes which are involved in the AMC (activated methyl cycle). AMC is a major metabolic pathway which recycles SAM, the chemical moiety that acts as the major donor of methyl group in these cellular reactions (Miller & Duerre, 1968; Schauder et al., 2001). The methyl group which was released from SAM gets activated in the reaction and then its incorporation into an acceptor molecule results in a toxic intermediate molecule, S-adenosylhomocysteine (SAH), which is transformed to S-ribosylhomocysteine (SRH) by Pfs (Fig. 3). Afterwards, two groups of independent researchers have shown that LuxS catalyzes the cleavage reaction of SRH to produce homocysteine and another compound which could not identified at that time with AI-2 activity (Winzer et al., 2002b; Schauder et al., 2001).

Although in vitro synthesis of AI-2 autoinducer molecules can be done utilizing purified LuxS and Pfs proteins (which are enzyme catalyzed reactions) where SAH is the precursor molecule (Schauder et al., 2001; Winzer et al., 2002b), characterizing the signals chemical nature was still a daunting task. Later it was observed that the LuxS's chemical product generated after reaction is a linear molecule DPD which is very unstable. That is why the identification of this signal using conventional biochemical and chemical techniques remained unsuccessful (Fig 3). Moreover, DPD has a tendency to spontaneously cyclize and produce its different isomers in solution which

raises another question- which isoform of DPD was sensed by the autoinducer AI-2 sensing bacterial species? (Fig. 4) (Chen et al., 2002). This puzzle was solved with an unorthodox approach where *V. harveyi* bacteria's LuxP receptor's high affinity was exploited to trap the ligand and later detect it. This complex which comprised of LuxP and the ligand was the first structure discovered that had an AI-2 activity. This molecule encompassed S-THMF-borate (S-2-methyl-2,3,3,4 tetrahydroxytetrahydrofuranborate) that is a cyclic and borated structure of AI-2 autoinducer molecule (Chen et al., 2002). Another shock arose after recognizing an enantiomer of DPD which was significantly non-identical to the previous forms, a ligand without any borate which was R-THMF (R-2-methyl2,3,3,4-tetrahydroxytetrahydrofuran) that was found in the crystal structure of another AI-2 receptor molecule LsrB. AI-2 receptors of LsrB type are found in *Salmonella typhimurium* (Miller et al., 2004).Though in all of the AI-2-producing bacterium which have been studied by this time, the signal synthesizing enzyme, the biosynthetic pathway being used and chemical moieties produced are all the same, the studies conducted show that the molecule which is being finally identified by these bacteria may vary. In spite of this, communication among the different species which were AI-2 dependent stayed as a possibility as the distinct DPD isomers in solution remain in balance and can quickly convert among the different isoforms (Miller et al., 2004; Meijler et al., 2004). This has been experimentally illustrated: culturing *Escherichia coli* and *V. harveyi* together, which activate S-THMFborate and R-THMF (shown in Figure 4), respectively, contributes to modifications in cell development which are AI-2-dependent, in case of both species based on each bacterium's capacity to synthesize and identify the stimulus (Xavier & Bassler, 2005b).

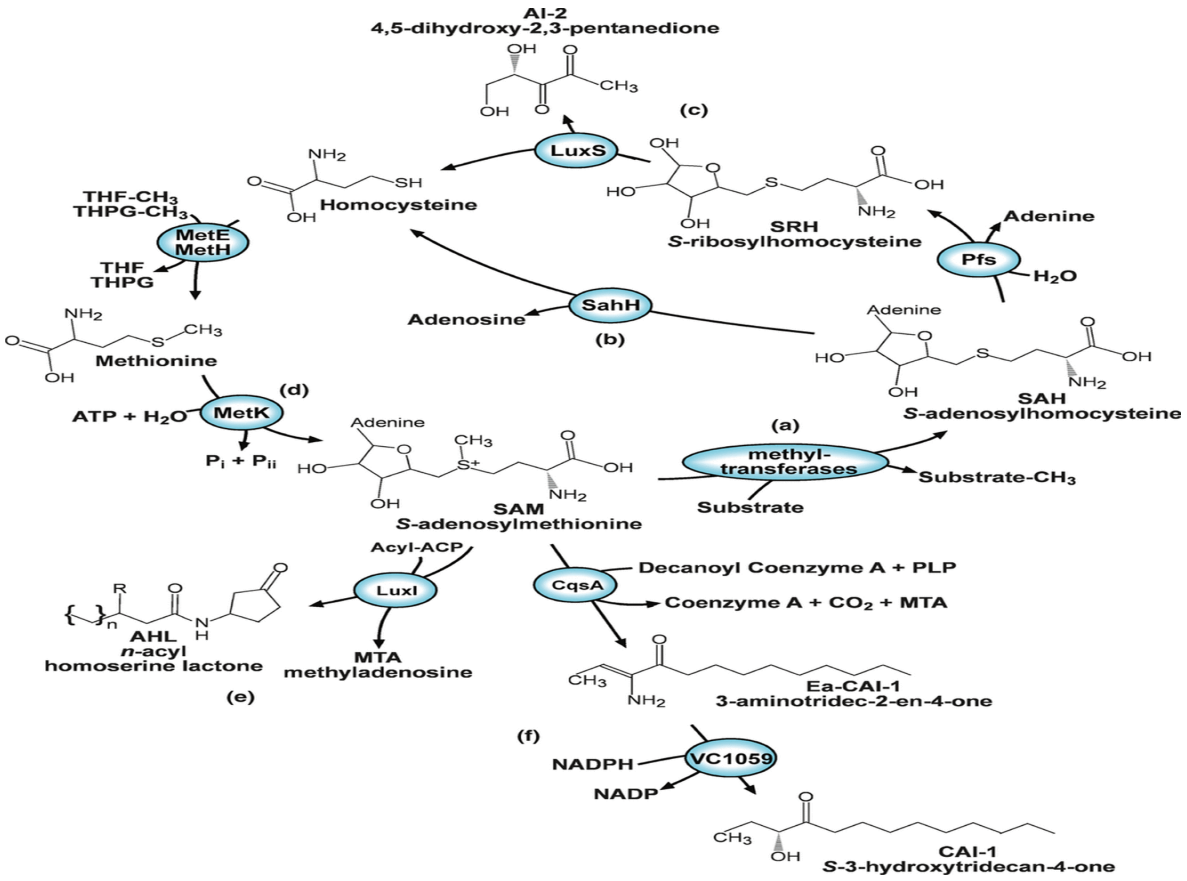

Figure 3: Activated Methyl Cycle (AMC) and Autoinducer synthesis. (Pereira, Thompson, & Xavier, 2013)

(a) A methyl group transfer from SAM to a molecule that accepts it produces SAH, an intermediate which is toxic in nature. (b) SahH-enzyme containing organisms directly transform SAH to homocysteine. (c) LuxS enzyme users catalyze this reaction to produce AI-2 in two separate steps: first, Pfs coverts SAH to yield SRH and the byproduct of this reaction is adenine, then LuxS transforms SRH to DPD (AI-2 molecules linear form) and homocysteine. (d) Through a sequence of responses incorporating methionine, regeneration of SAM from homocysteine. THF-CH3 represents N-methyl tetrahydrofolate and THPG represents 5-methyltetrahydropteroyl glutamate. (e) To produce AHL autoinducer, LuxI-type proteins utilize two substrates- Acyl-ACP and SAM. (f) Autoinducer CAI-1 of *Vibrio cholerae* is also made from decanoyl Coenzyme and SAM; it happens through a multi-stage reaction which requires VC1059 and CsqA, which is an enzyme similar to PLP (pyridoxal phosphatase)-aminotransferase. *V. harveyi* uses a homologue of CqsA that yields Z-3-aminoundec-2-en-4-one from octanoyl CoA. (Pereira, Thompson, & Xavier, 2013)

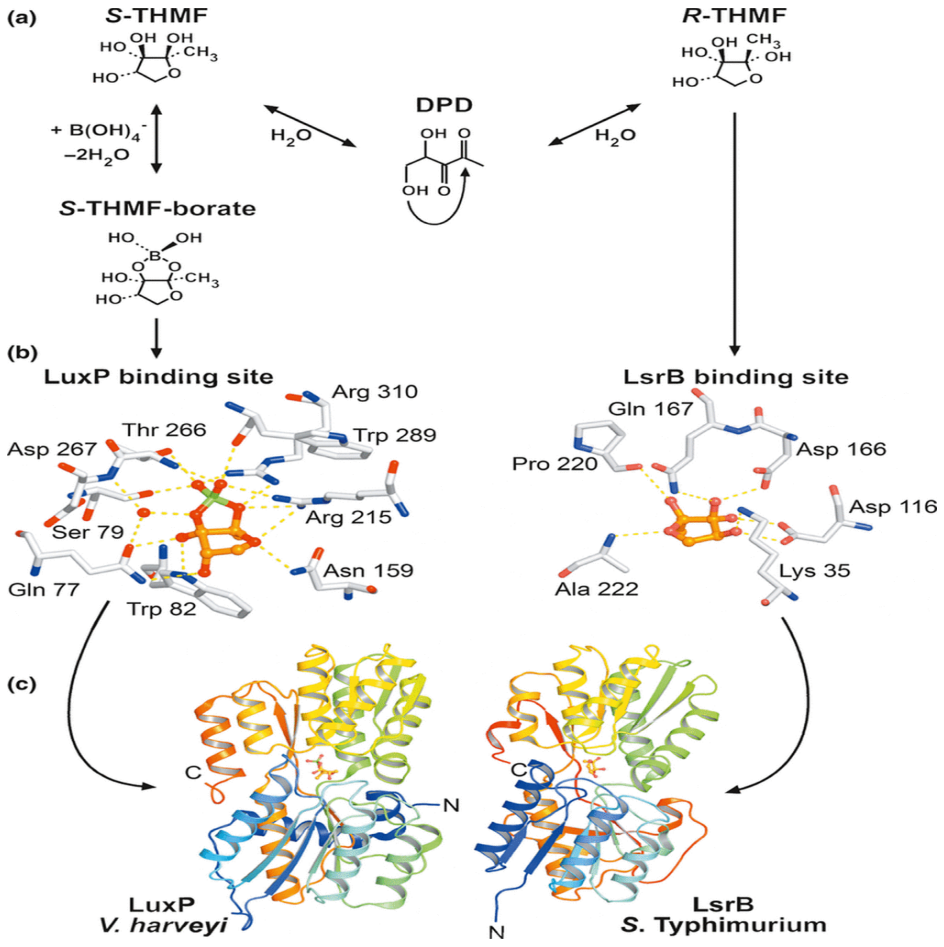

Figure 4: Molecules and receptors involved in AI-2 signaling. (Pereira, Thompson, & Xavier, 2013)

(a) S-THMF and R-THMF (identified by *S.Typhimurium*) are synthesized by spontaneous cyclization and hydration from LuxS product DPD which occurs in solution. S-THMF further reacts with boron (when boron is available) to form S-THMF-borate in the existence of boron (Vibrio harveyi detects this molecule). (b) Different interactions coordinate these AI-2 ligands (salt bridges or hydrogen bonds, shown by dashed lines of yellow color) with distinct amino acid molecules at their corresponding binding locations at the receptor: LsrB which contains R-THMF from *S. typhimurium* and LuxP which contains S-THMF borate that comes from the bacterium *V. harveyi*. These two receptor's design from the two bacteria, LuxP and LsrB are very similar and they are (c) presented here by ribbon diagrams which are colored in rainbow order that makes the visualization from the N-terminal to the C-terminal much easier. (Pereira, Thompson, & Xavier, 2013)

1.4 AI-2 Involved in Signaling as well as Metabolism

As demonstrated below, autoinducer molecule AI-2 has been shown to play a clear part in quorum sensing in some species of bacteria (Table 1). Since LuxS is both AI-2 synthase and an enzyme in AMC (Fig. 3), a vital key metabolic pathway, it is equally feasible that this molecule is simply a metabolic byproduct in other species. LuxS inactivation may lead in alterations in gene expression as a result of faulty signaling, metabolism of methionine, recycling of homocysteine, or accumulation of SAM metabolism intermediates. Therefore, it is highly essential to discriminate between these metabolic and signaling flaws in order to determine whether AI-2 is a genuine signaling molecule or it is solely a product of metabolism produced alongside other products in a specified species. One of the key investigational strategies for distinguishing among the luxS deletion's dual effects is the method of signal complementation. (Winzer et al., 2003; Hardie & Heurlier, 2008; Vendeville et al., 2005). Due to the recent development of several organic synthesis techniques for DPD (Meijler et al., 2004; Frezza et al., 2005; Smith et al., 2009; Kadirvel et al., 2010; Ascenso et al., 2011), and this product is nowadays commercially accessible (from Omm Scientific), thus performing signal complementation much easily.

In different cases, the application of preparations of pure DPD to complement defects in luxS mutant bacteria has effectively proved the function of AI-2 as a molecule for quorum sensing. Table 1 summarizes these research and reports in which the sensing mechanism for AI-2 was elucidated. Table 1 contains the details of further research in which faults were detected when luxS was disrupted, but the connection with modified signaling might not have been demonstrated definitively.

Although LuxS enzyme is only a part of the AMC in some species, the autoinducer AI-2 molecules which are produced by this enzyme, could be used as an information source by other bacterial species who live in that environment alongside the AI-2 producing bacteria, demonstrating the difficulty of ascertaining how AI-2 and LuxS influence behavior of bacteria at both species level and community level.

Past the discussion about classifying AI-2 as a signaling molecule or metabolite, there may be other consequences for the link between production of AI-2 and SAM. Living organisms of all type uses SAM as the principal donor of methyl group needed for cell growth, development and chemotaxis in many vital methylation responses. It also donates several different chemical groups

that are essential for a multitude of cellular functions; for instance- phospholipid and vitamin biosynthesis (Parveen & Cornell, 2011; Fontecave et al., 2004). Since AI-2 is a result of SAM metabolism, it is a viable candidate which can provide information about the cell's metabolic status. (Winans, 2002; Xavier & Bassler, 2003). Furthermore, the widespread utilization of SAM in methylation reactions results in a steady manufacturing of SRH. SRH is the substrate which is responsible for LuxS reaction. This provides a way for AI-2 manufacturing at a very low cost, thus allowing analysis of cellular activity economically (Winans, 2002; Keller & Surette, 2006; Xavier & Bassler, 2003; Winzer et al., 2003). It is striking that SAM can be used as a precursor to synthesize multiple autoinducers alongside AI-2. As illustrated in Fig. 3, SAM also is a chemical moiety used for AHL and CAI-1 synthesis (cholera autoinducer-1) in spite of them having a dissimilar chemical arrangement. It may not be a coincidence that all of these three classes of autoinducers are SAM derivatives, and it is very appealing to postulate that the cause behind this prevalent association is the benefit of incorporating core metabolism along with QS.

Table 1. Autoinducer AI-2 Mediated Functions

Species	AI-2 Mediated Functions	Receptor of Ai-2	Reference
Vibrio harveyi	1.Bioluminescence, 2.colony morphology, 3.siderophore production, 4a.biofilm formation, 4b.type III secretion and 5.metalloprotease Production	LuxP	Bassler et al. (1993, 1994), Lilley & Bassler (2000), Chen et al. (2002), Mok et al. (2003), Henke & Bassler (2004a, b), Waters & Bassler (2006)
Vibrio cholerae	Biofilms, protease and virulence factor production, and competence	LuxP	Jobling & Holmes (1997), Miller et al. (2002), Zhu et al. (2002), Hammer & Bassler (2003), Antonova & Hammer (2011)
Escherichia coli EHEC	Chemotaxis towards AI-2, motility and HeLa cell attachment	LsrB‡	Bansal et al. (2008)
Escherichia coli K12	Biofilm formation and motility† AI-2 incorporation and chemotaxis towards AI-2 LsrB	LsrB‡ LsrB	Xavier & Bassler (2005a), Gonzalez Barrios et al. (2006), Hegde et al. (2011)
Actinobacillus pleuropneumoniae	Biofilm formation†, adherence to host cells and growth in iron-limited medium	Unknown	Li et al. (2011)
Borrelia burgdorferi	Increased expression of the outer surface lipoprotein VlsE†	Unknown	Babb et al. (2005)
Aggregatibacter actinomycetemcomitans	Biofilm formation	LsrB and RbsB	Shao et al. (2007a,b)
Actinomyces naeslundii and Streptococcus oralis	Mutualistic biofilm formation	Unknown	Rickard et al. (2006)

Haemophilus influenzae strain 86-028NP	AI-2 incorporation and biofilm formation	RbsB	Armbruster et al. (2011)
Mycobacterium avium	Biofilm formation†	Unknown	Geier et al. (2008)
Pseudomonas aeruginosa	Production of Virulence factor	Unknown	Duan et al. (2003)
Helicobacter pylori	Motility	Unknown	Rader et al. (2007), Shen et al. (2010), Rader et al. (2011)
Moraxella catarrhalis	Biofilm formation and antibiotic resistance†	Unknown	Armbruster et al. (2010)
Staphyloccocus aureus	Capsular polysaccharide gene expression and survival rate in human blood and macrophages	Unknown	Zhao et al. (2010)
Salmonella enterica ssp. enterica serovar Typhimurium	Pathogenicity island 1 gene expression and invasion into eukaryotic cells AI-2 incorporation	LsrB‡ LsrB	Taga et al. (2001, 2003), Miller et al. (2004), Choi et al. (2007, 2012)
Sinorhizobium meliloti	AI-2 incorporation	LsrB	Pereira et al. (2008)
Streptococcus anginosus	Susceptibility to antibiotics	Unknown	Ahmed et al. (2007)
Staphylococcus epidermidis	Expression of phenol-soluble modulin peptides, acetoin dehydrogenase, gluconokinase, bacterial apoptosis protein LrgB, nitrite extrusion protein and fructose PTS system subunit	Unknown	Li et al. (2008)
Streptococcus intermedius	Haemolytic activity, biofilm formation and susceptibility to antibiotics	Unknown	Ahmed et al. (2008, 2009)
Streptococcus pneumoniae	Biofilm formation	Unknown	Vidal et al. (2011)
Streptococcus gordonii and *Streptococcus oralis*	Mutualistic biofilm formation Unknown Saenz et al. (2012)	Unknown	Saenz et al. (2012)

1.5 Interspecies Interactions

LuxR-type transcription factors comprise of two domains, a signal-binding N-terminal domain and a DNA-binding C-terminal domain. Simple homology searches cam be perfromed in order for identifying LuxR homologues from genomic sequences. A Search of the genome of *Salmonella typhimurium* disclosed the first instance of what is now called LuxR homologues orphan (Ahmer et al., 1998) or solo (Subramoni & Venturi, 2009) (Fig. 5A). The *S. typhimurium* genetic sequence has a luxR counterpart called sdiA which lacks a luxI gene and AHLs (Ahmer et al., 1998; Smith & Ahmer, 2003) is not produced by *S. typhimurium*. Many Gram-negative microbes have luxR-type genes and lack luxI-type genes, and many more Gram-negative bacteria have more luxR-type genes than genes of luxI-type. In *S. typhimurium's* situation, SdiA reacts to AHLs generated by other bacteria, resulting in particular genes being activated. *Escherichia coli* also has a sdiA gene. The *E. coli* SdiA has been reported to respond to AHLs and mammalian host- produced small molecules *E. coli* SdiA was reported to react to tiny molecules generated by mammalian host and AHLs. (Nguyen, et al., 2015; Hughes et al., 2010). These latter trials given the first indication that some LuxR orphan homologues could be engaged in directly sensing the host environment instead of serving as QS signal receptors (Fig. 5a).

Some orphan LuxR proteins can react to self-produced AHLs, such as QscR in the opportunistic pathogen *P. aeruginosa* (Chugani & Greenberg, 2014), while others are responding to signals that are self-produced non AHL signals. Two instances of the latter include Photorhabdus genus members. One species utilizes a LuxR orphan protein to identify α-pyrones produced by itself, whereas the other identifies dialkylresorcinols and cyclohexanediones produced by itself (Whiteley, Diggle & Greenberg, 2017) (Fig. 1b). Finally, in some plant-associated bacteria, there is a group of orphan LuxR homologues that activate the transcription of particular genes in reaction to the plant's tiny molecules (Schaefer, et al., 2016). The origin of such tiny molecules continues to be elusive and the plant microbiota may actually produce them. The LuxR orphan counterparts and our current understanding of them are bringing this study area to an interface with progress in microbiome studies, and findings of this subset of LuxR counterparts are a quickly evolving area (Whiteley, Diggle & Greenberg, 2017).

Developments in mammalian gut microbiome studies are starting to show how QS can affect the structure of microbiome species, how QS study could lead to ways of controlling infectious diseases including cholera, and how host bacteria could develop processes to regulate bacterial QS and thus transform their microbiomes. For instance, it has lately been shown that the AI-2 molecule generated by many bacterial species promotes Firmicutes colonization of the gut over Bacteroidetes, and commensal bacterium's production of Al-2 can restrict *Vibrio cholerae* infections (Bäumler & Sperandio, 2016). Even though the relationships of mamalian guts bacteria are known to be more complicated than the two-partner squid symbiosis, it becomes apparent that the participation of QS in these microbiomes can provide a chance to intervene in intestinal dysbiosis (Whiteley, Diggle & Greenberg, 2017).

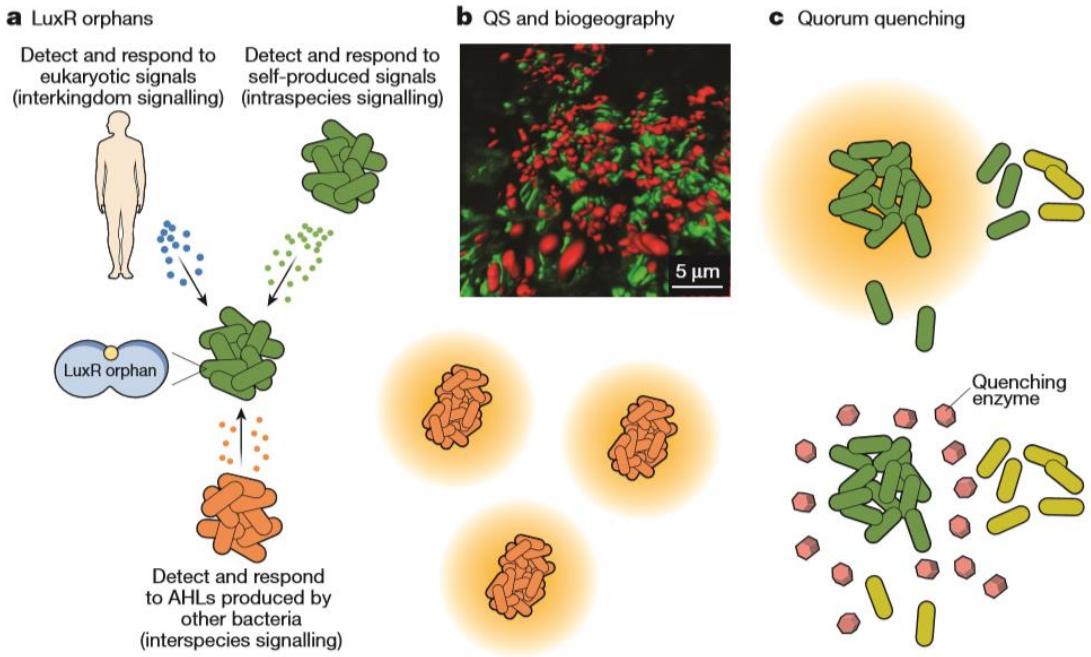

Figure 5: Factors that affect QS in natural environments.

a, LuxR orphans (solos) can detect and respond to signals produced by eukaryotic hosts, self, or other microbial species. b, Top, aggregates of two *P. aeruginosa* strains (green, strain PA14; red, strain PAO1) in synthetic cystic fibrosis sputum109. Bottom, diagram of *P. aeruginosa* aggregates (three clusters of red rods) spatially separated and socially isolated (yellow halos represent QS signals and QS-controlled exoproducts). c, Top, diagram of a high-density aggregate of QS positive cells (green) secreting a diffusible QS signal into the surrounding environment (orange halo). The signal can activate QS in nearby cells (green) but not in more distant cells (yellow). Bottom, quorum quenching (orange hexagons), either by the action of enzymes or due to environmental conditions, degrades the QS signal and limits the ability of an aggregate to induce QS in nearby cells.

1.6 Quorum Sensing, Biofilms and Biogeography of Microbes

Biofilms are described as bacterial clusters of high density that are most of the times connected to surfaces and embedded in a polymeric matrix of extracellular nature (Costerton, 1987). Compared to their planktonic (free-living) peers, biofilm cells are much different with several distinctive characteristics, such as having increased resistance to antibiotics. From 1980 to 2000, significant research regarding biofilms in nature were conducted and this encouraged the establishment of many experimental laboratories. These systems were leveraged by several organizations to conduct studies on QS in biofilms. One popular laboratory biofilm model is the flow-cell (Crusz, et al., 2012), where a *CLSM* (confocal laser scanning microscopy) is used to generate images of biofilms that are growing on glass coverslips which cover small channels. To provide nutrition to the biofilms, a nutrient medium is continually pumped through these channels. Several groups of researchers have demonstrated using flow-cell technologies that QS can affect the development of biofilms and the ability of the biofilm community to resist antimicrobial treatments as well (Parsek & Greenberg, 2005). QS is critical to constructing a biofilm in some instances, whereas in other instances it is essential to dismantle the biofilms. The discovery of connection between QS and the development of biofilm resulted in a wide range of research to evaluate how microbial social behaviors influence this significant method of growth. However, soon it was found that this connection relies on environmental conditions. Without any doubt it can be said that environmental signals and intercellular communication interact with each other. This gives rise to another question- whether this interaction between biofilms and quorum sensing is only present in a flow cell or this interaction is even possible in the natural ecosystem?

Three-dimensional arrangement of biofilm in flow cells does not always replicate the structure and composition of a biofilm in natural habitats (Bjarnsholt, 2013; Roberts, Kragh, Bjarnsholt, & Diggle, 2015). Truly, there are a few normally existing microbial biofilms that are made up of micrometer-sized, high-density aggregates that contain hundreds to thousands of cells. The spatial organization of these micrometre-scale aggregates are highly remarkable and for the microbial communities own fitness this biogeography is extremely important (Stacy et al., 2014). As the biofilms grown in flow cells share a lot of traits with natural aggregates such as increased antibiotic resistance, scientists hypothesized that quorum sensing is associated with aggregate formation. Several bacteria have been studied such as *E. coli*, *Burkholderia thailandensis, Pantoea ananatis,*

Rhodobacter sphaeroides and these studies support this hypothesis. In both cases of *R. Sphaeroides* and *P. ananatis*, it was observed that creation of large aggregates is inhibited by quorum sensing and the mechanism of this process is unidentified. On the other hand, quorum sensing encourages large aggregate formation in case of *B. thailandensis* and *E. coli*. In *E. coli*, chemotaxis of internal cells towards aggregates that produce autoinducer AI-2 promotes aggregate development (Laganenka, Colin & Sourjik, 2016).

While QS tends to serve some part in formation of aggregate, it is uncertain whether it is essential for precise spatial arrangement of aggregate and how the aggregate formation affects QS. In order to get answers of all these concerns, the behaviors which are controlled by quorum sensing and how these behaviors are related to aggregate size must be understood along with the effective distance of signals which an aggregate produces. Over the previous 10 to 15 years, the elucidation of the amount of cells needed to achieve a quorum has been constantly pursued. According to predictions of the QS hypothesis, even if single bacterial cells are confined in extremely small aqueous volumes like femto to pico-liter volumes, evidence has been found that quorum sensing can be sensed by single cells. However, it was not possible to discover any possible benefit of single cell QS (Boedicker, Vincent, & Ismagilov, 2009; Carnes, et al., 2009). Moreover, a major drawback of using these confined systems is that there is no transfer of nutrients from outside and as a result the proper growth of bacteria is lacking here. With the continued development of technologies in laser printing and microfluidics, it is now feasible to confine small numbers of bacterial species in hydrogel traps of pico-liter-size and it has allowed to reveal previously unwanted questions. There has been a study conducted using *V. harveyi,* where bacterial cells were trapped and 25 microliter diametered aggregates showed QS of robust scale whereas the ones with diameter below 10 microliter demonstrated poor QS-controlled gene expression (Gao, et al., 2016). Similar findings have been noted for P. aeruginosa, contained in microscopic ' lobster traps' for bacteria and these traps were produced in micro-3D printers. It has been shown that as few as 500 P. aeruginosa cells generate the QS-controlled pyocyanin exo-product when restricted within eight-pico-liter pits, suggesting that in an open system aggregates of this magnitude can initiate social behaviors (Connell, Kim, Shear, Bard, & Whiteley, 2014).

The subsequent stage of knowing the communication concerns among bacteria is whether communication between aggregates is possible or not. How far can two aggregates be from each

other and still communicate? What could be the distance of calling QS signals? Multiple theoretical surveys were conducted to answer this point and the overall view is that interaction is unlikely at ranges higher than 10–100 μm (Boyer & Wisniewski-Dyé, 2009; Decho, Frey, & Ferry, 2011). An empirical research provided a random collection of experiments to evaluate communication among spatially structured aggregates (Connell, Kim, Shear, Bard, & Whiteley, 2014). Laser-printing was used to trap aggregates of *P. aeruginosa* and these aggregates were separated by a distance of 8 μm. How aggregates of separate sizes were able to interact amongst themselves through AHL QS was evaluated. Signaling over this range of 8-μm would necessitate signal-generating aggregates containing a minimum of 2,000 cells. Though aggregates of *P. aeruginosa* exceeding 2,000 bacterial cells have been seen in natural habitats, including the aggregates found in chronic infections (Bjarnsholt, 2013; Kragh et al., 2014), most of the aggregates are usually smaller in populations and are often located more than 8 μm apart. These information indicate that QS of *P. aeruginosa* may mainly operate as an intra-aggregate communication method in some settings. Squid and *V. fischeri* have a symbiotic relationship and this example offers a distinctive chance to answer the queries of inter-aggregate signaling of a host-associate in a natural habitat. According to many researchers there is still much to be learned about how biogeography on a micrometer scale impacts bacterial relationships. This will be extremely important as scientists are starting to manipulate microbial populations by influencing quorum sensing like the gut microbiome of humans whether by a chemical approach or a probiotic method (Whiteley, Diggle, & Greenberg, 2017).

1.7 Quorum quenching in nature and as a therapy

Given that large numbers of natural microbial colonies are poly microbial and spatially organized, it is essential to reflect on how the evolution method of signaling is being shaped by ecological relationships between species. Fundamental aspects involves abiotic and biotic components disrupting QS by signal degradation (Fig. 5c), a procedure known as quorum quenching89. Signal deterioration may lead from environmental chemical features, like pH, or the activity of microbial or animal-produced enzymes. Two major kinds of AHL-degrading bacteria, lactonases and acylases were defined in the latter scenario. Lactonases hydrolyze an AHL's HSL ring to produce the respective acyl homoserines (Dong, et al., 2001), while the AHL amide bond is cleared by

acylases to create the respective fatty acid and homoserine lactone (Lin, et al., 2003). Considering the complexity of spatial framework and signal calling distance, establishing the consequence of quorum quenching on natural microbial colonies continues to be a major challenge for the upcoming time. For instance, are quorum quenching enzymes generated for the purpose of competition, collaboration or the personal advantage of cell production? Lactonases (the enzyme family of paraoxonase (PON), well known for their capacity of hydrolyzing organophosphate toxins and low quantity lipoproteins, being possessed by human is also remarkable (Li, Wang, Mulchandani, & Ge, 2016). The activity of PON lactonase is regarded as the inherited act and Drosophila PONs are emerged to perform host protection. Such enzymes may contribute to the host with a way of microbiome manipulation by modulating microbial social interactivity (Elias & Tawfik, 2012; Li, Wang, Mulchandani, & Ge, 2016).

Despite the real tasks of quorum quenching enzymes, it represents an appealing and developing way to the increasing amount of antimicrobial-resistant diseases. A latest study assigned by the UK Government guessed that antimicrobial resistance (AMR) could stimulate an extra 10 million fatalities per year by 2050, and an increasing loss of US$ 100 trillion to the world's GDP96. Instead of destroying pathogens, Quorum quenching enzymes and other QS-blocking methods obstruct the generation of virulence agents and these anti-virulence factors have been suggested to transmit reduced selective forces leading to the growth of impenetrable mutants (Allen, Popat, Diggle, & Brown, 2014) than standard antibiotics. The foundation for QS obstruction as a therapeutic proposition belongs to the 1990s, when an Australian macro-algae *Delisea pulchra* generated brominated furanone was shown to antagonize AHL-controlled phenotypes in a number of bacterialspecies (Givskov et al., 1996). Halogenated furanones have been shown to be efficient in vitro, leading to clearance of *P. aeruginosa* from infected mice's lungs (Hentzer et al., 2003). A number of tiny molecule blockers of AHL QS have been recognized in subsequent research, many of which are found to have powerful activity in laboratory culture as compared to several numerous bacterial germs (Givskov et al., 1996; Hentzer et al., 2003; Christensen, Grove, Booker, & Greenberg, 2013; Starkey, et al., 2014).

What illnesses can QS inhibition treat? Doubtlessly, we want to be capable of treating maladies that are not solved through present treatments. The persistent *P. aeruginosa* lung infections that afflict individuals with inherited disease cystic fibrosis have been an attractive objective and

focusing on persistent *P. aeruginosa* infections is a clear illustration of basic study and therapeutic improvement being interdependent and moving forward in parallel. These persistent diseases are not solved by antibiotics, and there are bacterial accumulation in the persistent microbial colonies observed in the lungs of individuals affected with cystic fibrosis (Kragh, et al., 2014). As QS triggers a battery of *P. aeruginosa* virulence agents and *P. aeruginosa* QS mutants decreased virulence in animal models, LasR, the 'master' QS signal recipient became the focal point of tiny molecule blocker screens (Oloughlin, et al., 2013; Gerdt, Mcinnis, Schell, Rossi, & Blackwell, 2014). Further ecological studies have demonstrated that many large numbers of patients harbor mutants of *P. aeruginosa* LasR (Bjarnsholt, et al., 2010). These ecological researches were certainly disheartening, though further analysis indicated that majority LasR mutant CF isolates have adopted a second QS system, the RhlR system, to substitute LasR in ways which are ambiguous (Feltner, et al., 2016). Maybe a RhlR blocker or a blocker aiming at both RhlR and LasR, could be a suitable treatment for cystic fibrosis, and one has been noted in recent days (Yang, et al., 2005). The moral is that to understand how, when or what to aim with a QS inhibitor, we need ongoing fundamental research about QS in natural environments.

CHAPTER 2

Methodology

2.1 *E. coli* Bacterial Strains and Their Phages

E. coli strains were collected from the environmental water samples and *E. coli* strain 0157:H7 and Shiga-toxin producing *E. coli* (STEC) strain was collected from BRAC Universities Mathematics and Natural Sciences Departments Laboratory. These *E. coli* strains were used in the plaque assays and also for isolation and culture of *E. coli* phages from the environmental water samples. The water samples were collected on a routine basis from different points of Buriganga River, Turag River and from Gulshan Lake. These water samples were quickly taken into our laboratory at Mohakhali and filtered to remove the dirt and debris from the water. For growth and isolation of bacterial cells, the sample waters were filtered with Whatman filter paper and 5 ml of filter water was mixed with 5 ml LB and placed in shaker incubator at 37 degrees Celcius and subjected to shaking at 120 rpm for 4 hours. After four hours of incubation 300 microliters of bacterial solution was taken and it was serially diluted to 10^{-7} times. From these diluted bacterial solutions 300 microliters sample were spread on Macconkey agar plates at different concentrations. From the different plates, the number of bacteria obtained after overnight incubation was counted and a few of them were stocked from each sample. On the other hand, to isolate phages from the environmental water samples, the filtered water was further filtered by a 0.22-micron syringe filter to exclude all the bacteria. *E. coli* host cells were cultured to reach their log phase and these young culture cells were mixed with 5ml the syringe filtered water. The mixture was placed in the shaker incubator for four hours at 37 degrees Celcius and subjected to shaking at 120 rpm for the enrichment of the phages. To check the presence of phage in the enriched mixture, soft agar plaque assay was used where presence of phages leads to clear zones in the lawn commonly known as a plaque. The phages obtained was then later further enriched and stocked.

2.2 Soft Agar Plaque Assay

In order to quantify the number of phage particles we have used the soft agar plaque assay method which was previously described by Faruque et al. (Faruque, et al., 2005). Suitable indicator strains have been used in this process. To measure the susceptibility of various *E. coli* bacterial strains to their corresponding bacteriophages, nascent bacterial cells were grown (cells grown in fresh LB

for 3 to 4 hours) in a shaker (around 160 rpm) at 37°C. Then 500 µl of each nascent bacterial strain were taken and mixed with soft agar of 3.5ml volume (soft agar contains 0.8% Bactoagar, Difco along with Nutrient broth). After that, 100 µl phage solution was added to the previous mixture which contained around 3.2×10^2 phage (it was determined previously using *E. coli* strain 0157:H7 as the indicator strain) and the mixture was laid quickly on top of a Luria agar plate before the soft agar solidified. The culture plates were then incubated overnight at 37°C. After incubation the number of plaques were counted to measure the degree of phage susceptibility of the different bacterial strains. The different number of phage plaques counted on the different soft agar plates indicated the varying degrees of phage susceptibility of the different strains which were grown either in the presence or absence of autoinducers.

2.3 Preparation of Spent Media containing Autoinducers

Vibrio cholerae cultures containing cloned cqsA gene which codes for CAI-1 synthase or another gene luxS that codes for AI-2 synthase were used to prepare the spent medium containing CAI-1 and AI-2. These genes are present as recombinant constructs in the form of a plasmid. To be more precise, pJZ176 holds the cqsA gene inside it and on the other hand pJZ346 carries the luxS gene under the control of a IPTG inducible lac promoter which is cloned in pTAC and all of these have been previously discussed by another author (Higgins, et al., 2007). To prepare the spent medium in laboratory, we cultured the bacterial cells overnight and afterwards that overnight culture of the recombinant *E. coli* was diluted 100 times in a fresh LB medium containing Ampicillin (50 µg/mL) and then it was sub-cultured for another 3 to 4 hours (until OD_{600} =0.5 to 1). Cells were precipitated using centrifugation for 15 minutes at 4500 xg and the supernatant liquid was disposed and then the pellets were resuspended in fresh LB medium without any antibiotics. Afterwards the culture flask was incubated in a shaker at 37 degrees Celsius and allowed to grow for another 6-8 hours. Then the liquid was divided into aliquots and subjected to vigorous centrifugation for about 20 minutes at 6000 xg to precipitate the cells. Then the culture supernatant which contained the autoinducer was taken in a syringe and filtered via a 0.22 micron syringe filter to remove all the bacterial cells. The filtrate contained autoinducer without any bacterial cells.

2.4 Phage Adsorption Assay

The adsorption of separate bacteriophages to *E. coli* strain 0157:H7 was studied. For this purpose, the bacterial strain was grown under different conditions- i.e- either in the presence or absence of autoinducers and later subjected to the experiment which has been formerly used and discussed by Tu and colleagues (Tu, Long, Svenningsen, Wingreen, & Bassler, 2010). To perform this experiment first *E. coli* strain was cultured overnight in LB broth medium and then it was diluted 100 times in fresh LB medium which contained 50% (v/v) spent autoinducer medium. Autoinducer CAI-1 and autoinducer AI-2 were used for this experiment and the cells were grown to reach the log phage of their lifecycle. Around 2.5×10^8 bacterial cells in liquid broth was mixed with a lower number of phages (around 10^7 pfu) to check the absorption profile of the phages to indicator bacterial strains. As control for this experiment, LB liquid broth was mixed with the phages without any indicator bacterial strain. The parallel mixtures were then kept in incubator at 30°C for no more than 20 minutes. Then to determine the number of phages that were not adsorbed, the bacterial mixture was centrifugated to precipitate the bacterial cells and the supernatant liquid was filtered via a 0.22 micrometer syringe filter. The filtrate contained only the non-adsorbed phages and to determine their number they were undergone serial dilution and the diluted samples were then plated on the indicator strains lawn. The plates were then incubated at 37°C for 16 hours and then the number of phage plaques were counted.

2.5 Assessment of Phage Resistance of Bacteria

The incidence of emergence of phage resistant bacterial strains which are occurring due to the presence or absence of autoinducers during their growth has been measured by a procedure which was described by Zahid et al. (Zahid, et al., 2008). At first, the *E. coli* strains were grown overnight and then diluted 100 times in fresh LB medium containing 50% (v/v) spent culture medium which either had autoinducer AI-2/CAI-1 or no autoinducer at all . To produce the autoinducers recombinant *Vibrio cholera* strains were cultured to their log phase and the method is described above. The bacterial cells were precipitated by centrifugation and further washed using LB to remove any leftover protease. The cells were then re-suspended in LB and then placed on a nylon membrane filter paper. This filter paper was then placed on top of a LB agar medium and incubated

at 37°C for 6 hours. Afterwards, when the small bacterial colonies became visible, the nylon membrane filter was removed and then placed upside down on top of a phage inoculated LB agar medium to learn about the nature of the bacterial colonies. The agar plate had a very high concentration of phage, around 10^9 phage particles per plate. This type of phage inoculated plates is called phage challenge plates. The nylon membrane was then removed and the phage inoculated plate which now has the bacteria from the membrane filter is now incubated overnight at 37°C. As a result, the bacterial strains which were phage sensitive either got lysed by the phages or could not proliferate due to the phages, but the phage resistant strains proliferated and formed large colonies on the plate. To ensure that the bacterial colonies were not a result of phage inactivation by protease enzymes produced by the bacteria, the representative bacterial strains were taken and sub-cultured and retested by growing them on a high concentration of phages to further confirm the phage resistance (Zahid, et al., 2008; Hoque, et al., 2016).

CHAPTER 3

Results

4.1 Autoinducer AI-2 Enhance Phage Resistance of *E. coli*

In order to analyze the effects of autoinducers AI-2 and CAI-1 on *E. coli* bacteria directly regarding their phage susceptibility, soft agar plaque count method was used on the lawn of the host *E. coli* bacteria. The *E. coli* bacteria were grown with exogenous autoinducers. These autoinducers were produced by recombinant bacteria grown in liquid LB broth and then filtered spent culture media was used alongside fresh LB media to culture host bacteria for lawn culture. Overnight cultures of host *E. coli* cells were diluted to 100 times and then they were mixed with 50% v/v spent culture medium containing autoinducers either AI-2 or CAI-1 which were produced by recombinant *V. cholerae* cells. These cells were grown to their log phage and only their young cells were taken for later assay. It was observed that treatment with autoinducer AI-2 by addition of spent media of recombinant *V. cholerae* to 50% v/v made the *E. coli* host cells much more resistant to their corresponding phage cells. The plaque counts found in lawn culture of untreated cells (control tested under completely identical conditions) and the cells treated with autoinducer CAI-1 showed huge number of plaques and it was significantly higher than that of the pretreated cells. Moreover, this resistance was not observed when the host was grown with the spent media of *V. cholerae* cells containing the empty vector. Furthermore, when the host was grown with the spent media of *V. cholerae* cells producing autoinducer CAI-1, there was no enhancement of phage resistance than the control grown in identical conditions. To further prove the results were due to the modulating effects of the autoinducers, mutant strains of *E. coli* incapable of quorum sensing can be further tested.

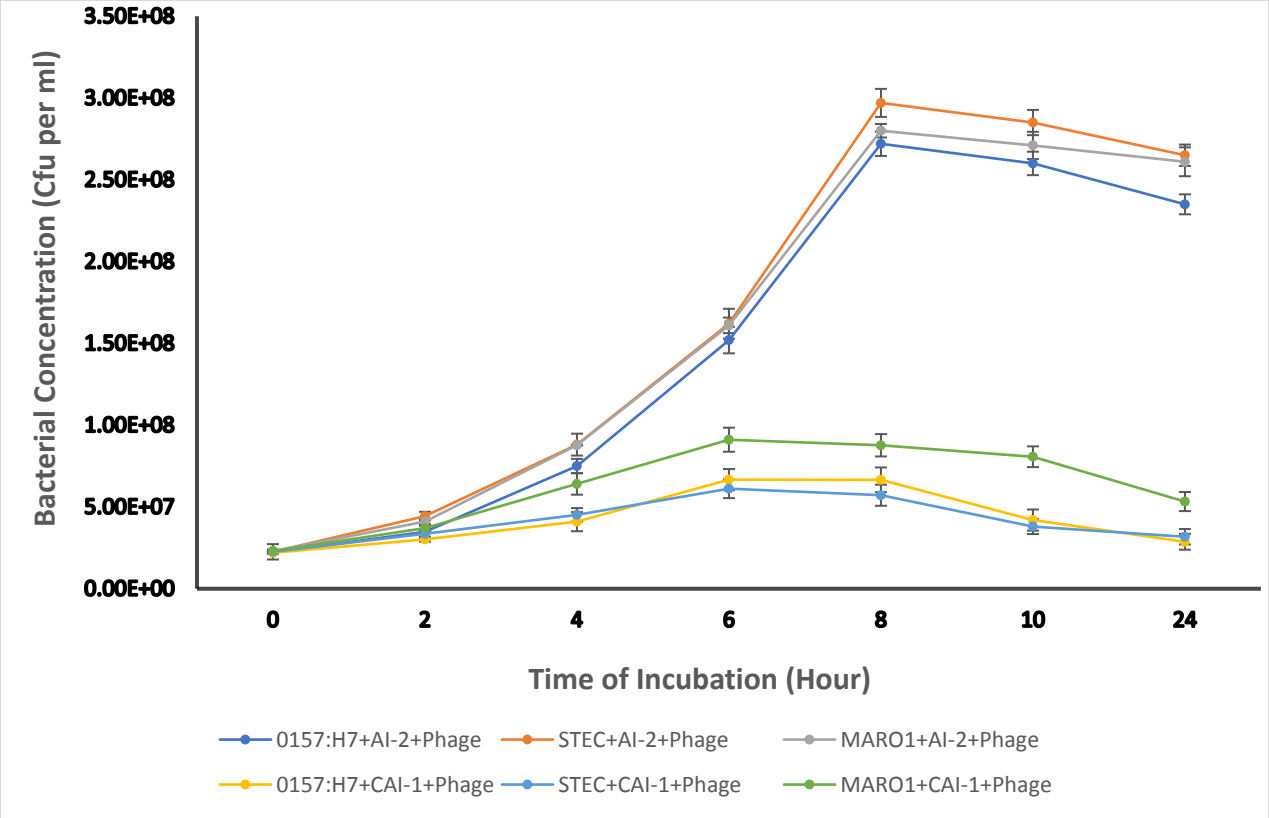

Figure 6: Effect of Autoinducers AI-2 and CAI-1 (Exogenous) on Phage-Bacterial Growth Kinetics where susceptible *E. coli* bacteria is co-cultured with its corresponding bacteriophage in terms of colony forming units (cfu) per ml

E. coli bacterial cells were cultured in liquid LB medium which was supplemented by exogenous autoinducer solution of autoinducer AI-2 and CAI-1. Autoinducer solution was prepared by syringe filtering recombinant *Vibrio cholerae* cells cultured in LB medium, parallel controls were also prepared. It was seen that autoinducer AI-2 significantly enhanced the survival rate of *E. coli* bacterial cells against its corresponding phages. On the other hand, autoinducer CAI-1 had little impact on resistance of parent *E. coli* strains.

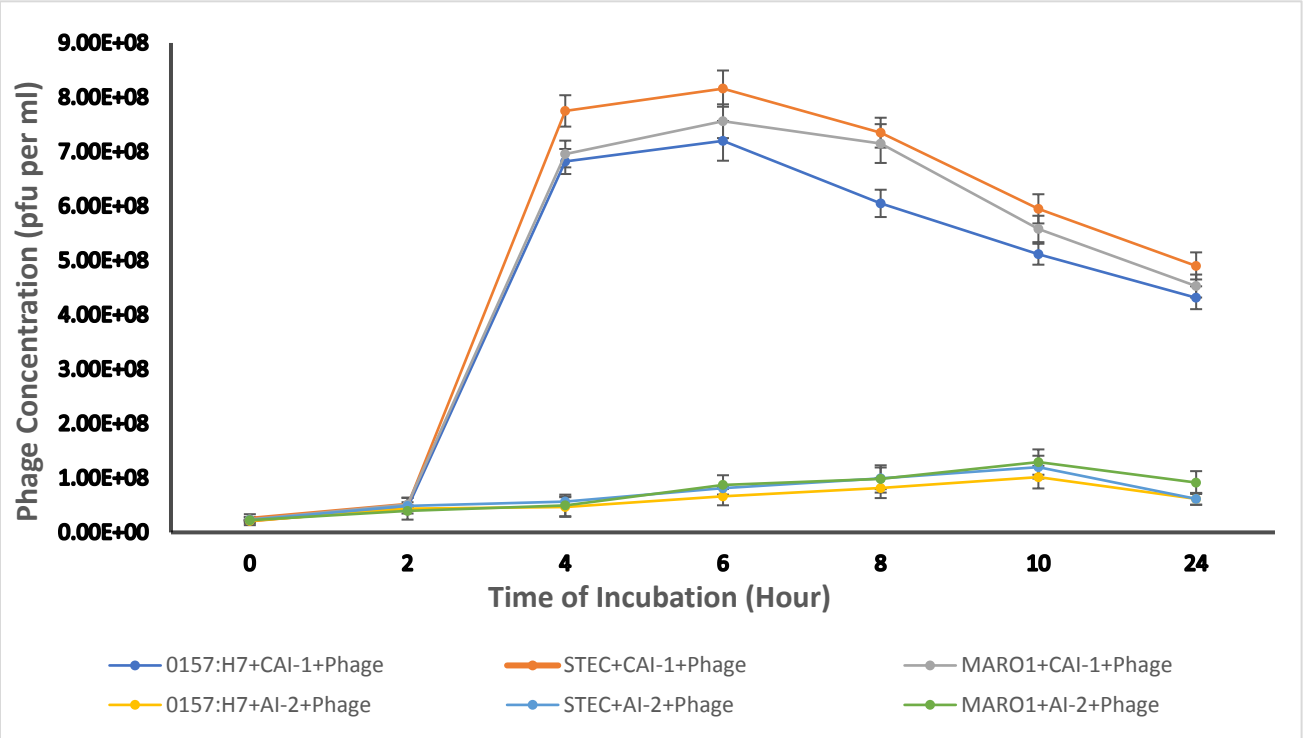

Figure 7: Effect of Autoinducers AI-2 and CAI-1 (Exogenous) on Phage-Bacterial Growth Kinetics where susceptible *E. coli* bacteria is cocultured with its corresponding bacteriophage in terms of plaque forming units (pfu) per ml

E. coli bacterial cells were cultured in liquid LB medium which was supplemented by exogenous autoinducer solution of autoinducer AI-2 and CAI-1. Autoinducer solution was prepared by syringe filtering recombinant *Vibrio cholerae* cells cultured in LB medium, parallel control were also prepared. It was observed that phage concentration is significantly reduced when grown in addition with autoinducer AI-2. However, in presence of autoinducer CAI-1 phage growth was not hampered.

4.2 Emanation of Phage Resistant *E. coli* Amplified by Quorum Sensing

The emergence of phage resistant bacterial cells was mostly due to the quorum mediated alterations in the bacterial cells and to prove that *E. coli* strain 0157:H7 and other environmental and reference strains were grown in the presence and absence of exogenous autoinducers and the rate at which the resistant strains emerged were calculated. For this purpose, an assay developed by Zahid et al. was used to measure the rate of emergence of phage resistant *E. coli* cells. It was found that growing *E. coli* cells in LB broth in the presence of exogenous autoinducers (through the addition of spent medium) produced resistant bacterial cells of the same strain at an extent which was far more than growing than in the absence of exogenous autoinducers. The frequency of finding resistant bacterial derivatives for *E. coli* strain 0157:H7 had been seen significantly high when they were grown in the presence of exogenous autoinducer AI-2 which was produced by *V. cholerae* strain 1545 (a mutant with a chromosomal integration AI-2 synthase gene). On the other hand, when the bacterial cells were grown in the presence of autoinducer CAI-1 or without any autoinducer, the rate of obtaining resistant derivatives was significantly lesser than cells pretreated with AI-2. As autoinducer CAI-1 is specific for *V. cholerae* it was considered as a negative control for *E. coli*. *V. cholerae* strain 1503 that produces AI-2 in low quantities produced resistant derivatives of *E. coli* much less than *V. cholerae* strain 1545 as it produces more than hundred times AI-2 compared to 1503 and it resulted in a huge difference visible in the naked eyes. Because of this huge difference strain 1545 was used to produce in these experiments.

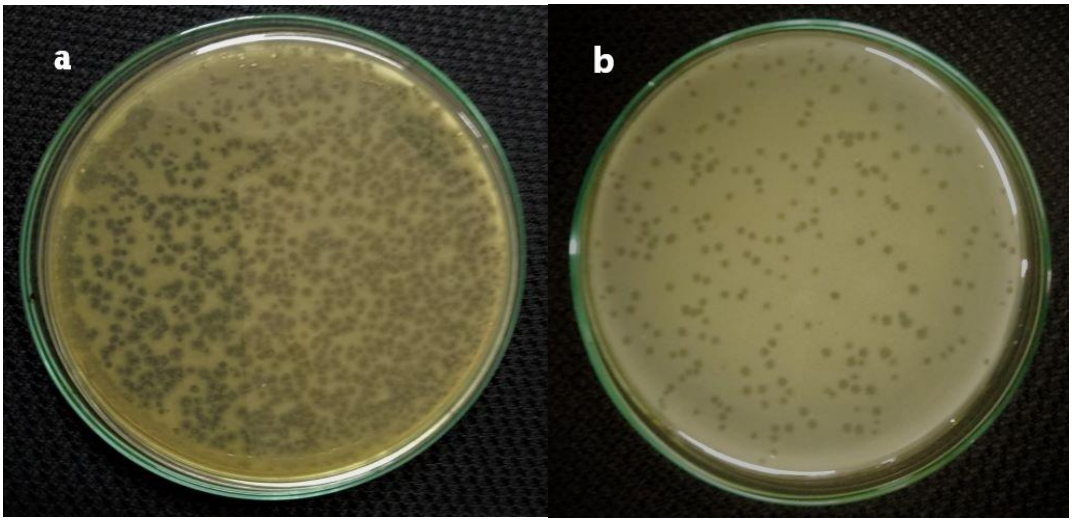

Figure 8: Plaques produced by *E. coli* bacteriophage on a lawn of *E. coli* strain 0157:H7 and its derivatives grown after autoinducer AI-2 treatment

a) Control plate

b) Autoinducer AI-2 treated plate

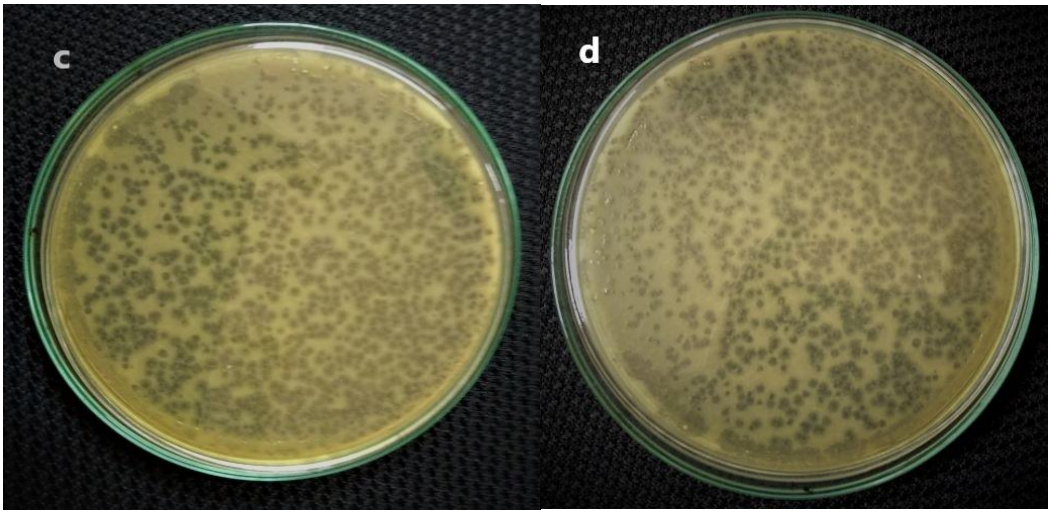

Figure 9: Plaques produced by *E. coli* bacteriophage on a lawn of *E. coli* strain 0157:H7 and its derivatives grown after autoinducer CAI-1 treatment

c) Control plate

d) Autoinducer CAI-1 treated plate

E. coli cells were grown to their logarithmic phage in LB broth in presence of 50% (v/v) spent culture medium supernatant of recombinant *V. cholerae* strains having genes for autoinducer AI-2 and CAI-1 synthesis and produce autoinducers either in huge quantities or do not produce autoinducers at all. These bacteria after growth to their log phage and not exceeding their young state, were mixed with a known titer of phage and then to produce a lawn culture they were overlaid on a LA plate. The resulting plaques found on the lawn of the bacteria treated with spent culture medium supernatant containing autoinducer AI-2 (b) and CAI-1 (d) and control plates a, c which showed plaques formed on the indicator bacterial strain that had been treated with spent culture medium supernatant of recombinant *V. cholerae* that do not produce autoinducer at all.

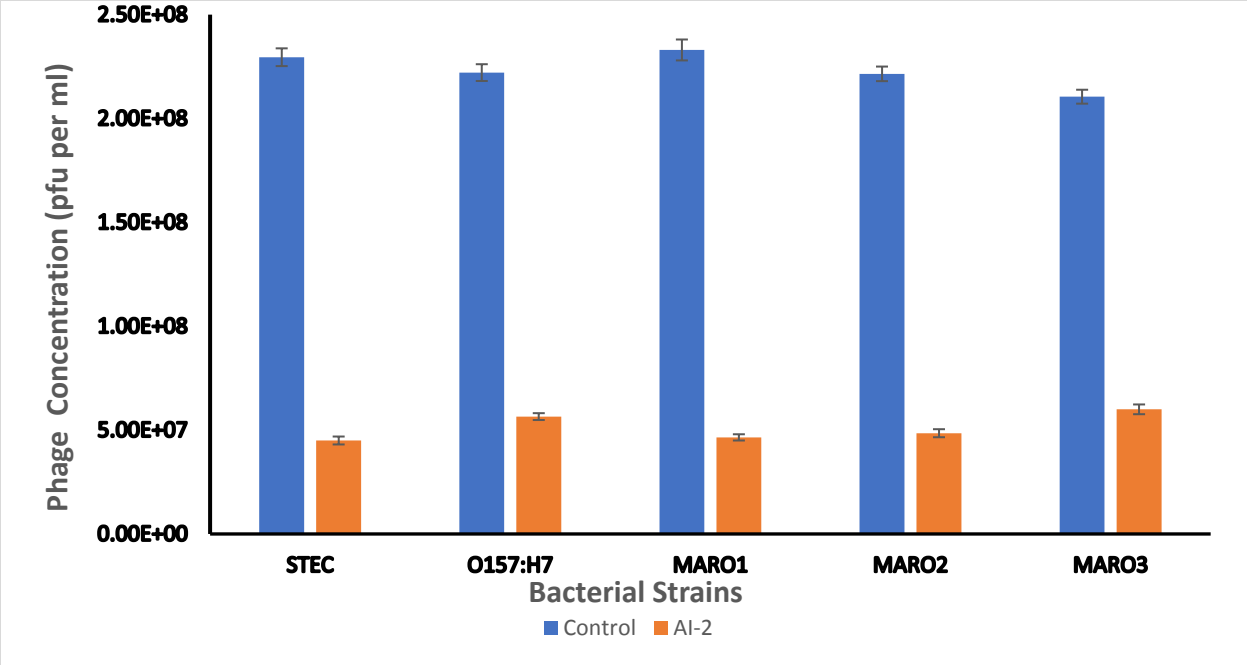

Figure 11: Effect of AI-2 on PFU (Plaque Forming units) per ml

E. coli cells were grown to their logarithmic phage in LB broth in presence of 50% (v/v) spent culture medium supernatant of recombinant *V. cholerae* strains which have genes for autoinducer AI-2. Parallel controls were also cultured and then both were tested via soft agar plaque assay. Subsequent pfus were then counted for the different bacterial strains. Here the blue columns represent control and yellow columns represent the AI-2 treated *E. coli*.

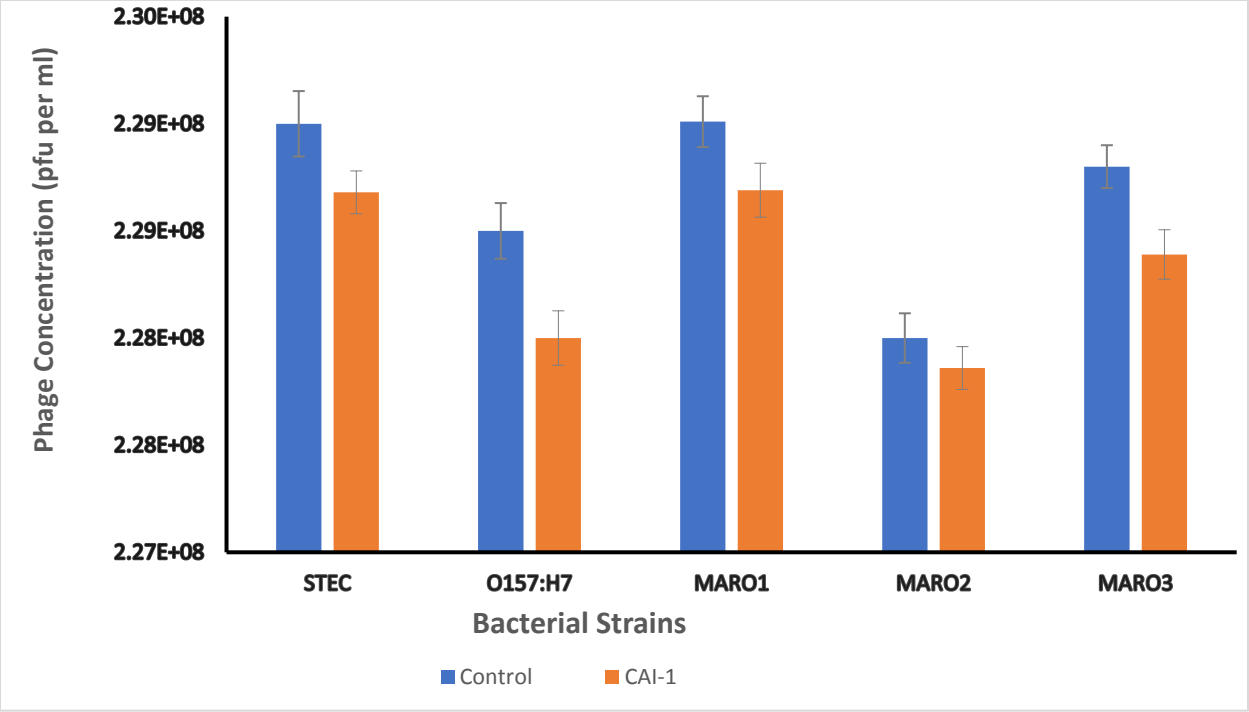

Figure 12: Effect of CAI-1 on PFU (Plaque Forming units) per ml

E. coli cells were grown to their logarithmic phage in LB broth in presence of 50% (v/v) spent culture medium supernatant of recombinant *V. cholerae* strains which have genes for autoinducer CAI-1. Parallel controls were also cultured and then both were tested via soft agar plaque assay. Subsequent pfus were then counted for the different bacterial strains. Here the blue columns represent control and yellow columns represent the CAI-1 treated *E. coli*.

4.3 Phage Adsorption on *E. coli* Cell Surface Affected by Autoinducer Treatment

Adsorption of phage on the surface of the host bacterial cells which is mediated by different receptors is a crucial step for a bacteria's phage susceptibility. To analyze phage susceptibility and how it is related with the receptors where the phages bind to, the *E. coli* bacterial strain 0157:H7 was grown in the presence or absence of exogenous autoinducers. The bacterial strain was tested against ten phages to whom it was susceptible. In the following figure no six, it was observed that autoinducer treatment change the property of phage adsorption on the bacterial strain 0157:H7. The bacterial strain treated with AI-2 showed the lowest adsorption and it was a drastic reduction of phage adsorption compared to control. When treated with autoinducer CAI-1, there was no change in their adsorption pattern. These results suggested that the reduction of phage susceptibility after autoinducer treatment is caused by a reduced adsorption of phages on the bacterial surface.

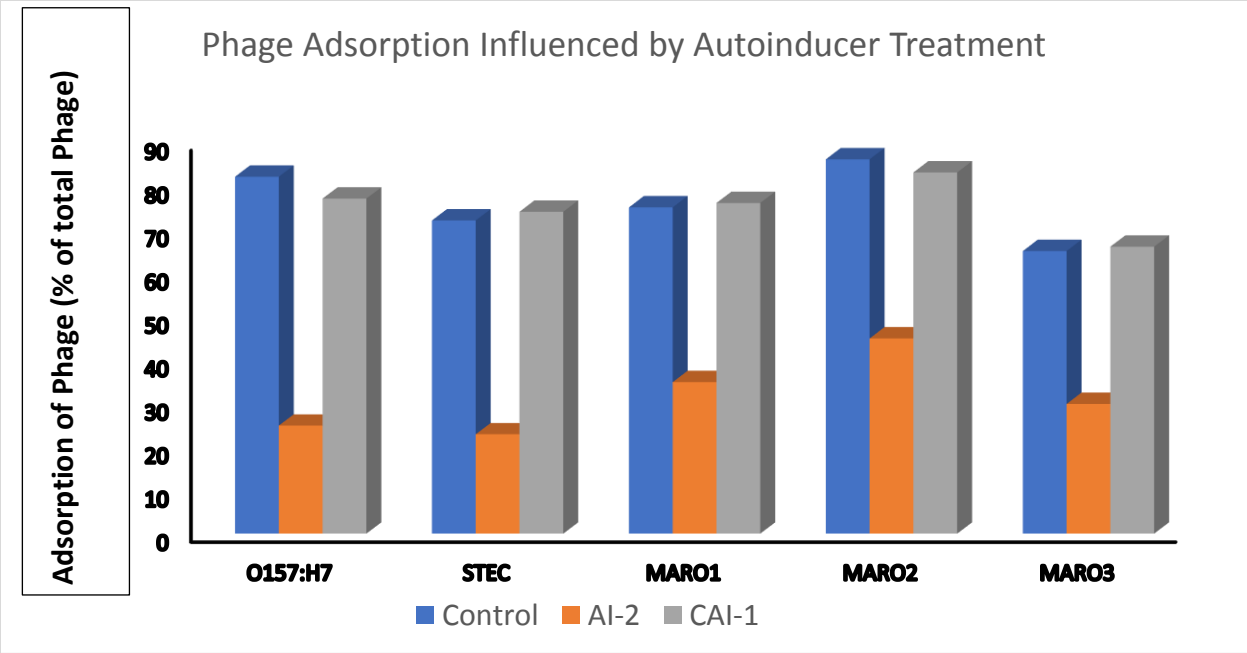

Figure 13: Adsorption of different *E. coli* phages to various *E. coli* strains (including environmental strains which were collected/ MAR strains) which were either pretreated with exogenous autoinducer in the form of culture supernatant of recombinant bacteria producing AI-2 or CAI-1 in 50% v/v ratio.

In order to check the percentage of phage adsorption to *E. coli* bacterial cells, around 2.5×10^8 bacterial cells were mixed with10^7 pfu of corresponding *E. coli* phage. Parallel samples as well as controls were also prepared and incubated for 20 minutes at 30 °C. After that the concentration of the non-adsorbed phages were determined by excluding the adsorbed phages using a syringe filter of 0.22 micron. The percentage of different phages being adsorbed in different treatment conditions are given in the figure.

4.4 Phage Resistant *E. coli* bacteria show Resistance for many Generations

The *E. coli* cells that became resistant after autoinducer AI-2 treatment were then sub-cultured on Macconkey agar plates and single colonies were isolated. They were sub-cultured every day using Macconkey agar plates and single colonies were then inoculated in fresh LB medium for growing them to their log phase to check their resistance. Phage resistance were tested on a regular basis via soft agar plaque assay. It was found that the cells that once turned resistant remain resistant to their corresponding phages for long period of time. In this study the resistant colonies were subcultured 30 times in two months and they still remained resistant to their corresponding phages till the last time their phage resistance was tested.

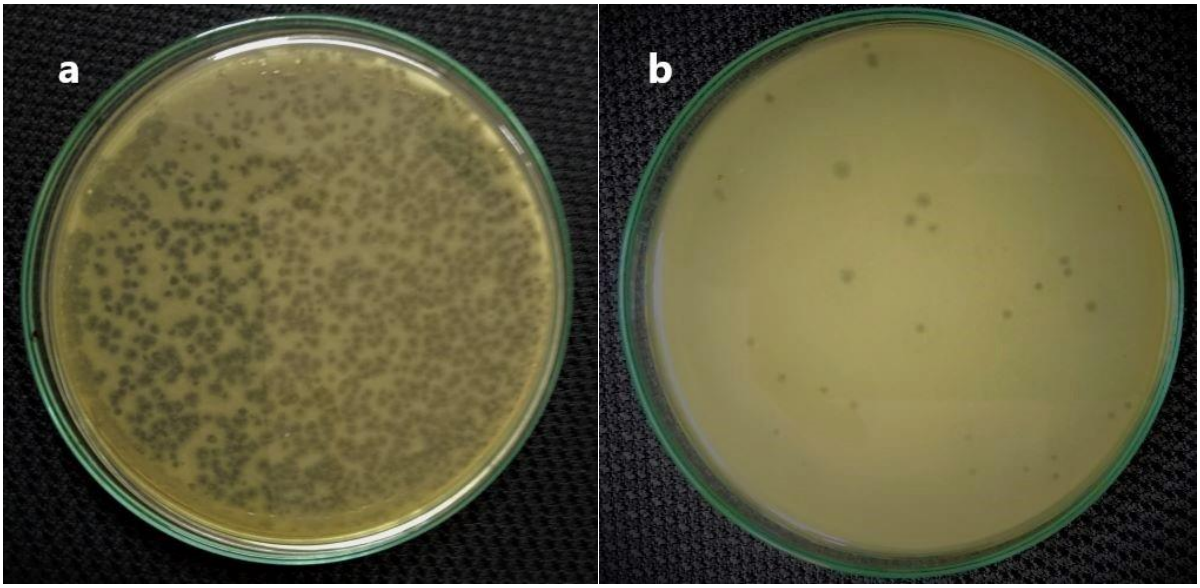

Figure 14: Comparison of the number of Pfus (Plaque forming units) of Resistant (AI-2 treated) and Normal (Control) *E. coli* Bacteria

a) Plaque forming units found in Normal (Control) *E. coli* Bacteria

b) Plaque forming units found in Resistant (AI-2 treated) *E. coli* Bacteria

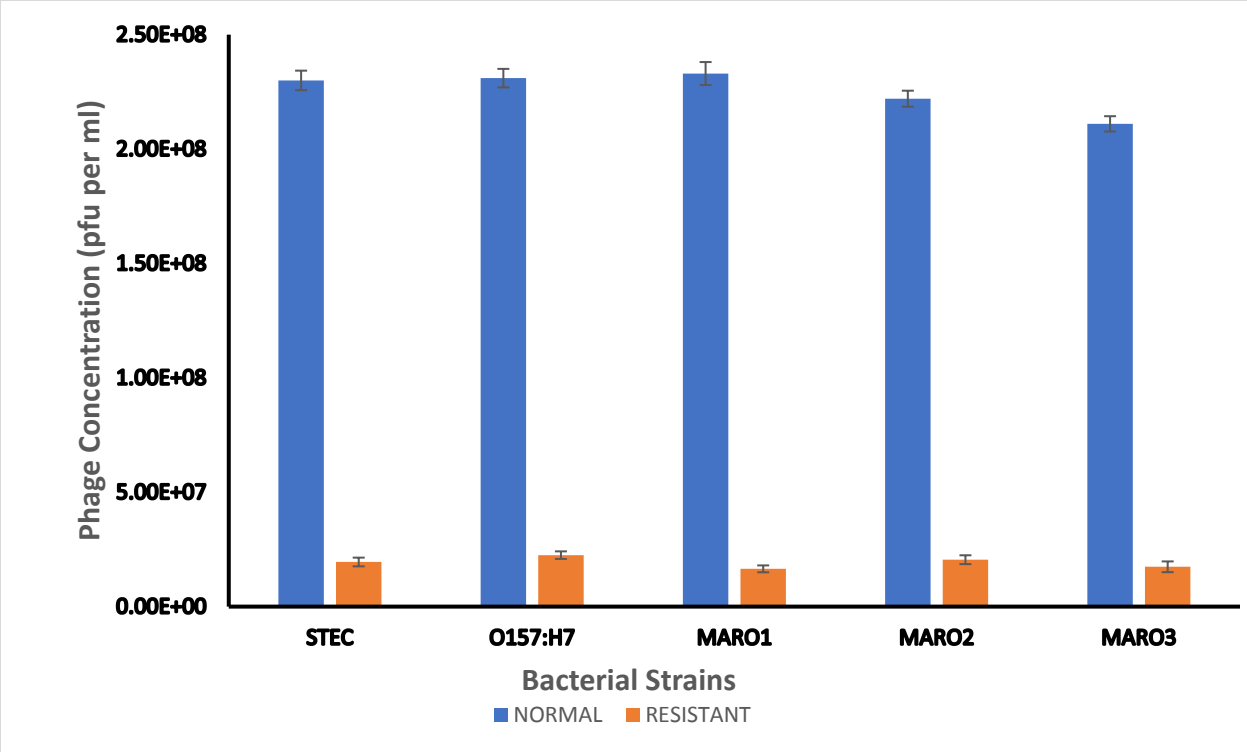

Figure 15: Comparisons on Number of Plaque Forming Units of Resistant and Normal *E. coli* Species

Resistant single colonies which were found after autoinducer AI-2 treatment were picked and cultured in LB for testing the number of Pfus and normal *E. coli* cells were cultured in parallel under similar conditions. The number of Plaque forming units for both resistant and normal *E. coli* cells are shown in this graph. Here, no of Pfus for normal, untreated bacteria are shown in blue and for resistant bacteria it is shown in yellow. Drastic change in phage predation was observed for autoinucer AI-2 treated resistant bacterial cells.

CHAPTER 4

Discussion

The results obtained in this experiment have great implications in comprehension of phage-bacterial relationships as well as how *E. coli* cells adapt and live in the aquatic environment and also in the intestine of living organisms. According to multiple studies, it was seen in case of *Vibrio cholerae* that phage predation of pathogenic cholera strains end the cholera epidemic (a).Hoque et al, 2005; b). Faruque, et al., 2005; Nelson, Harris, Morris, Calderwood, & Camilli, 2009). Since some cholera strain survive in significant number which return in the next cholera season to start another epidemic, it indicates that these survivor strains have some underlying mechanisms of resistance to survive their phage predation. In this similar way, *E. coli* strains might also survive predation by their phages and they might also deploy diverse mechanisms. In this study, the results found suggested that in high cell density *E. coli* cells have modified some genetic or regulatory responses which make them resistant against their corresponding phages. Though in high cell density conditions whether in the gut of the patients or in the natural aquatic environment the bacterial cells should provide a huge opportunity for the growth of *E. coli* phages, the actual scenario is a lot different due to quorum sensing and other novel mechanisms. The results obtained in this experiment correlate with similar research conducted on *Vibrio cholera* O1 strains which was described by Hoque et al. (Hoque et al, 2005) However, maintaining phage resistance may interfere with other physiological properties that help the bacteria to survive in different environments as ecologically fit (e. g- capable of replication in the host body). This following experiment has shed light on many of the underlying mechanisms using which *E. coli* cells survive phage predation by distinct extracellular and intracellular mechanisms.

In this experiment it was found that growing *E. coli* cells in the presence of autoinducer AI-2 made more cells immune to their corresponding bacteriophages. Though it was not possible to reveal the underlying mechanism by which these bacterial cells were developing their phage resistance, it might be attributed to some epigenic change or some form of genetic change that altered their receptor protein expression. For example, a nitrogenous base in a gene can be methylated which will alter its transcription nature or in case of *Vibrio cholera* there occurs a change in O1 antigen expression. According to Seed et al., genetic phage variation for *Vibrio cholera* phages which is considered a result of slipped strand mispairing at homopolymeric nucleotide stretches in two genes- manA and wbeL, controls the production of O1 antigen (Seed, et al., 2012). These two genes are essential for the biosynthesis of *Vibrio cholera* O1 antigen (Seed, et al., 2012). To prove whether these mispairing rates change due to their exposure to autoinducers will need further

studies to prove. These mispairings thus mutate genes selectively and eventually evolved these coding sequences which are more susceptible to mistakes by polymerases that frequently make mistakes in replication and most probably is driving the phage variation phenomenon in this way.

It has been reported in a previous study that if there is an insertion of transposon within the gene of cAMP or CRP (cAMP receptor protein) of *Vibrio cholera* O1 strain, the bacteria become more vulnerable to predatory phages than their parent strains (Zahid, et al., 2010). Moreover, it has been found that CRP is involved in CAI-1 production and in this way participates in the quorum sensing modulation (Liang, Pascual-Montano, Silva, & Benitez, 2007). The reason behind the susceptibility of these mutant bacterial species with transposon insertions can be explained by the phage resistance conferred by the autoinducers that has been tested in this experiment, the pathway which is silenced in the cAMP and CRP mutants.

The autoinducer CAI-1 is mostly used by Vibrio species, but autoinducer AI-2 is used by most bacterial species. So, any AI-2 molecules which has been produced in the human gut or natural environment can be used by the *E. coli* bacterial species and lead to or at least contribute to some extent to their phage resistance against *E. coli* phages. The communication of different species of bacteria might also be affecting the phage resistance of bacteria and further studies should be conducted to reveal these novel interactions and phage resistance across different bacterial species.

To survive the constant threat of phage predation, bacteria have developed a wide array of mechanisms (Labrie, Samson, & Moineau, 2010). Among these separate defensive mechanisms, CRISPR-Cas (clustered regularly interspaced short palindromic repeats/CRISPR-associated proteins) adaptive immune systems and restriction endonucleases are one of the most important ones (Seed, Lazinski, Calderwood, & Camilli, 2013; Barrangou, et al., 2007). Moreover, bacterial cells live in the natural environment mostly as biofilms (Hammer & Bassler, 2004; Zhu & Mekalanos, 2003) and inside these biofilms the cell density is very high. From this research experiment, we are proposing that the bacterial cells within the biofilms exhibit phage resistance modulated by quorum sensing. In a recent study conducted on *E. coli* K12 strain it was seen that AHL (N-acyl L-Homoserine Lactones) molecules (which is a typical quorum sensing molecule used mostly by the gram-negative bacterial community) confer resistance to lambda ($_{\gamma}$) phages when they are supplemented in the culture media of *E. coli* K12 strain (Høyland-Kroghsbo, Mærkedahl, & Svenningsen, 2013). Furthermore, in case of *Vibrio anguillarum*, AHL involved in

quorum sensing regulates the type of defense strategy which is going to be deployed. On the other hand, the *E. coli* cells which remain inside biofilms in high cell density conditions might stay as resistant to their predatory phages and survive in significant numbers to seed the next generation of bacterial cells. Inaccessibility of the phages inside the biofilms might also be a cause behind the phage resistance of *E. coli* and other bacterial cells in biofilms. Kamruzzaman et al. showed that *Vibrio cholera* cells stay as clumps of dormant cells known as CVEC (conditionally viable environment cells) cells and they resuscitate to their normal conditions when exposed to autoinducers, which also make them transiently resistant to *Vibrio cholera* phages (Kamruzzaman, et al., 2010; Bari, et al., 2013). In case of *E. coli* the same form of resistance is possible, but according to this experiment the *E. coli* cells which turned phage resistant when grown in presence of exogenous autoinducer AI-2 remained phage resistant for a significant period of time and even in *Vibrio cholera* there is no concrete evidence whether the conferred phage is transient and further studies should be conducted to prove that. This duration of acquired phage resistance after autoinducer treatment will play a significant role in case of phage therapy of *E. coli* cells and for any other bacteria on which phage therapy is intended to be used.

Nowadays, the interest in phages has been renewed to a huge extent to utilize phages to treat bacterial infections; a method popularly known as phage therapy. Recently it has been observed that, antibiotic treatments are losing their efficacy because of growing antibiotic resistance. To solve this complication, multiple phages have been tested to treat multi-drug resistant bacterial infection and some such experimental treatments have even seen significant success. For example, Dedrick et al. treated a cystic fibrosis patient who was infected by drug resistant *Mycobacterium abscessus* with a cocktail of three phage (Dedrick, et al., 2019) after a lung transplantation with a It is being predicted that the results which have been obtained in this experiment will have some relevance in phage therapy of *E. coli* infections of gastrointestinal tract. The bacterial pathogens which are enteric in nature divide and colonize in the intestine of living organisms and there they have a very high cell density. One such pathogen is *Vibrio cholera*. In this high cell density-state it is assumed that these bacterial cells have a high degree of phage resistance and if phage therapy is used under these circumstances it will most probably have its efficacy reduced. So, to enhance the effectiveness of phage therapy, additional incorporation of quorum modulators might be necessary. For example, quorum quenching molecules can be added to hamper the phage resistance of bacteria which is conferred by quorum sensing. On top of that, use of quorum modulators can

have unwanted effects as well. In case of *Vibrio cholera* O1 strain, it has been observed that quorum quenching may lead to enhanced production of virulence factor (Higgins, et al., 2007). As a result, the application of quorum modulators has to be chosen carefully for the intended pathogen. In conclusion, it can be said that quorum sensing plays a huge role to induce phage resistance in *E. coli* bacteria and it will have significant implications in modifying phage therapy to treat bacterial infections.

CHAPTER 5

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