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HALMOS COLLEGE OF NATURAL SCIENCES AND OCEANOGRAPHY

A tale of two theories: using an engineered strain of *E. coli* to bridge the gap between quorum sensing and diffusion sensing

By

Cortney E. Wilson

Submitted to the Faculty of
Halmos College of Natural Sciences and Oceanography
in partial fulfillment of the requirements for
the degree of Master of Science with a specialty in:

Biological Sciences

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Thesis of
Cortney E. Wilson

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Masters of Science:

Biological Sciences

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Abstract

Cooperation is a trait that is found at all levels of biological organization. Interestingly, cooperation appears to occur in bacteria that produce small, easily diffusible molecules called autoinducers. To understand why bacteria produce these autoinducers, the scientific community has focused on one predominant theory called quorum sensing. Under this theory, bacteria produce autoinducers so they can sense the density of the population. Once a sufficiently high population density is reached, autoinducers initiate the production of a costly gene product that serves to benefit the population. In contrast, a competing theory called diffusion sensing suggests that autoinducers are used by the individual cells and are not used for cooperation. Here, the production of the autoinducer serves as a mechanism to sense environmental conditions. If the environmental conditions are favorable, a costly gene product is produced. To what extent, and under what conditions, are each of these opposing theories valid remains to be identified. In this thesis, an engineered strain of *Escherichia coli* was used to identify the conditions under which quorum sensing and diffusing sensing can be observed. It was discovered that, depending upon the frequency at which the spatial distribution of the autoinducer and bacteria was disrupted, the population of engineered bacteria displayed hallmarks of either quorum sensing or diffusion sensing. Specifically, when the spatial distribution was disturbed at high or low frequency, quorum sensing was observed. However, when spatial distribution was disturbed at an intermediate frequency, diffusion sensing was observed. Understanding how these disturbances affect survival in bacteria may result in novel treatments for bacterial infections. In more general applications, it may be exploited in the development of alternative mechanisms for controlling invasive species or aid in species reintroduction.

Keywords: *quorum sensing, diffusion sensing, synthetic biology, cooperation*

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List of Abbreviations

AHL: acyl homoserine lactone

3OC6-HSL: 3-oxo-hexanoyl-homoserine lactone

3OC12-HSL: 3-oxo-dodecanoyl-homoserine lactone

C4-HSL: butanoyl-homoserine lactone

AIPs: autoinducing peptides

ABC: ATP-binding cassette

RNA: ribonucleic acid

GFP: green fluorescent protein

fL: femtoliter

DNA: deoxyribonucleic acid

RBS: ribosomal binding site

mRNA: messenger ribonucleic acid

MOPS: 3-(N-morpholino) propanesulfonic acid

LB: Luria-Bertani

IPTG: isopropyl β-D-1-thiogalactopyranoside

OD_{600}: Cell density/Optical density at wavelength 600 nm

CFU: colony forming unit

atc: anhydrotetracycline

FITC: fluorescein isothiocyanate

a.u.: arbitrary units

Introduction

Cooperation in biology

Natural selection favors genes that increase an organism's chance of survival so it can reproduce, passing on these favorable genes in the process. This suggests that the world should be dominated by selfish behaviors. Contrary to this assumption, cooperation can be found at all levels of biological organization: genes cooperate within genomes, organelles cooperate within eukaryotic cells, cells cooperate to form multicellular organisms, and individuals cooperate to form societies (S A West, Griffin, & Gardner, 2007a). For a behavior to be considered cooperative, it must provide a benefit to another individual, and it must have partially evolved because of this benefit (S A West et al., 2007a; S A West, Griffin, & Gardner, 2007b). While cooperation is observed in many multicellular organisms, it is also observed in single celled organisms such as bacteria. This has led to a need for research regarding the adaptive significance of cooperation in bacteria.

The adaptive significance of cooperation

Understanding how cooperation has evolved has challenged evolutionary biologists since natural selection favors selfish behaviors and uncooperative individuals (Brockhurst, Habets, Libberton, Buckling, & Gardner, 2010; Diggle, Gardner, West, & Griffin, 2007; Griffin, West, & Buckling, 2004). However, cooperation can be seen in multiple species where the benefit for the individual is not to procreate, but to aid the colony or population in survival (e.g., kin selection). For example, social insects, such as bees and ants, have sterile workers that contribute to the fitness of the colony although they have no chance of reproducing themselves (C. M. Waters & Bassler, 2005). The same can be said for

meerkats, in which groups of 30 adults live together and are divided into the dominant male and female, who do most of the breeding, and the subordinates, who help with raising the offspring (S A West et al., 2007a). This can be explained by Hamilton's theory in which the individuals that cooperate within a community promote the inheritance of their own genes when they contribute to the fitness of closely related kin (Kreft, 2004; C. M. Waters & Bassler, 2005). A similar mechanism is found in bacteria.

Bacteria display a stunning array of behaviors that facilitate cooperation so that they can perform actions such as dispersal, foraging, biofilm formation, chemical warfare, signaling, and reproduction (Velicer & Yu, 2003; Velicer, 2003; Stuart A West, Diggle, Buckling, Gardner, & Griffin, 2007; Stuart A West, Griffin, Gardner, & Diggle, 2006). These behaviors are a result of the secretion of extracellular molecules, called autoinducers or 'public goods' into the environment (Driscoll, Espinosa, Eldakar, & Hackett, 2013; Sanchez & Gore, 2013).

The bacterium *Myxococcus xanthus* provides an example in which individuals within the colony sacrifice themselves for the good of the population (C. M. Waters & Bassler, 2005). *M. xanthus* is a soil dwelling bacterium that, when nutrients become scarce, produce spores that can survive nonvegetatively for long periods of time while being dispersed to new environments (Velicer & Yu, 2003; C. M. Waters & Bassler, 2005). To produce these spores, a large percent of the population must endure a lethal differentiation event to form structures that function to promote spore generation and dispersal (C. M. Waters & Bassler, 2005). This is achieved through the detection of amino acids acting as communication molecules that are produced by *M. xanthus* when starvation conditions are also present, leading to the production of a spore-filled fruiting body (Bassler & Losick,

2006; Shapiro, 1998). *M. xanthus* also demonstrates a socially dependent swarming behavior that allows the population to pursue bacterial prey that can be visualized as behavior similar to how wolves hunt in packs (Crespi, 2001; Diggle et al., 2007).

Biofilm formation demonstrates another behavior found in bacteria that mimics cooperative behaviors found in higher-level organisms. Bacteria use autoinducers to form biofilms (T. F. C. Mah & O'Toole, 2001) which can serve as sites for offspring production, similar to hives, nests, or burrows seen in higher organisms (Crespi, 2001). Furthermore, biofilms found on human teeth provide an environment that allows for both intra and inter-species interactions (Diggle et al., 2007).

Understanding why cooperation occurs is of paramount importance as several theories suggest that cooperation should not be maintained in a population (Diggle et al., 2007; S A West et al., 2007b). Costly cooperative strategies make the population more vulnerable to exploitation by "cheaters" that do not participate in cooperation, but take advantage of the benefits produced by the cooperating individuals (Celiker & Gore, 2012; Diggle et al., 2007). This problem is termed the "tragedy of the commons." The tragedy of the commons is an evolutionary paradox in which the short-term interests of individuals can lead to negative long-term consequences for a population. Here, the selfish actions of individuals within a population can lead to the collapse of resources, which in turn can result in the extinction of a population (Hardin, 2009; Lampert & Tlusty, 2011; MacLean, 2007; Rankin, Bargum, & Kokko, 2007). While the tragedy of the commons has been most widely studied in the context of evolutionary biology, it has also been observed in other fields, widely ranging from ecology to economics.

Protecting shared common resources (or public goods) from the acts of selfish individuals (or cheaters) is difficult due to the competitive advantage that cheaters receive as compared to their non-cheating (or cooperator) counterparts (Kreft, 2004). Specifically, cheaters are afforded an economic advantage while passing the cost on to the remainder of the cooperating population. Such a scenario is prevalent in evolutionary biology. Here, a cooperating population produces a freely sharable 'public good' that benefits individuals that participate in cooperation, and thus benefits the entire population (Pai, Tanouchi, & You, 2012). The production of the public good comes at a cost, which may be manifested as increased injury, a metabolic burden, or death. Such populations are prone to infiltration by cheaters, which can take advantage, but not produce, the public good (Hibbing, Fuqua, Parsek, & Peterson, 2010). Furthermore, as the cheaters do not produce the public good, they do not pay the 'cost' of cooperation, and thus have a fitness advantage (Rankin et al., 2007).

Cell-to-cell communication in bacteria

Bacteria were thought to be self-contained entities that lacked the sophistication of higher organisms which are organized into multicellular groups and communicate amongst one another (Greenberg, 2003b). This idea has since become obsolete, as it is now known that bacteria can organize into groups and communicate amongst themselves, as well as with their eukaryotic hosts (Henke & Bassler, 2004). It is now widely accepted that bacteria produce and respond to chemical signals, and these coordinated actions lead to group activities (Greenberg, 2003a; Schertzer, Boulette, & Whiteley, 2009). This includes secretion of virulence factors (Antunes, Ferreira, Buckner, & Finlay, 2010; Henke & Bassler, 2004; Ji, Beavis, & Novick, 1995), biofilm formation (Greenberg, 2003a, 2003b;

T. F. C. Mah & O'Toole, 2001; T.-F. Mah et al., 2003), bioluminescence (Miller & Bassler, 2001), sporulation (De Kievit & Iglewski, 2000), and antibiotic resistance (T.-F. Mah et al., 2003; Stewart & Costerton, 2001).

Bacteria communication occurs through the use of chemical signals referred to as autoinducers. As the population of bacteria increases in density, the autoinducers produced by the bacteria also increase in density. Once the bacteria reach a particular density, and the autoinducer is at a sufficiently high concentration, gene expression is altered (C. M. Waters & Bassler, 2005).

The theory of quorum sensing

In spite of the "tragedy of the commons," cell communication can still persist in bacterial populations, even in the presence of cheaters (Dandekar, Chugani, & Greenberg, 2012; Keller & Surette, 2006b; Stevens, Schuster, & Rumbaugh, 2012). This suggests that communication is of paramount importance in some bacteria, thus demonstrating its adaptive usefulness. Previous studies (Skindersoe et al., 2008; Walters & Sperandio, 2006; Widder, 2010) have examined the specific adaptive significance of communication in bacteria and have attempted to develop theories to explain its importance.

One theory as to why bacteria communicate, and thus cooperate, is quorum sensing. Quorum sensing is the process used by bacteria to regulate gene expression in response to changes in cell population density (Darch, West, Winzer, & Diggle, 2012; Ji et al., 1995; Miller & Bassler, 2001; Pai et al., 2012; Platt & Fuqua, 2010; Stuart A. West, Winzer, Gardner, & Diggle, 2012) through the secretion and detection of small molecules that are present within a population (Schertzer et al., 2009; Stuart A. West et al., 2012). Through

this process, bacteria can express traits that are energetically expensive, but only when the benefit to the host will be maximized (Pai et al., 2012; Rutherford & Bassler, 2012). Quorum sensing is viewed as a mechanism to coordinate these cooperative behaviors in bacteria (Diggle et al., 2007; Schertzer et al., 2009). More specifically, it allows bacteria to assess cell density, and only after a threshold density has been obtained, allows for subsequent cooperation (Diggle et al., 2007; Lampert & Tlusty, 2011; Ross-Gillespie, Gardner, Buckling, West, & Griffin, 2009). This is because many cooperative behaviors would not be advantageous at low cell densities, so a communicative signal is used to inform the population when there are sufficient individuals present (Diggle et al., 2007; Platt & Fuqua, 2010). As such, *quorum sensing is viewed as a predominantly social, or cooperative, trait.*

Vibrio fischeri is a bioluminescent marine bacterium that has a symbiotic relationship with a variety of eukaryotic hosts and is the most intensely studied in terms of quorum sensing (Antunes et al., 2010; Miller & Bassler, 2001). It has often been considered the paradigm for quorum sensing in gram-negative bacteria (C. M. Waters & Bassler, 2005). *V. fischeri* was initially found in the light organ of the Hawaiian bobtail squid, *Euprymna scolopes*, and it was observed that for the organ to emit light, the bacteria needed to reach a sufficiently high cell density (Alberghini et al., 2009; Miller & Bassler, 2001; E. Waters et al., 2003). In this example, the squid used the light to avoid predation by using the light to counter-illuminate itself (Miller & Bassler, 2001; C. M. Waters & Bassler, 2005). This counter-illumination prevented a shadow from being cast beneath the squid on nights when the light from the moon and stars illuminates the ocean water (Miller & Bassler, 2001). In other examples, such as in the fish *Monocentris japonicus*, the light is

used to attract mates (Miller & Bassler, 2001). The uses of the light produced by *V. fischeri* vary depending on the organism.

In *V. fischeri*, quorum sensing is controlled by two regulatory proteins, LuxI and LuxR, which together regulate the expression of the luciferase operon, *luxICDABE* (C. M. Waters & Bassler, 2005). This is the case regardless of the host that harbors the bacteria to produce light (Miller & Bassler, 2001). LuxI is the autoinducer synthase enzyme, which is responsible for producing AHL (Miller & Bassler, 2001; C. M. Waters & Bassler, 2005), specifically 3-oxo-hexanoyl-homoserine lactone (3OC6-HSL) (Schuster, Joseph Sexton, Diggle, & Peter Greenberg, 2013). LuxR has dual functions; it binds to the autoinducer and, bound with AHL, activates transcription of the *luxICDABE* operon (Miller & Bassler, 2001; C. M. Waters & Bassler, 2005). As AHL is produced, it diffuses freely across the cell membrane, increasing in concentration in the environment as the cell density increases (Schuster et al., 2013; C. M. Waters & Bassler, 2005). Once the concentration of AHL reaches a critical threshold, it is bound by LuxR (Miller & Bassler, 2001; C. M. Waters & Bassler, 2005). This in turn activates the expression of the targeted gene (Figure 1). The *luxI* gene is also regulated by quorum sensing (Miller & Bassler, 2001; Schuster et al., 2013; C. M. Waters & Bassler, 2005) and is activated by the AHL-bound LuxR providing hysteresis to the system (Schuster et al., 2013). This configuration allows the environment to be flooded with the AHL signal, creating a positive feedback loop; the entire population is switched into the quorum sensing mode and light is produced (C. M. Waters & Bassler, 2005) (Figure 1).

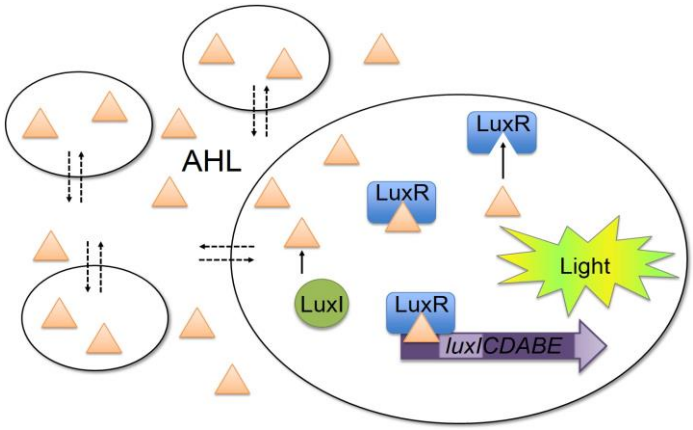

Figure 1: The general mechanism of the LuxI/LuxR quorum sensing in *Vibrio fischeri*

In *V. fischeri*, quorum sensing is controlled by two regulatory proteins, LuxI (green circle) and LuxR (blue rectangles). LuxI produces the autoinducer AHL (orange triangles). As the density of the population of bacteria increases, so does the concentration of AHL. Once the cell population (and AHL) reaches a high enough density, the AHL binds to the receptor LuxR. LuxR then attaches to the promoter, altering gene expression, and produces light from the *luxICDABE* gene (Miller & Bassler, 2001; C. M. Waters & Bassler, 2005).

The autoinducer that is used for quorum sensing varies amongst bacterial species. In gram-negative bacteria, most of the quorum sensing circuits that have been identified resemble the canonical system that is found in *V. fischeri* (Miller & Bassler, 2001). Another bacterium, which uses quorum sensing and that has been extensively studied, is *Pseudomonas aeruginosa*. *P. aeruginosa* is an opportunistic pathogen that has the ability to affect a range of hosts, including plants, insects, and mammals (Schuster et al., 2013). Humans that have compromised immune systems are particularly vulnerable to *P. aeruginosa*, which colonizes in the lungs of people with cystic fibrosis (Camilli & Bassler, 2006). *P. aeruginosa* has two complete AHL regulated circuits, LasR-LasI and RhlR-RhlI; both circuits are composed of a receptor resembling LuxR and a synthase like LuxI (Schuster et al., 2013; Williams & Cámara, 2009). The autoinducer that is produced by LasR is a 3-oxo-dodecanoyl-homoserine lactone (3OC12-HSL) while the one produced by RhlI is a butanoyl-homoserine lactone (C4-HSL) (Schuster et al., 2013). Many of the products that are produced by these systems are considered to be virulence factors because of the damage caused to the tissues of the host (Schuster et al., 2013; Van Delden & Iglewski, 1998).

Gram-positive bacteria also regulate a number of processes using quorum sensing. However, they employ a different type of autoinducer than those used by Gram-negative bacteria. Gram-positive bacteria use autoinducing peptides (AIPs) as their signaling molecules (Miller & Bassler, 2001; Rutherford & Bassler, 2012; E. Waters et al., 2003). As with Gram-negative bacteria, these signals are specific to the bacterium that uses it (C. M. Waters & Bassler, 2005). This signal gets transported out of the cell through an ATP-binding cassette (ABC) transporter (Kleerebezem, Quadri, Kuipers, & de Vos, 1997; Miller & Bassler, 2001). Once the extracellular level of the AIPs reaches a sufficiently high concentration, it binds to a two-component histidine kinase receptor located in the cell membrane (Miller & Bassler, 2001; Rutherford & Bassler, 2012). This two-component regulatory system uses phosphorylation to transfer information. This system is composed of a sensor and response-regulator protein to form a mechanism for signal transduction in bacteria (Kleerebezem et al., 1997). The sensor kinase autophosphorylates on a conserved histidine residue then transfers the phosphoryl group to the response-regulator protein, which is also phosphorylated (Miller & Bassler, 2001). Changes in gene expression within the cell subsequently ensue and often involve the expression of virulence factors such as toxins.

There are also examples that demonstrate how quorum sensing is maintained in the presence of cheating populations. Some mutants that form within a population of *V. fischeri* are incapable of luciferase production. These cheater bacteria are outcompeted by the wild-type, cooperative bacteria in the host, indicating that the host may possess a policing mechanism that eliminates cheaters (C. M. Waters & Bassler, 2005). Another example can be found in the bacterium *Agrobacterium tumefaciens*, a plant pathogen that induces the

development of tumors on wound sites of the host plant (Joint, Allan Downie, & Williams, 2007; C. M. Waters & Bassler, 2005). *A. tumefaciens* uses quorum sensing to induce virulence factors that allow for the formation of crown gall tumors on the wound sites of host plants (Joint et al., 2007). Interestingly, as the bacterial density increases, depleting the nutrients available, there is an increase in bacterial conjugation, which in turn leads to a higher copy number of the Ti plasmid, a plasmid that is critical in the infection process (C. M. Waters & Bassler, 2005). Quorum sensing is required for both of these events to occur, and in turn, most of the bacteria receive copies of the plasmid before they disseminate and become infective in a new location (C. M. Waters & Bassler, 2005).

These are only a few examples of quorum sensing. As there are many systems that require a high cell density to express a costly trait, this has given rise to the assumption that quorum sensing is a cooperative trait. For the population to work as a group, a sufficiently large population needs to be present, and only then will a costly product be produced from which all of the cells will benefit.

The theory of diffusion sensing

While the majority of the scientific community has accepted that cell communication in bacteria is a cooperative trait, in recent years alternative theories have been gaining ground. One opposing theory, diffusion sensing, suggests that the function of autoinducers is to enable individual cells to sense how quickly the molecules secreted by the cell diffuse away (Redfield, 2002; Von Bodman, Willey, & Diggle, 2008; Stuart A. West et al., 2012) (Figure 2). The theory of diffusion sensing was first introduced by Redfield in 2002. In her manuscript, she points out that the genes for quorum sensing would

evolve only if the cells that are investing the individual resources for the good of the population reproduced better that the cells that used these goods selfishly. Maintaining cooperative genes therefore would be difficult, especially in mixed populations, because selection will favor for any selfish cells that can reap the benefits without having to produce the costly good (i.e., the tragedy of the commons) (Redfield, 2002). As such, communication in bacteria is likely an adaptive strategy to benefit the individual cell and not the population (Alberghini et al., 2009).

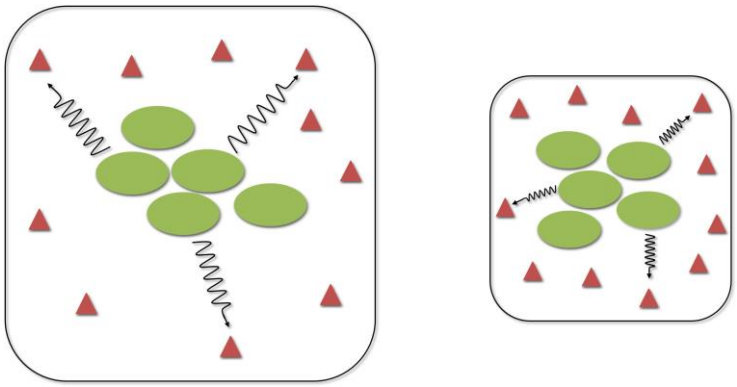

Figure 2: The central tenant of the diffusion sensing theory

The theory of diffusion sensing suggests that the function of the autoinducer is to aid the cells in sensing how quickly the autoinducer diffuses away. Here, environmental factors such as temperature, spatial structure, or viscosity, control this diffusion rate and thus determine the potential benefit of producing a costly exoproduct. The cells (green ovals) on the left have AHL (red triangles) diffusing away quicker than the cells on the right. Therefore, the cells on the left will not produce a costly product, while the cells on the right, sensing more of the AHL in their environment, will (Redfield, 2002; Von Bodman et al., 2008; Stuart A. West et al., 2012).

One key prediction of diffusion sensing is that the environment should dictate whether an autoinducer regulated costly product is made by the individual cell (Williams, Winzer, Chan, & Cámara, 2007). This contrasts to quorum sensing where the production of a costly product is solely dependent upon cell density, and not the environment (Williams et al., 2007). Environmental factors that can vary include temperature, pH, osmolarity, nutrient availability, spatial structure and diffusion rate of substances in the environment (Williams et al., 2007).

Several studies have produced results that support the diffusion sensing theory. *Staphylococcus aureus* is a Gram-positive bacterium that causes local and systemic infections in mammals that can range from minor infections to life-threatening conditions such as toxic shock syndrome and endocarditis (Shompole et al., 2003). Establishment of a *S. aureus* infection within a host involves attachment, a coordination of host defense elusion, and tissue invasion, all of which involve several independent virulence factors that fall under the control of the Agr-regulatory system (Shompole et al., 2003). The authors were able to demonstrate that the *S. aureus* Agr-regulatory system can be induced after internalization within epithelial cells. The endosomal compartment provides a restricted microenvironment. This allows for the secreted autoinducers to increase in concentration to saturation levels using a single bacterium or a small cluster of bacterial cells. The autoinducers, which were previously thought to be used to sense population density, are instead believed to be involved in assessing the frequency at which secreted molecules diffuse from the cell(s). Once enough of the autoinducer accumulates in this microenvironment, it initiates a cascade of signaling events to activate the virulence factors that are required to escape from the endosome. This data indicates that Agr-mediated regulation can support the diffusion sensing hypothesis because it can be induced by a single bacterium or a small bacterial cluster (Shompole et al., 2003). To experimentally confirm that induction was occurring inside the endosome, the authors used the RNAIII promoter to drive the expression of green fluorescent protein (GFP). The expression of GFP was observed as early as one hour post invasion, meaning that induction occurred while the bacteria were still in the endosome and that activation of the RNAIII promoter occurred without the presence of bacterial clusters (Shompole et al., 2003).

In another example using *S. aureus*, Carnes et al. (2010) were able to demonstrate adaption and survival through self-induction, which resulted in genetic reprogramming in isolated individual organisms. The authors were able to observe activation of autoinducer regulated genes in isolated, individual cells using a matrix that was designed at a sufficiently small physical scale where the overall cell density surpassed the reported activation threshold. Using this system, where *S. aureus* was individually contained within a small volume, it could sense and respond to confinement as the autoinducer accumulated, allowing for activation of the regulatory system. Their results demonstrate that under certain conditions, induction can occur independently of both cellular density and spatial distribution of cells, confirming one experimental condition of the diffusion sensing hypothesis (Carnes et al., 2010).

Using *P. aeruginosa*, Boedicker et al. (2009) demonstrated that they could initiate changes in gene expression due to cell communication using one to three cells. To investigate how these changes in gene expression could be initiated by a small group of cells, they used a previously described microfluidic technique (Park, Hur, Kwon, Park, & Suh, 2006). Once the bacteria were confined to volumes of approximately 100 fL, these small groups of *P. aeruginosa* were able to initiate expression of genes regulated through cell-to-cell communication (Boedicker, Vincent, & Ismagilov, 2009). These results accentuate that a few cells can induce such genes when restricted to a confined environment and that confinement may play a role in the association between virulence in pathogens and cell-to-cell communication (Boedicker et al., 2009).

Significance of understanding cooperation and communication in bacteria

Microbes offer numerous opportunities for ecologists and evolutionary biologists who study social evolution (Stuart A West et al., 2007). First, a significant amount of microbiology requires an evolutionary explanation (Stuart A West et al., 2007). Cooperation is abundant in the natural world, therefore the mechanisms that maintain it must exist, especially since the spread of cheaters is so prevalent (Von Bodman et al., 2008). Communication and cooperation in bacteria are very important. For example, in the bacterium *P. aeruginosa*, 6-10% of its genes are controlled by cell-cell communication systems (Stuart A West et al., 2007). To better understand the evolution of cooperation, explaining it in microbes may provide valuable insights (Stuart A West et al., 2007).

Second, the development of the social evolution theory was to aid in the explanation of known behaviors in animals such as insects, birds, and mammals (Stuart A West et al., 2007). For example, a familiar feature of social cooperation demonstrated by macroorganisms is the concept of a shared shelter, which is also seen in microbes in biofilm formation (Crespi, 2001). Another form of cooperation in many species involves food acquisition. Some vertebrates work together to subdue prey larger than themselves while smaller invertebrates coordinate mass attacks. In some bacteria (*M. xanthus* for example) this behavior can be observed when they attack other, larger bacteria for consumption (Crespi, 2001). Because microbes have such a wide variety of social behaviors, this presents a unique opportunity to test how these behaviors can be applied to other taxa (Stuart A West et al., 2007). Gaining a better understanding of how these cooperative behaviors have evolved may elucidate key evolutionary steps that led us to the complexity of multicellular organisms (Celiker & Gore, 2013).

Third, microbes are incredibly amenable to evolutionary theory experimentation as they have short generation times. This makes long-term evolution experiments more practical and altering gene expression (from cooperators to cheaters) is relatively easy (Celiker & Gore, 2013; Stuart A West et al., 2007).

Fourth, the social behaviors of microbes impact human lives in many ways. Cooperative behaviors found in microbes can harm humans, livestock, and agricultural crops through infection, while at the same time provide beneficial services by breaking down waste or though symbiotic relationships with other taxa (Stuart A West et al., 2007). Most cellular cooperation involves the production of a public good that benefits the other cells in the population (Celiker & Gore, 2013). These public goods include autoinducers, antibiotics, siderophores, extracellular enzymes, and exopolysaccharides (Celiker & Gore, 2013). Secretion of these products come at a cost to the producer, ranging from a small metabolic burden to cell death (Celiker & Gore, 2013).

Synthetic biology

While there exist several bacterial systems that communicate using autoinducers, these natural systems are complex. As such, it is challenging to study communication alone without interference from additional confounding variables such as larger, regulatory networks that may be involved in communication or downstream processes, cell-to-cell variability, the presence of natural cheaters, etc. Often, conclusions drawn from natural systems may not represent the entire story due to these and other confounding factors.

Synthetic biology offers an alternative to studying biological phenomena in natural systems through the creation of functional devices and systems, using standardized

biological building blocks (Weber & Fussenegger, 2012). Driven by mathematical modeling, synthetic biology involves engineering desired behaviors using genetic components, which are assembled into a gene circuit. The gene circuit is then placed into the 'chassis' of choice, which powers the circuit, thus implementing the desired behavior. Due to ease of manipulation and high variable growth conditions, *E. coli* have, to date, been the preferred chassis of choice. Indeed, through using *E. coli*, many different behaviors have been engineered, such as switches (Atkinson, Savageau, Myers, & Ninfa, 2003; Gardner, Cantor, & Collins, 2000; Hasty, McMillen, & Collins, 2002) and oscillators (Danino, Mondragón-Palomino, Tsimring, & Hasty, 2010; Prindle et al., 2011; Stricker et al., 2008). Since experiments using natural systems are often challenging due to the complexity involved, using engineered bacteria is advantageous in that such organisms are equipped with components that can be easily altered.

While synthetic systems have been designed for use in medicine (Brown, West, Diggle, & Griffin, 2009) and industry (Brenner, You, & Arnold, 2008), it is becoming increasingly recognized that these synthetic systems can be used to study ecological and evolutionary dynamics (Tanouchi, Smith, & You, 2012). Using engineered bacteria to study cooperation, or other applications to ecological/evolutionary relationships, has several advantages over the use of natural systems. The study of cooperation in natural systems is often challenging due to multiple interacting factors in the natural environment that cannot be controlled (Horswill, Stoodley, Stewart, & Parsek, 2007; Riccione, Smith, Lee, & You, 2012). These factors may serve to obscure the true behavior(s) governing cooperation, or its maintenance and loss, in the system. Using engineered bacteria serves to reduce this complexity. First, engineered bacteria operate using well-defined

components that can be readily perturbed and controlled. Second, such systems operate in well-defined environments, which can be readily controlled, thus reducing confounding variables. Third, these systems operate on much shorter time scales than natural environments allowing behaviors to be observed over the course of hours (as opposed to days or years) and can allow for multiple experiments, thus increasing repeatability (Tanouchi, Smith, et al., 2012). Indeed, engineering bacteria have been used to study several important relationships in cooperation.

To create a synthetic circuit, it is important to consider how a natural genetic circuit is comprised to control specific behaviors (Purnick & Weiss, 2009). For example, *E. coli* cells are made up of thousands of genes, but to express all of these genes all of the time would be costly. As such, many genes are expressed only when they are needed by the bacterium. This is possible because these genes are divided up into separate units that are controlled (or activated) by external signals. In nature, bacteria cells would use these signals to activate or repress the targeted gene (Jusiak & Daniel, 2014). It is through this design, where genes can be independently controlled, that the concept of the genetic circuit was formed.

Most gene circuits are made up of four main biological parts (Endy, 2005), each of which play a critical role in the function of the gene circuit (Figure 3). These parts are modular and act independently of other cellular processes (Sprinzak & Elowitz, 2005) allowing for each part to be mixed and matched, but have defined functions. For example, a promoter will drive the expression of the gene located downstream from it, regardless of what that gene may be (Jusiak & Daniel, 2014). The result is a novel gene circuit that displays the desired behavior intended by the designer. Because many of these are

preexisting parts found within natural systems (Purnick & Weiss, 2009), one must take care that there is minimal ‘cross talk’ between these novel genes and with other genes within the cell (Jusiak & Daniel, 2014). Minimizing cross talk allows for greater control of the new circuit (Wang, Kitney, Joly, & Buck, 2011).

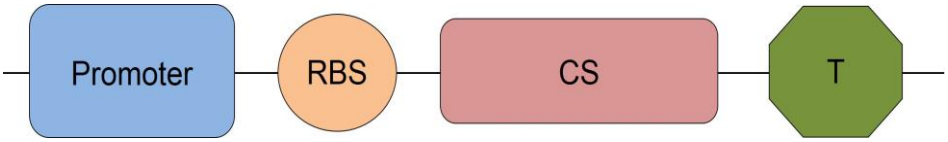

Figure 3: The core features of a gene circuit.

The four main biological parts of a gene circuit. The promoter activates expression of the gene. The ribosomal binding site (RBS) binds to the mRNA to initiate protein expression. The coding sequence (CS) contains the genetic information encoding the protein to be produced. The terminator (T) stops RNA polymerase transcription. Each part in the gene circuit can be interchanged to create unique combinations, which may result in novel behaviors.

Each of the pieces that make up a gene circuit are incredibly diverse, but contain core features that denote its functionality. At the beginning of the gene circuit is the promoter (Figure 3). Promoters are DNA sequences, which when found by RNA polymerase, serve to control the frequency and location of transcription initiation (Harley & Reynolds, 1987). This involves the integration of an external factor, including sugar or sugar analogs (Lutz & Bujard, 1997), autoinducers (You, Cox, Weiss, & Arnold, 2004), or specific wavelengths of light (Olson, Hartsough, Landry, Shroff, & Tabor, 2014), that allow the promoter to function similar to that of a light switch, turning the circuit on or off (Figure 4A). These signals serve to activate, or repress, RNA polymerase adhesion to the promoter. Once RNA polymerase is bound, it can transcribe the genetic information downstream from the promoter (Figure 4B). Ideally, the promoter is tunable, meaning that it can achieve different output levels through small changes in the signal (Jusiak & Daniel, 2014).

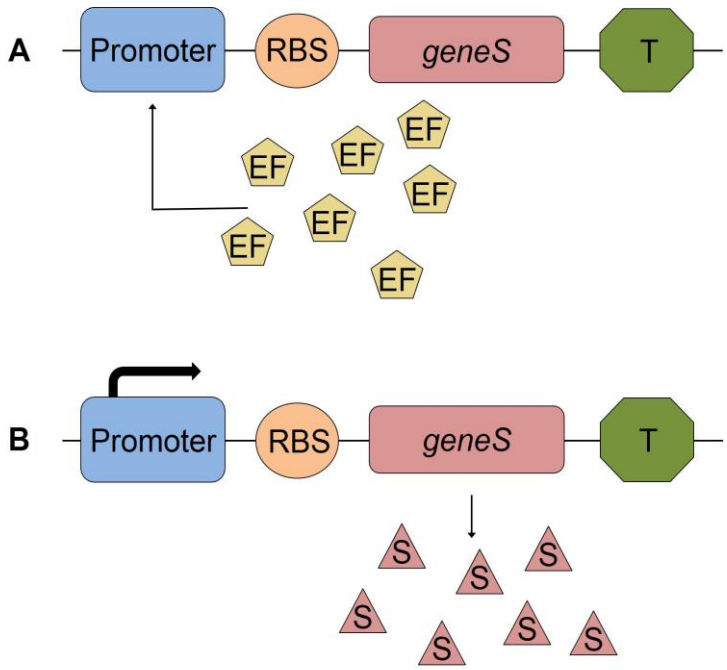

Figure 4: Activation of the promoter through external factors leads to transcription of a desired protein

(A) The promoter is activated by an external factor (EF) which may vary, from specific wavelengths of light, sugars, or molecules produced by the cell.
(B) Once activated, RNA polymerase is free to bind to the promoter, which in turn allows the genetic information (*geneS*) downstream to be transcribed, producing the desired protein (S).

Directly downstream from the promoter is the ribosomal binding site (RBS) (Figure 3). This is the region where the ribosome binds to the mRNA to initiate protein translation, controlling the translation initiation rate and the level of protein expression (Salis, Mirsky, & Voigt, 2009). Following the RBS is the protein coding sequence, containing the genetic information of the protein that is to be produced (Figure 3). Of the four main parts of the gene circuit, the protein coding sequence is often the most varied and the most critical, as it is often directly responsible in producing the desired behavior. There can be multiple protein coding sequences under the control of the promoter, both in natural systems, as is observed in the *lac* operon (Davies & Jacob, 1968), or in synthetic systems, where the first sequence produces the desired behavior while the second contains a fluorescent protein that is used to verify the activation of the promoter (Zaslaver et al., 2006). A terminator is located at the end of the gene circuit that stops RNA polymerase transcription and is essential for proper expression of genes (Peters, Vangeloff, & Landick, 2011) (Figure 3).

Objectives and Hypothesis

The objective of this thesis was to use a strain of engineered bacteria, constructed using the principles of synthetic biology, to examine the conditions that allow the observation of the core predictions of quorum sensing and diffusion sensing. The spatial distribution of the cells and the diffusion rate of the autoinducer were both manipulated as both have been predicted to affect, or not to affect, cell-to-cell communication. Four central hypotheses were developed:

1) *Quorum sensing and diffusion sensing are not competing theories. The core predictions of both theories can be observed in a single, bacterial system given the appropriate environmental conditions.*

2) *Spatial distribution of the bacteria and diffusion rate of the autoinducer will impact the ability of the engineered bacteria to successfully cooperate.*

3) *Initiation of the autoinducer activated gene expression will be impacted by the frequency at which the spatial distribution of AHL and bacteria are altered.*

4) *The stability of the autoinducer and initial positioning of the cells can be perturbed independently, thus allowing the autonomous contribution of both parameters to cooperate.*

Materials and Methods

Strains and growth conditions

A previously engineered strain of *Escherichia coli*, strain DH5αPRO (Clontech, Mountain View, CA), was used in this study (unless otherwise indicated). All experiments were performed in modified M9 (minimal growth) medium [1X M9 salts (48 mM $NaHPO_4$, 22 mM KH_2PO_4, 862 mM NaCl, 19mM NH_4Cl), 0.4% glucose, 2% casamino acids (Teknova, Hollister, CA), 0.05% thiamine (Alfa Aesar, Ward Hill, MA), 2 mM $MgSO_4$, 0.1 mM $CaCl_2$] buffered to pH 7.0 or 7.4 with 100 mM 3-(N-morpholino) propanesulfonic acid (MOPS) (Amresco, Solon, OH), with or without 0.2% or 0.4% agar (Alfa Aesar, Ward Hill, MA). 25 μg/mL chloramphenicol (Alfa Aesar, Heysham, England) and 50 μg/mL kanamycin (Amresco, Solon, OH) were added to the medium before each experiment. The medium was then overlaid with mineral oil (Fisher Scientific, Fair Lawn, NJ) to prevent evaporation. Single colonies were inoculated into 5 mL Luria-Bertani (LB) broth (MP Biomedicals, Solon, OH) containing 25 μg/mL chloramphenicol and 50 μg/mL kanamycin and incubated for 24 hours. Induction of the gene circuit was achieved by the addition of 1 mM isopropyl β-D-1-thiogalactopyranoside (IPTG) (Promaga, Madison, WI). Cell density was obtained at an optical density measured at a wavelength of 600 nm (OD_{600}) at 37°C in a PerkinElmer Victor X4 (Waltham, MA) microplate reader. Shaking took place in 5, 20, or 60 minute intervals for 10 seconds using a linear pattern with a diameter of 0.1 mm. Colony forming unit (CFU) counts were performed on LB solid medium containing chloramphenicol and kanamycin as previously described (Smith et al., 2014).

Determining dispersal rate

Experiments to determine dispersal rate were conducted by modifying the amount of agar (0%, 0.2%, or 0.4%) that was added to the medium. This experiment was performed using a cell chamber (Ibidi, Martinsried, Germany) made of uncoated hydrophobic glass that contained 150 μL of M9 medium (containing various agar concentrations) with both openings of the cell chamber overlaid with 15 μL of mineral oil to prevent evaporation. A plasmid containing *gfp(mut3b)* (Cormack, Valdivia, & Falkow, 1996) under the regulation of a $P_{tet0\text{-}1}$ promoter (Lutz & Bujard, 1997) was transformed into *E. coli* strain DH5αPRO using a Zymo Z-competent transformation kit (as per manufacturer's specifications, Genesee Scientific, San Diego, CA). Single colonies were grown overnight in 5 mL of LB medium with 25 μg/mL chloramphenicol at 37°C. The cells were resuspended the following day in M9 medium containing 25 μg/mL chloramphenicol and 100 ng/mL of anhydrotetracycline (atc) (Acros Organics, Geel, Belgium). The cells were allowed to incubate for an additional 3 hours to induce GFP expression. 10 μL of the culture was inoculated at one end of the cell chamber. Cell placement was observed using an Olympus IX73P2F fluorescent microscope with a 25X objective lens using a DP-80 camera (Olympus Microscopes, Center Valley, PA). The chamber was then allowed to incubate at 37°C without shaking. Cell movement was observed 2 hours later for cultures in 0% agar, and 12 hours later for cultures in both 0.2% and 0.4% agar. The cellSens software (Olympus Microscopes) was used to quantify the distance that the bacteria had travelled. Distances were averaged from 6 replicates.

Cell shaking assay

Single colonies of *E. coli* strain DH5αPRO expressing *gfp*(*mut3b*) were grown overnight in LB medium with 25 μg/mL chloramphenicol. The following day, the cells were resuspended into M9 medium containing 25 μg/mL chloramphenicol and 100 ng/mL atc and were shaken at 37°C for 3 hours to induce GFP expression. 190 μL of M9 medium (containing various agar concentrations) was added to the cell chamber and 5 μL of the bacterial strain was inoculated into one side of the chamber. The cell chamber was left at room temperature for 10 minutes whereupon the initial position of the cells was examined under an Olympus fluorescent microscope at 25X magnification using a DP-80 camera. The cell chamber was then placed in the microplate reader and shaken once as previously described. The cell chamber was removed and the displacement of the cells was measured using an Olympus microscope and the cellSens software. Distances were averaged from three replicates.

Growth and translation rate

To verify that shaking the plate at different frequencies was not affecting growth or translation rates, single colonies of *E. coli* strain DH5αPRO expressing *gfp*(*mut3b*) were grown overnight in LB medium with 25 μg/mL chloramphenicol. To induce the circuit, the cells were diluted 100-fold in M9 medium with 25 μg/mL chloramphenicol and 100 ng/mL atc. 200 μL of the diluted culture was plated in triplicate in a 96 well plate in both OFF (without atc) and ON (with atc) conditions. The wells were overlaid with 70 μL mineral oil to prevent evaporation. The cells were then incubated at 37°C for 48 hours in a Victor

X4 microplate reader and shaken at 5, 20, or 60 minute intervals. For the cells that were shaken every 5 or 20 minutes, the plate was read every 20 minutes for OD_{600} and GFP; for cells that were shaken every 60 minutes, the plate was read for OD_{600} and GFP every 60 minutes. To specifically examine translation rate, the GFP(mut3b) signal (a.u.) was normalized by OD_{600}.

AHL detector assay

A previously described AHL detector strain (Song, Payne, Gray, & You, 2009) was used to quantify AHL diffusion. The 3OC6HSL detector strain is implemented in TOP10F' and contains two plasmids; one harboring a P_{lac} promoter driving expression of *luxR* (p15a, KanR) and the second harboring a P_{lux} promoter driving *gfp*(UV) (ColE1, CmR). This detector strain was grown overnight in LB with 50 μg/mL kanamycin and 25 μg/mL chloramphenicol. 25 mL of M9 medium containing different percentages of agar (0%, 0.2%, and 0.4%) was made and 25 μg/mL chloramphenicol, 50 μg/mL kanamycin, 1 mM IPTG, and 25 μL of overnight cell culture was added to the medium once it cooled to approximately 50°C. 200 μL of this culture was them added to a cell chamber. After the medium was allowed to solidify, 1 μL of 20 μM 3OC6HSL was added to one end of the cell chamber. The cell chambers were incubated at 37°C for 5 hours whereupon the position of the cells was recorded from two areas of the cell chamber. The first was roughly 1 cm from the end of the cell chamber where 3OC6HSL was first introduced, and the second was roughly 5 cm from where 3OC6HSL was introduced. The cells were imaged using the fluorescein isothiocyanate (FITC) channel. The exposure and gain were kept constant for each image and condition (exposure 3.75 seconds, 0 gain). Bottom threshold was set to

5000 arbitrary units (a.u.) in all experiments to remove background fluorescence. GFP intensity was quantified in random cells from each of three images taken per experiment. Cell fluorescence was quantified using cellSens software and determined using the following equation:

Corrected fluorescence = (fluorescence x area) – (background fluorescence x area)

The exact same shape was used in the quantification of cell and background fluorescence. The average of all corrected fluorescence values were calculated and outliers removed (those that were outside one standard deviation of the average). The average was plotted from 5 replicates.

Critical threshold experiments

Cultures were grown overnight in 5 mL LB medium with 25 μg/mL chloramphenicol and 50 μg/mL kanamycin. A 10-fold dilution series was performed in M9 medium. CFUs were measured at the beginning of each experiment for all dilutions. 190 μL of M9 medium with different agar densities (0%, 0.2%, and 0.4%) in both OFF (no IPTG) and ON (1 mM IPTG) conditions was plated into a 96 well plate (REF 25-104; Olympus, San Diego, CA). Each well was overlaid with 70 μL mineral oil to prevent evaporation. 10 μL of each dilution was added to the center of the well. For the samples grown in the ON condition, 1 mM of IPTG was added to the sample and vortexed prior to adding it to the medium containing IPTG (the ON condition). The plate was then incubated in a Victor X4 microplate reader at 37°C for 48 hours. The plate was shaken linearly at different frequencies (1 shake/hr, 3 shakes/hr, 12 shakes/hr) for 10 seconds. Measurements

were taken at OD_{600} every 60 (for cells shaken 1/hr) or 20 minutes (for cells shaken 3 or 12/hr). A minimum of three replicates were averaged for each data point.

To examine how altering in the initial distribution of the cells in the well affected cooperation (mixed population experiments), cultures were grown overnight in 5 mL LB with 25 μg/mL chloramphenicol and 50 μg/mL kanamycin and a 10-fold dilution series was performed in M9 medium. CFUs were measured at the beginning of each experiment of all dilutions. 950 μL of M9 medium with different agar densities (0%, 0.2%, and 0.4%) containing 1 mM IPTG was added to 50 μL of each culture dilution that also contained 1 mM IPTG and vortexed. 200 μL of the vortexed culture was added to a 96 well plate and the well was overlaid with 70 μL of mineral oil. The plate was incubated in a Victor X4 microplate reader at 37°C for 48 hours. The plate was shaken linearly every 20 minutes. Measurements were taken at $OD_{600.}$ A minimum of three replicates were averaged for each data point.

Statistical analysis

For the experiments where measurements were taken to demonstrate cell movement (diffusion with different agar densities and plate shaking with different agar densities), a two-tailed t-test was used and the measurements compared.

To determine the first initial density at which cooperation was significantly inhibited, thus resulting in little to no growth (C_{CRIT}), a two-tailed t-test was used to determine if the OD_{600} values observed at 48 hours were statistically different from zero. In cases where the OD_{600} value was less than 0.01, the value was set to zero as this is below

the detectability level of the microplate reader and likely represents minor differences in background readings. This assumption has been used in previous studies to carry out similar analyses (Smith et al., 2014). C_{CRIT} was reported as the first initial density where the OD_{600} value at 48 hours was not statistically greater than zero ($P \leq 0.05$).

To determine if growth rates or translation rates were different in the various shaking frequencies, the area of the log phase for growth and translation were located by visually examining the growth curves and by then fitting linear lines through the selected data such that $R^2 > 0.95$. Following this, the slope of each of the lines was calculated and a two-tailed t-test was performed. Rates were considered to not be statistically significant if $P < 0.05$. Note that the different frequencies were compared against each other.

Results

Engineered bacteria that require cooperation to survive

The objective of this thesis is to investigate quorum sensing and diffusion sensing theories in a synthetic bacterial system. To achieve this, a bacterial strain that requires communication as a facet of its survival was needed. In recent years, Allee effects have become increasingly studied because of the potential role that they may play in extinctions, of already endangered, rare or dramatically declining species, invasive species (Taylor & Hastings, 2005), reintroduction biology (Dai, Vorselen, Korolev, & Gore, 2012), as well as in the study of epidemiology and infectious diseases (Smith et al., 2014). As Allee effects have been recognized to be a common cause of extinction in low-density populations, understanding this effect is imperative to developing strategies to manage species invasion, establishing guidelines for species introduction for biological control, and for conservation

of endangered species (Robinet et al., 2008). In some species, the ability to cooperate determines growth and survival. While first reported in goldfish, obligate cooperation is now observed in many species, including *Danaus plexippus*, *Vincetoxicum rossicum,* and in *V. cholera* pathogenesis (Smith et al., 2014). In addition to the tragedy of the commons, the Allee effect is now being widely recognized as a mechanism by which cooperative organisms can go extinct, yet it remains substantially less explored.

Named after the ecologist Warder C. Allee, Allee effects are defined as the positive relationship between any fitness component of a species and the density of the population (Stephens, Sutherland, & Freckleton, 1999; Taylor & Hastings, 2005; Tobin et al., 2009; Tobin, Berec, & Liebhold, 2011). For a species with an Allee effect, cooperation is only initiated once a sufficiently high density of individuals is reached. Once cooperation is initiated, the species can survive. Otherwise, if the density of individuals is too low, cooperation is not initiated, or is insufficient, the population goes extinct (Smith et al., 2014). This could be in response to not finding a mate or species' inability to colonize empty sites (Ferdy & Molofsky, 2002). Thus, for cooperative species, as the individuals in a population decrease, the benefits gained through cooperation may diminish disproportionately, decreasing one or more of the components of individual fitness (Angulo, Rasmussen, Macdonald, & Courchamp, 2013). Indeed, the engineered bacteria used in this study have the core behavior of the Allee effect as they require cooperation to survive. The initial density of the bacterial population determines if cooperation will be successful.

The engineered bacteria that were used in this study were previously designed and used in Smith et al. (2014). The circuit was designed to display the bistable growth seen in

species with an Allee effect (Keitt, Lewis, & Holt, 2001), where the growth of the population depends upon the initial density of the population. If the initial density is sufficiently high, growth occurs. However, if the initial density is below a critical threshold, C_{CRIT}, growth is significantly reduced or does not occur. To engineer these bacteria, two systems were used. The first was the LuxR/LuxI quorum sensing system from *V. fischeri* (Miller & Bassler, 2001), which allows for the cells to communicate with each other. The second was the CcdA/CcdB toxin/antitoxin module that controls population survival (Figure 5). To induce the system, 1 mM IPTG was introduced to the medium, activating the $P_{lac/ara}$ and P_{lac} promoters. Activation of the $P_{lac/ara}$ and P_{lac} promoters drives the expression of both the LuxR/LuxI system, and *ccdB*, respectively, which causes death by inhibiting DNA replication (Dao-Thi et al., 2005). However, CcdB can be inhibited by CcdA, which is controlled by the quorum-sensing module. AHL is synthesized by *luxI*, and can readily diffused across the cell membrane. Here, the concentration of AHL in the medium increases as the population of bacteria increases. Once the AHL reaches a sufficiently high concentration, it activates the P_{lux} promoter, driving the expression of *ccdA*, which inhibits CcdB, and rescues the population (Smith et al., 2014) (Figure 5).

An experimental framework to study quorum sensing and diffusion sensing

To observe facets of quorum sensing and diffusion sensing in the same system, an experimental framework was designed where three critical aspects of cell communication could be perturbed. These three aspects were 1) the diffusion rate of the autoinducer, 2) the stability of the autoinducer, and 3) the spatial distribution of the engineered bacteria. To realize these requirements, an experimental design was devised in which the bacteria were seeded into the center of a 96 well microplate and allowed to diffuse. Altering the

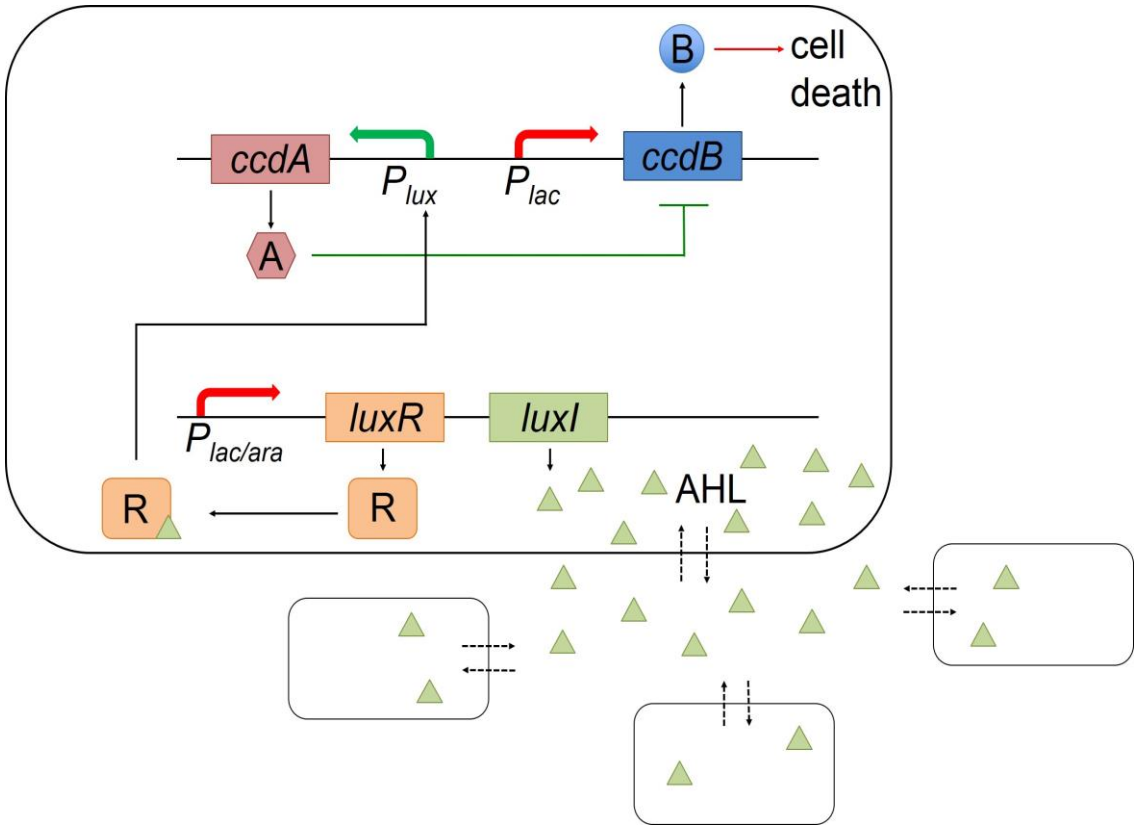

Figure 5: A synthetic gene circuit that confers an Allee effect to a population of bacteria.

This circuit was designed so the growth of the population was dependent on the initial density being above a threshold density (C_{CRIT}). This was achieved through the use of two systems. The first was the LuxR/LuxI quorum sensing system from *Vibrio fischeri*, which produced AHL (green triangles) needed for the cells to activate the desired gene expression. The second was the CcdA/CcdB toxin/antitoxin module, which controlled population survival. The circuit was induced with 1 mM IPTG, which activated the $P_{lac/ara}$ and P_{lac} promoters, driving the expression of the LuxR/LuxI system and CcdB, respectively. *luxI* (green rectangle) synthesized the autoinducer AHL, which could readily diffuse across the cell membrane, while *luxR* (orange rectangle) synthesized a receptor (R, orange square). CcdB will lead to cell death, but can be inhibited by CcdA, the antitoxin. Once there was a sufficient amount of AHL produced, it would bind to the receptor, which would activate the P_{lux} promoter, driving the expression of CcdA. CcdA could then inhibit CcdB, and the population would be rescued (Smith et al., 2014).

concentration of the agar in the medium controlled the rate of diffusion. As the concentration of the agar was increased, the diffusion rate of both the autoinducer and the bacteria would decrease. To realize the second condition, the stability of the autoinducer was altered by changing the pH in the medium. Decreasing the pH in the medium slowed the degradation of the autoinducer, making it more stable in the environment. To realize

the third condition, the microplate was then shaken at different time intervals to examine how disturbances affected the ability of the population to cooperate.

Increasing agar concentration decreases dispersal

One previously described mechanism to alter spatial distribution of bacteria is by varying the percentage of agar in the medium (Ben-Jacob et al., 1994). To determine how increasing the percentage of the agar in medium would affect the dispersal rate of bacteria, a strain of *E. coli* that expresses a green fluorescent protein (GFP) in response to the addition of atc was used. These bacteria were seeded into a cell chamber containing medium with different concentrations of agar (0%, 0.2%, and 0.4%). The initial position of the cells was recorded using a fluorescent microscope. The cells were then allowed to incubate undisturbed at 37°C. After 2 hours for cultures in 0% agar and 12 hours for cultures in 0.2% and 0.4% agar, the positions of the cells were again observed using the fluorescent microscope. The dispersal rate was determined by taking the average of the total distance the cells moved for each of the agar concentrations, and dividing it by the number of hours over which the experiment occurred.

As the agar concentration in the medium was increased, it was observed that the dispersal rate of the cells decreased (Figure 6). Cells that were placed in a medium with 0% agar (purple) were able to disperse quickly, and moved on average 644.23 μm/hour (± 521.25 μm/hour). When the agar concentration was increased to 0.2% agar (blue), the cell dispersal decreased, with the average dropping to 11.98 μm/hour (± 9.08 μm/hour). Increasing in agar concentration to 0.4% agar (red) resulted in a further decrease in cell dispersal to 0.92 μm/hour (± 0.64 μm/hour).

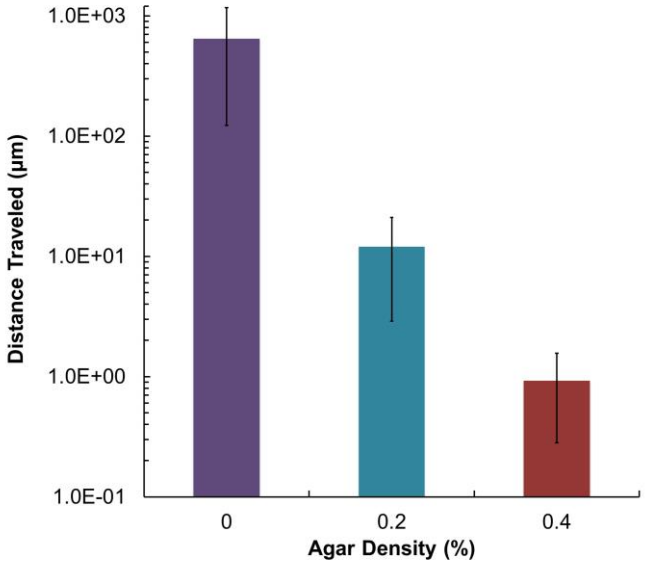

Figure 6: Increasing agar percentage in the medium decrease bacterial dispersal rate

It was confirmed that as the agar concentration increased from 0%, to 0.2%, to 0.4%, the dispersal rate of the cells decreased. Using a strain of *E. coli* that expresses GFP in response to atc, the distance (µm) that the bacteria travelled over time in an undisturbed environment was measured. Cells grown in the presence of 0% agar (purple) traveled the furthest (644.23 µm/hour (± 521.25) µm/hour). The distance decreased as agar concentration increased with the cells moving 11.98 µm/hour (± 9.08 µm/hour) in 0.2% agar (blue) while cells grown in 0.4% agar (red) traveled the least (0.92 µm/hour (± 0.64) µm/hour). Standard deviation derived from six replicates. *P*-values ≤ 0.03 amongst all conditions.

Shaking the microplate disturbs the spatial structure of the bacteria.

To verify that shaking the microplate reader caused movement of the cells, a strain of *E. coli* that expressed GFP was inoculated into a cell chamber containing M9 medium with various agar densities. Once an initial image was taken under a fluorescent microscope, the cells were placed on the microplate reader and shaken. The cells were given 10 minutes to settle before being examined again under the fluorescent microscope. The distance that the bacteria moved in the different agar concentrations was measured using the cellSens software. It was observed that the shaking altered the spatial positioning of the cells (Figure 7). As the agar density increased, the ability of the cells to be moved by the shaking of the microplate reader decreased. Specifically, we found that with 0% (purple), 0.2% (blue), and 0.4% agar (red), the cells were moved 1625.98 (± 616.37) µm, 92.49 (± 23.29) µm, and 11.93 (± 3.60) µm, respectively. *P*-values were ≤ to 0.05 amongst all conditions.

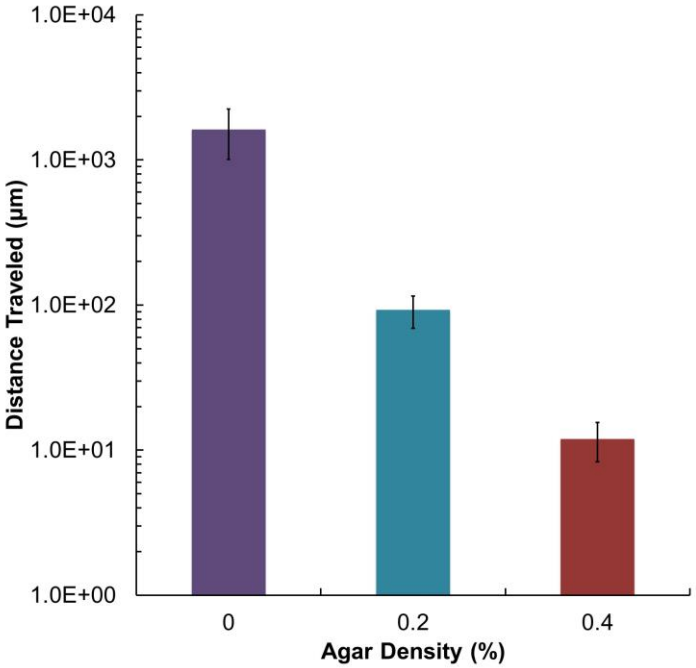

Figure 7: Increasing agar percentage in the medium decreases movement of bacterial cells shaken in the microplate

Agar concentration affected the distance that the cells moved when shaken. Here, the shaking feature of the microplate reader was used to disturb the spatial arrangement of the cells. Using a strain of *E. coli* that expressed GFP in response to atc, it was observed that the greatest movement in bacteria in cells shaken in 0% agar (1625.98 (± 616.37) µm) (purple). As the density of the agar was increased to 0.2% (blue) and 0.4% (red), the distance that cells moved due to shaking of the microplate decreased (92.49 (± 23.29) µm, and 11.93 (± 3.60) µm, respectively) Standard deviation derived from three replicates. *P*-values ≤ 0.05 amongst all conditions.

Confirmation that shaking frequency does not affect growth or translation rate

Previous studies have shown that certain conditions can affect growth (Brophy & Voigt, 2014; Tan, Marguet, & You, 2009) or translation (Tanouchi, Pai, Buchler, & You, 2012) rates of synthetic bacteria, impacting the functionality of the circuit, and thus the behavior of the bacteria. As such, verification was needed to ensure that shaking the plate at different frequencies did not affect either the growth or translation rates in the engineered bacteria. To do this, a strain of bacteria that contained an atc inducible *gfp* construct was used in order to examine both growth and translation rates. Neither the growth rate nor translation rate was statistically different for any of the shaking frequencies that were examined.

Specifically, for growth rate an increase in OD_{600}/min of 4×10^{-4} ($\pm 1 \times 10^{-4}$), 3×10^{-4} ($\pm 1 \times 10^{-4}$) and 4×10^{-4} ($\pm 1 \times 10^{-4}$) was detected when cells were shaken at 1/hr, 3/hr and

12/hr, respectively (Figure 8, left panel). When all three growth rates were compared amongst each other, all *P*-values were ≥ 0.47. For translation rate (Figure 8, right panel), the values were normalized and were found to be 541.7 a.u./min (± 73.4), 398.0 a.u./min (± 29.8), and 398.3 a.u./min (± 50.4) when the cells were shaken at 1/hr, 3/hr and 12/hr, respectively. When all three translation rates were compared amongst each other, all *P*-values were ≥ 0.12.

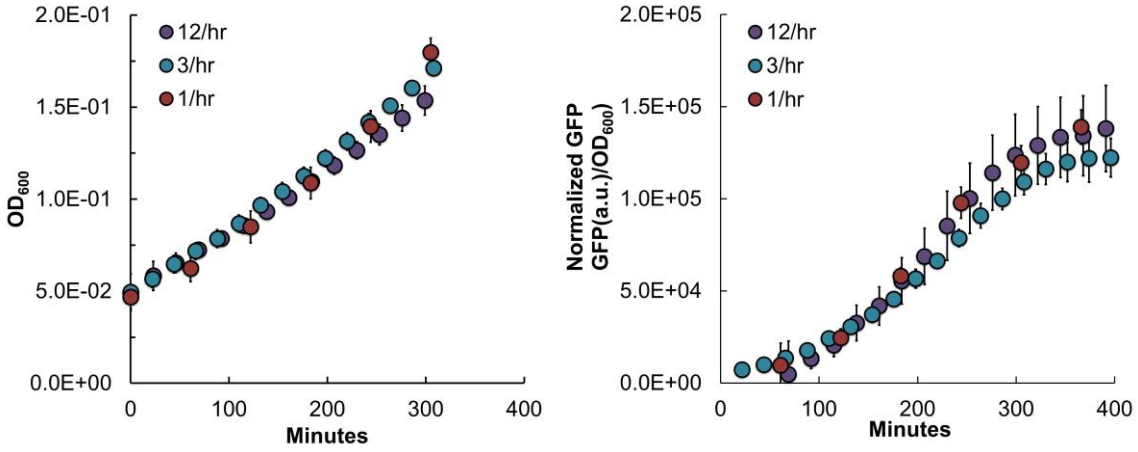

Figure 8: Growth and translation rates of the engineered bacteria were not affected by shaking of the microplate

Left panel: Growth rate was not affected by the shaking of the microplate reader. Cells that were shaken 3/hr (blue circles) and 12/hr (purple circles) were measured at OD_{600} every 20 minutes while the cells shaken 1/hr (red circles) were read every 60 minutes. No statistical difference was found in growth rate for all 3 conditions ($P \geq 0.48$ for all comparisons). In both panels, standard deviation from three replicates.
Right panel: Translation rate was also unaffected by the shaking of the plate reader under all three conditions. GFP was normalized to OD_{600} and no statistical difference in translation rate was observed for all three conditions ($P \geq 0.13$ for all comparisons).

AHL diffusion decreases as agar concentration increases

The impact that different agar densities in the medium had on the diffusion of AHL was examined by using a previously published detector strain that expresses GFP(UV) in response to the specific AHL used by the engineered bacteria, 3OC6HS (Song et al., 2009). Previous studies found that increasing the agar percentage in growth medium resulted in a

decrease in diffusion rate of small molecules (Kümmerli, Griffin, West, Buckling, & Harrison, 2009). In this study it was noted that as the agar percentage increased in the medium, the intensity of GFP(UV) expression approximately 1 cm away from the initial point of inoculation decreased (Figure 9). Specifically, it was seen that with 0%, 0.2%, and 0.4% agar in the medium, the GFP(UV) fluorescence measured 221.3 a.u. (± 43.8), 107.84 a.u. (± 64.5) and 70.32 a.u. (± 53.5), respectively, at ~1 cm away from the initial inoculation point of AHL. Similar trends were observed ~5 cm further in the channel, where the GFP(UV) fluorescence measured 24.09 a.u. (± 10.22), 14.13 a.u. (± 6.02) and 6.84 a.u. (± 2.73) for 0%, 0.2%, and 0.4%, respectively. When averages were compared within each point on the slide, the *P* values were always less than or equal to 0.021. Given that expression from the P_{lux} promoter is dependent upon the concentration of AHL detected (Collins, Arnold, & Leadbetter, 2005), it can be surmised that this decrease in intensity was a result of less 3OC6HSL in the cell chamber at both locations, because of a decreased diffusion rate due to increased agar concentration. The cells with AHL were incubated for five hours prior to performing a measurement to ensure sufficient expression of LuxR and AHL-induced expression of GFP(UV).

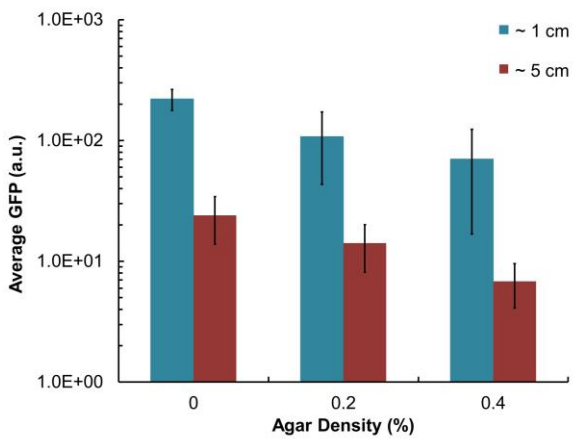

Figure 9: Increasing agar percentage in the medium decreased the diffusion rate of AHL

It was observed that increasing the agar concentration in the medium decreased the diffusion rate of AHL. A detector strain was used that expressed GFP in response to AHL that was inoculated into one end of a cell chamber. GFP fluorescence was recorded ~1 cm (blue bars) and ~5 cm (red bars) from where AHL was introduced into the chamber. The cells at ~1 cm showed more fluorescence for all conditions compared to the cells at ~5 cm and the fluorescence decreased as agar concentration increased. *P*-values ≤ 0.021 for all comparisons. Standard deviation from five replicates.

The effect of pH on the stability of AHL

Several previous studies have found that increasing pH decreases the stability of AHL (Englmann et al., 2007; You et al., 2004). Furthermore, a previous study has shown that increasing pH increases C_{CRIT} of the engineered bacteria in this study (Smith et al., 2014). These changes in pH did not affect cell physiology or circuit functionality as they were measured in Smith et al., 2014 using fluorescent reporters and growth rate assays. As such, the impact of pH on AHL stability was not directly measured in this study; instead, the data from the previous studies was utilized.

Verification of circuit functionality

Verification was needed to ensure that the engineered bacteria functioned as desired in the experimental framework. Towards this end, the engineered bacteria were grown in a 96 well plate in both the ON (with IPTG) and OFF (without IPTG) conditions at different initial cell densities. The bacteria were grown in a microplate reader at 37°C for 48 hours and cell density was measured at OD_{600} every 20 minutes (following shaking). The cells that were induced with IPTG (ON condition) displayed a strong Allee effect, where growth was significantly reduced at initial densities that were less than ~10^5 CFU/mL, (or C_{CRIT}) (Figure 10). Experimentally, we defined C_{CRIT} as the first initial density where the final OD_{600} was not statistically different than zero. Wells that were inoculated with cultures that contained initial densities greater than ~10^5 CFU/mL displayed substantial increases in OD_{600}, which was indicative of growth. For the cells grown without IPTG (OFF condition), OD_{600} increased in all of the wells regardless of the initial cell density.

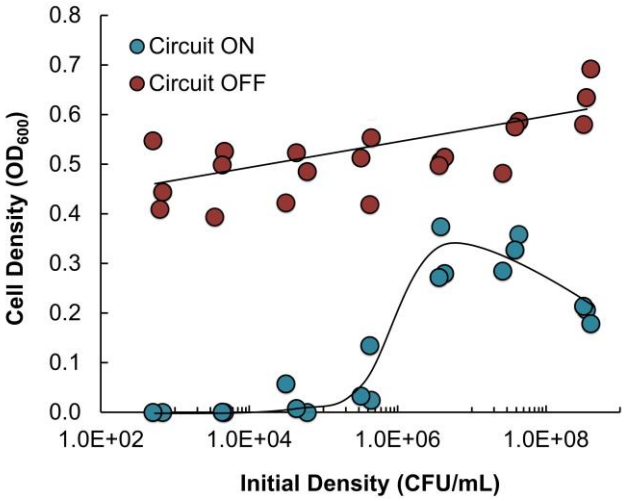

Figure 10: Verification of circuit functionality in the experimental setup

When the engineered bacteria were grown in the OFF condition (without IPTG, red circles), the cells grew regardless of the initial cell density. However, when the bacteria were grown in the ON condition (1 mM IPTG, blue circles), significant growth was not detected below ~10^5 CFU/mL (C_{CRIT}, P = 0.06). Wells that were inoculated with an initial density more than ~10^5 CFU/mL survived. Lines drawn as a guide.

Shaking affects population survival

The impact that changing the agar concentration in the medium, as well as how the shaking frequency (e.g., the spatial distribution of cells) affected population survival, was examined. The cells were inoculated into the center of a 96 well plate containing media with different percentages of agar (0%, 0.2%, and 0.4%) in both the ON (with IPTG) and OFF (without IPTG) conditions. The cells were grown in a microplate reader at 37°C for 48 hours and OD_{600} (e.g., cell density) was measured every 20 minutes (3/hr) (following shaking). For the cells grown in the ON condition, C_{CRIT} (Figure 11, top panel, circles with thicker borders) varied depending on the density of the medium. Cells grown in medium with 0% agar had a C_{CRIT} that averaged 3.93 x 10^5 (± 6.42 x 10^4) CFU/mL (Figure 11, top panel, purple circles). When the agar percentage in the medium was increased to 0.2% agar, the C_{CRIT} was reduced to an average of 4.47 x 10^4 (± 1.46 x 10^4) CFU/mL (Figure 11, top panel, blue circles). A further increase in the percentage of agar in the medium (0.4%) resulted in a further decrease in C_{CRIT} to 5.70 x 10^3 (± 1.42 x 10^3) CFU/mL (Figure 11, top

panel, red circles). For the cells grown without IPTG (OFF condition), OD_{600} increased in all of the wells regardless of the initial cell density (Figure 11, bottom panel). When the values of C_{CRIT} were compared amongst each other, the *P* values were always less or equal to 0.04. That is, C_{CRIT} was statistically different for cells grown in medium containing the different agar concentrations.

Next, the frequency at which the microplate was shaken was reduced to every 60 minutes (1/hr) and OD_{600} was quantified over 48 hours. When the bacteria were grown in the ON condition, it was found that C_{CRIT} was the same for all of the cells, regardless of the agar concentration that it was grown in (Figure 12, top panel, circles with thicker boarders). Specifically, cells grown in medium with 0%, 0.2% and 0.4% agar had C_{CRIT} of 3.96×10^4 ($\pm 2.05 \times 10^4$) CFU/mL (purple circles), 4.78×10^4 ($\pm 7.72 \times 10^3$) CFU/mL (blue circles), and 4.20×10^4 ($\pm 1.14 \times 10^4$) CFU/mL (red circles), respectively. For the cells grown without IPTG (OFF condition), OD_{600} increased regardless of the initial cell density (Figure 12, bottom panel). When C_{CRIT} values were compared amongst each other, the *P*-values were always greater than or equal to 0.44 indicating that all values of C_{CRIT} were statistically equivalent.

The frequency at which the microplate was shaken was increased to every 5 minutes (12/hr), and a similar trend was seen. That is, in the ON condition, C_{CRIT} was the same for all bacterial cells grown in each medium (Figure 13, top panel, circles with thicker borders). Specifically, the C_{CRIT} values for medium containing 0%, 0.2%, and 0.4% agar were 3.76×10^4 ($\pm 3.51 \times 10^3$) CFU/mL (purple circles), 4.48×10^4 ($\pm 1.33 \times 10^4$) CFU/mL (blue

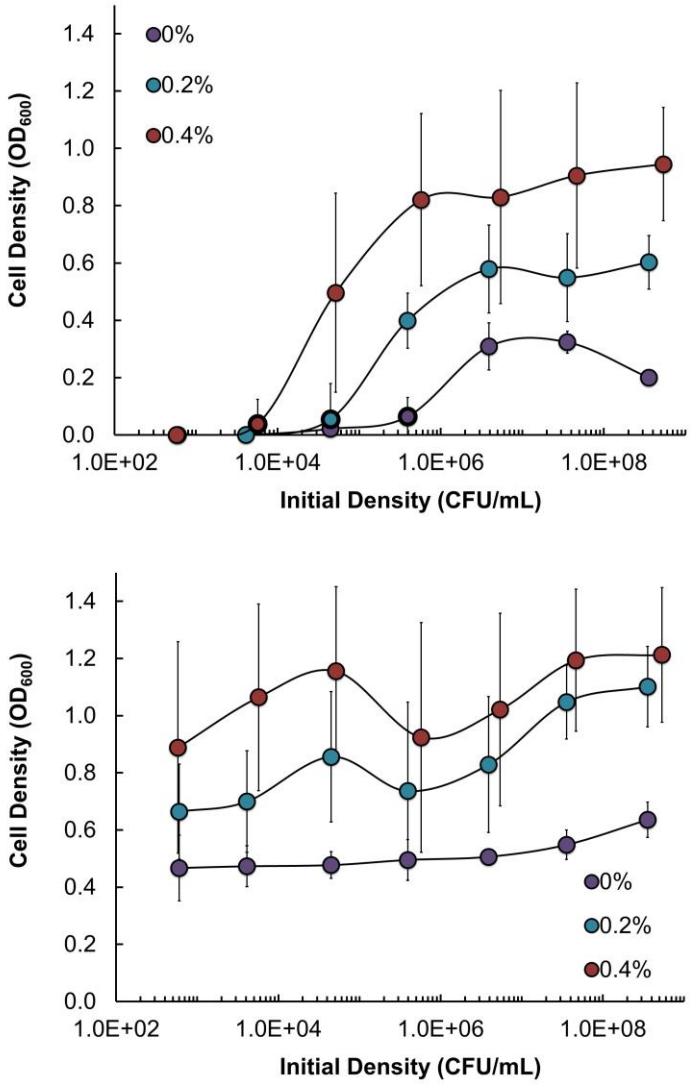

Figure 11: The concentration of agar in the medium affects bacteria survival when the cells are shaken at an intermediate frequency

The engineered bacteria were diluted in a 10-fold serial dilution and inoculated into the center of a 96 well plate containing different densities of agar (0%, 0.2%, and 0.4%) in both the OFF (without IPTG) and ON (with 1mM IPTG) conditions. The cells were then shaken 3/hr (intermediate shaking frequency). When the cells were grown in the ON condition (top panel), C_{CRIT} (circles with thicker boarders) was observed and was varied dependent on the agar density. Cells grown in 0% agar (purple circles) had the highest C_{CRIT} (average 3.93×10^5 ($\pm$ 6.43×10^4) CFU/mL). As the agar density was increased to 0.2% (blue circles), the C_{CRIT} decreased (average 4.47×10^4 ($\pm$ 1.46×10^4) CFU/mL). Further increasing the agar density to 0.4% (red circles) caused C_{CRIT} to decrease once again (average 5.70×10^3 ($\pm$ 1.42×10^3) CFU/mL). *P*-values were ≤ 0.04 for all C_{CRIT} values. When the cells were grown in the OFF condition (bottom panel), growth was observed at all initial cell densities, regardless of the agar density. Standard deviation derived from a minimum of three replicates. Lines drawn as a guide.

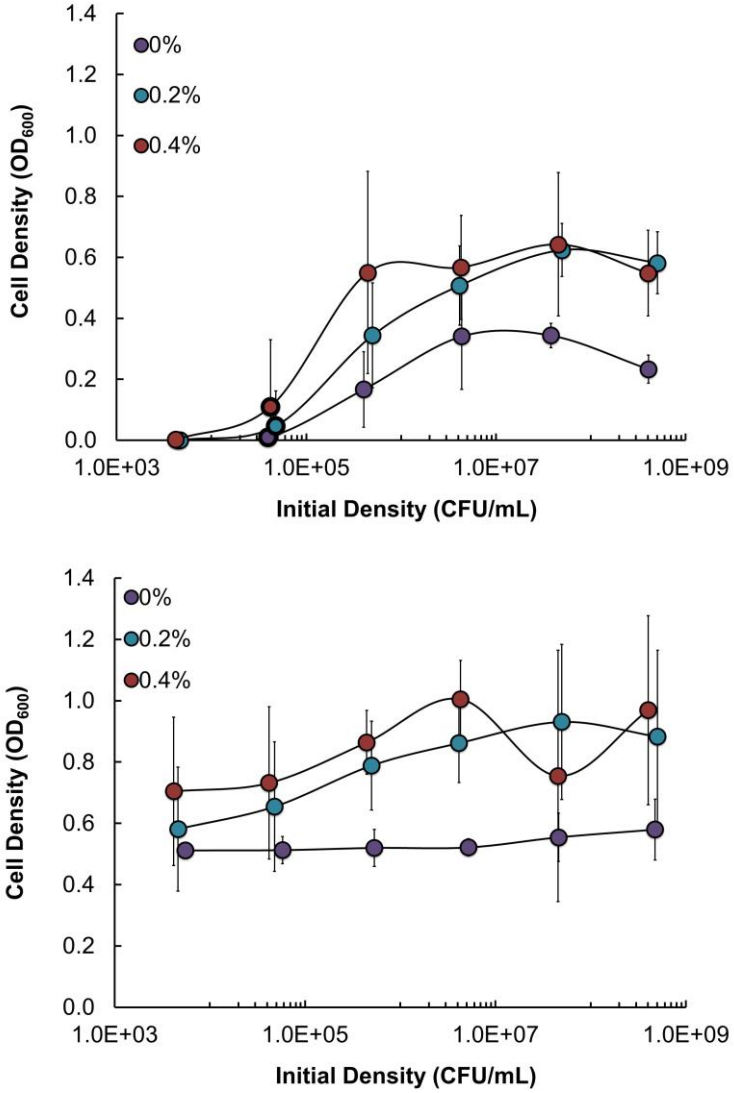

Figure 12: C_{CRIT} was equivalent for bacteria grown in all agar densities when shaken at low frequency

The engineered bacteria were diluted in a 10-fold serial dilution and inoculated into the center of a 96 well plate containing different densities of agar (0%, 0.2%, and 0.4%) in both the OFF (without IPTG) and ON (with 1mM IPTG) conditions. The cells were then shaken 1/hr (low shaking frequency) then read at OD_{600}. In the ON condition, C_{CRIT} (top panel, circles with thicker borders) was observed and found to be statistically equivalent at all agar densities (*P*-values ≥ 0.44). Specifically, in 0% agar (purple circles), C_{CRIT} was found to be on average 3.96×10^4 ($\pm 2.05 \times 10^4$) CFU/mL, in 0.2% agar (blue circles), C_{CRIT} was found to be on average 4.78×10^4 ($\pm 7.72 \times 10^3$) CFU/mL, and in 0.4% agar (red circles), C_{CRIT} was found to be 4.20×10^4 ($\pm 1.14 \times 10^4$) CFU/mL. In the OFF condition (bottom panel), the cells grew at all initial cell densities and in all agar concentrations. Standard deviation derived from a minimum of three replicates. Lines drawn as a guide.

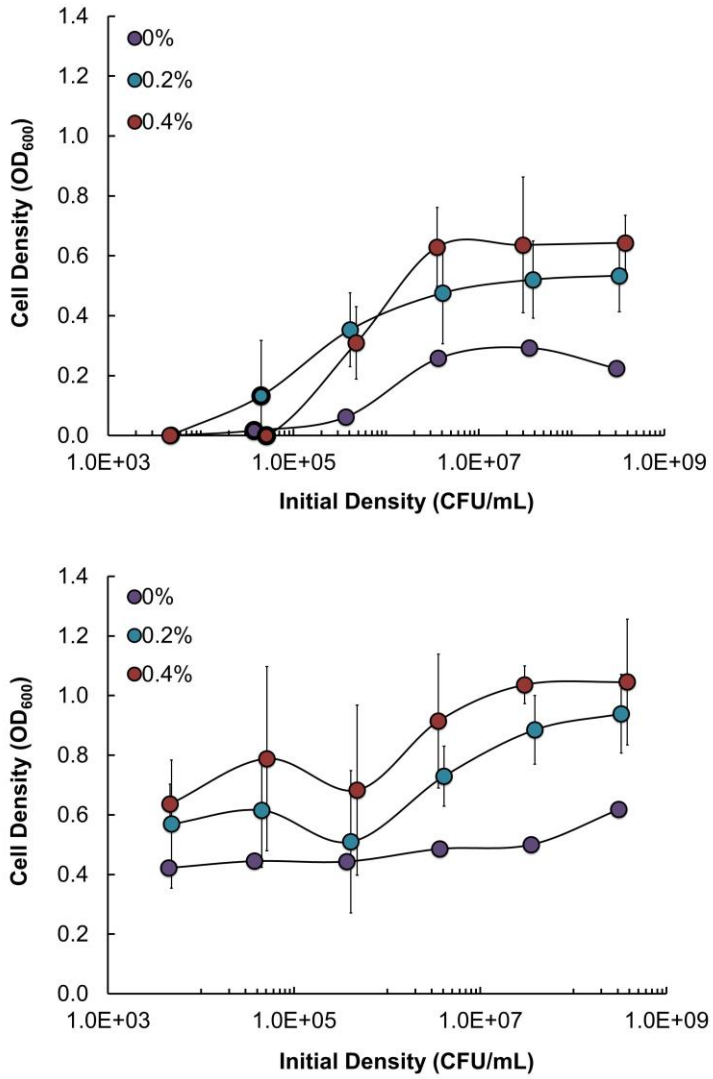

Figure 13: C_{CRIT} was equivalent for bacteria grown in all agar densities when shaken at high frequency

The engineered bacteria were diluted in a 10-fold serial dilution and inoculated into the center of a 96 well plate containing different densities of agar (0%, 0.2%, and 0.4%) in both the OFF (without IPTG) and ON (with 1mM IPTG) conditions. The cells were then shaken 12/hr (high shaking frequency) then read at OD_{600}. In the ON condition, C_{CRIT} (top panel, circles with thicker borders) was observed and found to be statistically equivalent for all agar densities (*P*-value ≥ 0.44). Specifically, in 0% agar (purple circles), C_{CRIT} was found to be on average 3.76×10^4 ($\pm 3.51 \times 10^3$) CFU/mL, in 0.2% agar (blue circles), C_{CRIT} was found to be on average 4.48×10^4 ($\pm 1.33 \times 10^4$) CFU/mL, and in 0.4% agar (red circles), C_{CRIT} was found to be 5.10×10^4 ($\pm 1.84 \times 10^4$) CFU/mL. Growth was observed in all of the wells where bacteria were grown in the OFF condition (bottom panel), regardless of the agar concentration or initial cell density. Standard deviation derived from a minimum of three replicates. Lines drawn as a guide.

circles), and 5.10 x 10^4 (± 1.84 x 10^4) CFU/mL (red circles) respectively. When C_{CRIT} values were compared amongst each other, the *P*-values were always greater than or equal to 0.44, indicating statistical equivalence amongst all C_{CRIT} values. Similarly, when the C_{CRIT} values for the bacterial cells grown at a low shaking frequency (1/hr) were compared to those grown at a high shaking frequency (12/hr), C_{CRIT} values were again statistically equivalent (*P*-values were greater than or equal to 0.61). For the cells grown without IPTG (OFF condition), OD_{600} increased regardless of the initial cell density (Figure 13, bottom panel).

Overall, when comparing C_{CRIT} for all agar densities and shaking frequencies (Figure 14), it was found that C_{CRIT} for low (1/hr) and high (12/hr) shaking frequencies were statistically equivalent for all agar densities (*P*-value ≥ 0.66). This result was consistent with the central prediction of the quorum sensing theory, which states that cell density is the sole factor initiating expression of autoinducer-regulated genes. However, when C_{CRIT} for all agar densities were compared at the intermediate (3/hr) shaking frequency, it was found that C_{CRIT} varied amongst the different agar densities (*P*-value ≤ 0.04). This result was more consistent with the theory of diffusion sensing, in which environmental factors (bacterial dispersal and AHL diffusion) dictate expression of autoinducer regulated genes (Figure 14).

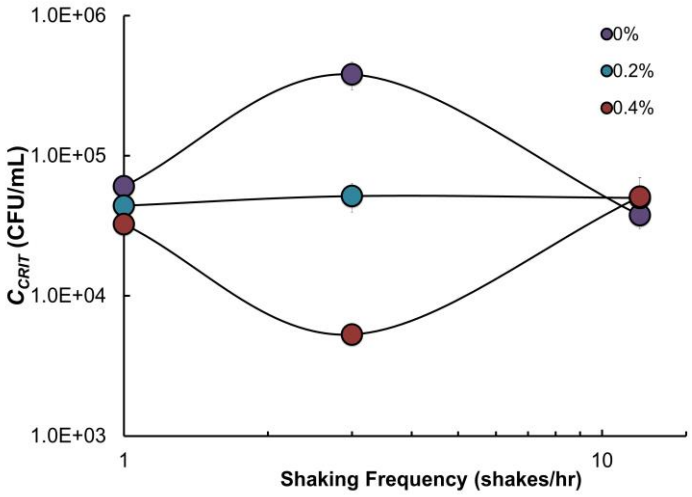

Figure 14: Summary figure showing C_{CRIT} values of engineered bacteria grown in medium with different agar densities and at different shaking frequencies demonstrated the core facets of both quorum sensing and diffusion sensing

When all C_{CRIT} values were compared amongst each other, C_{CRIT} was observed to be statistically equivalent for all agar concentrations (0%, 0.2%, and 0.4%) at low shaking frequency (1/hr) (3.96×10^4 CFU/mL, 4.78×10^4 CFU/mL, and 4.20×10^4 CFU/mL) and at high shaking frequency (12/hr) (3.76×10^4 CFU/mL, 4.48×10^4 CFU/mL, and 5.10×10^4 CFU/mL). *P*-values for these conditions were always greater than or equal to 0.44. At intermediate shaking frequency (3/hr), C_{CRIT} was dependent upon agar concentration, where highest agar concentration (0.4%) had the lowest C_{CRIT} (5.70×10^3 CFU/mL). As the agar concentration was decrease to 0.2% and 0%, C_{CRIT} increased to 4.47×10^4 CFU/mL and 3.93×10^5 CFU/mL, respectively. *P*-values amongst these three conditions were always less than or equal to 0.04. Standard deviation from a minimum of three replicates. Lines drawn as a guide. C_{CRIT} was determined from data in Figures 11, 12, and 13.

Increasing the stability of AHL reduces C_{CRIT} at low and intermediate shaking frequency.

The role that access to AHL had on the above trends was examined. One mechanism to perturb access to AHL, but not cell physiology or circuit functionality, was to modify the pH of the medium. Disrupting AHL access might lead to a better understanding as to why C_{CRIT} varies at intermediate shaking frequency, but not at low or high shaking frequency. To perturb AHL access, the pH of the growth medium was decreased from pH 7.4 to pH 7.0. This served to stabilize AHL and has been previously shown to decrease C_{CRIT} (Smith et al. 2014).

Cells were inoculated into the center of a 96 well plate and grown in the ON condition (1 mM IPTG) in a microplate reader at 37°C for 48 hours. OD_{600} (e.g., cell density) was measured every 20 minutes (3/hr). At the intermediate shaking frequency, a decrease in C_{CRIT} was observed in bacteria grown in 0% and 0.2% agar, while bacteria grown in 0.4% agar exhibited no change in C_{CRIT}. Specifically, C_{CRIT} (Figure 15, circles with thicker border) decreased in the medium when the agar concentration was 0% and 0.2% to 4.73 x 10^4 (± 1.56 x 10^4) CFU/mL (Figure 15, purple circles) and 4.80 x 10^3 (± 1.41 x 10^2) CFU/mL (Figure 15, blue circles), respectively. However, there was no change in C_{CRIT} in the 0.4% agar concentration (4.80 x 10^3 (± 1.41 x 10^2) CFU/mL) (Figure 15, red circles).

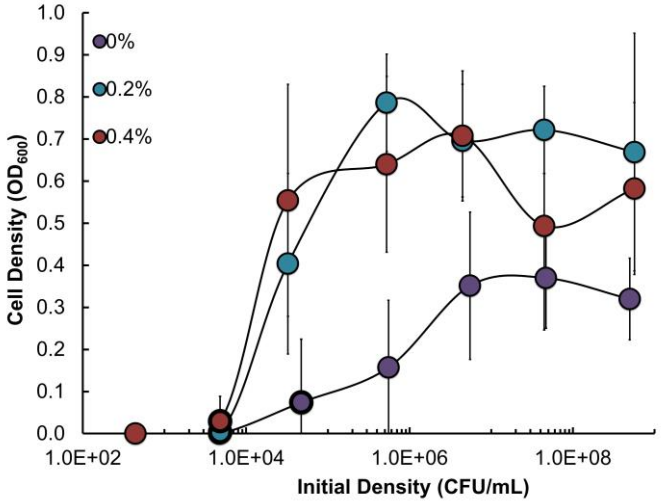

Figure 15: Stabilization of AHL reduced C_{CRIT} in cells grown in 0% agar and 0.2% agar, but had no effect on C_{CRIT} in cells grown in 0.4% agar when shaken at intermediate frequency

The engineered bacteria were diluted in a 10-fold serial dilution and inoculated into the center of a 96 well plate containing different densities of agar (0%, 0.2%, 0.4%) in the ON (1mM IPTG) condition in M9 medium buffered to a pH of 7.0. The cells were shaken 3/hr (intermediate shaking frequency) then read at OD_{600}. The bacteria grown in 0% agar had a 10-fold decrease in C_{CRIT} (circles with thicker borders) with an average of 4.73 x 10^4 (± 1.56 x 10^3) CFU/mL (purple circles) when compared to the bacteria grown in M9 media with a higher pH (7.4). The cells grown in 0.2% agar also had a 10-fold decrease in C_{CRIT} with an average of 4.80 x 10^3 (± 1.41 x 10^2) CFU/mL (blue circles). Bacteria grown in medium with 0.4% agar had no change in C_{CRIT} (4.80 x 10^3 (± 1.41 x 10^2) CFU/mL, red circles). Standard deviation derived from a minimum of three replicates. Lines drawn as a guide.

When the cells were shaken at low frequency (1/hr), C_{CRIT} (Figure 16, circles with thick borders) decreased only in the cells grown in 0.4% agar, while cells grown in the 0% and 0.2% agar remained unchanged when compared to cells grown in medium with a pH 7.4. That is, C_{CRIT} for cells grown in medium containing 0.4% agar decreased to 5.59 x 10^3 (± 1.19 x 10^3) CFU/mL (Figure 16, red circles), while 0% and 0.2% agar remained unchanged with 7.60 x 10^4 (± 4.36 x 10^4) CFU/mL (Figure 16, purple circles) and 6.04 x 10^4 (± 3.68 x 10^4) CFU/mL, respectively (Figure 16, blue circles). The *P*-value for cells grown in medium with pH 7.0 at 0% and 0.2% agar was 0.57, indicating that they were statically equivalent.

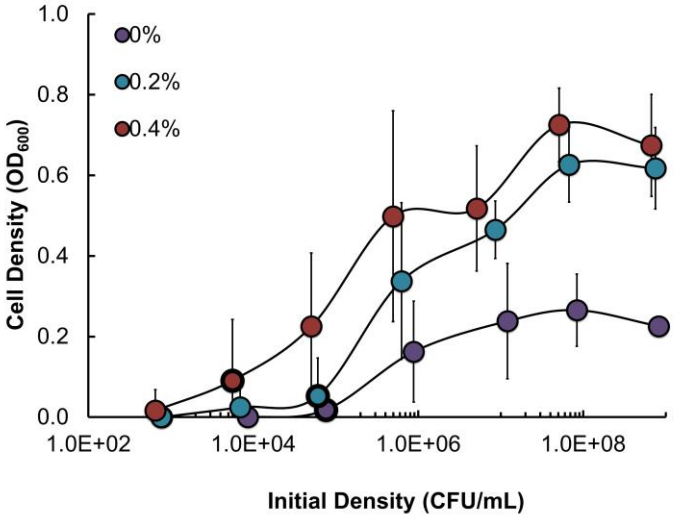

Figure 16: Stabilization of AHL decreased C_{CRIT} in bacteria grown in 0.4% agar but had no effect on bacteria grown in 0% agar and 0.2% agar when shaken at low frequency.

The engineered bacteria were diluted in a 10-fold serial dilution and inoculated into the center of a 96 well plate containing different agar densities (0%, 0.2%, and 0.4%) in the ON (with 1mM IPTG) condition in M9 media buffered to pH 7.0. The cells were shaken 1/hr (low shaking frequency) then read at OD_{600}. For the cells shaken in 0% and 0.2% agar, C_{CRIT} (circles with thicker borders) was found to have not changed. That is, C_{CRIT} was 7.60 x 10^4 (± 4.36 x 10^4) CFU/mL for cells grown in 0% agar (purple circles) and 6.04 x 10^4 (± 3.68 x 10^4) CFU/mL (blue circles) for 0.2% agar. C_{CRIT} was increased for bacteria grown in 0.4% agar to 5.59 x 10^3 (± 1.19 x 10^3) CFU/mL (red circles). Standard deviation derived from a minimum of three replicates. Lines drawn as a guide.

Interestingly, C_{CRIT} remained the same when the cells were shaken at high frequency (12/hr) (Figure 17, circles with thicker borders). Here, with media containing 0%, 0.2%, and 0.4% agar, C_{CRIT} values were 4.70 x 10^4 (± 6.27 x 10^3) CFU/mL (Figure 17, purple circles), 4.68 x 10^4 (± 5.80 x 10^3) CFU/mL (Figure 17, blue circles), and 3.70 x 10^4 (± 1.76 x 10^4) CFU/mL (Figure 17, red circles) respectively. When C_{CRIT} values were compared, the *P*-values were always greater than or equal to 0.20 indicating that all values of C_{CRIT} were statistically equivalent amongst each other.

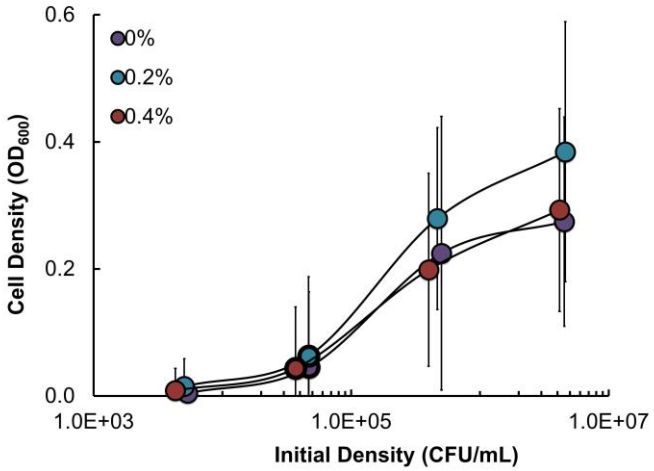

Figure 17: Stabilization of AHL had no effect on C_{CRIT} for bacteria shaken at high frequency

The engineered bacteria were diluted in a 10-fold serial dilution and inoculated into the center of a 96 well plate containing different agar densities (0%, 0.2%, and 0.4%) in the ON (1mM IPTG) condition in M9 media buffered to pH 7.0. The cells were shaken 12/hr (high shaking frequency) then read at OD_{600}. At this shaking frequency, C_{CRIT} (circles with thicker borders) remained statistically equivalent at all agar densities (*P*-value ≥ 0.20). Specifically, in 0% agar, C_{CRIT} was found to be on average 4.70 x 10^4 (± 6.27 x 10^3) CFU/mL (purple circles), in 0.2% agar, C_{CRIT} was found to be on average 4.68 x 10^4 (± 5.80 x 10^3) CFU/mL (blue circles), and in 0.4% agar, C_{CRIT} was found to be 3.70 x 10^4 (± 1.76 x 10^4) CFU/mL (red circles). These values were also statistically equivalent to the bacteria grown at the same shaking frequency in pH 7.4 (*P*-value ≥ 0.05). Standard deviation derived from a minimum of three replicates. Lines drawn as a guide.

Overall, when AHL was stabilized in the medium by reducing the pH from 7.4 to 7.0, C_{CRIT} was not affected evenly amongst shaking frequencies or agar concentrations (Figure 18). For cells shaken at low frequency (1/hr), C_{CRIT} was affected only in the cells

grown in the medium with 0.4% agar (*P*-value = 0.02 for values compared between pH 7.0 and 7.4). When C_{CRIT} values were compared at low shaking frequency between the cells grown in 0% and 0.2% agar in both pH 7.0 and pH 7.4, those *P*-values were always greater than or equal to 0.20, indicating that these values were statistically equivalent amongst all growth conditions. While the trend observed at pH 7.4 was indicative of that of quorum sensing, where the cell density depicted survival, the change in an environmental factor (pH), led to a decrease in one of the agar conditions (0.4%), leading to the assumption that a transition from quorum sensing to diffusion sensing was occurring.

The opposite trend was observed in bacteria shaken at intermediate frequency (3/hr, Figure 18). For cells grown in intermediate shaking frequency at pH 7.0, a decrease in C_{CRIT} for both 0% and 0.2% agar (*P*-values were 0.01 and 0.04, respectively) was seen, while bacteria grown in 0.4% agar had no difference in C_{CRIT} (*P*-value = 0.29) when compared to values obtained at pH 7.4. Different C_{CRIT} values for each agar density was detected when the cells were grown in a medium with pH 7.4, which is indicative of the facets of diffusion sensing. Being able to perturb these values by stabilizing AHL further supports the theory of diffusing sensing, as altering environmental factors has affected C_{CRIT} for bacteria grown in 0% and 0.2% agar. Additionally, it was found that C_{CRIT} for 0.2% and 0.4% agar were statistically equivalent (*P*-value = 1.00) in this condition.

At high shaking frequency, changing the pH to 7.0 had no effect on C_{CRIT} for bacterial cells grown in any of the agar densities (Figure 18). The C_{CRIT} was statistically equivalent at all agar densities (*P*-value $\geq$ 0.34). When these shaking frequencies were compared to those grown in a medium with a pH of 7.4, there was still no statistical difference between these values (*P*-values $\geq$ to 0.05). At this shaking frequency, neither

agar density nor pH has an effect on C_{CRIT}, suggesting that quorum sensing dominates at high shaking frequency.

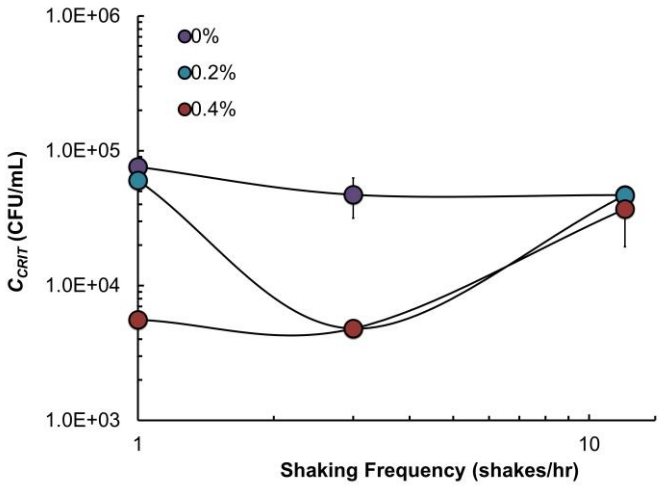

Figure 18: C_{CRIT} was not affected evenly when AHL was stabilized

When all C_{CRIT} values were compared amongst each other, it was observed that the C_{CRIT} values were not affected evenly amongst shaking frequencies nor agar concentrations. At low shaking frequency (1/hr), C_{CRIT} decreased for cells grown in 0.4% agar to 5.59×10^3 ($\pm 1.19 \times 10^3$) CFU/mL, while bacteria grown in either 0% or 0.2% agar had no statistical difference in C_{CRIT} (7.60×10^4 ($\pm 4.36 \times 10^4$) CFU/mL and 6.04×10^4 ($\pm 3.68 \times 10^4$) CFU/mL, respectively) between each other or when compared to cells grown in the same conditions at pH 7.4 (P-values ≥ 0.20). Increasing the shaking to intermediate frequency (3/hr) decreased C_{CRIT} for both 0% and 0.2% agar (4.73×10^4 ($\pm 1.56 \times 10^4$) CFU/mL and 4.80×10^3 ($\pm 1.41 \times 10^2$) CFU/mL, respectively, while bacteria grown in 0.4% agar remained the same when compared to cells grown in the same condition at pH 7.4, where C_{CRIT} was 4.80×10^3 ($\pm 1.41 \times 10^2$) CFU/mL (P-value = 0.29). At high shaking frequency (12/hr), C_{CRIT} for 0%, 0.2%, and 0.4% agar were statistically equivalent (4.70×10^4 ($\pm 6.27 \times 10^3$) CFU/mL, 4.68×10^4 ($\pm 5.80 \times 10^3$) CFU/mL, and 3.70×10^4 ($\pm 1.76 \times 10^4$) CFU/mL, respectively) when compared amongst each other in media with both pH 7.0 and pH 7.4 (P-value ≥ 0.05). Standard deviations were derived from a minimum of three replicates. Lines drawn as a guide.

Mixed population experiments

The affect that the initial placement of the bacteria had on population survival was then examined. In the medium with 0% agar, the cells were dispersed throughout the environment every time the plate was shaken, but for bacteria grown in either 0.2% agar or 0.4% agar, this was not the case. Under these conditions, despite the shaking action caused by the microplate reader, these higher agar densities kept the majority of the bacteria fairly restricted to the central area of the well where they were first inoculated. By mixing the cells throughout the media, it was hypothesized that this change in the initial placement

of the bacteria could have an effect on cell survival. Given that previous results had indicated that survival of the engineered bacteria, or C_{CRIT}, was most affected at intermediate shaking frequency, this condition was explicitly examined.

To disperse the bacteria equally throughout the medium, the cells were mixed into the medium before it had (in the case of 0.2% and 0.4% agar) solidified. As an internal control, concurrent experiments were preformed where the cells were inoculated into the center of the well (as described in previous experiments). When the bacteria were shaken at intermediate frequency (3/hr), it was observed that there was no change in C_{CRIT} in cells grown in 0% agar or 0.2% agar (Figure 19). However, when cells were grown in medium with 0.4% agar, C_{CRIT} increased ~10-fold (relative to when cells were inoculated into the center of the well). Specifically, C_{CRIT} was 3.25 x 10^5 (± 7.07 x 10^3) CFU/mL, 3.78 x 10^4 (± 2.99 x 10^3) CFU/mL, and 3.70 x 10^4 (± 5.57 x 10^3) CFU/mL for cells grown in 0%, 0.2%, and 0.4% agar, respectively. For bacteria grown in medium with 0.2% and 0% agar, it was noted that the value of C_{CRIT} was statistically equivalent (P-value ≥ 0.21) when bacteria were either initially well mixed in the medium or when the bacteria were

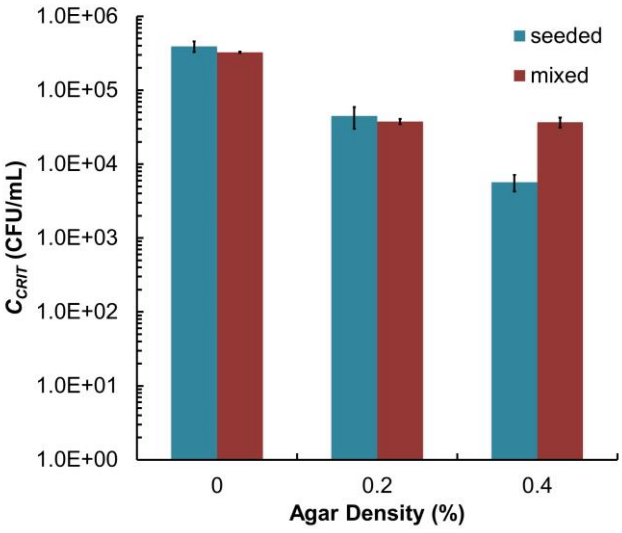

Figure 19: Mixing the bacteria in medium caused an increase in C_{CRIT} for bacteria grown in 0.4% agar

Bacteria were mixed with medium containing different agar densities (0%, 0.2%. and 0.4% agar) in the ON condition (1 mM IPTG) before they were added to the well. It was observed that there was no statistical difference in C_{CRIT} with bacteria grown in 0% and 0.2% agar in the well mixed condition (3.25 x 10^5 (± 7.07 x 10^3) CFU/mL and 3.78 x 10^4 (± 2.99 x 10^3) CFU/mL, respectively) when compared with the bacteria grown in the same agar conditions where they were inoculated into the center of the well (P-value ≥ 0.21). For bacteria grown in media with 0.4% agar, an increase was observed in C_{CRIT} (3.70 x 10^4 (± 5.57 x 10^3) CFU/mL). Standard deviation derived from a minimum of three replicates.

inoculated into the center of the well. For bacteria grown in medium with 0.4% agar, the value of C_{CRIT} increased to 3.70×10^4 ($\pm 5.57 \times 10^3$) CFU/mL) (P-value = 0.01).

Discussion

One of the central hypotheses of this thesis is that quorum sensing and diffusion sensing are not competing theories, and that the core predictions of both theories can be seen in a single bacterial system when the appropriate conditions are present. The theory of quorum sensing states that bacteria regulate gene expression in response to cell density (Darch et al., 2012; Ji et al., 1995; Miller & Bassler, 2001; Pai et al., 2012; Platt & Fuqua, 2010; Stuart A. West et al., 2012) through the use of autoinducers (Schertzer et al., 2009; Stuart A. West et al., 2012). Overall, quorum sensing is viewed as a social behavior, as a sufficient amount of bacteria are required to be present in order to initiate cooperation to alter gene expression. The theory of diffusion sensing suggests that the role of autoinducers is not used for cooperation, but instead to enable individual cells to sense how quickly the molecules secreted by the cell diffuse away (Redfield, 2002; Von Bodman et al., 2008; Stuart A. West et al., 2012). A key prediction of diffusion sensing is that the environment, and not cell density, should dictate whether a costly product should be produced by the individual cell (Williams et al., 2007). Factors that influence environmental conditions, and thus diffusion sensing, include changes in temperature or pH, nutrient availability, spatial structure, and diffusion rate (Williams et al., 2007).

To investigate this central hypothesis, a previously engineered strain of bacteria (Smith et al., 2014) was used, where survival was dependent upon an autoinducer. For the cells to survive, a sufficiently high concentration of autoinducer was required.

Furthermore, an experimental set up was designed where environmental factors could be readily perturbed, to assess whether or not these environmental factors had any effect on autoinducer activated gene expression. After verifying the functionality of the engineered cells (Figure 10), it was determined that changing the density of the agar in the medium affected bacterial dispersal and AHL diffusion. It was observed that by increasing the percentage of agar in the medium from 0% to 0.2% to 0.4%, the dispersal rate of the bacteria and the diffusion rate of AHL decreased, which affected the survival rate of the engineered bacteria. However, this reduction was unlikely to be equal, as AHL and bacteria move through the medium at different speeds. Specifically, AHL, being a molecule much smaller than the bacterium, should always diffuse faster than the bacterium. Overall, it was surmised that the results were predominantly driven by the variance in the spatial positioning between AHL and bacteria. With less spatial variance, C_{CRIT} would be lower. Similarly, with high spatial variance, C_{CRIT} would be higher.

The bacteria were first grown in medium with a pH of 7.4. When the cells were shaken at low frequency (1/hr), it was found that C_{CRIT} was statistically equivalent for all agar densities (Figure 14). This was likely due to near equal variance in the spatial distribution of AHL and bacteria. That is, despite differences in AHL diffusion rate and bacterial dispersal rate, the relative positions of bacteria and AHL were near equal amongst all conditions. It was proposed that under this condition of infrequent disturbance that the AHL produced by each bacterium remained closer to that bacterium, and this, in addition to the AHL produced by their relatively undisturbed neighboring cells, was sufficient to activate *ccdA* expression. Thus, overall, each bacterium effectively encountered a high amount of AHL. When the spatial position of the cells and AHL was moved, the long time

interval until the next disturbance offered the opportunity for the cells to rebuild the AHL concentration to levels high enough to eventually initiate cooperation. As such, when the cells were shaken at a low shaking frequency, the bacteria were able to access a local AHL concentration.

Next, high shaking frequency (12/hr) of the bacteria was examined to observe the effect that increased disturbance had on the survival rate of the bacteria. Again, it was found that C_{CRIT} was statistically equivalent for cells grown in all agar densities (Figure 14). This C_{CRIT} was also statistically equivalent to the cells grown in the low shaking frequency experiments, and similarly, it was hypothesized that this was because the variance between the spatial distribution of AHL and the bacteria were nearly equal for all agar densities. However, unlike the previous experiment (low shaking frequency) in which the bacteria were able to take advantage of AHL because it was able to build up over a long period of time within the vicinity of the cells, the frequent shaking allowed the AHL to be well mixed within the environment. This increased the likelihood that each bacterium had access to sufficient AHL. This effect contrasts to what was observed in the experiments at low shaking frequency, where each bacterium used more of the AHL produced by itself and by its neighboring cells. At high shaking frequency, the bacteria had a greater chance of encountering AHL produced from cells from anywhere within the microplate well, as under this shaking frequency, the contents of the microplate well were disturbed often enough that AHL was well mixed throughout the environment. As such, at high frequency, the bacteria were able to access a global AHL concentration.

When the cells were shaken at either low (1/hr) or high (12/hr) frequency, C_{CRIT} was statistically equivalent for all agar densities, supporting the theory of quorum sensing,

as the density of the bacterial population dictated when gene expression was altered to produce the costly product (*ccdA*) that rescued the population. Additionally, this activation of the autoinducer regulated gene was independent of environmental conditions (diffusion and dispersal). That is, the same C_{CRIT} was observed in all of the agar densities and depended solely on cell density. However, at intermediate shaking frequency, environmental conditions affected the autoinducer activated expression of *ccdA*.

When shaken at an intermediate frequency (3/hr), C_{CRIT} was statistically different for cells grown in all of the agar densities. Here, the cells grown in 0.4% agar had the lowest C_{CRIT}, while the cells grown in 0% agar had the highest (Figure 14). The cells in 0.2% agar had a C_{CRIT} that was statistically equivalent to cells shaken at either high or low frequency. It was hypothesized that during intermediate shaking frequency the variance in the spatial distribution of AHL and the cells were no longer equal in the three agar densities. When grown in medium with 0.4% agar, the spatial variance between AHL and bacteria was the lowest. Here, the combination of intermediate shaking frequency and 0.4% agar in the medium was the most optimal condition for the bacteria to access AHL. As this was the lowest C_{CRIT} that was observed, it was theorized that this may be the optimal point for this system. Conversely, when grown in 0% agar, the spatial variance between AHL and bacteria was the highest. Under this condition, it was speculated that the bacteria were less successful at accessing the AHL they produced or of the AHL produced by the population. That is, the combination of the intermediate shaking frequency and 0% agar required a higher population density of bacteria to build up a sufficient concentration of the AHL within the local vicinity, as was observed in the low shaking frequency experiments. At the same time, the intermediate shaking frequency was not frequent enough to sufficiently mix

the population and grant access to the global AHL available throughout the environment. Overall, at intermediate shaking frequency, population survival was dependent upon environmental conditions, as these were used to perturb C_{CRIT}, and thus satisfied the core tenant of diffusion sensing.

Next, the pH in the environment was decreased from pH 7.4 to pH 7.0. This caused the degradation rate of AHL to decrease, making more of it available in the medium for the cells. All things being equal, a decrease in AHL degradation should effectively decrease the spatial variance between the bacteria and AHL for all shaking frequencies and all agar densities as more AHL will be available in the environment. That is, as there will be more AHL in the system at any given time, bacteria stand to effectively encounter AHL at any position on the plate.

The bacteria were grown in a medium with a pH of 7.0 at all agar concentrations (0%, 0.2%, and 0.4%) and shaken at low frequency (1/hr). It was observed that the cells grown in both 0% and 0.2% agar were statistically equivalent to the cells grown in the same agar densities with a pH of 7.4 (Figure 18). As such, it appeared that the stabilization of AHL had no effect on these conditions; this suggests that the spatial variance between AHL and bacteria was not decreased with the increase of AHL that was available within the environment so as to affect C_{CRIT}. However, for the cells grown in 0.4% agar, a decrease in C_{CRIT} was observed (as compared to the same experiment at pH 7.4). These results suggest that the spatial variance was reduced under this condition. Due to the decreased degradation rate of AHL coupled with very low dispersal of the bacteria (due to the high agar concentration), it was surmised that AHL increased around the cells significantly faster, thus reducing C_{CRIT} for this condition. Here, it was also noted, since an

environmental condition was again influencing C_{CRIT}, the system was transitioning from quorum sensing, which was seen when the cells were grown in pH 7.4, to diffusion sensing.

Interestingly, at high shaking frequency, C_{CRIT} was again found to be statistically equivalent for cells grown in all agar densities (Figure 18). Furthermore, C_{CRIT} was statistically equivalent to C_{CRIT} when AHL was less stable and degraded faster (pH 7.4). These results suggest that the bacteria and AHL were moving frequently enough that the increase in AHL does not offer any additional benefit to the cells because the spatial variance between AHL and the bacteria remained the same. That is, although there was effectively more AHL available within the environment, the frequent perturbation via shaking prevents any additional benefit to the cells in this condition. At high shaking frequency, regardless of any changes that were made in any of the environmental conditions, quorum sensing dominated.

When cells were grown at an intermediate shaking frequency (3/hr), a change in C_{CRIT} was observed in two of the three agar densities (Figure 18). The cells grown in 0.4% agar displayed no change in C_{CRIT}, having a C_{CRIT} that was statistically equivalent to the cells grown in the same condition in a medium with pH 7.4. Again, it is proposed that the variance between the spatial distribution of the cells and AHL was at its optimal point. The cells grown in both 0% and 0.2% agar displayed a reduction in C_{CRIT}. This influx of additional AHL via reduced degradation served to lessen the spatial variance between the spatial distribution of the cells and AHL, thus decreasing C_{CRIT}. This again supported the theory of diffusion sensing, as perturbing an environmental condition affected C_{CRIT}.

The above experiments were initiated using bacteria inoculated into the center of the well, which would initially serve to decrease the spatial variance. In contrast, beginning experiments using equally distributed, or well-mixed, bacteria would serve to initially increase the spatial variance between bacteria and AHL. In agreement with the proposed mechanism, this would serve to increase C_{CRIT} under some conditions. Because most of the variance in C_{CRIT} occurred in experiments where the cells were shaken at intermediate frequency, this was the only frequency considered for these experiments. As expected, the cells grown in mixed medium with 0% agar had a C_{CRIT} that was statistically equivalent to that of cells were seeded (inoculated into the center of the well) into the medium (Figure 19). This was also observed in cells grown in 0.2% agar. Although the cells were dispersed throughout the environment, the spatial variance between AHL and the bacteria remained close enough to that of cells that were seeded, which resulted in a C_{CRIT} that was statistically equivalent between both of these conditions. For cells grown in 0.4% agar, an increase in C_{CRIT} was observed when the cells were evenly distributed in the environment, meaning that more bacteria were required for the population to survive. The spatial variance between AHL and bacteria was increased due to the distance between each of the bacteria in the medium. This made it harder for the bacteria to access AHL from its neighbors and to utilize all of the AHL that it produced.

Overall, these results served to support a theory that the spatial variance between bacteria and AHL was critical in determining successful activation of autoinducer regulated genes. It appeared that if passive movement (dispersal and diffusion) dominated (low shaking frequency), or if active movement dominated (high shaking frequency), the spatial variance conforms despite changes to environmental conditions (dispersal, diffusion,

autoinducer degradation rate). Here, quorum sensing was observed. However, if passive movement and active movement had non-dominating roles (intermediate shaking frequency), spatial variance, and thus in this case C_{CRIT}, varies with the environmental conditions. Here, diffusion sensing was observed.

The ability of bacteria to communicate is essential for their survival in many instances. Communication allows bacteria to work together, regulating gene expression as a single unit, and providing them with greater opportunity to adapt to changes within their environment. This adaption is vital, as natural environments are heterogeneous and subjected to frequent disturbances. In turn, understanding how these disturbances affect survival in bacterial species may aid in the prediction of potential outcomes at a grander scale. These disturbances are important, as they play a critical role in maintaining biodiversity. Moreover, this understanding may contribute to novel ways of addressing species reintroduction or controlling invasive species.

Presently, there is a public health crisis, as the emergence of once treatable bacterial infections are now resistant to the antibiotics that were considered to be a last line of defense. As these antibiotics are losing their efficacy due to the increased incidence of resistant bacterial strains, new treatment options must be developed to combat these diseases. Many bacteria utilize cooperation to colonize their hosts (Keller & Surette, 2006a), evade the immune system (Ackermann et al., 2008), and resist antibiotics (Allen et al., 2010; Martínez, 2008), but the core parameters that lead to effective disruption have yet to be explored. This research has demonstrated that disturbance of spatial distribution offers a possibility to enhance or detract growth. This may offer new prospects for research to develop more effective treatment for these diseases.

Conclusion

Two competing theories exist as to why bacteria communicate using autoinducers; quorum sensing and diffusion sensing. Using a genetically engineered strain of bacteria, the core predictions of both quorum sensing and diffusion sensing were observed by predominantly changing the frequency at which the spatial structure of the bacteria and AHL were perturbed. Overall, this analysis has shown that both theories can be observed in a single bacterial species. These results have implications for a range of issues. This data suggests that how often a system is disturbed has a significant impact on species survival. Strategies that intervene with this ability may have an impact on how to control or possibly eradicate invasive species. At the opposite end of the spectrum, this may aid in the survival of reintroduced species. These results may also contribute to the development of novel treatments for bacterial infections, as the treatments currently available are becoming less effective.

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