A SYNTHETIC QUORUM SENSING SYSTEM REVEALS INTERACTION BETWEEN EXTRACELLULAR MATRIX AND QUORUM SENSING MOLECULES

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DEDICATION

I would like to dedicate this work to everybody who helped me finish my PhD work, including members of my graduate committee: Elliott Ross, Vanessa Sperandio, Jen Liou; all current and former Süel lab members: Munehiro Asally, Tolga Çağatay, Dong-yeon Lee, Anna Kuchina, Mark Kittisopikul, Jintao Liu, Arthur B. Prindle, Jacqueline Humphries. Special thanks to my mentor Gürol Süel for his guidance and support.

A SYNTHETIC QUORUM SENSING SYSTEM REVEALS INTERACTION BETWEEN EXTRACELLULAR MATRIX AND QUORUM SENSING MOLECULES

by

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The University of Texas Southwestern Medical Center at Dallas, 2015

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Even though bacteria are unicellular organisms, they commonly reside in structured communities known as biofilms. One of the defining characteristics of biofilms is the presence of an extracellular matrix (ECM) that encapsulates all cells within the community and provides the biofilm with structural integrity. The production and degradation of ECM components are often regulated by quorum sensing (QS), a prevailing cell-cell communication method between bacterial cells. Quorum sensing allows bacteria to communicate with each other by secreting and sensing small molecules called quorum signals. The literature suggests that the ECM may affect diffusion of quorum molecules through a physical connection between these processes. However, since QS regulates ECM expression, ECM expression and QS are tightly coupled and cannot be perturbed independently. Here we constructed a synthetic QS system in *Bacillus subtilis* to

overcome this limitation and investigate whether ECM production affects QS signals, by quantitatively measuring the synthetic QS response in biofilm communities and single cells. Specifically, we constructed a synthetic quorum-sensing system with designated “Sender” and “Receiver” cells in *Bacillus subtilis*. This synthetic QS system allowed us to uncouple and independently investigate ECM production and QS in both biofilms and single cells. Our results showed that ECM-producing cells have a higher gene expression response to QS signals. The enhanced QS response suggests a private benefit for ECM-producing cells, which may indicate another mechanism to balance the cost of ECM production and constrain ECM production cheaters in biofilms.

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PRIOR PUBLICATIONS

Zhang F, Kwan A, Xu A, Süel GM (2015) A Synthetic Quorum Sensing System Reveals a Potential Private Benefit for Public Good Production in a Biofilm. *PLoS One* 10(7):e0132948.

Kuchina A, Espinar L, Cagatay T, Balbin AO, **Zhang F**, Alvarado A, Garcia-Ojalvo J, Süel GM (2011) Temporal competition between differentiation programs determines cell fate choice. *Mol Syst Biol* 7: 557

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agr	accessary gene regulator
AHL	acyl-homoserine lactones
AI-2	Autoinducer-2
c-di-GMP	bis-(3'–5')-cyclic dimeric guanosine monophosphate
CFP	Cyan Fluorescent Protein
ComA~P	phosphorylated ComA
CSF	Competence and sporulation factor
ECM	Extracellular Matrix
eDNA	Extracellular DNA
PQS	2-heptyl-3,4-dihydroxyquinoline
QS	Quorum Sensing
SipW	signal peptidase W
Spo0A~P	phosphorylated Spo0A
WT	wild type
YFP	Yellow Fluorescent Protein

Chapter 1: Introduction and literature review

1.1 Biofilms

Biofilms are multicellular communities formed by unicellular organisms such as bacteria. Biofilms can form at the air-solid, air-liquid or liquid-solid interfaces. A self-produced extracellular matrix (ECM) allows biofilm bacterial cells to attach to surfaces and stick together. Biofilms can be composed of single or multiple species. Even biofilms made up of a single species can differentiate into distinct cell types.

1.1.1 History of biofilm study

The existence of bacterial biofilms was first described in the 1680s. Antony van Leeuwenhoek described a massive accumulation of bacteria sticking to the surface of his teeth in his reports to the Royal Society of London (Dobell 1932). Leeuwenhoek's work was the first publication about bacterial biofilms. Of course, in the report, he neither came up with the name 'biofilm', nor realized the architecture of those bacteria was much more complicated than simple passive accumulation.

After Leeuwenhoek's reports, there was a dark age of roughly three hundred years, when scientists paid little attention to biofilms. In 19th century, Robert Koch invented the classic method of studying microbial organisms – the liquid culture of a single species (Blevins and Bronze 2010). This method helped scientists achieve extraordinary success in understanding pathogen associated human diseases. Therefore, for the entire 19th century and

early 20th century, scientific researchers were fascinated with the 'pure culture paradigm' and thus did not prioritize research on microorganism consortia. However, in the middle of 20th century, the 'pure culture paradigm' started to reach its limit. Scientists realized that pure cultures do not represent the natural living state of bacteria in most cases. Even the planktonic state is not the major state in the life cycle of most bacteria.

Biologists or ecologists who studied water bodies were the first group of scientists who went back to investigate the aggregation of bacteria. When they studied biofouling on the bottom of ships, they found out that bacteria growing in the slimes caused such biofouling (Angst 1923). Soon they discovered that the biofilm state, instead of the planktonic state, is the main living state of water bacteria (Henrici 1933).

In early years, biofilms were given various names, such as "slime layers" (Geesey et al. 1977). The term 'biofilm' was first used by Mack WN, et al in their publication titled "Microbial film development in a trickling filter" in 1975 (W. N. Mack, Mack, and Ackerson 1975). Eventually, 'biofilm' became the most frequently used term among researchers to describe such structured microbial communities.

In the beginning of 21th century, research articles on biofilms started to increase exponentially (**Figure 1**). With improvements in related techniques, scientists were able to observe biofilms with higher resolution to gain insights. The early studies of biofilms focused on describing the morphology of biofilms formed in various environments (McCoy et al. 1981; Marrie, Nelligan, and Costerton 1982; Pedersen 1982; Kinner, Balkwill, and Bishop 1983; Eighmy, Maratea, and Bishop 1983)**.** As research progressed, studies reported reagents or surfaces that were capable of altering biofilm morphology (Turakhia, Cooksey, and

Characklis 1983; Gristina and Costerton 1985; Hsieh, Lion, and Shuler 1985; Camper et al. 1985). With advances in molecular biology techniques, researchers started to screen random gene mutations in order to reveal the genes mediating biofilm formation (Cochran et al. 2000; Fournier and Hooper 2000; Rashid et al. 2000; D. Mack et al. 1994). In recent years, studies on biofilms have diverged in several interesting directions, such as configuration and interaction between species in multispecies biofilms (Hojo et al. 2009; Alpkvista and Klapper 2007; Moons, Michiels, and Aertsen 2009), structure and function of extracellular matrix components in biofilms (A. G. Stöver and Driks 1999b; Romero et al. 2014; Romero et al. 2011), and growth or metabolism dynamics in biofilms (J. Liu et al. 2015; Asally et al. 2012).

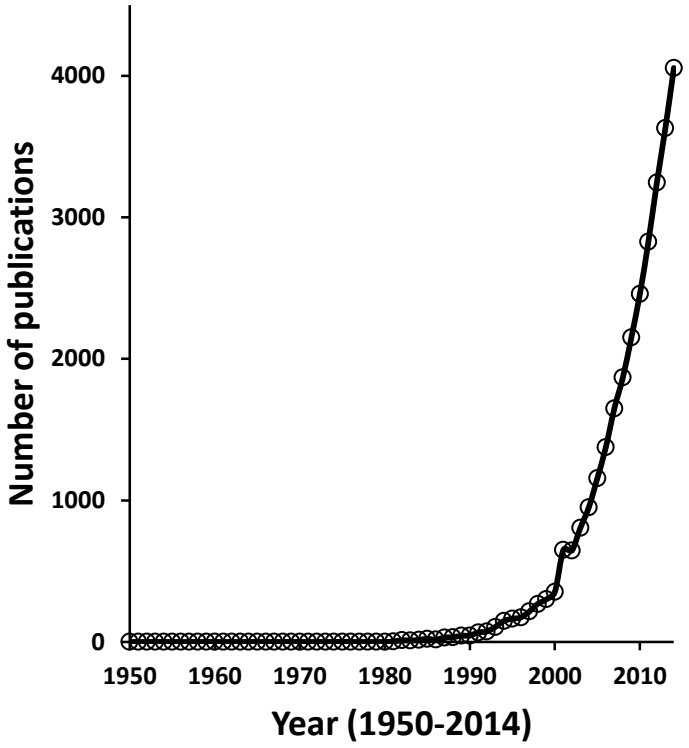

Figure 1. Number of publications on biofilms in each year from 1950 to 2014.
Data from papers indexed by PubMed with key word of 'biofilm'. Research published on biofilms started to arise around 1990, and increased dramatically after 2000. However, since

the term ‘biofilm’ was first introduced in 1975, using publications contains the keyword ‘biofilm’ to represent biofilm studies from 1950 to 2014 might introduce artifacts to some extent. Moreover, since other terms such as ‘slime layers’ had been used to describe biofilms, the number of publications on biofilms was underestimated, especially in early years.

1.1.2 Biofilm formation and its regulation

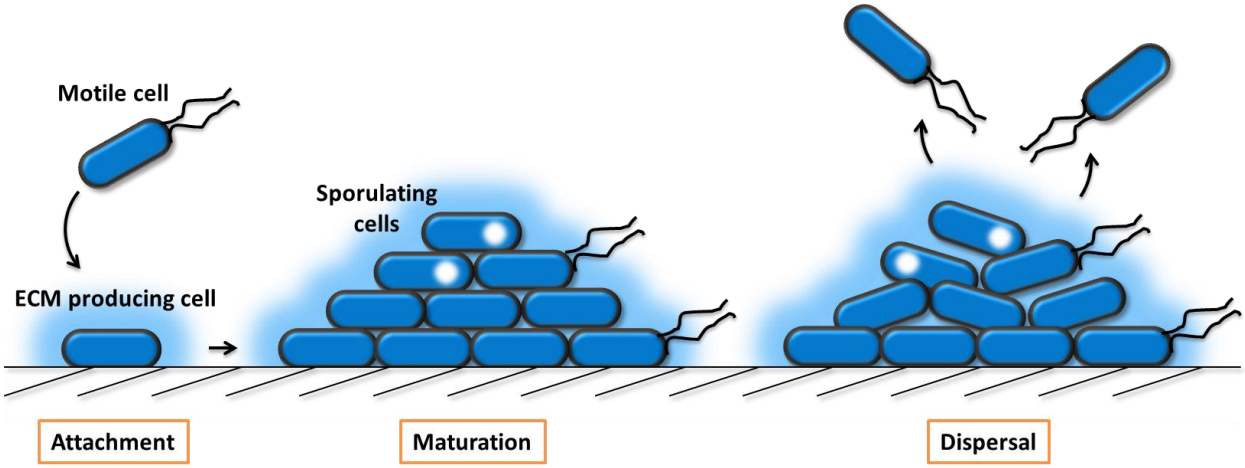

Figure 2. Life cycle of the biofilm.
Illustration of the stages of biofilm formation using *Bacillus subtilis* as an example. Generally, biofilm formation goes through three stages: attachment, maturation and dispersal. In the attachment stage, motile cells deflagellate and differentiate into ECM producing cells, allowing those cells to attach to the surface. In the maturation stage, the attached cells proliferate and differentiate into even more cell types such as sporulating cells. Different types of cells cooperate and form a mature biofilm with a 3D structure. In the last dispersal stage, biofilm cells go back to the motile state and disperse from the old biofilm, seeking a better environment.

Biofilm formation starts with the initial attachment of motile cells to a suitable surface. At this stage, motile cells lose their flagella, differentiating into ECM producing cells. The ECM attaches these cells to the surface and sticks all cells together while they proliferate. When the attached cells grow into a big enough colony, cells begin to differentiate into distinct cell types. This self-organized growth and development eventually

leads to a mature biofilm with multiple cooperating cell types and characteristic morphology such as wrinkles. In an aging biofilm, cells become extremely crowded and may suffer from nutrient or oxygen limitation. At this stage, biofilm cells may return to the motile state and disperse from the biofilm, looking for better niches in order to survive.

Biofilm development is guided by a variety of environmental signals. Nutrient availability is one of the key factors that control biofilm formation. Limitation of certain nutrients can induce biofilm formation for some bacteria. For example, lack of sugars such as glucose induces biofilm formation of *Escherichia coli* and *B. subtilis* (Jackson, Simecka, and Romeo 2002; Stanley et al. 2003). Indole, a poor carbon and nitrogen source, has been shown to be a stimulator of biofilm formation in several gram-negative bacteria including *E. coli, Klebsiella oxytoca,* and *Haemophilus influenza* under nutrient-depleted conditions (Martino et al. 2003; Newton and Snell 1964). In these cases, forming a microbial community allows cells to cooperate and get through the harsh environment together. However, in some other cases, bacteria form biofilms under nutrient-rich environments. For instance, a sugar-rich environment strongly induces biofilm formation in *Vibrio cholera, Staphylococcus epidermidis, Staphylococcus aureus* and *Streptococcus mutans* (Kierek and Watnick 2003; Dobinsky et al. 2003; Y. Lim et al. 2004; Shemesh, Tam, and Steinberg 2007). Perhaps these cells can only afford the high energy cost of ECM production when nutrients are plentiful. In addition to sugar availability, the quantity of some inorganic molecules also controls biofilm formation. Limitation of iron, a scarce nutrient for bacteria, can inhibit biofilm formation in *Pseudomonas aeruginosa* and *V. cholera* (Mey, Craig, and Payne 2005; P. K. Singh et al. 2002; Banin, Vasil, and Greenberg 2005). Likewise, insufficient phosphate supply inhibits

biofilm formation in *Pseudomonas aurofaciens* and *Pseudomonas fluorescens* (Monds et al. 2007; Monds, Silby, and Khris Mahanty 2001). Moreover, quorum sensing signals can also affect biofilm development in various bacteria. Such effects of quorum sensing will be discussed in 1.3.3 QS and biofilm.

To process these environmental signals, cells employ a complex gene regulation network. Such networks integrate various input signals to decide the fate of cells and guide biofilm dynamics.

Many gram-negative bacteria use the ubiquitous secondary messenger bis-(3'–5')-cyclic dimeric guanosine monophosphate (c-di-GMP) as the central regulator that controls biofilm formation (Simm et al. 2004; D. a. D'Argenio and Miller 2004). In c-di-GMP signaling cascades, the intracellular level of the c-di-GMP molecule is controlled by proteins possessing either GGDEF or EAL domains. Proteins with a GGDEF domain act as nucleotide cyclases to synthesize c-di-GMP from two GTP molecules. Proteins with an EAL domain, on the other hand, are phosphodiesterases responsible for c-di-GMP degradation (Tal et al. 1998). c-di-GMP binds to its effectors, either proteins or RNAs, altering the structure and output function of the effectors (Boyd and O'Toole 2012). Mutations in GGDEF-type proteins generally decrease biofilm formation, whereas mutations in EAL-type proteins do the opposite (Hoffman et al. 2005). In both *P. fluorescens* and *P. aeruginosa*, the GGDEF-type response regulator WspR is involved in wrinkled biofilm formation (D. A. D'Argenio et al. 2002; Spiers et al. 2003). Moreover, several studies have shown that the amount of intracellular c-di-GMP is proportional to the amount of biofilm formed (Beyhan et

al. 2006; Hickman, Tifrea, and Harwood 2005; Kuchma et al. 2007; B. Lim et al. 2006; Merritt et al. 2007; Simm et al. 2004; Tischler and Camilli 2004).

Another common signaling pathway that bacteria adopt to process environmental signals is the phosphorelay. The phosphorelay is a multi-stage phosphoryl group transferring process that requires histidine kinases. The phosphorelay signaling cascade starts by sensing environmental signals via transmembrane histidine kinases. Upon activation, the histidine kinases undergo auto-phosphorylation, followed by direct or indirect phosphoryl group transfer to the response regulators that control biofilm related genes. Examples of such signaling cascades include the GacS/GacA system in *P. aeruginosa* and the KinA/Spo0A system in *B. subtilis* (Goodman et al. 2004; Hamon and Lazazzera 2001). To ensure precise spatial and temporal regulation of biofilm formation, such complicated gene regulation networks generally involve many proteins and employ various regulatory methods. I will further describe such complex regulation networks in section 1.5.2 Regulation on biofilm formation in *B. subtilis*.

1.1.3 Impact of biofilms on humans

Biofilms influence human life in many ways. Biofilms are found on almost all surfaces, including natural, medical and industrial surfaces. Given their ubiquity in the environment, the study of biofilm formation is of great importance to human beings.

In natural environments, the majority of bacteria live in biofilms (R. M. Donlan and Costerton 2002). Biofilms are of great value in an ecological sense. Biofilms in water bodies play an important role in the carbon cycle (Lyon and Ziegler 2009). Biofilms in soil are

involved in critical process of nitrogen fixation, making nitrogen accessible to plants (Burmølle, Kjøller, and Sørensen 2011; Desai, Assig, and Dattagupta 2013).

In industry, biofilms are often a source of damage. Biofouling is a typical problem caused by biofilms. Microorganisms gather on submerged surfaces, such as ship bottoms, and form biofilms that cover those surfaces, significantly reducing the hydrodynamic performance of those vessels (Holm et al. 2004). Government and industry spend billions of US dollars every year to prevent and control marine biofouling [1]. In addition, biofilms often clog pipes and tubes, causing huge financial loss (Hall-Stoodley and Stoodley 2009). On the other hand, biofilms can be beneficial for industry. Biofilms are often used in waste water treatment systems (R. Singh, Paul, and Jain 2006). Biofilms can potentially be a source of microbial fuel as well (Erable et al. 2010).

In medical settings, biofilms frequently build up on surfaces of medical devices such as implants, causing chronic infections that are difficult to cure (Hall-Stoodley, Costerton, and Stoodley 2004; R M Donlan 2008; Hatt and Rather 2008). Additionally, biofilms are a common cause of persistent infections (J. W. C. P. S. Stewart and Creenberg 1999). Infections on teeth, skin and urinary tracts, are often associated with biofilms (Hatt and Rather 2008). The existence of highly resistant cell types such as persisters and spores renders antibiotics ineffective in biofilm related infections, making the infections extremely hard to treat (P. S. Stewart and William Costerton 2001; R. M. Donlan and Costerton 2002; K. Lewis 2005).

[1] http://www.birmingham.ac.uk/generic/ambio/about/biofouling-problem.aspx

1.2 Extracellular matrix

The existence of extracellular matrix (ECM) is the deterministic property of biofilms. ECM can comprise over ninety percent of the dry mass in biofilms (Flemming and Wingender 2010). In biofilms, a subpopulation of dedicated ECM producing cells synthesizes and secretes ECM. The secreted ECM holds all biofilm cells together and attaches the biofilm to suitable surfaces, which is essential for biofilm integrity.

1.2.1 Chemical components of ECM

ECM generally contains exopolysaccharides, proteins and DNA. Some other components such as lipids and humic substances may also be included (Flemming and Wingender 2010). Even with similar ingredients, biofilms can vary greatly in the structure and percentage of individual components.

Exopolysaccharide is the first component of ECM discovered that has demonstrated ubiquity. Exopolysaccharide has been found in almost all biofilm forming species whose ECM has been isolated and characterized (Flemming and Wingender 2010). In many bacteria, exopolysaccharide is an indispensable constituent of ECM. Exopolysaccharide-defective mutations lead to limited or even no biofilm formation in almost all biofilm forming bacteria (S S Branda et al. 2001; Danese, Pratt, and Kolter 2000; Ma et al. 2009; Watnick and Kolter 1999). Most exopolysaccharides identified are linear or branched long polymers weighing between 0.5-2x10^6 daltons (Flemming and Wingender 2010). These polymers form a complex ECM network and attach cells to the network (Sutherland 2001). The monomers that constitute exopolysaccharides vary dramatically between species, and even between strains of the same species (Vaningelgem et al. 2004). Most exopolysaccharides are

heteropolysaccharides consisting of a mixture of sugar residues. The most common kinds of exopolysaccharides in ECM are polymer of β-1,6-N-acetyl-D-glucosamine (PGA) and cellulose (Karatan and Watnick 2009).

ECM may contain considerable amounts of protein, even exceeding the amount of exopolysaccharides. Some of these extracellular proteins are structural proteins such as cell surface-associated proteins and extracellular carbohydrate-binding proteins. These proteins stabilize the exopolysaccharide network by crosslinking sugar fibers, as well as attaching cells to the exopolysaccharide network (Romero et al. 2014; Absalon, Ymele-leki, and Watnick 2012; de Jong et al. 2009). Other than these structural proteins, the ECM also carries a large number of protein enzymes. These enzymes conjugate, modify or degrade ECM components, maintaining the dynamic stability of the biofilm (Houot and Watnick 2008; J. B. Kaplan 2010). In addition, ECM degradation enzymes may be involved in the biofilm dispersal process (S. F. Lee, Li, and Bowden 1996; Stoodley et al. 2002). During starvation, the ECM degradation enzymes can also degrade the ECM to release nutrients such as carbon, nitrogen and other elements for biofilm cells (K. I. M. Lewis et al. 2001).

Although researchers detected extracellular DNA (eDNA) in the ECM, they initially did not consider eDNA as a functional component of ECM, rather they thought it was a side effect of cell lysis. However, the importance of eDNA for biofilms was established in the work of Whitchurch, C.B. *et al* , which reported that the addition of DNase I, which degrades eDNA, inhibited biofilm formation of *Pseudomonas aeruginosa* (Whitchurch et al. 2002). eDNA can be a major structural component of ECM in some species such as *Staphylococcus aureus* (Izano et al. 2008). However, it can also be a minor component in some other even

closely related species such as *Staphylococcus epidermidis* (Izano et al. 2008).The role and localization of eDNA in ECM varies from species to species. Cell lysis is a defined source of eDNA in several species such as *S. epidermids* and *P. aeruginosa* (Molin and Tolker-Nielsen 2003; Steinberger and Holden 2005). Active excretion of DNA has also been suggested since eDNA can be distinctly different from genomic DNA (Böckelmann et al. 2006). eDNA assists initial attachment and surface aggregation onto surfaces through acid-base interactions in *S. epidermids* (Das et al. 2010). eDNA also plays a role in resistance to antimicrobial substrates (Lewenza 2013; Mulcahy, Charron-Mazenod, and Lewenza 2008; Johnson et al. 2013).

1.2.2 Regulation on ECM production

ECM production is under tight control. Since ECM is the deterministic property of the biofilm, cells usually regulate ECM production or degradation to direct biofilm formation. The master regulator of *B. subtilis* biofilm formation, SinR, directly controls the transcription of genes encoding ECM synthases (Chu et al. 2006; Kearns et al. 2005). In *P. aeruginosa*, phosphorylated GacA activates the transcription of sRNAs that binds to CsrA, derepressing ECM producing genes (Goodman et al. 2004). c-di-GMP also regulates ECM production. GGDEF-type proteins CelR1 and CelR2 are involved in cellulose production in *Rhizobium leguminosarum* bv. trifolii (Ausmees et al. 1999). The EAL-type protein DGC controls cellulose production in *Gluconacetobacter xylinus* (Tal et al. 1998).

Activation of ECM production usually correlates with suppression of motility. For example, the alternative sigma factor AlgT activates ECM production and at the same time inhibits flagellar gene expression in *P. aeruginosa* (Tart, Blanks, and Wozniak 2006). EpsE,

a glycosyltransferase involved in exopolysaccharide production by *B. subtilis*, also interacts with the flagellar motor switch protein, FliG, to inhibit flagellar rotation and motility (Blair et al. 2008; Guttenplan, Blair, and Kearns 2010).

1.2.3 Function of ECM

ECM has three main functions: 1) forming the scaffold for biofilm cells; 2) creating an 'internal' microenvironment for biofilm cells; 3) protecting biofilm cells from outside threats.

ECM forms a network with various cross-linked polymers. This ECM network immobilizes biofilm cells and keeps them in a fixed position relative to neighboring cells. Thus in the biofilm, mechanical forces play a much more important role than in the liquid culture, where cells are all far away from each other and have little physical interaction. For example, the wrinkle morphology of *B. subtilis* biofilm forms through mechanical forces. Localized cell death coupled with the rigidity provided by ECM spatially focuses mechanical forces, lifting up the film of cells to form wrinkles (Asally et al. 2012). Furthermore, ECM is responsible for surface adhesion and cell immobilization at desirable locations (Das et al. 2010).

ECM creates an 'internal' microenvironment for biofilm cells, which can be very different from the 'external' environment. ECM retains numerous extracellular enzymes and nutrients close to biofilm cells, forming a versatile external digestion system as well as keeping nutrient pools near the cells (Flemming and Wingender 2010). Furthermore, ECM keeps biofilm cells in close proximity. The extremely short distance between neighboring

cells permits intense intercellular communication. Cells in biofilms can even communicate directly through intercellular nanotubes (Dubey and Ben-Yehuda 2011).

ECM forms a diffusion barrier between the 'internal' environment and 'external' environment. This barrier protects the biofilm from antimicrobials. For example, The BslA proteins form a hydrophobic layer on *B. subtilis* biofilm surfaces (Kobayashi and Iwano 2012). *B. subtilis* biofilms are extremely non-wetting and impenetrable to gas as a result of the ECM composition and rough surface topography, conferring great defense capability against antimicrobials spreading through water or air (Epstein et al. 2011). In addition, ECM can protect biofilm cells from invasion of external bacterial cells (Nadell et al. 2015).

1.3 Quorum sensing

Quorum sensing (QS) is a prevailing cell-cell communication method between bacterial cells. In the quorum sensing process, bacterial cells communicate with each other by secreting and sensing small molecules called quorum signals.

1.3.1 History of QS study

Quorum sensing was discovered during studies of bioluminescence in *Vibrio fischeri*, a marine bacteria that form a mutualistic symbiosis with the Hawaiian bobtail squid, *Eupryma scolopes* (Geszvain and Visick 2006). In the light organs located at the bottom of the squid, *V. fischeri* grows to high cell densities. At night, the luminescence produced by *V. fischeri* mimics moonlight, hiding the shadow of the squid from its prey (Verma and Miyashiro 2013). When growing *V. fischeri* in liquid culture, people found that these

bacterial cells only produced luminescence upon reaching a certain cell density (K H Nealson and Hastings 1979). Initially the cell density dependent luminescence was hypothesized to be caused by the existence of a luminescence inhibitor in the culture media. As cell density increased, inhibition of luminescence would be depleted by cells (Kempner and Hanson 1968). But later this hypothesis was proved to be wrong when the real activator, the autoinducer, was discovered (Kenneth H. Nealson, Platt, and Hastings 1970; a. Eberhard 1972). In the following decade, scientists uncovered a lot about this quorum sensing system in *V. fischeri*, including the chemical structure of the autoinducer, and genes involved in this quorum sensing system (A. Eberhard et al. 1981; Engebrecht, Nealson, and Silverman 1983). Now this AHL-based QS system in *V. fischeri* has become the classic model of quorum sensing.

People named the system "quorum sensing" based on the hypothesis that the function of such a system is sensing population density. This hypothesis is intuitive because the QS system only activates when the population density reaches a critical threshold. However, some researchers are skeptical about the purpose of this system. The first skepticism came from Rosemary Redfield, who stated that it is equally fair to assume that quorum sensing is actually diffusion sensing (Redfield 2002). Cells may secrete molecules to measure the diffusion rate of its surroundings. This article started a long-standing debate between opinion holders of these two hypotheses, that continues today (West et al. 2012; Hense et al. 2007).

1.3.2 Paradigm of QS

During quorum sensing, bacterial cells secrete small molecules called quorum molecules into the environment. Cells also sense the existence of quorum molecules and

respond to them through changes in behavior (Figure 3). The basal level of quorum molecule production is low. When the cell density is low, the quorum molecule concentration is also low. As the cell density increases, accumulated quorum molecules reach the critical threshold that induces the production of the quorum molecule itself. The autoinducing production of quorum signals rapidly increases its concentration in the media, activating downstream responses and shifting the behavior of the whole population.

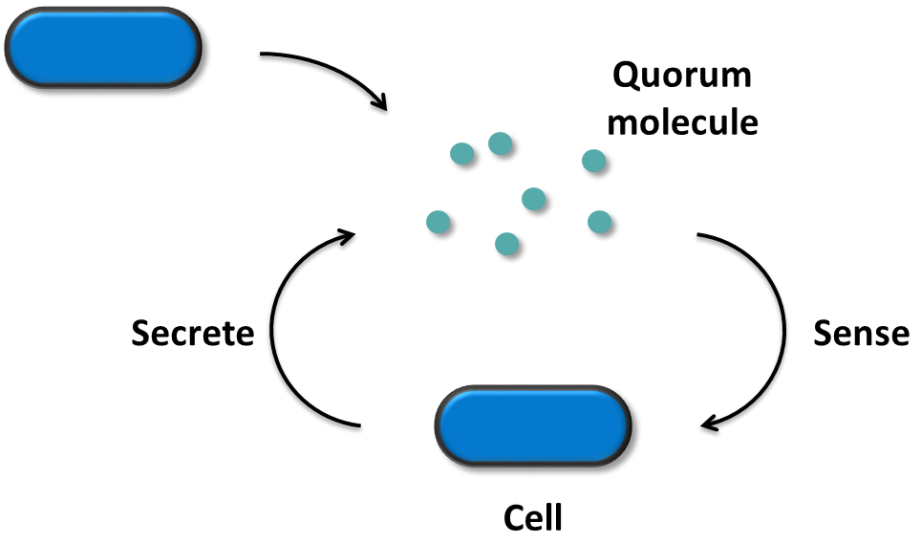

Figure 3. Quorum sensing in bacteria.
Cells secrete quorum molecules out into the environment. Cells also sense the existence of quorum molecules secreted by themselves or other cells.

Gram-negative bacteria generally evolve small molecules as their quorum signals. The first described QS system is the bioluminescence controlling system in *V. fischeri* (K H Nealson and Hastings 1979). It is also the most studied QS system in gram-negative bacteria. In *V. fischeri*, the *luxICDABE* operon encodes luciferase enzymes LuxCDABE that produce light, and an autoinducer synthase LuxI that synthesizes the quorum molecule acyl-

homoserine lactone (AHL), N-(3-oxohexanoyl)-homoserine lactone (Engebrecht, Nealson, and Silverman 1983; A. Eberhard et al. 1981; C. Y. Lee et al. 1993; Engebrecht and Silverman 1984). Upon production, AHL can diffuse freely in or out of the cell membrane (H. B. Kaplan and Greenberg 1985). When AHL reaches a certain concentration threshold, it binds to the LuxR receptor. Formation of the LuxR-AHL complex activates the transcription activity of LuxR by exposing its DNA binding domain (Engebrecht, Nealson, and Silverman 1983). This LuxR-AHL complex activates transcription of the *luxICDABE* operon, increasing light production as well as AHL synthesis (Hanzelka and Greenberg 1995; Schaefer et al. 1996; A M Stevens, Dolan, and Greenberg 1994; Ann M. Stevens et al. 1999; Ann M. Stevens and Greenberg 1997).

LuxIR-type QS systems are prevalent in gram-negative bacteria (Manefield and Turner 2002). Examples include the LasI/LasR system in *Pseudomonas aeruginosa*, the TraI/TraR system in Agrobacterium *tumefaciens,* and the ExpI/ExpR system in *Erwinia carotovora* (Morohoshi et al. 2004; Fuqua and Winans 1994; Ochsner and Reiser 1995). However, each species evolves its own unique AHL. AHL signals share the same core backbone but differ in the fatty acyl side chains. The side chain diversity determines the specificity of each AHL (Watson et al. 2002). Each species' LuxR possesses a specific binding pocket that only fits the AHL produced by the same species (Vannini et al. 2002; R. Zhang et al. 2002). Therefore, LuxIR-type systems are designed for intraspecies communication. Even if multiple types of AHL molecules coexist in the environment, bacteria of one species only respond to AHL signals produced by the same species.

QS systems in gram-positive bacteria usually adopt short peptides as quorum molecules. These quorum peptides mature by cleavage and modification of large precursors (L. Zhang et al. 2002). Peptides cannot freely diffuse through the cell membrane as AHL does. Thus, these quorum peptides are actively exported by specific or general oligopeptide permeases (Lazazzera et al. 1999). Peptide maturation and exportation can occur sequentially or simultaneously. Peptide receptors on the membrane sense the existence of quorum molecules outside the membrane. Upon binding to quorum peptides, the receptors autophosphorylate, then activate corresponding transcription regulators that control downstream gene transcription. The receptor and its corresponding transcription regulator usually form a two-component system. Extensively studied QS examples in gram-positive bacteria include the ComD/ComE system in *Streptococcus pneumonia*, the ComP/ComA system in *B. subtilis*, and the AgrC/AgrA system in *Staphylococcus aureus* (Cvitkovitch 2001; Magnuson, Solomon, and Grossman 1994; Richard P Novick and Geisinger 2008). The accessory gene regulator (*agr*) system in *Staphylococcus epidermidis*, which is similar to the agr system in *S. aureus*, is described in detail in section 2.1.2 Agr system in *Staphylococcus epidermidis*.

QS systems normally confer intraspecies communication, with exceptions. Autoinducer-2 (AI-2) is the most studied quorum sensing signal that potentially mediates interspecies communication. AI-2 production has been detected in many bacterial species including both gram-positive and gram-negative bacteria (Bassler 1999; Surette, Miller, and Bassler 1999). Moreover, the AI-2 synthase, LuxS, is present in over half of sequenced bacterial genomes (Waters and Bassler 2005). AI-2 affects biofilm formation, motility,

virulence factor production and many other processes in various bacterial species (Geier et al. 2008; Duan et al. 2003; Rader et al. 2007). These evidences suggest that AI-2 may mediate interspecies communication (Schauder and Bassler 2001; K. B. Xavier and Bassler 2003; Bassler 1999). Together with species-specific quorum sensing systems, this AI-2 interspecies quorum sensing system allows cells to distinguish self from non-self (Pereira, Thompson, and Xavier 2013).

1.3.3 Function of QS

The main function of QS is controlling the production of various metabolites, such as degradation enzymes, antibiotics and virulence factors. Most QS controlled metabolites are secreted out to the environment. Since the secreted metabolites can benefit the whole population, including cells that do not produce these products, these metabolites are called public goods. Production of public goods is often only viable when the population density is high and every cell in the population contributes to production. A cell density dependent QS response is capable of achieving these two requirements. QS-controlled public goods production is exemplified as follows: PhrG and PhrH control production of degradative enzymes through the DegS-DegU two-component system in *B. subtilis* (M Ogura et al. 2001; Mäder et al. 2002; Mitsuo Ogura et al. 2003; Kunst et al. 1997); AIP in *S. aureus* and AHL in *P. aeruginosa* induce production of virulence factors (Abdelnour et al. 1993; Pearson et al. 2000); QS systems control production of antibiotics in *B. subtilis*, *Burkholderia thailandensis*, *Serratia plymuthica* and many other bacteria (Auchtung, Lee, and Grossman 2006; Seyedsayamdost et al. 2010; X. Liu et al. 2007).

QS can also provide private benefits to cells by regulating the production of intracellular enzymes. As discussed before, the AHL based quorum system induces production of intracellular luciferase enzymes LuxCDABE involved in bioluminescence reaction in *V. fischeri*, which allows the bacteria to form a mutualistic relationship with squids (Engebrecht, Nealson, and Silverman 1983; A. Eberhard et al. 1981; C. Y. Lee et al. 1993; Engebrecht and Silverman 1984). In *P. aeruginosa*, LasR induces intracellular metabolic enzymes that control adenosine metabolism, providing benefits for cells that respond to QS signals (Dandekar, Chugani, and Greenberg 2012). Some other intracellular behaviors are also controlled by QS signals in many bacteria (Schuster et al. 2003; Zhao et al. 2014; Vivant et al. 2014).

QS also contributes to cell differentiation. ComX and CSF regulate differentiation of *B. subtilis* into competent cells or sporulating cells (Magnuson, Solomon, and Grossman 1994; Perego and Hoch 1996; Solomon, Lazazzera, and Grossman 1996). The involvement of QS in biofilm formation sometimes depends on the effect of QS on cell fate determination. The role of QS in biofilm formation is described in the next section, 1.3.4 QS and biofilms.

1.3.4 QS and biofilms

One QS system can play different roles during different stages of biofilm formation or under different conditions. The *agr* system in *S. aureus* represses surface adhesions that mediate cell attachment to a host matrix (Yarwood and Schlievert 2003). However, depending on the culture conditions, *agr* mutants can have positive, negative, or even neutral effects on biofilm formation, indicating the complicated role of the *agr* system during biofilm formation (Yarwood et al. 2004; Pratten et al. 2001; Vuong et al. 2000). In addition, the *agr*

system activates dispersal of *S. aureus* biofilms, detaching cells from the biofilm with the aid of extracellular proteases (Boles and Horswill 2008).

Homologue QS systems in different species can play different or even opposite roles. LuxS is necessary for biofilms formed on human gallstones by *Salmonella enterica* (Prouty, Schwesinger, and Gunn 2002). In contrast, the *lusS* mutant of *Helicobacter pylori* forms biofilms more efficiently compared to the wild type (Cole et al. 2004). In *Streptococcus mutans*, a mutation in *luxS* causes changes in the biofilm architecture, forming a looser, hive-like biofilm (Wen and Burne 2004).

Multiple QS systems affecting biofilm formation may coexist in the same bacteria. For example, the addition of enzymatically synthesized AI-2 stimulates biofilm formation in *E. coli*, meanwhile the addition of AHL represses biofilm formation (J. Lee, Jayaraman, and Wood 2007; González Barrios et al. 2006).

One interesting link between QS and biofilms is that both of them reveal the social aspect of bacteria. Both communication and community-building were once considered to be features possessed only by eukaryotes. However, the existence of QS and biofilms blurs the boundaries between prokaryotes and eukaryotes as social organisms. Research on QS and biofilms may uncover the evolution of these social traits from prokaryotes to eukaryotes.

1.4 Interaction between ECM and QS in biofilms

1.4.1 Effect of QS on ECM

As discussed in the above section, QS involves in various aspects of biofilm formation. In most cases, QS functions by controlling production or degradation of ECM. In

V. cholera, the quorum signal AI-2 represses exopolysaccharide production through derepression of HapR, which in turn suppresses the *vps* operon encoding exopolysaccharide synthases (Hammer and Bassler 2003). In *B. subtilis*, ComX activates ECM production by inducing production of surfactin that activates ECM synthesis (López et al. 2009). In *P. aeruginosa*, the AHL quorum sensing system promotes biofilm dispersal by reducing the synthesis of a major ECM component encoded in the *pel* operon (Ueda and Wood 2009). Seemingly in contrast, this QS system in *P. aeruginosa* also induces the release of extracellular DNA, another component of the ECM (Allesen-Holm et al. 2006).

1.4.2 Effect of ECM on QS

There is some evidence indicating that ECM may affect diffusion of quorum molecules. The detected concentration of 3-oxoacyl homoserine lactones, a quorum signal in AHL family, is more than 20-fold higher within the biofilm than in the surrounding environment (Charlton et al. 2000). This result indicates that biofilms may accumulate QS molecules. Moreover, a recent *in vitro* study demonstrated that the quorum sensing molecules 2-heptyl-3,4-dihydroxyquinoline (PQS) and N-(3-oxododecanoyl)-L-homoserine lactone are transiently associated with functional ECM amyloids in *P. aeruginosa*, further validating the potential interaction between ECM and QS signals (Seviour et al. 2015). However, whether this transient association of QS signals and ECM affects the response of ECM-producing cells *in vivo* remains unclear and will be tested in Chapter 4.

1.5 *Bacillus subtilis* biofilms and QS

B. subtilis is a commonly used gram-positive bacteria model system. Initially, *B. subtilis* was used as an alternative system to *E. coli* because *B. subtilis* lived a more complex

life cycle, which enriched knowledge on the living states of bacteria. In particular, the process of sporulation, which results in the production of highly thermoresistant spores, had attracted much attention. In the past two decades, *B. subtilis* caught researchers' attention again due to the intriguing wrinkle morphology biofilms present on air-solid interfaces as shown in **Figure 4** (S S Branda et al. 2001). Furthermore, people found that such biofilms contain multiple coexisting cell types, including but possibly not limited to motile cells, matrix producing cells, spores, competent cells and cannibal cells (Lopez and Kolter 2010). Those cell types were found to be spatially organized during biofilm forming process (Vlamakis et al. 2008).

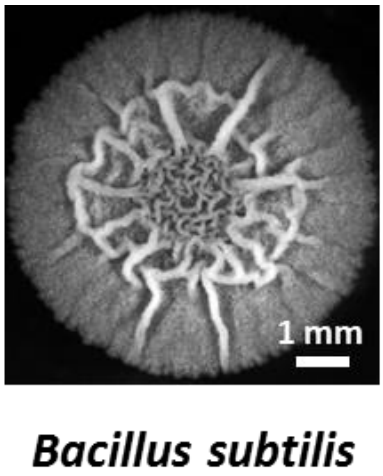

Figure 4. The top view of a three-day-old *B. subtilis* biofilm.

1.5.1 ECM of B. subtilis biofilm

Exopolysaccharides and amyloid-like protein fibers are the main components of the *B. subtilis* ECM.

Exopolysaccharides of *B. subtilis* are synthesized by proteins encoded in the *eps* operon, consisting of 15 genes, *epsABCDEFGHIJKLMNO*. Mutation of genes in the *eps* operon, such as *epsH* deletion, leads to severe biofilm formation defects (S S Branda et al. 2001). Bioinformatic analysis suggests that the potential role of each *eps* gene product is as follows: EpsA and B may regulate the chain length of exopolysaccharides; EpsC is predicted to be a nucleotide sugar synthase; EpsD, E, F, H, J, L and M are glycosyltransferases that build up the polysaccharide chains; EpsG facilitates polymerization of exoplysaccharides; EpsK is involved in sugar export (Lemon et al. 2008). Among all these *eps* genes, only a subset of genes has been studied experimentally. EpsE is the most thoroughly studied *eps* gene (Blair et al. 2008; Ren et al. 2004; Nagorska et al. 2010; Nagórska et al. 2008). EpsE functions as a glycosyltransferase but also interacts with the flagellar motor switch protein, FliG, inhibiting flagellar rotation and cell motility (Blair et al. 2008; Guttenplan, Blair, and Kearns 2010). This remarkable dual functionality ensures that cells reduce their motility when starting matrix production. In addition to the 15 genes in *eps* operon, there are another two genes, *pgcA* and *gtaB*, that are involved in the same pathway of exopolysaccharide production (Steven S Branda et al. 2004; Lazarevic et al. 2005). Furthermore, genes responsible for the synthesis of UDP-galactose, the sugar precursor of exopolysaccharide, are also necessary for biofilm formation (Yunrong Chai et al. 2012).

The structural protein component of *B. subtilis* biofilms is synthesized by the products of a three-gene operon *tapA-sipW-tasA* (Steven S Branda et al. 2004). Among these three genes, *tasA* encodes a protein TasA that forms the majority of structural ECM protein. TasA is known to assemble into long amyloid-like fibers (L. Chai et al. 2013). Purified TasA

monomers aggregate spontaneously and form fibers 10 to 15 nm in width and variable length (Romero et al. 2010). These cross-linked amyloid-like fibers form a network that holds biofilm cells together. Another structural component is the TapA protein encoded by *tapA*. Even though the amount of TapA protein is 100 times less than TasA protein in ECM, TapA serves as an important linker that anchors cells to the TasA fiber network (Romero et al. 2011). TapA is also critical for the proper assembly of TasA fibers (Romero et al. 2011). The last member of the *tapA* operon, *sipW*, encodes a type I signal peptidase W (SipW). SipW recognizes the N-terminal signaling peptides on the TasA and TapA precursors, facilitating their secretions by cleaving the signaling peptides (Tuteja 2005; Tjalsma et al. 1998; A. G. Stöver and Driks 1999a; a G. Stöver and Driks 1999). In addition to TasA and TapA, another protein, BslA, also contributes to ECM. BslA proteins polymerize and form a thin layer on the biofilm surface, rendering high surface hydrophobicity to the biofilm (Kobayashi and Iwano 2012).

1.5.2 Regulation on biofilm formation in B. subtilis

Biofilm formation in *B. subtilis* is under control of a complex gene regulation network. In the motile state, expression of both *eps* and *tapA* operons are repressed by a master regulator, SinR (Kearns et al. 2005; Chu et al. 2006). Both SinI and SlrR can bind to SinR, forming a complex that derepresses ECM production (Bai, Mandic-Mulec, and Smith 1993; Chu et al. 2008). SinI expression is regulated by the concentration of phosphorylated Spo0A (Spo0A~P) (Grandvalet, Gominet, and Lereclus 2001). The promoter of *sinI* contains two binding sites for Spo0A~P, one activator of high affinity to Spo0A and one repressor of low affinity to Spo0A~P (Y Chai et al. 2008). Therefore, intermediate levels of Spo0A~P in

a given cell promote ECM production, whereas high levels of Spo0A~P inhibit ECM production and promote sporulation (Fujita et al. 2005). This mechanism ensures cells committed to sporulation do not waste energy on ECM production. Spo0A is phosphorylated by at least four histidine kinases (KinA, KinB, KinC and KinE) through a phosphorelay (Jiang et al. 2000). This phosphorelay starts by activation of histidine kinases, goes through Spo0F and Spo0B, and eventually passes the phosphoryl group to Spo0A (Rhaese, Hoch, and Groscurth 1977; Hirochika et al. 1981; Grossman 1991). Those four histidine kinases respond to environmental changes capable of triggering biofilm formation (Grossman 1991; Shemesh and Chaia 2013; L. Wang et al. 2001). SlrR forms a double negative feedback loop with SinR: free SinR represses the expression of SlrR, while SlrR binds to SinR and titrates the free SinR out (Kobayashi 2008; Yunrong Chai, Kolter, and Losick 2009). This double negative feedback regulation circuit ensures that the free SinR level is locked in either the low or high state, resulting in a sharp distinction between the ECM producing state and non-producing state (Yunrong Chai et al. 2010).

1.5.3 QS in B. subtilis

As in other gram-positive bacteria, *B. subtilis* employs short peptides as quorum signals. There are two known families of quorum signals in *B. subtilis*, namely ComX and Phr.

ComX is the first QS signal found in *B. subtilis*. Initially it was found to trigger competence (Magnuson, Solomon, and Grossman 1994). Later on researchers found that ComX is also involved in sporulation. The structure of ComX remained a mystery until 2005 (Okada et al. 2005). ComX purified from *B. subtilis* strain RO-E-2 is a peptide containing six

amino acids (Gly-Ile-Phe-Trp-Glu-Gln) with the Trp residue modified by a geranyl group (Okada et al. 2005). ComX is expressed from a three-gene operon, ComQXP. Once translated, ComX is modified and secreted outside of the cell by the membrane protein ComQ. Another membrane protein ComP serves as the receptor for extracellular ComX. Upon binding to ComX, ComP undergoes auto-phosphorylation and activates the transcription factor ComA by transferring the phosphoryl group to it. The phosphorylated ComA activates several downstream genes, such as *comS* and *spo0A*, which control competence and sporulation.

The Phr family is another group of QS signals in *B. subtilis*. The Phr family are a series of five-amino-acid peptides, including PhrA, PhrC, PhrE, PhrF, PhrG, PhrI and PhrK (Solomon, Lazazzera, and Grossman 1996; McQuade, Comella, and Grossman 2001). Precursors of Phr peptides are secreted and processed outside of the cell, forming mature five-amino-acid quorum peptides (Lanigan-Gerdes et al. 2007; Lanigan-Gerdes et al. 2008). The mature Phr peptides are actively transported into the cell by an oligopeptide permease Opp (Lazazzera, Solomon, and Grossman 1997; Solomon et al. 1995; Solomon, Lazazzera, and Grossman 1996; Perego and Hoch 1996). Upon entering the cell, Phr peptides activate their corresponding receptors, Rap phosphatases, which belong to a family of aspartyl-phosphate phosphatases (Reizer et al. 1997). Rap phosphatases and Phr peptides are encoded in the same signaling cassette, called *rap phr* cassettes. Once activated by their corresponding Phr, the Rap phosphatases control the activity of downstream response regulators through dephosphorylation, regulating cellular behaviors such as competence and sporulation (Pottathil and Lazazzera 2003a). PhrC, also known as competence and

sporulation factor (CSF), is the first peptide found in the Phr family and also the most thoroughly studied one (Solomon et al. 1995).

Chapter 2: Construction of a synthetic QS system in undomesticated *Bacillus subtilis*

2.1 Introduction

Many synthetic QS systems have been engineered in gram-negative bacteria, especially in *E. coli* (Zargar et al. 2015; Z. Wang et al. 2014; Hong et al. 2012; Marchand and Collins 2013). However, no synthetic cell-cell communication system has been developed in gram-positive bacteria. The gram-positive model organism *B. subtilis* forms biofilms comprised of distinct cell types. This cellular differentiation appears to be spatially organized. QS has been suggested to play a critical role in the spatial organization of cellular differentiation. Therefore, developing a functional synthetic QS system in undomesticated biofilm communities may aid in uncovering the principles that govern the spatial organization of cell types within biofilms as well as investigating various other questions regarding biofilm development.

To construct a synthetic QS system in *B. subtilis*, we transplanted the Auto-Inducing-Peptide (AIP) based QS system (*agr* system) from *Staphylococcus epidermidis* into the biofilm forming *B. subtilis* NCIB 3610 strain (**Figure 5**). We chose the *S. epidermidis* QS system because of the following four main reasons: (1) *S. epidermidis* is a gram-positive bacteria, which increases the likelihood that this QS system could be functional in gram-positive *B. subtilis* cells. (2) The *S. epidermidis* QS system is well characterized and simple (Richard P Novick and Geisinger 2008). It involves only four

components (AgrA-D) that are expressed from a single operon. The *agrD* gene encodes the AIP peptide and AgrB is responsible for posttranslational modification and export of AIP (L. Zhang et al. 2002). AgrC is the AIP receptor that activates AgrA, the transcription factor that promotes expression of the *agr* operon driven by the P*2* promoter, as well as gene expression of downstream targets driven by P*3* promoters (R P Novick et al. 1995; R P Novick et al. 1993). (3) The *S. epidermidis* AIP is a short peptide containing 8 amino acids (Otto et al. 1998) (**Figure 6**) and is similar in size to the major QS peptides in *B. subtilis* that range in size from 5-6 amino acids (Pottathil and Lazazzera 2003b; Solomon, Lazazzera, and Grossman 1996). (4) There is no known *agr* system in *B. subtilis*, suggesting that the transplanted QS system would not interfere with native *B. subtilis* QS.

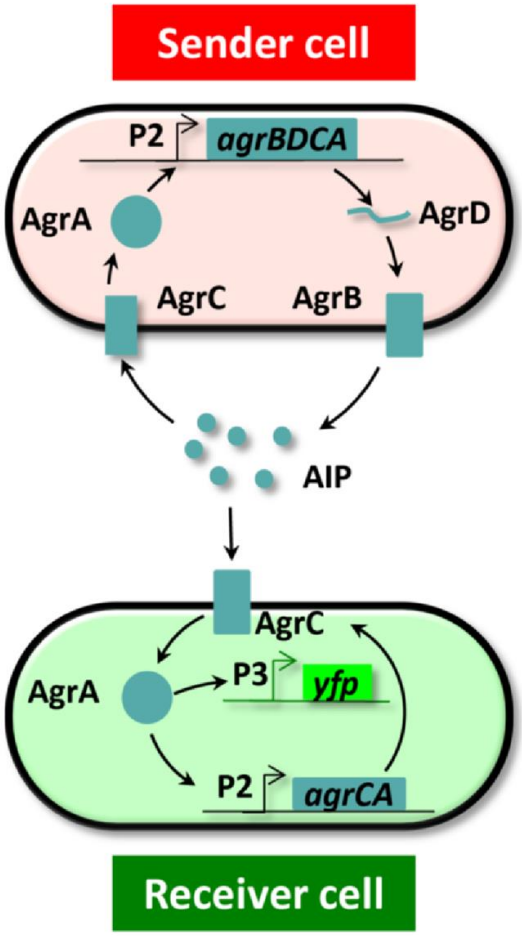

Figure 5. Diagram of the synthetic agr systems in Sender cells (top) and Receiver cells (bottom).
The Sender cell contains the entire *agr* operon (P*2-agrBDCA*) and can thus both secrete and sense AIP, the autoinducing peptide. The Receiver cell, on the other hand, carries P*2-agrCA* and P*3-yfp* and thus can respond to AIP by expressing YFP.

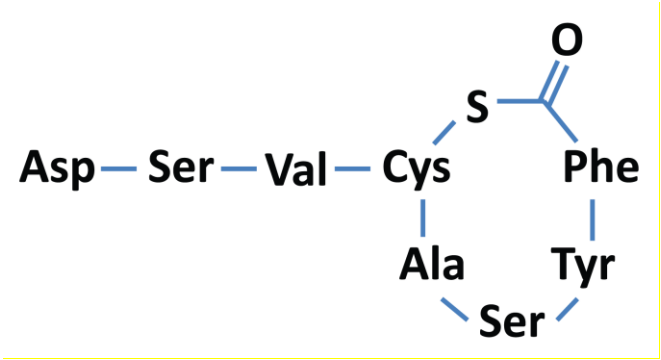

Figure 6. Structure of AIP in *Staphylococcus epidermidis* (Otto et al. 1998)**.**
It has a thioester linkage between carboxyl-terminus and the middle cysteine.

2.2 Materials and Methods

2.2.1 Strain constructions

All *Bacillus subtilis* strains used in this study were derived from NCIB3610 (a gift from the laboratory of Wade Winkler, University of Maryland, College Park, MD (Irnov and Winkler 2010)), an undomesticated wild type strain. Strains are listed in **Table 1**.

Table 1. List of strains.

Strain	Genotype	Source
	Bacillus subtilis	
NCIB3610		a gift from the laboratory of Wade Winkler
WT Receiver	*GltA::P3-yfp* (Neo^R) *AmyE::P2-agrCA* (Spec^R) *SacA::PrpsD-cfp* (CM^R)	This study
ΔepsH Receiver	*epsH::tet* (Tet^R) *GltA::P3-yfp* (Neo^R) *AmyE::P2-agrCA* (Spec^R)	This study
ΔtapA op Receiver	*tapA-sipW-tasA::cat* (CM^R) *GltA::P3-yfp* (Neo^R) *AmyE::P2-agrCA* (Spec^R)	This study
WT Sender	*AmyE:: PrpsD-mCherry* (Spec^R)	This study

	SacA::P2-agrBDCA(CM^R)	
Sender with optimal AIP yield	PY79 *GltA:: PrpsD-mCherry-PrpsD-spsB* (Neo^R) *AmyE:: PrpsD-agrBDCA* ($Spec^R$) *SacA:: P2-agrBDCA* (CM^R)	This study
WT Receiver (no constitutive marker)	*GltA::P3-yfp* (Neo^R) *AmyE::P2-agrCA* ($Spec^R$)	This study
ΔepsH Sender (with P*3-yfp)*	*epsH::tet* (Tet^R) *GltA::P3-yfp* (Neo^R) *SacA::P2-agrBDCA-PrpsD-cfp* (CM^R)	This study
WT Sender (with P*3-yfp)*	*GltA::P3-yfp* (Neo^R) *SacA::P2-agrBDCA* (CM^R) *AmyE::PrpsD-cfp* ($Spec^R$)	This study
WT Receiver with P*yqxM-cfp*	*GltA::P3-yfp* (Neo^R) *AmyE:: PyqxM-cfp* (CM^R) *SacA::P2-agrCA* ($Spec^R$)	This study
P*rpsD-yfp* P*yqxM-cfp*	*AmyE:: PyqxM-cfp* (CM^R) *SacA:: PrpsD-yfp* ($Spec^R$)	This study
WT with P*rpsD-cfp*	*AmyE:: PrpsD-cfp* ($Spec^R$)	This study
WT with P*srf-lacZ*	*AmyE:: Psrf-lacZ* (CM^R)	This study
ΔepsH with P*srf-lacZ*	*epsH::tet* (Tet^R) *AmyE:: Psrf-lacZ* (CM^R)	This study
Staphylococcus epidermidis		
ATTC 14990		ATCC

Either the *agr* system or a fluorescent protein fused to the *rpsD* promoter were integrated into *B. subtilis* using a standard chromosomal integration method. First, target genes were generated through fusion PCR and cloned into chromosomal integration vectors. Then the resulting constructs were confirmed by direct sequencing. The *agr* system was PCR amplified using Phusion high-fidelity DNA polymerase (NEB) and appropriate primers from *Staphylococcus epidermidis* ATTC 14990. Vectors designed for integration into the *gltA*,

amyE and *sacA* loci were pGlt-Kan (ECE173, constructed by R Middleton and obtained from the Bacillus Genetic Stock Center), pDL30 (a kind gift from Jonathan Dworkin, Columbia University), and pSac-Cm (Middleton and Hofmeister 2004) (ECE174, constructed by R Middleton and obtained from the Bacillus Genetic Stock Center) respectively. Then, vectors containing target genes were transformed into *B. subtilis* using a standard one-step transformation procedure (Jarmer et al. 2002). Strains with targeted integration were confirmed through colony PCR with appropriate primers.

2.2.2 Promoter definition

P*2*: Primers were designed based on the promoter sequence described in a previous study (Otto et al. 1998) and PCR from *Staphylococcus epidermidis* ATTC 14990. This P*2* promoter is located from 1689507 to 1689972 (direct orientation) on *Staphylococcus epidermidis* ATCC 12228 chromosome.

P*3*: Primers were designed based on the promoter sequence described in a previous study (Otto et al. 1998) and PCR from *Staphylococcus epidermidis* ATTC 14990. This P*3* promoter is located from 1689610 to 1689810 (complementary orientation) on *Staphylococcus epidermidis* ATCC 12228 chromosome.

P*rpsD*: chromosomal sequence 2853257 to 2853567 (direct orientation) on *B. subtilis* chromosome. Previous literature has reported this promoter to be constitutively highly expressed (Jester et al. 2003).

2.2.3 Growth condition

Cells were grown in Luria-Bertani (LB) broth (EMD) or on LB agar plates at 37 ℃. When appropriate, antibiotics were added at the following concentration: 9 μg/mL neomycin (Fisher BioReagents), 5 μg/mL chloramphenicol (Sigma-Aldrich), 300 μg/mL spectinomycin (Sigma-Aldrich), and 100 μg/mL ampicillin (Sigma-Aldrich).

MSgg medium was used when testing cells' response to AIP. MSgg medium containing 5 mM potassium phosphate (pH 7.0, Fisher BioReagents), 100 mM MOPS (pH 7.0, Sigma-Aldrich), 2mM $MgCl_2$ (Fisher BioReagents), 700 μM $CaCl_2$ (Fisher BioReagents),50 μM $MnCl_2$ (Sigma-Aldrich), 100 μM $FeCl_3$ (Sigma-Aldrich), 1 μM $ZnCl_2$ (Sigma-Aldrich),2 μM thiamine (Fisher BioReagents), 0.5% glycerol (Sigma-Aldrich) and 0.5% glutamate (Sigma-Aldrich)(S S Branda et al. 2001).

2.2.4 Synthesis of S. epidermidis AIP

S. epidermidis AIP was synthesized by Protein Chemistry Technology Core in University of Texas Southwestern Medical Center. The synthesis method was previously described by Otto M, *et al*. (Otto et al. 1998).

2.2.5 Dose response curve measurement

Receiver strains were grown on LB agar plates with appropriate antibiotics at 37 ℃ overnight. For each strain, a single colony was inoculated and grew in 1mL plain MSgg liquid medium overnight. The cell cultures were then diluted 100-fold in plain MSgg liquid medium and grew until OD reached 1. 50 μL cultures were added to a clear bottom 96 well plate with pre-added MSgg liquid medium containing increasing concentrations of AIP. The

plate was incubated in the shaker for 1h before measuring OD and fluorescence intensity simultaneously using Infinite 200 microplate reader (TECAN).

2.2.6 Conditioned media preparation

The conditioned media preparation method is based on previous work of Magnuson R., *et al.* (Magnuson, Solomon, and Grossman 1994). Wild type *B. subtilis* was grown overnight on plain LB agar plates at 37 ℃. A single colony was inoculated and grown in 1mL plain MSgg liquid medium for overnight. The cell culture was then diluted 200-fold in plain MSgg liquid medium and grew until OD reached 1.6. After spinning down to get rid of cells, the supernatant was filtered and stored in -80 ℃.

To optimize AIP yield, the Sender with optimal AIP yield was used in supernatant preparation. As specified in Table S1, the Sender with optimal AIP yield contains two copies of *agr* operon, one is driven by P*2* and the other is driven by P*rpsd*. In addition, this sender contains one copy of *spsB* expressed from P*rpsD*. SpsB is a membrane peptidase that can remove the N-terminal leader of AIP (Kavanaugh, Thoendel, and Horswill 2007), thus facilitating the release of AIP.

2.2.7 Single cell imaging

Movies and snapshots were acquired with an IX71 inverted fluorescence microscope (Olympus), IX83 inverted fluorescence microscope (Olympus) or DeltaVision microscopy imaging system (GE Healthcare). Images were taken every 40 min. Single cell movies were taken with a 100x objective lens (UPLFLN 100x/1.3, Olympus) while growing 10-fold diluted cell cultures on an MSgg pad at 30 ℃.

Response of single cells to AIP was tested in liquid culture containing 1 μM AIP. *B. subtilis* strains were grown overnight on LB agar plates with appropriate antibiotics at 37 ℃. For each strain, a single colony was inoculated and grew in 1mL plain MSgg liquid medium overnight. The cell cultures were then diluted 100-fold in plain MSgg liquid medium and grew until OD reached 1. Cultures from different strains were mixed together at a certain ratio and incubated with 1 μM AIP. After 1h, 1μL culture was spotted on a solid MSgg pad (1.5% agar, dried overnight) and dried for a while. Pads were then flipped onto glass bottom petri dishes for single cells imaging.

2.2.8 Single cell segmentation

ImageJ (National Institutes of Health, http://imagej.nih.gov/ij/) and MATLAB (MathWorks) were used for image processing and analysis. The detailed image analysis methods for biofilms and single cells are described below respectively. Single cells were segmented based on phase contrast images using the analyze particle function in ImageJ or image processing and statistics toolboxes in MATLAB, as previously described (Süel et al. 2006). When mixing WT Receiver and ECM-deficient Receiver cells together, WT Receiver cells were marked by P*rpsD-cfp*, which enabled us to distinguish between these two cell types.

2.3 Results

We began by testing the modularity and functionality of the *agr* system after it was transformed into *B. subtilis*. Specifically, we constructed two types of synthetic QS systems in *B. subtilis*, namely "Receiver" and "Sender" cells (**Figure 5**). The Sender cells contain the

entire *agr* operon (*agrA,B,C* and *D*) and are capable of sensing and secreting AIP. In contrast, Receiver cells can only respond to AIP, but not synthesize it as they lack the necessary genes. Receiver cells only contain the AgrC receptor and downstream AgrA transcription factor that regulates expression of the P*3* promoter. Therefore, we can use Receiver cells to measure the response to AIP by monitoring expression of a fluorescent protein reporter YFP from the P*3* promoter (P*3-yfp*).

Initially we constructed a Sender strain with only AgrBD, the AIP production module, by integrating P*rpsD-agrBD*. However, this strain did not secrete enough AIP to induce the response in Receiver cells. To amplify the production of AIP, we introduced the auto-inducing circuit with the receiving module into the Sender strain. The auto-inducing property of such Sender strain increases its AIP yield greatly. Therefore, we eventually used this Sender strain in this study. Another advantage of using such Sender strain is that its circuit structure mimics the nature behavior of the QS system.

2.3.1. Receiver cells can respond to chemically synthesized AIP

Quantitative fluorescence microscopy measurements confirmed that *B. subtilis* Receiver cells containing the synthetic QS system could indeed respond to extracellular AIP (**Figure 7A**). In particular, we find that addition of 100 nM chemically synthesized AIP induced a nearly 5-fold increase in P*3* expression (**Figure 7B**). We also obtained the AIP dose response curve for P*3* expression in Receiver cells (**Figure 7C**). Receiver cells begin to elicit a detectable response to approximately 70 nM extracellular AIP. The response increased with increasing AIP concentrations and surprisingly did not saturate even with

addition of 10 μM AIP, a concentration 3 orders of magnitude higher than the minimal activating concentration.

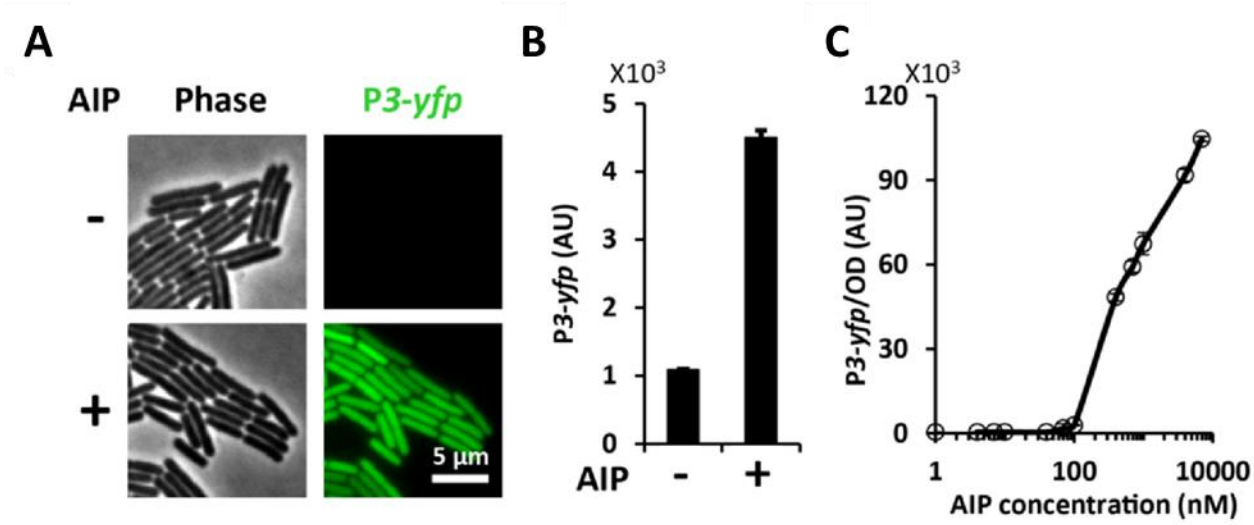

Figure 7. Receiver cells can respond to chemically synthesized AIP.
(**A**) Snapshots of Receiver cells grown on MSgg pads with 0 (-) or 100nM (+) chemically synthesized AIP. *yfp* expression from P*3* is shown in green. (**B**) Mean P*3-yfp* fluorescence intensity from individual Receiver cells with 0 (-) or 100 nM (+) AIP (mean ± SEM, n=78 cells, $p<0.0001$). (**C**) Dose response curve of Receiver cells to AIP in shaking liquid culture (mean ± SEM, n=2).

2.3.2 Sender cells secrete functional AIP

Next we tested the ability of Sender cells to synthesize and secrete functional AIP. Specifically, Sender cells were grown in LB for 3 hours after which the supernatant was collected and added to Receiver cells (**Figure 8A**). Exposure of Receiver cells to supernatant collected from Sender cells triggered P*3* expression in Receiver cells (**Figure 8A and B**). Therefore, Sender cells secrete sufficient functional AIP to trigger a QS

response in Receiver cells. In contrast, supernatant collected from genetically unmodified *B. subtilis* cells did not elicit a response in Receiver cells (**Figure 8A and B, Figure 26**). This data also shows that *B. subtilis* cells do not express any peptide that can stimulate the *agr* system, further indicating the lack of interference between the synthetic and natural QS systems. Together, these data indicate that the synthetic QS is functional in both *B. subtilis* Sender and Receiver cells and performs as intended.

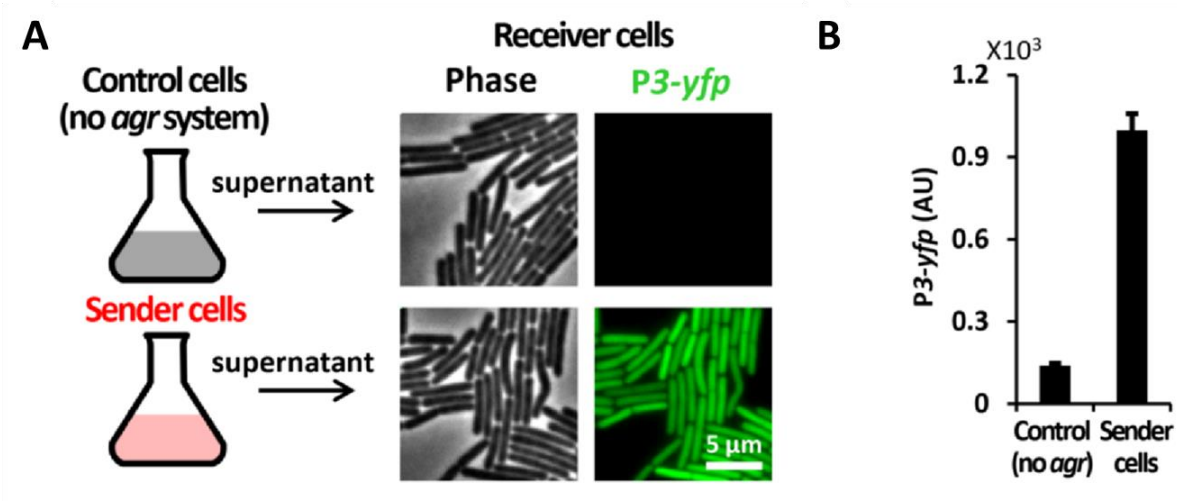

Figure 8. Sender cells secrete functional AIP.
(**A**) Images of Receiver cells grown on MSgg pad supplemented with 50% conditioned media from control cells (no *agr* system) or Sender cells. (**B**) Mean P*3-yfp* fluorescence intensity in Receiver cells grown with conditioned media from control cells or Sender (mean ± SEM, n=97 cells, $p<0.0001$).

2.3.3 Integration of the synthetic system does not interfere with biofilm development process

Before utilize the synthetic QS system in the biofilm, we performed two control experiments to determine adverse effects of *S. epidermidis* gene integrations or AIP peptide addition to *B. subtilis* biofilm morphology. Results show that chromosomal integration of *agr* genes into *B. subtilis* does not affect biofilm formation (**Figure 9**). Furthermore, extracellular addition of 10 μM AIP does not alter *B. subtilis* biofilm development or morphology, suggesting that native ECM production is not affected by the presence of *S. epidermidis* AIP (**Figure 10**). Together, these data show that the *S. epidermidis agr* system in Receiver cells is functional and does not interfere with native *B. subtilis* biofilm development.

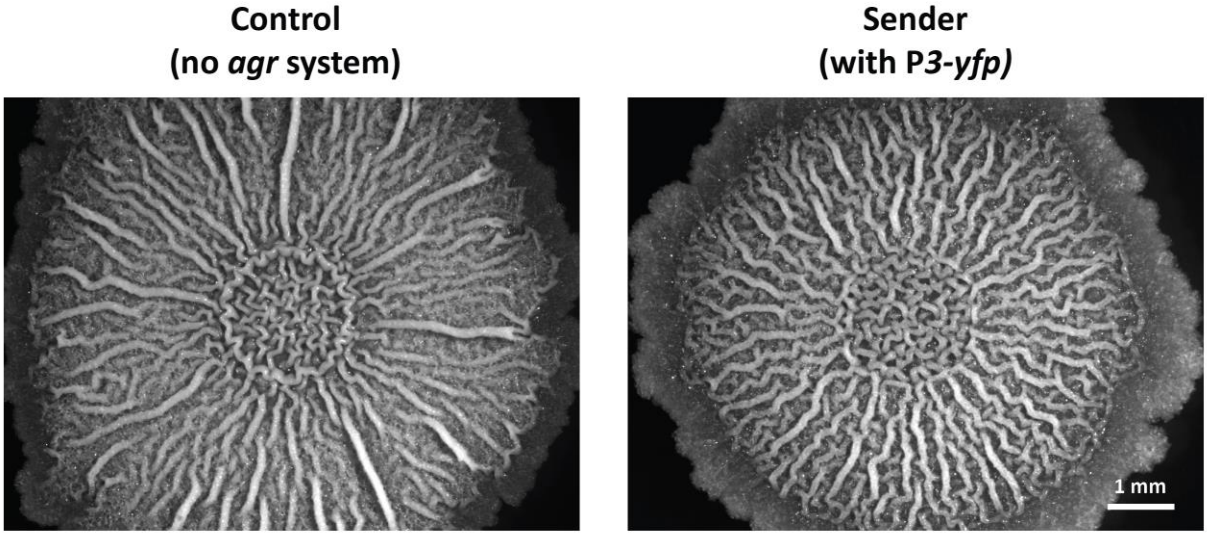

Figure 9. Chromosomal integration of *agr* genes into *B. subtilis* does not affect biofilm formation.
Four-day-old biofilm structure of control strain (no *agr* system integrated) and Sender strain (with P*3-yfp).*

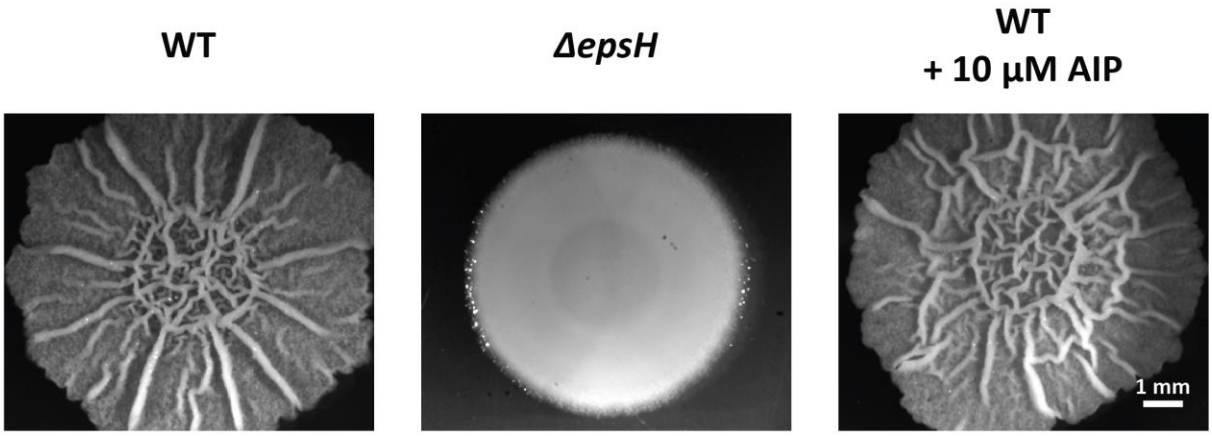

Figure 10. AIP has no significant effects on *B. subtilis* biofilm formation.
WT cells were grown on MSgg plates with 0 or 10 μM AIP for 3 days. Then the bright field image was taken from the top and compared to *ΔepsH* cells grown on MSgg plates for 3 days.

2.4 Discussion

The system constructed in this work represents the first synthetic QS system engineered into gram-positive bacteria. Marchand N. and Collins C. constructed another synthetic QS system in *Bacillus megaterium* (Marchand and Collins 2015). Their work published after us represents another example of synthetic cell-cell communication systems in gram-positive bacteria.

Synthetic cell-cell communication systems have been used a lot in industry (Hong et al. 2012; W. Zhang and Nielsen 2015). In bioprocessing, the microbial consortia include multiple groups of cells performing distinct functions. As cell-cell communication systems offer us a way to efficiently control the cell growth, our synthetic systems allowed us to design the temporal cooperation of different cell groups for higher efficiency. and downstream effectors can help divide work in microbial consortia. Another advantage of the synthetic QS system is its autoinducing property that allows cells to produce a large amount

of target proteins, typically several-fold higher than the amount obtained using conventional IPTG induction system(Studier 2005; Weber and Fussenegger 2007).

Gram-positive bacteria are often utilized in industry for food or enzyme production. Compared with gram-negative bacteria, gram-positive bacteria secrete a large number of proteins into the surrounding environment, which makes product purification much simpler (Freudl 1992). In particular, the gram-positive bacteria *B. subtilis* is generally recognized as a safe strain in contrast to the well-studied gram-negative bacteria *E. coli* (Westers, Westers, and Quax 2004). Due to such safety consideration, *B. subtilis* is highly preferred over *E. coli* in food industry. Therefore, building a synthetic QS system in *B. subtilis* can provide a controlled yet dynamic new system for industrial fermentation.

Chapter 3: ECM enhances QS response

3.1 Introduction

Even though bacteria are unicellular organisms, they predominantly reside in structured communities known as biofilms (O'Toole, Kaplan, and Kolter 2000; Bryers 2008). One of the defining characteristics of biofilms is the presence of an extracellular matrix that encapsulates all cells within the community and equips the biofilm with structural integrity (S S Branda et al. 2005; Flemming and Wingender 2010). The major ECM constituents of most biofilms, for example those formed by *Bacillus subtilis*, are polysaccharides and amyloid-like protein filaments (Romero et al. 2014; Romero et al. 2010; S S Branda et al. 2006; S S Branda et al. 2001).

There is some evidence suggesting that ECM may affect the diffusion of quorum molecules as discussed in section 1.4.2 Effect of ECM on QS. Specifically, a recent *in vitro* study indicated that quorum sensing molecules could transiently associate with the ECM (Seviour et al. 2015). Therefore, the ECM may enrich QS signals locally, increasing the availability of the QS signal to cells. However, it remains unclear whether the transient association of QS signals and the ECM affects the response of ECM-producing cells *in vivo*. Here we investigated whether ECM producing cells may locally concentrate QS signals, by quantitatively measuring a synthetic QS signaling system in biofilm communities and single cells

ECM expression and QS are tightly coupled and cannot be perturbed independently, since the native QS regulates ECM expression (Hammer and Bassler 2003; Waters et al. 2008; Hammer and Bassler 2009; Shank and Kolter 2011; Lopez et al. 2009; López et al.

2009; Sakuragi and Kolter 2007) (**Figure 11**). Therefore, we used the orthogonal (inert) synthetic QS system in *B. subtilis* constructed in Chapter 2 to study how the response to QS signals may be affected by ECM production (**Figure 11**). This system consists of dedicated "Sender" and "Receiver" cells that can secrete or respond to QS signals, respectively. This synthetic QS system provides the unique opportunity to study QS without interfering with native *B. subtilis* QS or ECM production (**Figure 11**). Investigating synthetic QS in the background of ECM gene deletion strains revealed that ECM expressing cells are more responsive to QS signals. Additionally, we find that the ECM produced by cells retains QS peptides *in vivo*, enhancing the QS response. Finally, we confirm that results from our synthetic QS system also apply to the native QS response of *B. subtilis*.

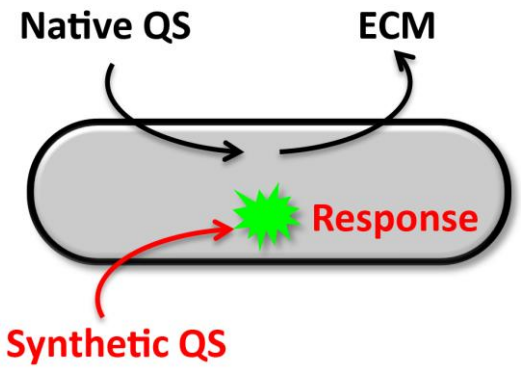

Figure 11. Native QS vs. synthetic QS.
An orthogonal (inert) synthetic QS system was constructed in *B. subtilis*. This synthetic QS system does not genetically regulate ECM production, thus enabling us to independently study how the response to QS signals may be affected by ECM production.

3.2 Materials and Methods

3.2.1 Strain construction

All *Bacillus subtilis* strains used in this study were derived from NCIB3610 (a gift from the laboratory of Wade Winkler, University of Maryland, College Park, MD (Irnov and Winkler 2010)), an undomesticated wild type strain. Strains are listed in **Table 1**.

The *ΔepsH* strain is a kind gift from the Roberto Kolter laboratory (Harvard Medical School, Cambridge, MA) (S S Branda et al. 2001). All other strains harboring *epsH* deletion were constructed based on this *ΔepsH* strain. To construct the *ΔtapA* operon strain, upstream and downstream regions of the *tapA* operon were cloned into per449, a generic integration vector constructed for integration into the gene of interest (kind gift from Wade Winkler, University of Maryland, College Park, MD). The resulting vector was then transformed into *B. subtilis*.

3.2.2 Biofilm Formation

The biofilm formation assay is adapted from previous protocols (S S Branda et al. 2001). *B. subtilis* strains were grown on LB agar plates with appropriate antibiotics at 37 ℃ for overnight. For each strain, a single colony was picked and grown in 1mL LB liquid culture at 37 ℃ for 6h until saturation. When needed, cultures from different strains were mixed together at a certain ratio and diluted 5000-fold with MSgg medium. Then 1μL culture was spotted on MSgg solid plate (1.5% agar, 3 mm thickness, dried overnight). During the imaging process, cells were incubated at 30 ℃.

3.2.3 Microscopy

Movies and snapshots were acquired with an IX71 inverted fluorescence microscope (Olympus), IX83 inverted fluorescence microscope (Olympus) or DeltaVision microscopy imaging system (GE Healthcare). Biofilm movies were taken from the bottom with a 2.5x objective lens (MPLFLN 2.5x/0.08, Olympus) to film the whole biofilm or a 10x objective lens (UPLFLN 10x/0.3, Olympus) to zoom in on certain regions. Images were taken every 40 min. Top-view of biofilms was acquired by a Retiga 2000R digital camera (QImaging) via an SZX10 fluorescent stereomicroscope (Olympus).

3.2.4 Biofilm image segmentation and quantification

In a mixed biofilm, regions of each strain were segmented out based on fluorescence images of constitutive markers. P*rpsD* fused with a fluorescent protein (CFP or mCherry) were integrated into each strain except one. For strains containing P*rpsD* driven fluorescent proteins, a fluorescent image was taken accordingly. Then this fluorescent image was processed using ImageJ to determine the region of the strain in two steps. First, the edge was detected using the Otsu method in auto local threshold function. This algorithm assumes that the image contains two classes of pixels that follow bi-modal histogram. It calculates the threshold that minimizes the intra-class variance of the two classes. Second, the fluorescence positive region was determined by the analyze particle function. After the region of each fluorescently marked strain was determined, the rest of the area was assigned to the one strain without a fluorescent marker.

After segmentation, the distance of each pixel within Receiver microcolonies to the edge of Sender microcolonies was calculated. In Figure 12, the average P3-*yfp* fluorescent intensity of all pixels within every 12.9μm (10 pixels) distance was calculated and plotted against its distance from the edge of the Sender microcolony for WT Receiver and *ΔepsH* Receiver, respectively. The average P3-*yfp* fluorescence intensity of WT Receiver and *ΔepsH* Receiver within 12.9μm from the edge of Sender microcolony (grey region in **Figure 12**) was calculated and normalized according to the P3-*yfp* fluorescent intensity of WT Receiver. This relative P3-*yfp* intensity of WT Receiver and *ΔepsH* Receiver was calculated for multiple locations in different movies and then the mean was shown in **Figure 13D**. Similarly, relative P3-*yfp* fluorescence intensity in the region of each strain was measured and the mean was calculated across different movies in **Figure 16C**.

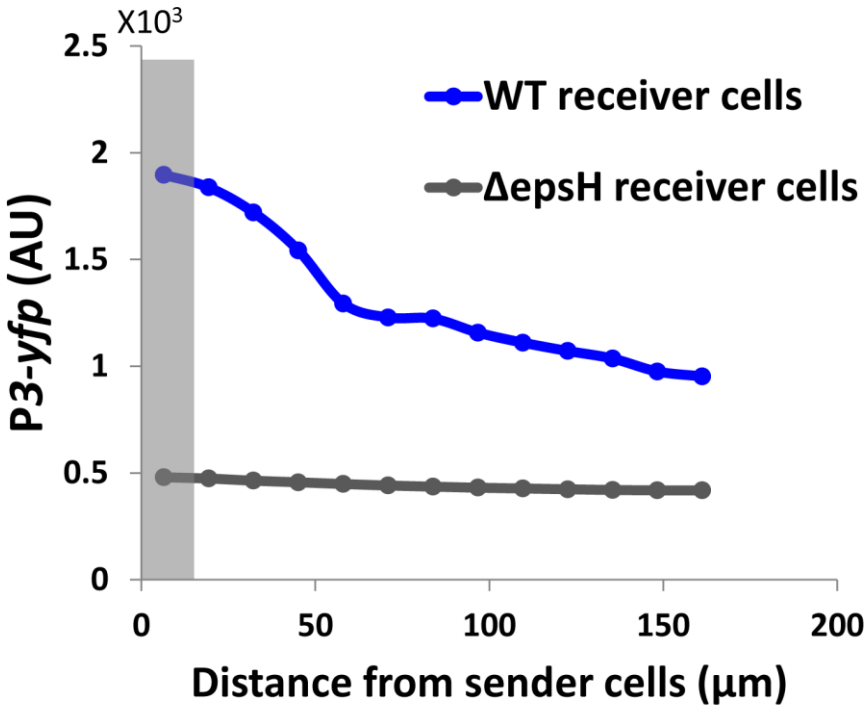

Figure 12. Average P3-*yfp* intensity plotted against the distance from Sender.

Average P*3-yfp* intensity of WT and *ΔepsH* Receiver cells in Figure 13 plotted against the distance from Sender. The relative P*3-yfp* intensity in Figure 13D is calculated with the fluorescence intensity in the grey bar.

3.2.5 Density measurement

Cell density was determined using bright field images as previously described and validated by A. Seminara *et al.* (Seminara et al. 2012). Relative cell density was defined as $-\log(I/I_0)$, where I is the average image intensity in the region of a certain strain and I_0 is the average image intensity in regions outside of the biofilm.

3.2.6 β-galactosidase assay

Cells were grown on LB agar plates with appropriate antibiotics at 37 ℃ overnight. For each strain, a single colony was inoculated and grown in 1mL plain MSgg liquid medium overnight. The cell cultures were then diluted 200-fold in plain MSgg liquid medium and grown until OD reached 0.1. The cell culture and the conditioned media were mixed by 1:1 ratio and incubated for 1h, after which the A600 of each samples was measured and recorded. We spun down 750 μL cell culture and resuspended the cell pellet in 250 μL Z buffer containing 60 mM Na_2HPO_4 (Macron Fine Chemicals), 40 mM NaH_2PO_4 (Sigma-Aldrich), 10 mM KCl (Sigma-Aldrich), 1 mM $MgSO_4$ (BioExpression), 50 mM β-mercaptoethanol (a gift from the laboratory of Kit Pogliano, University of California, San Diego, CA) with pH adjusted to 7.0). To lyse the cells, we added lysozyme (Sigma-Aldrich) to 0.5 mg/mL and incubated samples for 10 min at 37 ℃, followed by addition of 0.08% Triton X-100 (AMERESCO) and thorough vortexing. After adding another 250 μL of Z buffer , the samples were prewarmed at 30 ℃ for 10min. After

prewarming, we mixed 100 μL prewarmed 4 mg/mL ONPG (in Z buffer, a gift from the laboratory of Kit Pogliano, University of California, San Diego, CA). with the samples and keep them at 30 ℃ until they turned light yellow. The reaction was terminated by addition of 250 μL 1M Na_2CO_3 (Sigma-Aldrich) and the reaction time was recorded. At this point, A420 of each sample was recorded. β-galactosidase activity was calculated as A420 * 1000/ [reaction time (min) * 0.75 (mL) * A600]. In this equation, the enzyme activity was normalized against the cell density by dividing A600. '1000' was a scale factor used to make the numbers easier to compare.

3.3 Results

3.3.1 ECM enhances QS response in biofilms

We investigated directly in biofilm communities whether detection of QS signals would be affected by ECM expression. Specifically, we grew mixed biofilms that contained Sender cells together with two types of Receiver cells, where one Receiver strain was deficient in ECM production (**Figure 13A**). The ECM deficient Receiver strain was obtained by deleting the *B. subtilis epsH* gene (**Table 1**). The *epsH* gene encodes a critical enzyme necessary for extracellular polysaccharide synthesis and it has been shown that *ΔepsH* cells are deficient in ECM production and biofilm development (S S Branda et al. 2001). We grew mixed biofilms that were comprised of adjacent clusters of Sender and Receiver strains (**Figure 13B**). We then measured the QS response of Receiver cells with and without the *epsH* gene to AIP secreted by an adjacent cluster of Sender cells (**Figure 13C**). Results show that the *ΔepsH* strain has approximately four-fold lower QS response

(P*3-yfp*) compared to the Receiver strain with an intact *epsH* gene (**Figure 13D**). Control experiments show that despite the reduced response to AIP, the *ΔepsH* Receiver strain was still functional and capable of exhibiting an AIP dependent dose response curve (**Figure 14**). This result indicates that the reduced response in *ΔepsH* Receiver strain was not because its *agr* system had any potential damages.Furthermore, we confirmed that the difference in the response between Receiver strains was not due to differences in cell density (**Figure 15**). These findings suggest that ECM deficient cells have a reduced response to AIP-mediated QS and are therefore less effective in sensing signals from distant cells.

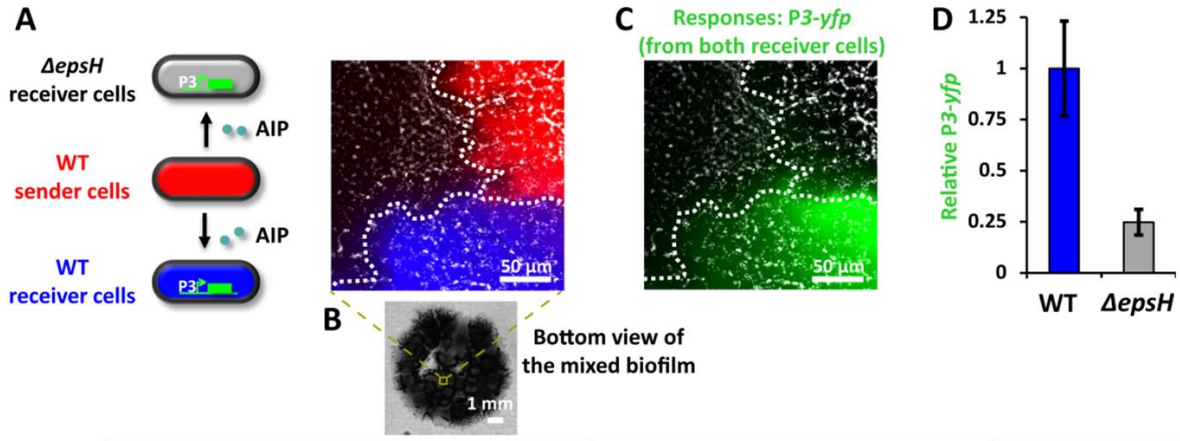

Figure 13. The *ΔepsH* strain has lower QS response compared to the WT Receiver strain.
(**A**) A local region in a mixed biofilm where clusters of WT Sender cells (red), WT Receiver cells (blue) and *ΔepsH* Receiver cells (grey) merge. Sender cells are marked by P*rpsD-mCherry* and WT Receiver cells are marked by P*rpsD-cfp*, where P*rpsD* is a constitutive promoter. In this experiment, WT Sender cells (red) secrete AIP that diffuses to nearby WT (blue) and *ΔepsH* (grey) Receiver cells. Both Receiver cells respond to AIP by expressing YFP. (**B**) Bottom view of the mixed biofilm. (**C**) P*3-yfp* overlaid on bright field image showing the same biofilm region as in **A**. (**D**) Relative mean P*3-yfp* intensity of WT Receiver cells and *ΔepsH* Receiver cells (mean ± SEM, n=3, $p<0.09$).

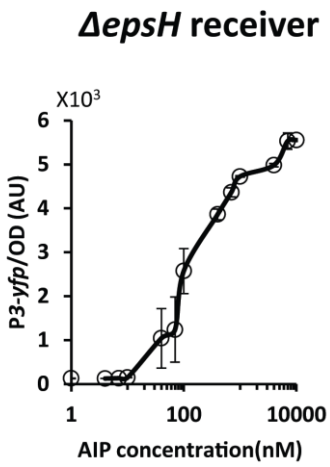

Figure 14. *ΔepsH* Receiver strain also exhibits an AIP dependent dose response curve. Dose-response curve of *ΔepsH* Receiver cells to AIP (mean ± SEM, n=2).

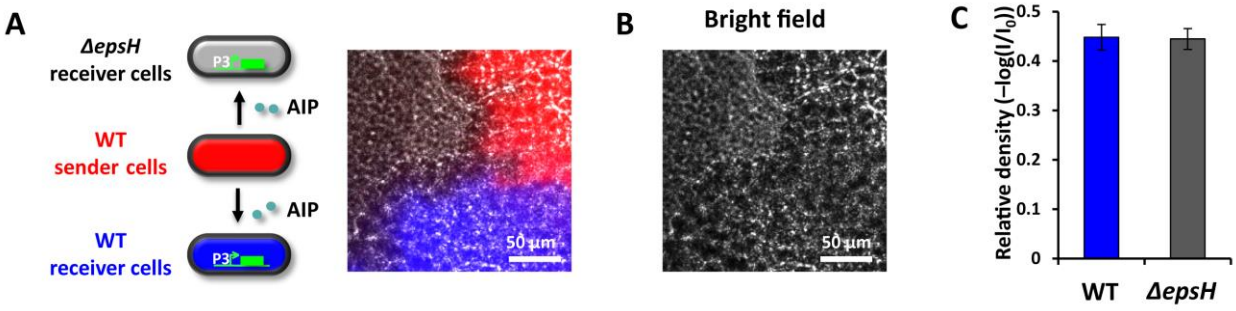

Figure 15. Cell densities in regions of the two Receiver strains are comparable. (**A**) A local region in a mixed biofilm where clusters of Sender cells, *ΔepsH* Receiver cells and WT Receiver cells merge. Sender cells are marked by P*rpsD-mCherry* (red) and WT Receiver cells are marked by P*rpsD-cfp* (blue), where P*rpsD* is a constitutive promoter. This is the same figure as Fig.13A. (**B**) Bright field image of the same biofilm region as in A. (**C**) Relative cell density (–log(I/I_0)) of WT Receiver cell clusters and *ΔepsH* Receiver cell clusters (mean ± SEM, n=3).

As the distance between Sender and Receiver cells anti-correlates with the response to the QS signal (**Figure 12**), we asked whether the above-described deficiency in QS response of ECM-deficient Receiver cells would be alleviated if these cells also produce the QS signal. Interestingly, we found that even if *ΔepsH* cells are the ones to produce the AIP, the more distant WT Receiver cells exhibited a higher QS response. Specifically, we grew mixed biofilms that contained *ΔepsH* Sender cells and wild type Receiver cells (**Figure 16A**). We find that the *ΔepsH* strain had approximately two-fold lower QS response compared to the wild type Receiver strain (**Figure 16B and C**). Even though *ΔepsH* Sender cells would experience a higher local AIP concentration, distant wild type Receiver cells generated a higher QS response. Therefore, even when the QS signal is not required to travel among cells, ECM deficient cells have a reduced response to locally made QS signals. These data suggest that the higher QS response of ECM producing cells constitutes a private property that is independent of whether cells respond to their own AIP signal or those expressed by distant cells.

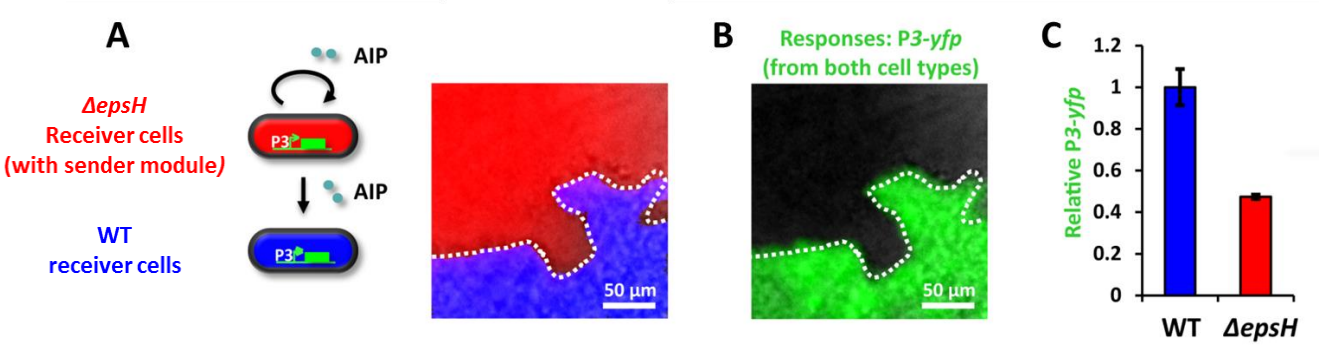

Figure 16. Even if *ΔepsH* cells are the ones to produce the AIP, the more distant WT Receiver cells exhibited a higher QS response.
(**A**) A local region in a mixed biofilm where clusters of *ΔepsH* Receiver cells (with sender module, red) and WT Receiver cells (false colored as blue) merge. *ΔepsH* Receiver cells (with sender module) are marked by P*rpsD-cfp*. In this experiment, *ΔepsH* Receivercells (red) secrete AIP which induces a response in the *ΔepsH* Receivercells (red) themselves. Meanwhile some AIP will diffuse to nearby WT Receiver cells and trigger a response. (**B**) P*3-yfp* overlaid on bright field image showing the same biofilm region as in **A**. (**C**) Relative mean P*3-yfp* intensity of WT Receiver cells and *ΔepsH* Receiver cells (with P*3-yfp*) (mean ± SEM, n=3, $p<0.05$).

3.3.2 ECM enhanced QS response does not extend to directly adjacent cells

To directly test the finding that enhanced QS appears to be limited exclusively to ECM producing cells, we performed experiments at the single cell level. In particular, we wanted to determine if ECM expression could enhance the QS response of directly adjacent cells. We thus turned to experiments in mixed microcolonies that enabled quantitative measurement of the QS response in single cells (**Figure 17A**). Results show that the QS response of WT Receiver cells, capable of ECM production, is clearly higher comparing to the response measured in the nearest physically adjacent *ΔepsH* Receiver cells (**Figure 17B**).

Furthermore, the low QS response of *ΔepsH* Receiver cells did not decrease further as a function of increasing distance from the WT Receiver cells (**Figure 18**). Together, these results indicate that enhanced QS response in ECM producing cells is indeed a private property at the single cell level that does not extend to adjacent cells.

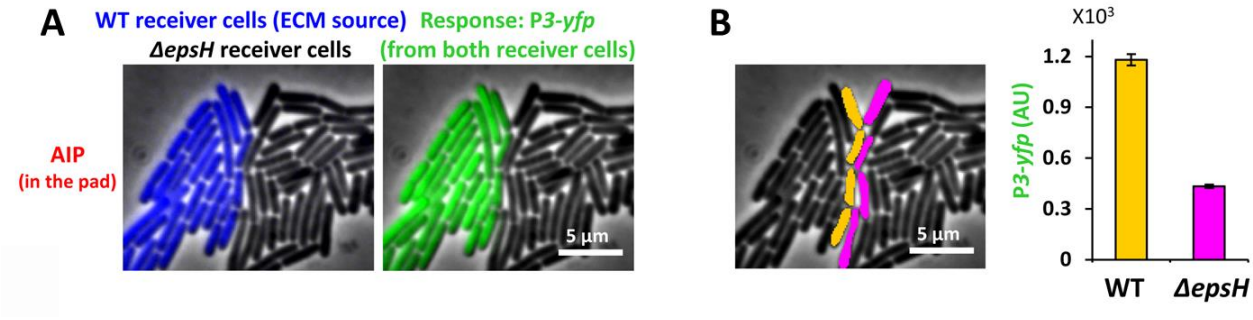

Figure 17. QS response of WT Receiver cells is higher compared to the nearest physically adjacent *ΔepsH* Receiver cells.
(**A**) A snapshot of WT (blue) and *ΔepsH* (grey) Receiver cells growing near each other on an MSgg agar pad containing 100nM AIP. WT Receiver cells are marked by P*rpsD-cfp*. The response, P*3-yfp*, is shown in green on the right panel. (**B**) Response of WT (false colored as orange) and *ΔepsH* Receiver cells (false colored as magenta) on the interface where these two strains merge (mean ± SEM, n=39 cells, $p<0.0001$).

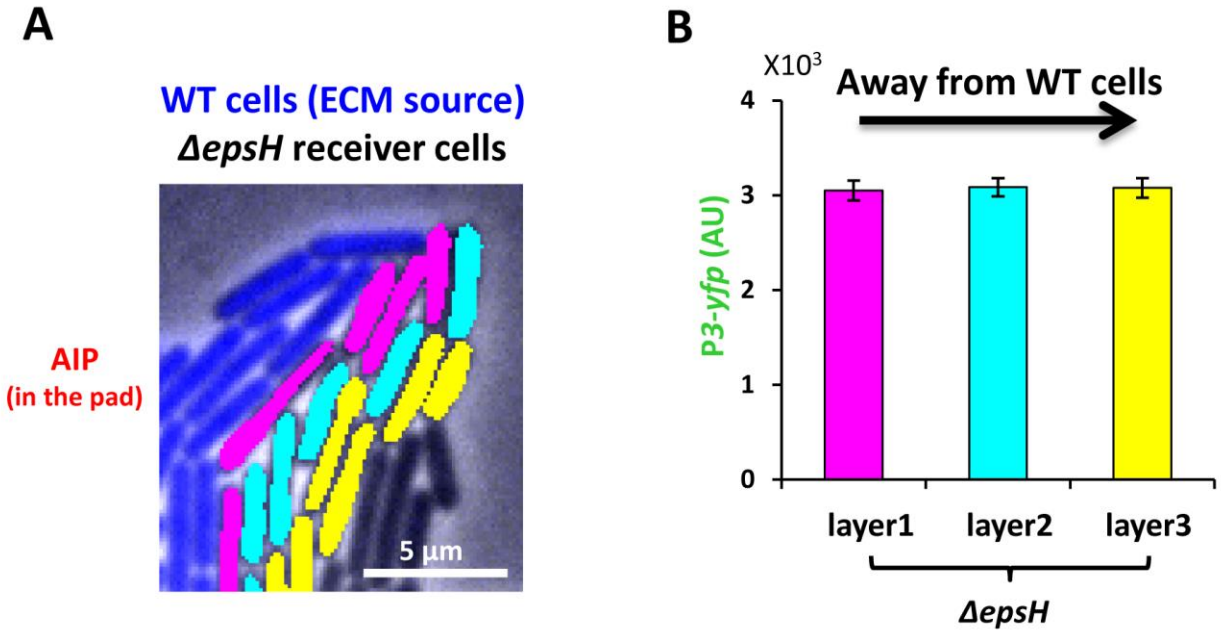

Figure 18. The low QS response of *ΔepsH* Receiver cells did not decrease further as a function of increasing distance from the WT Receiver cells.
(**A**) *ΔepsH* Receiver cells grown nearby WT cells (P*rpsD-cfp*, blue) on the MSgg agar pad containing 100nM AIP. *ΔepsH* Receiver cells with different distance from the border of WT cells are false colored with magenta (layer 1), cyan (layer 2) and yellow (layer 3). (**B**) Response (P*3-yfp*) of these three layers of *ΔepsH* Receiver cells (mean ± SD, n=49 cells).

3.3.3 ECM production dependent QS response also affects single cells in liquid cultures

Finally we investigated whether the reduced QS response observed in ECM deficient strains was limited to structured communities such as biofilms, or could even be observed in liquid cultures. Accordingly, we grew an equal mixture of wild type and *ΔepsH* Receiver strains for one hour in liquid culture that was supplemented with 1µM of chemically synthesized AIP (**Figure 19A**). We then imaged single cells sampled from this liquid culture. Consistent with our previous results, quantitative fluorescence microscopy showed

that ECM deficient *ΔepsH* Receiver cells have a lower response to AIP than wild type Receiver cells, even when grown together in shaking liquid cultures (**Figure 19B**).

Could the reduced QS response be specifically linked to the deletion of the *epsH* gene, or perhaps be a general consequence of ECM deficiency? To address this question, we repeated the same experiment using an equal mixture of wild type and a *tapA* operon deletion strain (**Table 1**). The *tapA* operon encodes another critical component of the ECM, namely proteins that can form amyloid-like protein filaments (S S Branda et al. 2006). We find that similar to *ΔepsH* Receiver cells, the *tapA* operon deleted Receiver cells also exhibit a reduced QS response compared to wild type Receiver cells (**Figure 19C**). Therefore, regardless of whether the ECM deficiency is caused by lack of exopolysaccharides or amyloid-like protein fibers, the QS response to AIP is reduced in both types of ECM deficient cells. We also ruled out the possibility that the reduced QS response observed in ECM deficient cells was due to a general problem with protein expression by measuring the activity of a ribosomal gene promoter (P*rpsD*) in wild type and ECM deficient cells (**Figure 20**). Collectively, these results show that the reduced QS response is not tied to the deletion of any particular gene, but appears to be caused by an overall deficiency in ECM production. Furthermore, the reduced QS response in ECM deficient strains not only arises in the context of the biofilm community or microcolonies, but appears to be a single cell level property that even applies in liquid cultures.

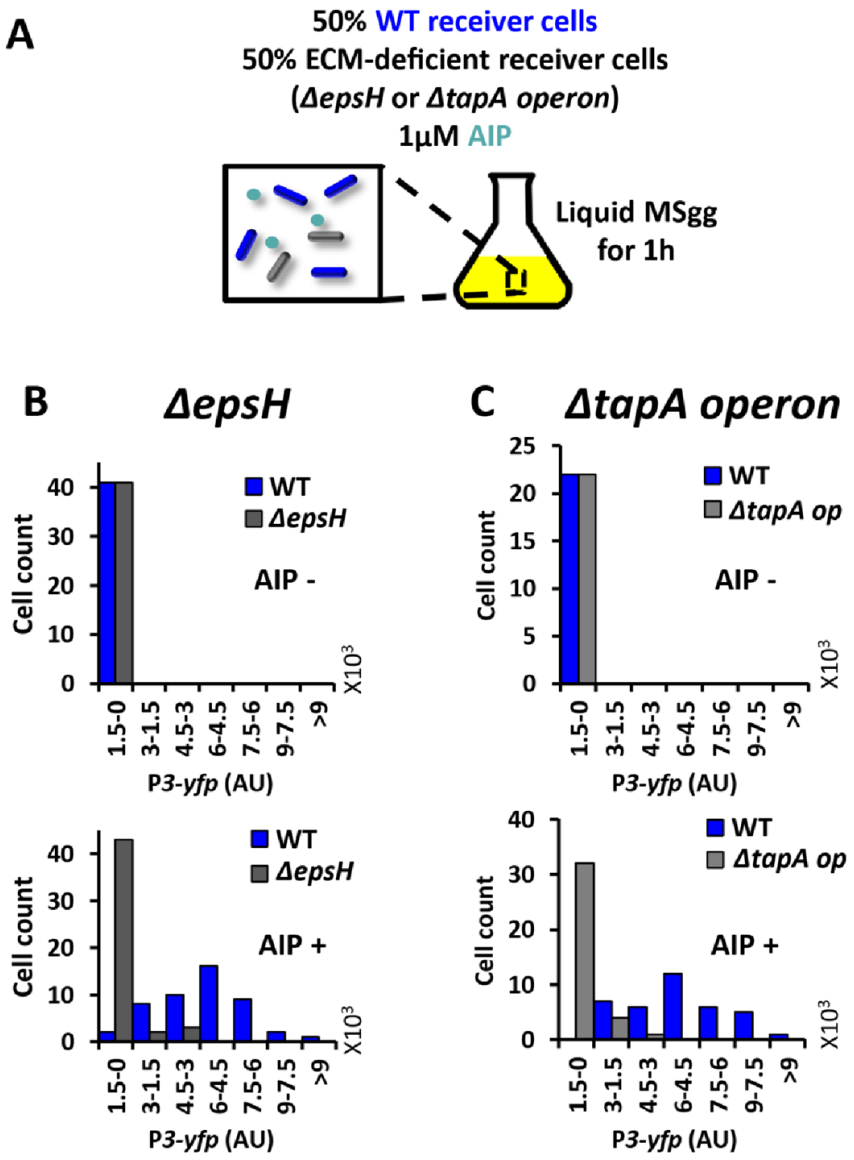

Figure 19. ECM enhances response of producer cells to AIP even in liquid culture. (**A**) Illustration of experimental setup for B-C. ECM-deficient Receiver cells (*ΔepsH* or *ΔtapA operon*) and WT Receiver cells (P*rpsD-cfp*, blue) are growing together in liquid culture containing 0 or 1 μM AIP for 1h before the response (P*3-yfp*) is quantified. (**B**) Histograms of P*3-yfp* fluorescence intensity from *ΔepsH* Receiver cells and WT Receiver cells (n=41 cells for the upper panel and n=48 cells for the bottom panel). The expression of P*3-yfp* was measured for individual cells from microscopic snapshots. (**C**) Histograms of P*3-yfp* fluorescence intensity from *ΔtapA operon* Receiver cells and WT Receiver cells (n=22 cells for the upper panel and n=40 cells for the bottom panel). The expression of P*3-yfp* was measured for individual cells from microscopic snapshots.

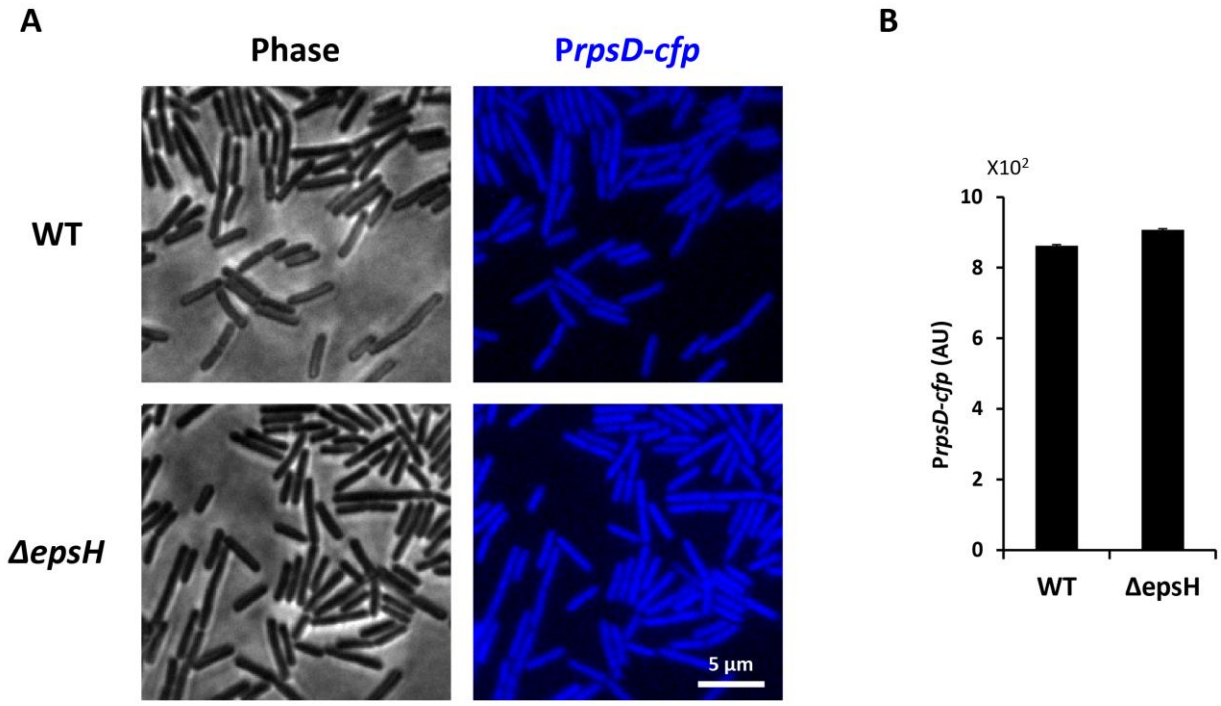

Figure 20. Fluorescence intensity of P*rpsD-cfp* in *ΔepsH* and WT cells are comparable. (**A**) Snapshot of WT and *ΔepsH* cells grow in MSgg liquid culture. P*rpsD-cfp* is shown in green. (**B**) P*3-yfp* fluorescence intensity in WT and *ΔepsH* cells (mean ± SEM, n=20 cells).

3.3.4 ECM concentrates extracellular AIP in the vicinity of ECM producing cells

Motivated by the recent *in vitro* study that indicated transient physical binding of QS molecules to the ECM (Seviour et al. 2015), we wanted to test whether the elevated QS response we observed was due to local concentration of AIP by the ECM. To determine whether AIP accumulates near ECM producing cells, we added chemically synthesized AIP to wild type cells capable of ECM production. This culture was mixed, spun down and washed three times to eliminate unbound AIP. We then subjected the culture to mild

sonication in order to release AIP that may have been retained by the ECM back into the media. In parallel, we subjected *ΔepsH* cells to the same protocol to serve as a control. The supernatant collected after sonication from both WT and *ΔepsH* cells was added to WT Receiver cells and their QS response was measured (**Figure 21A**). We found that WT Receiver cells elicited a higher QS response when exposed to the supernatant from WT cells compared to that obtained from *ΔepsH* cells (**Figure 21B**). This result provides a mechanistic explanation for the enhanced QS response of ECM producing cells, by indicating that the ECM concentrates AIP in the vicinity of cells and therefore increases the likelihood of binding to receptors on the membrane (**Figure 21C**).

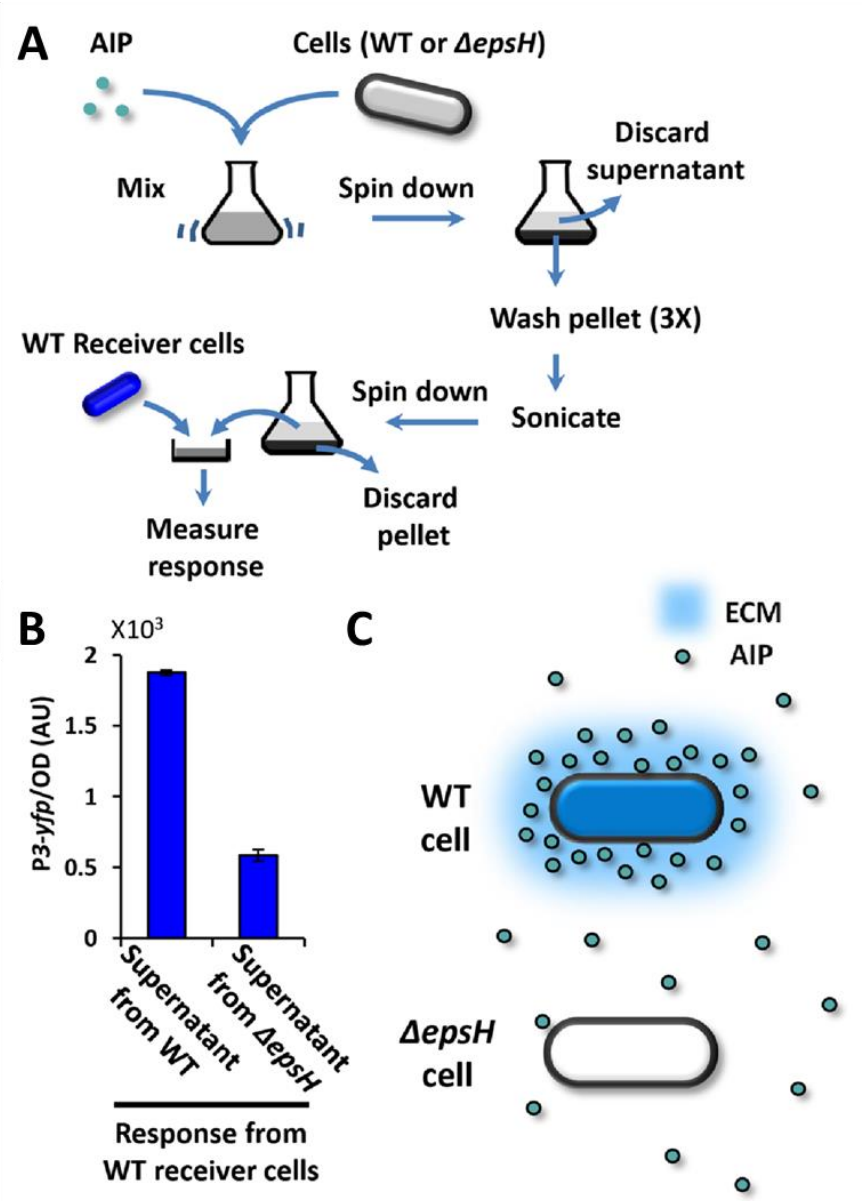

Figure 21. ECM concentrates extracellular AIP in the vicinity of ECM producing cells. (**A**) Scheme of the experimental setup in E. (**B**) Response of WT Receiver cells to AIP extracted from WT cells or *ΔepsH* cells (mean ± SEM, n=4, $p<0.0001$). According to the dose response curve in Figure 7C, the concentration difference between AIP extracted from WT cells and *ΔepsH* cells is roughly 70 nM. (**C**) A diagram illustrates that WT cells concentrate AIP in their vicinity, while *ΔepsH* cells do not.

3.3.5 The QS response positively correlates with natural cell-to-cell variation in ECM production

The amount of ECM produced by individual cells can vary (Vlamakis et al. 2008; Lopez and Kolter 2010). Therefore, we tested whether the natural cell-to-cell variation that is inherent to ECM production would affect the synthetic QS response. Specifically, we introduced into the Receiver strain a second fluorescent protein reporter to measure the natural cell-to-cell variation in *tapA* expression (P*tapA-cfp*). Single cell analysis of the two-color reporter strain for P*3* and P*tapA* expression shows that the QS response in Receiver cells positively correlates with the natural variation in *B. subtilis* ECM expression (**Figure 22**). In contrast, the control experiment showed that variation in P*tapA-cfp* expression did not correlate with expression of the P*rpsD* promoter for a ribosomal gene (**Figure 22**). Therefore, in addition to the reduced QS response observed in ECM gene deletion strains, response to the QS signal is also affected by the natural variation in ECM expression that is inherent to B. *subtilis*.

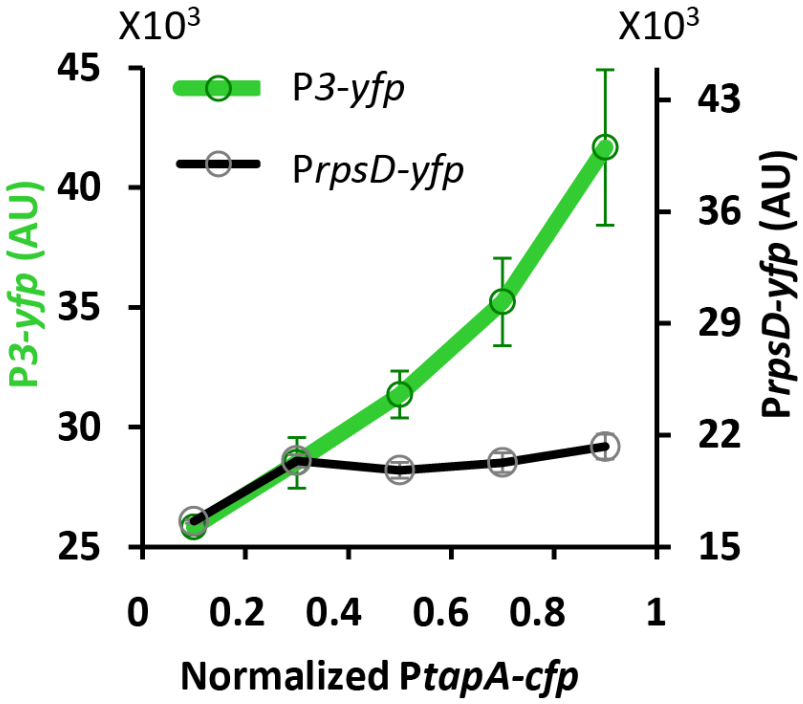

Figure 22. The QS response positively correlates with natural cell-to-cell variation in ECM production.
Average P*3-yfp* and P*rpsD-yfp* as a function of normalized P*tapA* activity (mean ± SEM, total n=646 cells). WT Receiver cells containing P*tapA-cfp* were grown in liquid MSgg culture with 1μM AIP. The expression from the reporters P*tapA-cfp* and P*3-yfp* were measured for individual cells from microscopic snapshots. Relationship between P*tapA-cfp* and P*rpsD-yfp* were measured accordingly using the strain containing P*tapA-cfp* and P*rpsD-yfp*.

3.3.6 ECM elevates response to native B. subtilis quorum sensing signals as well

We asked whether our results obtained using a synthetic QS system would also be relevant to the native QS response of *B. subtilis*. Accordingly, we used what is known as conditioned, or spent media that naturally contains QS signals expressed by *B. subtilis* cells, such as ComX, and then monitored expression of P*srf*, a *B. subtilis* native QS responsive gene promoter (Magnuson, Solomon, and Grossman 1994). We find that upon addition of

conditioned media containing QS signals, WT cells display a higher expression of P*srf-lacZ* compared to *ΔepsH* cells (**Figure 23**). This result shows that the amplitude of the native QS response also depends on ECM production, indicating that results obtained with our synthetic QS system may be generally applicable to native QS systems in *B. subtilis*.

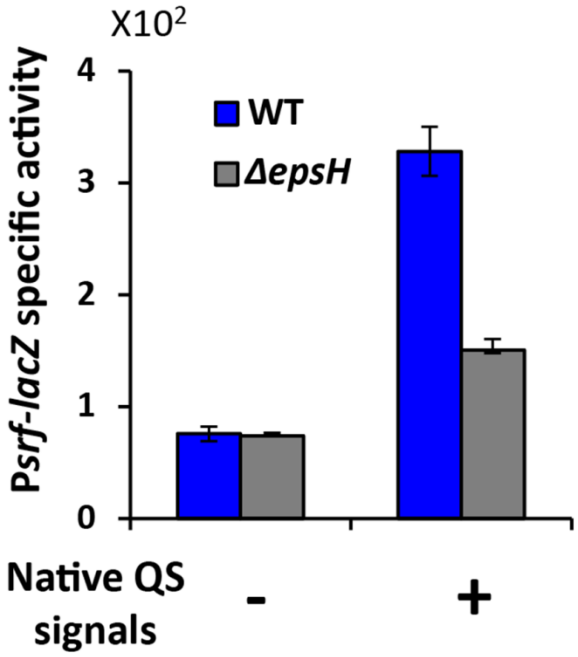

Figure 23. ECM elevates response to native *B. subtilis* quorum sensing signals as well. Response of WT and *ΔepsH* to conditioned media, which contains native quorum sensing signals that activate P*srf*. Response are measured by P*srf-lacZ* (mean ± SEM, n=6, $p<0.01$ for native QS signals + group).

3.4 Discussion

It has been shown that only a subpopulation of cells synthesizes and secretes these ECM components in *B. subtilis* biofilms (Vlamakis et al. 2008; Lopez, Vlamakis, and Kolter 2009) (**Figure 24**). Those ECM producer cells are clearly burdened with a cost at a time of

environmental stress and high cell density. This cost is evident by the observation that an ECM-deficient strain can outcompete the ECM producing strain in a mixed culture (van Gestel et al. 2014). However, all cells within the biofilm, even those that do not contribute to ECM production, are believed to benefit from the ECM protection (Rainey and Rainey 2003; Borenstein et al. 2013; Hibbing et al. 2010). This provokes the question of how the subpopulation of cells that are burdened with ECM production can be sustained within the biofilm community (Nadell and Bassler 2011; Brockhurst, Buckling, and Gardner 2007; J. B. Xavier and Foster 2007; van Gestel et al. 2014; Drescher et al. 2014; Schluter et al. 2015). It has been argued that expression of ECM by a subpopulation of cells may constitute a primitive form of altruism in bacteria (Lopez, Vlamakis, and Kolter 2009). However, our results suggest that ECM producers could also enjoy a private benefit, countering the cost of this public good production.

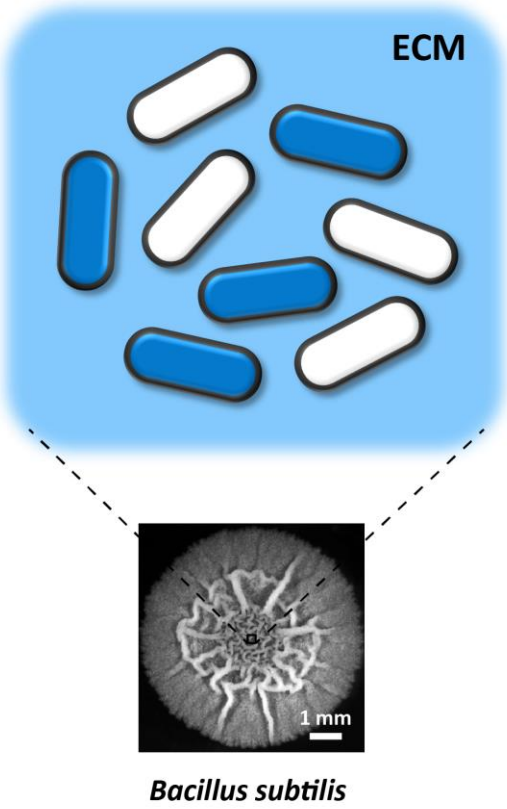

Figure 24. Cost-benefit dilemma for ECM producers in biofilms.
In biofilms, only a subgroup of cells produces and secretes ECM. However, once secreted, ECM benefits all cells, including non-producers. The bottom picture is the top view of a three-day-old biofilm.

The results presented in this study indicate that the cost of ECM expression may be balanced by an enhanced private response to QS signaling (**Figure 25**). As the name implies, QS refers to a collective process by which individual cells can sense global population density. While QS contains information about the population, the response to a QS signal is an intracellular process that can be considered "private" to the cell. In fact, recent studies have shown that response to QS can give rise to an advantageous metabolic response in individual cells (Dandekar, Chugani, and Greenberg 2012; Vivant et al. 2014; Schuster et al.

2003; Zhao et al. 2014). Therefore, our findings together with results published by other groups suggest a possible private benefit for ECM production. In particular, our data revealed that ECM expressing cells might perceive changes in global cell density earlier and respond accordingly. Earlier detection of an environmental change such as crowding can be beneficial by speeding up stress responses that, for example, induce changes in metabolic activity. Furthermore, ECM deficient cells exhibit a lower response not only to signals produced by distant cells (paracrine), but also self-made signals (autocrine). Therefore, ECM expression appears to promote both paracrine and autocrine mediated QS signaling. Balancing the cost of public good production with a private benefit would allow ECM producing cells to be more easily sustained within the biofilm and also reduce the ability of cheater cells to dominate the community.

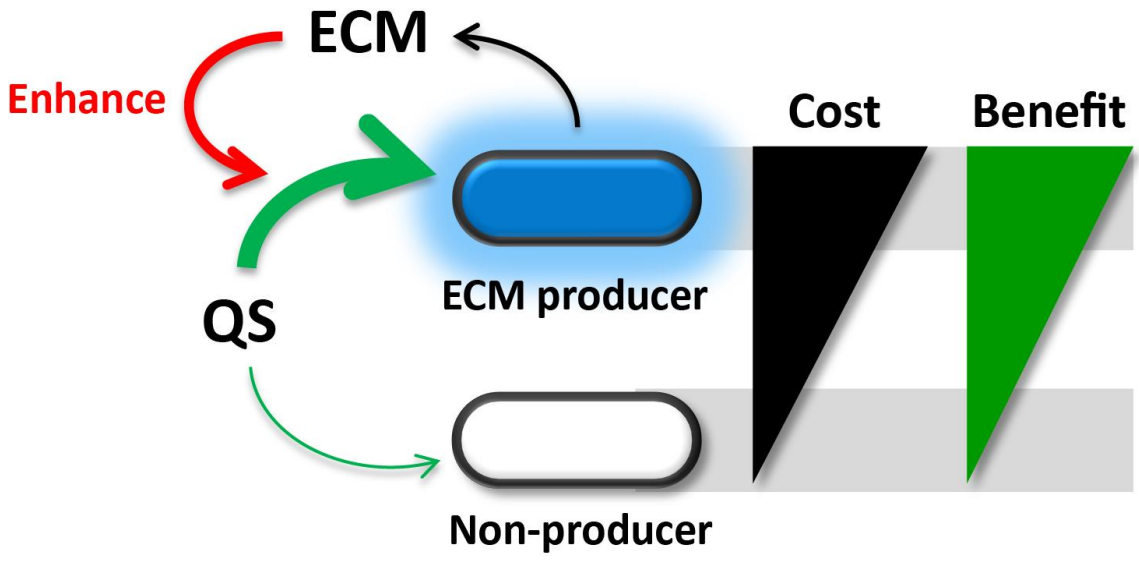

Figure 25. Model of potential private benefit of the enhanced QS response.
ECM production can exclusively benefit producer cells by enhancing their response to QS, thereby compensating for the cost of ECM production.

The enhanced QS response, however, may not provide a private benefit in all conditions. For example, cells can respond to some QS signals by producing and secreting public goods, such as virulence factors and degradative enzymes (Rutherford and Bassler 2012; Comella and Grossman 2005). If the ECM elevates the response to such QS signals, the response could place an additional burden on ECM producing cells. Furthermore, we note that the relationship between the ECM and QS described here may vary depending on the chemical characteristics and composition of the ECM and QS molecules. As suggested by a previous study (Seviour et al. 2015), hydrophobicity can for example determine the affinity between the ECM and QS signals. If a QS molecule is hydrophilic, rather than attracting it, ECM may even prevent the QS from approaching cells. Therefore, the chemical nature of QS molecules and/or the composition of the ECM may provide bacterial cells with an additional means to tune their QS response.

Chapter 4: Conclusion and future directions

In this work, we studied how ECM affects quorum sensing in biofilms. Since the native QS regulates ECM production in *B. subtilis*, ECM and native QS are tightly coupled and cannot be perturbed independently. This dependency causes difficulty in revealing the potential effect of ECM on QS. Therefore, we constructed an orthogonal (inert) synthetic QS system in Chapter 2 by transplanting the Auto-Inducing-Peptide (AIP) based QS system (*agr* system) from *Staphylococcus epidermidis* into *B. subtilis* to study how the response to QS signals may be affected by ECM production. In Chapter 2, we constructed the synthetic *agr* system in the biofilm forming *B. subtilis* NCIB 3610 strain. We verified the modularity and functionality of the constructed synthetic system in dedicated "Sender" and "Receiver" cells by demonstrating that: (1) The Receiver cells, which contain the AgrC receptor and downstream AgrA transcription factor that regulates expression of the P*3* promoter, can respond to chemically synthesized AIP by expression YFP fused to the P*3* promoter. (2) The Sender cells containing the entire *agr* operon (*agrA,B,C* and *D*) can synthesize and secrete functional AIP. (3) Integration of the synthetic system does not interfere with the normal biofilm development process in *B. subtilis*, suggesting that the synthetic QS systems should have no effect on ECM production. In Chapter 3, we tested the effect of ECM on the synthetic QS response in biofilms and found that ECM enhances QS response. ECM deficient Receiver strains have lower QS response compared to the WT Receiver strain in biofilms. This enhanced QS response in ECM producing cells is a private property at the single cell level that does not extend to even adjacent cells in biofilms. We further

demonstrated that the ECM production dependent QS response also affects single cells in liquid cultures. ECM concentrates AIP in the vicinity of cells. This may increase the likelihood of binding between AIP and receptors on the membrane. In addition to the reduced QS response observed in ECM gene deletion strains, response to the QS signal is also affected by the natural variation in ECM expression that is inherent to *B. subtilis*. Moreover, ECM elevates response to native *B. subtilis* QS signals as well, indicating that results obtained with our synthetic QS system may be generally applicable to native QS systems in *B. subtilis*.

Our results show that ECM producing cells have an elevated response to QS signals. But the hypothesis that the enhanced QS response provides a private benefit for ECM producing cells remains to be tested. In the future, we can test this hypothesis by investigating genes regulated by native *B. subtilis* QS signals, especially those genes related to production of intracellular enzymes that may provide private benefits (such as metabolic advantage) to the producer cells. We can study whether this private benefit helps constrain ECM production cheaters in biofilm.

The development of a functional synthetic QS system in undomesticated biofilm communities may help address various questions regarding biofilm development. ECM does not distribute evenly in biofilm. Since ECM physically affects QS molecules, heterogeneous distribution of ECM may leads to heterogeneous distribution of QS molecules. Furthermore, QS molecules with different chemical characteristics may interact differently with the ECM. Therefore, uneven ECM distribution may lead to a more complicated spatial distribution pattern of different QS molecules. The combination of different QS molecules can locally

determine cell fate. Thus, interactions between ECM and QS may help uncover the principles that govern the spatial organization of cell types within biofilms.

In addition, the *B. subtilis* synthetic QS system we constructed may be used in industry. *B. subtilis* is a species of high industrial relevance. Synthetic QS systems also have a lot of potential application in industry. Introduction of a synthetic QS system into *B. subtilis* provides a useful tool for controlled industrial fermentation. In the future, we could express proteins of commercial values under the control of P*3*. We could consider optimizing the synthetic QS system to increase the yield of its downstream products. As our system is modulated and easy to manipulate, we can redesign the topology of the synthetic system to fulfill various needs in bioprocessing.

Appendix A: Supplementary figure

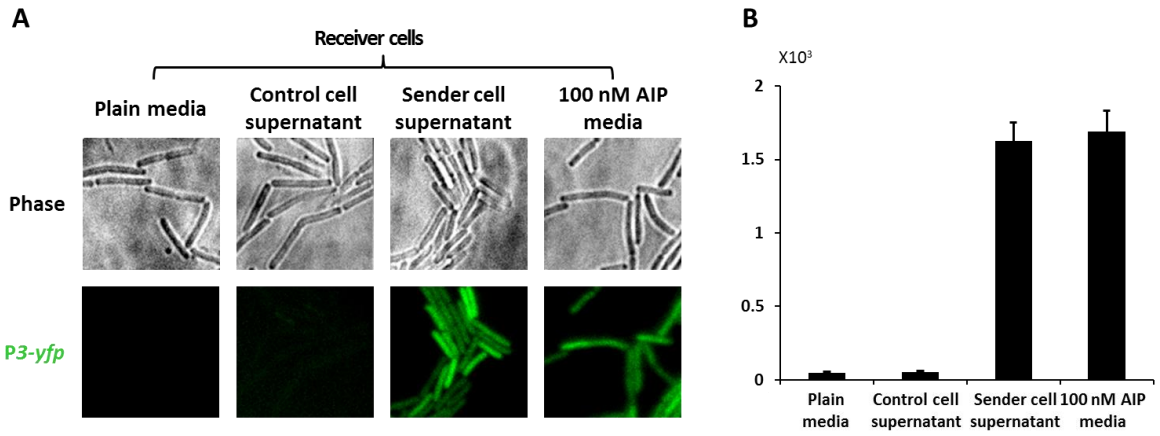

Figure 26. Sender cells secrete functional AIP.
(A) Images of Receiver cells grown on MSgg pad supplemented with 50% plain MSgg media, conditioned media from control cells (no *agr* system) or Sender cells, or MSgg media with 100 nM AIP. (**B**) Mean P*3-yfp* fluorescence intensity in Receiver cells grown with different media (mean ± SEM, n=29 cells).

Reference

Abdelnour, A., S. Arvidson, T. Bremell, C. Ryden, and A. Tarkowski. 1993. "The Accessory Gene Regulator (agr) Controls Staphylococcus Aureus Virulence in a Murine Arthritis Model." *Infection and Immunity* 61 (9): 3879–3885. doi:10.1016/j.bjid.2014.03.005.

Absalon, Cedric, Patrick Ymele-leki, and Paula I Watnick. 2012. "Platform for Protein Delivery The Bacterial Biofilm Matrix as a Platform for Protein Delivery MATRIX" 3 (4): 1–5. doi:10.1128/mBio.00127-12.Updated.

Allesen-Holm, Marie, Kim Bundvig Barken, Liang Yang, Mikkel Klausen, Jeremy S. Webb, Staffan Kjelleberg, Søren Molin, Michael Givskov, and Tim Tolker-Nielsen. 2006. "A Characterization of DNA Release in Pseudomonas Aeruginosa Cultures and Biofilms." *Molecular Microbiology* 59 (4): 1114–1128. doi:10.1111/j.1365-2958.2005.05008.x.

Alpkvista, Erik, and Isaac Klapper. 2007. "A Multidimensional Multispecies Continuum Model for Heterogeneous Biofilm Development." *Bulletin of Mathematical Biology* 69 (2) (February 22): 765–789. doi:10.1007/s11538-006-9168-7. http://link.springer.com/10.1007/s11538-006-9168-7.

Angst, EC. 1923. "The Fouling of Ships Bottoms by Bacteria." *Report, Bureau Construction and Repair. United States Navy Department, Washington, DC*.

Asally, Munehiro, Mark Kittisopikul, Pau Rué, Yingjie Du, Zhenxing Hu, Tolga Çağatay, Andra B Robinson, Hongbing Lu, Jordi Garcia-Ojalvo, and Gürol M Süel. 2012. "Localized Cell Death Focuses Mechanical Forces during 3D Patterning in a Biofilm." *Proceedings of the National Academy of Sciences of the United States of America* 109 (46) (November 13): 18891–6. doi:10.1073/pnas.1212429109. http://www.pubmedcentral.nih.gov/articlerender.fcgi?artid=3503208&tool=pmcentrez&rendertype=abstract.

Auchtung, Jennifer M, Catherine a Lee, and Alan D Grossman. 2006. "Modulation of the ComA-Dependent Quorum Response in Bacillus Subtilis by Multiple Rap Proteins and Phr Peptides." *Journal of Bacteriology* 188 (14) (July): 5273–85. doi:10.1128/JB.00300-06. http://www.pubmedcentral.nih.gov/articlerender.fcgi?artid=1539962&tool=pmcentrez&rendertype=abstract.

Ausmees, Nora, Hans Jonsson, Stefan Höglund, Hans Ljunggren, and Martin Lindberg. 1999. "Structural and Putative Regulatory Genes Involved in Cellulose Synthesis in Rhizobium Leguminosarum Bv. Trifolii." *Microbiology* 145 (5): 1253–1262. doi:10.1099/13500872-145-5-1253.

Bai, U., I. Mandic-Mulec, and I. Smith. 1993. “SinI Modulates the Activity of SinR, a Developmental Switch Protein of Bacillus Subtilis, by Protein-Protein Interaction.” *Genes and Development* 7 (1): 139–148. doi:10.1101/gad.7.1.139.

Banin, Ehud, Michael L Vasil, and E Peter Greenberg. 2005. “Iron and Pseudomonas Aeruginosa Biofilm Formation.” *Proceedings of the National Academy of Sciences of the United States of America* 102 (31): 11076–11081. doi:10.1073/pnas.0504266102.

Bassler, Bonnie L. 1999. “How Bacteria Talk to Each Other: Regulation of Gene Expression by Quorum Sensing.” *Current Opinion in Microbiology*. doi:10.1016/S1369-5274(99)00025-9.

Beyhan, Sinem, Anna D. Tischler, Andrew Camilli, and Fitnat H. Yildiz. 2006. “Transcriptome and Phenotypic Responses of Vibrio Cholerae to Increased Cyclic Di-GMP Level.” *Journal of Bacteriology* 188 (10): 3600–3613. doi:10.1128/JB.188.10.3600-3613.2006.

Blair, Kris M, Linda Turner, Jared T Winkelman, Howard C Berg, and Daniel B Kearns. 2008. “A Molecular Clutch Disables Flagella in the Bacillus Subtilis Biofilm.” *Science (New York, N.Y.)* 320 (5883) (June 20): 1636–8. doi:10.1126/science.1157877. http://www.ncbi.nlm.nih.gov/pubmed/18566286.

Blevins, Steve M., and Michael S. Bronze. 2010. “Robert Koch and the ‘Golden Age’ of Bacteriology.” *International Journal of Infectious Diseases*. doi:10.1016/j.ijid.2009.12.003.

Böckelmann, Uta, Andrea Janke, Ramona Kuhn, Thomas R Neu, Jörg Wecke, John R Lawrence, and Ulrich Szewzyk. 2006. “Bacterial Extracellular DNA Forming a Defined Network-like Structure.” *FEMS Microbiology Letters* 262 (1) (September): 31–8. doi:10.1111/j.1574-6968.2006.00361.x. http://www.ncbi.nlm.nih.gov/pubmed/16907736.

Boles, Blaise R, and Alexander R Horswill. 2008. “Agr-Mediated Dispersal of Staphylococcus Aureus Biofilms.” *PLoS Pathogens* 4 (4) (April): e1000052. doi:10.1371/journal.ppat.1000052. http://www.pubmedcentral.nih.gov/articlerender.fcgi?artid=2329812&tool=pmcentrez&rendertype=abstract.

Borenstein, David Bruce, Yigal Meir, Joshua W Shaevitz, and Ned S Wingreen. 2013. “Non-Local Interaction via Diffusible Resource Prevents Coexistence of Cooperators and Cheaters in a Lattice Model.” *PloS One* 8 (5) (January): e63304. doi:10.1371/journal.pone.0063304. http://www.pubmedcentral.nih.gov/articlerender.fcgi?artid=3656920&tool=pmcentrez&rendertype=abstract.

Boyd, Chelsea D., and George a. O'Toole. 2012. "Second Messenger Regulation of Biofilm Formation: Breakthroughs in Understanding c-Di-GMP Effector Systems." *Annual Review of Cell and Developmental Biology* 28 (1): 439–462. doi:10.1146/annurev-cellbio-101011-155705.

Branda, S S, F Chu, D B Kearns, R Losick, and R Kolter. 2006. "A Major Protein Component of the Bacillus Subtilis Biofilm Matrix." *Molecular Microbiology* 59 (4): 1229–1238. doi:10.1111/j.1365-2958.2005.05020.x. http://www.ncbi.nlm.nih.gov/pubmed/16430696.

Branda, S S, J E Gonzalez-Pastor, S Ben-Yehuda, R Losick, R Kolter, and J E Gonz áez-Pastor. 2001. "Fruiting Body Formation by Bacillus Subtilis." *Proceedings of the National Academy of Sciences of the United States of America* 98 (20) (September 25): 11621–6. doi:10.1073/pnas.191384198. http://www.pubmedcentral.nih.gov/articlerender.fcgi?artid=58779&tool=pmcentrez&rendertype=abstract.

Branda, S S, S Vik, L Friedman, and R Kolter. 2005. "Biofilms: The Matrix Revisited." *Trends in Microbiology* 13 (1): 20–26. doi:10.1016/j.tim.2004.11.006. http://www.ncbi.nlm.nih.gov/pubmed/15639628.

Branda, Steven S, Jos éEduardo Gonz áez-Pastor, Etienne Dervyn, S Dusko Ehrlich, Richard Losick, and Roberto Kolter. 2004. "Genes Involved in Formation of Structured Multicellular Communities by Bacillus Subtilis." *Journal of Bacteriology* 186 (12) (June): 3970–9. doi:10.1128/JB.186.12.3970-3979.2004. http://www.pubmedcentral.nih.gov/articlerender.fcgi?artid=419949&tool=pmcentrez&rendertype=abstract.

Brockhurst, Michael a, Angus Buckling, and Andy Gardner. 2007. "Cooperation Peaks at Intermediate Disturbance." *Current Biology : CB* 17 (9) (May 1): 761–5. doi:10.1016/j.cub.2007.02.057. http://www.ncbi.nlm.nih.gov/pubmed/17379522.

Bryers, James D. 2008. "Medical Biofilms." *Biotechnology and Bioengineering* 100 (1) (May 1): 1–18. doi:10.1002/bit.21838. http://www.pubmedcentral.nih.gov/articlerender.fcgi?artid=2706312&tool=pmcentrez&rendertype=abstract.

Burmølle, Mette, Annelise Kjøller, and Søren J. Sørensen. 2011. "Biofilms in Soil." In , 70–75. doi:10.1007/978-90-481-3585-1_260. http://link.springer.com/10.1007/978-90-481-3585-1_260.

Camper, Anne K, Mark W Lechevallier, Susan C Broadaway, and Gordon A Mcfeters. 1985. "Growth and Persistence of Pathogens Carbon Filters Granular Activated:" 1378–1382.

Chai, Liraz, Diego Romero, Can Kayatekin, Barak Akabayov, Hera Vlamakis, Richard Losick, and Roberto Kolter. 2013. “Isolation, Characterization and Aggregation of a Structured Bacterial Matrix Precursor.” *The Journal of Biological Chemistry* (1) (April 30): 1–21. doi:10.1074/jbc.M113.453605. http://www.ncbi.nlm.nih.gov/pubmed/23632024.

Chai, Y, F Chu, R Kolter, and R Losick. 2008. “Bistability and Biofilm Formation in Bacillus Subtilis.” *Molecular Microbiology* 67 (2): 254–263. doi:10.1111/j.1365-2958.2007.06040.x. http://www.ncbi.nlm.nih.gov/pubmed/18047568.

Chai, Yunrong, Pascale B Beauregard, Hera Vlamakis, Richard Losick, and Roberto Kolter. 2012. “Galactose Metabolism Plays a Crucial Role in Biofilm Formation by Bacillus Subtilis.” *mBio* 3 (4) (January): e00184–12. doi:10.1128/mBio.00184-12. http://www.pubmedcentral.nih.gov/articlerender.fcgi?artid=3419520&tool=pmcentrez&rendertype=abstract.

Chai, Yunrong, Roberto Kolter, and Richard Losick. 2009. “Paralogous Antirepressors Acting on the Master Regulator for Biofilm Formation in Bacillus Subtilis.” *Molecular Microbiology* 74 (4): 876–887. doi:10.1111/j.1365-2958.2009.06900.x.

Chai, Yunrong, Thomas Norman, Roberto Kolter, and Richard Losick. 2010. “An Epigenetic Switch Governing Daughter Cell Separation in Bacillus Subtilis.” *Genes and Development* 24 (8): 754–765. doi:10.1101/gad.1915010.

Charlton, T S, R de Nys, a Netting, N Kumar, M Hentzer, M Givskov, and S Kjelleberg. 2000. “A Novel and Sensitive Method for the Quantification of N-3-Oxoacyl Homoserine Lactones Using Gas Chromatography-Mass Spectrometry: Application to a Model Bacterial Biofilm.” *Environmental Microbiology* 2 (5) (October): 530–41. http://www.ncbi.nlm.nih.gov/pubmed/11233161.

Chu, F, D B Kearns, S S Branda, R Kolter, and R Losick. 2006. “Targets of the Master Regulator of Biofilm Formation in Bacillus Subtilis.” *Molecular Microbiology* 59 (4): 1216–1228. doi:10.1111/j.1365-2958.2005.05019.x. http://www.ncbi.nlm.nih.gov/pubmed/16430695.

Chu, F, D B Kearns, A McLoon, Y Chai, R Kolter, and R Losick. 2008. “A Novel Regulatory Protein Governing Biofilm Formation in Bacillus Subtilis.” *Molecular Microbiology* 68 (5): 1117–1127. doi:10.1111/j.1365-2958.2008.06201.x. http://www.ncbi.nlm.nih.gov/pubmed/18430133.

Cochran, W L, S J Suh, G A McFeters, and P S Stewart. 2000. “Role of RpoS and AlgT in Pseudomonas Aeruginosa Biofilm Resistance to Hydrogen Peroxide and Monochloramine.” *Journal of Applied Microbiology* 88 (3) (March): 546–53. http://www.ncbi.nlm.nih.gov/pubmed/10747236.

Cole, Sheri P., Julia Harwood, Richard Lee, Rosemary She, and Donald G. Guiney. 2004. "Characterization of Monospecies Biofilm Formation by Helicobacter Pylori." *Journal of Bacteriology* 186 (10): 3124–3132. doi:10.1128/JB.186.10.3124-3132.2004.

Comella, Natalia, and Alan D Grossman. 2005. "Conservation of Genes and Processes Controlled by the Quorum Response in Bacteria: Characterization of Genes Controlled by the Quorum-Sensing Transcription Factor ComA in Bacillus Subtilis." *Molecular Microbiology* 57 (4) (August): 1159–74. doi:10.1111/j.1365-2958.2005.04749.x. http://www.ncbi.nlm.nih.gov/pubmed/16091051.

Cvitkovitch, D G. 2001. "Genetic Competence and Transformation in Oral Streptococci." *Critical Reviews in Oral Biology and Medicine : An Official Publication of the American Association of Oral Biologists* 12 (3): 217–243. doi:10.1177/10454411010120030201.

D'Argenio, David A., M. Worth Calfee, Paul B. Rainey, and Everett C. Pesci. 2002. "Autolysis and Autoaggregation in Pseudomonas Aeruginosa Colony Morphology Mutants." *Journal of Bacteriology* 184 (23): 6481–6489. doi:10.1128/JB.184.23.6481-6489.2002.

D'Argenio, David a., and Samuel I. Miller. 2004. "Cyclic Di-GMP as a Bacterial Second Messenger." *Microbiology* 150 (8): 2497–2502. doi:10.1099/mic.0.27099-0.

Dandekar, Ajai a, Sudha Chugani, and E Peter Greenberg. 2012. "Bacterial Quorum Sensing and Metabolic Incentives to Cooperate." *Science (New York, N.Y.)* 338 (6104) (October 12): 264–6. doi:10.1126/science.1227289. http://www.pubmedcentral.nih.gov/articlerender.fcgi?artid=3587168&tool=pmcentrez&rendertype=abstract.

Danese, P. N., L. a. Pratt, and R. Kolter. 2000. "Exopolysaccharide Production Is Required for Development of Escherichia Coli K-12 Biofilm Architecture." *Journal of Bacteriology* 182 (12) (June 15): 3593–3596. doi:10.1128/JB.182.12.3593-3596.2000. http://jb.asm.org/cgi/doi/10.1128/JB.182.12.3593-3596.2000.

Das, Theerthankar, Prashant K. Sharma, Henk J. Busscher, Henny C. Van Der Mei, and Bastiaan P. Krom. 2010. "Role of Extracellular DNA in Initial Bacterial Adhesion and Surface Aggregation." *Applied and Environmental Microbiology* 76 (10): 3405–3408. doi:10.1128/AEM.03119-09.

De Jong, Wouter, Han a B Wösten, Lubbert Dijkhuizen, and Dennis Claessen. 2009. "Attachment of Streptomyces Coelicolor Is Mediated by Amyloidal Fimbriae That Are Anchored to the Cell Surface via Cellulose." *Molecular Microbiology* 73 (6) (September): 1128–40. doi:10.1111/j.1365-2958.2009.06838.x. http://www.ncbi.nlm.nih.gov/pubmed/19682261.

Desai, Mahesh S, Karoline Assig, and Sharmishtha Dattagupta. 2013. “Nitrogen Fixation in Distinct Microbial Niches within a Chemoautotrophy-Driven Cave Ecosystem.” *The ISME Journal* 7 (12) (December): 2411–2423. doi:10.1038/ismej.2013.126. http://www.nature.com/doifinder/10.1038/ismej.2013.126.

Dobell, Clifford. 1932. *Antony Van Leeuwenhoek and His “Little Animals.”*

Dobinsky, Sabine, Kathrin Kiel, Holger Rohde, Katrin Bartscht, J. K M Knobloch, Matthias A. Horstkotte, and Dietrich Mack. 2003. “Glucose-Related Dissociation between icaADBC Transcription and Biofilm Expression by Staphylococcus Epidermidis: Evidence for an Additional Factor Required for Polysaccharide Intercellular Adhesin Synthesis.” *Journal of Bacteriology* 185 (9): 2879–2886. doi:10.1128/JB.185.9.2879-2886.2003.

Donlan, R M. 2008. “Biofilms on Central Venous Catheters: Is Eradication Possible?” *Current Topics in Microbiology and Immunology* 322 (January): 133–61. http://www.ncbi.nlm.nih.gov/pubmed/18453275.

Donlan, R. M., and J. W. Costerton. 2002. “Biofilms: Survival Mechanisms of Clinically Relevant Microorganisms.” *Clinical Microbiology Reviews* 15 (2) (April 1): 167–193. doi:10.1128/CMR.15.2.167-193.2002. http://cmr.asm.org/cgi/doi/10.1128/CMR.15.2.167-193.2002.

Drescher, Knut, Carey D Nadell, Howard a Stone, Ned S Wingreen, and Bonnie L Bassler. 2014. “Solutions to the Public Goods Dilemma in Bacterial Biofilms.” *Current Biology : CB* 24 (1) (January 6): 50–5. doi:10.1016/j.cub.2013.10.030. http://www.pubmedcentral.nih.gov/articlerender.fcgi?artid=3935403&tool=pmcentrez&rendertype=abstract.

Duan, Kangmin, Carol Dammel, Jeffrey Stein, Harvey Rabin, and Michael G. Surette. 2003. “Modulation of Pseudomonas Aeruginosa Gene Expression by Host Microflora through Interspecies Communication.” *Molecular Microbiology* 50 (5): 1477–1491. doi:10.1046/j.1365-2958.2003.03803.x.

Dubey, Gyanendra P, and Sigal Ben-Yehuda. 2011. “Intercellular Nanotubes Mediate Bacterial Communication.” *Cell* 144 (4) (February 18): 590–600. doi:10.1016/j.cell.2011.01.015. http://www.ncbi.nlm.nih.gov/pubmed/21335240.

Eberhard, A, A L Burlingame, C Eberhard, G L Kenyon, K H Nealson, and N J Oppenheimer. 1981. “Structural Identification of Autoinducer of Photobacterium Fischeri Luciferase.” *Biochemistry* 20 (9): 2444–2449. doi:10.1021/bi00512a013.

Eberhard, a. 1972. “Inhibition and Activation of Bacterial Luciferase Synthesis.” *Journal of Bacteriology* 109 (3): 1101–1105.

Eighmy, T Taylor, Denise Maratea, and Paul L Bishop. 1983. “Electron Microscopic Examination” 45 (6): 1921–1931.

Engebrecht, J, K Nealson, and M Silverman. 1983. “Bacterial Bioluminescence: Isolation and Genetic Analysis of Functions from Vibrio Fischeri.” *Cell* 32 (3): 773–781. doi:10.1016/0092-8674(83)90063-6.

Engebrecht, J, and M Silverman. 1984. “Identification of Genes and Gene Products Necessary for Bacterial Bioluminescence.” *Proceedings of the National Academy of Sciences of the United States of America* 81 (13): 4154–4158. doi:10.1073/pnas.81.13.4154.

Epstein, Alexander K, Boaz Pokroy, Agnese Seminara, and Joanna Aizenberg. 2011. “Bacterial Biofilm Shows Persistent Resistance to Liquid Wetting and Gas Penetration.” *Proceedings of the National Academy of Sciences of the United States of America* 108 (3) (January 18): 995–1000. doi:10.1073/pnas.1011033108. http://www.pubmedcentral.nih.gov/articlerender.fcgi?artid=3024672&tool=pmcentrez&rendertype=abstract.

Erable, Benjamin, Narcis M Duţeanu, M M Ghangrekar, Claire Dumas, and Keith Scott. 2010. “Application of Electro-Active Biofilms.” *Biofouling* 26 (1) (January): 57–71. doi:10.1080/08927010903161281. http://www.ncbi.nlm.nih.gov/pubmed/20390557.

Flemming, Hans-Curt, and Jost Wingender. 2010. “The Biofilm Matrix.” *Nature Reviews. Microbiology* 8 (9) (September): 623–33. doi:10.1038/nrmicro2415. http://www.ncbi.nlm.nih.gov/pubmed/20676145.

Fournier, B, and D C Hooper. 2000. “A New Two-Component Regulatory System Involved in Adhesion, Autolysis, and Extracellular Proteolytic Activity of Staphylococcus Aureus.” *Journal of Bacteriology* 182 (14) (July): 3955–64. http://www.pubmedcentral.nih.gov/articlerender.fcgi?artid=94580&tool=pmcentrez&rendertype=abstract.

Freudl, R. 1992. “Protein Secretion in Gram-Positive Bacteria.” *Journal of Biotechnology*. doi:10.1016/0168-1656(92)90072-H.

Fujita, Masaya, Jos éEduardo Gonz ález-pastor, Eduardo Gonza, and Richard Losick. 2005. “High- and Low-Threshold Genes in the Spo0A Regulon of Bacillus Subtilis High- and Low-Threshold Genes in the Spo0A Regulon of Bacillus Subtilis †” 187 (4). doi:10.1128/JB.187.4.1357.

Fuqua, W. C., and S. C. Winans. 1994. “A LuxR-LuxI Type Regulatory System Activates Agrobacterium Ti Plasmid Conjugal Transfer in the Presence of a Plant Tumor Metabolite.” *Journal of Bacteriology* 176 (10): 2796–2806.

Geesey, G. G., W. T. Richardson, H. G. Yeomans, R. T. Irvin, and J. W. Costerton. 1977. "Microscopic Examination of Natural Sessile Bacterial Populations from an Alpine Stream." *Canadian Journal of Microbiology* 23 (12) (December): 1733–6. doi:10.1139/m77-249. http://www.nrcresearchpress.com/doi/abs/10.1139/m77-249.

Geier, Henriette, Serge Mostowy, Gerard A. Cangelosi, Marcel A. Behr, and Timothy E. Ford. 2008. "Autoinducer-2 Triggers the Oxidative Stress Response in Mycobacterium Avium, Leading to Biofilm Formation." *Applied and Environmental Microbiology* 74 (6): 1798–1804. doi:10.1128/AEM.02066-07.

Geszvain, Kati, and Karen L Visick. 2006. "Roles of Bacterial Regulators in the Symbiosis between Vibrio Fischeri and Euprymna Scolopes." *Progress in Molecular and Subcellular Biology* 41: 277–290.

González Barrios, Andrés F., Rongjun Zuo, Yoshifumi Hashimoto, Li Yang, William E. Bentley, and Thomas K. Wood. 2006. "Autoinducer 2 Controls Biofilm Formation in Escherichia Coli through a Novel Motility Quorum-Sensing Regulator (MqsR, B3022)." *Journal of Bacteriology* 188 (1): 305–316. doi:10.1128/JB.188.1.305-316.2006.

Goodman, Andrew L., Bridget Kulasekara, Arne Rietsch, Dana Boyd, Roger S. Smith, and Stephen Lory. 2004. "A Signaling Network Reciprocally Regulates Genes Associated with Acute Infection and Chronic Persistence in Pseudomonas Aeruginosa." *Developmental Cell* 7 (5): 745–754. doi:10.1016/j.devcel.2004.08.020.

Grandvalet, C., M. Gominet, and D. Lereclus. 2001. "Identification of Genes Involved in the Activation of the Bacillus Thuringiensis inhA Metalloprotease Gene at the Onset of Sporulation." *Microbiology* 147 (7): 1805–1813.

Gristina, A G, and J W Costerton. 1985. "Bacterial Adherence to Biomaterials and Tissue. The Significance of Its Role in Clinical Sepsis." *The Journal of Bone and Joint Surgery. American Volume* 67 (2) (February): 264–73. http://www.ncbi.nlm.nih.gov/pubmed/3881449.

Grossman, A D. 1991. "Integration of Developmental Signals and the Initiation of Sporulation in B. Subtilis." *Cell* 65 (1): 5–8. doi:10.1016/0092-8674(91)90353-Z.

Guttenplan, Sarah B., Kris M. Blair, and Daniel B. Kearns. 2010. "The EpsE Flagellar Clutch Is Bifunctional and Synergizes with EPS Biosynthesis to Promote Bacillus Subtilisbiofilm Formation." *PLoS Genetics* 6 (12): 1–12. doi:10.1371/journal.pgen.1001243.

Hall-Stoodley, Luanne, J William Costerton, and Paul Stoodley. 2004. "Bacterial Biofilms: From the Natural Environment to Infectious Diseases." *Nature Reviews. Microbiology* 2

(2) (February): 95–108. doi:10.1038/nrmicro821. http://www.ncbi.nlm.nih.gov/pubmed/15040259.

Hall-Stoodley, Luanne, and Paul Stoodley. 2009. “Evolving Concepts in Biofilm Infections.” *Cellular Microbiology* 11 (7) (July): 1034–43. doi:10.1111/j.1462-5822.2009.01323.x. http://www.ncbi.nlm.nih.gov/pubmed/19374653.

Hammer, Brian K, and Bonnie L Bassler. 2009. “Distinct Sensory Pathways in Vibrio Cholerae El Tor and Classical Biotypes Modulate Cyclic Dimeric GMP Levels to Control Biofilm Formation.” *Journal of Bacteriology* 191 (1) (January): 169–77. doi:10.1128/JB.01307-08. http://www.pubmedcentral.nih.gov/articlerender.fcgi?artid=2612459&tool=pmcentrez&rendertype=abstract.

Hammer, Brian K., and Bonnie L. Bassler. 2003. “Quorum Sensing Controls Biofilm Formation in Vibrio Cholerae.” *Molecular Microbiology* 50 (1) (August 20): 101–104. doi:10.1046/j.1365-2958.2003.03688.x. http://doi.wiley.com/10.1046/j.1365-2958.2003.03688.x.

Hamon, M A, and B a Lazazzera. 2001. “The Sporulation Transcription Factor Spo0A Is Required for Biofilm Development in Bacillus Subtilis.” *Molecular Microbiology* 42 (5) (December): 1199–1209. http://www.ncbi.nlm.nih.gov/pubmed/11886552.

Hanzelka, B. L., and E. P. Greenberg. 1995. “Evidence That the N-Terminal Region of the Vibrio Fischeri luxR Protein Constitutes an Autoinducer-Binding Domain.” *Journal of Bacteriology*.

Hatt, J K, and P N Rather. 2008. “Role of Bacterial Biofilms in Urinary Tract Infections.” *Current Topics in Microbiology and Immunology* 322 (January): 163–92. http://www.ncbi.nlm.nih.gov/pubmed/18453276.

Henrici, A T. 1933. “Studies of Freshwater Bacteria: I. A Direct Microscopic Technique.” *Journal of Bacteriology* 25 (3) (March): 277–87. http://www.pubmedcentral.nih.gov/articlerender.fcgi?artid=533461&tool=pmcentrez&rendertype=abstract.

Hense, Burkhard a, Christina Kuttler, Johannes Müller, Michael Rothballer, Anton Hartmann, and Jan-Ulrich Kreft. 2007. “Does Efficiency Sensing Unify Diffusion and Quorum Sensing?” *Nature Reviews. Microbiology* 5 (3) (March): 230–9. doi:10.1038/nrmicro1600. http://www.ncbi.nlm.nih.gov/pubmed/17304251.

Hibbing, Michael E, Clay Fuqua, Matthew R Parsek, and S Brook Peterson. 2010. “Bacterial Competition: Surviving and Thriving in the Microbial Jungle.” *Nature Reviews. Microbiology* 8 (1) (January): 15–25. doi:10.1038/nrmicro2259.

Hickman, Jason W, Delia F Tifrea, and Caroline S Harwood. 2005. “A Chemosensory System That Regulates Biofilm Formation through Modulation of Cyclic Diguanylate Levels.” *Proceedings of the National Academy of Sciences of the United States of America* 102 (40): 14422–14427. doi:10.1073/pnas.0507170102.

Hirochika, H., Y. Kobayashi, F. Kawamura, and H. Saito. 1981. “Cloning of Sporulation Gene spoOB of Bacillus Subtilis and Its Genetic and Biochemical Analysis.” *Journal of Bacteriology* 146 (2): 494–505.

Hoffman, Lucas R, David A D’Argenio, Michael J MacCoss, Zhaoying Zhang, Roger A Jones, and Samuel I Miller. 2005. “Aminoglycoside Antibiotics Induce Bacterial Biofilm Formation.” *Nature* 436 (7054): 1171–1175. doi:10.1038/nature03912.

Hojo, K., S. Nagaoka, T. Ohshima, and N. Maeda. 2009. “Bacterial Interactions in Dental Biofilm Development.” *Journal of Dental Research* 88 (11) (November 1): 982–990. doi:10.1177/0022034509346811. http://jdr.sagepub.com/cgi/doi/10.1177/0022034509346811.

Holm, Eric R, Michael P Schultz, Elizabeth G Haslbeck, Walter J Talbott, and Andrew J Field. 2004. “Evaluation of Hydrodynamic Drag on Experimental Fouling-Release Surfaces, Using Rotating Disks.” *Biofouling* 20 (4-5): 219–226. doi:10.1080/08927010400011245.

Hong, Seok Hoon, Manjunath Hegde, Jeongyun Kim, Xiaoxue Wang, Arul Jayaraman, and Thomas K Wood. 2012. “Synthetic Quorum-Sensing Circuit to Control Consortial Biofilm Formation and Dispersal in a Microfluidic Device.” *Nature Communications* 3 (January): 613. doi:10.1038/ncomms1616. http://www.pubmedcentral.nih.gov/articlerender.fcgi?artid=3272573&tool=pmcentrez&rendertype=abstract.

Houot, Laetitia, and Paula I. Watnick. 2008. “A Novel Role for Enzyme I of the Vibrio Cholerae Phosphoenolpyruvate Phosphotransferase System in Regulation of Growth in a Biofilm.” *Journal of Bacteriology* 190 (1): 311–320. doi:10.1128/JB.01410-07.

Hsieh, K E Ming, Leonard W Lion, and Michael L Shuler. 1985. “Bioreactor for the Study of Defined Interactions of Toxic Metals and Biofilms” 50 (5): 1155–1161.

Irnov, Irnov, and Wade C Winkler. 2010. “A Regulatory RNA Required for Antitermination of Biofilm and Capsular Polysaccharide Operons in Bacillales.” *Molecular Microbiology* 76 (3) (May): 559–75. doi:10.1111/j.1365-2958.2010.07131.x. http://www.ncbi.nlm.nih.gov/pubmed/20374491.

Izano, Era a, Matthew a Amarante, William B Kher, and Jeffrey B Kaplan. 2008. “Differential Roles of Poly-N-Acetylglucosamine Surface Polysaccharide and

Extracellular DNA in Staphylococcus Aureus and Staphylococcus Epidermidis Biofilms." *Applied and Environmental Microbiology* 74 (2) (January): 470–6. doi:10.1128/AEM.02073-07. http://www.pubmedcentral.nih.gov/articlerender.fcgi?artid=2223269&tool=pmcentrez&rendertype=abstract.

Jackson, Debra W., Jerry W. Simecka, and Tony Romeo. 2002. "Catabolite Repression of Escherichia Coli Biofilm Formation." *Journal of Bacteriology* 184 (12): 3406–3410. doi:10.1128/JB.184.12.3406-3410.2002.

Jarmer, Hanne, Randy Berka, Steen Knudsen, and Hans H Saxild. 2002. "Transcriptome Analysis Documents Induced Competence of Bacillus Subtilis during Nitrogen Limiting Conditions." *FEMS Microbiology Letters* 206 (2) (January 10): 197–200. doi:10.1111/j.1574-6968.2002.tb11009.x. http://www.ncbi.nlm.nih.gov/pubmed/11814663.

Jester, Brian C, Jeffrey D Levengood, Herv éRoy, Michael Ibba, and Kevin M Devine. 2003. "Nonorthologous Replacement of Lysyl-tRNA Synthetase Prevents Addition of Lysine Analogues to the Genetic Code." *Proceedings of the National Academy of Sciences of the United States of America* 100 (24) (November 25): 14351–6. doi:10.1073/pnas.2036253100. http://www.pubmedcentral.nih.gov/articlerender.fcgi?artid=283595&tool=pmcentrez&rendertype=abstract.

Jiang, M, W Shao, M Perego, and J A Hoch. 2000. "Multiple Histidine Kinases Regulate Entry into Stationary Phase and Sporulation in Bacillus Subtilis." *Molecular Microbiology* 38 (3) (November): 535–542. http://www.ncbi.nlm.nih.gov/pubmed/11069677.

Johnson, Lori, Shawn R Horsman, Laetitia Charron-Mazenod, Amy L Turnbull, Heidi Mulcahy, Michael G Surette, and Shawn Lewenza. 2013. "Extracellular DNA-Induced Antimicrobial Peptide Resistance in Salmonella Enterica Serovar Typhimurium." *BMC Microbiology* 13 (1): 115. doi:10.1186/1471-2180-13-115. http://www.pubmedcentral.nih.gov/articlerender.fcgi?artid=3724500&tool=pmcentrez&rendertype=abstract.

Kaplan, Heidi B, and E P Greenberg. 1985. "Diffusion of Autoinducer Is Involved in Regulation of the Vibrio Fischeri Luminescence System." *Journal of Bacteriology* 163 (3): 1210–1214.

Kaplan, J B. 2010. "Biofilm Dispersal: Mechanisms, Clinical Implications, and Potential Therapeutic Uses." *Journal of Dental Research* 89 (3): 205–218. doi:10.1177/0022034509359403.

Karatan, Ece, and Paula Watnick. 2009. “Signals, Regulatory Networks, and Materials That Build and Break Bacterial Biofilms.” *Microbiology and Molecular Biology Reviews : MMBR* 73 (2) (June): 310–47. doi:10.1128/MMBR.00041-08. http://www.pubmedcentral.nih.gov/articlerender.fcgi?artid=2698413&tool=pmcentrez&rendertype=abstract.

Kavanaugh, Jeffrey S, Matthew Thoendel, and Alexander R Horswill. 2007. “A Role for Type I Signal Peptidase in Staphylococcus Aureus Quorum Sensing.” *Molecular Microbiology* 65 (3) (August): 780–98. doi:10.1111/j.1365-2958.2007.05830.x. http://www.ncbi.nlm.nih.gov/pubmed/17608791.

Kearns, Daniel B, Frances Chu, Steven S Branda, Roberto Kolter, and Richard Losick. 2005. “A Master Regulator for Biofilm Formation by Bacillus Subtilis.” *Molecular Microbiology* 55 (3) (February): 739–49. doi:10.1111/j.1365-2958.2004.04440.x. http://www.ncbi.nlm.nih.gov/pubmed/15661000.

Kempner, E. S., and F. E. Hanson. 1968. “Aspects of Light Production by Photobacterium Fischeri.” *Journal of Bacteriology* 95 (3): 975–979.

Kierek, Katharine, and Paula I. Watnick. 2003. “Environmental Determinants of Vibrio Cholerae Biofilm Development.” *Applied and Environmental Microbiology* 69 (9): 5079–5088. doi:10.1128/AEM.69.9.5079-5088.2003.

Kinner, Nancy E, David L Balkwill, and Paul L Bishop. 1983. “Light and Electron Microscopic Studies of Microorganisms Growing in Rotating Biological Contactor Biofilms” 45 (5): 1659–1669.

Kobayashi, Kazuo. 2008. “SlrR/SlrA Controls the Initiation of Biofilm Formation in Bacillus Subtilis.” *Molecular Microbiology* 69 (6): 1399–1410. doi:10.1111/j.1365-2958.2008.06369.x.

Kobayashi, Kazuo, and Megumi Iwano. 2012. “BslA(YuaB) Forms a Hydrophobic Layer on the Surface of Bacillus Subtilis Biofilms.” *Molecular Microbiology* 85 (1) (July): 51–66. doi:10.1111/j.1365-2958.2012.08094.x. http://www.ncbi.nlm.nih.gov/pubmed/22571672.

Kuchma, Sherry L., Kimberly M. Brothers, Judith H. Merritt, Nicole T. Liberati, Frederick M. Ausubel, and George A. O’Toole. 2007. “BifA, a Cyclic-Di-GMP Phosphodiesterase, Inversely Regulates Biofilm Formation and Swarming Motility by Pseudomonas Aeruginosa PA14.” In *Journal of Bacteriology*, 189:8165–8178. doi:10.1128/JB.00586-07.

Kunst, F, N Ogasawara, I Moszer, A M Albertini, G Alloni, V Azevedo, M G Bertero, et al. 1997. “The Complete Genome Sequence of the Gram-Positive Bacterium Bacillus Subtilis.” *Nature* 390 (6657): 249–256. doi:10.1038/36786.

Lanigan-Gerdes, Sara, Geraldine Briceno, Alek N. Dooley, Kym F. Faull, and Beth A. Lazazzera. 2008. “Identification of Residues Important for Cleavage of the Extracellular Signaling Peptide CSF of Bacillus Subtilis from Its Precursor Protein.” *Journal of Bacteriology* 190 (20): 6668–6675. doi:10.1128/JB.00910-08.

Lanigan-Gerdes, Sara, Alek N Dooley, Kym F Faull, and Beth a Lazazzera. 2007. “Identification of Subtilisin, Epr and Vpr as Enzymes That Produce CSF, an Extracellular Signalling Peptide of Bacillus Subtilis.” *Molecular Microbiology* 65 (5) (September): 1321–33. doi:10.1111/j.1365-2958.2007.05869.x. http://www.ncbi.nlm.nih.gov/pubmed/17666034.

Lazarevic, Vladimir, Blazenka Soldo, Noël Médico, Harold Pooley, Sierd Bron, and Dimitri Karamata. 2005. “Bacillus Subtilis A-Phosphoglucomutase Is Required for Normal Cell Morphology and Biofilm Formation.” *Applied and Environmental Microbiology* 71 (1): 39–45. doi:10.1128/AEM.71.1.39-45.2005.

Lazazzera, B a, I G Kurtser, R S McQuade, and a D Grossman. 1999. “An Autoregulatory Circuit Affecting Peptide Signaling in Bacillus Subtilis.” *Journal of Bacteriology* 181 (17) (September): 5193–200. http://www.pubmedcentral.nih.gov/articlerender.fcgi?artid=94022&tool=pmcentrez&rendertype=abstract.

Lazazzera, B a, J M Solomon, and a D Grossman. 1997. “An Exported Peptide Functions Intracellularly to Contribute to Cell Density Signaling in B. Subtilis.” *Cell* 89 (6) (July 13): 917–25. http://www.ncbi.nlm.nih.gov/pubmed/9200610.

Lee, C. Y., R. B. Szittner, C. M. Miyamoto, and E. A. Meighen. 1993. “The Gene Convergent to luxG in Vibrio Fischeri Codes for a Protein Related in Sequence to RibG and Deoxycytidylate Deaminase.” *Biochimica et Biophysica Acta - Bioenergetics* 1143 (3): 337–339. doi:10.1016/0005-2728(93)90206-U.

Lee, Jintae, Arul Jayaraman, and Thomas K Wood. 2007. “Indole Is an Inter-Species Biofilm Signal Mediated by SdiA.” *BMC Microbiology* 7: 42. doi:10.1186/1471-2180-7-42.

Lee, Song F., Yung H. Li, and George H. Bowden. 1996. “Detachment of Streptococcus Mutans Biofilm Cells by an Endogenous Enzymatic Activity.” *Infection and Immunity* 64 (3): 1035–1038.

Lemon, K P, A M Earl, H C Vlamakis, C Aguilar, and R Kolter. 2008. “Biofilm Development with an Emphasis on Bacillus Subtilis.” *Current Topics in Microbiology and Immunology* 322: 1–16. http://www.ncbi.nlm.nih.gov/pubmed/18453269.

Lewenza, Shawn. 2013. “Extracellular DNA-Induced Antimicrobial Peptide Resistance Mechanisms in Pseudomonas Aeruginosa.” *Frontiers in Microbiology*. doi:10.3389/fmicb.2013.00021.

Lewis, K. 2005. “Persister Cells and the Riddle of Biofilm Survival.” *Biochemistry. Biokhimiia* 70 (2) (February): 267–74. http://www.ncbi.nlm.nih.gov/pubmed/15807669.

Lewis, K I M, K I M Lewis, T H E Usual Suspects, and T H E Usual Suspects. 2001. “Riddle of Bio Lm Resistance.” *Society* 45 (4): 999–1007. doi:10.1128/AAC.45.4.999.

Lim, Bentley, Sinem Beyhan, James Meir, and Fitnat H. Yildiz. 2006. “Cyclic-diGMP Signal Transduction Systems in Vibrio Cholerae: Modulation of Rugosity and Biofilm Formation.” *Molecular Microbiology* 60 (2): 331–348. doi:10.1111/j.1365-2958.2006.05106.x.

Lim, Yong, Malabendu Jana, Thanh T. Luong, and Chia Y. Lee. 2004. “Control of Glucose- and NaCl-Induced Biofilm Formation by Rbf in Staphylococcus Aureus.” *Journal of Bacteriology* 186 (3): 722–729. doi:10.1128/JB.186.3.722-729.2004.

Liu, Jintao, Arthur Prindle, Jacqueline Humphries, Marçal Gabalda-Sagarra, Munehiro Asally, Dong-yeon D. Lee, San Ly, Jordi Garcia-Ojalvo, and Gürol M. Süel. 2015. “Metabolic Co-Dependence Gives Rise to Collective Oscillations within Biofilms.” *Nature* (July 22). doi:10.1038/nature14660. http://www.nature.com/doifinder/10.1038/nature14660.

Liu, Xiaoguang, Mohammed Bimerew, Yingxin Ma, Henry Müller, Marianna Ovadis, Leo Eberl, Gabriele Berg, and Leonid Chernin. 2007. “Quorum-Sensing Signaling Is Required for Production of the Antibiotic Pyrrolnitrin in a Rhizospheric Biocontrol Strain of Serratia Plymuthica.” *FEMS Microbiology Letters* 270 (2): 299–305. doi:10.1111/j.1574-6968.2007.00681.x.

Lopez, D, M A Fischbach, F Chu, R Losick, and R Kolter. 2009. “Structurally Diverse Natural Products That Cause Potassium Leakage Trigger Multicellularity in Bacillus Subtilis.” *Proceedings of the National Academy of Sciences of the United States of America* 106 (1): 280–285. doi:10.1073/pnas.0810940106. http://www.ncbi.nlm.nih.gov/pubmed/19114652.

Lopez, D, and R Kolter. 2010. “Extracellular Signals That Define Distinct and Coexisting Cell Fates in Bacillus Subtilis.” *FEMS Microbiology Reviews* 34 (2): 134–149.

doi:10.1111/j.1574-6976.2009.00199.x. http://www.ncbi.nlm.nih.gov/pubmed/20030732.

Lopez, D, H Vlamakis, and R Kolter. 2009. "Generation of Multiple Cell Types in Bacillus Subtilis." *FEMS Microbiology Reviews* 33 (1): 152–163. doi:10.1111/j.1574-6976.2008.00148.x. http://www.ncbi.nlm.nih.gov/pubmed/19054118.

López, Daniel, Hera Vlamakis, Richard Losick, Roberto Kolter, and D Lopez. 2009. "Paracrine Signaling in a Bacterium." *Genes & Development* 23 (14) (July 15): 1631–1638. doi:10.1101/gad.1813709. http://www.ncbi.nlm.nih.gov/pubmed/19605685.

Lyon, David R., and Susan E. Ziegler. 2009. "Carbon Cycling within Epilithic Biofilm Communities across a Nutrient Gradient of Headwater Streams." *Limnology and Oceanography* 54 (2): 439–449. doi:10.4319/lo.2009.54.2.0439.

Ma, Luyan, Matthew Conover, Haiping Lu, Matthew R Parsek, Kenneth Bayles, and Daniel J Wozniak. 2009. "Assembly and Development of the Pseudomonas Aeruginosa Biofilm Matrix." *PLoS Pathogens* 5 (3) (March): e1000354. doi:10.1371/journal.ppat.1000354. http://www.pubmedcentral.nih.gov/articlerender.fcgi?artid=2654510&tool=pmcentrez&rendertype=abstract.

Mack, D, M Nedelmann, A Krokotsch, A Schwarzkopf, J Heesemann, and R Laufs. 1994. "Characterization of Transposon Mutants of Biofilm-Producing Staphylococcus Epidermidis Impaired in the Accumulative Phase of Biofilm Production: Genetic Identification of a Hexosamine-Containing Polysaccharide Intercellular Adhesin." *Infection and Immunity* 62 (8) (August): 3244–53. http://www.pubmedcentral.nih.gov/articlerender.fcgi?artid=302952&tool=pmcentrez&rendertype=abstract.

Mack, W N, J P Mack, and A O Ackerson. 1975. "Microbial Film Development in a Trickling Filter." *Microbial Ecology* 2 (3) (September): 215–26. doi:10.1007/BF02010441. http://www.ncbi.nlm.nih.gov/pubmed/24241336.

Mäder, U., H. Antelmann, T. Buder, M. K. Dahl, M. Hecker, and Georg Homuth. 2002. "Bacillus Subtilis Functional Genomics: Genome-Wide Analysis of the DegS-DegU Regulon by Transcriptomics and Proteomics." *Molecular Genetics and Genomics* 268 (4): 455–467. doi:10.1007/s00438-002-0774-2.

Magnuson, R, J Solomon, and a D Grossman. 1994. "Biochemical and Genetic Characterization of a Competence Pheromone from B. Subtilis." *Cell* 77 (2) (April 22): 207–16. http://www.ncbi.nlm.nih.gov/pubmed/8168130.

Manefield, Mike, and Sarah L. Turner. 2002. "Quorum Sensing in Context: Out of Molecular Biology and into Microbial Ecology." *Microbiology*.

Marchand, Nicholas, and Cynthia H Collins. 2013. “Peptide-Based Communication System Enables Escherichia Coli to Bacillus Megaterium Interspecies Signaling.” *Biotechnology and Bioengineering* 110 (11) (November): 3003–12. doi:10.1002/bit.24975. http://www.ncbi.nlm.nih.gov/pubmed/23775238.

Marchand, Nicholas, and Cynthia H. Collins. 2015. “Synthetic Quorum Sensing and Cell-Cell Communication in Gram-Positive Bacillus Megaterium.” *ACS Synthetic Biology*: 150723125644007. doi:10.1021/acssynbio.5b00099. http://pubs.acs.org/doi/abs/10.1021/acssynbio.5b00099.

Marrie, T J, J Nelligan, and J W Costerton. 1982. “A Scanning and Transmission Electron Microscopic Study of an Infected Endocardial Pacemaker Lead.” *Circulation* 66 (6) (December): 1339–41. http://www.ncbi.nlm.nih.gov/pubmed/7139907.

Martino, P Di, R Fursy, L Bret, B Sundararaju, and R S Phillips. 2003. “Indole Can Act as an Extracellular Signal to Regulate Biofilm Formation of Escherichia Coli and Other Indole-Producing Bacteria.” *Canadian Journal of Microbiology* 49 (7): 443–449. doi:Doi 10.1139/W03-056.

McCoy, W. F., J. D. Bryers, J. Robbins, and J. W. Costerton. 1981. “Observations of Fouling Biofilm Formation.” *Canadian Journal of Microbiology* 27 (9) (September): 910–7. doi:10.1139/m81-143. http://www.nrcresearchpress.com/doi/abs/10.1139/m81-143.

McQuade, R. S., N. Comella, and A. D. Grossman. 2001. “Control of a Family of Phosphatase Regulatory Genes (phr) by the Alternate Sigma Factor Sigma-H of Bacillus Subtilis.” *Journal of Bacteriology* 183 (16): 4905–4909. doi:10.1128/JB.183.16.4905-4909.2001.

Merritt, Judith H., Kimberly M. Brothers, Sherry L. Kuchma, and George A. O’Toole. 2007. “SadC Reciprocally Influences Biofilm Formation and Swarming Motility via Modulation of Exopolysaccharide Production and Flagellar Function.” In *Journal of Bacteriology*, 189:8154–8164. doi:10.1128/JB.00585-07.

Mey, Alexandra R., Stephanie A. Craig, and Shelley M. Payne. 2005. “Characterization of Vibrio Cholerae RyhB: The RyhB Regulon and Role of ryhB in Biofilm Formation.” *Infection and Immunity* 73 (9): 5706–5719. doi:10.1128/IAI.73.9.5706-5719.2005.

Middleton, Rebecca, and Antje Hofmeister. 2004. “New Shuttle Vectors for Ectopic Insertion of Genes into Bacillus Subtilis.” *Plasmid* 51 (3) (May): 238–45. doi:10.1016/j.plasmid.2004.01.006. http://www.ncbi.nlm.nih.gov/pubmed/15109830.

Molin, Søren, and Tim Tolker-Nielsen. 2003. “Gene Transfer Occurs with Enhanced Efficiency in Biofilms and Induces Enhanced Stabilisation of the Biofilm Structure.”

Current Opinion in Biotechnology 14 (3) (June): 255–261. doi:10.1016/S0958-1669(03)00036-3. http://linkinghub.elsevier.com/retrieve/pii/S0958166903000363.

Monds, Russell D., Peter D. Newell, Robert H. Gross, and George A. O'Toole. 2007. "Phosphate-Dependent Modulation of c-Di-GMP Levels Regulates Pseudomonas Fluorescens Pf0-1 Biofilm Formation by Controlling Secretion of the Adhesin LapA." *Molecular Microbiology* 63 (3): 656–679. doi:10.1111/j.1365-2958.2006.05539.x.

Monds, Russell D., Mark W. Silby, and H. Khris Mahanty. 2001. "Expression of the Pho Regulon Negatively Regulates Biofilm Formation by Pseudomonas Aureofaciens PA147-2." *Molecular Microbiology* 42 (2): 415–426. doi:10.1046/j.1365-2958.2001.02641.x.

Moons, Pieter, Chris W. Michiels, and Abram Aertsen. 2009. "Bacterial Interactions in Biofilms." *Critical Reviews in Microbiology* 35 (3) (August): 157–168. doi:10.1080/10408410902809431. http://www.tandfonline.com/doi/full/10.1080/10408410902809431.

Morohoshi, Tomohiro, Tadanori Inaba, Norihiro Kato, Kinya Kanai, and Tsukasa Ikeda. 2004. "Identification of Quorum-Sensing Signal Molecules and the LuxRI Homologs in Fish Pathogen Edwardsiella Tarda." *Journal of Bioscience and Bioengineering* 98 (4): 274–281. doi:10.1263/jbb.98.274.

Mulcahy, Heidi, Laetitia Charron-Mazenod, and Shawn Lewenza. 2008. "Extracellular DNA Chelates Cations and Induces Antibiotic Resistance in Pseudomonas Aeruginosa Biofilms." *PLoS Pathogens* 4 (11). doi:10.1371/journal.ppat.1000213.

Nadell, Carey D, and Bonnie L Bassler. 2011. "A Fitness Trade-off between Local Competition and Dispersal in Vibrio Cholerae Biofilms." *Proceedings of the National Academy of Sciences of the United States of America* 108 (34) (August 23): 14181–5. doi:10.1073/pnas.1111147108. http://www.pubmedcentral.nih.gov/articlerender.fcgi?artid=3161532&tool=pmcentrez&rendertype=abstract.

Nadell, Carey D, Knut Drescher, Ned S Wingreen, and Bonnie L Bassler. 2015. "Extracellular Matrix Structure Governs Invasion Resistance in Bacterial Biofilms." *The ISME Journal* 9 (8): 1700–1709. doi:10.1038/ismej.2014.246. http://www.nature.com/doifinder/10.1038/ismej.2014.246.

Nagorska, K, A Ostrowski, K Hinc, I B Holland, and M Obuchowski. 2010. "Importance of Eps Genes from Bacillus Subtilis in Biofilm Formation and Swarming." *Journal of Applied Genetics* 51 (3): 369–381. doi:10.1007/BF03208867.

Nagórska, Krzysztofa, Krzysztof Hinc, Mark A. Strauch, and Michał Obuchowski. 2008. "Influence of the σB Stress Factor and yxaB, the Gene for a Putative Exopolysaccharide Synthase under σB Control, on Biofilm Formation." *Journal of Bacteriology* 190 (10): 3546–3556. doi:10.1128/JB.01665-07.

Nealson, K H, and J W Hastings. 1979. "Bacterial Bioluminescence: Its Control and Ecological Significance." *Microbiological Reviews* 43 (4): 496–518.

Nealson, Kenneth H., Terry Platt, and J. Woodland Hastings. 1970. "Cellular Control of the Synthesis and Activity of the Bacterial Luminescent System." *Journal of Bacteriology* 104 (1): 313–322.

Newton, Wm. Austin, and Esmond E. Snell. 1964. "CATALYTIC PROPERTIES OF TRYPTOPHANASE, A MULTIFUNCTIONAL PYRIDOXAL PHOSPHATE ENZYME." *Proceedings of the National Academy of Sciences of the United States of America* 51 (3): 382–389. doi:10.1073/pnas.51.3.382.

Novick, R P, S J Projan, J Kornblum, H F Ross, G Ji, B Kreiswirth, F Vandenesch, and S Moghazeh. 1995. "The Agr P2 Operon: An Autocatalytic Sensory Transduction System in Staphylococcus Aureus." *Molecular & General Genetics : MGG* 248 (4) (August 30): 446–58. http://www.ncbi.nlm.nih.gov/pubmed/7565609.

Novick, R P, H F Ross, S J Projan, J Kornblum, B Kreiswirth, and S Moghazeh. 1993. "Synthesis of Staphylococcal Virulence Factors Is Controlled by a Regulatory RNA Molecule." *The EMBO Journal* 12 (10) (October): 3967–75. http://www.ncbi.nlm.nih.gov/pubmed/413679.

Novick, Richard P, and Edward Geisinger. 2008. "Quorum Sensing in Staphylococci." *Annual Review of Genetics* 42 (January): 541–64. doi:10.1146/annurev.genet.42.110807.091640. http://www.ncbi.nlm.nih.gov/pubmed/18713030.

O'Toole, G, Heidi B Kaplan, and Roberto Kolter. 2000. "Biofilm Formation as Microbial Development." *Annual Review of Microbiology* 54 (January): 49–79. doi:10.1146/annurev.micro.54.1.49. http://www.ncbi.nlm.nih.gov/pubmed/11018124.

Ochsner, U A, and J Reiser. 1995. "Autoinducer-Mediated Regulation of Rhamnolipid Biosurfactant Synthesis in Pseudomonas Aeruginosa." *Proceedings of the National Academy of Sciences of the United States of America* 92 (14): 6424–6428. doi:10.1073/pnas.92.14.6424.

Ogura, M, H Yamaguchi, Yoshida Ki, Y Fujita, and T Tanaka. 2001. "DNA Microarray Analysis of Bacillus Subtilis DegU, ComA and PhoP Regulons: An Approach to

Comprehensive Analysis of B.subtilis Two-Component Regulatory Systems." *Nucleic Acids Research* 29 (18): 3804–3813. doi:10.1093/nar/29.18.3804.

Ogura, Mitsuo, Kana Shimane, Kei Asai, Naotake Ogasawara, and Teruo Tanaka. 2003. "Binding of Response Regulator DegU to the aprE Promoter Is Inhibited by RapG, Which Is Counteracted by Extracellular PhrG in Bacillus Subtilis." *Molecular Microbiology* 49 (6): 1685–1697. doi:10.1046/j.1365-2958.2003.03665.x.

Okada, Masahiro, Isao Sato, Soo Jeong Cho, Hidehisa Iwata, Toshihiko Nishio, David Dubnau, and Youji Sakagami. 2005. "Structure of the Bacillus Subtilis Quorum-Sensing Peptide Pheromone ComX." *Nature Chemical Biology* 1 (1) (June): 23–4. doi:10.1038/nchembio709. http://www.ncbi.nlm.nih.gov/pubmed/16407988.

Otto, Michael, Roderich Süßmuth, Günther Jung, Friedrich Götz, and R Süssmuth. 1998. "Structure of the Pheromone Peptide of the Staphylococcus Epidermidis Agr System." *FEBS Letters* 424 (1-2) (March 6): 89–94. doi:10.1016/S0014-5793(98)00145-8. http://www.ncbi.nlm.nih.gov/pubmed/9537521.

Pearson, James P., Matthew Feldman, Barbara H. Iglewski, and Alice Prince. 2000. "Pseudomonas Aeruginosa Cell-to-Cell Signaling Is Required for Virulence in a Model of Acute Pulmonary Infection." *Infection and Immunity* 68 (7): 4331–4334. doi:10.1128/IAI.68.7.4331-4334.2000.

Pedersen, Karsten. 1982. "Method for Studying Microbial Biofilms in Flowing-Water Systems" 43 (1): 6–13.

Perego, M, and J A Hoch. 1996. "Cell-Cell Communication Regulates the Effects of Protein Aspartate Phosphatases on the Phosphorelay Controlling Development in Bacillus Subtilis." *Proceedings of the National Academy of Sciences of the United States of America* 93 (4) (February 20): 1549–53. http://www.pubmedcentral.nih.gov/articlerender.fcgi?artid=39978&tool=pmcentrez&rendertype=abstract.

Pereira, Catarina S., Jessica a. Thompson, and Karina B. Xavier. 2013. "AI-2-Mediated Signalling in Bacteria." *FEMS Microbiology Reviews* 37 (2): 156–181. doi:10.1111/j.1574-6976.2012.00345.x.

Pottathil, Mridula, and Beth A Lazazzera. 2003a. "The Extracellular Phr Peptide-Rap Phosphatase Signaling Circuit of Bacillus Subtilis." *Frontiers in Bioscience : A Journal and Virtual Library* 8 (1-3) (January 1): d32–45. doi:10.2741/913. http://www.ncbi.nlm.nih.gov/pubmed/12456319.

———. 2003b. “The Extracellular Phr Peptide-Rap Phosphatase Signaling Circuit of Bacillus Subtilis.” *Frontiers in Bioscience : A Journal and Virtual Library* 8 (1-3) (January 1): d32–45. doi:10.2741/913. http://www.ncbi.nlm.nih.gov/pubmed/12456319.

Pratten, Jonathan, Simon J. Foster, Pan F. Chan, Michael Wilson, and Sean P. Nair. 2001. “Staphylococcus Aureus Accessory Regulators: Expression within Biofilms and Effect on Adhesion.” *Microbes and Infection* 3 (8): 633–637. doi:10.1016/S1286-4579(01)01418-6.

Prouty, A. M., W. H. Schwesinger, and J. S. Gunn. 2002. “Biofilm Formation and Interaction with the Surfaces of Gallstones by Salmonella Spp.” *Infection and Immunity* 70 (5): 2640–2649. doi:10.1128/IAI.70.5.2640-2649.2002.

Rader, Bethany A., Shawn R. Campagna, Martin F. Semmelhack, Bonnie L. Bassler, and Karen Guillemin. 2007. “The Quorum-Sensing Molecule Autoinducer 2 Regulates Motility and Flagellar Morphogenesis in Helicobacter Pylori.” *Journal of Bacteriology* 189 (17): 6109–6117. doi:10.1128/JB.00246-07.

Rainey, Paul B, and Katrina Rainey. 2003. “Evolution of Cooperation and Conflict in Experimental Bacterial Populations” 425 (September): 72–74. doi:10.1038/nature01942.1.

Rashid, M H, K Rumbaugh, L Passador, D G Davies, A N Hamood, B H Iglewski, and A Kornberg. 2000. “Polyphosphate Kinase Is Essential for Biofilm Development, Quorum Sensing, and Virulence of Pseudomonas Aeruginosa.” *Proceedings of the National Academy of Sciences of the United States of America* 97 (17) (August 15): 9636–41. doi:10.1073/pnas.170283397. http://www.pubmedcentral.nih.gov/articlerender.fcgi?artid=16917&tool=pmcentrez&rendertype=abstract.

Redfield, Rosemary J. 2002. “Is Quorum Sensing a Side Effect of Diffusion Sensing?” *Trends in Microbiology* 10 (8) (August): 365–70. http://www.ncbi.nlm.nih.gov/pubmed/12160634.

Reizer, J, A Reizer, M Perego, and M H Saier. 1997. “Characterization of a Family of Bacterial Response Regulator Aspartyl-Phosphate (RAP) Phosphatases.” *Microbial & Comparative Genomics* 2 (2): 103–111. doi:10.1089/omi.1.1997.2.103.

Ren, Dacheng, Laura A. Bedzyk, Peter Setlow, Stuart M. Thomas, Rick W. Ye, and Thomas K. Wood. 2004. “Gene Expression in Bacillus Subtilis Surface Biofilms with and Without Sporulation and the Importance of yveR for Biofilm Maintenance.” *Biotechnology and Bioengineering* 86 (3): 344–364. doi:10.1002/bit.20053.

Rhaese, H. J., J. A. Hoch, and R. Groscurth. 1977. “Studies on the Control of Development: Isolation of Bacillus Subtilis Mutants Blocked Early in Sporulation and Defective in Synthesis of Highly Phosphorylated Nucleotides.” *Proceedings of the National Academy of Sciences* 74 (3) (March 1): 1125–1129. doi:10.1073/pnas.74.3.1125. http://www.pnas.org/cgi/doi/10.1073/pnas.74.3.1125.

Romero, Diego, Claudio Aguilar, Richard Losick, and Roberto Kolter. 2010. “Amyloid Fibers Provide Structural Integrity to Bacillus Subtilis Biofilms.” *Proceedings of the National Academy of Sciences of the United States of America* 107 (5) (February 2): 2230–4. doi:10.1073/pnas.0910560107. http://www.pubmedcentral.nih.gov/articlerender.fcgi?artid=2836674&tool=pmcentrez&rendertype=abstract.

Romero, Diego, Hera Vlamakis, Richard Losick, and Roberto Kolter. 2011. “An Accessory Protein Required for Anchoring and Assembly of Amyloid Fibres in B. Subtilis Biofilms.” *Molecular Microbiology* 80 (5) (June): 1155–68. doi:10.1111/j.1365-2958.2011.07653.x. http://www.pubmedcentral.nih.gov/articlerender.fcgi?artid=3103627&tool=pmcentrez&rendertype=abstract.

———. 2014. “Functional Analysis of the Accessory Protein TapA in Bacillus Subtilis Amyloid Fiber Assembly.” *Journal of Bacteriology* 196 (8) (April): 1505–13. doi:10.1128/JB.01363-13. http://www.ncbi.nlm.nih.gov/pubmed/24488317.

Rutherford, Steven T, and Bonnie L Bassler. 2012. “Bacterial Quorum Sensing: Its Role in Virulence and Possibilities for Its Control.” *Cold Spring Harbor Perspectives in Medicine* 2 (11) (November): 1–26. doi:10.1101/cshperspect.a012427. http://www.pubmedcentral.nih.gov/articlerender.fcgi?artid=3543102&tool=pmcentrez&rendertype=abstract.

Sakuragi, Yumiko, and Roberto Kolter. 2007. “Quorum-Sensing Regulation of the Biofilm Matrix Genes (pel) of Pseudomonas Aeruginosa.” *Journal of Bacteriology* 189 (14) (July): 5383–6. doi:10.1128/JB.00137-07. http://www.pubmedcentral.nih.gov/articlerender.fcgi?artid=1951888&tool=pmcentrez&rendertype=abstract.

Schaefer, Amy L., Brian L. Hanzelka, Anatol Eberhard, and E. P. Greenberg. 1996. “Quorum Sensing in Vibrio Fischeri: Probing Autoinducer-LuxR Interactions with Autoinducer Analogs.” *Journal of Bacteriology* 178 (10): 2897–2901. doi:10.1046/j.1365-2958.1999.01261.x.

Schauder, S., and B. L. Bassler. 2001. “The Languages of Bacteria.” *Genes and Development*. doi:10.1101/gad.899601.

Schluter, Jonas, Carey D Nadell, Bonnie L Bassler, and Kevin R Foster. 2015. "Adhesion as a Weapon in Microbial Competition." *The ISME Journal* 9 (1) (January): 139–49. doi:10.1038/ismej.2014.174. http://www.ncbi.nlm.nih.gov/pubmed/25290505.

Schuster, Martin, C Phoebe Lostroh, Tomoo Ogi, and E P Greenberg. 2003. "Identification, Timing, and Signal Specificity of Pseudomonas Aeruginosa Quorum-Controlled Genes: A Transcriptome Analysis." *Journal of Bacteriology* 185 (7) (April): 2066–79. doi:10.1128/JB.185.7.2066. http://www.pubmedcentral.nih.gov/articlerender.fcgi?artid=151497&tool=pmcentrez&rendertype=abstract.

Seminara, A, T E Angelini, J N Wilking, H Vlamakis, S Ebrahim, R Kolter, D A Weitz, and M P Brenner. 2012. "Osmotic Spreading of Bacillus Subtilis Biofilms Driven by an Extracellular Matrix." *Proceedings of the National Academy of Sciences of the United States of America* 109 (4): 1116–1121. doi:10.1073/pnas.1109261108. http://www.ncbi.nlm.nih.gov/pubmed/22232655.

Seviour, Thomas, Susan Hove Hansen, Liang Yang, Yin Hoe Yau, Victor Bochuan Wang, Marcel R Stenvang, Gunna Christiansen, et al. 2015. "Functional Amyloids Keep Quorum Sensing Molecules in Check." *The Journal of Biological Chemistry* (January 13). doi:10.1074/jbc.M114.613810. http://www.ncbi.nlm.nih.gov/pubmed/25586180.

Seyedsayamdost, Mohammad R., Josephine R. Chandler, Joshua A V Blodgett, Patricia S. Lima, Breck A. Duerkop, Ken Ichi Oinuma, E. Peter Greenberg, and Jon Clardy. 2010. "Quorum-Sensing-Regulated bActobolin Production by Burkholderia Thailandensis E264." *Organic Letters* 12 (4): 716–719. doi:10.1021/ol902751x.

Shank, Elizabeth Anne, and Roberto Kolter. 2011. "Extracellular Signaling and Multicellularity in Bacillus Subtilis." *Current Opinion in Microbiology* 14 (6) (December): 741–747. doi:10.1016/j.mib.2011.09.016. http://www.ncbi.nlm.nih.gov/pubmed/22024380.

Shemesh, Moshe, and Yunrong Chaia. 2013. "A Combination of Glycerol and Manganese Promotes Biofilm Formation in Bacillus Subtilis via Histidine Kinase KinD Signaling." *Journal of Bacteriology* 195 (12): 2747–2754. doi:10.1128/JB.00028-13.

Shemesh, Moshe, Avshalom Tam, and Doron Steinberg. 2007. "Expression of Biofilm-Associated Genes of Streptococcus Mutans in Response to Glucose and Sucrose." *Journal of Medical Microbiology* 56 (11): 1528–1535. doi:10.1099/jmm.0.47146-0.

Simm, Roger, Michael Morr, Abdul Kader, Manfred Nimtz, and Ute Römling. 2004. "GGDEF and EAL Domains Inversely Regulate Cyclic Di-GMP Levels and Transition from Sessibility to Motility." *Molecular Microbiology* 53 (4): 1123–1134. doi:10.1111/j.1365-2958.2004.04206.x.

Singh, Pradeep K, Matthew R Parsek, E Peter Greenberg, and Michael J Welsh. 2002. “A Component of Innate Immunity Prevents Bacterial Biofilm Development.” *Nature* 417 (6888): 552–555. doi:10.1038/417552a.

Singh, Rajbir, Debarati Paul, and Rakesh K Jain. 2006. “Biofilms: Implications in Bioremediation.” *Trends in Microbiology* 14 (9) (September): 389–97. doi:10.1016/j.tim.2006.07.001. http://www.ncbi.nlm.nih.gov/pubmed/16857359.

Solomon, J M, B a Lazazzera, and a D Grossman. 1996. “Purification and Characterization of an Extracellular Peptide Factor That Affects Two Different Developmental Pathways in Bacillus Subtilis.” *Genes & Development* 10 (16) (August 15): 2014–2024. doi:10.1101/gad.10.16.2014. http://www.genesdev.org/cgi/doi/10.1101/gad.10.16.2014.

Solomon, J M, R Magnuson, a Srivastava, and a D Grossman. 1995. “Convergent Sensing Pathways Mediate Response to Two Extracellular Competence Factors in Bacillus Subtilis.” *Genes & Development* 9 (5) (March 1): 547–558. doi:10.1101/gad.9.5.547. http://www.genesdev.org/cgi/doi/10.1101/gad.9.5.547.

Spiers, Andrew J., John Bohannon, Stefanie M. Gehrig, and Paul B. Rainey. 2003. “Biofilm Formation at the Air-Liquid Interface by the Pseudomonas Fluorescens SBW25 Wrinkly Spreader Requires an Acetylated Form of Cellulose.” *Molecular Microbiology* 50 (1): 15–27. doi:10.1046/j.1365-2958.2003.03670.x.

Stanley, Nicola R., Robert A. Britton, Alan D. Grossman, and Beth A. Lazazzera. 2003. “Identification of Catabolite Repression as a Physiological Regulator of Biofilm Formation by Bacillus Subtilis by Use of DNA Microarrays.” *Journal of Bacteriology* 185 (6): 1951–1957. doi:10.1128/JB.185.6.1951-1957.2003.

Steinberger, R E, and P a Holden. 2005. “Extracellular DNA in Single- and Multiple-Species Unsaturated Biofilms.” *Applied and Environmental Microbiology* 71 (9) (September): 5404–10. doi:10.1128/AEM.71.9.5404-5410.2005. http://www.pubmedcentral.nih.gov/articlerender.fcgi?artid=1214645&tool=pmcentrez&rendertype=abstract.

Stevens, A M, K M Dolan, and E P Greenberg. 1994. “Synergistic Binding of the Vibrio Fischeri LuxR Transcriptional Activator Domain and RNA Polymerase to the Lux Promoter Region.” *Proceedings of the National Academy of Sciences of the United States of America* 91 (26): 12619–12623. doi:10.1073/pnas.92.8.3631a.

Stevens, Ann M., Nobuyuki Fujita, Akira Ishihama, and E. P. Greenberg. 1999. “Involvement of the RNA Polymerase ??-Subunit C-Terminal Domain in LuxR-Dependent Activation of the Vibrio Fischeri Luminescence Genes.” *Journal of Bacteriology* 181 (15): 4704–4707.

Stevens, Ann M., and E. P. Greenberg. 1997. “Quorum Sensing in Vibrio Fischeri: Essential Elements for Activation of the Luminescence Genes.” *Journal of Bacteriology* 179 (2): 557–562. doi:10.1111/j.1462-2920.2008.01755.x.

Stewart, J W Costerton Philip S, and E P Creenberg. 1999. “Bacterial Biofilms : A Common Cause of Persistent Infections” 25 (May): 1318–1323.

Stewart, Philip S, and J William Costerton. 2001. “Antibiotic Resistance of Bacteria in Biofilms.” *The Lancet* 358 (9276) (July): 135–138. doi:10.1016/S0140-6736(01)05321-1. http://linkinghub.elsevier.com/retrieve/pii/S0140673601053211.

Stoodley, P, K Sauer, D G Davies, and J W Costerton. 2002. “Biofilms as Complex Differentiated Communities.” *Annual Review of Microbiology* 56: 187–209. doi:10.1146/annurev.micro.56.012302.160705.

Stöver, a G, and a Driks. 1999. “Regulation of the Synthesis of the B. Subtilis Transition Phase, Spore-Associated Antibacterial Protein TasA.” *J. Bacteriol.* 181 (17): 5476–5481.

Stöver, Axel G, and Adam Driks. 1999a. “Control of Synthesis and Secretion of the Bacillus Subtilis Protein YqxM These Include : Control of Synthesis and Secretion of the Bacillus Subtilis Protein YqxM” 181 (22): 1–6.

———. 1999b. “Secretion, Localization, and Antibacterial Activity of TasA, a Bacillus Subtilis Spore-Associated Protein.” *Journal of Bacteriology* 181 (5) (March): 1664–72. http://www.pubmedcentral.nih.gov/articlerender.fcgi?artid=93559&tool=pmcentrez&rendertype=abstract.

Studier, F William. 2005. “Protein Production by Auto-Induction in High Density Shaking Cultures.” *Protein Expression and Purification* 41 (1): 207–234. doi:10.1016/j.pep.2005.01.016.

Süel, Gürol M, Jordi Garcia-Ojalvo, Louisa M Liberman, and Michael B Elowitz. 2006. “An Excitable Gene Regulatory Circuit Induces Transient Cellular Differentiation.” *Nature* 440 (7083) (March 23): 545–50. doi:10.1038/nature04588. http://www.ncbi.nlm.nih.gov/pubmed/16554821.

Surette, M G, M B Miller, and B L Bassler. 1999. “Quorum Sensing in Escherichia Coli, Salmonella Typhimurium, and Vibrio Harveyi: A New Family of Genes Responsible for Autoinducer Production.” *Proceedings of the National Academy of Sciences of the United States of America* 96 (4): 1639–1644. doi:10.1073/pnas.96.4.1639.

Sutherland, I. W. 2001. “Biofilm Exopolysaccharides: A Strong and Sticky Framework.” *Microbiology* 147 (1): 3–9. doi:10.1051/lait:2001108.

Tal, Rony, Hing C. Wong, Roger Calhoon, David Gelfand, Anna Lisa Fear, Gail Volman, Raphael Mayer, et al. 1998. “Three Cdg Operons Control Cellular Turnover of Cyclic Di-GMP in Acetobacter Xylinum: Genetic Organization and Occurrence of Conserved Domains in Isoenzymes.” *Journal of Bacteriology* 180 (17): 4416–4425.

Tart, Anne H., Michael J. Blanks, and Daniel J. Wozniak. 2006. “The AlgT-Dependent Transcriptional Regulator AmrZ (AlgZ) Inhibits Flagellum Biosynthesis in Mucoid, Nonmotile Pseudomonas Aeruginosa Cystic Fibrosis Isolates.” *Journal of Bacteriology* 188 (18): 6483–6489. doi:10.1128/JB.00636-06.

Tischler, Anna D., and Andrew Camilli. 2004. “Cyclic Diguanylate (c-Di-GMP) Regulates Vibrio Cholerae Biofilm Formation.” *Molecular Microbiology* 53 (3): 857–869. doi:10.1111/j.1365-2958.2004.04155.x.

Tjalsma, Harold, Albert Bolhuis, Maarten L. Van Roosmalen, Thomas Wiegert, Wolfgang Schumann, Cees P. Broekhuizen, Wim J. Quax, Gerard Venema, Sierd Bron, and Jan Maarten Van Dijl. 1998. “Functional Analysis of the Secretory Precursor Processing Machinery of Bacillus Subtilis: Identification of a Eubacterial Homolog of Archaeal and Eukaryotic Signal Peptidases.” *Genes and Development* 12 (15): 2318–2331. doi:10.1101/gad.12.15.2318.

Turakhia, M H, K E Cooksey, and W G Characklis. 1983. “Influence of a Calcium-Specific Chelant on Biofilm Removal” 46 (5): 1236–1238.

Tuteja, Renu. 2005. “Type I Signal Peptidase: An Overview.” *Archives of Biochemistry and Biophysics* 441 (2) (September 15): 107–11. doi:10.1016/j.abb.2005.07.013. http://www.ncbi.nlm.nih.gov/pubmed/16126156.

Ueda, Akihiro, and Thomas K. Wood. 2009. “Connecting Quorum Sensing, c-Di-GMP, Pel Polysaccharide, and Biofilm Formation in Pseudomonas Aeruginosa through Tyrosine Phosphatase TpbA (PA3885).” *PLoS Pathogens* 5 (6): 1–15. doi:10.1371/journal.ppat.1000483.

Van Gestel, Jordi, Franz J Weissing, Oscar P Kuipers, and Akos T Kovács. 2014. “Density of Founder Cells Affects Spatial Pattern Formation and Cooperation in Bacillus Subtilis Biofilms.” *The ISME Journal* 8 (10) (October 3): 2069–79. doi:10.1038/ismej.2014.52. http://www.ncbi.nlm.nih.gov/pubmed/24694715.

Vaningelgem, F., M. Zamfir, F. Mozzi, T. Adriany, M. Vancanneyt, J. Swings, and L. De Vuyst. 2004. “Biodiversity of Exopolysaccharides Produced by Streptococcus Thermophilus Strains Is Reflected in Their Production and Their Molecular and Functional Characteristics.” *Applied and Environmental Microbiology* 70 (2) (February 6): 900–912. doi:10.1128/AEM.70.2.900-912.2004. http://aem.asm.org/cgi/doi/10.1128/AEM.70.2.900-912.2004.

Vannini, Alessandro, Cinzia Volpari, Cesare Gargioli, Ester Muraglia, Riccardo Cortese, Raffaele De Francesco, Petra Neddermann, and Stefania Di Marco. 2002. "The Crystal Structure of the Quorum Sensing Protein TraR Bound to Its Autoinducer and Target DNA." *EMBO Journal* 21 (17): 4393–4401. doi:10.1093/emboj/cdf459.

Verma, Subhash C., and Tim Miyashiro. 2013. "Quorum Sensing in the Squid-Vibrio Symbiosis." *International Journal of Molecular Sciences* 14 (8): 16386–16401. doi:10.3390/ijms140816386.

Vivant, Anne-Laure, Dominique Garmyn, Laurent Gal, and Pascal Piveteau. 2014. "The Agr Communication System Provides a Benefit to the Populations of Listeria Monocytogenes in Soil." *Frontiers in Cellular and Infection Microbiology* 4 (November) (November 6): 1–7. doi:10.3389/fcimb.2014.00160. http://www.frontiersin.org/Cellular_and_Infection_Microbiology/10.3389/fcimb.2014.00160/abstract.

Vlamakis, Hera, Claudio Aguilar, Richard Losick, and Roberto Kolter. 2008. "Control of Cell Fate by the Formation of an Architecturally Complex Bacterial Community." *Genes & Development* 22 (7) (April 1): 945–53. doi:10.1101/gad.1645008. http://www.ncbi.nlm.nih.gov/pubmed/18381896.

Vuong, C, H L Saenz, F Götz, and M Otto. 2000. "Impact of the Agr Quorum-Sensing System on Adherence to Polystyrene in Staphylococcus Aureus." *The Journal of Infectious Diseases* 182 (6): 1688–1693. doi:10.1086/317606.

Wang, L., C. Fabret, K. Kanamaru, K. Stephenson, V. Dartois, M. Perego, and J. A. Hoch. 2001. "Dissection of the Functional and Structural Domains of Phosphorelay Histidine Kinase A of Bacillus Subtilis." *Journal of Bacteriology* 183 (9): 2795–2802. doi:10.1128/JB.183.9.2795-2802.2001.

Wang, Zhaoshou, Xin Wu, Jianghai Peng, Yidan Hu, Baishan Fang, and Shiyang Huang. 2014. "Artificially Constructed Quorum-Sensing Circuits Are Used for Subtle Control of Bacterial Population Density." *PLoS ONE* 9 (8). doi:10.1371/journal.pone.0104578.

Waters, Christopher M, and Bonnie L Bassler. 2005. "Quorum Sensing: Cell-to-Cell Communication in Bacteria." *Annual Review of Cell and Developmental Biology* 21: 319–346. doi:10.1146/annurev.cellbio.21.012704.131001.

Waters, Christopher M, Wenyun Lu, Joshua D Rabinowitz, and Bonnie L Bassler. 2008. "Quorum Sensing Controls Biofilm Formation in Vibrio Cholerae through Modulation of Cyclic Di-GMP Levels and Repression of vpsT." *Journal of Bacteriology* 190 (7) (April): 2527–36. doi:10.1128/JB.01756-07. http://www.pubmedcentral.nih.gov/articlerender.fcgi?artid=2293178&tool=pmcentrez&rendertype=abstract.

Watnick, Paula I, and Roberto Kolter. 1999. “Steps in the Development of a Vibrio Cholerae El Tor Bio ® Lm” 34: 586–595.

Watson, William T., Timothy D. Minogue, Dale L. Val, Susanne Beck Von Bodman, and M. E A Churchill. 2002. “Structural Basis and Specificity of Acyl-Homoserine Mactone Signal Production in Bacterial Quorum Sensing.” *Molecular Cell* 9 (3): 685–694. doi:10.1016/S1097-2765(02)00480-X.

Weber, Wilfried, and Martin Fussenegger. 2007. “Inducible Product Gene Expression Technology Tailored to Bioprocess Engineering.” *Current Opinion in Biotechnology*. doi:10.1016/j.copbio.2007.09.002.

Wen, Zezhang T., and Robert A. Burne. 2004. “LuxS-Mediated Signaling in Streptococcus Mutans Is Involved in Regulation of Acid and Oxidative Stress Tolerance and Biofilm Formation.” *Journal of Bacteriology* 186 (9): 2682–2691. doi:10.1128/JB.186.9.2682-2691.2004.

West, Stuart A, Klaus Winzer, Andy Gardner, and Stephen P Diggle. 2012. “Quorum Sensing and the Confusion about Diffusion.” *Trends in Microbiology* 20 (12) (December): 586–94. doi:10.1016/j.tim.2012.09.004. http://dx.doi.org/10.1016/j.tim.2012.09.004.

Westers, Lidia, Helga Westers, and Wim J. Quax. 2004. “Bacillus Subtilis as Cell Factory for Pharmaceutical Proteins: A Biotechnological Approach to Optimize the Host Organism.” *Biochimica et Biophysica Acta - Molecular Cell Research* 1694 (1-3 SPEC.ISS.): 299–310. doi:10.1016/j.bbamcr.2004.02.011.

Whitchurch, Cynthia B, Tim Tolker-nielsen, Paula C Ragas, and John S Mattick. 2002. “Extracellular DNA Required for Bacterial Biofilm Formation.” *Science (New York, N.Y.)* 295 (5559) (February 22): 1487. doi:10.1126/science.295.5559.1487. http://www.ncbi.nlm.nih.gov/pubmed/11859186.

Xavier, Joao B, and Kevin R Foster. 2007. “Cooperation and Conflict in Microbial Biofilms.” *Proc. Natl. Acad. Sci. USA* 104 (3) (January): 876–81. doi:10.1073/pnas.0607651104.

Xavier, Karina B., and Bonnie L. Bassler. 2003. “LuxS Quorum Sensing: More than Just a Numbers Game.” *Current Opinion in Microbiology*. doi:10.1016/S1369-5274(03)00028-6.

Yarwood, Jeremy M, Douglas J Bartels, Esther M Volper, and E Peter Greenberg. 2004. “Quorum Sensing in Staphylococcus Aureus Biofilms.” *Journal of Bacteriology* 186 (6) (March): 1838–50. doi:10.1128/JB.186.6.1838. http://www.pubmedcentral.nih.gov/articlerender.fcgi?artid=355980&tool=pmcentrez&rendertype=abstract.

Yarwood, Jeremy M., and Patrick M. Schlievert. 2003. “Quorum Sensing in Staphylococcus Infections.” *Journal of Clinical Investigation*. doi:10.1172/JCI200320442.

Zargar, Amin, David N Quan, Milad Emamian, Chen Yu Tsao, Hsuan-Chen Wu, Chelsea R Virgile, and William E Bentley. 2015. “Rational Design of ‘Controller Cells’ to Manipulate Protein and Phenotype Expression.” *Metabolic Engineering* (April 20): 1–8. doi:10.1016/j.ymben.2015.04.001. http://www.ncbi.nlm.nih.gov/pubmed/25908186.

Zhang, Linsheng, Lillian Gray, Richard P Novick, and Guangyong Ji. 2002. “Transmembrane Topology of AgrB, the Protein Involved in the Post-Translational Modification of AgrD in Staphylococcus Aureus.” *The Journal of Biological Chemistry* 277 (38) (September 20): 34736–42. doi:10.1074/jbc.M205367200. http://www.ncbi.nlm.nih.gov/pubmed/12122003.

Zhang, Rong-guang, Katherine M Pappas, Jennifer L Brace, Paula C Miller, Tim Oulmassov, John M Molyneaux, John C Anderson, James K Bashkin, Stephen C Winans, and Andrzej Joachimiak. 2002. “Structure of a Bacterial Quorum-Sensing Transcription Factor Complexed with Pheromone and DNA.” *Nature* 417 (6892): 971–974. doi:10.1038/nature10294.

Zhang, Weiwen, and David R Nielsen. 2015. *Synthetic Biology Applications in Industrial Microbiology*. doi:10.3389/978-2-88919-342-4. http://www.frontiersin.org/books/Synthetic_biology_applications_in_industrial_microbiology/384.

Zhao, Kelei, Yi Li, Bisong Yue, and Min Wu. 2014. “Genes as Early Responders Regulate Quorum-Sensing and Control Bacterial Cooperation in Pseudomonas Aeruginosa.” *PloS One* 9 (7) (January): e101887. doi:10.1371/journal.pone.0101887. http://www.pubmedcentral.nih.gov/articlerender.fcgi?artid=4090235&tool=pmcentrez&rendertype=abstract.