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Quorum Sensing in Archaea

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Abstract

Bacteria coordinate cell density dependent behaviors by communicating through chemical intermediaries in a process called quorum sensing. In a bacterial culture, individual cells will constitutively produce signal molecules, termed autoinducers, and export them into the environment. When the concentration of autoinducers reaches a threshold, the cells sense that they are in a specific situation, which requires the upregulation of certain genes. This upregulation causes the bacteria to produce proteins that allow them to take part in a coordinated population-wide behavior.

In bacteria that are naturally competent, or capable of importing DNA from the environment, the expression of competence genes is often regulated by quorum sensing. Recent evidence has shown that the archaean *Haloferax volcanii* is naturally competent, which leads to the hypothesis that *H. volcanii* may use quorum sensing as a regulatory mechanism as well. In the domain Archaea, quorum sensing is a phenomenon that has received very little investigation and no concrete conclusions have been drawn yet.

Comparative genomics studies and nutritional competence growth studies with *H. volcanii* in conditioned media were performed to gain further insight into quorum sensing in archaea. Comparative proteomic studies suggest that a peptide autoinducer export protein may also function in archaea. The conditioned media growth studies suggest that an autoinducer susceptible to heat treatment may regulate competence in *H. volcanii.*

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Table of Contents

List of Tables

List of Figures

1. Introduction

1.1. Cell to Cell Chemical Mediated Communication

Until the late 1960's, it was believed that prokaryotes adapted to their surroundings by responding to a range of environmental conditions such as nutrient availability, changes in temperature and salinity. However, there were certain traits present in populations of cells that seemed to depend on the presence of other cells. Further investigation over several decades lead to the discovery of how cells detect one another through chemical intermediates, through the process termed quorum sensing (Miller and Bassler 2001).

It is now know that in a significant number of bacterial species, quorum sensing is an extremely important decision making mechanism based on the cell density of the community. The chemical signal, the autoinducer, is constitutively synthesized by bacteria at low levels, and secreted into the environment. As the population of bacteria reproduces, the number of autoinducers secreted into the media increases proportionally. At a high cell density, a threshold amount of autoinducers are present in the environment, and is sensed by individual bacteria by specific autoinducer receptors present in either the cytoplasm or the membrane. With the activation of these receptors, the individual cells sense they are in dense community of bacteria and respond by transcribing genes associated with high cell density behavior. Also, when cells are confined to a small volume, the autoinducers have limited diffusion and the threshold concentration is reached quicker and triggers the respective genes faster (Miller and Bassler 2001).

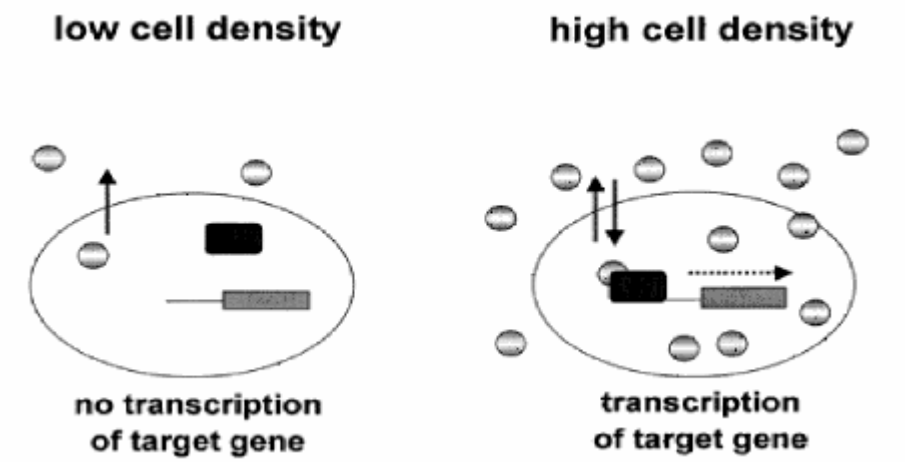

Figure 1.1: Basic Diagram of Quorum Sensing: At a low cell density, cells produce autoinducers and secrete them into the environment. At this concentration however, the autoinducers fail to initiate transcription of target genes. At a high cell density, the concentration of autoinducers is higher, and will initiate a reaction from the cells causing transcription of the target genes. Image from Bitan-Banin, (2002)

1.1.1. Bioluminecence in *Vibrio fischeri*

Quorum sensing was first discovered in the Gram negative marine bacterium *Vibrio fischeri*, which can be found living free in the ocean or as a symbiont in the light organ of some species of fish and squid. When living in the light organs of marine animals, *V. fischeri* is bioluminescent, and produces light that helps its hosts. For example, the squid *Euprymna scolopes* uses the light to help evade predation, whereas the fish *Monocentris japonicus* uses the light for attracting mates. In return, *V. fischeri* is provided with a nutrient rich environment in which to grow. However, when the bacteria are free living in the ocean, they do not produce any light (Miller and Bassler 2001).

This same regulation of bioluminescence can be seen in a laboratory; when cells are at low density in a culture, they do not produce any light but after multiple rounds of cell replication, the culture will start to illuminate suggesting that bioluminescence was regulated by the growth of the cells themselves. However, when fresh cells are inoculated

into “conditioned media" (media removed from a bioluminescent culture) there is no lag in the induction of bioluminescence in the new cells. This indicates that something present in the media, and not the physical presence or density of the cells induced bioluminescence (Kempner 1967). In 1981, the first autoinducer was purified from a culture of *Photobacterium fischeri*. Using nuclear magnetic resonance spectroscopy, infrared spectroscopy, and mass spectrometry, the structure of the autoinducer was determined to be *N*-(3-oxohexanoyl)-3-aminodihydro-2(3H)-furanon, or *N*-(p-ketocaproy1) homoserine lactone (Eberhard 1981).

1.1.2. Genetic Transfer in Streptococcal Species

One of the most famous experiments in the history of biological research is the Griffith experiment from 1928 (Griffith 1928). Griffith found that dead virulent *Strepotococcus pneumoniae* cells were able to transform a similar nonvirulent strain into the virulent strain. Griffith described a “transforming principle” in the virulent strain that was able to change the nonvirulent cells into the virulent strain and make them deadly. A few years later it was discovered that this ability to transform was not a constant property, and was subject to some form of regulation (Dawson and Sia 1931). Although these experiments are most famous for the discovery of DNA as the genetic material (Avery, MacLeod et al. 1979), or Griffiths transforming principle, it also paved the way for characterizing quorum sensing.

A few decades after the discovering DNA was the transforming principle, more work began to explain the observations of Dawson and Sia. It was found that cells could

only transform DNA, or become competent, at particular cell densities (Tomasz 1964). Furthermore, this induction appeared to be dependent on extracellular factors, then called competence activators (Tomasz 1966). For several more decades, the identity of the competence activators could not be confirmed, but was predicted to most likely be a protein. It was not until 1995 that the structure of the competence activator was determined to be a peptide containing 17 amino acid residues (Havarstein, Coomaraswamy et al. 1995).

1.1.3. Difference in Quorum Sensing in Different Bacteria Classes

Bacteria were traditionally classified into two groups: the Gram positive, and Gram negative bacteria. Historically, bacteria were placed into either category based on the results of a Gram stain, which would color them purple if they were Gram positive, or pink if they were Gram negative. The physiological reason for this is that Gram positive bacteria have a single cell membrane surrounded by a thick wall of peptidoglycan that allows the binding of dyes in specific conditions, whereas Gram negative bacteria have a thin wall of peptidoglycan surrounded by two different cell membranes. However, more significant than the difference in color is the physiological difference between the cells and the implications for various cellular processes, such as quorum sensing and natural competence that follow different pathways in each class of bacteria even though the overall behavior is similar.

1.1.4. LuxR-LuxI Regulation in Gram Negative Bacteria

Due to it being the first well studied model for quorum sensing, most of what is known today regarding quorum sensing in Gram negative bacteria was learned by studying *V. fischeri.* As *V. fischeri* cells grow from a low-density culture, they produce the enzyme LuxI, the autoinducer synthase, at a basal level. This low level of LuxI production results in a low level of autoinducer production (Eberhard 1981). When growing in the limited space of the light organ of the host organism, the autoinducer increases in concentration quite rapidly. As the autoinducer concentration in the organ increases, more autoinducer diffuses back into the cytoplasm of the cells and binds to LuxR. This binding activates LuxR and makes it recruit RNA polymerase to the luxICDABE operon, resulting in the upregulation of the bioluminescence genes (Stevens, Dolan et al. 1994). This same mechanism for regulating quorum sensing is common amongst most other quorum sensing gram negative organisms and can regulate traits as diverse as virulence in *Pseudomonas aeruginosa* and antibiotic production in *Erwinia cartovora* (Miller and Bassler 2001).

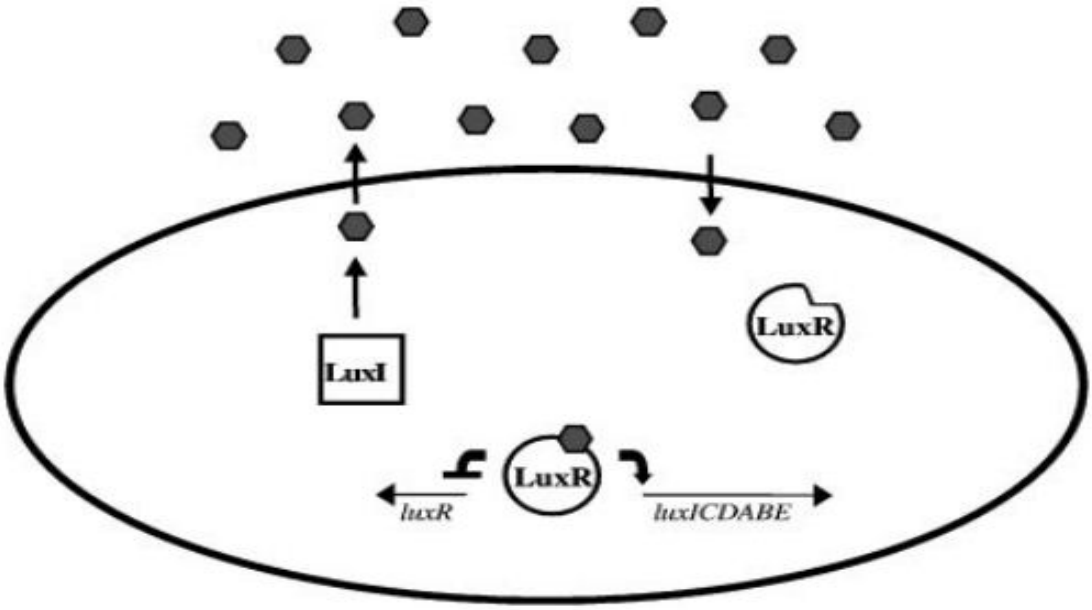

Figure 1.2: Quorum Sensing in Gram Negative Bacteria: In Gram negative bacteria, homoserine lactones are produced by LuxI and diffuse out of the cell. When the concentration accumulates in the environment, and enough diffuse back into the cell, they bind and activate LuxR. LuxR becomes activated recruits RNA polymerase to transcribe the other lux genes. Image from Miller and Bassler, 2001.

1.1.5. Peptide Pheromones in Gram Positive Bacteria

Although Gram positive bacteria regulate gene expression in an autoinducer mediated, cell density dependent manner similar to that of Gram negative bacteria, the actual mechanisms are quite different between the two classes of bacteria. After discovering a competence activator was necessary for transformation in *S. pneumoniae,* much work was done to purify and characterize the molecule used by Gram positive bacteria. It was estimated that the molecule was some sort of peptide or protein, due to the inactivation of the competence activator by proteases. However, steps to further purify the CF from media simply disrupted the biological activity of the molecule

(Havarstein 1999). It was not until 1990 when real progress was made towards identifying the competence activator.

First, the nucleotide sequence of a gene necessary for competence, ComA, was determined by genetic mapping. The sequence of ComA was compared to known sequences and was found to be very similar to an *Escherichia coli* secretary protein and other ATP-dependant transporters. This gave the researchers a clue that ComA likely functioned in secreting something that was important for competence induction (Hui 1991). Several years later, ATP-binding cassette (ABC) transporters were linked to the export of anti-microbial peptides. Most peptides that are exported through ABC transporters contain a glycine-glycine repeat that marks the position of processing during export. It was also determined that the N-terminal region of the ABC transporters are catalytic domains responsible for the cleaving the peptides at the double glycine marker (Havarstein, Coomaraswamy et al. 1995). Based on homology, ComA was predicted to have the same N-terminal catalytic domain thus narrowing the search for the CF to small peptides containing double glycine markers. Using this strategy, a purification technique was devised to isolate the proposed peptide. This technique worked and a 17-residue cationic peptide with sequence H-Glu-Met-Arg-Leu-Ser-Lys-Phe-Phe-Arg-Asp-Phe-Ile-Leu-Gln-Arg-Lys-Lys-OH was isolated and named the competence stimulating peptide (CSP) (Havarstein, Coomaraswamy et al. 1995). When isolated and added to a non-competent culture, the CSP was able to stimulate competence in *S. pneumonia.* Furthermore, a chemically synthesized peptide of the same sequence had the same biological activity as the purified CSP. (Havarstein, Coomaraswamy et al. 1995). These

experiments showed that the peptide alone was sufficient for inducing competence in wild-type cells.

The peptide sequence was traced back to the genome and was found to be processed from the nonfunctional ComC structural protein. This processing is performed by the N-terminal catalytic domain of ComA prior to its export. Sequencing the up and downstream regions of the ComC gene fortuitously revealed a histidine kinase (ComD), and a response regulator (ComE) within the same operon (Pestova, Havarstein et al. 1996). Together ComD and ComE make a two-component regulatory system, a signal transduction pathway common in bacteria. ComD and ComE were found to have an important role to quorum sensing through genetic manipulation (Pestova, Havarstein et al. 1996). Shortly after this discovery, it was confirmed that ComD is the receptor for the CSP (Havarstein 1996). This system of CSP's activating two component regulatory systems is common throughout a wide variety of Gram positive bacteria and regulates behaviors such as biofilm formation, virulence and sporulation (Kleerebezem, Quadri et al. 1997).

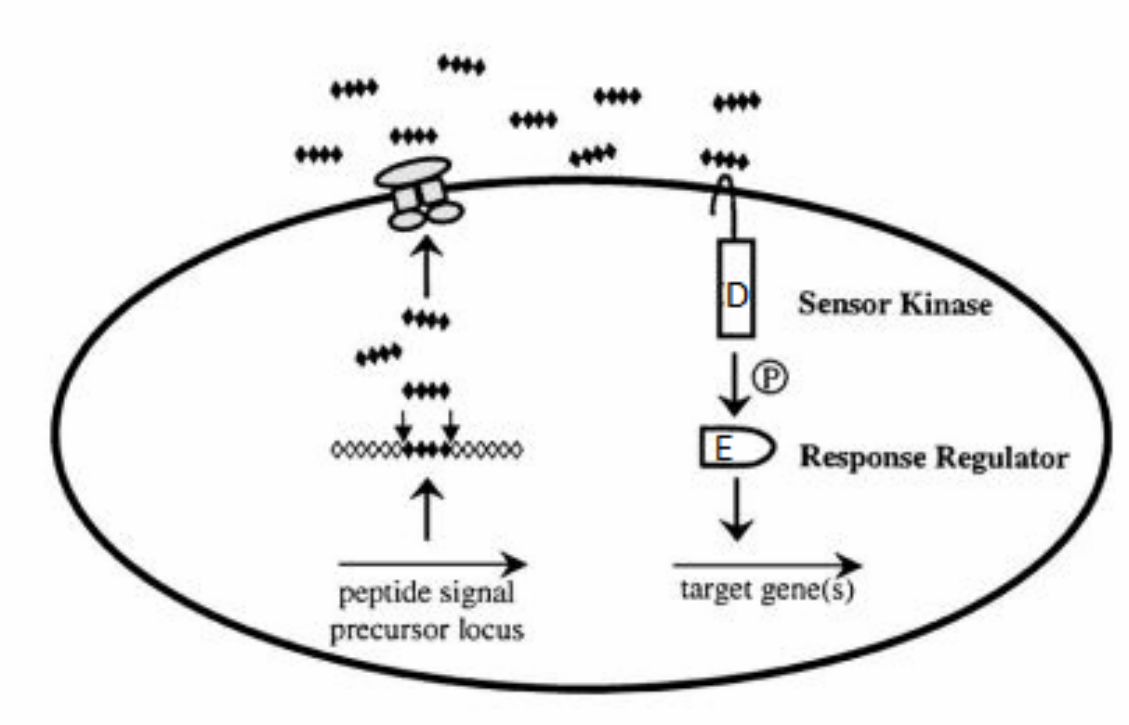

Figure 1.3: Quorum Sensing in Gram Positive Bacteria. Gram positive bacteria synthesize signal peptide precursors inside the cell. The precursor gets processed into the signal peptide and exported from the cell. When peptides accumulate, they bind to and activate the sensor kinase, which activates the response regulator to initiate transcription. Image from Miller and Bassler, 2001.

1.1.6. Quorum Sensing and Cell Survival

As a population of cells reaches a high density, several changes need to happen in the population in order to assure its survival. The Gram positive bacterium *Bacillus subtilis* utilizes quorum sensing as a way to make important decisions regarding cell life and death. When living in a crowded community, *B. subtilis* will sporulate, become competent, or try to continue growing as it has. Both sporulation and competence are controlled by quorum sensing and play an important survival role while in unfavorable environments (Lazazzera 1999).

As a culture of *B. subtilis* reaches high density, approximately 1-10% of the cells will become competent. There are two proposed reasons why competence is dependant

on population density. First, in a higher density population, it is more likely that there is exogenous DNA present in the media (due to cell lysis, DNA secretion, etc.). By restricting competence activation to high cell densities, it increases the likeliness that DNA will be available for uptake(Lazazzera 1999). In fact, in *S. pneumonia* and related species, naturally competent cells induce the lysis of cells that are not competent in order to generate more DNA in the environment for uptake (Steinmoen et al., 2002).

Secondly, quorum sensing could indicate that neighboring cells are of the same species and promote the uptake of more closely related DNA in an effort to prevent the uptake of possibly harmful DNA sequences. Both *B. subtilis* and *S. pneumonia* regulate competence through quorum sensing, but do not regulate the sequence of the DNA they take up, whether it is from closely related or highly divergent species. Other naturally competent species such as *Neisseria gonorrhoeae* and *Haemophilus influenzae* take in DNA in a sequence specific manner. This is done by having DNA uptake signal sequences marked throughout the genome, and then requiring DNA uptake sequence recognition before DNA can be imported (Chen 2004). Naturally competent Gram positive bacteria do not use DNA uptake sequences, but most likely need to regulate the DNA that is imported from the environment. Quorum sensing may replace the role of sequence specific uptake by promoting uptake when surrounded by cells of the same species.

In addition to nutrient starvation, sporulation is also dependent on high cell density (Grossman and Losick 1988). The main role of sporulation is to prevent cell death when starved of nutrients by becoming metabolically dormant and resistant to environmental factors. *B. subtillus* uses quorum sensing as a way to detect high cell

density, which in turn indicates the likelihood of high competition for nutrients. If the cells do not use quorum sensing as a regulator of sporulation, they would sporulate prematurely and unnecessarily since nutrients could become available soon with little competition for them. Since sporulation requires a significant amount of time and energy, it should only be used as a last resort, and quorum sensing is used to insure that it is.

In *B. subtilis*, there are a variety of secreted peptides that are used to direct cell differentiation. The most important of these are ComX and the Competence and Sporulation Factor (CSF). When ComX accumulates in the media it activates the membrane histidine kinase ComP, which in turn phosphorylates the response regulator ComA. ComA then induces transcription of several different regions, including the srfA operon which in turn activates ComK, which initiates the transcription of more than forty competence genes. CSF on the other hand acts in a more complicated manner. Between 1 and 5 nM concentration, CSF will induce competence, and above 20 μM, CSF will induce sporulation. CSF primarily functions by inactivating RapC, which prevents RapC from inactivating the ComA~P complex. These complicated quorum sensing regulated mechanisms allows for *B. subtilis* to differentiate when challenged by adverse environmental conditions to insure the survival of a portion of the population until conditions become more favorable (Lazazzera 1999).

1.2. Archaea

For a long time it was widely believed that all life could be classified as either eukaryotes or as prokaryotes. This is not the case anymore. In the 1970's Carl Woese

discovered that prokaryotes could be divided into two different groups (Domains), the Bacteria and Archaea, by comparing sequences of the 16S rRNA gene (Woese and Fox 1977) and Peter Gogarten then showed through rooting the tree of life that Archaea are more closely related to eukaryotes than they are to the other "prokaryote" lineage, demonstrating the term prokaryote is a poor taxonomic term (Gogarten et al., 1989). Although the distinction between the three domains (Bacteria, Archaea and Eukarya) was originally made based on the 16S rRNA gene sequences, further research has shown that archaea are unique for many more reasons, yet have a several traits in common with both bacteria and eukaryotes.

Archaea are best known for their extremophilic nature. Many archaea were originally discovered in extreme environments where it was thought life could not survive, such as in high temperatures, high salt concentrations, extremely acidic or basic pH, and high pressures (Konings, Albers et al. 2002). The name archaea was actually used to describe these organisms because they were thought to live in environments resembling that of the early Earth. However, it is now known today that bacteria often inhabit the same extreme environments where the archaea were first discovered, and archaea inhabit common microbial communities such as the oceans and even the human gut (Herndl, Reinthaler et al. 2005; Gill, Pop et al. 2006).

One property of extremophilic archaea that allows for them to survive in harsh conditions is their unique membrane structure. Both eukaryotes and bacteria have lipid bilayer membranes. Individual lipids are composed of non-polar fatty acyl chains that are ester linked to polar head groups. These lipids are oriented so the non-polar chains face to the inside of the membrane, while the polar head groups interact with the aqueous interior

and exterior of the cell. On the other hand, archaeal fatty acyl chains and head groups are instead linked by ether linkages. Furthermore, in certain species of archaea, such as the acidophiles and thermophiles, the inward facing acyl chains stretch from one head to the other, forming a so-called monolayer instead of a bilayer (Konings, Albers et al. 2002). Membranes composed of lipids that are linked by ethers as opposed to esters are sterically more stable. This increased stability of archaeal membranes allows for them to thrive in diverse conditions.

The first insight to the similarities shared between archaea and eukaryotes was their susceptibility and immunity to various antibiotics. Both archaea and eukaryotes are sensitive to 80S ribosome directed antibiotics, such as anisomycin, as well as aphidicolin, an inhibitor of the bacterial DNA polymerase. This hinted that archaea and eukaryotes share similarities in the machinery of replication, transcription and translation. Specifically, the DNA polymerase and the RNA polymerase are structurally very similar between eukaryotes and archaea. Also, archaea and eukaryotes both use TATA boxes as a transcription regulatory mechanism, whereas bacteria do not (Brown and Doolittle 1997).

At a glance there are obvious similarities between archaea and bacteria, such as the basic physiology. Organisms from both domains share similar cell size, and lack nuclei and membrane bound orgenells. Bacteria and archaea also share similar genome organizational features. This includes having one large circular chromosome, as well as several additional plasmids containing a variety of other genes. In both domains, genes are commonly organized into operons and are regulated by similar mechanisms (Brown and Doolittle 1997). Besides these physical traits, archaea and bacteria also tend to

occupy similar niches and while fighting for survival, encounter similar problems. This aspect of life for prokaryotes potentially drives evolution in both species in a parallel direction as they create similar solutions to ensure their continued existence.

Because archaea share much in common with the other two domains, understanding their evolution specifically can answer questions about the evolution of eukaryotes and bacteria as well. However, the amount of research being performed on archaea is smaller than in the other domains, leaving much left to discover.

1.2.1. Halophilic Archaea

Extremely halophilic archaea are unique organisms that are obliged to live in environments with high salt concentrations, such as natural salt lakes, salterns or even on salted meats. Most haloarchaeal species grow optimally at a NaCl concentration of 2-4 M, but some can live in saturated salt water and in solid salt crystals.

One of the most useful haloarchaeal models is *Haloferax volcanii*. In 1975, *H. volcanii* (then called *Halobacterium volcanii*) was isolated from mud at the northern end of the Dead Sea (Mullakhanbhai 1975). In contrast to the other haloarchaeal model, *Halobacterium* sp. NRC-1, *H. volcanii* utilizes simple sugars, such as glucose and synthesizes all of its required amino acids, which allows for easy manipulation on defined media while culturing *H. volcanii*. Due to the ability to culture *H. volcanii* in defined media, a range of genetic and biochemical tools have been developed in order to manipulate the organism. These include a pop-in/pop-out gene knockout system (Bitan-Banin, Ortenberg et al. 2003), inducible promoters (Large 2007), transformation

protocols (Cline, Schalkwyk et al. 1989), and protein purification techniques specific for *H. volcanii* (Allers 2010). Furthermore, the genome of *H. volcanii* was recently published and is readily available bioinformatics analysis (Hartman, Norais et al. 2010). With its ease in culturing, multitude of genetic tools, and the availability of its genome, *H. volcanii* serves as an excellent model for haloarchaea specifically, as well as the entire domain Archaea.

1.3. Natural Competence in Archaea

Since the study of archaea started there have only been a handful of reports of natural competence in various archaeal species. The first two reports came in 1987 from separate groups studying different methanogenic (archaea common in wetlands and animal guts that produce methane) strains. One group took strains of *Methanococcus voltae* that were unable to synthesize their own histidine, purine, and vitamin B_{12}, making them auxotrophic for those chemicals. They were able to abolish this auxotrophy by culturing the mutant strains with DNA isolated from wild-type strains (Bertani and Baresi 1987). The other group took a different approach and introduced DNA from a strain of *Methanobacterium thermoautotrophicum* that was resistant to the drug 5-flourouracil to a culture of wild-type cells (Worrell, Nagle et al. 1988). Using this method, they were able to introduce the drug resistance to the wild-type strain by simply adding DNA to the culture.

More recently, it was found that *Pyrococcus furiosus* is also naturally competent. The *pyrF* uracil biosynthesis gene was first mutated to become nonfunctional, and then

was restored by introducing DNA encoding the gene to the media. *P. furiosus* was able to take in the DNA molecule as genomic DNA, as a plasmid insert, or as a PCR fragment, and undergo transformation of the gene to restore the ability to synthesize uracil (Lipscomb, Stirrett et al. 2011).

1.3.1. Evidence for Natural Competence in *H. volcanii*

The observations of natural competence in the methanogenic archaea and in *P. furiosus* were made while trying to design genetic manipulation systems for their respective organisms. However, in the case of haloarchaea the prospects of natural competence and its relationship to the high recombinogenic nature of haloarchaea are of interest. For instance, it has been seen that levels of diversity in populations of *Halorubrum* were greater than sexually reproducing animals (Papke, Koenig et al. 2004).

It has been shown that *H. volcanii* is able to utilize DNA as a source of nutrition. DNA is made of carbon, phosphorus, and nitrogen, all of which can be utilized by *H. volcanii* when grown in starvation media devoid of these nutrients. However, it appears that DNA is most efficiently used as a source of phosphorus because it grows to a significantly higher density when provided additional sources of nitrogen and carbon, but negligibly different when a phosphorus source is added as well (Figure 1.4). Also, the rate and maximum cell density are dependent on the concentration of supplemented DNA, with higher concentrations yielding cultures that achieve higher cell densities more quickly.

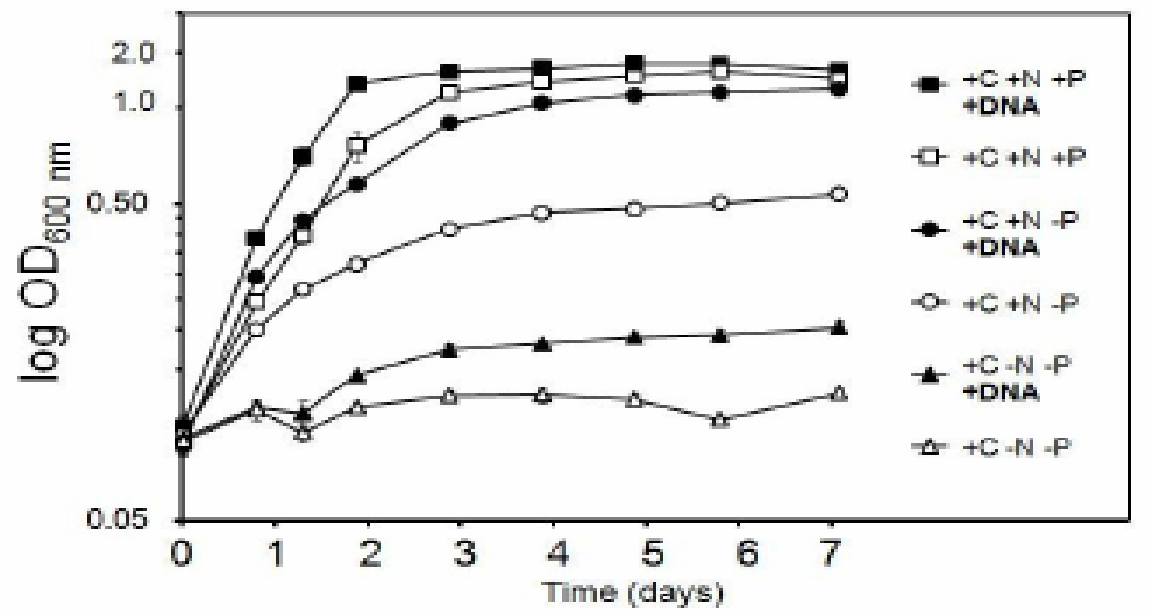

Figure 1.4: Growth of *H. volcanii* when Provided Different Nutrients in Addition to DNA: When provided a source of carbon, nitrogen and phosphorus, the addition of DNA has almost no effect on growth. When phosphorus is absent, the addition of DNA greatly increases the growth back to a normal rate. When nitrogen and phosphorus are absent, DNA addition restores some of the growth of the culture, but not to the same level as when all nutrients are provided. Image from Chimileski et al., 2011.

1.3.2. Methylation Biases DNA Uptake in *H. volcanii*

Most Gram positive bacteria do not regulate which types of DNA they take up, whether it be from divergent or similar species, whereas most Gram negative bacteria use DNA uptake sequences to assure that DNA that is imported is from closely related species. At first, *H. volcanii* appeared to regulate uptake similarly to Gram negative bacteria, in that they could consume DNA from closely related species, but rejected DNA from divergent sources, such as *E. coli*. However, the *H. volcanii* genome did not appear to contain DNA uptake sequences like competent Gram negative bacteria do. Instead, it was discovered that the methylation status of DNA played a significant role in its uptake. When supplemented with unmethylated DNA from *E. coli* (from *dam-/dcm-* mutant), *H.*

volcanii cultures grew to a higher density than when supplemented with either wild-type *E. coli* DNA or even conspecific DNA (Figure 1.5). It is not clear exactly how the methylation detection mechanism works, but methylation patterns may serve as a regulatory mechanism to prevent the uptake of possibly detrimental DNA, such as DNA from divergent organisms or improperly methylated DNA.

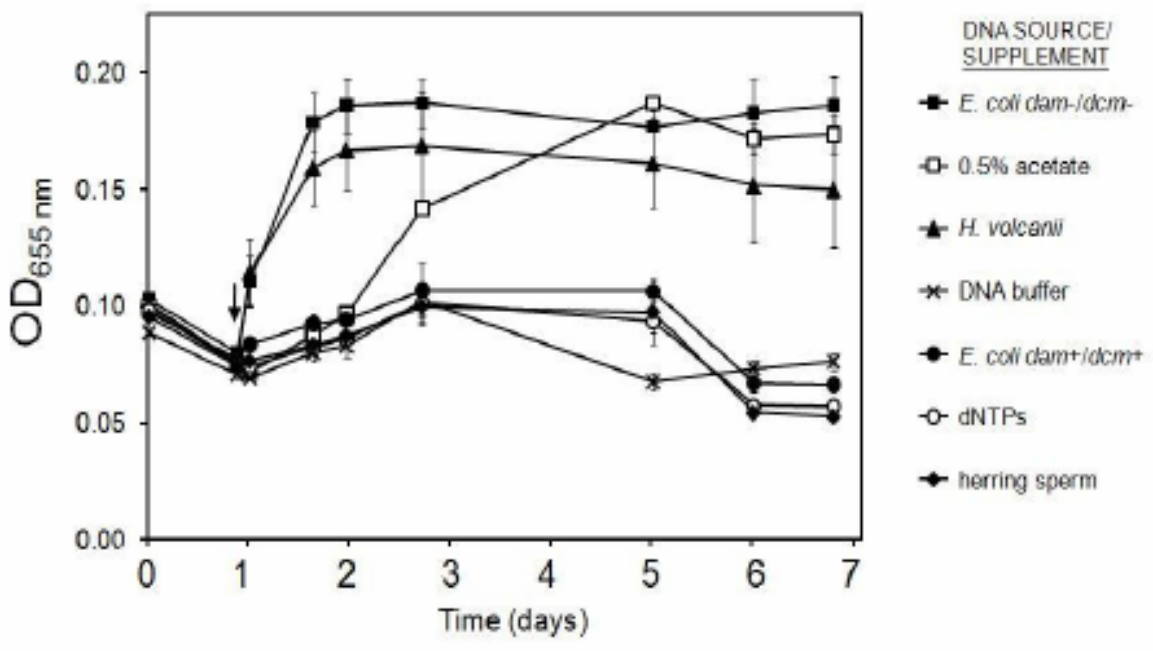

Figure 1.5: **Growth on DNA is Dependant on Methylation:** When grown in starvation media devoid of carbon, nitrogen and phosphorus the addition of conspecific DNA, acetate and *E. coli dam-/dcm-* unmethylated DNA yields low levels of growth. The addition of dNTP's, herring sperm DNA, and wild-type *E. coli* methylated DNA does not improve growth when compared to the DNA buffer addition control. Image from Chimileski et al., 2011.

1.4. Natural Competence and Quorum Sensing

With examples of natural competence in archaea surfacing, it is beginning to look as if it may not be an isolated trait and could have extremely important implications for the evolution of archaea. Based on the wealth of information known about natural competence in bacteria, it leads to questions regarding similarities and differences

between bacterial and archaeal natural competence. Because quorum sensing is important for regulating natural competence in a large number of naturally competent bacteria, it may also be the case in archaea. For this reason, studying quorum sensing in archaea could provide additional insight into natural competence, as well as other population controlled behaviors.

1.4.1. Published Quorum Sensing Evidence for Archaea

As of now, there has only been one paper published that provides initial evidence for quorum sensing in any archaean. The archaean in which this behavior was observed was the halophilic *Natronococcus occultus* (Paggi, Martone et al. 2003). An extracellular protease is secreted from the organism as it approaches late exponential phase, and into the stationary phase of growth. Protease secretion is commonly monitored by quorum sensing in bacteria, leading to the idea that some extracellular proteases are possibly regulated by the same quorum sensing mechanism in archaea.

When conditioned media extracted from high density cultures was added to low density cultures, the protease was synthesized and secreted into the media by the cells. The presence of the protease was detected after the addition of conditioned media by western blotting using an antibody raised against the protease. This lead to the idea that some quorum sensing molecule may be present in the media of the dense culture that promoted the synthesis of the protease in the dilute culture. To test the identity of the autoinducer, the biosensor *Agrobacterium* was used to detect the presence of homoserine lactones in the conditioned media. The condition media elicited a response in the

biosensor similar to the response when a positive control (a Gram negative quorum sensing bacterium) is used, leading the researchers to conclude that *N. occultus* communicates through homoserine lactones. These homoserine lactones are at least responsible for the initiation of protease synthesis and secretion, and possibly other behaviors as well (Paggi, Martone et al. 2003).

1.4.2. Quorum Sensing in *H. volcanii*

Around the same time the *N. occultus* paper was published, a thesis was written that described initial evidence supporting quorum sensing in *H. volcanii* (Bitan-Banin 2002). First, *E. coli* plasmids containing the archaeal *hdrA* gene, which encodes an enzyme essential for thymidine synthesis, were artificially transformed into *H. volcanii* WR445 strain cells that lack the *hdrA* gene. Because the transformed plasmids are from *E. coli*, they do not contain archaeal promoters, and therefore can only be expressed when the gene recombines with the archaeal genome downstream from an active promoter. The transformed cells were plated onto selective media that did not contain thymidine, and colonies that grew were transferred to liquid media. The assumption here is that if the gene was integrated into the chromosome downstream from a promoter that is activated by quorum sensing, the cells will not be able to grow until the threshold amount of quorum sensing molecules is present. In a colony on solid media, there is very little diffusion of secreted molecules, and the threshold concentration is met quickly, allowing for the transcription of the *hdrA* gene without much delay. However, in a low density liquid culture, the quorum sensing molecules diffuse more readily and there is a

significant lag in achieving the required concentration to initiate transcription of density dependant genes. If the *hdrA* gene is located downstream of a promoter that is induced by quorum sensing molecules, the cells will not be able to grow until autoinducers accumulate in the media and induce its transcription.

Using this method, three strains were isolated that exhibited an extended lag phase when grown in the absence of supplemented thymidine, but a normal growth curve when thymidine was provided in the media. This suggested that the *hdrA* gene was integrated downstream of three quorum sensing induced promoters. Furthermore, adding conditioned media to low density cultures abolished the extended lag phase, indicating that something present in the media of high density cultures stimulated the activation of these promoters. After sequencing the genes located downstream from these quorum sensing induced promoters in wild-type cells, it was seen that they were not transcribed in low density cultures unless conditioned media was added, as indicated by northern blot analysis.

Attempts were then made to characterize the molecule that was responsible for inducing the transcription of density dependent genes. First, it was found that the autoinducer precipitated between 75% and 80% ethanol. Next, the conditioned media was run through filters with a cutoff of 3,000 Daltons, and it was found that the flow through had the same biological activity as the ordinary conditioned media, indicating that the molecule is smaller than 3,000 Daltons. The autoinducer was then retained on hydroxylapatite columns, which lead to the conclusion that it contained phosphate. Based on this hypothesis, alkaline phosphatase was used and found to destroy the biological activity of the autoinducer. Based on these chemical observations, it was proposed that

the signal molecule may be a phosphate ester. If it is indeed a phosphate ester, then its activity is not only coordinated by its rate of synthesis, but also by its inactivation by phosphatases.

1.5. Objective of the Thesis

Although there have been a few accounts of quorum sensing in archaea, they are not concrete proof that the phenomonon occurs in archaea. Furthermore, the observations reported seem to point in different directions regarding the identity of the type of molecule used by archaea to coordinate density specific behavior. This thesis will attempt to provide additional insight into quorum sensing in archaea and design a method to test for the presence of autoinducers made by *H. volcanii.*

2. Materials and Methods

2.1. Bioinformatics

2.1.1 Comparative Genomics Study Protein Selection

To represent quorum sensing genes from Gram negative bacteria, LuxI and LuxR from *V. fisheri* were selected. *V. fisheri* is the most common model to study quorum sensing in Gram negative bacteria. LuxI and LuxR are the autoinducer synthase and autoinducer receptor, and therefore represent the most important two quorum sensing proteins for Gram negative bacteria.

For Gram positive bacteria, ComA, ComC, ComD and ComE from *S. pneumoniae* was selected. ComA is the exporter and processor of the ComC peptide responsible for competence induction is *S. pneumoniae*. ComD and ComE make up the two-component regulatory system that binds the peptide and initiates competence induction. Based on the nutritional competence seen in *H. volcanii* and given the common role of quorum sensing in its regulation, it seems possible that similar proteins could be involved in autoinducer peptide secretion in archaea. Also selected are ComX and PhrC from *B. subtillus*. These were selected because they are the precursor proteins for the two secreted peptide autoinducers in *B. subtilus*, ComX and CSF.

2.1.2. BLASTs

Basic Local Alignment Search Tool (BLAST) was used to compare the sequences on known quorum sensing genes from bacteria with the genomes of specific archaea. The archaea chosen and the reason why are outlined in table 2.1. BLAST's were performed at http://blast.ncbi.nlm.nih.gov/Blast.cgi (Stephen F. Altschul 1997; Stephen F. Altschul 2005).

Table 2.1: Archaea Selected for Comparative Genomics Study.

Species	Reasons
Haloferax volcanii DS2	Nutritional competence observed (Chimileski et al, 2011). Strain is available in the lab.
Methanococcus voltae A3	Genetic transformation has been observed (Bertani and Baresi, 1987).
Natronomonas pharaonis DSM 2160	Evidence of quorum sensing has been published for *Natronococcus occultus*, however no sequence is avaiable. *N. pharaonis* was selected due to its relation and genome availability.

2.2. Conditioned Media Growth Study

2.2.1. Strains and Culture Conditions

The *H. volcanii* strain used in this research is the Wild-type DS2 strain isolated from the Dead Sea as described in Mullakhanbhai and Larsen, 1975. The two media types used were the rich, undefined H media, and the defined HV-Minimal media (HV-Min). The H media contained (per 1 liter H_2O) 150 g NaCl, 36.9 g $MgSO_4*7H_2O$, 5 g yeast extract, 5 mL of 1 M KCL solution, 1.8 mL of 75-mg/L $MnCl_2$ solution, and 50 mL of 1 M Tris-HCl (pH 7.2). After autoclaving and cooling, 5 mL of 1 M CaCl was filter sterilized and added.

HV-Min contained (per 800 mL H_2O) 115.2 g NaCl, 16.8 g $MgSO_4*7H_2O$, 14.4 g $MgCl_2*6H_2O$, 3.36 g KCl, and 33.6 mL 1M Tris-HCl (pH 7.2). After autoclaving and cooling, the following additional supplements were added: 3.4 mL of 60% sodium lactate Solution, .2 mL glycerol, 4 mL of 1M NH_4Cl solution, 1.6 mL of .5M potassium phosphate buffer (pH 7.5), .1 mL of 6.4 mg/mL Thiamine solution, .1 mL of .8 mg/mL Biotin solution, .8 mL of Trace element solution and 2.4 mL of 1M $CaCl_2$ solution. For some applications of nutritional competence experiments, potassium phosphate was often excluded from the media.

E. coli dam-/dcm- strains were used for collection of DNA. *E. coli* were cultured in LB media containing (per 1 L H_2O) 10 g NaCl, 10 g Tryptone, and 5 g Yeast Extract. For all applications, ampicillin was added to a final concentration of 100 μg/ml.

2.2.2. *E. coli* DNA Purification

E. coli Δ*dam-/dcm-* strains were transformed using the PTA131 plasmid to make cells resistant to ampicillin to ensure quality of cultures. Transformed cells were plated onto selective LB AMP plates and grown overnight at 37ºC. Colonies were then picked and inoculated into 1 liter of LB AMP and grown overnight at 37º with shaking at 150 RPM. Next, cells cultures were centrifuged of at 15,000 RPM for 15 minutes. Pelleted cells were resuspended into a smaller volume and lysed using Promega SV Miniprep Kit. Lysed cellular components were precipitated in ethanol overnight at -20ºC. Precipitated components were subjected to 3-4 rounds of phenol/chloroform extraction to obtain pure DNA. The pure DNA was again precipitated in ethanol overnight and resuspended in a 10 mM Tris DNA buffer at a concentration between 2500 ng/μL and 3000 ng/μL.

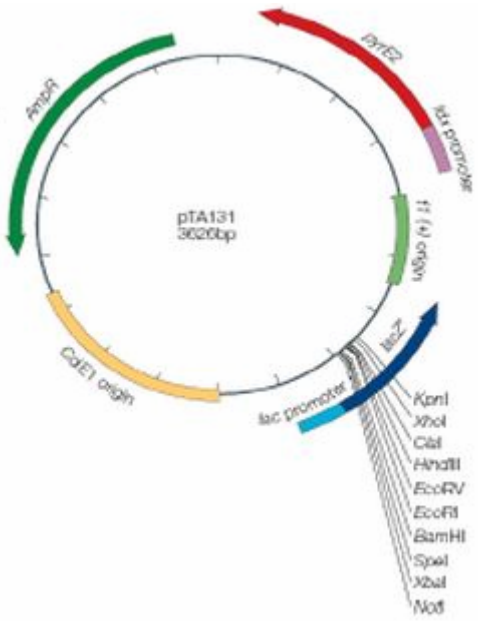

Figure 2.1: PTA131 Plasmid Map

2.2.3. Conditioned Media Preparation

H. volcanii cells were grown from a dilute culture to the mid log phase (OD_{600} ~ .4) in H media. At this point, cells were centrifuged at 15,000 RPM for 5

minutes and resuspended in Hv-Min lacking phosphate buffer. The centrifugation and resuspension step was repeated 3 times to eliminate the presence of any residual H media. The cells were resuspended for the last time in Hv-Min and set to an OD_{600}= 1.5. The cultures were placed in a shaker at 42ºC for 24 hours. After this time, the cells were pelleted by centrifugation at 15000 RPM for 5 minutes, the media was collected and the cells were discarded.

2.2.4. **Conditioned Media Growth Studies**

H. volcanii cells were grown from a dilute culture to the mid log phase (OD_{600} ~ .4) in H media. They were washed three times in the same way as described in conditioned media preparation. Half of the cells were then resuspended in fresh HV-Min to an OD_{620} ~ .04 while the other half were resuspended in conditioned media to an OD_{620} ~ .04. Each culture was distributed into 9 180 µL aliquots in a 96-well cell culture plate. Also added to the plate was 200 µL of fresh HV-Min and 200 µL of conditioned media not inoculated with cells to be used as blanks. Three wells for each culture were supplemented with either 20 µL of *E. coli* dam-/dcm- DNA (2800 ng/µL), 20 µL of DNA buffer (10 mM Tris), or 20 µL of 1 M sodium acetate. The 96 well plate was then placed into a Fischer Multiscan FC plate reader, which was set to incubate at 37ºC, shake the plate at a low speed, and take readings every twenty minutes for 90 hours.

2.2.5. Initial Characterization of the Autoinducer

The *H. volcanii* putative autoinducer reported by Bitan-Binan was ethanol soluble. To confirm this observation, and create a method to allow for enzymatic testing of the conditioned media, an ethanol precipitation was performed on the conditioned media immediately upon harvest. 100 µL of 3 M sodium acetate was added to 1 mL of conditioned media, followed by 3 mL of cold (-20ºC) ethanol. The media was chilled at -20ºC for 6 hours and then spun in a centrifuge at 15,000 RPM for 5 minutes to pellet the precipitate. The precipitate was then resuspended in fresh conditioned media at either 2X, 1X, or .5X the original concentration of the conditioned media. Cells were then resuspended in this media and a growth study was performed as outlined in the section above.

To test if the putative autoinducer is stable at high temperatures, conditioned media was collected and then heated at 80ºC in a water bath for 20 minutes. After the heating, the media was briefly placed at 4ºC to get the temperature back to room temperature. Cells were then resuspended in this media and a growth study was performed.

3. Results

3.1. Comparative Genomics Study

The results in this section show the best BLAST hits from comparing the sequences of known bacterial quorum sensing proteins to all of the proteins in the proteomes of the selected archaea. In other words, the program will find archaeal proteins that show sequence similarity to the query proteins and perform statistical analyses to show how strong the similarities are and where they occur in the sequence.

The statistics presented from the BLAST analysis are the scores, the query coverage and the E value. The E value represents the expected number of matches with the same amount of similarity due to chance alone. This means that if the E value is high, the detected homology is likely due to random chance, and could be obtained using random sequences. Likewise, a low E value indicates the similarity is most likely due to common descent. An E value smaller than 10^{-4} is considered significant for suggesting an ancestral linkage between the two sequences. Query coverage represents the percentage of detectable homology between the identified and queried protein. For example, if the query coverage is 75%, then 75% of the sequence shows some homology. The scores are a culmination of E value, query coverage and other parameters that give a value for how significant the detected homology is.

The following figures of this section use a color coded representation to show the scores along the length of the query protein. The best BLAST hits in each organism are

represented by horizontal lines underneath the red line marked “query.” The color coded scale is above that and indicates scores along the length of the query protein. When the scores are under 40, the lines appear black, when the scores are between 40 and 50 they appear blue and so on. The lines correspond to the descriptions in the table immediately below that give the name of the protein in each organism. In other words, the line on top corresponds to the top result presented in the corresponding table along with its statistical data.

3.1.1. Gram Negative Quorum Sensing Proteins

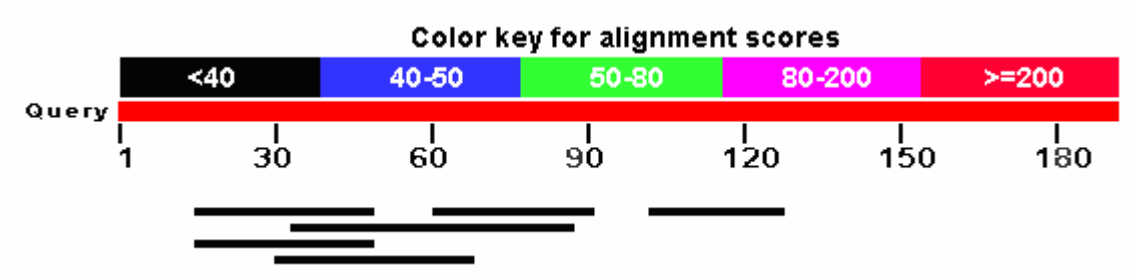

Figure 3.1.1: BLASTp of *V. fischeri* LuxI: Low score indicates that almost no significant homology was found. Query coverage was low and suggests that possible detected homology only occurs for no more than 28% of the sequence. The high E values suggest any detected homology is most likely due to random chance. Therefore, no significant homology was detected between any protein from the selected archaeal genomes and LuxI from *V. fischeri*.

Table 3.1.1: BLASTp of *V. fischeri* LuxI:

Description	Max score	Total score	Query coverage	E value
cobalt chelatase [Natronomonas pharaonis DSM 2160]	29.3	29.3	17%	0.32
dihydroxy-acid dehydratase [Natronomonas pharaonis DSM 2160]	26.2	26.2	28%	2.3
cobalamin biosynthesis protein [Haloferax volcanii DS2]	26.2	26.2	17%	2.6
threonine ammonia-lyase [Haloferax volcanii DS2]	25	25	20%	5.8

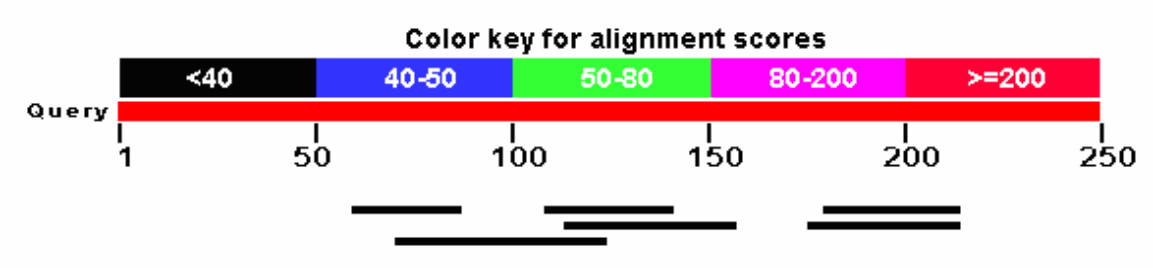

Figure 3.1.2: BLASTp of *V. fischeri* LuxR: The low scores indicate that almost no significant homology was found. Query coverage was low and suggests that possible detected homology only occurs for no more than 21% of the sequence. High e values suggest that this low amount of homology isn't unique, and is most likely due to random chance. Therefore, no significant homology was detected between any protein from the selected archaeal genomes and LuxR from *V. fischeri*.

Table 3.1.2: BLASTp of *V. fischeri* LuxR

Description	Max score	Total score	Query coverage	E value
hypothetical protein NP3842A [Natronomonas pharaonis DSM 2160]	25	25	11%	9.4
periplasmic copper-binding protein [Methanococcus voltae A3]	25.4	25.4	17%	6.4
histidinol-phosphate aminotransferase [Natronomonas pharaonis DSM 2160]	25.4	25.4	21%	6.9

There was no significant homology detected between LuxI and LuxR from *V. fischeri*, and any proteins from *H. volcanii*, *N. pharaonis*, and *M. voltae*. The scores and query coverage were both low, and the E values seen were extremely high in every case indicating that the detected homology was most likely due to random chance. Based on this bioinformatic data, it seems unlikely that the selected archaea have any proteins that are evolutionary similar to the two key quorum sensing genes in *V. fischeri*.

3.1.2. Gram Positive Quorum Sensing Proteins

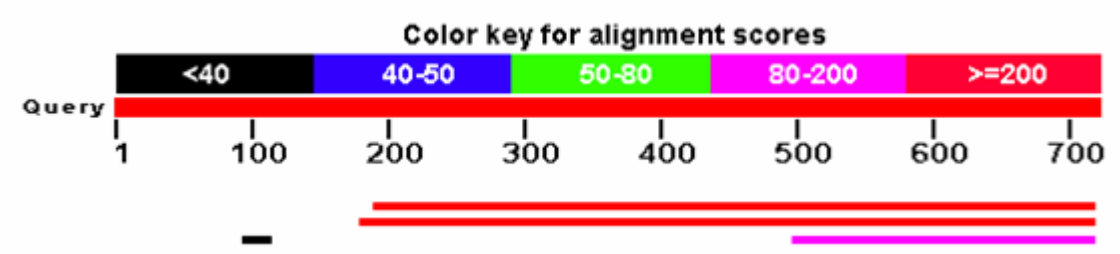

Figure 3.2.1: BLASTp of *S. pneunomiae* ComA: As indicated by the query coverage, significant homology was detected for 73% and 74% of the length of hypothetical ABC transporters in *H. volcanii* and *N. pharaonis*, respectively, with scores reaching over 200 and an E value that suggests a near zero possibility of the homology being random. The length of the *H. volcanii* homolog is 645 amino acids and the homolog for *N. pharaonis* is 641 amino acids long. The identified homolog of *M. voltae* is an ABC transporter subunit that has only 241 homologous amino acids.

Table 3.2.1: BLASTp of *S. pneunomiae* ComA

Description	Max score	Total score	Query coverage	E value
ABC transporter ATP-binding/permease [Haloferax volcanii DS2]	238	238	73%	1.00E-63
ABC-type transport system ATP-binding protein [Natronomonas pharaonis DSM 2160]	214	214	74%	4.00E-56
phosphate ABC transporter, ATPase subunit [Methanococcus voltae A3]	86.7	86.7	30%	8.00E-18

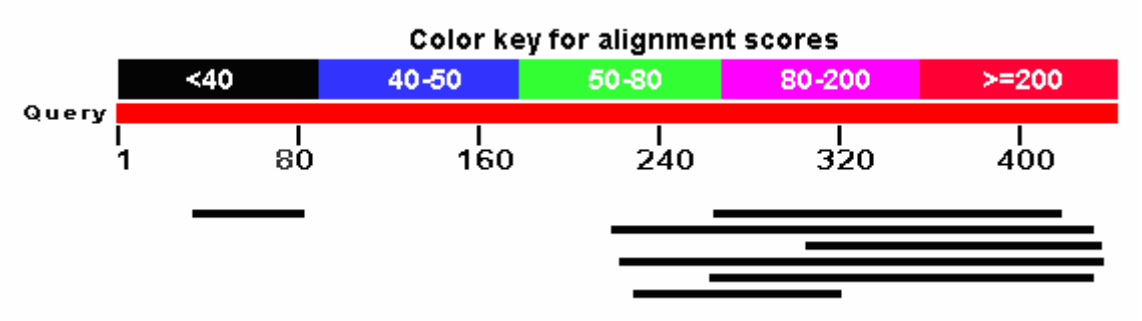

Figure 3.2.2: BLASTp of *S. pneunomiae* ComD: The low scores indicate that almost no significant homology was found. Query coverage was low and suggests that possible detected homology only occurs for no more than 48% of the sequence. High e values suggest that this low amount of homology isn't unique, and is most likely due to random chance. Therefore, no significant homology was detected between any protein from the selected archaeal genomes and ComD from *S. pneumoniae*.

Table 3.2.2: BLASTp of *S. pneunomiae* ComD.

Description	Max score	Total score	Query coverage	E value
HTR-like protein [Haloferax volcanii DS2]	30.8	30.8	34%	0.35
restriction system mrr-like protein [Natronomonas pharaonis DSM 2160]	29.3	29.3	11%	0.99
signal-transducing histidine kinase/response regulator [Natronomonas pharaonis DSM 2160]	28.9	28.9	48%	1.2
signal-transducing histidine kinase [Natronomonas pharaonis DSM 2160]	27.7	27.7	29%	2.9
signal-transducing histidine kinase-like protein [Haloferax volcanii DS2]	26.9	26.9	48%	4.4

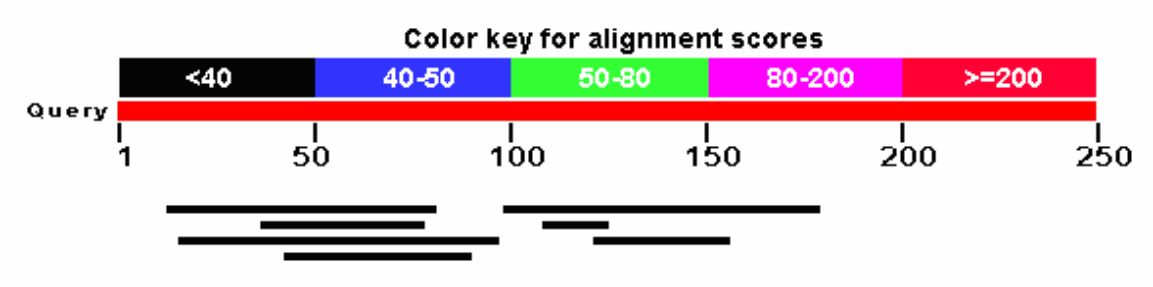

Figure 3.2.3: BLASTp of *S. pneunomiae* ComE: The low scores indicate that almost no significant homology was found. Query coverage was low and suggests that possible detected homology only occurs for no more than 32% of the sequence. High e values suggest that this low amount of homology isn't unique, and is most likely due to random chance. Therefore, no significant homology was detected between any protein from the selected archaeal genomes and ComE from *S. pneumoniae*.

Table 3.2.3: BLASTp of *S. pneunomiae* ComE.

Description	Max score	Total score	Query coverage	E value
TatD-related deoxyribonuclease [Methanococcus voltae A3]	28.9	28.9	32%	0.59
dehydrogenase/ reductase 7 [Natronomonas pharaonis DSM 2160]	26.2	26.2	6%	4.1
cytidylate kinase [Haloferax volcanii DS2]	25.4	25.4	27%	6.7
hypothetical protein Mvol_0892 [Methanococcus voltae A3]	25.4	25.4	16%	7.1

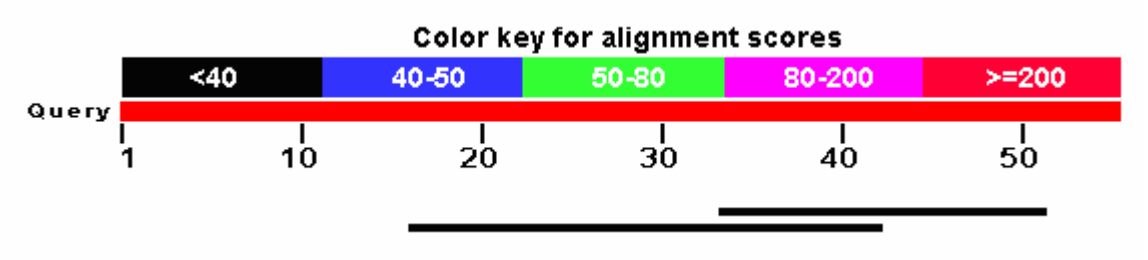

Figure 3.2.4: BLASTp of *B. subtillus* ComX: The low scores indicate that almost no significant homology was found. Query coverage was low and suggests that possible detected homology only occurs for no more than 47% of the sequence. High e values suggest that this low amount of homology isn't unique, and is most likely due to random chance. Therefore, no significant homology was detected between any protein from the selected archaeal genomes and ComX from *B. subtillus.*

Table 3.2.4: BLASTp of *B. subtillus* ComX.

Description	Max score	Total score	Query coverage	E value
4Fe-4S ferredoxin iron-sulfur binding domain protein [Methanococcus voltae A3]	23.1	23.1	32%	6.7
hypothetical protein NP4114A [Natronomonas pharaonis DSM 2160]	22.7	22.7	47%	9.7

PhrC

The BLAST program was not able to detect any significant homology between archaeal proteins and PhrC from *B. subtillus*.

ComC

The BLAST program was not able to detect any significant homology between archaeal proteins and ComC from *S. pnuemoniea*.

After comparing all of the targeted Gram positive quorum sensing proteins with the proteomes of *H. volcanii, N. pharaonis,* and *M. voltae*, the only quorum sensing

protein found with significant homology was ComA from *S. pneumoniae*. Figure 3.2.1. and table 3.2.1. show extremely significant homology for the C-terminus 73% and 74% ends of two ABC transporter proteins in *H. volcanii* and *N. pharaonis*, as well as the C-terminus 30% of an ABC transport subunit in *M. voltae*. The scores were high in every case, and the low E-values indicate that the detected homology was not likely by chance.

For all other target Gram positive quorum sensing proteins compared, the max score never rose above 31, and the high E values observed suggest that the homology seen was most likely by chance. Based on this bioinformatic evidence, it seems likely that the only targeted Gram positive quorum sensing protein with significant homology in the peptide exporter and processor ComA from *S. pneumonia*.

3.2. Conditioned Media Stimulated Growth on DNA

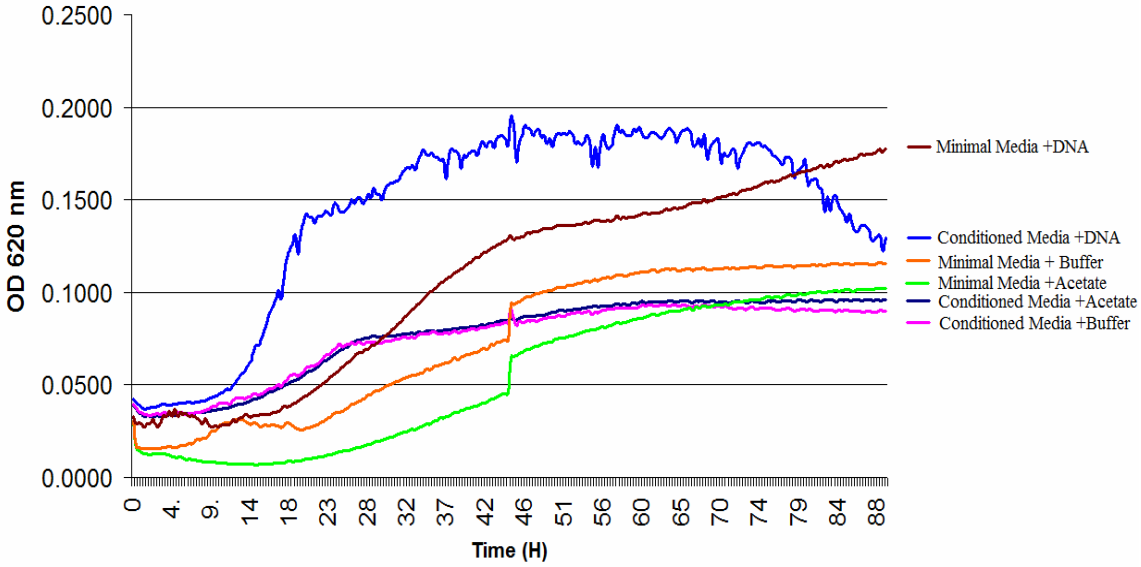

Figure 3.4.1: Effect of conditioned media on nutritional competence in *H. volcanii*. Growth of *H. volcanii* cultures in either fresh minimal media or conditioned media was observed by measuring absorbance at 620 nm over 90 hours every 20 minutes. The cultures were suspended in either conditioned media or fresh HV-Min media and supplemented with sodium acetate, *E. coli dam-/dcm-* DNA, or DNA buffer. The legend on the right of the figure indicates which media and supplement combination is represented by which colored line. All lines represent the average of three replicates. The sudden increase in OD at the 45th hour is an artifact of adjusting the plate reader.

To test for the presence of autoinducers in the media of high density cultures responsible for competence induction, cells were suspended in conditioned media harvested from high density cultures. If autoinducers are present in the media, a low density culture suspended in conditioned media should be able to utilize DNA sooner, and at a higher efficiency than a culture suspended in fresh HV-Min that has no autoinducers present.

It was seen that all cultures suspended in conditioned media started growing around the same time. However, the culture supplemented with DNA grew at a much quicker rate and achieved a significantly higher cell density than the cultures

supplemented with acetate or DNA buffer. This same behavior was also seen in the HV-Min cultures, in that the culture supplemented with DNA grew at a faster rate and to a higher cell density. This was expected because DNA serves as the only source of phosphorus in the media in both cases.

The important observation from this experiment is that the culture suspended in conditioned media and supplemented with DNA grew at the highest rate. This indicates that there may have been autoinducers present in the conditioned media that allowed for quicker and more efficient utilization of the supplemented DNA than the HV-Min culture devoid of possible autoinducers.

3.2.1. Initial Characterization of Possible Autoinducer

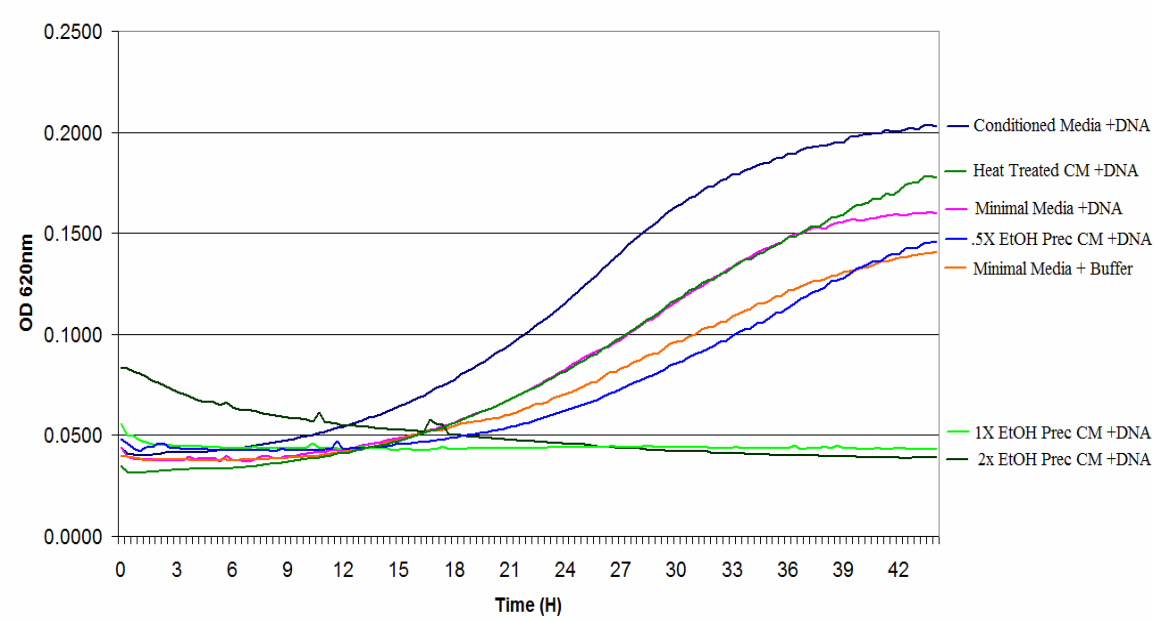

Figure 3.5.1: Effect of treated conditioned media on nutritional competence in *H. volcanii*. Growth of *H. volcanii* cultures in either fresh minimal media (pink and orange lines) or conditioned media (dark blue line) was observed by measuring absorbance at 620 nm every 20 minutes for a total of 45 hours. Also, conditioned media was subjected to either ethanol precipitation (neon green, dark green, and blue lines) or a heat treatment at 80ºC for 20 minutes (green). Cultures were supplemented with either *E. coli* dam-/dcm- DNA or DNA buffer. The lines for the ethanol precipitation conditioned media cultures are the average of two replicates, and all other lines are the average of three replicates.

To investigate the properties of the putative autoinducers, conditioned media was subjected to either heat treatment or ethanol precipitation. The heat treated conditioned media was heated at 80ºC for 20 minutes and cooled to room temperature before it was used to resuspend cells. To test solubility in ethanol, an ethanol precipitation was performed (as described in materials and methods) on conditioned media. The precipitate was then resuspended in fresh HV-Min at either 2X, 1X, or .5X the original concentration of the conditioned media.

The original observation that conditioned media enhanced the ability to utilize exogenous DNA was confirmed. Cells resuspended in conditioned media and

supplemented with DNA grew to a higher density and at a faster rate than cells resuspended in HV-Min with supplemented DNA. Ethanol precipitated conditioned media that was reconcentrated is fresh HV-Min at either 2X or 1X the original concentration appeared lethal because the average OD decreased. The ethanol precipitated conditioned media reconcentrated at .5X the original concentration grew at approximately the same rate as the HV-Min culture supplemented with DNA, indicating that the putative autoinducer did not precipitate in the ethanol. The heat treated conditioned media culture supplemented with DNA grew at approximately the same rate as the Hv-Min culture, indicating the heat treatment eliminated the effect of the conditioned media.

4. Discussion

4.1. Comparative Genomics Study

To gain an initial insight into possible quorum sensing proteins in archaea, a comparative genomics study was performed to identify any proteins in archaea that may function similarly to known quorum sensing proteins in model bacteria. There are currently eighty-five available archaeal genomes in the NCBI database. The three archaea chosen for this study were *H. volcanii*, *N. pharaonis* and *M. voltae* for the reasons outlined in table 3.1.

4.1.1. Gram Negative Quorum Sensing Proteins

The two proteins selected to represent quorum sensing proteins from Gram negative bacteria were LuxI and LuxR from *V. fischeri*. Quorum sensing was first discovered in the *Vibrio* genus, and *V. fischeri* has risen to become the most popular model in studying quorum sensing in Gram negative bacteria. LuxI is the autoinducer synthase enzyme responsible for the production of homoserine lactone autoinducers. LuxR is the receptor protein that binds the autoinducer and recruits the RNA polymerase to transcribe the remaining lux genes. The LuxI-LuxR quorum sensing regulation system is found throughout quorum sensing Gram negative bacteria and is responsible for most of the population based behaviors seen in Gram negative bacteria.

After comparing the protein sequences for LuxI and LuxR to all known proteins in the chosen archaeal proteomes, no significant homology was found. This same result was seen for the remaining archaeal proteomes as well (data not shown). This suggests that if archaea use quorum sensing, they most likely don't use the same LuxI-LuxR mechanism as Gram negative bacteria. In Paggi et al, 2003, the authors believe they found evidence for homoserine lactone based quorum sensing in *N. pharaonis*. If *N. pharaonis* does it fact use homoserine lactones, the proteins involved in making and processing them are likely highly divergent from the Lux proteins found in Gram negative bacteria.

4.1.2 Gram Positive Quorum Sensing Proteins

The proteins selected from Gram positive bacteria were ComA, ComD, ComE and ComC from *S. pneumoniae*, as well as ComX and PhrC from *B. subtillus*. ComA is the peptide processor and exporter that cleaves the signal peptide from the ComC precursor peptide and exports it into the environment. ComD is the histidine kinase that binds the peptide autoinducer and phosphorylates the response regulator ComE. Upon activation, ComE initiates the transcription of the late competence genes. ComX and PhrC are the precursor proteins of the ComX and CSF signal peptides in *B. subtillus* that drive differentiation in a population of cells.

The only significant homology detected between the selected archaeal proteomes and the targeted quorum sensing proteins in Gram positive bacteria was detected for ComA of *S. pneumoniae*. Both *N. pharaonis* and *H. volcanii* had a protein that showed

very strong homology for amino acids 200-700 of ComA. When compared to the homolog in *H. volcanii*, the 30% of the amino acids were identical, 52% of the amino acids had similar properties, and there were 3% gaps. *M. voltae* only had a protein that showed homology along the C-terminal 200 amino acids. The length of ComA in *S. pneumoniae* is 717 amino acids. The homologous protein in both *N. pharaonis* and *H. volcanii* is an ABC transporter of length 641 and 645 amino acids, respectively, and the homolog in *M. voltae* appears to be a smaller subunit of an ABC transporter 221 amino acids long. In all three cases, the N-terminal domain seems quite divergent, while the rest of the proteins are very similar. In *S. pneumoniae*, the N-terminal 150 amino acids of ComA make up the catalytic domain of the protein, while the rest is primarily involved in the transport of the processed peptide (Ishii, Yano et al. 2006). The catalytic domain cleaves the 17 amino acid autoinducer peptide at a glycine-glycine repeat (a mechanism common throughout many bacteria) away from the ComC precursor protein. Since this N-terminal domain is the divergent portion in the archaeal homologs, it seems possible that these proteins are involved in peptide export, but either lacks the catalytic capabilities, or process peptides in a different way than in *S. pnuemoniae.*

The lack of homology between the peptide autoinducer precursor proteins indicates that if peptides are used, they're cleaved from different proteins and are of a different sequence. This makes sense because autoinducers are specific for the organisms that produces them, or at least for closely related organisms, and therefore it should be expected that there is no homology between known bacterial autoinducer peptides and possible archaeal ones.

The lack of homology between ComD and ComE and archaeal proteins suggests that response to autoinducers is not performed by the same proteins. If archaea do use peptide autoinducer mediated quorum sensing to communicate with one another, they likely use a unique two component regulatory system, or a different signal transduction mechanism altogether. The lack of homology here does not provide negative proof, but rather says that more research needs to be done before quorum sensing response in archaea is completely understood.

4.2. Conditioned Media Growth Studies

4.2.1. DNA can serve as the Source of Phosphorus for *H. volcanii*

These growth studies confirmed the previous reports that *H. volcanii* can use double stranded, unmethylated DNA as a nutrient (Chilleski 2011). Specifically, it was seen that when cells were suspended in media lacking phosphorus, they were able to grow when provided DNA. The cultures that were supplemented either DNA buffer or acetate exhibited an extended lag phase and a much slower rate of growth due to the lack of phosphorus in the media, suggesting DNA was necessary for efficient growth.

4.2.2. Conditioned Media Accelerated Growth on DNA

Cells that were resuspended in conditioned media that had previously contained a dense culture (OD_{600}=1.6) for 24 hours had an increase in growth after approximately

eight hours while the cells suspended in fresh Hv-Min remained in lag phase until about sixteen hours after resuspension. However, the cells that were suspended in the conditioned media and supplemented with DNA exhibited a very drastic increase in growth while the other conditioned media cultures had a much milder spike. After this initial increase, the growth of the conditioned media cultures all seemed to plateau and reach a stationary phase. However, the culture supplemented with DNA did so at a much higher cell density because of the initial extreme growth rate. The fresh HV-Min culture supplemented with DNA exhibited the expected growth curve, as it had a short lag period, followed by a period of growth and then reached stationary phase at a higher cell density than the conditioned media culture did after about eighty hours.

The optimal condition for rapid growth in this experiment was using conditioned media for resuspension of cells, and supplementing DNA. The data suggests that there is something present in the conditioned media that allowed the cultures to exit lag phase sooner than the control and accelerated the ability for cells to utilize DNA as a source of phosphorus. Based on the evidence and what is known in bacteria, it seems likely that the dense culture of archaea used for conditioning the media secreted autoinducers. These autoinducers may have been responsible for the initial spike seen in all three conditioned media samples, but a byproduct of metabolism or any other change in the media could be responsible for the initial spike. However, when supplemented with DNA, the cultures grew at an extremely rapid rate, indicating that something is triggering the induction of competence and allowing the cells to use DNA. If there were not autoinducers present in the conditioned media, it would be expected that the cells in the fresh HV-Min culture

with DNA would grow the fastest. This is because the fresh HV-Min would be full of nutrients whereas the conditioned media would be partially depleted.

4.2.3. Conditioned Media Effect is Lost upon Treatment

Heating the conditioned media at 80ºC for twenty minutes made the conditioned media lose its effect of accelerating growth in *H. volcanii*. This suggests that the putative autoinducer in the conditioned media is vulnerable to heat. However, the experiment should be repeated still to confirm this observation.

The ethanol precipitated conditioned media that was reconcentrated in HV-Min to 2X and 1X the original concentration appeared lethal. This was most likely due to a flaw in the design of the experiment. Either there was residual ethanol left in the samples before they were resuspended in fresh HV-Min, or certain elements of the media were overconcentrated to a lethal dosage. Since the .5X concentrated conditioned media was still able to grow, it indicates that either of these possibilities is likely since diluting either ethanol or excess nutrients would reduce the lethality of the media. The experiment will be redone to see if the putative autoinducer precipitates in ethanol. This will be done by adding an additional wash step to insure the ethanol is completely evaporated and by adjusting the concentrations of the nutrients in the media used for resuspension until the cell growth matches the controls. If the autoinducer does precipitate in ethanol it will make classification of the autoinducer easier by allowing resuspension in various buffers. Suspending the putative autoinducers will partially purify it, and make performing certain tests, such as protease inactivation, easier.

4.3. The Nature of the Autoinducer Remains Unknown

As of now, no autoinducer in archaea has been identified. The only published paper regarding quorum sensing in archaea suggests that *N. pharaonis* uses homoserine lactones, similarly to Gram negative bacteria (Paggi, Martone et al. 2003). However, the bioinformatics presents in this thesis suggest that if this is true, then the homoserine lactones would be produced and sensed by proteins highly divergent or completely different from LuxI and LuxR from *V. fischeri*. Unfortunately, no follow up research to purify and identify the autoinducer has been published by those authors in the past eight years.

The thesis that investigated quorum sensing in *H. volcanii* predicted that phosphoesters are used as autoinducers (Bitan-Banin 2002). The most important observation that led to this conclusion is that phosphatase negated the effect of the conditioned media when the media was treated prior to resuspension. Also, they found that the autoinducer is heat stable, which is a different observation than what was seen in this thesis. The bioinformatics method used in this thesis cannot be used to refute the claim that archaea use an unknown form of quorum sensing mediated by phophoesters because there are no proteins currently known to be involved in phosphoester mediated quorum sensing, and therefore there are no protein sequences that can be used to compare to.

The strongest evidence seen using the bioinformatics method was the significant homology with ComA from *S. pneumoniae*. This indicated that there is a protein in many archaea, and specifically in *H. volcanii*, that probably functions similarly in the export of

a peptide that may be an autoinducer. Since ComA, is essential for the processing and export of the *S. pneumoniae* autoinducer, it seems likely that it could have the same function in *H. volcanii*. Current experiments are underway to perform the same growth study using *comA* deletion mutant in *H. volcanii* to see if the spike in growth caused by the conditioned media can be eliminated by using the conditioned media from a *comA* deletion mutant.

The most important accomplishment of this research was the design of a method to test quorum sensing in *H. volcanii* based on its ability to use DNA as a nutrient. When provided conditioned media, *H. volcanii* can grow much faster on DNA than cultures resuspended in fresh HV-Min. This means that some signal in cultures of *H. volcanii* in inducing the upregulation competence genes and allowing them to use DNA more efficiently than common substrates. Using this method, a multitude of experiments can be designed to test the characteristics of the putative autoinducer, such as it's stability in heat and solubility in ethanol. After characterizing the autoinducer further, it can be purified using a process such as HPLC and then identified using mass spectrometry.

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