Shewanella oneidensis MR-1 cell-to-cell signaling and its influences on biogeochemical processes

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Abstract

The goal of this project is to decipher the quorum sensing (cell-to-cell signaling) abilities of *Shewanella oneidensis* MR-1, a Gram-negative bacterium well known for its ability to use geologic substrates, such as Fe and Mn oxides, for respiratory purposes. Overall our results show that *S. oneidensis* cannot utilize either an acyl-homoserine lactone (AHL) or AI-2 quorum sensing signal, despite previous work that indicated that it produced an AHL that would enhance it ability to growth in certain anaerobic environments. Using a variety of quorum sensing signal sensors, no evidence could be found that *S. oneidensis* has a typical AHL signal. An *in silco* analysis of the genome also produced little evidence that *S. oneidensis* has the genes to accept or relay an AHL signal. *S. oneidensis* can produce a luminescence response in the AI-2 reporter strain, *Vibrio harveyi* MM32. This luminescence response is abolished upon deletion of *luxS*, the gene responsible for catalyzing AI-2. Deletion of *luxS* also affected biofilm formation. Within 16 hours of growth in a biofilm flow-through reactor, the *luxS* mutant had an inhibited ability to initiate biofilm formation. After 48 hours of growth, the mutant's biofilm had developed similarly to wild-type. The addition of synthetic AI-2 did not restore the mutant's ability to initiation biofilm formation, which led to the conclusion that AI-2 is not likely used as a quorum sensing signal in *S. oneidensis* for this phenotype. Because of the involvement of LuxS in the activated methyl cycle (AMC) in other organisms, growth on various sulfur sources was examined. A mutation in *luxS* produced a reduced ability to growth with methionine as the sole sulfur source. Methionine is a key metabolite used in the AMC to produce a methyl source in the cell and homocysteine. This data suggests that LuxS is important in metabolizing methionine and the AMC in *S. oneidensis*.

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Attributions

Below is a description of the colleagues who greatly contributed to the work presented in this dissertation and it briefly defines what valuable contributions they made.

Prof. Michael F. Hochella, Jr. (Dept. of Geosciences, Virginia Tech) is the primary advisor who was responsible for funding all of this work. He provided scientific guidance and writing assistance on this entire dissertation.

Assoc. Prof. Ann M. Stevens (Dept of Biological Sciences, Virginia Tech) provided scientific guidance, molecular microbiology equipment, and assisted writing chapters 2 and 3.

Dr. Steven D. Brown (Biosciences Division, Oak Ridge National Laboratory) provided scientific guidance of the microarray data collected in chapter 3. He is responsible for funding the equipment costs of the microarray data. He also assisted in writing chapter 3.

Dr. Haakrho J. Yi (Biosciences Division, Oak Ridge National Laboratory) provided assistance in collecting RNA used for microarray analysis and aided in microarray hybridization used in chapter 3.

Dr. Stanton L. Martin (Bioinformatics Research Center, North Carolina State University) provided assistance with the statistical software that was used to process the microarray data. He also assisted in writing chapter 3.

Prof. Gill G. Geesey (Department of Microbiology, Montana State University) provided equipment and scientific advice on developing a biofilm flow through system. He also assisted in writing chapter 3.

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Chapter 1 - Introduction

Mineral microbe interactions

For approximately 3.8 billion years, microorganisms and geologic constituents have been living in close relation. This equates to nearly 83% of the history of the Earth. Over this time, these two natural systems have evolved together, while each a played a unique role relative to the other. Numerous researchers have concluded that rock and mineral surfaces played a key role in the transition of the Earth from abiotic to biotic (e.g. 20, 31, 45, 101). Through metabolic pathways like photosynthesis, nitrogen fixation, and methanogenesis, microorganisms have played a role in atmospheric chemistry (51). Microorganisms can also use rocks and minerals as sources of essential nutrients like iron, sulfur, potassium, and magnesium (e.g. 88, 89). In doing so, microbes play a key role in the "evolution" of rocks by driving certain weathering reactions, which can alter the rock cycle (3, 8, 89, 104). Similarly, microbes affect the speciation of metals in the environment by producing complexing agents or by enzymatic reactions (see reviews, 2, 81, 85). The driving force behind some of these reactions is biological energy flow via respiration. As long as a microbe can gain energy from a reaction, the reaction will be thermodynamically favored.

Shewanella oneidensis MR-1 is one of the most intensely studied bacteria of environmental relevance. It is a Gram-negative facultative anaerobe that has the ability to use a variety of terminal electron acceptors, such as iron, manganese, chromium, uranium, fumarate, nitrate, sulfur, and oxygen, to name several (66, 79). It has even been previously proposed that *S. oneidensis* produces specific cell surface proteins that it uses to recognize and bind to specific mineral surfaces (67, 68). The dominating theory is that *S. oneidensis* can interact with these substrates through direct contact of the cell membrane and its numerous components (proteins, lipids, polysaccharides) (79, 91, 92). It should also be mentioned that others have proposed that direct contact is not necessary for metal oxide reduction and that these interactions can be driven by indirect contact via soluble organic shuttles (64, 86). It seems likely however, that cellular binding would also enhance these nonenzymatic reduction mechanisms (e.g. quinones that serve as extracellular electron shuttles) by increasing the concentration of the compound at the oxide

surface. Currently there is ongoing research within a number of groups to gain a better understanding of the mineral-microbe interactions that occur during dissimilatory metal reduction.

Not only can microbes utilize essential nutrients from Earth materials, they can also use toxins as a source of energy. Dissimilatory metal reducing bacteria (DMRB), like *Shewanella oneidensis* MR-1, can employ toxins like hexavalent chromium and uranium(VI) as terminal electron acceptors (81, and references therein). Certain anaerobic bacteria can even exploit man-made dioxin derivates as terminal electron acceptors (15). The versatility that certain microbes exhibit to utilize pollutants for energetic purposes, as well as making the pollutant less soluble or toxic, has made them candidates for bioremediation. Bioremediation is a simpler, cheaper, and more environmentally friendly method to sequester toxins in the environment. One setback of bioremediation is the uncertainty about fundamental questions directed towards how microbes will interact with their environment, abiotic or biotic. Numerous research groups around the world focus on examining the interactions between microbes and abiotic constituents; however, little research has been completed that addresses how bacterial cell-cell communication or quorum sensing may affect the fate of environmental contaminants or other geochemical processes.

Quorum sensing

Quorum sensing is defined as the ability of bacteria to communicate with one another via diffusible signal molecules to induce a cellular function (known as a phenotypic response). This phenomenon, originally termed autoinduction, was first found to control competency, the ability of an organism to take up free DNA molecules from the environment in *Streptococcus pneumoniae* (110), and luminescence in *Vibrio fischeri* (83). The term "quorum sensing" was not coined until 1994 (41) and the early work on autoinduction was met with a great deal of skepticism (80). The model was not widely accepted until its genetic elements were verified in the 1980s (26, 27). In Gram-negative bacteria, quorum sensing works as a mode of communication because the autoinducer can diffuse away from its cell of origin to other cells; at

certain concentrations of autoinducer in the environment, the autoinducer along with receptor proteins can induce the transcription of specific genes.

Currently, there are well over 50 different species that have the ability to use quorum sensing (38, 76). It has even been speculated that most bacteria have a communication system that involves signal production and response (38). In addition, the functions of quorum sensing have greatly expanded from competency and luminescence. Quorum sensing has now been linked to virulence factors, protein production, swarming, siderophore production, biofilm formation, plasmid conjugation, antibiotic synthesis, and stationary phase growth (see reviews, 39, 56, 117, 120). As the literature cited above shows, quorum sensing can control numerous genes in a variety of different bacteria, however, understanding the biochemical mechanisms that controls these genes is quite difficult. The best way to illustrate some of the mechanisms that are controlled by quorum sensing is to examine the model systems of *V. fischeri* and *V. harveyi*, which will be discussed in further detail in the following sections.

Quorum sensing in *Vibrio fischeri*. In nature, *V. fischeri* can be a free living marine bacterium or it can live in a symbiotic association with several eukaryotic hosts, including *Monocentris japonicus* (Japanese pinecone fish) (94) and *Euprymna scolopes* (Hawaiian near shore squid) (14). In each of the two mentioned eukaryotes, *V. fischeri* is selectively cultured in the organism's light organ. The symbiotic benefit for the bacteria is that the eukaryote provides a nutrient rich environment for *V. fischeri* and in return *V. fischeri* produces light. The Hawaiian squid uses the luminescence for protection from predators by producing counter-illumination (93, 115). This illumination helps the squid to avoid casting a shadow from the moon during the night. The Japanese pinecone fish uses the luminescence for mating purposes, warding off predators, and attracting prey (82).

The first two genes identified as being involved in quorum sensing in *V. fischeri* were *luxR* and *luxI* (26, 27). *luxI* is responsible for autoinducer synthesis. In *V. fischeri* the autoinducer molecule is N-3-oxo-hexanoyl-L-homoserine lactone (3-oxo-C6-HSL) (25), which is an acyl-homoserine lactone (acyl-HSL), the most common signaling molecule found in Gram-negative bacteria (38-40, 76, 120). The material needed to synthesize acyl-HSLs are S-adenosylmethionine (SAM) and fatty acid derivatives (78, 95), which are also used for other functions in the microbial cell. After synthesis, certain autoinducers (e.g., short chained acyl-

HSLs) can diffuse passively through the cell membrane to the external environment where the molecules can passively diffuse into neighboring bacteria (50). Some bacteria synthesize autoinducer molecules with longer chained acyl-HSLs that are more hydrophobic and therefore have to be transported out of the cell by an efflux pump (28, 90, 118).

LuxR is a transcription activator that is activated by binding an autoinducer molecule. The LuxR protein is only found inside the cell. The protein has two important domains. The amino-terminal domain, which is responsible for binding of autoinducers (43, 98, 100) and the carboxy-terminal domain, which contains a helix-turn-helix (HTH) motif that functions to bind to DNA (18, 103). When LuxR comes into contact with its corresponding autoinducer (3-oxo-C6-HSL), a conformation changes occurs that allows DNA binding and causes the protein to bind with another LuxR/acyl-HSL forming a homodimer (19, 111, 118, 127), which is necessary for transcriptional activation.

At low cell densities (free living marine environment), *V. fischeri* produces a basal level of autoinducer, which is not enough to activate transcription of the bioluminescence genes (Fig. 7). However, at high cell densities more autoinducer is synthesized, which in turn increases the concentration of autoinducer that in turn activates the transcription of the bioluminescence genes (Fig. 8). For example, in the light organ of the Hawaiian squid *V. fischeri* cell density levels can reach 10^{11} cells per ml (87). As the cell density of *V. fischeri* increases over time, the amount of autoinducer also increases such that at a certain cell density the concentration of autoinducer is high enough to initiate the steps in quorum sensing. The autoinducer and LuxR bind to each other and this causes LuxR to undergo a conformation change that causes the formation of a multimeric protein complex. This dimer can now bind at the activator site (*lux* box) of the *lux* operon and increase transcription of the *lux* operon (102, 126). The *lux* operon contains six structural genes (*luxABCDEG*) that code for the enzymes that activate bioluminescence and the *luxI* gene (26, 27).

V. fischeri also has the ability to produce a second and potentially third putative autoinducer. The second autoinducer is *N*-octanoyl HSL (C8-HSL) and it is synthesized by the AinS protein (42, 44, 59, 69, 71). AinS shows no homology to the LuxI protein family, but it does show homology to LuxM from the quorum sensing system of *Vibrio harveyi* (4, 6). It has been proposed that the AinS system is a second quorum sensing system that regulates early

colonization factors. As cell density increases, the AinS system is believed to be overtaken by the LuxRI system, which then activates luminescence (70, 71, 114).

The third putative autoinducer protein, LuxS, was discovered by examining the genome of *V. fischeri* (69). In *V. harveyi*, LuxS is responsible for producing the signal molecule furanosyl borate diester (AI-2) (17). *V. harveyi* uses both LuxM, LuxS, and CqsA as signal molecules to regulate three separate two-component sensory relay systems that function to control the transcription of $LuxR_{Vh}$ (not homologous to *luxR* from *V. fischeri*) (47, 77).

Quorum sensing in *Vibrio harveyi.* *V. harveyi* and *V. fischeri* are two closely related bacteria that share many similarities but have traits that are specific to each species. Both can be free living marine bacteria but *V. fischeri* can also live in a symbiotic relationship. *V. harveyi* can either be a constituent of a biofilm in marine animals and/or a pathogen to marine animals (82). Both bacteria use quorum sensing to regulate luminescence but the mechanisms that activate quorum sensing have unique qualities. *V. fischeri* uses a combination of the AinS and LuxRI system to regulate the expression of luminescence. *V. harveyi* uses a multi-channeled system to regulation luminescence (4, 5), colony morphology (77), type III secretion (46), and the production of siderophores (65), polysaccharides (65), and metalloproteases (77). The multi-channeled quorum sensing system is composed of three separate systems that work in parallel to regulate the traits mentioned above. Each of the three systems has a separate signal molecule and receptor. To explain how the *V. harveyi* quorum sensing system works in its entirety, the three systems (System 1 – 3) will be described separately and then it will be explained how they function in concert to regulate the quorum sensing genes.

The *luxMN* genes are responsible for the production of the signal molecule and receptor protein for System 1 in *V. harveyi*. The protein LuxM synthesizes the signal molecule N-(3-hydroxybutanoyl) homoserine lactone (AI-1) (16) and is homologous to AinS (4, 6). LuxM shows no homology to LuxI, but each is produced through similar biosynthetic pathways (4). AI-1 is produced inside the cell and passively diffuses out of the cell and into the environment surrounding the bacterium. The receptor protein of AI-1 is LuxN, which is a membrane bound two-component hybrid sensor kinase (4). LuxN, a relative to sensor kinases of two component regulatory systems, is a hybrid senor kinase because it has the ability to be a senor kinase and a response regulator (4).

At low cell density, there are low amounts of AI-1 in the environment surrounding the bacteria. As a result no signal molecule is bound to LuxN. This causes LuxN to autophosphorylate and subsequently transfer the phosphate to another protein LuxU (35-37) (the discussion of Systems 1, 2, & 3 will stop at LuxU and the topic of LuxU will be continued after all three systems are introduced). At high cell density, the concentration of AI-1 builds up in the surrounding environment. Unlike the signal for *V. fischeri*, AI-1 does not diffuse back into the cytoplasm of the cell to bind with LuxN. LuxN contains a membrane bound domain that binds with AI-1 in the periplasm. The binding of AI-1 causes LuxN to switch from a protein kinase to a protein phosphatase (35), which in turn causes LuxU to dephosphorylate.

The genes involved in System 2 are *luxSQP* (17, 97, 105). The protein LuxS is responsible for producing the signal molecule (AI-2) for System 2. The signal molecule AI-2 is not an acyl-HSL, it is a furanone that contains boron (3A-methyl-5,6-dihydro-furo(2,3-D)(1,3,2)dioxaborole-2,2,6,6A-tetraol) (17). There are two receptor proteins for System 2: LuxQ and LuxP (5). LuxQ, similar to LuxN, is a membrane bound two-component hybrid sensor kinase (4). LuxP is similar to a periplasmic ribose binding protein (5) and is the initial receptor for AI-2. Upon binding, a complex, LuxP/AI-2, is formed, which then binds to LuxQ (5).

At low cell density, there are low amounts of AI-2 in the environment surrounding the bacterium and therefore no binding of the signal molecule to LuxP. This causes LuxQ to autophosphorylate and subsequently transfer phosphate to LuxU (35-37). At high cell density, AI-2 builds up in environment. Similar to system 1, AI-2 diffuses into the periplasm (not to the cytoplasm of the cell), where it can bind with LuxP. The complex, LuxP/AI-2, will then bind to LuxQ and cause the protein to switch from a protein kinase to a protein phosphatase (35). LuxQ, now functioning as a protein phosphatase, will then dephosphorylate LuxU.

System 3 is a newly discovered quorum sensing system in *V. harveyi* (47). The signal molecule for System 3 (CA-1) is produced inside the cell by CqsA (47). The structure of CA-1 is still unknown. The receptor for this system is CqsS (47). Like the receptors in Systems 1 and 2, CqsS is a membrane bound two-component hybrid sensor kinase. The signal molecule produced by CqsA is received by CsqS in the cell's periplasm and phosphate is either transferred to (low cell density) or hydrolysed from (high cell density) LuxU (47).

All three quorum sensing systems of *V. harveyi* converge on the phosphorelay protein LuxU (4, 5, 47). Each of the three systems can either transfer phosphate to (phosphorylate) or

remove phosphate from (dephosphorylate) LuxU. LuxU is a phosphorelay protein that can then pass the signal (e.g., phosphate) to its response regulatory, LuxO (6, 35-37), which is the end target of the phosphorelay signal cascade that was originally initiated with the receptor protein of each system. When LuxO is active (low cell density/ phosphorylated), it works along with RpoN (σ^{54}) to activate the transcription of small regulatory RNAs (sRNAs) (61, 65). These sRNAs work together with the RNA chaperone Hfq to destabilize the mRNA responsible for the translation of $LuxR_{Vh}$ (61, 65). This prevents the production of $LuxR_{Vh}$, thus preventing the activation of the genes involved with quorum sensing in *V. harveyi* (46, 61, 73, 99). When LuxO is inactive (high cell density/dephosphorylated), no sRNAs are produced so the expression of LuxR is not blocked and the genes involved in quorum sensing are activated (61, 65).

LuxS and its implications for inter-species communication

The AI-2 autoinducer signal of *V. harveyi* has been shown to be involved in the regulation of the quorum sensing regulated phenotype of luminescence. In addition, it may also be a part of a more complex communication network that involves different bacterial species. It is thought that the AI-2 signal molecule (whose synthesis is catalyzed by LuxS) is used for interspecies communication because *luxS* homologues have been found in a wide variety of Gram-negative and Gram-positive proteobacteria (105). Since *luxS* homologues are found in both types of bacteria, it has also been proposed that AI-2 may be an ancient form of autoinducer molecule that was used prior to the evolutionary split of these two major groups (96). This broad conservation could also be related to the involvement of LuxS in the biosynthetic pathway called the activated methyl cycle (AMC) (97, 121). The AMC generates homocysteine, methionine, adenosine, and S-adenosylmethionine (SAM), a major methyl donor source in the cell (reviewed in 113). This metabolic role of LuxS has resulted in much debate on whether a mutation in this gene affects certain phenotypes because of a lack of quorum sensing abilities or the disrupting of a biosynthetic pathway (23, 113, 122).

The ability of AI-2 to act as an interspecies communication molecule may also help to explain why *V. harveyi* has three different sets of signal molecules and receptor proteins. In nature, *V. harveyi* grows in a complex ecosystem containing numerous species of bacteria (82).

The ability to sense AI-2 from other species of bacteria allows *V. harveyi* to evaluate its neighbors and change its behavior to be more competitive (96). Gaining a competitive edge in a mixed population of bacteria would be a great benefit for *V. harveyi*. Nevertheless, numerous bacterial species possess LuxS genes that are involved in the formation of the complex environment of biofilms (1, 34, 75, 119, 125).

Quorum sensing and the environmental geosciences

At first glance, it might appear that quorum sensing has few ties directly to the geosciences. However, on closer inspection it becomes apparent that some of the functions that quorum sensing regulates, such as biofilm formation, exopolysaccharide production, siderophore synthesis, and iron cycling are indeed important in our understanding of many geochemical processes.

One way quorum sensing can affect geochemical processes is by controlling biofilm structure and/or formation. This affects geochemistry because it will influence the distribution and number of chemically active sites available for aqueous cation sorption. It is well known that biofilms are complex microbial environments that play an active role in the fate of many aqueous chemical complexes (e.g. 11, 32, 33, 60, 107-109, 112). They have even been known to sorb many different toxic heavy metals, like lead (109), copper, uranium, cadmium, zinc, and nickel (33, and references therein).

Biofilms can be defined as aqueous, surface associated, heterogenic communities of bacteria within a matrix of extracellular polymeric substances. The polymeric substances are composed of simple and complex lipids, proteins, and polysaccharides (32). These substances within a biofilms mediate the reactions between the biofilm and the aqueous complexes, especially toxic heavy metals. The anionic functional groups from these polymeric substances, like carboxyl, phosphoryl, and sulfate, provide sorption sites most likely responsible for cation interactions (106).

The architecture of biofilms is also important to its function. For example, biofilms have channels and pores that can aid in the transport of nutrients within a community (21, 62). The structure of biofilms can also protect the community by acting as a diffusion barrier to keep

harmful substances away from the community (49, and references therein). In a study by Davies et al. (22), the structure of a biofilm produced by *P. aeruginosa* was shown to effect its ability to resist the biocide sodium dodecyl sulfate. Interestingly enough, this study also found that *P. aeruginosa* used cell-to-cell signaling to control the morphology of its biofilms. Mutants of *P. aeruginosa* missing its quorum sensing signal producing gene (*lasI*) produced flat undifferentiated biofilms that were more susceptible to sodium dodecyl sulfate.

Similar to biofilm formation, if quorum sensing is involved in the regulation of polysaccharide production it can affect the amount of sorption sites available for aqueous cation sorption. Researchers have looked specifically at the ability of the components of the outer membrane of a bacterium, not within the structure of a biofilm, to affect the fate of aqueous chemical complexes (e.g. 9, 10, 12, 13, 29, 30, 52, 123, 124). This research has solidified the importance of the interface of between cell surfaces and aqueous solutions. Whether it is the peptidoglycan and/or teichoic acid of a Gram-positive bacterium or the lipopolysaccharide layer in a Gram-negative bacterium, the functional groups of the associated constituents are responsible for sorption of most complexes. Again similarly to biofilms, anionic functional groups, like carboxyl, hydroxyls, and phosphoryl, are responsible for cation sorption (12, 13, 29, 124).

Quorum sensing has also been shown to control siderophore production in certain bacteria. This is important to the geochemical processes because siderophore production could affect mineral dissolution and metal contaminant fate (see references below). Siderophores are soluble, organic ligands used by bacteria to chelate ferric iron and transport it into the cell (72, 116, and references therein). Siderophores are important to bacteria because most inorganic iron compound are insoluble and iron is an essential microbial nutrient. Iron is a constituent of cytochromes and iron-sulfur proteins, which are key components of respiration. Siderophores are excreted by bacteria and can extract ferric iron from most iron compounds because of their high affinity for ferric iron (116). To obtain the iron within the cell, ferrisiderophores (siderophores-ferric iron complexes) are recognized by specific receptor proteins on the outer membrane of Gram-negative bacteria. Since a ferrisiderphore is too large to pass through the outer membrane porins, transportation into the cytoplasm is dependent on the TonB complex (116, and references therein). The TonB complex is a group of three proteins (TonB, ExbB, and

ExbD) that couples the transportation of ferric iron into the cell with the use of the proton motive force.

Siderophores affinity for ferric iron is so high that it can even extracts ferric iron from iron bearing minerals, like hematite and goethite. The iron extract from iron bearing minerals can be used in cellular processes (88), or aid in the dissolution of iron bearing minerals (48, 53, 63, 74). Not only do siderophore affect the fate of iron in the environment, they have also been implicated in effecting contaminant fate and transport of toxic metals (Pb, Cu, Cd, Zn) and radionuclides in soils (57, 58, 84). In each of the previous studies, siderophores were found to bind metals that are not iron associated.

Quorum sensing may even be involved in iron cycling in the environment. In De Windt et al. (24), it was proposed that the addition of an acyl-HSL increased the consumption of hydrogen and iron corrosion by *S. oneidensis*. In another experiment, the addition of an autoinducer was shown to aid the recovery of starvation in ammonium oxidizing bacteria (7). If quorum sensing could increase the rate of metabolism, especially in bacteria like *S. oneidensis*, it could affect iron cycling by aiding the processes of iron mineral dissolution and precipitation. Contrary to this argument, Lies et al. (64) found that the addition of a *S. oneidensis* culture's supernatant was not able to increase the amount of iron-bead reduction in their experiments. This study was looking for evidence to support that *S. oneidensis* could reduce iron indirectly. Their results do not necessarily prove that *S. oneidensis* does not use quorum sensing to regulate the production of proteins used for direct iron reduction.

Future work

The main conclusion from this study is that *S. oneidensis* cannot utilize an acyl-homoserine lactone (AHL) or AI-2 quorum sensing despite previous work that indicated that it produced an AHL that would enhance it ability to growth in certain anaerobic environments. A question that still remains is why a mutation in *luxS* creates a problem in biofilm development, especially if it is not related to quorum sensing (24). Currently, the conclusion that the involvement of AI-2 in biofilm formation is related to quorum sensing is being reevaluated throughout the literature. Even the involvement of AHL signals in biofilms is under scrutiny

(54, 55). The biological complexities as well as all the environmental conditions (flow rate, carbon source, and substrate) that can control biofilm development make solving this dilemma problematic. It seems unlikely that the role *luxS* plays in the AMC is large enough to regulate biofilm formation. Additionally, if the medium is enriched in a variety of sulfur sources (e.g. LB medium), this disruption of the AMC should not be important in the cell, especially since the enzyme 5'-methylthioadenosine/S-adenosylhomocysteine nucleosidase (MTA/SAHase) is sufficient enough to remove the toxic AMC intermediate, S-adenosylhomocysteine (SAH). The answer to this question may lie in how a *luxS* mutation can alter the SAM methyltransferase reaction. Altering the major methylation reaction in the cell could have a variety of phenotypic affects from chemotaxis to altering cell division.

In the cases where it is demonstrated that AI-2 can regulate biofilm development through quorum sensing, the next frontier will be to try to test the function of AI-2 as an interspecies signal. The greatest advantage of utilizing an interspecies signal molecule may be apparent in a complex environment like a biofilm. The main evidence for AI-2 to be an interspecies signal is from *luxS* sequence similarity and the ability of AI-2 from other species to induce certain phenotypes like luminescence. The ability of AI-2 to induce or inhibit biofilm formation from one bacterium to another in a biofilm has not yet been established. Not only does this have importance to the quorum sensing field, but to the field of environmental microbiology since most biofilms in the environment are the result of multiple species.

Structure of dissertation

This dissertation consists of two chapters based on experimental results, as well as an appendix. Chapter 2, entitled "Analysis of acyl-homoserine lactone production in *Shewanella oneidensis* MR-1", describes a search for the ability to utilize quorum sensing in *S. oneidensis* MR-1. This project was in collaboration with Ann Stevens at Virginia Tech. The appendix contains supplemental data from microarray analysis. Chapter 3, entitled "*Shewanella oneidensis* MR-1 LuxS involvement in biofilm development and sulfur metabolism", will be submitted to the journal *Applied and Environmental Microbiology*. This project describes the role LuxS plays in *Shewanella oneidensis* MR-1. This chapter was written in collaboration with

Steve Brown and Yi Harrko at Oak Ridge National Lab, and Stan Martin at North Carolina State, Gill Geesey at Montana State University, and Ann Stevens at Virginia Tech.

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Chapter 2 - Analysis of acyl-homoserine lactone production in *Shewanella oneidensis* MR-1

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Abstract

The quorum sensing abilities of *Shewanella oneidensis* MR-1 have been examined by *in silico* analysis and by various biochemical experiments. Results indicate that *S. oneidensis* does not utilize Gram-negative acyl-homoserine lactone (AHL) signaling. A variety of AHL reporter strains were used in an attempt to detect AHL production. No evidence was produced to support that *S. oneidensis* can produce an AHL signal. Additionally, *S. oneidensis* has little genomic evidence that would support the presents of AHL synthase, a LuxR-type transcription regulator, or the presence of the genes needed to receive the LuxS/AI-2 signal when compared to two known systems: the Lux phosphorelay and the Lsr system.

Introduction

Currently well over 50 different bacterial species have been identified that have the ability to use quorum sensing (13, 20, 44). Cellular functions stimulated by quorum sensing such as competency (40) and luminescence (31) have been known for approximately 30 years. More recently, a variety of phenotypes including virulence factors (41), protein and siderophore production (11, 37), antibiotic synthesis (1, 46), and biofilm formation (2, 8, 29, 45, 48) have been shown to be controlled by quorum sensing in a variety of species. While most quorum sensing signals (autoinducers) share common organic structures, Gram-positive bacteria typically

utilize modified oligopeptides (21, 22) and Gram-negative proteobacteria utilize acyl-homoserine lactones (AHL) (9, 15): Autoinducers vary in their structures so as to interact with species specific cellular receptors. Because LuxS, the enzyme responsible for catalyzing the formation of the AI-2 family of signals, is conserved in Gram-positive and Gram-negative bacteria, it has lead to the conclusion that the AI-2 signal molecule is used for interspecies communication (38).

In a study that focused on the relationship between hydrogen consumption and anoxic iron corrosion (10), it was proposed that *Shewanella oneidensis* MR-1 could produce an AHL. In the study, it was determined that adding the filter-sterilized supernatant from a cell culture grown at high cell density to a culture having low cell density resulted in an increased consumption of hydrogen and increased oxidation of Fe(0) to Fe(II). When the supernatant was tested for the presence of an AHL using a green fluorescent protein (GFP) biosensor system, an AHL was found but its identity was not determined. Contrary to these results, Lies et al. (24) found that the addition of the supernatant from an *S. oneidensis* culture was not able to increase the amount of iron reduction in their experiments.

S. oneidensis, a Gram-negative dissimilatory metal reducing bacterium (DMRB), is an important environmental microorganism because of its unique respiratory system. It has the ability to respire on numerous substances such as O_2, NO_3^-, TMAO, DMSO, fumarate, Fe-and Mn-oxides, and even toxic metals like Cr(VI) and U(VI) (26, 30). The regulatory pathway and molecular mechanism that allows DMRB to transfer electrons to solid phase Fe and Mn-oxides to gain energy is still poorly understood. If *S. oneidensis* could use quorum sensing to affect its rate of respiration, it would aid in the understanding of how DMRB regulate their electron transport system.

Currently *S. oneidensis* has no known AHL autoinducers or phenotypic response(s) regulated by an AHL autoinducer signal. It does have a putative *luxS* gene (18) and its ability to produce AI-2 has recently been established (5). The goal of the study is to identify if *S. oneidensis* has any quorum sensing signals other than AI-2. If quorum sensing could increase the rate of metabolism, especially in bacteria like *S. oneidensis*, it could affect numerous processes relevant to biogeochemistry like metal and organic contaminant fate, biofilm formation, and mineral dissolution and precipitation.

Materials and Methods

Bacterial strains, plasmids, and media. A list of all bacteria and plasmids used in this study can be found in Table 3-1. Most cultures were grown on Luria-Bertani (LB) medium (33) at 30° C or 37° C. *Vibrio harveyi* strains BB120 and MM32 were grown on AB medium (16) at 30° C. When necessary, supplements or antibiotics were added at the following concentrations (ug/ml): ampicillin (Ap) 100, chloramphenicol (Cm) 10, kanamycin (Km) 100 or 50, or tetracycline (Tc) 10.

Autoinducer Assays. Two broad range AHL reporter strains were used to test if *S. oneidensis* could produce an AHL. The first was *Agrobacterium tumefaciens* NTL4 (pCF218)(pCF372) (14). It was grown in liquid overnight and then was crossed-streaked on agar-based LB medium with a positive control (AHL producing), *A. tumefaciens* KYC6 (pCF218) (14) and *S. oneidensis*. The reporter stain, *A. tumefaciens* NTL4, can detect a wide range of AHLs (14, 49). The reporting system for this strain utilizes the *lacZ* gene, and in the presence of an AHL, LacZ is synthesized, which in turn cleaves the X-gal compound resulting in a blue color. The second broad range AHL reporter strain that was used is *Escherichi coli* MG1655 (pJWP01s) (6). In the presence of certain AHLs, *E. coli* MG1655 (pJWP01s) activates the production of a stable GFP. *Escherichi coli* MG1655 (pJWP01s) was grown overnight and then diluted and grown to an approximate O.D. 600 of 0.4. Then filtered supernate of overnight experimental cultures where added in a variety of ratios. The reporter and supernate mix was then incubated at 30°C. Fluorescence was monitored over a period of 4-6 hours by adding 200 µl of each sample mix to a well in a 96 well plate and analysis was done with a fluorometer (Tecan, Spectrafluor Plus).

V. harveyi BB886 was used as qualitative reporter strain to identify the presence the 4-carbon chained autoinducer N-(3-hydroxybutanoyl) homoserine lactone (AI-1) (4). The reporter strain was grown for 16 hours and then diluted 1:2500. 90 µl of the diluted reporter was then added to a 96 well plate. Cultures of *V. harveyi* BB120 (produces N-(3-hydroxybutanoyl) homoserine lactone) (4) and *S. oneidensis* were grown to both mid-log and stationary phase and 1ml of the culture was centrifuged and filter sterilized. Then 10 µl of the filtered supernatant

was added to the reporter stain. The plates were incubated at 30° C and the luminescence of both reporter strains was monitored using a luminometer (Beckman Coulter, LD400) for 18 hours.

Results and Discussion

Searching for an AHL signal. To evaluate the hypothesis that *S. oneidensis* utilizes AHL quorum sensing, an *in silico* genomics approach was used to find evidence that would demonstrate *S. oneidensis* has the ability to use this regulatory network. Genomic analysis was preformed with the BLAST (Basic Local Alignment Search Tool; www.ncbi.nlm.nih.gov) program and the SDSC Biology Workbench (San Diego Supercomputer Center Biology Workbench; http://workbench.sdsc.edu/) to search for genes displaying sequence features characteristic of known quorum sensing genes. A variety of AHL synthase homologues were used in BLAST searches: *V. fischeri luxI* and *ainS*, *V. harveyi luxM*, *P. aeruginosa lasI* and *rhlI*, and *Pantoea stewartii esaI.* These searches produced no genes in *S. oneidensis* with sequence similarity to any of the above mentioned *luxI* homologues.

Even though BLAST searches did not produce any AHL synthase homologues, three separate experiments were performed to identify the physical presence of autoinducer molecules using AHL reporter strains. The two broad range AHL reporters, *A. tumefaciens* NTL4 (pCF218)(pCF372) (data not shown) and *E. coli* MG1655 (pJWP01s) (Figure 2-1), produced no evidence for the presence of an AHL signal in *S. oneidensis* supernatant. Because the two broad range AHL reporter strains have trouble sensing AHLs with a four carbon chain, a third reporter strain (*V. harveyi* BB886) assay was completed. *V. harveyi* BB886 can sense the presence of the four carbon chain AHL of *V. harveyi*. This third assay also failed to provide evidence for the physical presence of an AHL (data not shown).

E. coli MG1655 (pJWP01s), a GFP based reporter strain, did show that *S. oneidensis* supernatant produced levels of GFP production slightly above background (Figure 2-1). Similar results from a GFP based reporter strain were seen in the hydrogen consumption and anoxic iron corrosion study (10). It is our conclusion that the *E. coli* MG1655 (pJWP01s) GFP production from *S. oneidensis* supernatant is not considered an AHL response because it was approximately three orders of magnitude less than known AHL producers like *V. fischeri* MJ1 and *Pseudomonas aeruginosa* PAO1 (Figure 3-1A). The slightly above background level GFP

production may have resulted from an excreted metabolite produced by *S. oneidensis* that was in the supernatant. Moreover, the hypothesis that increased hydrogen consumption and anoxic iron corrosion from addition of spent culture could instead be explained by a metabolite in the spent culture supernate, not a quorum sensing signal. Further evidence for extracellular metabolites has been found in many DMRD, including *S. oneidensis* (27, 32, 43).

***S. oneidensis* LuxR family proteins.** *S. oneidensis* has seven putative transcription regulatory genes that have been labeled as being in the LuxR family of proteins: TIGR locus SO 0351, SO 0864, SO 1860, SO 2648, SO 2725, SO 3305, and SO 4624. These results do not necessarily prove that *S. oneidensis* has an AHL dependent transcription regulator. LuxR-type quorum sensing regulator proteins are part of the NarL-FixJ superfamily (15). This superfamily consists mostly of DNA binding two component–type response regulators (19). LuxR-type homologues have two important domains: the N-terminal domain, which is responsible for binding of autoinducers (17, 34, 35), and the C-terminal domain, which contains a helix-turn-helix (HTH) motif that functions to bind to DNA (7, 36). The N-terminal domains of LuxR-type homologues have no sequence similarities to the NarL-FixJ superfamily (19).

Sequence comparison was conducted on SDSC Biology Workbench to delineate whether any of the seven putative LuxR family proteins of *S. oneidensis* are homologues to LuxR-type quorum sensing regulators. When compared to LuxR-type regulators, the seven LuxR family proteins have sequence similarities that range from 16-21%. This is not unlike other known LuxR-type homologues, which only have 18–23% end to end sequence similarity (13, 36). When comparing the sequences of the seven LuxR family proteins to other known LuxR-type homologues (*P. aeruginosa* LasR and RhlR, *P. stewartii* EsaR, *Aeromonas salmonicida* AsaR, and *Yersinia enterocolitica* YenR), only three amino acid residues are conserved in the C-terminus region. These three residues are in the NarL HTH region (12). In addition, Stevens and Greenburg (36) compared the sequence of 15 different LuxR-type homologues and found that there are seven amino acid residues spread throughout the N and C-terminus regions that are absolutely conserved in all the examined LuxR homologues. Therefore, the seven LuxR family proteins in *S. oneidensis* are not similar to LuxR-type proteins that are AHL dependent transcription regulators. The reason that the seven proteins were placed in the LuxR family of proteins is most likely not because of their involvement in quorum sensing but because of their

HTH sequence similarity. This can be visualized by examining the Phylip-format dendrogram tree that was produced by ClustalW (Biology Workbench). The seven *S. oneidensis* proteins have a close relationship to NarL but the LuxR-type proteins make their own branch of the tree (Figure 2-2).

AI-2 pathways. There are two well-studied pathways that can receive the AI-2 signal and use it to regulate phenotypic responses. The Lux system in *Vibro spp*. consists of a periplasmic protein binding the signal to initiate a phosphorelay cascade that aids in regulating bioluminescence (23, 28, 38). The second system in *Salmonella typhimurium* and *E. coli* transports and phosphorylates the AI-2 signal into the cell via the Lsr ATP binding cassette transporter and once inside the cell, the phosphorylated signal interacts with the responses regulator LsrR (39, 42, 47). BLAST searches did not provide evidence that any of the genes in the Lsr system were also in *S. oneidensis*. For the Lux system, numerous proteins with high homology were found for two proteins in the AI-2 pathway: LuxQ and LuxO. Even though these two proteins are essential for AI-2 signaling, they do not comprise enough of the system to initiate signaling alone and there is no obvious *V. harveyi* LuxR homologue. In *V. harveyi*, LuxQ is a senor kinase (3) and LuxO is a sigma 54 dependent response regulator (25) both of which have highly conserved domains that could be the reason for BLAST searches to find LuxO and LuxQ genes with high homology in *S. oneidensis*.

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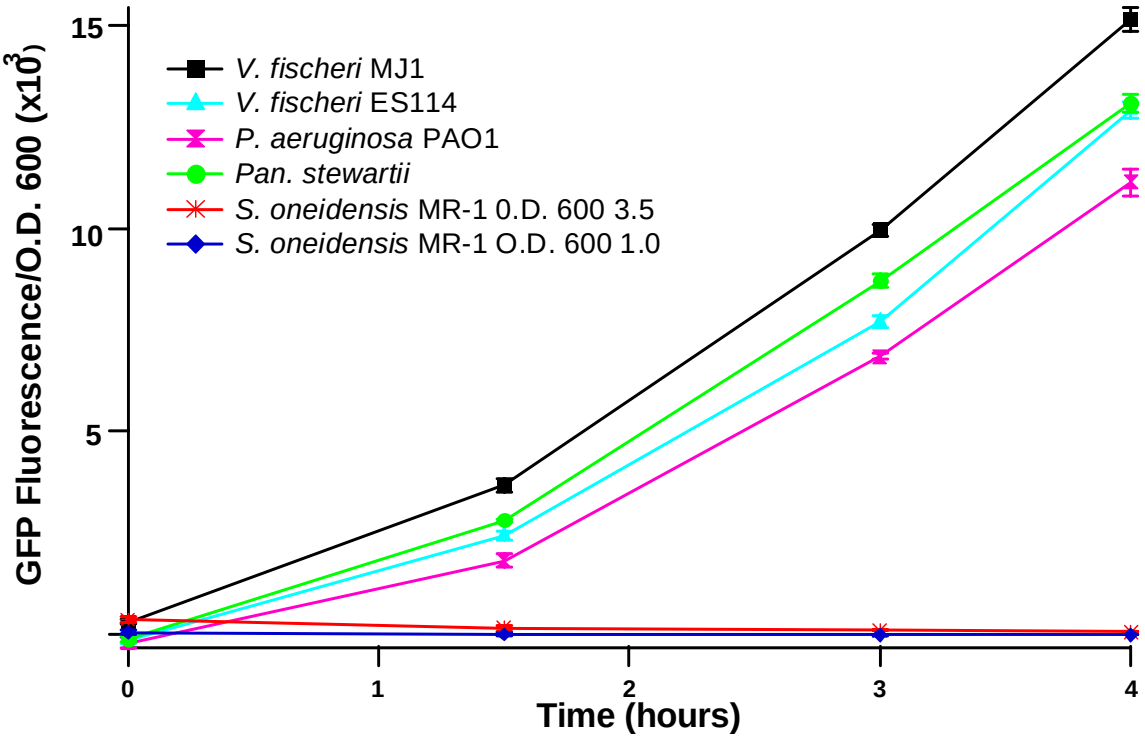

Figure 2-1. Represents the GFP production of an *E. coli* AHL reporter strain with the addition of filtered supernate from *S. oneidensis* at O.D. 600 of 3.5 and 1.0 and other known AHL producers: *V. fischeri* ES 114 and MJ1, *P. aeruginosa* PAO1, and *Pan. stewartii*. Data representative of experiments done in triplicate.

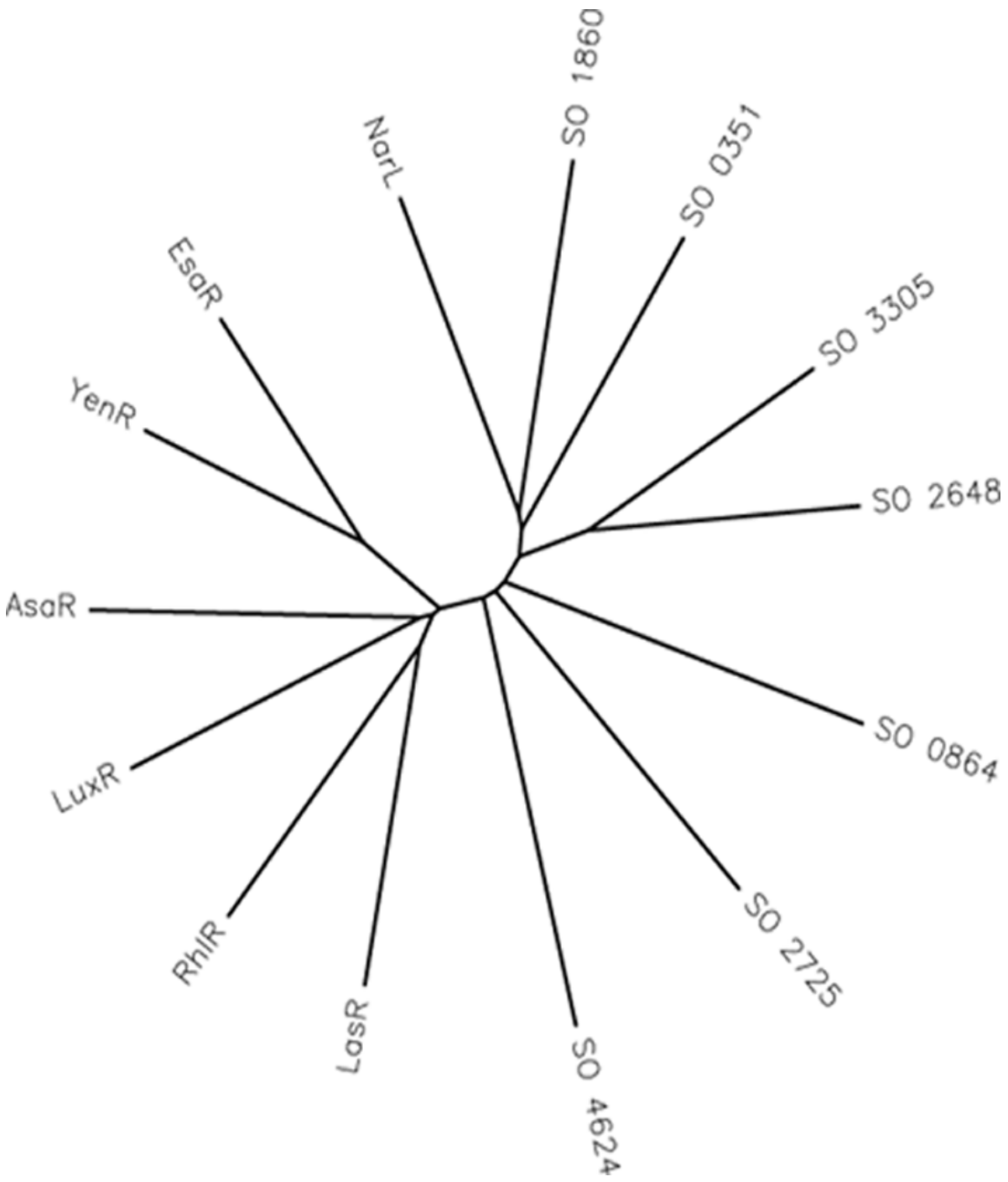

Figure 2-2. Dendrogram tree showing the relationship between known LuxR-type proteins, *S. oneidensis* LuxR family proteins, and NarL.

Table 2-1. Strains used in this study.

Bacterial Strain	Relevant Features	Source or reference
Escherichia coli		
DH5α	*E. coli* cloning strain (AHL/*luxS*-)	Invitrogen
MG1655 (pJWP01s)	Broad range AHL reporter strain	(8)
MG1655 (pJWP02)	AHL positive control	(8)
Shewanella oneidensis		
MR-1	Wild type	(32)
Agrobacterium tumefaciens		
NTL4 (pCF218)(pCF372)	Broad range AHL reporter strain	(16)
KYC6 (pCF218)	AHL producer	(16)
Vibrio harveyi		
BB120	Wild type AHL and AI-2 producer	(5)
BB886	AHL (4C) reporter strain	(5)
Vibrio fisheri		
ES114	Wild type AHL producer	(7)
MJ1	Wild type AHL producer	(35)
Pseudomonas aeruginosa		
PAO1	Wild type AHL producer	A.M. Stevens

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Chapter 3 - *Shewanella oneidensis* MR-1 LuxS involvement in biofilm development and sulfur metabolism

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Abstract

The role of LuxS in *Shewanella oneidensis* MR-1 has been examined by transcriptomic profiling, biochemical and physiological experiments. Results indicate that a mutation in *luxS* inhibits initial biofilm development, not by altering quorum sensing abilities, but by disrupting the activated methyl cycle (AMC). *S. oneidensis* wild-type (WT) can produce a luminescence response in the AI-2 reporter strain, *Vibrio harveyi* MM32. This luminescence response is abolished upon deletion of *luxS*. Deletion of *luxS* also inhibited biofilm formations in static and flow through conditions. Confocal images showed that the *luxS* mutant had an inhibited ability to initiate biofilm formation. The mutant's biofilm had fewer microcolonies and had less surface coverage compared to WT after 16 hours of growth. The mutant's biofilm was visually similar to WT following 48 hours of growth. The addition of synthetic AI-2 did not restore the mutant's ability to initiation biofilm formation, which led to the conclusion that AI-2 is not likely used as a quorum sensing signal to regulate biofilm development in *S. oneidensis*. Because of the involvement of LuxS in the AMC, growth on various sulfur sources was examined. A mutation in *luxS* produced a reduced ability to grow with methionine as the sole sulfur source. Methionine is a key metabolite used in the AMC to produce a methyl source in the cell and homocysteine.

The data suggests that LuxS is important to metabolizing methionine and the AMC in *S. oneidensis*.

Introduction

The AI-2 family of quorum sensing signals is unique because LuxS, the enzyme responsible for catalyzing the formation of these signals, is conserved in both Gram-positive and Gram-negative proteobacteria. This broad genetic conservation has led to the conclusion that AI-2 is used for interspecies communication (40). In addition to being implicated with quorum sensing, LuxS is involved in the activated methyl cycle (AMC) (36, 50). This metabolic role of LuxS has resulted in much debate on whether a mutation in this gene affects certain phenotypes because of a lack of quorum sensing abilities or the disrupting of a biosynthetic pathway (8, 44, 51).

The AMC generates homocysteine, methionine, adenosine, and S-adenosylmethionine (SAM), a major methyl donor source in the cell (reviewed in 44). The conversion of SAM to S-adenosylhomocysteine (SAH) results in the methylation of DNA, RNA, proteins, and metabolites. To complete the cycle, SAH, a toxic metabolite, is then converted to S-ribosylhomocysteine (SRH) and then SRH is converted to homocysteine and 4,5-dihydroxy-2,3-pentanedione (DPD) by LuxS. DPD can then spontaneously cyclize into an AI-2 type signal. Two known AI-2 structures are (2S,4S)-2-methyl-2,3,3,4-tetrahydroxytetrahydrofuran-borate (*Vibrio harveyi*) (6) and (2R,4S)-2-methyl-2,3,3,4-tetrahydroxytetrahydrofuran (*Salmonella enterica* serovar Typhimurium) (30). DPD has no known function in the cell besides producing the quorum sensing signal AI-2.

Quorum sensing involving the AI-2 signal has been linked to a variety of different phenotypes from luminescence to biofilm formation and virulence factors in a variety of organisms (20, 46, 48). Certain phenotypes like biofilm formation could affect numerous processes relevant to biogeochemistry, like metal and organic contaminant fate and mineral precipitation. Recently it has been proposed that an important environmental microorganism, *Shewanella oneidensis* MR-1, has the ability to produce an acyl-homoserine lactone (AHL) (9) and the AI-2 quorum sensing signals (3). *S. oneidensis* is an important environmental bacterium

because of its unique ability to respire on numerous substances such as O_2, NO_3^-, TMAO, DMSO, fumarate, Fe-and Mn-oxides, Cr(VI) and U(VI) (25, 32). It has been suggested that *S. oneidensis* uses an AHL to enhance metabolism, under specific growth conditions (9). Contrary to these results, the addition of the supernatant from an *S. oneidensis* culture (23) or a mutation in *luxS* (4) was not able to affect iron reduction rates under the experimental conditions of each study.

To date, no known AHL autoinducers have been identified in *S. oneidensis* and no physiological traits are known to be regulated by an autoinducer in this bacterium. The present study examines what role LuxS may play in *S. oneidensis* and whether this could be related to quorum sensing or a biosynthetic pathway.

Materials and Methods

Bacterial strains, plasmids, and media. *S. oneidensis* MR-1 (32) and *Escherichia coli* DH5α (Invitrogen) or WM3064 (William Metcalf, personal communication) were grown on Luria-Bertani (LB) medium (35) at 30° C or 37° C, respectively. *Vibrio harveyi* strains BB120 and MM32 were grown on AB medium (17). Anaerobic growth experiments were grown on a minimal medium (MM) (16) or LM (31) with 20 mM lactate and a terminal electron acceptor (10 mM fumarate, 10 mM trimethylamine n-oxide (TMAO), or 5 mM Fe_2O_3). Iron reduction was quantified by analyzing soluble Fe (II) using the ferrozine assay (39). For growth experiments in sulfur limiting conditions, M9 medium (35) was prepared and all sulfate salts were exchanged for comparable chloride salt. The medium was supplemented with 20 mM lactate, 15 μM thiamine, and 50 μM cysteine, 100 μM homocysteine, 100 μM methionine, or 200 μM K_2SO_4. When necessary, supplements or antibiotics were added at the following concentrations (μg/ml): ampicillin (Ap) 100, chloramphenicol (Cm) 10, diaminopimelic acid (DAP) 100, kanamycin (Km) 100 or 50, or tetracycline (Tc) 10.

Mutagenesis. The construction of a *luxS* gene deletion in *S. oneidensis* was completed using a mutagenesis procedure described elsewhere (11). All molecular work was performed by standard methods (35). Briefly, PCR-amplified 631 bp upstream and 702 bp downstream

fragments of *luxS* were inserted into the suicide vector pJK100 (11). The resulting plasmid p*luxS*:UD was electroporated into a conjugal donor strain, *E. coli* WM 3064. The mating experiments involved concentrating 1.0 ml of *E. coli* WM 3064 p*luxS*:UD and 0.5 ml of *S. oneidensis* into 100 µl and the resulting mixture of cells was spotted onto a LB plate and allowed to grow at 30° C overnight. Successful double crossover integration of the suicide vector produced *S. oneidensis* p*luxS*:UD strains that exchanged *luxS* for a Km resistant gene. The chromosomal inserted resistance cassette was then removed by conjugating pCM157 (27), a Cre recombinase vector, into *S. oneidensis* p*luxS*:UD via *E. coli* WM 3064. Removal of the resistance cassette was possible because of the *loxP* sites that flanked the inserted Km resistance gene. The resulting strain, *S. oneidensis* Δ*luxS* pCM157, was then cured of pCM157. The final in-frame deletion mutant had a 72 base pair scar sequence, contained no antibiotic markers and was named S. *oneidensis* DL13. The mutant was verified via DNA sequencing (data not shown). For complementation, *S. oneidensis luxS* was PCR-amplified and ligated into pBBR1MCS-3 (21) producing p*luxS*. The plasmids p*luxS* and p519*ngfp* (GFP-plasmid for confocal microscopy) (28) were conjugated into *S. oneidensis* strains as described above.

Autoinducer Assays. *V. harveyi* MM32 was used as qualitative reporter strain to identify the presence of AI-2 (30). The reporter strain was grown for 16 hours and then diluted 1:5000. 90 µl of the diluted reporter was then added to a 96 well plate. Cultures of *V. harveyi* BB120 (AI-2^{+}) (2), *E. coli* DH5α (AI-2^{-}), and *S. oneidensis* were grown to O.D. 600 of 1.5 and 3.0, respectively. Then 1ml of the culture was centrifuged and filter sterilized and 10 µl of the filtered supernatant was added to the reporter stain. The plates were incubated at 30° C and the luminescence of the reporter strain was monitored using a luminometer (Beckman Coulter, LD400) for 18 hours.

In vitro AI-2 synthesis. 5'-methylthioadenosine/S-adenosylhomocysteine nucleosidase (MTA/SAHase) and LuxS were purified and used in a two step reaction to enzymatically convert S-adenosylhomocysteine (SAH, Sigma) to AI-2. This protocol has been described elsewhere (49). Briefly, IPTG was used to induce the expression of pProEX HT-mtan and pProEX-LuxS (49) in *E. coli* DH5α for his-tag purification of MTA/SAHase and LuxS, respectively. The cells were lysed using BugBuster protein extraction solution (Novagen EMD Biosciences) and the

desired proteins were retained with Ni-NTA His-Bind resin (Novagen EMD Biosciences). Purified MTA/SAHase was incubated with 2 mM SAH at 37°C for 1 hour in an anaerobic chamber. MTA/SAHase was removed by a centrifugal filter (Centricon, Millipore) and the remaining solution was then incubated with LuxS for an additional 2 hours. Since homocysteine and AI-2 have a 1:1 stoichiometry in the reaction, the resulting solution was analyzed for homocysteine concentration by adding Ellman's reagent and measuring the absorbance at 412 nm. Additional verification of AI-2 activity came from adding various concentrations of synthetic AI-2 to the reporter strain V. *harveyi* MM32 (procedure described above).

Microarray analysis. Overnight LB cultures of WT and DL13 were subcultured into fresh medium and grown to an O.D. 600 of 1.5 in duplicate. The cells were rapidly centrifuged, cell pellets snap frozen in liquid nitrogen and stored at -20°C. RNA isolation, microarray construction, hybridization, scanning, image quantification, and data analysis were performed as previously described (5, 15, 45). Statistical analysis was done with JMP Genomics 3.0 software (SAS Institute, Cary, NC). The raw data were log 2 transformed and imported into the software for analysis. A distribution analysis and data correlation analysis were done as a quality control step. The overlayed kernel density estimates derived from the distribution analysis allowed the visualization of sources of variation based on strain, as well as variation attributed to technical factors such as array and dye. The data were subsequently normalized using a standard normalization technique. A mixed model analysis of variance was done to determine differential expression levels between the MR-1 and LuxS bacterial strains. To control the false discovery rate a testing correction was applied at an alpha level of 0.05.

Oxidative stress. To determine the effect of oxidative stress on a *luxS* mutation, cultures were grown overnight, subcultured into fresh medium, and grown to an OD 600 of 1.5. The cells were diluted to O.D. 600 of 0.5 and hydrogen peroxide was added at various concentrations (5, 10, 20, 40 mM). The O.D. 600 was monitored over a 6 hour period.

Biofilm plate assay. 96 well microtiter plate assays were conducted as an initial method to quantify biofilm growth under static conditions. The protocol has been described elsewhere (33, 34). Briefly, cells were grown overnight in MM medium. Then the cells were diluted to an

O.D. 600 of 0.01 and 175 µl were added to each well. If desired, concentrations of AI-2 (0.1, 1, 5, 10 µM) or homocysteine (10 µM) were also added. AI-2 concentration ranges were chosen by comparing *V. harveyi* MM32 luminescence response of *S. oneidensis* supernatant to known concentrations of AI-2 as described above. *S. oneidensis* WT cell free supernatant and an AI-2 concentration of 10 uM produced a similar luminescence response in the reporter strain *V. harveyi* MM32. The plates were then incubated at 30° C and monitored over a three day period. To process the plates, 10 µl of 0.5% crystal violet was used to stain the cells, which adhered to the wells, for 10 minutes. The wells were then washed and the dye was extracted with methanol. The extracts absorbance was quantified at 570 nm.

Flow-through biofilm assay. Aerobic biofilms were grown using a standard flow-through system (7, 42, 43). The system consisted of a MasterFlex L/S® pump, MasterFlex® silicone tubing, bubble trap, and a three channel flow chamber (dimension: 40 mm length x 4 mm width x 1 mm depth; BioCentrum-DTU). The flow chamber was covered with a glass slide (Fisher Brand #1.5 24x50 mm) and sealed with silicon glue. All components were autoclaved except the flow chamber which was sterilized with 10% H_20_2. The system was equilibrated with LB growth medium for at least 5 hours before inoculation. WT and mutant cells were grown in LB to an O.D. 600 of 1.0 and then diluted to an O.D. 600 of 0.03. To inoculate the system, media flow through the system was stopped and 1-2 mls of culture was injected into the flow chamber. The chamber was placed glass side down to increase cell attachment to the glass slide. After 1 hour the chamber was inverted and flow was restored to an approximate flow rate of 112 µl/min. For chemical AI-2 supplementation, 5 µM of synthetic AI-2 was supplemented to the medium. Images were collected with a Zeiss LSM510 confocal microscope 40X water immersion and 10X planar objective. Image analysis was performed by Zeiss image browser.

Results and Discussion

***luxS* mutagenesis depletes AI-2 activity.** A BLAST search identified a LuxS homologue (SO1101) in the genome of *S. oneidensis* with 78% (132/169) amino acid identity and 89% similarity (152/169) to LuxS in *V. harveyi*. A SO1101 gene replacement was

constructed to produce *S. oneidensis* DL13 so that the physiological role of the *S. oneidensis* LuxS could be examined. Supernatants from *S. oneidensis* wild type (WT) and DL13 cultures were examined for AI-2 production via the *V. harveyi* MM32 reporter strain method (Figure 3-1). After 15 hours, the assays revealed that the supernate from WT produced an AI-2 induced luminescence response in the reporter strain at levels similar to the positive control *V. harveyi* BB120. DL13 generated near background levels of luminescence, similar to *E. coli* DH5α (*luxS*-). AI-2 production was restored to WT levels in the mutant strain upon complementation via a plasmid encoded *S. oneidensis* native *luxS*, DL13 pl*uxS*.

Microarray analysis of *luxS* mutation. Gene expression of WT and DL13 cells at an O.D. 600 of 1.5 were analyzed via microarrays to determine what global affects a *luxS* mutation may have on transcriptome expression. Gene expression profiles meeting our significance criteria can be found in Table 3-1 and are presented as arithmetic means. A complete list of the microarray data can be found in the supplemental material (Appendix 1). The genes have been place in four broad phenotypic categories: metabolism, protein and nutrient transport, biofilm development, and stress response. Overall large expression differences were not seen but the p-values show that some differences are statistically significant. The biggest expression difference was only a 6-fold increase (SO3483: small hypothetical protein) in the WT; the majority of the genes differentially expressed had increased expression in WT (or a decrease in DL13). Some of the broad categories identified in this study have also been seen in other organisms. Microarray data collected from *luxS* mutants in *E. coli* and *Streptococcus mutans* also showed differential gene expression of genes related to biofilm formation and stress response (10, 41). The *E. coli* study also showed expression differences solute transport genes.

A *luxS* mutation did not cause a large difference in global gene expression in *S. oneidensis*. Depending on the bacterial species, a *luxS* mutation has been known to cause large (10, 41) or small (14) differences in global gene regulation. This may have been in part due to having biological duplicates or experimental conditions. The data could also suggest that a *luxS* mutation may be more important for post translation modification. This is reasonable hypothesis because of the involvement of LuxS in the AMC, is a major methyl donor source in the cell.

***luxS* does not affect oxidative stress response or anaerobic growth.** The largest microarray category affected by a loss of *luxS* is related to stress response. This category has genes with antioxidant activity, stress response chaperones and transcriptional activators, and DNA and RNA metabolism and repair. The hypothesis that a *luxS* mutation influences stress response, particularly oxidative stress, seemed likely because this same trait has been seen in other organisms (22, 47). However, no differences were seen between the growth rate of WT and DL13 to various concentrations of H_2O_2 (data not shown).

Varying results have previously been presented on whether *S. oneidensis* can use extracellular quorum sensing signals to enhance metabolism (9, 23). Microarray data also suggests that a *luxS* mutation may be involved with certain metabolic pathways (Table 3-1). The array data contained genes implicated with the activated methyl cycle (AMC), amino acid synthesis, the TCA cycle, and electron transport. The involvement of *luxS* in the AMC will be described later. The influence of *luxS* on broad metabolism was examined under aerobic and anaerobic conditions to compare the growth rates of WT and DL13. The resulting growth curves showed that the *luxS* mutation had little effect on aerobic or anaerobic growth with soluble (fumarate and TMAO) or insoluble (Fe_2O_3) terminal electron acceptors (data not shown). These findings are also support Bretschger et al. (4) that a *luxS* mutation causes no significant differences in iron reduction in *S. oneidensis*.

***luxS* mutation influences biofilm development.** Initial *S. oneidensis* biofilm assays were conducted under static conditions using a 96 well microtiter plate. After one day of growth, DL13 has a slight (10%) decreased ability to form a biofilm when compared to WT as measured by reading the absorbance of the plate staining at 570 nm (Figure 3-2). Upon three days of growth, DL13 biofilm had only 66% the biomass compared to WT. These experiments suggest that a mutation in *luxS* decreases *S. oneidensis* ability to initiate early surface colonization.

Various concentrations of synthetic AI-2 were added to the biofilm plate assay to test whether AI-2 acts as a quorum sensing signal in *S. oneidensis*. The concentration ranges of AI-2 was taken from comparing the relative luminescence response of *V. harveyi* MM32 to the addition of WT supernatant and known concentrations of AI-2. AI-2 did not restore DL13 biofilm forming abilities to WT levels after one or three days of growth. To verify if AI-2 inhibits overall cell growth, aerobic planktonic growth experiments were conducted. No aerobic

growth differences were seen between DL13 and WT with the addition of AI-2 (data not shown). The addition of homocysteine, a byproduct of the AI-2 synthesis, was also examined to show that AI-2 was affecting biofilm formation and not a reaction byproduct. Homocysteine had no negative affect on biofilm growth for DL13 or WT.

The influence of LuxS on *S. oneidensis* biofilms was also evaluated under flow conditions. WT biofilms covered the majority of the glass slide and a high density of three dimensional tower-like structures was visible after 16 hours (Figure 3-3 A). Similar structures have also been seen by other studies examining *S. oneidensis* biofilm formation (42, 43). In comparison, DL13 showed a low density of small microcolonies forming on the surface. However, DL13 and WT biofilms were similar upon 48 hours of growth (Figure 3-3 B). The addition of 5 μM AI-2 did not restore DL13 biofilm forming abilities to WT levels after 16 hours of growth (Figure 3-3 A, right).

Continuous flow and static biofilms experiments support the conclusion that a mutation in *luxS* inhibits initial cell adhesion and/or initial biofilm development. The microarray data also showed a decrease in DL13 gene expression associated with motility and chemotaxis and pilin biosynthesis. Both motility and pilin production are both known to play a role in *S. oneidensis* biofilm development (43). The observation that a *luxS* mutation can alter initial biofilm development has also been seen in *S. mutans* and *Klebsiella pneumonia* (1, 29). Under the experimental conditions examined, LuxS is important for biofilm development but the addition of AI-2 did not restore the biofilm defect. This supports the conclusion that the quorum sensing signal AI-2 is not used to regulate biofilm development.

LuxS impacts the activated methyl cycle. Growth under sulfur limited conditions was examined to determine if a mutation in *luxS* disrupts the AMC and homocysteine recycling. Cells were grown in M9 medium with a variety of sole sulfur species: K_2SO_4, cysteine, homocysteine, or methionine. Growth on K_2SO_4, cysteine, or homocysteine produced no growth differences between WT and DL13 (Figure 3-4). DL13 showed consistent growth reduction compared to WT when grown with methionine as the sole sulfur source.

The growth difference seen between *S. oneidensis* WT and DL13 with methionine as the sole sulfur source is likely to be related to the disruption of the AMC and the mutant's inability to recycle homocysteine and potentially participate in methylation reactions. Similarly growth

deficiencies in *luxS* mutants of *Bacillus subtilis* and *Staphylococcus aureus* under sulfur limiting conditions have been linked to the disruption of the AMC (13, 18). Additional support for this conclusion comes from microarray data that produced evidence that DL13 had a reduction in genes involved with SAM-dependent methyltransferase (*menG*-2) and the flux of adenosine (*coaD*), both are related to the AMC. The growth difference seen between WT and DL13 on methionine is an additional link of the role of LuxS in metabolism rather than quorum sensing.

Since a *luxS* mutation in *S. oneidensis* can cause problems with methionine metabolism within the AMC, there is a potential for this mutation to also affect the SAM methyltransferase reaction. Altering the cell's ability to participate in methyltransferase reactions could have broad cellular repercussions and could be the reason LuxS is important for biofilm development. SAM, the major methyl donor in the cell, has been called the second most used (first being ATP) enzyme substrate (24). DNA methylation can alter the cell cycle, gene regulation, virulence, and DNA repair (19, 26). Post translational methylation can be used to alter or broaden the function of proteins. One such example is CheR, a chemotaxis methyltransferase that utilizes SAM as a methyl donor (12, 37, 38).

The AI-2-metabolism debate. Over 60 different bacterial species possess *luxS*. The seemingly ubiquitousness of *luxS* could be explained because it is an interspecies signal producer or a necessary gene in a biosynthetic pathway. The toxic AMC intermediate SAH can be detoxified from the cell by a one step reaction catalyzed by SAH hydrolase or a two step process that involves Pfs, LuxS, and the production of AI-2. One could argue that the benefit of utilizing the two step reaction is that the AI-2 signal is produced. Also, one SAM methyl donation event produces one molecule of AI-2, which makes it a good candidate for a cell density signal and metabolic activity (50, 51). Together this makes a case for AI-2 being a potential signal. Within the *Vibrio* species, the link between quorum sensing and *luxS* is well defined. Outside this genus, the debate between AI-2 quorum sensing and metabolism still endures. Direct evidence that links the AI-2 signal to *luxS*-related phenotypes would help resolve this debate (44, 48).

Our findings show that LuxS is involved in *S. oneidensis* biofilm development, but it does not use the AI-2 quorum sensing signal to regulate biofilm development or act as a global gene regulator. We have also produced evidence that LuxS is important in *S. oneidensis* for methionine metabolism, mostly likely through the AMC. This supports the argument that having

luxS or even the ability to create a positive AI-2 reporter strain response does not necessarily mean the bacteria have the ability to utilize quorum sensing. A question that still remains is why a *luxS* mutation can cause slower biofilm development. The biofilm medium used in our experiments was too nutrient rich to extrapolate any specific differences in metabolism to biofilm development. A metabolic defect could directly influence biofilm formation or related processes like methylation and post translational modification could play a significant role.

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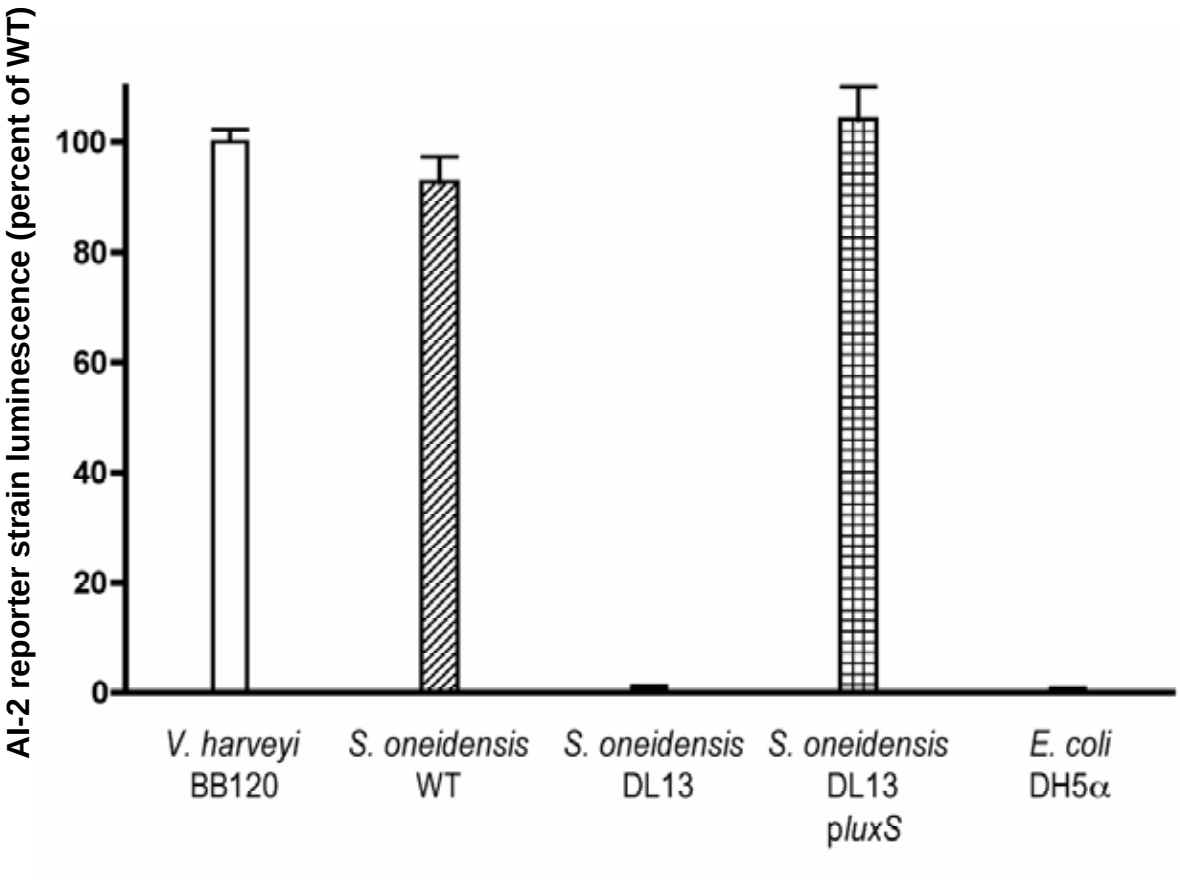


Figure 3-1. Luminescence response of *V. harveyi* MM32 to AI-2 from filtered supernatant from *V. harveyi* BB120 (AI-2+), *E. coli* DH5α (AI-2-), *S. oneidensis* WT, *S. oneidensis* DL13 (Δ*luxS*), and DL13 p*luxS* (plasmid encoded *luxS*). Raw luminescence value for *V. harveyi* BB120 is 9535. Experiment was done in triplicate with 8 replicates. Error bars represent standard error of the mean (SEM) (n=8).

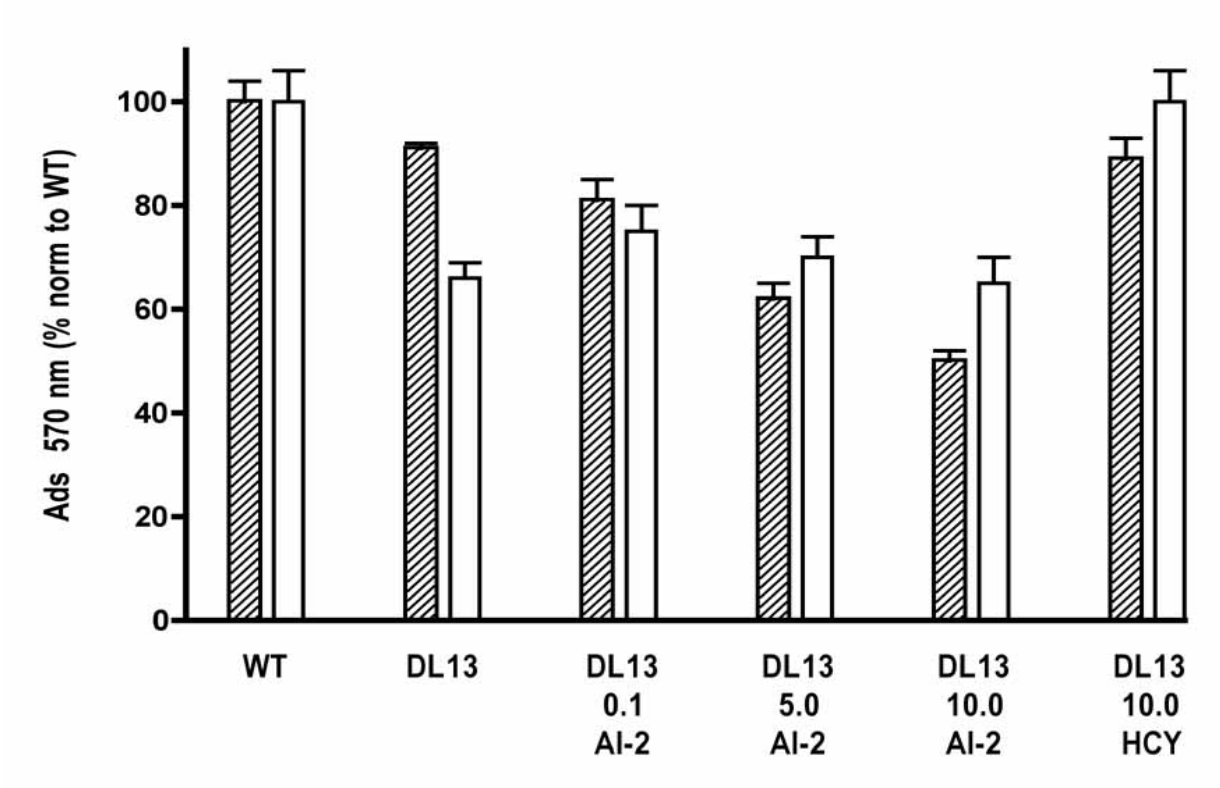
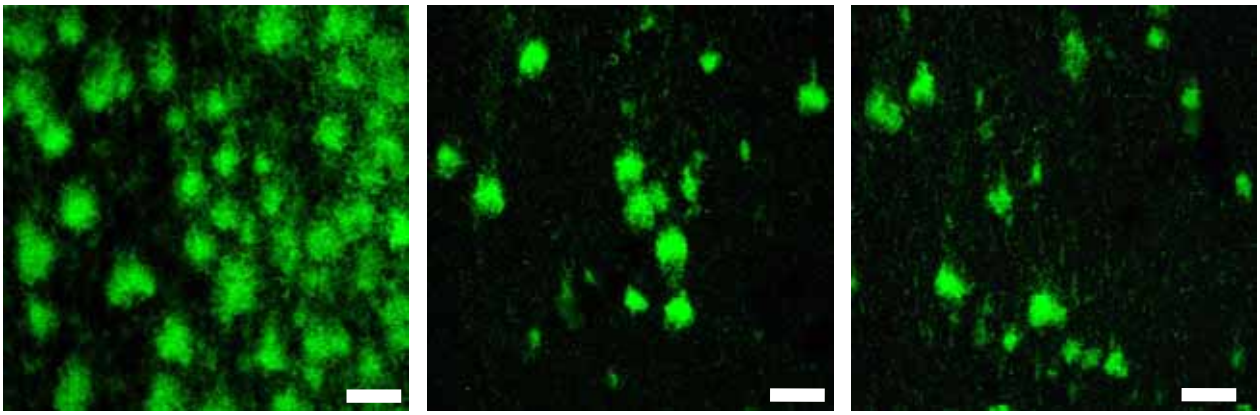


Figure 3-2. Biofilm microtiter plate assay showing one (hatched box) and three (white box) days of DL13 biofilm growth. Varying concentrations of synthetic AI-2 (0, 0.1, 5, 10 μm) and 10 μM homocysteine were added. The data is normalized to WT absorbance at 570 nm values of each day. The raw absorbance value of WT day 1 is 0.439 and day three is 0.609. Experiment was completed in duplicate with 6 replicates. Error bars represent SEM (n=6).

A.



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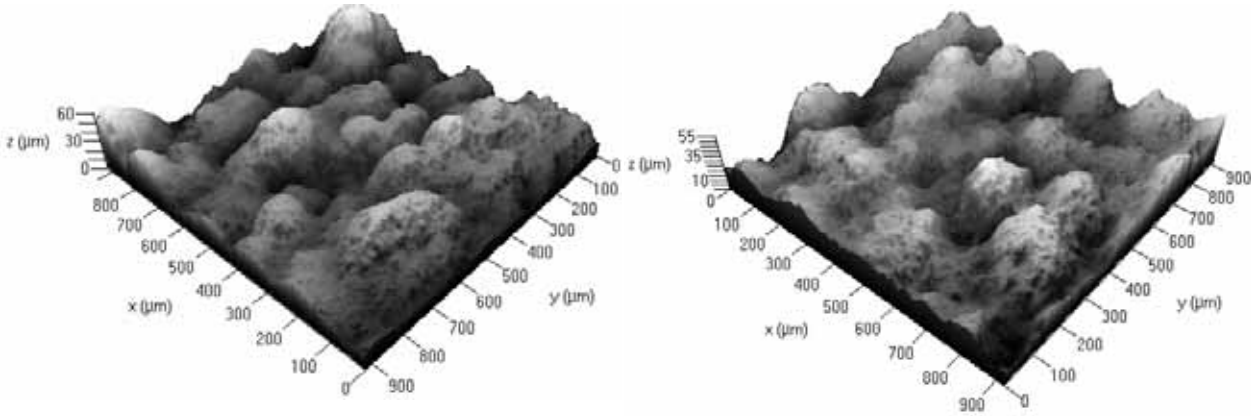

Figure 3-3. (A) Two dimensional confocal images after 16 hours of biofilm growth. *S. oneidensis* WT (left), DL13 (middle), and DL13 (right) supplemented with 5 µM AI-2. The scale bars represent 100 µm.

Figure 3-3. (B) Three dimensional projections of confocal images after 48 hours of growth. *S. oneidensis* WT (left) and DL13 (right). All images taken with a 10X objective lens.

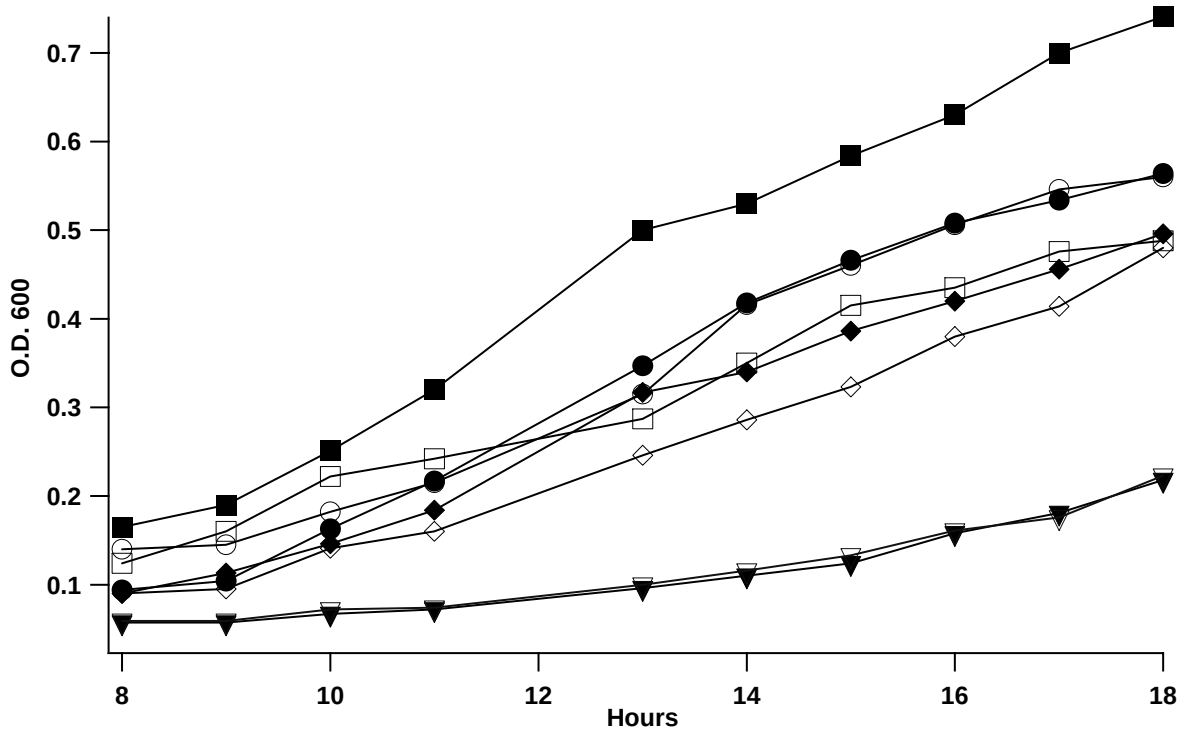

Figure 3-4. Growth curves of *S. oneidensis* WT (filled shapes) and DL13 (open shapes) on various sources of sulfur. Triangle: cysteine. Diamond: K_2SO_4. Circle: homocysteine. Box: methionine. The data from hours 0-7 are not shown because under these conditions there was long lag phase and little growth was seen. At time 0, the O.D. 600 was 0.03. Data representative of experiments done in triplicate.

Table 3-1. *S. oneidensis* MR-1 genes showing gene expression compared to Δ*luxS* during exponential growth.

Gene ID	Gene	General Role	Induction ratio (fold) [a]	-log$_{10}$ p-value [b]
Metabolism				
SO3286	*cydA*	Cytochrome d ubiquinol oxidase	3.7	8.3
SO1234	*torE*	TMNO reductase activity	1.6	2.9
SO2362	*ccoQ*	Cytochrome c oxidase	1.3	6.8
SO2363	*ccoO*	Cytochrome c oxidase	1.3	4.9
SO1929	*sdhB*	Succinate dehydrogenase	1.2	5.5
SO4197	*menG*-2	SAM-dependent methyltransferase	1.1	3.1
SO4684	*coaD*	Adenylyltransferase	1.3	3.2
SO3519	*glnB*-2	Glutamate family biosynthesis	-1.2	3.1
SO4233	*leuD*	Pyruvate family biosynthesis	-1.1	3.9
Transport and Binding				
SO2766		Degradation of proteins	4.6	5.8
SO0251	*secY*	Preprotein translocase	1.2	6.4
SO0262	*ccmB*	Heme exporter protein	1.2	3.6
SO0263	*ccmA*	Heme exporter protein	1.1	2.9
SO4203	*tatB*	Sec-independent protein translocase	1.1	3.2
SO3965	*ptsO*	Sugar phosphotransferase	1.3	3.0
SO0139	*ftn*	Ferric iron binding protein	1.3	7.5
SO1827	*exbD2*	TonB system transport protein	1.2	3.5
SO2233		ATPase transmembrane activity	2.1	3.1
SO2373		Bcr/CflA family, resistance transporter	2.0	4.0
SO2750	*tolR*	Transporter activity	1.4	4.6
SO4317		Type I secretion target	1.5	6.6
Biofilm Development				
SO3202	*cheW*-3	Chemotaxis and motility	1.3	3.2
SO3251	*cheR*-2	Chemotaxis and motility	1.2	4.1
SO4106	*mshB*	Adhesion (MSHA pilin protein)	1.2	5.1
Stress Response				
SO2756		Antioxidant activity	2.3	4.8
SO3341		AhpC/TSA family, antioxidant activity	1.5	4.1
SO0452	*trxC*	Thioredoxin 2	1.2	3.4
SO3718		DsbA family oxidoreductase	1.2	3.1
SO0703	*groES*	Chaperonin	1.2	4.4
SO1455		RadC family, DNA metabolism	1.5	3.7
SO2087	*ihfA*	Integration host factor, DNA metabolism	1.2	5.3
SO2401	*ihfB*	Integration host factor, DNA metabolism	1.2	6.5
SO2780	*rpmF*	Ribosomal protein L32	2.6	2.8
SO0006	*rnpA*	Ribonuclease P protein	1.3	3.4
SO0225	*rpoC*	DNA-dependent RNA polymerase	1.2	5.1
SO0256	*rpoA*	DNA-dependent RNA polymerase	1.2	4.9
SO0082		MerR family, DNA interactions	1.1	3.4

[a] Relative gene expression is presented as the mean ratio of the fluorescence intensity of MR-1 cells to that of Δ*luxS* cells.

[b] Each gene showed significant differential expression as described in the Materials and Methods. P-values are represented as a $-\log_{10}$ number.

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Appendix 1. Table of the entire *S. oneidensis* wildtype vs. Δ*luxS* microarray data set.

Gene ID	*Gene*	Mainrole	Induction ratio (fold) [(a)]	-log$_{10}$ p-value [(b)]
SO0756	*aroG*	Amino acid biosynthesis	-0.9	4.3
SO1625	*dapD*	Amino acid biosynthesis	1.1	3.1
SO4055	*metL*	Amino acid biosynthesis	-0.8	3.5
SO3519	*glnB-2*	Amino acid biosynthesis	-1.2	3.1
SO2068	*hisF*	Amino acid biosynthesis	-0.8	4.5
SO2072	*hisC*	Amino acid biosynthesis	-0.8	3.7
SO2073	*hisD*	Amino acid biosynthesis	-0.8	3.2
SO4233	*leuD*	Amino acid biosynthesis	-1.1	3.9
SO2221	*pabB*	Biosynthesis of cofactors, prosthetic groups, and carriers	-0.8	4.0
SO1031		Biosynthesis of cofactors, prosthetic groups, and carriers	-0.8	3.9
SO3653	*ispB*	Biosynthesis of cofactors, prosthetic groups, and carriers	-0.9	4.0
SO4197	*menG-2*	Biosynthesis of cofactors, prosthetic groups, and carriers	1.1	3.1
SO4684	*coaD*	Biosynthesis of cofactors, prosthetic groups, and carriers	1.3	3.2
SO1634	*cdsA*	Fatty acid and phospholipid metabolism	1.1	3.3
SO1640	*fabZ*	Fatty acid and phospholipid metabolism	1.2	4.0
SO0220	*rplK*	Protein synthesis	1.3	3.6
SO0233	*rplW*	Protein synthesis	1.2	3.3
SO0237	*rpsC*	Protein synthesis	1.2	2.8
SO0238	*rplP*	Protein synthesis	1.2	3.6
SO0239	*rpmC*	Protein synthesis	1.2	7.9
SO0240	*rpsQ*	Protein synthesis	1.2	5.7
SO0247	*rplR*	Protein synthesis	1.3	7.1
SO0250	*rplO*	Protein synthesis	1.2	4.9
SO0254	*rpsK*	Protein synthesis	1.3	3.8
SO1360	*rplS*	Protein synthesis	1.2	2.8
SO2780	*rpmF*	Protein synthesis	2.6	2.8
SO3928	*rpsR*	Protein synthesis	1.2	2.9
SO3565	*cpdB*	Purines, pyrimidines, nucleosides, and nucleotides	-0.8	4.2
SO1797		DNA metabolism	1.1	3.3
SO1455		DNA metabolism	1.5	3.7
SO2087	*ihfA*	DNA metabolism	1.2	5.3
SO2401	*ihfB*	DNA metabolism	1.2	6.5
SO2506	*uvrB*	DNA metabolism	-0.9	3.6
SO2514	*nth*	DNA metabolism	1.1	3.2
SO2790	*sbcB*	DNA metabolism	-0.8	6.2

SO2524		Central intermediary metabolism	-0.8	3.7
SO3513		Central intermediary metabolism	-0.9	3.2
SO3498		Energy metabolism	-0.8	3.1
SO2459	*cga*	Energy metabolism	-0.9	5.4
SO0452	*trxC*	Energy metabolism	1.2	3.4
SO2362	*ccoQ*	Energy metabolism	1.3	6.8
SO2363	*ccoO*	Energy metabolism	1.3	4.9
SO3286	*cydA*	Energy metabolism	3.7	8.3
SO3623		Energy metabolism	1.4	3.2
SO3920	*hydA*	Energy metabolism	-0.8	5.0
SO4144		Energy metabolism	-0.7	4.9
SO1237		Energy metabolism	1.2	4.5
SO2450	*susB*	Energy metabolism	-0.9	3.9
SO4458		Energy metabolism	-0.8	6.7
SO1929	*sdhB*	Energy metabolism	1.2	5.5
SO0082		Regulatory functions	1.1	3.4
SO0997		Regulatory functions	-0.9	3.0
SO2847		Regulatory functions	-0.8	2.8
SO3305		Regulatory functions	-0.9	2.8
SO4312		Regulatory functions	-0.9	3.1
SO1669	*tyrR*	Regulatory functions	-0.9	3.9
SO2543		Signal transduction	-0.8	3.9
SO3999		Signal transduction	-0.9	5.0
SO4478	*cpxA*	Signal transduction	-0.9	2.9
SO0358		Transcription	1.2	3.9
SO0225	*rpoC*	Transcription	1.2	5.1
SO0256	*rpoA*	Transcription	1.2	4.9
SO0006	*rnpA*	Transcription	1.3	3.4
SO0187		Protein fate	-0.7	3.8
SO0606	*hflC*	Protein fate	1.1	3.8
SO0959	*pepA-1*	Protein fate	-0.9	5.0
SO3302		Protein fate	-0.9	3.1
SO3391		Protein fate	-0.9	3.3
SO0251	*secY*	Protein fate	1.2	6.4
SO0262	*ccmB*	Protein fate	1.2	3.6
SO0263	*ccmA*	Protein fate	1.1	2.9
SO4203	*tatB*	Protein fate	1.1	3.2
SO0703	*groES*	Protein fate	1.2	4.4
SO3718		Protein fate	1.2	3.1
SO1334	*lgt*	Protein fate	-0.9	3.1
SO3870		Protein fate	-0.9	2.9
SO0858		Transport and binding proteins	-0.7	4.6
SO4654	*cysW-2*	Transport and binding proteins	-0.9	3.0

SO3965	*ptsO*	Transport and binding proteins	1.3	3.0
SO0139	*ftn*	Transport and binding proteins	1.3	7.5
SO1827	*exbD2*	Transport and binding proteins	1.2	3.5
SO3914		Transport and binding proteins	-0.9	4.2
SO0802		Transport and binding proteins	-0.8	4.3
SO1918		Transport and binding proteins	-0.8	3.0
SO0160		Transport and binding proteins	-0.9	3.0
SO0539		Transport and binding proteins	-0.8	4.0
SO2233		Transport and binding proteins	2.1	3.1
SO3078		Transport and binding proteins	-0.8	3.0
SO0207	*murI*	Cell envelope	-0.9	3.9
SO4097	*mreC*	Cell envelope	-0.9	4.8
SO1102		Cell envelope	-0.8	2.8
SO1234	*torE*	Cell envelope	1.6	2.9
SO2570		Cell envelope	-0.9	3.4
SO4334		Cell envelope	-0.9	4.9
SO2324	*cheW-2*	Cellular processes	-0.8	3.3
SO3202	*cheW-3*	Cellular processes	1.3	3.2
SO3251	*cheR-2*	Cellular processes	1.2	4.1
SO2756		Cellular processes	2.3	4.8
SO3341		Cellular processes	1.5	4.1
SO0135		Cellular processes	-0.9	3.2
SO4106	*mshB*	Cellular processes	1.2	5.1
SO4317		Cellular processes	1.5	6.6
SO2373		Cellular processes	2.0	4.0
SO2750	*tolR*	Cellular processes	1.4	4.6
SO3484		Cellular processes	-0.8	5.2
SO3485		Cellular processes	-0.7	4.3
SO4274		Cellular processes	-0.8	4.7
SO0674		Other categories	-0.8	5.6
SO0675		Other categories	-0.8	2.9
SO2685		Other categories	-0.8	3.7
SO2948		Other categories	1.2	4.4
SO1974		Other categories	-0.9	3.4
SO2129		Other categories	-0.8	4.0
SO2654		Other categories	-0.8	3.3
SO0413		Unknown function	-0.9	3.0
SO0632	*hrpB*	Unknown function	-0.9	3.0
SO0715		Unknown function	-0.9	3.0
SO1670		Unknown function	-0.9	3.0
SO1936		Unknown function	1.2	2.9
SO3497		Unknown function	-0.9	3.5
SO1000		Unknown function	-0.8	3.2
SO1570		Unknown function	-0.9	2.8
SO2309	*crcB*	Unknown function	1.2	5.1
SO3325		Unknown function	1.3	4.8

SO3611		Unknown function	-0.8	2.8
SO4040		Unknown function	-0.7	4.1
SO4672	*glpE*	Unknown function	1.2	3.5
SO2032			1.2	6.3
SOA0040			1.2	5.8
SOA0044			0.9	3.7
SOA0048			0.8	3.7
SOA0132			0.8	5.0
SOA0150			1.4	4.3
GR_SO2031			1.7	8.3
GR_SOA0115			1.4	5.0
SO0086		Hypothetical proteins	1.8	3.1
SO0115		Hypothetical proteins	0.9	3.0
SO0156		Hypothetical proteins	1.1	3.3
SO0203		Hypothetical proteins	0.9	4.5
SO0290		Hypothetical proteins	1.2	3.6
SO0489		Hypothetical proteins	1.5	4.2
SO0563		Hypothetical proteins	1.2	4.4
SO0595		Hypothetical proteins	1.1	4.8
SO0670		Hypothetical proteins	0.9	3.3
SO0679		Hypothetical proteins	0.7	3.2
SO0680		Hypothetical proteins	0.9	2.8
SO0681		Hypothetical proteins	0.7	2.8
SO0710		Hypothetical proteins	0.7	6.9
SO0730		Hypothetical proteins	0.7	3.6
SO0758		Hypothetical proteins	1.3	6.5
SO0890		Hypothetical proteins	0.9	3.0
SO0910		Hypothetical proteins	0.9	2.9
SO0971		Hypothetical proteins	1.1	3.7
SO1003		Hypothetical proteins	0.9	3.4
SO1182		Hypothetical proteins	1.1	3.3
SO1222		Hypothetical proteins	0.8	5.1
SO1365		Hypothetical proteins	1.2	4.0
SO1419		Hypothetical proteins	0.8	3.3
SO1589		Hypothetical proteins	0.8	2.9
SO1595		Hypothetical proteins	0.9	2.9
SO2024		Hypothetical proteins	0.9	3.4
SO2027		Hypothetical proteins	0.9	4.0
SO2058		Hypothetical proteins	1.1	4.4
SO2084		Hypothetical proteins	1.6	2.7
SO2166		Hypothetical proteins	1.5	5.0
SO2184		Hypothetical proteins	0.7	2.8
SO2206		Hypothetical proteins	0.9	3.2
SO2249		Hypothetical proteins	1.3	4.5
SO2334		Hypothetical proteins	1.5	5.4
SO2382		Hypothetical proteins	1.5	6.2
SO2526		Hypothetical proteins	1.4	4.1

SO2673	Hypothetical proteins	0.8	3.4
SO2682	Hypothetical proteins	0.9	3.1
SO2712	Hypothetical proteins	0.9	2.8
SO2765	Hypothetical proteins	1.4	2.8
SO2873	Hypothetical proteins	1.7	3.2
SO2904	Hypothetical proteins	1.5	2.9
SO2936	Hypothetical proteins	0.8	3.2
SO2972	Hypothetical proteins	1.1	2.8
SO2998	Hypothetical proteins	0.8	3.4
SO3027	Hypothetical proteins	1.1	2.8
SO3086	Hypothetical proteins	1.6	2.9
SO3482	Hypothetical proteins	6.6	7.2
SO3526	Hypothetical proteins	1.2	6.2
SO3591	Hypothetical proteins	1.1	4.7
SO3618	Hypothetical proteins	1.1	3.8
SO3643	Hypothetical proteins	1.2	3.3
SO3656	Hypothetical proteins	2.3	7.2
SO3724	Hypothetical proteins	0.6	3.8
SO3856	Hypothetical proteins	1.1	3.2
SO3955	Hypothetical proteins	1.1	3.9
SO4038	Hypothetical proteins	1.1	2.8
SO4067	Hypothetical proteins	2.4	3.3
SO4117	Hypothetical proteins	0.7	2.8
SO4137	Hypothetical proteins	2.2	4.5
SO4188	Hypothetical proteins	1.3	3.7
SO4270	Hypothetical proteins	0.9	2.9
SO4275	Hypothetical proteins	0.8	2.9
SO4594	Hypothetical proteins	1.2	4.3
SO4656	Hypothetical proteins	1.1	5.2
SO0119	Hypothetical proteins	1.1	4.3
SO0335	Hypothetical proteins	1.4	2.9
SO0336	Hypothetical proteins	0.8	3.2
SO0591	Hypothetical proteins	0.8	2.8
SO0908	Hypothetical proteins	0.8	3.0
SO0964	Hypothetical proteins	1.5	4.6
SO1169	Hypothetical proteins	1.1	3.4
SO1657	Hypothetical proteins	2.6	4.4
SO1939	Hypothetical proteins	1.2	3.7
SO2064	Hypothetical proteins	0.9	3.4
SO2365	Hypothetical proteins	1.2	2.9
SO2385	Hypothetical proteins	0.8	3.7
SO2523	Hypothetical proteins	0.7	3.5
SO2622	Hypothetical proteins	1.1	3.1
SO2663	Hypothetical proteins	0.8	3.3
SO2721	Hypothetical proteins	1.1	2.9
SO2766	Hypothetical proteins	4.6	5.8
SO2800	Hypothetical proteins	1.1	2.9
SO2821	Hypothetical proteins	1.1	4.2
SO2976	Hypothetical proteins	1.1	3.0
SO3104	Hypothetical proteins	1.2	5.9

SO3159	Hypothetical proteins	0.8	4.0
SO3184	Hypothetical proteins	0.9	3.5
SO3192	Hypothetical proteins	1.1	3.3
SO3355	Hypothetical proteins	1.1	3.4
SO3357	Hypothetical proteins	0.8	4.1
SO3366	Hypothetical proteins	1.2	6.5
SO3764	Hypothetical proteins	1.2	3.0
SO3985	Hypothetical proteins	0.8	2.8
SO4143	Hypothetical proteins	0.8	2.8
SO4169	Hypothetical proteins	0.7	2.9
SO4455	Hypothetical proteins	1.2	3.4
SO4504	Hypothetical proteins	1.2	3.0
SO4641	Hypothetical proteins	1.2	3.0

[(a)] Relative gene expression is presented as the mean ratio of the fluorescence intensity of MR-1 cells to that of Δ*luxS* cells.

[(b)]Each gene showed significant differential expression as described in the Materials and Methods. P-values are represented as a $-\log_{10}$ number.