ABSTRACT

Title of Document: QUORUM SENSING IN BACTERIA

                            ASSOCIATED WITH MARINE SPONGES

                            MYCALE LAXISSIMA AND *IRCINIA*

                            STROBILINA

                            Jindong Zan, Doctor of Philosophy, 2013

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                            Environmental Science

Sponges can form close associations with microbes that in some cases comprise up to 30% of the biomass of the sponge, which we hypothesized would provide an ideal environment for quorum sensing. I isolated 420 bacterial strains from two marine sponge species and screened these isolates for acyl-homoserine lactone (AHL) production. Results showed that isolates from the *Silicibacter-Ruegeria* (SR) subclade of the *Roseobacter* group are the dominant AHL producers. Production of these signaling compounds was consistently observed in isolates obtained from different sponge individuals during different seasons. The SR-type strain *Ruegeria* sp. KLH11 was isolated from tissue of the sponge *Mycale laxissima*. Chemical analysis of the AHLs produced by *Ruegeria* sp. KLH11 showed them to be predominantly composed of a mixture of long chains AHLs with 3-OH substitutions. Two pairs of *luxR* and *luxI* homologues and one

solo *luxI* homologue were identified and designated as *ssaRI*, *ssbRI* and *sscI* (<u>s</u>ponge-associated <u>s</u>ymbiont locus <u>A</u>, <u>B</u> and <u>C</u>, *luxR* or *luxI* homologue). SsaI directs synthesis of predominantly 3-oxo-AHLs whereas SsbI and SscI specify 3-OH-AHL derivatives. Wild type *Ruegeria* sp. KLH11 cultures are dominated by SsbI or SscI-specified AHLs. Mutation of either *ssaR* or *ssaI* results in loss of swimming motility, flagellar production and flagellin synthesis whereas mutation of *ssbR* or *ssbI* had no effect on these characteristics and no detectable phenotype. In wild type cultures, flagella are produced only in late stage growth. The non-essential phosphorelay *cckA-chpT-ctrA* system acts downstream of s*saRI* to control flagellar motility. Mutants of *ssaI* and *ssaR* showed increased biofilm formation while mutants of *ssbI* and *ssbR* were not affected in biofilm formation, and this is not due solely to the loss of motility. The results showed the presence of AHL molecules similar to those specified by SsaI in sponge tissues and that the *ssaI* gene is actively expressed *in situ*, revealed by RT-PCR. We have established *Ruegeria* sp. KLH11 as a model to study the complex symbiotic relationships between sponges and microbes.

QUORUM SENSING IN BACTERIA ASSOCIATED WITH MARINE
SPONGES *MYCALE LAXISSIMA* AND *IRCINIA STROBILINA*

By

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DEDICATION

I dedicate my thesis to my wife Yue Liu, Ph.D, mother Jianfang Han, and

father Shengfa Zan for their endless love and support.

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settle down when I first joined the laboratory, and Dr. Naglaa Mohamed, who taught me many important laboratory techniques and has been a good friend. I would also like to give my thanks to Jeanette Davis for all her support and encouragement over the past five years. I thank Dr. Leah C. Blasiak for helping edit my writing many times and Ryan J. Powell for many useful scientific conversations. Jan Vicente is specially acknowledged for his help in sponge collection. I am also grateful to Dr. Michael Hibbing and Miss. Jing Xu from Dr. Fuqua's laboratory, who taught me how to do biofilm assays and real-time PCR.

Statement of Contribution

Elisha M. Cicirelli identified the *ssaRI* and *ssbRI* systems and Okhee Choi identified the *sscI* gene. Elisha M. Cicirelli, Stephanie Kroll and Okhee Choi created the strains and plasmids that start with EC, SK and OKC, respectively. Also, they performed most of the experiments with crude organic extract of KLH11 cultures. Jason E. Heindl performed the flagellar stain experiment and provided three expression constructs that start with JEH, described in Chapter 4. All these four colleagues are from Department of Biology, Indiana University.

Hiruy Sibhatu, Charis L. Uhlson, Christina L. Wysoczyski, Robert C. Murphy, and Mair. E.A. Churchill performed all the mass spectrometric experiments. Christina L. Wysoczynski performed the organic extraction from sponge tissues. All these colleagues are from University of Colorado, Denver.

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Chapter 1. Introduction and literature review

1.1. Marine sponges

1.1.1 Sponge body structure and reproduction

Sponges belong to the phylum *Porifera* and are considered to be the most ancient clade of the metazoans, with a fossil record dating back about 580 million years (Vacelet and Donadey, 1977; Taylor *et al.*, 2007). Sponges are the morphologically simplest metazoans and have very simple body structures. In general, sponges have three distinct layers in their body plans as shown in Figure 1: the outer layer is the pinacoderm, the inner layer is the choanoderm, consisting of flagellated chambers and the middle region is the mesohyl (Hentschel *et al.*, 2012). Sponges are filter feeders and they obtain their nutrition by filtering the surrounding water through small holes called ostia on their surface (Fig. 1). After passage through flagellated chambers, the filtered water is almost sterile on discharge and is pumped out through the main water channel termed the osculum. The size, number and shape of the ostia vary from one species to another (Taylor *et al.,* 2007).
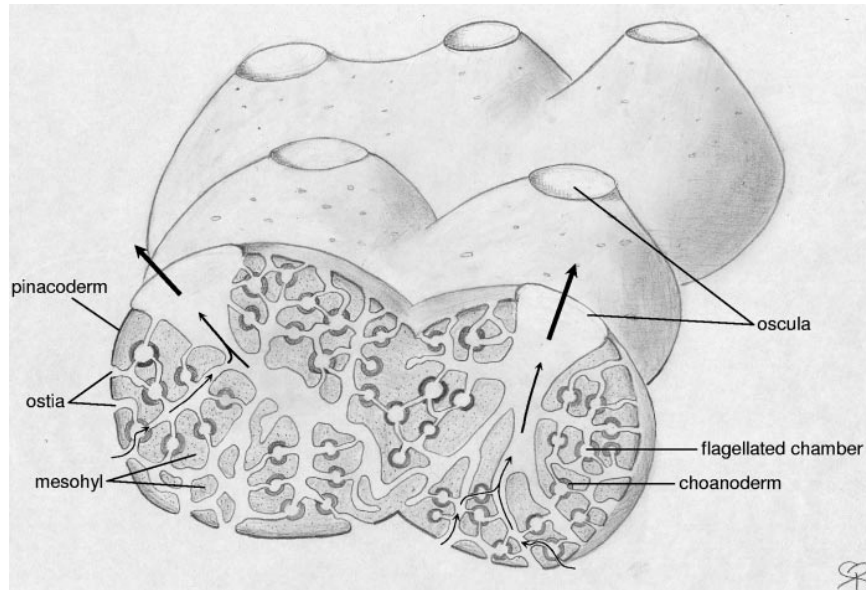
Figure 1.1. **Schematic display of a sponge body structure.** Adapted from Taylor *et al*. (2007) with permission. The arrows indicate the direction of the water flow.

Sponges are able to reproduce asexually or sexually (Levi, 1957). There are generally three different mechanisms of asexual reproduction: 1) Budding. A set of cells can form on the external portion of the sponge body. This set of cells can develop into a complete sponge and breaks off from the original sponge and eventually becomes an independent individual. 2) Gemmule formation. This process is similar to budding but differs in that the gemmule is formed within the sponge and has a protective layer around it, which can help it survive even in harsh environments. 3) Regeneration. This refers to the process in which a sponge is broken into different pieces that are able to develop into new sponges.

Sponges have both female and male reproductive organs, but they very rarely self-fertilize. A sponge produces sperm in the mesohyl layer. The sperm will

develop to the gamete stage before they are pumped into the surrounding water through the sponge osculum. Water currents can bring these gametes into the neighboring sponges through ostia where the sperm is able to contact the eggs produced by a different individual. The fertilized eggs can be either released immediately or undergo limited development in the mother sponge, to be relased as larvae into the surrounding water, which can settle onto the solid surface and proceed to develop into independent sponges (Bergquist, 1978).

1.1.2 Sponge taxonomy

Sponges have a wide global distribution, can be found in almost all aquatic habitats and often are the dominant members of benthic communities (Taylor *et al.*, 2007; Hentschel *et al.*, 2012). There are about 8,500 described species in the phylum *Porifera* with an estimated total species number of 15,000 (Hooper and van Soest, 2002; van Soest *et al.*, 2012). The majority of sponges are from the oceans although some sponge species live in fresh water. The phylum *Porifera* consists of four classes: Demospongiae, Homoscleromorpha, Hexactinellida and Calcarea. The class Demospongiae is the largest and it has 12 orders and roughly 7,000 species (about 83% of all the sponge species) (van Soest *et al.*, 2012). The class Homoscleromorpha has the smallest number of representatives with only one order and 87 species (roughly 1% of all the sponge species). Members of the class Calcarea have spicules made of calcium carbonate in the form of calcite or aragonite while members of Hexactinellida are often referred to as glass sponges because they have a skeleton made of four to six-pointed siliceous spicules. An online database, World Porifera Database

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(WPD) (http://www.marinespecies.org), categorizes the known sponge species and is organized in a fully hierarchical system to promote the stability of sponge names, which provides a useful resource for sponge taxonomy (van Soest *et al*., 2012).

Traditionally, sponge taxonomy relies on characterizing the skeleton, mainly focusing on the size, form and location of spicules. However, this is challenging because of the high phenotypic plasticity due to the lack of basic organ and tissue differentiation (van Soest *et al*., 2012). Recently, identification of sponges by molecular methods has proved to be very useful in sponge taxonomy. The Sponge Barcoding Project (SBP) was initiated aiming to cover all sponge taxa, including the four classes described above and also sponges from both marine and freshwater environments. Phylogenetic markers such as the 28S rRNA gene, the mitochondrial cytochrome oxidase subunit 1 (CO1) gene and flanking regions can serve as suitable tools for identifying sponges. However, there are limitations when applying DNA barcoding to taxonomically understudied groups and this approach can fail to reveal new species that may clearly be morphologically different especially if only a single gene is used in the phylogenetic analysis (Meyer and Paulay, 2005; Hickerson *et al*., 2006).

1.1.3 Genomes of marine sponges

To better understand the evolution and physiology of marine sponges, a group of scientists chose the Great Barrier Reef demosponge *Amphimedon queenslandica* as the model sponge for which to obtain a draft genome sequence (Srivastava *et al.*, 2010). They elegantly used the embryos and larvae

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from a single mother for genomic DNA extraction to minimize DNA contamination of microbial origin from the microbes associated with adult sponges. Sanger shotgun sequencing was utilized to obtain about 9-fold coverage of the genome. Their results predict that the *Amphimedon queenslandica* genome encodes about 30,000 coding sequences (CDS), similar to that of the human genome. About 18,000 of these CDS have identifiable homologues in other organisms. Overall, the genome shows remarkable similarity in terms of content, structure and organization to other animal genomes (Srivastava *et al*., 2010). With genome sequence available, now it is possible to answer some fundamental questions such as what makes an animal, including some of the genetic characteristics of the last common ancestor of multicellular life (Srivastava *et al*., 2010). It is technically challenging to sequence marine sponge genomes, because marine sponges harbor many millions of microbes in their tissues. It is critical to avoid microbial contamination in order to produce accurate sponge genome sequences.

1.2. Marine sponge microbiology

Marine sponges can harbor highly diverse and abundant microbial communities and in some cases up to 30%-40% of the sponge biomass is derived from these microbes, including bacteria, archaea and single cell eukaryotes (Vacelet, 1975; Webster and Taylor, 2012). Sponges can be categorized into either High Microbial Abundance (HMA) or Low Microbial Abundance (LMA) sponges based on the abundance of associated microorganisms (Hentschel *et al*., 2006). In the early days, researchers used

electron microscopy (EM) to study the associated microorganisms. For example, Vacelet and Donadey (1977) observed a high abundance of microorganisms in sponge tissues by EM. With the advent of molecular techniques, such as PCR, Denaturing Gel Gradient Electrophoresis (DGGE) and 16S rRNA gene analysis, as well as next-generation high-throughput sequencing, the amazing diversity of bacteria associated with marine sponges is being revealed.   A relatively comprehensive survey of the available 16S rRNA gene sequences deposited in GenBank that were obtained from bacteria associated with marine sponges conducted (roughly 11,000) by Webster and Taylor (2012) shows bacterial sequences mainly belong to 16 different phyla as shown in Figure 1.2. The top three most abundant phyla are *Proteobacteria*, *Actinobacteria* and *Chloroflexi*. It is certain that more and more sequences belonging to different phyla will be recovered as the bacterial communities associated with more and more sponges are analyzed by high–throughout sequencing. Of note, the Department of Energy of the United States currently is funding a collaborative project named the Earth Microbiome Project (EMP) (http://www.earthmicrobiome.org/), which aims to characterize the global microbial taxonomic and functional diversity that are likely beneficial to the planet and humans and a large proportion of the sequencing efforts will be directed towards microbial communities associated with marine sponges.
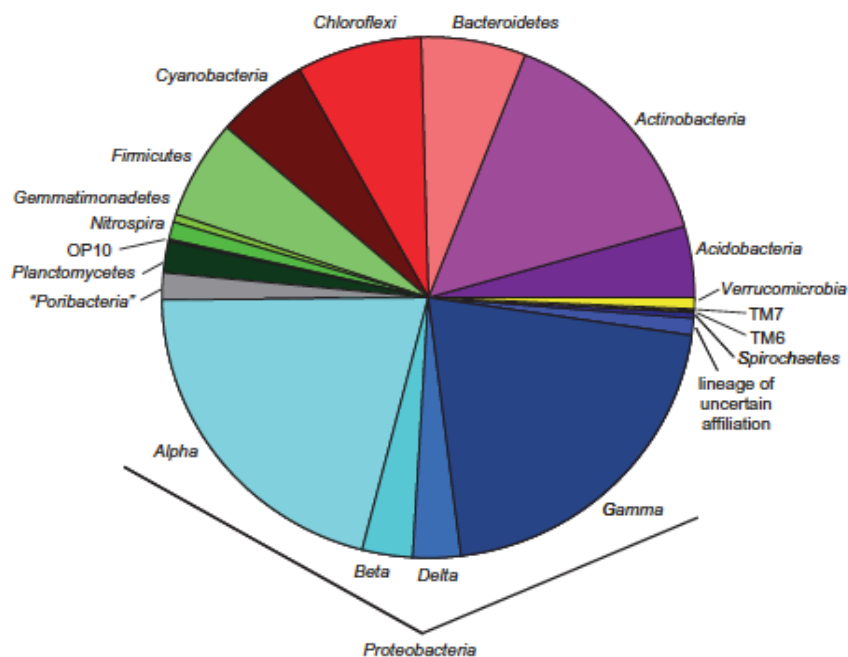
Figure 1.2. Phyogenetic distribution of sponge-associated bacteria. Adapted from Webster and Taylor (2012) with permission. A total of 11,284 16S rRNA gene sequences were obtained from GenBank in September 2010. Bacterial phyla that have fewer than 10 sequences obtained from sponges were excluded for clarity, including: *Chlamydiae, Deferribacteres, Deinococcus-Thermus, Epsilonproteobacteria, Fusobacteria, Lentisphaerae,* OP11*, Tenericutes,* and WS3*.*

Hentschel *et al*. (2002) first defined sponge-specific bacterial clusters: "the sequences of each cluster are more closely related to each other than to a sequence from non-sponge sources". Many different sponge-specific clusters have been reported since then. A candidate phylum *Poribacteria,* in which members showed <75% identity on the16S rRNA gene sequence to known bacteria phyla and were found only in sponges was proposed (Fieseler *et al.,* 2004). Thus, the existence of sponge-specific bacteria became a paradigm in

this field (Taylor, *et al*., 2007). However, with the development of next-generation sequencing techniques, deep sequencing enabled a better understanding of these sponge-specific clusters. Webster *et al*. (2010) first reported the recovery of sequences corresponding to sponge-specific clusters in samples from surrounding seawater at extremely low abundance by using 454-pyrosequencing methods. Taylor *et al*. (2012) conducted a comprehensive study in which 173 previously described sponge specific clusters were compared to over 12 million different 16S rRNA sequences from different ecological environments and results show that 77 of these clusters (ca. 44%) can be recovered from seawater or sediments although in general at very low abundance, suggesting the role of a "seed bank" of bacteria present at low abundance in the surrounding environment for colonizing the sponges and thus providing strong evidence for horizontal acquisition of these sponge symbionts. Furthermore, the candidate phylum *Poribacteria* can also be found in a variety of non-sponge environments (Taylor *et al*., 2012). Taken together, it is questionable if sponge-specific clusters indeed exist; it seems more likely that this paradigm reflected the inadequacy of earlier sequencing approaches to detect bacteria present in the surrounding environment at very low abundance.

One of the central questions in sponge microbiology is how the sponges acquire sponge-specific symbionts (Taylor *et al*., 2007). Two modes of bacterial transmission mechanisms have been proposed: vertical and horizontal. Vertical transmission refers to the transfer of these symbionts between generations through larvae. The evidence for vertical transmission of sponge-associated

bacteria first came from electron microscopy studies (de Caralt *et al.*, 2007).

Many subsequent studies utilized fluorescence in situ hybridization (FISH) to examine the distribution of bacteria in sponge larvae and found strong evidence of vertical transmission (Enticknap *et al.*, 2006; Sharp *et al.*, 2007). In addition, studies that compared the bacterial compositions between the adult sponges and the larvae or embryos showed overlap in the bacterial communities in these two life stages, suggesting vertical transmission (Schmitt *et al.*, 2008; Lee *et al.*, 2009). Horizontal transmission refers to the acquisition of bacteria from the surrounding seawater or other non-sponge environments. Many of the previously characterized sponge-specific clusters now have been found in the surrounding seawater or even sediments at extremely low abundance (Taylor *et al.*, 2012), which strongly suggest that these bacteria can be acquired horizontally. Even for the bacteria for which vertical transmission has been experimentally shown, it is difficult to exclude the possibility that they can also be horizontally recruited.

A central question in the field of sponge microbiology is "What are the functions of the bacterial symbionts associated with sponges?" The vast majority of microorganisms in the water that pass through sponges are trapped and digested and the water pumped out from the main exhalent water channel, the osculum, is nearly sterile (Taylor *et al.*, 2007). Symbiotic relationships between sponges and microorganisms are considered to contribute to the health and nutrition of sponge. However, little evidence exists showing the contribution of symbiotic microbes to sponge health or survival (Webster and Blackall, 2009).

Exceptions are the translocation of photosynthate from cyanobacteria to the host sponge and a decrease in the health status of sponge associated with loss of cyanobacteria (Wilkinson and Fay, 1979). It is worth pointing out that much circumstantial evidence points toward the significance of the symbionts to the sponge heath (Webster and Blackall, 2009). One good example is the role of symbionts in nitrogen fixation, a process whereby microorganisms convert atmospheric dinitrogen gas into biologically usable ammonium. Sponges that live in the nutrient-poor coral reef environments are a reasonable ecological niche for nitrogen-fixing bacteria because there could be an unbalanced carbon and nitrogen ratio, which is caused by photosynthetically derived carbohydrates provided to sponges since these compounds are rich in carbon but scarce in nitrogen (Wilkinson, 1983). Mohamed *et al*. (2008a) determined the $\partial^{15}N$ values in two shallow water marine sponges: *Mycale laxissima and Ircinia strobilina*, and found a low value in *I. strobilina*, consistent with this sponge obtaining a substantial part of its fixed nitrogen through biological nitrogen fixation. Furthermore, the expression of *nifH* genes was examined in these two sponges by using reverse transcription- PCR (RT-PCR) and results showed that the *nifH* genes exclusively from cyanobacteria are actively expressed (Mohamed *et al.*, 2008a).

Sponges are sessile organisms and need effective defense mechanisms against predation, fouling and disease and thus it is possible that the associated microbial community may be involved in host defense (Taylor *et al.*, 2007). For example, Hentschel *et al*. (2001) found that 27 out of 238 bacterial strains

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isolated from the Mediterranean sponges *Aplysina aerophoba* and *Aplysina cavernicola* showed antibacterial activities and they also observed an interesting pattern: Gram-positive bacteria tended to show inhibitory effects on Gram-positive isolates whereas Gram-negative bacteria inhibited Gram-negative strains. Thakur *et al*. (2004) found that strains from the genus *Bacillus* with a close association with the host sponge *Ircinia fusca,* showed antimicrobial activity against fouling bacteria.

It is clear that our knowledge about the diversity of the microbes that live together with marine sponges is improving at a rapid pace; however our understanding of the functions of these bacteria and the nature of the symbioses is lagging behind. Major efforts need to be directed towards these aspects of bacteria-sponge symbiosis to further advance the development of this field.

1.3. Quorum sensing

Bacteria are unicellular organisms and they can exist in almost all ecological niches and play vital roles in the ecosystem. However, bacteria can also coordinate their group behavior through a process termed quorum sensing (QS), which enables bacteria to sense and perceive their population density through the use of diffusible signals (Fuqua *et al.*, 1994). About 40 years ago, seminal research on the control of bioluminescence production in *Vibrio fischeri* (previously named *Photobacterium fischeri*) found that bacteria can coordinate their group behavior by communicating with each other via diffusible chemical molecules (Nealson *et al.*, 1970). It was unclear what the molecule was at that time. In the 1980s, the chemical was discovered to be N-3-oxohexanoyl-L-

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homoserine lactone (3-oxo-C6-HSL) (Eberhard *et al.*, 1981). Generally, the signal molecules are called *N*-acylhomoserine lactones (AHLs). The enzyme that is responsible for the synthesis of 3-oxo-C6-HSL in *V. fischeri* is called LuxI and the receptor that can respond to this molecule is called LuxR. Since its discovery, the LuxI-LuxR type QS pathway has been reported in more than 100 different bacterial species (Ahlgren *et al.*, 2011), the vast majority of which belong to the ecologically diverse and abundant *Proteobacteria*. In the following sections, I review the progress of our understanding of bacterial quorum sensing.

1.3.1 General model of QS in *Proteobacteria*

The canonical LuxI-LuxR QS pathway was first dissected in the marine animal symbiont *V. fischeri,* a gammaproteobacterium (Engebrecht *et al.*, 1983). In the simplest model, the gene called *luxI* encodes the enzyme that is responsible for the synthesis of AHL molecules. The reason that the gene is called *luxI* is because in *V. fischeri* the QS pathway controls the luminescence (*lux*) system (Fuqua *et al.*, 2001). At low cell density, the concentration of the AHL is low and it can quickly diffuse across the cell membranes. As the cell density increases, the AHL molecules accumulate. Once the cell density and thus the concentration of the AHL reach a certain threshold, the AHL diffuses back into the cell and bind to its cognate receptor LuxR. The complex of LuxR and AHL turns on or turns off a certain set of genes and thus coordinates the group behavior (Fig.1.3) (Miller and Bassler, 2001). The set of phenotypes that are typically controlled by this process includes motility, biofilm formation,

bioluminescence, and production of antibiotics or secreted products such as virulence factors (Fuqua and Greenberg, 2002).
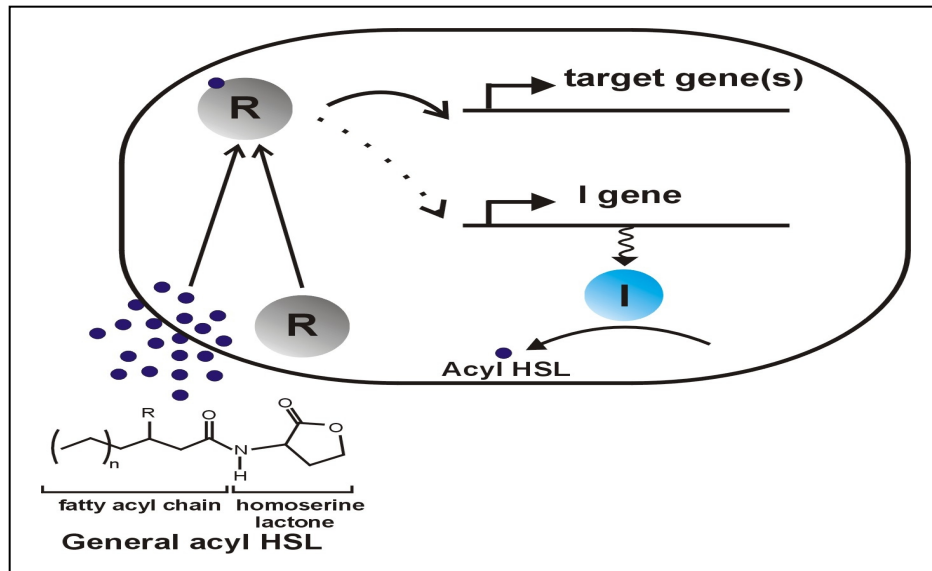
Figure 1.3. A general mode of QS in *Proteobacteria*. The I gene represents the *luxI* homologue. R represents the AHL receptor LuxR protein. The light blue solid circle represents the LuxI enzyme while the dark blue dots represent the AHL molecules. Stalked arrows indicate the transcription of the genes and the dotted line with arrow shows the positive feedback by the complex of the LuxR receptor and AHLs on the AHL synthase gene. The solid curved line with arrow depicts the function of R complex on the target genes. The left corner shows the basic structure of a typical AHL molecule. N can vary from 4 to 18. R can be oxo, OH or H. The figure is kindly provided by Dr. Clay Fuqua.

The majority of AHL synthases that have been discovered so far belong to the LuxI family, although a few other types of enzymes that can synthesize AHL have been reported in different species, such as LuxM, VanM and AinS in a few *Vibrio*

species (Churchill and Chen, 2011). The focus of this section is on the LuxI-type enzyme. The LuxM will be reviewed in Section 1.3.6 together with LuxS, the autoinducer-2 synthase since both of these two enzymes are best characterized in *Vibrio* species (Ng and Bassler, 2009). The substrates for LuxI-type enzyme are S-adenosylmethionine (SAM) and fatty acyl precursors conjugated to the acyl carrier protein (ACP). The methionine in SAM provides the homoserine moiety, which is conjugated to the acyl chain donated by acyl-ACP (Moré *et al.*, 1996; Schaefer *et al.*, 1996). It is worth mentioning that in a few species LuxI-type enzymes use CoA- rather than ACP-linked substrates for acyl-HSL synthesis (Lindemann *et al.*, 2011). The LuxI-type enzyme is roughly 180-230 amino acids (aa) in length with some exceptions (such as the Sil1 in *R. pomeroyi* DSS-3 and SsaI in *Ruegeria* sp KLH11 that are both about 280 aa in length) (Moran *et al.*, 2004; Zan *et al.*, 2012). The LuxI-type protein can be divided into two regions: the N-terminal region and the C-terminal region. The N-terminal region, responsible for the binding of the converted substrate SAM and catalysis, is the most conserved part of the enzyme and has eight invariable residues, Arg24, Phe29, Trp35, Asp46, Asp49, Arg70, Glu100 and Arg103 (the numbering is based on the LuxI in *V. fischeri*) (Churchill and Chen, 2011). In contrast, there is very low conservation in the C-terminal region, which is involved in the recognition of the acyl chain, the highly variable part of the acyl-ACP substrate (Churchill and Chen, 2011).

In most cases, the *luxI* gene is genetically linked with a cognate *luxR* gene, encoding the AHL receptor that is present in the cytoplasm (Subramoni and

Venturi, 2009). However, not all AHL receptors are in the cytoplasm. The

exception is the membrane bound AHL receptor LuxN in *V. harveyi,* which is

discussed in Section 1.3.6. Typically, LuxR-type AHL receptors have two

domains. The N-terminal domain contains the AHL-binding sites and the C-

terminal domain encodes the DNA binding motifs. These two domains are linked

together via a conformationally flexible linker. The N-terminal domain inhibits the

function of the DNA-binding domain when the receptor is in the apo-form, free of

AHL (Schuster and Greenberg, 2008). Binding to the cognate AHL can relieve

this inhibition and enable the LuxR receptor to multimerize, which has been

shown in several different bacterial species (Choi and Greenberg, 1992; Zhu and

Winans, 2001; Kiratisin *et al.*, 2002). Binding to AHL can also enhance the

resistance to protease-mediated degradation of LuxR (Zhu *et al.*, 2001). The

binding of AHL to its cognate receptor is a reversible process for LuxR in *V.*

fischeri while it is an irreversible process for LasR in *Pseudomonas aeruginosa*

or for TraR *in Agrobacterium tumefaciens.* The C-terminal domain of LuxR-type

proteins has a Helix-Turn-Helix motif (HTH) and is able to recognize a conserved

palindromic sequence element upstream of target genes (Stevens and

Greenberg, 1999). This DNA element is 18-22 bp in length and is named the *lux-*

type box, commonly found close to the regulated promoters. These sequences

share considerable similarity with the LuxR target sequences upstream of the *V.*

fischeri lux operon (the 20 bp sequence of which is

ACCTGTAGGATCGTACAGGT), which is centered 42.5 bp upstream from the

luxI transcriptional start site and -61.5 bp upstream of the *luxI* translational start

site (Devine *et al.*, 1989; Egland and Greenberg, 1999). Both half sites of the box are required for LuxR activation and the position of the box is also critical (Egland and Greenberg, 1999). A later study showed that six critical nucleotides define the minimal sequences of a *lux* box (Antunes *et al.*, 2008). In the plant-pathogen *A. tumefaciens*, the AHL receptor TraR is a transcriptional activator and regulates the conjugal transfer of the octopine type Ti plasmid at high cell density (Hwang *et al.*, 1995). In total, there are seven TraR-dependent promoters and four *tra* boxes that have been identified (Fuqua *et al.*, 1994; Fuqua and Winans, 1996). These four *tra* boxes are 18 bp in length and share high sequence similarity. TraR regulates its own expression but does not contain any recognizable *tra* boxes in its promoter region (Fuqua and Winans, 1996).

Several members of this LuxR family are able to fold, dimerize, bind to DNA, and regulate transcription in the absence of AHLs and are antagonized by their cognate AHLs (Tsai and Winans, 2010). AHLs disrupt the complexes between some of these proteins and their DNA binding sites in vitro (Tsai and Winans, 2010). All such proteins are fairly closely related and form a monophyletic clade. The best-characterized representative is the EsaR from the plant pathogen *Pantoea stewartii* (formerly *Erwinia stewartii*). This QS system regulates exopolysaccharide (EPS) production and EsaR and EsaI mutants display completely opposite regulatory effects on EPS production. The EsaR mutant overexpressed the EPS production whereas the EsaI mutant decreased its production (von Bodman and Farrand, 1995; von Bodman *et al.*, 1998). It was later discovered that apo-EsaR is able to bind to the promoter of the EPS

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synthesis gene *rcsA* to repress its expression (Minogue *et al.*, 2005). At high cell density, the AHL disrupts this binding and thus derepresses the *rcsA* expression. It is unclear whether or not the active apo-EsaR can bind to AHL. Interestingly, the mechanisms of how AHLs inactivate this type of LuxR proteins are unclear (Tsai and Winans, 2010).

1.3.2 Case studies of QS: *V. fischeri* and *P. aeruginosa*

1.3.2.1 *V. fischeri*

V. fischeri ES114, is the light organ symbiont of the Hawaiian bobtail squid, *Euprymna scolopes*, which can be maintained and reproduced under laboratory conditions (Stabb *et al.*, 2008). The *luxI* and *luxR* genes are adjacently arranged but transcribed separately. *luxI* is the first gene in the *lux* operon which contains another six genes (*luxCDABEG*) and LuxI synthesizes 3-oxo-C6-HSL. The LuxR protein complexed with 3-oxo-C6-HSL binds to the *lux* box described in Section 1.3.1 to create a positive feedback on the expression of the *luxI* gene. *V. fischeri* also has a *luxS* gene that can produce AI-2 and a homologue to *luxM* in *V. harveyi* described below in Section 1.3.6: the *ainS* gene that can produce 3-octanoyl-HSL (C8-HSL) (Kuo *et al.*, 1994; Gilson *et al.*, 1995; Hanzelka *et al.*, 1999). For the symbiosis, the squid selectively acquires *V. fischeri* horizontally from the surrounding water and the light production follows a diel cycle. At night, the *V. fischeri* grows to high cell density and turns on the *lux* operon resulting in light production within the light organ that harbors the symbiotic bacteria, which can benefit the animal by matching the intensity of the moon above to protect

them from predators by a process termed counter-illumination camouflage (McFall-Ngai and Ruby, 1991). At dawn, the squid releases ~ 95% of the bacteria from the light organ to maintain a low cell density. The squid hide themselves in the sand during the day, a situation in which light production is not required. The remaining bacteria grow during the day to reach a high cell density again in the mid-afternoon. The pattern repeats daily (Nyholm and McFall-Ngai, 2004). Paradoxically, the LuxI signal plays a critical role in the symbiosis while it has little effect on luminescence in culture, demonstrating the fact that artifacts under laboratory conditions can seriously obscure our views.

1.3.2.2 *P. aeruginosa*

P. aeruginosa is a ubiquitous and versatile gammaproteobacterium. It is an opportunistic pathogen and the dominant microorganism in chronic lung infection of cystic fibrosis patient (Lyczak *et al.*, 2000). There are two sets of *luxI-luxR* pathways in *P. aeruginosa*: the *lasIR* and the *rhlIR* systems. LasI produces 3-oxo-dodecanoyl-HSL (3-oxo-C12-HSL) and RhlI produces 3-butyryl-HSL (C4-HSL), which binds to their cognate LuxR type receptors LasR and RhlR, respectively (Pearson *et al.*, 1994; Pearson *et al.*, 1995). There is also a third distinct QS system in *P. aeruginosa* that involves the regulator MvfR and the *Pseudomonas* quinolone signal (PQS) (Mashburn and Whiteley, 2005). Both of the first two LuxI-LuxR systems have the typical positive feedback on the expression of the AHL synthase gene via the cognate receptor with AHLs. This positive feedback can lead to dramatic increase of the expression of the genes regulated by QS, such as the virulence genes, once a critical threshold of the

cell-density is reached (Pesci *et al.*, 1997). A transcriptomic study showed that the QS systems control ca. 6% of the total *P. aeruginosa* genes, including genes that encode the main virulence factors and antibiotics, such as rhamnolipid, elastase and pyocyanin (Schuster *et al.,* 2003).

It is generally considered that there is a regulatory hierarchy existing in this QS system. LasR initiates the regulatory system and then subsequently activates the transcription of *rhlR* and 3-oxo-C12-HSL can also block C4-HSL from binding to RhlR (Latifi *et al.*, 1996; Pearson *et al.*, 1997; Pesci *et al.*, 1997). However, results from a series of studies have challenged this view. Dekimpe and Déziel (2009) show that the activity of the *rhl* system is only delayed in *lasR* mutants into stationary phase and *rhlR* still shows significant expression in the *lasR* mutant although at lower levels compared to that in wild type culture. Furthermore, they show that the *rhl system* can also affect the factors that were previously thought to be controlled solely by the *las* system, including the *lasI* gene. Limmer *et al.* (2009) showed that RhlR plays a more complex role in pathogenesis than previously thought and RhlR and LasR mutants show opposite phenotypes in pathogenicity in a *Drosophila melanogaster* infection model, suggesting the absence of the positive regulatory hierarchy previously proposed (Pesci *et al.,* 1997). However, it is still possible that under certain environmental conditions the regulatory hierarchy still plays a critical role in *P. aeruginosa* pathogenicity.

Besides the two sets of LuxI-LuxR QS circuits, *P. aeruginosa* also has a third LuxR homologue, named QscR. QscR is not linked to any *luxI* homologue, thus

is called a LuxR solo (Fuqua, 2006). QscR can respond to LasI derived 3-oxo-

C12-HSL and regulates a set of genes distinct from those regulated by *lasIR-*

rhlIR systems (Lequette *et al.*, 2006). LuxR solos have subsequently been very

commonly found and are present in many different bacterial genomes, some of

which even have multiple LuxR solos (Subramoni and Venturi, 2009). Thus far,

investigations suggest that these LuxR solos can have diverse roles in intra- and

inter-species communication and some may even be involved in inter-domain

singaling (Subramoni and Venturi, 2009).

1.3.3 Quorum sensing in Roseobacters

The *Roseobacte*r clade is a group of bacteria that belong to the

Alphaproteobacteria and its members share greater than 89% 16S rRNA gene

sequence identity (Buchan *et al.*, 2005). It includes about 17 different genera

(Buchan *et al.*, 2005; Wagner-Döbler *et al.*, 2005). Members of this clade have a

diverse and broad ecological distribution but are exclusively restricted to marine

or hypersaline environments (Buchan *et al.*, 2005, Moran *et al.*, 2007).

Generally, roseobacters have been characterized as ecological generalists and

exhibit different lifestyle strategies, including heterotrophy, photoheterotrophy

and autotrophy (Moran *et al.*, 2004; Moran *et al.*, 2007). Roseobacters are

numerically abundant and these bacteria are estimated to account for about 20-

30% of the bacterial 16S rRNA genes in ocean surface waters. Furthermore,

many members are found to be associated with marine invertebrates (such as

marine sponges or corals), marine algae, dinoflagellates and seagrasses (Taylor

et al., 2004; Mohamed *et al.*, 2008c; Slightom and Buchan, 2009). *Ruegeria*

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pomeroyi DSS-3 (previously known as *Silicibacter pomeroyi* DSS-3) was the first member in this clade to have its genome sequenced (Moran *et al*., 2004) and currently 42 bacterial strains from this group have been sequenced (see www.roseobase.org for details). The number of genome sequences available for *Roseobacter* strains as well as other bacteria is increasing rapidly as the price of sequencing is plummeting. An important ecological function of members in this clade is in global sulfur cycling through metabolizing dimethylsulfoniopropionate (DMSP), an organic sulfur compound produced by marine phytoplankton (Moran *et al.*, 2012). The pathways and enzymes participating in the demethylation or cleavage pathways are comprehensively reviewed in Moran *et al*. (2012).

Marine environments that contain high nutrient concentration would be reasonably expected to host bacteria that can produce AHLs as bacteria could reach sufficiently high cell density for quorum sensing. These environments include marine snow, marine invertebrates and marine algae. Gram *et al*. (2002) first screened bacteria isolated from marine snow for AHL production and showed that three roseobacterial isolates from marine snow are able to produce AHL molecules detected by an *A. tumefaciens* AHL biological reporter system. Marine snow is made up of organic and inorganic particles and is rich in energy and nutrients. Bacteria can colonize marine snow and produce exoenzymes to utilize the nutrient sources available in the aggregated organic particles (Gram *et al.*, 2002). Taylor *et al*. (2004) found a bacterial strain from the genus *Ruegeria* isolated from a marine sponge that produced AHLs detected by reporter systems utilizing *Chromobacterium violaceum* CV026 and *A. tumefaciens* A136. In the

same study, organic extracts from 27 of 37 different marine invertebrates including marine sponges, corals, ascidians and bryozoans were showed to stimulate the short-chain AHL reporter (CV026), indicating the presence of short-chain AHLs in these invertebrates. The AHLs responsible for induction of the reporter systems in these studies by Gram *et al*. (2002) and Taylor *et al*. (2004) were not characterized.

Wagner-Döbler *et al*. (2005) screened 102 marine bacterial strains isolated from different marine environments for AHL production and found that 33 roseobacterial strains were able to significantly increase the fluorescence of their AHL reporter system, suggesting AHL production. The majority of these AHL-producing roseobacterial strains were isolated from either marine dinoflagellates or picoplankton. Mohamed *et al*. (2008c) found that isolates from the *Silicibacter-Ruegeria* (SR) subgroup of the *Roseobacter* clade are the dominant AHL producers from the cultivated isolates from two marine sponges, *M. laxissima* and *I. strobilina*. These marine sponge roseobacterial symbionts produce a mixture of short and long-chain length AHLs revealed by Thin-Layer Chromatography (TLC) coupled with an AHL biological reporter assay. However, the AHL profile of *Ruegeria* sp. KLH11 chosen as a representative of this group was analyzed in detail by mass spectrometry (MS) and the AHLs were predominantly 12-16 carbons whereas the short chain length AHLs detected in bioassyas were below the limits of sensitivity of the MS approach (Zan *et al*., 2012).

Among the 42 sequenced roseobacterial genomes deposited in the Roseobase (www.roseobase.org), a genomic resource for marine roseobacters, 33 of them encode *luxR-luxI* type homologues, including species from almost all of the 17 genera in the *Roseobacter* clade. Of note, the Hill laboratory has recently sequenced 17 SR bacterial strains isolated from marine sponges that can produce AHLs (Zan, Hill, Fuqua and Ravel, work in progress) detected by the *A. tumefaciens* KYC55 reporter (Zhu *et al.*, 2003). Once the annotation of these genomes is finished, substantial additional information on the LuxI-LuxR type QS pathways in these sponge symbionts will become available. In contrast to the growing body of knowledge about the genomes of these AHL producers, very few studies have been performed to examine the physiological and genetic characteristics of the QS pathway in these species. Efforts were first directed towards the free-living marine bacterium *R. pomeroyi* DSS-3 (Moran *et al*., 2004). Its genome encodes two LuxI-LuxR pathways, designated as SilI1/SilR1 and SilI2/SilR2. Both of the AHL synthases were expressed in *E. coli* and the AHL profile of SilI1 is very similar to that of the wild type culture of *R. pomeroyi* DSS-3 whereas SilI2 synthesizes different AHLs (Moran *et al*., 2004). Furthermore, the SilR1 increases the expression of the *silI1* expression in response to addition of exogenous AHL when examined in *A. tumefaciens* NTL4 (E. Cicirelli and C. Fuqua, unpublished). However, it is unclear what phenotypes these QS pathways in *R. pomeroyi* DSS-3 can regulate.

In recent years, novel non-AHL type QS molecules have been identified at a steady pace. For example, diffusible signal factor (DSF) cis-decenoic acid is a

QS molecule in *Xanthomonas* spp. (Ryan and Dow, 2008). Arguably, many

antibiotics probably function as signaling molecules since the concentration for

these molecules required to be effective as antibiotics has never been detected

in nature (Surette and Davies, 2008). One good example of a novel non-AHL

type QS molecule in *Roseobacter* is the sulfur-containing compound,

tropodithietic acid (TDA) produced in *Silicibacte*r sp. TM1040 discovered by the

Belas laboratory (Geng *et al.*, 2008). *Silicibacter* sp. TM1040 is an essential

symbiont for its dinoflagellate host *Pfiesteria piscicida* (Miller and Belas, 2006).

Surprisingly, *Silicibacter* sp. TM1040, closely related to *R. pomeroyi* DSS-3 and

*Ruegeri*a sp. KLH11, does not produce any detectable AHLs or encode any *luxI*

or *luxM* homologues although it encodes four *luxR* homologues in the genome

(Cicirelli *et al.*, 2008), which might be able to respond to exogenous AHLs

produced by other bacteria. Interestingly, its motility has been shown to be

important in the beginning phase of the symbiosis. TM1040 forms a biofilm on

the host surface when it associates with the host dinoflagellate (Miller and Belas,

2006). Using transposon mediated mutagenesis, 12 genes were identified as

critical for TDA biosynthesis and six of these genes are located on a

megaplasmid (Geng *et al.*, 2008). TDA shows a growth phase-dependent

production in static conditions with only very low levels of production under

shaking conditions (Geng and Belas, 2010). Furthermore, an electrophoresis

mobility shift assay showed that the LysR type regulator TdaA can bind to the

promoter of one of the TDA synthesis genes *tadC*, within the same operon as

tdaD and *tdaE*, although this binding is not stimulated by TDA in vitro (Geng and

Belas, 2011). However, addition of TDA to *Silicibacter* sp. TM1040 culture increases the expression of TDA synthesis genes most likely via TdaA (Geng et al., 2011). This type of positive feedback indeed resembles that of the expression of *luxI* homologues via LuxR protein complexed with AHLs. Furthermore, TDA-like molecules are produced by several different roseobacterial strains (Geng *et al*., 2010). Taken together, this provides convincing evidence supporting the role of TDA as a QS molecule. The involvement of TDA in the regulation of motility and biofilm formation and thus the symbiosis with the dinoflagellate warrants further investigation. Notably, the sponge symbiont *Pseudovibrio* sp. JE062, which is found associated with several different sponges (Enticknap *et al.*, 2006) also produces TDA (Geng *et al*., 2010) and the involvement of TDA-mediated QS in the symbiosis of Roseobacters with sponge hosts is also an interesting area for further exploration.

A regulatory link between LuxI-LuxR QS and TDA has been established in *Phaeobacter gallaeciensis* DSM 17395 (Berger *et al.*, 2011). The *luxI* homologue *pgaI* in this strain synthesizes 3-hydroxydecanoyl-HSL (3-OH-C10-HSL) and the cognate *luxR* homologue is called *pgaR*. There seems to be a minor effect of PgaR on the expression of *pgaI*. Insertion mutants of *pgaI* and *pgaR* cannot synthesize TDA and also lose the production of a yellow-brown pigment. The essential transcriptional regulator for TDA biosynthesis TdaA is under positive transcriptional control by the *pgaIR* system, in contrast to the constitutive expression of TdaA in *Silicibacter* sp. TM1040. The TDA itself is an autoinducer in DSM 17395 because addition of TDA can restore the expression

of the TDA synthesis genes and the production of the pigment in the *pgaI* insertion mutant background. Furthermore, it was suggested that the function of TDA as an autoinducer requires the presence of the regulator PgaR (Berger *et al.*, 2011). However, experimental evidence on the effect of TDA addition on the biosynthesis genes and pigment production in the *pgaR* mutant background is missing. Berger *et al.* (2011) hypothesize that the *pgaIR* QS system is located relatively high in the regulatory hierarchy and exerts its effects potentially through the regulator TdaA on the TDA synthesis genes and pigment production. However, it is unclear if *pgaIR* QS system directly or indirectly regulates the TDA system.

1.3.4 Novel AHL molecules

The length of the fatty acid chains in AHLs varies from 4 carbons to 18 carbons, although chain lengths with odd numbers of carbons are less common. The oxidation status of the third carbon can vary from fully oxidized to fully reduced. Furthermore, some species have one or two double bonds somewhere in the chain. All these factors produce the diversity of AHL molecules that can be employed by different bacterial species (Fuqua and Greenberg, 2002). The *Proteobacteria* contains about 1,534 bacterial species (http://www.earthlife.net/prokaryotes/proteo.html). It is intuitive to hypothesize that AHLs with novel structures different from those described above are likely to exist in some species. The anoxygenic phototrophic soil bacterium *Rhodopseudomonas palustris* CGA009 gave the first surprise (Schaefer *et al.,* 2008). Efforts to detect AHL activity by using traditional biological assays in this

species failed although a *luxI*-homologue *rpaI* is encoded in the genome. However, organic extracts of cultures of this strain grown in media with the substrate *p*-coumarate, a major plant aromatic monomer of lignin polymers, was able to trigger the *rpaI-lacZ* expression via RpaR, which can directly bind to the *rpaI* promoter to activate its expression (Hirakawa *et al.*, 2011). The active molecule was identified as *p*-coumaroyl-HSL (*p*C-HSL) showing typical growth-phase dependent production (Schaefer *et al.*, 2008). The *p*C-HSL like molecule can also be detected in *Bradyrhizobium* sp. and *R. pomeroyi* DSS-3 when grown in media supplemented with *p*-coumarate. The sponge symbiont *Ruegeria* sp. KLH11 has also been shown to be able to trigger RpaR-dependent expression of *rpaI-lacZ*. Notably, this activation is independent of the three LuxI homologues in the genome (See Chapter 3), indicating that a novel type of enzyme(s) is responsible for the synthesis. *Phaeobacter gallaeciensis* BS107 (also known as DSM 17395), a member of the *Roseobacter* clade, responds to the presence of *p*-coumarate produced by the microalga *Emiliania huxleyi* to control the production of algaecides and thus converts itself into an opportunistic pathogen of the host microalga (Seyedsayamdost *et al.*, 2011).

The *p*C-HSL like molecule in stem-nodulating photosynthetic *Bradyrhizobium* ORS278 was confirmed to be cinnamoyl-HSL, which lacks a hydroxyl group on the aromatic ring compared to *p*C-HSL. BraI synthesizes this molecule, although it does not require an exogenous source of cinnamate. BraI-BraR comprises the QS pathway in this strain (Algren *et al.*, 2011). Interestingly, this strain produces a very low level of cinnamoyl-HSL in culture and BraR can respond to picomolar

concentrations, although BraR can also respond to other non-cognate AHL in the range of nanomolar to micromolar concentrations (Algren *et al*., 2011). In a soybean symbiont *Bradyrhizobium japonicum* USDA110, the LuxI homologue BjaI is closely related to RpaI and BraI that synthesize aryl-HSL. Surprisingly, BjaI does not synthesize aryl-HSL but rather isovaleryl-HSL (IV-HSL), a branched-chain fatty AHL. Its cognate receptor BjaR can also respond to picomolar concentrations of IV-HSL, similar to that of BraR (Lindemann *et al.*, 2011). Overall, the discoveries of these three novel signaling molecules are important reminders of the diversity of molecules that can be synthesized by LuxI-type enzymes and also greatly extend the range of possibilities for AHL quorum sensing. Meanwhile it also raises the question of how many novel molecules have been overlooked in nature and reminds us that it is necessary to develop new reporter systems and new methods to detect potential novel signal molecules.

1.3.5 AHL-mediated QS in non-*Proteobacteria*

1.3.5.1 *Gloeothece* PCC6909

Gloeothece PCC6909 belongs to the phylum *Cyanobacteria* and can form biofilms and microcolonies. A protective layer of multilaminated sheath that forms around the colonies is considered to function as a diffusion barrier against toxic compounds and can also provide a conducive environment in which signal molecules can accumulate and thus trigger a quorum-sensing process (Meeks *et al.*, 1978; Sharif *et al.*, 2008). Using the AHL biological reporter strain *A.*

tumefaciens NTL4 (pZLR4) coupled with a TLC assay, Sharif *et al*. (2008) were able to show that this cyanobacterial strain produces an AHL-like molecule, which was confirmed to be N-octanoyl homoserine lactone (C8-HSL) by mass spectrometry. The production of the C8-AHL molecule displays a very typical pattern of autoinduction. Addition of this molecule to early-growth stage cells of *Gloeothece* PCC6909 can significantly affect about 15 proteins revealed by two-dimensional gel electrophoresis. These include the proteins RuBisCO, glutamate synthase, and chorismate synthase (Sharif *et al*., 2008). However, the molecular mechanism of the QS pathway is unknown. Whether this cyanobacterial strain contains the canonical LuxI-LuxR pathway remains elusive. This represents the first detailed characterization of AHL production in a non-*Proteobacteria* strain.

1.3.5.2. *Cytophaga-Flavobacterium-Bacteroides*

Wagner-Döbler *et al*. (2005) first showed that a *Flavobacterium* sp. strain was able to trigger the response of an AHL-biological reporter strain although they were not able to identify the potential AHL-like molecule in this strain. Subsequently, the presence of AHL molecules was confirmed in two strains belonging to the genus *Bacteroides,* both of which contain a mixture of several different types of AHLs (Huang *et al*., 2008)**.** Romero *et al*. (2010) reported the discovery of C4-HSL in the fish pathogen *Tenacibaculum maritimum,* a member of the *Cytophaga-Flavobacterium-Bacteroides* (CFB) phylum. Taken together with the finding of AHLs in the cyanobacterium *Gloeothece*, this suggests that AHL-mediated QS is not restricted only to the *Proteobacteria*. As more bacterial isolates from different phyla are screened, it is possible that an even broader

distribution of AHL-type QS will be found in a wide diversity of bacteria. However, genetic dissection of these QS pathways is still required to provide convincing data for the presence of LuxI-LuxR circuits in these non-*Proteobacteria* isolates.

1.3.5.3 AHLs in a methanogenic archaeon

Until recently, AHL type QS pathways were thought to exist only in bacteria, more specifically in gram-negative bacteria, although a putative homoserine lactone-like molecule was previously detected in the haloalkaliphilic archaeon *Natronococcus occultus* (Paggi *et al.*, 2003).   A recent remarkable study by scientists from China showed the production of carboxylated AHLs by a LuxI homologue in the methanogenic archaeon *Methanosaeta harundinacea* 6Ac (Zhang *et al.*, 2012). They observed a cell density dependent morphology change and then proceeded to probe the genome using bacterial *luxI* homologues. A CHASE 4 domain in the putative protein predicted to encode a multi-sensor signal transduction histidine kinase showed ca. 40% identity to the LuxI homologue AhlI in *Erwinia chrysanthemi.* The homologue was renamed as *filI*, showed a growth stage-dependent expression and was shown in vitro to be able to synthesize a new class of carboxylated AHLs. A few other methanogens likely also have *filI* homologues. The possible *luxR* homologue in this methanogen named as *filR* shows very low identity to bacterial *luxR* (Zhang *et al.*, 2012). The QS mechanism of this novel *filI-filR* pathway deserves further investigation.

1.3.6 QS in *V. harveyi* and *V. cholerae*: LuxM, CqsS and LuxS-mediated pathways

As described above, in a typical LuxI-LuxR QS pathway, LuxR is present in the cytoplasm. However, a separate class of membrane-bound AHL receptors has been identified in *V. harveyi*, a free-living marine bacterium but also an important pathogen of marine organisms (Austin and Zhang, 2006). The AHL in this case is synthesized by LuxM homologues. *V. harveyi* produces and responds to 3-OH-C4-HSL. The enzyme LuxM shows no homology to the LuxI-type enzyme but both can catalyze similar reactions (Bassler *et al.*, 1993; Bassler *et al.*, 1994). LuxM presumably uses SAM and acyl-ACPs or acyl-CoA substrate to synthesize the AHL while most LuxI homologues cannot use acyl-CoA as their substrate. LuxN is the cognate receptor of LuxM-derived AHLs (Freeman *et al.*, 2000). It is a membrane bound protein with dual functions. At low-cell density, it does not bind to its AHL and functions as a histidine kinase while at high-cell density it can function as a phosphatase when it is bound to AHLs (Wei *et al.*, 2012). The way that information is conveyed is the same as that of CqsS and LuxQ, receptors of CAI-1 and AI-2, and will be described in the following paragraph. In addition to AHL-based QS pathways, *V. harveyi* has another two sets of QS pathways: CAI-1 and Autoinducer-2 mediated, respectively. The CAI-1 was first reported in *V. cholerae*, the etiological agent of the disease cholera, and proved to be 3-hydroxytridecan-4-one. The enzyme that synthesizes it is called CqsA and uses SAM and acyl-CoA as substrates. *V. harveyi* also has a

CqsA homologue. The cognate receptor for this type of molecule is the membrane-bound CqsS (Higgins *et al*., 2007; Wei *et al*., 2012).

Autoinducer-2 (AI-2) is another well-known molecular cue and is generally considered to be an interspecies signal. Both Gram-positive and Gram-negative bacteria encode the *luxS* gene in their genomes. The molecular pathway has been extensively analyzed in *V. harveyi* and *V. cholerae*, where it is involved in regulation of bioluminescence and virulence-associated traits (Miller *et al.*, 2002; Henke and Bassler, 2004; Lenz *et al.*, 2004). The activated methyl cycle is a crucial metabolic pathway to recycle homocysteine from the major methyl donor *S*-adenosyl methionine. LuxS, a *S*-ribosylhomocysteinase, catalyzes part of the cycle and functions to convert *S*-ribosylhomocysteine to homocysteine; meanwhile, it can also, as a side reaction, synthesize 4,5-dihydroxy-2,3-pentanedione, the precursor of AI-2. 4,5-dihydroxy-2,3-pentanedione can spontaneously give rise to several furanone derivatives, collectively referred to as AI-2, which can freely diffuse across the cell membrane. The active AI-2 in *V. harveyi* requires the binding of metal boron, the only known case of a biological role for boron. However, in *E. coli* and *Salmonella typhimurium,* AI-2 signal does not require binding to boron to function probably due to limitation of this element in terrestrial environments (Ng and Bassler, 2009; references herein).

The mechanisms for the three different pathways in *V. harveyi* and the two different pathways in *V. cholerae* are similar and involve a phosphoryl group transfer-based signal relay system. At low cell density, the membrane bound receptors LuxN, LuxPQ and CqsA for *V. harveyi* and LuxPQ and CqsA for *V.*

cholerae cannot bind to their cognate signal molecules and thus function as histidine kinases to phosphorylate the protein LuxU and then transfer the phosphate group to the protein LuxO. The phosphorylated LuxO activates transcription of genes encoding five small regulatory RNAs called Qrr1-5 in *V. harveyi* and four in *V. cholerae* called Qrr1-4. Together with the RNA chaperone Hfq, the Qrr1-5 can bind to the mRNA of the master quorum-sensing regulator LuxR in *V. harveyi* and Qrr1-4 can bind to HapR, a homologue of LuxR (not homologous to AHL-responsive LuxR) in *V. cholerae*, which destabilizes the LuxR /HapR mRNA transcript. The phosphate group flow from LuxU to LuxO is reversed at high cell density when these membrane bound receptors bind to their cognate signals and function as phosphatases. Non-phosphorylated LuxO is inactive and thus no small RNAs are transcribed, which allows LuxR/HapR to be translated (Lenz *et al.*, 2004; Tu and Bassler, 2007; Tu *et al.*, 2008). LuxR can regulate bioluminescence, the genes encoding type III secretion apparatus and metalloproteases (Henke and Bassler, 2004; Pompeani *et al.*, 2008). In *V. cholerae*, HapR activates the expression of Hap protease at low cell-density while it inhibits biofilm formation and virulence factor production at high cell-density (Zhu and Mekalanos, 2003; Hammer and Bassler, 2003). However, it is worth pointing out that the dual roles of LuxS in metabolism and in AI-2 formation have led to controversy regarding its function in species other than *V. harveyi* or V. cholerae (Rezzonico and Duffy, 2008).

1.4. AHL-mediated inter-kingdom signaling: *Ulva*-AHL interaction

The molecular mechanisms of AHL-mediated communication have been examined extensively in many bacterial species, including both intra- and inter-species communication (Fuqua and Greenberg, 2002; Ng and Bassler, 2009). Moreover, convincing data have revealed that some eukaryotes can also sense and respond to AHLs. *Ulva intestinalis* is a green intertidal biofouling macroalga in the division Chlorophyta and contributes to the biofouling of man-made surfaces worldwide (Joint *et al.*, 2000). *U. intestinalis* can reproduce zoospores which are released from the thallus with timing often related to tidal cycles. Once these swimming zoospores find a favorable site for attachment, the zoospores firmly attach onto the surface permanently by secreting an adhesive glycoprotein and proceed to develop into mature individuals. However, if the surface is not satisfactory, zoospores will detach and swim to find another surface. The presence of bacterial biofilms on the surface is shown to be a determining factor in the choice of settlement or detachment for the spores. Pioneering work conducted by Joint *et al*. (2000) investigated the relationship between bacterial biofilms and the attachment of *Ulva* zoospores on glass slides and found a positive relationship between the number of zoospores that attach onto the surface and the number of bacteria present. A follow up study showed that AHLs in the fish pathogen *V. anguillarum* are involved in zoospore settlement (Joint *et al.*, 2002). *V. anguillarum* has *vanMN* and *vanIR* QS pathways. VanM synthesizes 3-C6-HSL and 3-OH-C6-HSL while VanI produces 3-oxo-C10-HSL

(Milton *et al.*, 2001; Milton, 2006). The biofilm of wild type *V. anguillarum* can effectively attract the spores while biofilms of *vanM* or *vanIM* mutants showed a substantial decrease in zoospore settlement (Joint *et al.*, 2002). It would be also very interesting to test how these AHL receptor mutants (*vanN* or *vanR*) affect the settlement. Furthermore, biofilms of the wild type *V. anguillarum* strain expressing the *Bacillus* lactonase AiiA that can degrade AHLs by opening the lactone ring showed decreasing abilities to attract spores (Joint *et al.*, 2002). To exclude the possibility that it is the known or unknown AHL-mediated phenotypes that participate in this settlement process, the authors provided more convincing data by expressing these AHL synthases in *E. coli* and showing an increase of zoospore settlement (Joint *et al.*, 2002). Provision of functional pure AHL molecules repeated the pattern obtained by expressing the gene heterologously (Joint *et al.*, 2002). Image analysis showed that zoospores indirectly bind to the bacterial microcolonies where AHL concentration is higher, which argues that the topology of microcolonies might play a role in the attachment process (Tait *et al.*, 2005). However killing the biofilm with UV treatment or the treatment of antibiotic chloramphenicol that can kill the cells but does not affect the physical properties, significantly affected the zoospore attachment, which suggests that the topology of the microcolonies is not important in the attachment process (Tait *et al.*, 2005). A control that adds the AHL back to these dead biofilms to test if it can restore the attachment would be a useful experiment to provide additional evidence to underscore the direct link between the AHL and attachment. An array of elegant experiments was performed by Wheeler *et al.* (2006) to understand the

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mechanism of the effect of AHLs on the settlement. AHLs were shown to act as a chemoattractant for *Ulva* zoospores through chemokinesis. Zoospores can accumulate around an AHL point source while the swimming speed dropped dramatically as they approach closely to the AHL source. The decrease in swimming speed was also confirmed in the presence of *V. anguillarum* biofilm producing AHL while not in the presence of an AHL production deficient biofilm (Wheeler *et al.*, 2006). However, it is unclear what molecular pathway is used by the zoospores to sense the presence of AHLs. To test whether the observed inter-kingdom signaling happens in situ, Tait *et al*. (2009) successfully showed the presence of AHLs (3-C8-HSL and 3-C10-HSL) on the surfaces of pebbles recovered from intertidal rock-pools where zoospores can colonize. The concentrations of these AHLs were roughly 600-pmol cm^{-2}. The bacterial composition of these surface associated communities was examined by 16S rRNA gene clone library analysis. The dominant members are *Alphaproteobacteria* and *Bacteroidetes*. More importantly, some of the environmental isolates from the original habitats are able to produce AHLs. Zoospores show enhanced settlement towards biofilms of bacteria that can produce AHLs while not to those that do not produce AHLs, providing convincing data that this interaction between bacteria and algae occurs in situ (Tait *et al.*, 2009)

1.5. Two-component systems

Two-component systems (TCS) are arguably the most dominant means that allow bacteria to sense and respond to their environment. A typical TCS consists

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of a membrane-bound histidine kinase (HK) that senses the signal and conveys

the signal input to a cognate response regulator (RR) though phosphorylation

(Fig. 1.4) (Capra and Laub, 2012).   In its simple version, the HK is membrane

bound and typically catalyzes an autophosphorylation reaction on the conserved

histidine residues. The phosphoryl group is transferred to a conserved aspartate

(D) on a cognate response regulator. In a more complicated version, the HK has

an additional domain called the receiver domain and thus is called a hybrid

histidine kinase. The phosphoryl group is still transferred to a conserved

aspartate residue in the receiver domain first but in the same HK protein. In turn,

the same group is transferred to the histidine residue in the intermediate protein

called a histidine phosphotrasferase and ultimately transferred to the conserved

aspartate residue in the cognate response regulator. This complicated version is

also called a phosphorelay system (Capra and Laub, 2012). The

phosphorylation on the aspartate residue of the response regulators would

induce conformational changes, which can lead to homodimerization of the

receiver domain and eventually cause transcriptional changes (Carroll *et al.*,

2009). The response regulator has an input receiver domain and an output

domain. There are many different types of output domain but most frequently

these are DNA binding domains (Laub and Goulian, 2007). The two described

versions of TCS are illustrated in Figure 1.4. When no signal is available, the HK

functions as the phosphatase and the flow of the phosphoryl group is reversed

and the response regulator is in an inactive status (Capra and Laub, 2012).
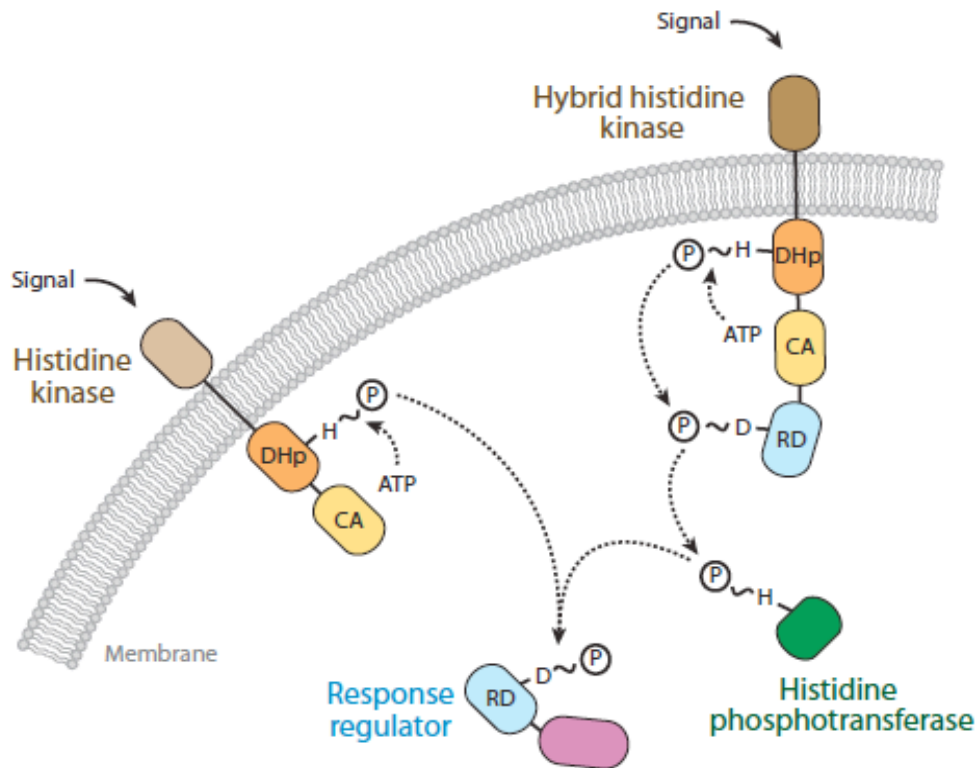
Figure 1.4. Organization and mechanism of two-component systems.

Adapted from Capra and Laub (2012) with permission. DHp=dimerization and histidine phosphotransfer, CA=catalytic and ATP binding. The name of each domain represented by the ellipse with different colors is shown next to it except for the purple one (most frequently a DNA binding domain).

Almost all the sequenced bacterial genomes encode two-component systems. The number of signaling proteins in these sequenced genomes roughly

scales with the genome size and also is related to the diversity of environments in which organisms live (Capra and Laub, 2012). How a single bacterial genome evolves to encode many TCSs and how the specificity of each system is determined have been extensively reviewed (Laub and Guolian, 2007; Capra and Laub, 2012). The two-component system mediated signal pathways have been implicated in the responses of bacteria to a variety of environmental signals and stimuli, such as nutrients, cellular redox state, quorum signals and others (Laub and Guolian, 2007). The *cckA* (histidine kinase)-*chpT* (phosphotransferase)-*ctrA* (response regulator) phosphorelay system is a good example that illustrates how the signal pathway works (Quon *et al.*, 1996; Jacobs *et al.*, 2003). It has been extensively examined in the bacterial development model *Caulobacter crescentus*, in which it coordinates DNA replication, cell division and polar morphogenesis and the response regulator CtrA is considered to be the cell cycle master regulator (Laub *et al.*, 2002; Iniesta *et al.*, 2006). The role of this system in regulating cell cycle has been implicated in several species while in some other bacteria species its role in regulating in cell cycle is obscure (Mercer *et al.*, 2010; Greene *et al.*, 2012). The example of LuxM, LuxPQ and CqsS in *V. harveyi* described above in Section 1.3.6 is another good example demonstrating the mechanisms of a two-component system. The difference is when these membrane-bound receptors bind to their cognate signals; it functions as a phosphatase not a kinase (Ng and Bassler, 2009).

1.6. Focus and objectives

Marine sponges can be favorable environments for bacteria where nutrient concentration can be high and bacteria can grow dense enough to allow bacterial QS processes to be important. My research dissects the molecular pathways of QS in a representative sponge symbiont and reveals the phenotypes that can be controlled by QS and in turn the exact molecular mechanisms for that control. Furthermore, a major part of my research effort is directed towards establishing an experimental model for studying the ecological role of QS in the symbiosis between bacteria-sponge. To fulfill these goals, my research objectives are:

I. Dissection of the QS pathways in the sponge symbiont *Ruegeria* sp. KLH11.

II. Understanding of the molecular pathways for flagellar swimming motility control by QS.

III. Establishment of the connection between laboratory understanding of QS and the role of QS in the sponge host.

IV. Development of *M. laxissima*/KLH11 as a model system for the study of bacterial colonization of sponges.

The results from my research will reveal for the very first time the architecture of the multiple QS pathways in a sponge symbiont that is also a member of the ecologically abundant and significant *Roseobacter* clade. Insights are also provided into the LuxI solo in bacteria. LuxR solos have been studied in many different bacterial species whereas only in a very few species has a LuxI solo been reported. Furthermore, the efforts devoted to establish an experimental

model to study the role of QS in the bacteria-sponge symbiosis provide a primary

model for the future to better understand the complex symbioses existing in

nature.

Chapter 2. A complex LuxR-LuxI type quorum sensing network in a roseobacterial marine sponge symbiont activates flagellar motility and inhibits biofilm formation

2.1. Abstract

Bacteria isolated from marine sponges, including the *Silicibacter-Ruegeria* (SR) subgroup of the *Roseobacter* clade, produce *N*-acylhomoserine lactone (AHL) quorum sensing signal molecules. This study is the first detailed analysis of AHL quorum sensing in sponge-associated bacteria, specifically *Ruegeria* sp. KLH11, from the sponge *Mycale laxissima*. Two pairs of *luxR and luxI* homologues were identified and designated *ssaRI,* and *ssbRI* (sponge-associated symbiont locus A, and B, *luxR* or *luxI* homologue). SsaI produced predominantly long-chain 3-oxo-AHLs and SsbI specified 3-OH-AHLs. Addition of exogenous AHLs to KLH11 increased the expression of *ssaI* but not *ssaR*, *ssbI* or s*sbR*, and genetic analyses revealed a complex interconnected arrangement between SsaRI and SsbRI systems. Interestingly, flagellar motility was abolished in the *ssaI* and *ssaR* mutants, with the flagellar biosynthesis genes under strict SsaRI control, and active motility occurring only at high culture density. Conversely, *ssaI* and *ssaR* mutants formed more robust biofilms than wild type KLH11. The coordination of motility and biofilm formation by AHL signaling could contribute to the decision between motility and sessility and that it also may facilitate acclimation to different environments including the sponge host.

2.2. Introduction

Over the past decade, culture-based and culture-independent techniques have frequently and consistently identified alpha-proteobacteria among the diverse bacteria from marine environments (Buchan *et al.*, 2005), including in association with marine sponges (Webster and Hill, 2001; Mohamed *et al.*, 2008c). More specifically, the *Roseobacter* clade, is estimated to account for 20-30% of the bacterial 16S rRNA in the ocean surface waters (Buchan *et al.*, 2005). Although a significant number of roseobacters are apparently free-living, many have been found associated with eukaryotic hosts, such as dinoflagellates and sponges (Miller and Belas, 2006; Mohamed *et al.*, 2008b; Slightom and Buchan, 2009), and in some cases, the symbiotic bacteria are essential for host survival. When nutrients are plentiful, roseobacters can associate with particulate organic matter or algal particles to form aggregates (Fenchel, 2001). For roseobacters the switch from a free-living state to a multicellular aggregate may involve chemical signaling (Gram *et al.*, 2002).

Marine sponges harbor complex and diverse bacterial communities, which in some cases is estimated as 30-40% of the sponge biomass (Vacelet, 1975; Vacelet and Donadey, 1977; Hentschel *et al.*, 2006). Although some bacteria serve as nutrients, there is evidence for stable symbionts, including several that are vertically transmitted (Enticknap *et al.*, 2006, Sharp *et al*., 2007; Schmitt *et al.*, 2008; Lee et al., 2009). Within the densely colonized sponge, there is also ample opportunity for both inter-species and intra-species signaling (Zan *et al.*, 2011b).

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Microbial signaling is common among dense microbial populations, such as those found within sponges. Quorum sensing (QS) allows bacteria to sense and perceive their population density through the use of diffusible signals (Fuqua *et al.*, 1994). The acyl-homoserine lactones (AHLs), that are widespread among *Proteobacteria*, are most often synthesized by enzymes of the LuxI family (Churchill and Chen, 2011).

AHLs have been identified in greater than a hundred different species and are among the best-studied signaling molecules in *Proteobacteria* (Ahlgren *et al.*, 2011). QS regulates a variety of cellular processes including bioluminescence, conjugal transfer, symbioses, virulence, and biofilm formation (Fuqua and Greenberg, 2002; Daniels *et al.*, 2004). Various sponge-associated roseobacters have been shown to synthesize AHLs (Taylor *et al.*, 2004; Mohamed *et al.*, 2008c). *Ruegeria* sp. KLH11, a sponge-associated member of the *Silicibacter–Ruegeria* subgroup of *Roseobacter* clade, produces at least six different AHLs detected using AHL-responsive biosensors (Mohamed *et al.*, 2008c). Examination of the QS circuits in KLH11 provides a window into the complex interbacterial signaling that is potentially at play within sponges. In this study, we report (i) the types and relative amounts of KLH11 AHLs (ii) isolation and genetic analyses of multiple genetically-linked *luxR* and *luxI* homologues, their cognate AHLs, and their quorum sensing networks, (iii) the control of flagellar swimming motility and biofilm formation by QS. This study represents the first detailed chemical and genetic analyses of QS in the *Roseobacter* clade.

2.3. Experimental procedures

2.3.1 Reagents, strains, plasmids, and growth conditions

All strains and plasmids used in this study are listed in Table 2.1 and all the primers used are listed in Table 2.2. Antibiotics were obtained from Sigma Chemical Co. (St. Louis, MO). Standards used for AHL analysis; D_3C6-HSL (N-[(3S)-tetrahydro-2-oxo-3-furanyl]-hexanamide-6,6,6-D3 ≥99% deuterated product), 3-oxo-C14-HSL, 3-oxo-C14:1 Δ7cis-(L)-HSL and 3-oxo-C16:1 Δ11cis-(L)-HSL were purchased from Cayman Chemical (Ann Arbor, Michigan). 3-OH-C12-HSL, 3-OH-C13-HSL and 3-OH-C14-HSL were synthesized as described previously (Gould *et al.*, 2006). Reagents used for sample derivatization and mass spectrometry analysis were: methoxyamine hydrochloride (MP Biomedicals, Solon, Ohio), bis (trimethylsily) tri-fluoroacetamide (Supelco, Bellefonte, PA). HPLC grade acetonitrile, HPLC grade methanol, and sodium acetate trihydrate were purchased from Fisher Scientific (Fair Lawn, New Jersey). The solid phase extraction cartridges were Strata-x 33u polymeric reversed phase 60 mg ml^{-1} from Phenomenex (Torrance, CA) or Sep-Pak plus, silica cartridges (Milford, MA).

DNA manipulations were performed by standard technique (Sambrook *et al.*, 1989) and restriction enzymes and Phusion^{TM} High-Fidelity DNA Polymerase were obtained from New England Biolabs (Ipswich, MA). Oligonucleotides were obtained from Integrated DNA Technologies (Coralville, IA) and DNA sequencing was performed on an ABI3700 automated sequencer. Sequence analysis was

performed with Vector NTI Advance 10 (Invitrogen Corp., Carlsbad, CA). KLH11 and KLH11-EC1 derivatives were grown in Marine Broth 2216 (MB2216) (BD, Franklin Lakes, NJ) at 28°C. *E. coli* strains were grown at 37°C in Luria-Bertani (LB) broth. *A. tumefaciens* strains were grown in AT minimal medium supplemented with 0.5% glucose and 15 mM $(NH4)_2SO_4$ (ATGN) (Tempé *et al*., 1977). Antibiotics were used at the following concentrations (μg ml^{-1}): (i) *E. coli* (ampicillin, Ap, 100; gentamicin, Gm, 25; kanamycin, Km, 25; spectinomycin, Sp, 100); (ii) KLH11 (Km 100; Rifampicin, Rif, 200; Gm 25; Sp100) (iii), *A. tumefaciens* (Gm 300; Sp 200).

Plasmids were introduced into KLH11 using either electroporation or conjugation, into *E. coli* using standard methods of transformation or electroporation (Sambrook *et al*., 1989) and into *A. tumefaciens* using a standard electroporation method (Mersereau *et al*., 1990). The methods to make electrocompetent cells of KLH11 were similar to that described by Piekarski *et al.* (2009). Briefly, mid-log phase ($OD_{600}\sim$ 0.5-0.6) of KLH11 was harvested by centrifugation at 5000 x g for 7 min. The supernatant was discarded and the cell pellets were gently washed with 10% ice-cold glycerol followed by centrifuged at 5000 x g for 7 min. The wash step was repeated twice. The cells were eventually resuspended in 30% ice-cold glycerol and stored at -80°C.

2.3.2 Preparation of AHL samples and analysis by RP-HPLC and ESI mass spectrometry

KLH11 derivatives were grown in MB 2216 with appropriate antibiotics at 28°C to stationary phase (OD_{600} ~2.0) in the presence of 5 g l^{-1} of Amberlite XAD 16 resin for 36 h. *E. coli* MC4100 expressing *ssaI* or *ssbI* was grown in LB at 37°C with 5 g l^{-1} of Amberlite XAD 16 resin to an OD_{600} of 0.6-0.8 and expression was induced 24 h by addition of 1 mM IPTG. Cells and resin were separated by centrifugation and extracted with 50 ml methanol and dried to 2 ml. 3 nanomoles of D_3-C6-HSL was added to each sample as an internal standard and a volume of 0.2 ml was purified using solid phase extraction methods as described previously (Gould *et al*., 2006).

The purified sample was dried down and resuspended in 38 µl solvent A (8.3 mM acetic acid-NH_4 pH 5.7) and 2 µl solvent B (methanol). This solution was injected on to a 50 x 3.00 mm 2.6µ C18 Kinetex Phenomenex column. A mobile phase gradient was generated from 5% B to 65% B in 5 min, then B was increased to 95% in 15 min and held for 8 min at a flow rate of 250 µl min^{-1}. The HPLC system was interfaced to the electrospray source of a triple quadrupole mass spectrometer (Sciex API2000, PE Sciex, Thornhill, Ontario, CA). Precursor ion-scanning experiments were performed in positive-ion mode with the third quadrupole set to monitor m/z 102.1 and the first quadrupole set to scan a mass range of 170 to 700 over 9 s. The collision cell and instrument parameters were as follows: ion spray voltage of 4200 V, declustering potential of 50 V and collision energy of 25 V with nitrogen as the collision gas.

2.3.3 Qualitative analysis and estimation of AHL quantities

The identification of AHL molecular species was carried out at the first level using the HPLC retention times for the specific ions that gave rise to m/z 102.1 (precursor ion scanning) corresponding to the available AHL reference standards. In all cases, co-injection of the authentic reference standard gave rise to an increase in the single HPLC peak corresponding to the correct AHL. For those AHLs for which reference material was not available, the observed precursor ion of m/z 102.1 was used to determine the $[M+NH_4]^+$ and therefore the molecular weight of a putative AHL. If this molecular weight corresponded to a saturated or monounsaturated AHL, then the retention time was compared to predicted retention time for this molecular species based on retention times of the reference AHLs. If the molecular weight corresponded to addition of an oxygen atom (keto or hydroxyl substituent, 14 and 16 u higher) to the saturated HSL series, then derivatization by trimethylsilylation (Clay and Murphy, 1979) and methoximation (Maclouf *et al.*, 1987) and reanalysis was performed to confirm the presence of an oxidized AHL molecular species. An increase in 72 u or 29 u in the observed $[M+NH_4]^+$ would correspond to the formation of a trimethylsilyl ether or methoxylamine derivative of a hydroxylated-AHL or keto-AHL, respectively.

The relative amount of each AHL species was estimated based on the ratio of the abundance of the transition $[M+NH_4]^+$ to m/z 102.1 divided by the abundance of the ion transition derived from the added internal standard (m/z 220.3 ->102.1), as previously described (Gould *et al.*, 2006). In separate experiments

this resulted in a linear relationship between the abundance ion ratios for the precursor ions of m/z 102.1 and quantity of AHL reference standards.

2.3.4 Genomic library screen of KLH11 QS genes

KLH11 genomic DNA was obtained using the BactozolTM DNA Isolation Kit from Molecular Research Center Inc. (Cincinnati, OH). The DNA was independently digested to completion by restriction enzymes *Hin*dIII and *Sal*I and the fragments were ligated into expression vector pBBR1-MCS5 (Kovach *et al.*, 1995) followed by electroporation into *E. coli* Electro-Ten Blue competent cells. Cells were plated onto LB plates with gentamycin selection and incubated at 37°C overnight. Plasmids were extracted from colonies pooled from a large number of plates. The mixed plasmids preparations from the *Hin*dIII and *Sal*I libraries were independently electroporated *en masse* into *A. tumefaciens* NTL4 (Zhu *et al.*, 1998). Transformants were plated on ATGN media plus appropriate antibiotics and X-gal (40 µg ml^{-1}). Blue colonies (harboring putative AHL synthases) were chosen for further analysis after growth at 28°C for 2-3 days.

The plasmids pECH100, pECH101, and pECS102 were identified as AHL+ transformants in the KLH11 genomic library screen and were used as the template for PCR amplification of *ssaI*, *ssbI*, and *ssaR*. To PCR amplify *ssbR*, the oligonucleotide specific for the 3' end of *ssbR* was designed from *de novo* sequence obtained from pECH101, isolated from the *Hin*dIII genomic library. However, only 62 bp of the *ssbR* sequence was carried on pECH101, due to a *Hin*dIII site at this position. *R. pomeroyi* DSS-3 has *silR2* homologous to *ssbR* (Moran *et al.*, 2004) and based on this sequence a primer designed to the gene

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presumptively flanking *ssbR* was generated, and used to PCR amplify the complete *ssbR* sequence from KLH11.

2.3.5 Directed mutation, *lacZ* fusions and complementation

For Campbell-type, recombinational mutagenesis internal gene fragments were generated and used to disrupt target genes (Kalogeraki and Winans, 1997). An internal portion of the *ssaI* gene was amplified from the pECH100 template using primers designated as 1 and 2 of *ssaI*. The partial *ssaI* fragment was gel purified and cloned into pGEM®-T Easy (Promega, Madison, WI), creating pEC103, which was confirmed through sequencing. For recombinational mutagenesis, pEC103 was digested with *Eco*RI and *Kpn*I, and the resulting *ssaI* fragment was ligated to a similarly digested R6K replicon, the pVIK112 suicide vector (Kalogeraki and Winans, 1997), creating pEC113. pEC113 was conjugated into KLH11 and transconjugants resistant to kanamycin (Km) were selected and confirmed by sequencing. The *ssbI* null mutant, designated as EC3, the *ssaR* null mutant EC4, and the *ssbR* null mutant EC5 were created similarly to EC2, each using the primers 1 and 2 of these genes and confirmed by sequencing. These plasmid insertions also create *lacZ* transcriptional fusions in the genes they disrupt. To study the effect of AHL on the *luxR*-type genes, a fragment of *ssaR* gene, about 500 bp ending at the stop codon was PCR amplified using primers ssaRintact F and ssaRintact R. The PCR amplicon was cloned into pGEM®-T Easy (Promega, Madison, WI) first and then subcloned into pVIK112, creating pJZ001. The pJZ001 was conjugated into KLH11 and transconjugants were selected and confirmed as described above. Thus, the

lacZ was fused into the 3' end of the *ssaR* gene, keeping *ssaR* intact. The same

approach was used to fuse *lacZ* into the 3' end of the *ssbR* gene while retaining

ssbR intact.

To generate in-frame deletions, a standard approach was utilized (Merritt *et al.*, 2007). For the *ssaI* gene, about 600 bp upstream of *ssaI* gene was PCR-amplified using primers ssaI D1and ssaI D2 and approximately 400 bp including 206 bp of *ssaI* encoding sequences and about 200 bp downstream of *ssaI* was PCR-amplified using primers ssaI D3 and ssaI D4 (Table 2.2). Primers ssaI D2 and ssaI D3 had complementary sequence at the 5' end to allow Splicing by Overlapping Extension (SOEing), as described previously (Merritt *et al.*, 2007). In brief, these two fragments were gel purified, followed by PCR amplification with primers ssaI D1 and ssaI D4 using an equal amount of the two fragments as templates. The PCR product of this amplification was gel purified and digested using restriction enzymes *Spe*I and *Sph*I. The digested PCR product was ligated into the *sacB* counter-selectable vector pNPTS138 (Hibbing and Fuqua, 2011), which was digested with the same combination of restriction enzymes. Derivatives of pNPTS138 were conjugated into *Ruegeria* sp. KLH11. The selection of the first crossover was performed by plating transconjugants onto MA 2216 plates with Rif and Km. The colonies that grew on this selective medium, but not on the same plates supplemented with 5% (w/v) sucrose were chosen and subcultured in MB 2216 without Km to allow for excision of the integrated plasmid, followed by plating on 5% sucrose MA2216 plates without Km. Sucrose resistant (Suc^R) Km^S colonies were selected. Deletion of the

targeted region was confirmed by PCR. Deletion of *ssbI* was performed in the same way using primers ssbI D1-ssbI D4. The double deletion of *ssaI* and *ssbI* was performed by deleting *ssbI* in the *ssaI* deletion strain using the same method as described above.

Controlled expression constructs of *ssaI*, *ssaR*, *ssbI* and *ssbR* were generated by PCR amplification of the coding regions of each gene using primers designated as 3 and 4 for each specific gene and genomic DNA of KLH11 as template (Table 2.2). The *E. coli lacZ* ribosomal binding site was engineered into the 5' primer of each gene. The PCR products were digested with the appropriate restriction enzyme(s) and ligated into the vector pBBR1-MCS5 (Kovach *et al.*, 1995). The insert carried by each construct was confirmed by sequencing.

2.3.6 Plasmid-borne promoter fusions and expression plasmids

The intergenic region upstream of the *ssaI* coding sequence and downstream of *ssaR* contains the presumptive *ssaI* promoter region and was PCR amplified from pECS102 using primers ssaI P1 and ssaI P4. The upstream and downstream oligos anneal 182 bp upstream, and 3 bp downstream of the *ssaI* translational start site, respectively. In addition, several plasmid-borne *ssaI* promoter deletions were also generated using PCR amplification to truncate the 5' sequences, at positions 79 bp and 63 bp upstream of the *ssaI* translational start site. These PCR products were cloned into pCR®2.1-TOPO® and their content was confirmed by DNA sequencing. The pCR®2.1-TOPO® derivatives were digested with EcoRI and *Pst*I, and the resulting fragments were ligated with

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equivalently digested pRA301 vector (Akakura and Winans, 2002). Similarly, the *ssbI* promoter and translational start site were amplified from KLH11 genomic DNA using primers ssbI P1 and ssbI P4 to generate an amplicon that extends 229 bp upstream and 3 bp downstream of the *ssbI* predicted translational start site. This amplicon was subsequently used to generate a plasmid-borne P_{ssbI}-*lacZ* fusion on pRA301. Plasmids P_{lac}-*ssaR* (pEC112) and P_{lac}-*ssbR* (pEC123) were introduced into the AHL⁻ *A. tumefaciens* NTL4 in combination with the compatible P_{ssaI}-*lacZ and* P_{ssbI}-*lacZ* plasmids to examine gene regulation patterns in a heterologous AHL⁻ host.

2.3.7 Preparation of log phase cell concentrates and β-galactosidase assays

β-galactosidase assays for both *A. tumefaciens* and KLH11 derivatives were performed as described previously (Miller, 1972). Mid-log phase *A. tumefaciens* cultures were diluted at 1:100 dilution to an OD600~0.01 and were supplemented with different concentration of 3-oxo-C16:1 Δ11-HSL. The cell suspension was thoroughly mixed and then aliquoted into 15 ml test tubes, incubated at 28°C for 24 h to an OD_{600}~0.4. Mid-log phase cultures were measured for OD_{600} and frozen at -20°C and used for subsequent β-galactosidase assays. Cultures of *Ruegeria* sp. KLH11 were prepared in a similar way. KLH11 was grown in MB 2216 supplemented with Km as required overnight and was diluted at 1:100 dilution to an OD600~0.01. 3-OH-C14-HSL and 3-oxo-C16:1 Δ11-HSL were added at the concentration of 20 µM and 2 µM, respectively. Mid-log phase of

KLH11 cultures was sampled and β-galactosidase assays were performed immediately.

2.3.8 AHL detection using an ultrasensitive *A. tumefaciens* reporter for KLH11 derivatives

AHLs were extracted from KLH11 cultures using dichloromethane, fractionated by reverse phase TLC, and detected using an ultrasensitive AHL bioreporter derived from *A. tumefaciens* (Zhu *et al.*, 2003, Mohamed *et al.*, 2008c). Five ml MB 2216 cultures from KLH11 were grown to an OD_{600} of 1.5 – 2.0, followed by extraction with an equal volume of dichloromethane. Culture pH was monitored and was within the range of 7.6 ± 0.2 at the time of harvesting (sterile Marine Broth 2216 is pH 7.6). Following centrifugation, the organic phase was removed and allowed to evaporate in a fume hood. Extracts were concentrated 1000 fold, normalized to an OD_{600} of 1.5 and resuspended in a final volume of approximately 5 µl of acidified (0.01%) ethyl acetate and loaded onto a C18 RP-TLC plate (Mallinckrodt Baker, Phillipsburg, NJ, USA). TLC plates were developed in a 60% methanol water mobile phase, dried, and overlaid with 100 ml of 0.6% ATGN media supplemented with 40 µg/ml X-gal and 1 ml of an OD_{600} = 12.0 suspension of the highly sensitive *A. tumefaciens* AHL reporter (*A. tumefaciens* KYC55 [pJZ372][pJZ384][pJZ410]) as previously described (Zhu *et al.*, 2003). TLC plate overlays were placed in a sealed container and incubated at 28°C for 16-48 hrs.

2.3.9 Motility assay, flagellar stain and immunodetection of flagellin

Bacterial swimming assays were performed using MB2216 with 0.25 % (w/v) agar. No antibiotics were added to the medium. Plates were inoculated at the center with freshly isolated colonies. 3-oxo-C16:1 Δ11-HSL was added to MB 2216 agar to 2 μM. Plates were placed in an air-tight container with a beaker containing 15 ml of K_2SO_4 to maintain constant humidity, and incubated 8 days at 28°C.

Flagellar stains were performed based on methods described by Mayfield and Inniss (1977) and were viewed by phase contrast microscopy. For immunoblotting with anti-flagellin antibodies 5 ml MB2216 cultures (wild type, *ssaI⁻* and *ssaR⁻*), were grown to late stationary phase. Culture volumes were normalized to an OD_{600} of 0.6, and portions of these were centrifuged at 716 x g for 12 min to gently separate cells and supernatant. Protein from the whole culture and supernatant fractions was precipitated with an equal volume of 100% trichloroacetic acid. Following vortexing and 15 min incubation on ice, samples were centrifuged (8,765 x g; 20 min) and washed with 1 ml of acetone. The whole culture, pellet, and supernatant fractions were resuspended in 1X SDS lysis buffer, boiled 10 min, and used in subsequent immunoblotting analysis.

Immunoblotting with anti-flagellin polyclonal antibodies raised against *C. crescentus* whole flagella (a gift from Y.V. Brun) was performed using a standard technique. Samples were separated by 15% SDS-PAGE at 100 V for 80-90 min, and transferred to the membrane (Osmonics, Westborough, MA) at 30 V for 40

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min using a semi-dry electrotransfer system. The membrane was blocked

overnight at 4°C in Blotto (1x TBS-T [Tris Buffered Saline], 1% Tween 20) and

5% dried milk). The polyclonal antibody was diluted 1:20,000 in 4 ml of Blotto

and incubated with the membrane for 45 min on a rocking shaker at room

temperature. The membrane was then washed three times for 5 min in 1X TBS-

T. Secondary antibody solution at 1:20,000 in 4 ml of Blotto was incubated with

the membrane and rocked 45 min at room temp. The blot was rinsed three times

for 5 min with 1X TBS-T, followed by 2 times in 1X TBS. Chemoluminescent

substrate, Supersignal West Pico Chemoluminescent Substrate (Pierce,

Rockford, IL) was used as the detection reagent. Equal parts of the

Luminol/Enhancer and Stable Peroxide Buffer were combined and pooled on top

of the membrane for ~5 min. Excess detection reagent was removed by blotting

on filter paper, and signal was detected by exposure to Kodak BioMax film

(Kodak).

To analyze flagellin biosynthesis across the KLH11 growth curve, samples

were taken at a range of times between mid-exponential phase through late

stationary phase. 100 ml cultures were grown at 28°C and samples were taken

for EC1 (wt KLH11) at OD_{600} of 0.5, 1.3, 1.8, and 2.2; strain EC2 (*ssaΓ*,) at OD_{600}

of 0.8, 1.3, 1.7, and 2.4. All samples were processed and analyzed by western

blotting and culture samples were also viewed under phase contrast microscopy

to evaluate swimming at these time points.

2.3.10 RNA extraction and quantitative reverse transcription PCR (qRT-PCR) analysis

Expression levels of *fliC* and *flaA* were measured using qRT-PCR with specific primers (Table 2.2). KLH11 and derivatives were grown in MB2216 to an OD_{600} of 1.8 and 1 ml RNA Protect Bacterial Reagent (Qiagen, Valencia, CA) was added to 0.5 ml culture. The mixture was kept at room temperature for 5 min and was centrifuged at 5000 x g for 10 min. The cell pellet was stored in at -20°C for RNA extraction. Total RNA was isolated using an RNeasy miniprep kit (Qiagen, Valencia, CA), with genomic DNA removed by TURBO DNase (Ambion, Austin, TX), and cDNA synthesized using qScript™ cDNA SuperMix according to the manufacturer's instructions (Quanta BioSciences, Gaithersburg, MD). The RT-PCR was carried with PerfeCTa™ SYBR® Green FastMix™ Low ROX. Reactions were performed on an Mx3000P qPCR system (Stratagene, Santa Clara, CA) using the following cycling parameters: 2 min at 95°C for initial denaturation, 40 cycles for 10 s cycles at 95°C, and 30 s for primer annealing, and primer extension at 60°C. Melt curves were performed to confirm the specificity of primers and the absence of primer dimers. Expression levels of *fliC* and *flaA* genes were normalized to the KLH11 vegetative sigma factor 70 gene (*rpoD*).

2.3.11 Biofilm assays

A standard coverslip biofilm assay was used to evaluate the impact of KLH11 QS on biofilm formation (Tomlinson *et al.*, 2010). Briefly, overnight cultures of different strains were inoculated at OD_{600}~0.06 in 3 ml MB 2216 in UV-sterilized

12-well polystyrene plates and biofilms were grown on sterile PVC coverslips suspended vertically in the wells. The 12-well plates were incubated statically at 28°C over three days. At specific time points, 200 µl of culture was removed to measure the turbidity. The adherent biomass was stained with 0.1% (w/v) crystal violet (CV) solution for 5-10 min and then rinsed gently with DI water to remove loosely attached cells. CV adsorbed to the biofilms was extracted with 33% acetic acid and its absorbance at 600 nm (A_{600}) was measured. These values were normalized by the OD_{600} of planktonic growth.

To examine inhibition of biofilm formation, wt KLH11 was grown in MB2216 at 28°C with shaking (200 rpm) to late stationary phase (55–60 h). Bacterial cells were removed from culture volumes of 300 ml by 2 rounds of centrifugation at 5000 x g for 10 min. The supernatant was filtered through a 0.22-µm filter and stored at -80 °C until ready for use. For the biofilm assays to which culture fluids were added the $\Delta ssaI$ mutant was grown in MB2216 overnight and then diluted to an OD_{600} ~ 0.06. 2X MB2216 was diluted with the appropriate amounts of culture fluids and water to ensure that there was always at least a 1X concentration of nutrient in the initial biofilm inoculum. Biofilm formation was detected at 48 h as described above.

2.3.12 Statistical analysis

Unpaired Student's *t* test was used to calculate P value in all chapters.

Table 2.1. Strains and plasmids used in Chapter 2.

Bacteria/Plasmids	Relevant feature[a]	Reference
E. coli Electro-Ten Blue	Standard alpha-complementation strain	Stratagene
E. coli DH5α/λpir	Strain for propagating R6K suicide plasmids	Lab collection
E. coli DH5α/λpir	Strain for propagating R6K suicide plasmids	Lab collection
E.coli XL-1 Blue	Standard alpha-complementation strain	Lab collection
E. coli TOP 10 F´	Standard alpha-complementation strain, *lacI*^{Q}	Qiagen
E. coli MC4100	K-12 derivative, Δ*lacZ*	Lab collection
A. tumefaciens NTL4	Ti plasmidless derivative, nopaline chromosomal background	(Zhu *et al.*, 1998)
A.tumefaciens KYC55	Ti plasmidless derivative, octopine chromosomal background	(Zhu *et al.*, 2003)
Ruegeria pomeroyi DSS-3	wild type	(Moran *et al.*, 2004)
KLH11	wild type	(Mohamed *et al.*, 2008c)
KLH11-EC1	Rif^R	This study
KLH11-EC2	*ssaI-lacZ*, null *ssaI*, Rif^R, Km^R	This study
KLH11-SK01	Δ*ssaI*, Rif^R	This study
KLH11-EC3	*ssbI-lacZ*, null *ssbI*, Rif^R, Km^R	This study
KLH11-SK02	Δ*ssaI* Δ*ssbI*, Rif^R	This study
KLH11-EC4	*ssaR-lacZ*, null *ssaR*, Rif^R, Km^R	This study
KLH11-EC5	*ssbR-lacZ*, null *ssbR*, Rif^R, Km^R	This study
KLH11-JZ1	*ssaR-lacZ*, wild type *ssaR*, Rif^R, Km^R	This study
KLH11-JZ2	*ssbR-lacZ*, wild type *ssbR*, Rif^R, Km^R	This study
KLH11-OKC9	*fliC-lacZ*, null *fliC*, Rif^R, Km^R	This study

pCR®2.1-TOPO®	PCR fragment cloning vector, Ap^R, Km^R	Invitrogen
pBBR1-MCS2	P_{lac} expression vector, Km^R	(Kovach *et al.*, 1995)
pBBR1-MCS5	P_{lac} expression vector, Gm^R	(Kovach *et al.*, 1995)
pGEM®T-Easy	PCR fragment cloning vector, Ap^R	Promega
pVIK112	R6K-based *lacZ* transcriptional fusion, integration vector, Km^R	(Kalogeraki and Winans, 1997)
pRA301	*lacZ* translational fusion vector	(Akakura and Winans, 2002)
pJZ372	P_{traI}-*lacZ* translation fusion, Tc^R	(Zhu *et al.*, 2003)
pJZ384	P_{T7}-*traR*, Sp^R	(Zhu *et al.*, 2003)
pJZ410	T7 polymerase expressing plasmid	(Zhu *et al.*, 2003)
pECH100	pBBR1-MCS5 derivative, 3 kb HindIII fragment containing *ssaI* and truncated *ssaR*, Gm^R	This study
pECH101	pBBR1-MCS5 derivative, 2.8 kb HindIII fragment containing *ssbI* and truncated *ssbR,* Gm^R	This study
pECS102	pBBR1-MCS5 derivative, 3.2 kb Sal I fragment containing *ssaI* and *ssaR*, Gm^R	This study
pEC103	pGEM®T-Easy derivative, carrying truncated *ssaI* fragment, Ap^R	This study
pEC104	pGEM®T-Easy derivative, carrying truncated *ssbI* fragment, Ap^R	This study
pEC105	pGEM®T-Easy derivative, carrying truncated *ssaR* fragment, Ap^R	This study
pEC106	pGEM®T-Easy derivative, carrying full length PCR-amplified *ssbR* fragment, Ap^R	This study
pEC107	pVIK112 derivative carrying truncated *ssaR* gene from pEC105, Km^R	This study
pEC108	pBBR1-MCS5 derivative carrying full length P_{lac}-*ssaI*, from pEC111, Gm^R	This study

pEC109	pBBR1-MCS5 derivation carrying full length P_{lac}-*ssbI*, from pEC110, Gm^R	This study
pEC110	pCR$^®$2.1-TOPO$^®$ derivative carrying full length PCR-amplified P_{lac}-*ssbI*, Km^R	This study
pEC111	pCR$^®$2.1-TOPO$^®$ derivative carrying full length PCR-amplified P_{lac}-*ssaI*, Km^R	This study
pEC112	pBBR1-MCS5 derivative, carrying full length P_{lac}-*ssaR* from pEC106, Gm^R	This study
pEC113	pVIK112 derivation carrying truncated *ssaI* gene from pEC103, Km^R	This study
pEC114	pCR$^®$2.1-TOPO$^®$ derivative, carrying PCR amplified P_{ssaI}, Ap/Km^R	This study
pEC115	pVIK112 derivative carrying truncated *ssbI* gene from pEC104, Km^R	This study
pEC116	pRA301 derivation, P_{ssaI}-*lacZ*, Sp^R	This study
pEC117	pGEM$^®$T-Easy derivative, carrying P_{ssbR} and *ssbR* Ap^R	This study
pEC118	pGEM$^®$T-Easy derivative, carrying P_{ssbI} and *ssbI* Ap^R	This study
pEC119	pGEM$^®$T-Easy derivative, carrying P_{ssbR} and *ssbR,* Ap^R	This study
pEC120	pGEM$^®$T-Easy derivative, carrying full length P_{lac}-*ssbR*, Ap^R	This study
pEC121	pRA301 derivative, P_{ssbI}-*lacZ*, Sp^R	This study
pEC122	pVIK112 derivative carrying truncated *ssbR* from pEC119, Km^R	This study
pEC123	pBBR1-MCS5 derivative, carrying full length P_{lac}-*ssbR*, from pEC120, Gm^R	This study
pEC124	pRA301 derivative, P_{ssaI}-*lacZ*, 5' promoter deletion, 79 bp with *lux* type box, Sp^R	This study

pEC127	pRA301 derivative, P_{ssaI}-lacZ, 5' promoter deletion, 63 bp fragment lacks lux type box, Sp^R	This study
pJZ001	pVIK112 derivative, ssaR gene with 5' truncation, to retain wt ssaR, Km^R	This study
pJZ002	pVIK112 derivative, ssbR gene with 5' truncation, to retain wt ssbR, Km^R	This study
pOKC9	pVIK112 derivative, fliC gene with 3' truncation, Km^R	This study

[a]Ap=ampicillin, Gm=gentamicin, Km=kanamycin, Rif=rifampicin, Sp=spectinomycin. Tc=tetracycline.

Table 2.2. Primers used in Chapter 2.

Primer name	Sequence [b](5'-3')	Restriction Enzyme
ssaI D1	ACTAGTCTATGGTGACGACTGGAAG	*Spe*I
ssaI D2	**GAATTCGTCAGTCAGTCA**GTTTCCCGTAATATTGGCTT	NA
ssaI D3	**TGACTGACTGACGAATTC**AGGCTGGCGAACTCAAGCCTG	NA
ssaI D4	GCATGCGACTACATTGTCGAGCTG	*Sph*I
ssbI D1	ACTAGTGCAATCAGGGTTATTCGATC	*Spe*I
ssbI D2	**GAATTCGTCAGTCAGTCA**CAACATGATTGTTCCCCTTGT	NA
ssbI D3	**TGACTGACTGACGAATTC**GCCTGACCTTGGTGGAAATTG	NA
ssbI D4	GCATGCGATACGGTGAATGGTCGTTGC	*Sph*I
ssaI 1	cggGAATTCATGTTCGAACTGCGCGCTCGGG	*Eco*RI
ssaI 2	gccGGTACCATCGCAGGGACCTTGCCCATC	*Kpn*I
ssaI 3	ggcCTCGAGC**TGA**AAC*AGGAAA*CAGCT**ATG**ATTTTGGTAGTTGATG	*Xho*I
ssaI 4	ggcGAATTCGGG**TCA**GGCCTCATGAGCAAAAGC	*Eco*RI
ssaR 1	cgcGAATTCTCAGCACCCTCCCCGAACAGG	*Eco*RI
ssaR 2	cgcGGTACCCGGCCCATTGCAAAATCTC	*Kpn*I
ssaR 3	gggCTCGAGG**TGA**AAC*AGGAAA*CAGCT**ATG**GATATTGTTGATCTCAGC	*Xho*I
ssaR 4	gggGAATTCGGC**TTA**ACCTGGGTAGATTAGCCC	*Eco*RI
ssbI 1	cgcGAATTCGGATGAGCTGCATAAATTTCCGG	*Eco*RI
ssbI 2	gccGGTACCAACGGAAATCCGGTCTCGCCCG	*Kpn*I
ssbI 3	ggcCTCGAGC**TGA**AAC*AGGAAA*CAGCT**ATG**TTGCGTTATTTATATGCGG	*Xho*I
ssbI 4	ggcGAATTCGGG**TCA**GGCGGAAAGCGCAAACCG	*Eco*RI
ssbR 1	ggcGAATTCTCGGTTCAGCTGCGCGATCGG	*Eco*RI
ssbR 2	cgcGGTACCTTCCGGTTGCCGGTTCTGGGC	*Kpn*I
ssbR 3	gcgCTCGAGG**TGA**AAC*AGGAAA*CAGCT**ATG**AGGCTTGCGCGCCCGCG	*Xho*I
ssbR 4	gggGAATTCGGC**TTA**AACGACTATTAATCCTCTGC	*Eco*RI
fliC 1	cgcgaattcAAGTCGGTCAACATGAACCTG	*Eco*RI

fliC 2	gccggtaccGTTGTCACGATCAAGCGAGGA	KpnI
fliCRT1	CGCAGAACCTGTCGACCGGT	NA
fliCRT2	GGTATCGCCTGCGGCCAATGT	NA
flaART1	GCACCGATGCATATGCGCAAGCT	NA
flaART2	TGAGCGATTGCAGCCGGGTT	NA
rpoDRT1	GACGCCTATCGCGGCCGT	NA
rpoDRT2	GCCGACCTGCGCCATATCGT	NA
ssaI P1	ccgGAATTCTGCACTAACCACACCTCAGGCCG	EcoRI
ssaI P2	gcgGAATTCTACGGGAAACCCCCAATAGATTCG	EcoRI
ssaI P3	gggGAATTCTAGATTCGCTGTGAAATCCGAG	EcoRI
ssaI P4	ccgCTGCAGAAT**CAT**GTTAACCCCCTTCG	PstI
ssbI P1	cggGAATTCTATAGCCGGGCACAGGTGGCGC	EcoRI
ssbI P2	cccCTGCAGCAA**CAT**GATTGTTCCCCTTGTCGT	PstI
aP1	CGGCACCATTCATGGCCATGT	NA
aP2	CCATTCGTCCCGACTGCAGC	NA
aP3	ATGATTTTGGTAGTTGATG	NA
aP4	GTCGCATAGGACACCGAGTT	NA
bP1	CCAATATGGCTTCACGACCT	NA
bP2	AATAACCCTGATTGCCCACA	NA
bP3	GGTAAACGAAGATGGCGAAG	NA
bP4	GAGCCGATCATGCGATAAAT	NA
ssaRintactF	GAATTCCAAGGCCTGCATCTGATCG	EcoRI
ssaRintactR	GGTACCTTAACCTGGGTAGATTAGCCC	KpnI
ssbRintactF	GAATTCTATCACCGCATTGATCCGG	EcoRI
ssbRintactR	GGTACCTTAAACGACTATTAATCCTCTGCTG	KpnI

2.4. Results

2.4.1 AHL synthesis and genetic isolation of *luxI* and *luxR* homologues from KLH11

The sponge symbiont *Ruegeria* sp. KLH11 has a very complex profile of AHLs as evaluated by bioassays and fractionation by thin layer chromatography (Fig. 2.1; Mohamed *et al.*, 2008c). This bioassay is highly sensitive, but has a bias towards short chain AHLs similar to the *A. tumefaciens* cognate 3-*N*-oxo-octanoyl-L-homoserine lactone (3-oxo-C8-HSL) (Zhu *et al.*, 1998). A less biased semi-quantitative chemical analysis of the KLH11 whole culture organic extracts was performed using high performance liquid chromatography coupled to tandem mass spectrometry (LC-MS/MS; Gould *et al.*, 2006). The dominant AHLs were found to be hydroxylated forms of tetradecanoyl (C14) species, saturated (3-OH-C14-HSL) and unsaturated (3-OH-C14:1-HSL), and hydroxylated dodecanoyl species (3-OH-C12-HSL) (Fig. 2.2A, Fig. 2.3A, Table 2.3). The shorter chain AHLs detectable in bioassays (Fig. 2.1), were not observed with this chemical analysis suggesting that they are in low relative abundance.

A genetic approach was employed to isolate KLH11 AHL synthase genes. Two plasmid-borne gene libraries, with KLH11 genomic DNA digested to completion with *Hin*dIII or *Sal*I, both with cleavage products averaging 4 kb, were ligated into the expression (P_{lac}) vector pBBR1-MCS5 (Kovach *et al.*, 1995) and then transformed *en masse* into an *A. tumefaciens* strain that does not

synthesize AHLs (Mohamed *et al.*, 2008c). This AHL sensitive strain responds to introduction of plasmid-borne AHL synthase genes and expresses an AHL-dependent *lacZ* fusion. A pool of several thousand transformants was screened on selective medium with no exogenous AHL and X-gal. Of the 16 blue colonies isolated, several produced a diffusible activity, which induced *lacZ* expression in closely adjacent colonies after extended incubation. From these presumptive AHL[+] transformants, insert fragments were sequenced, identifying two separate loci with similarity to *luxI* from *V. fischeri*, each adjacent and in tandem arrangement downstream of a *luxR* homologue (Fig. 2.4A and B). We designated these genes *ssaRI* and *ssbRI* (Sponge-associated symbiont locus A and locus B, respectively). The draft genome of KLH11 was completed during the course of this study (Zan *et al.*, 2011b) and 100% identical loci were identified; thus we refer to these Genbank Accession numbers for *ssaRI*, *ssbRI* and flanking genes (Fig. 2.4).

The KLH11 *ssaR-ssaI* and *ssbR-ssbI* genes are highly similar to the *silR1-silI1* and *silR-silI2* genes from *R. pomeroyi* DSS-3 (Moran *et al.*, 2004). SsaI is 71% identical to SilI1, and each has an unusual C-terminal extension (~60 aa) relative to other LuxI-type proteins. SsaR and SilR1 share 79% identity. SsbI shares 82% identity with SilI2 and SsbR is 74% identical to SilR2. Thus, it appears that SsaRI and SilRI1, and SsbRI and SilRI2, are orthologous, whereas the SsaRI and SsbRI systems are paralogous.

After completing the KLH11 genome sequence, a third presumptive AHL synthase was identified and named s*scI*. Although *sscI* had escaped our genetic

screen, it shares 81% identity to SsbI on the amino acid level, is of similar size

and is likely to be the result of a recent gene duplication (See Chapter 3).
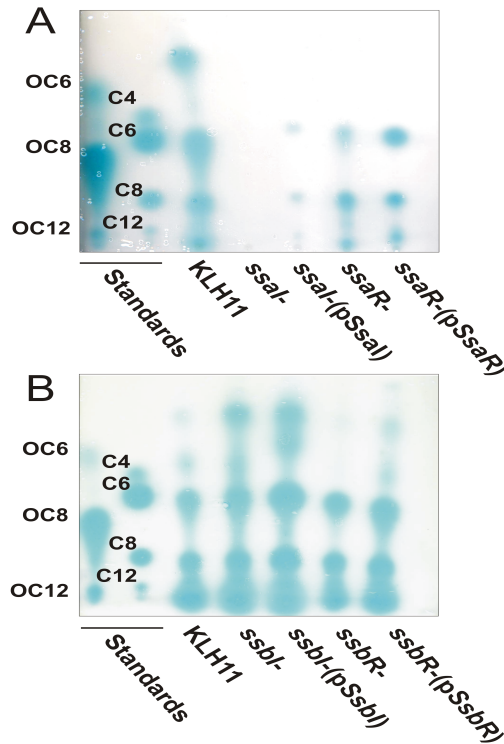
Figure 2.1. RP-TLC analysis of AHLs from KLH11 and QS mutants.

TLC plates were overlaid with the *A. tumefaciens* ultrasensitive AHL reporter

strain (Zhu *et al*. 2003). Mixtures of synthetic 3-oxo- and fully reduced AHL

standards were run on each plate in lanes 1 and 2 (labeled on plate). (A) KLH11

SsaRI mutants. KLH11, *ssaI⁻*, and *ssaR⁻* mutants and the plasmid-

complemented mutants are labeled. (B) KLH11 SsbRI mutants. KLH11, *ssbI⁻* and

ssbR⁻ mutants, and the plasmid-complemented mutants are labeled. AHL

standard concentrations are: Fully reduced, C4, 1 mM; C6, 500 µM; C8, 50 nM;

C10, 125 μM. 3-oxo derivatives, 3-oxo-C6, 50 nM; 3-oxo-C8, 42 nM; 3-oxo-C12, 68 μM. Figure provided by Elisha M. Cicirelli and Clay Fuqua.
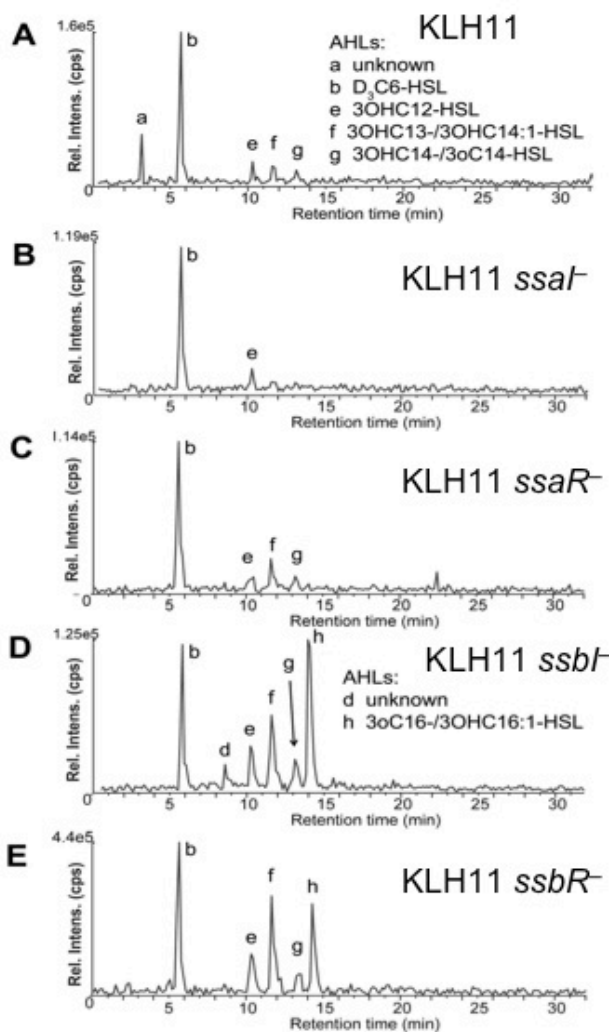
Figure 2.2. Chemical analysis of purified samples from KLH11 derivatives.

The products of reverse-phase chromatographic separation of AHLs extracted and purified from wild type KLH11 and mutants were examined using the precursor ion-scanning mode (transitions were monitored for precursor [M + H]$^+$ -> m/z 102.1). The peaks in the chromatograms (A) wt KLH11, (B) KLH11 *ssaI⁻*,

(C) KLH11 *ssaR⁻*, (D) KLH11 *ssbI⁻*, and (E) KLH11 *ssbR⁻* are labeled with lowercase lettering and include species defined in Table 2.3 and the AHLs noted. Figures are provided by Mair Churchill.
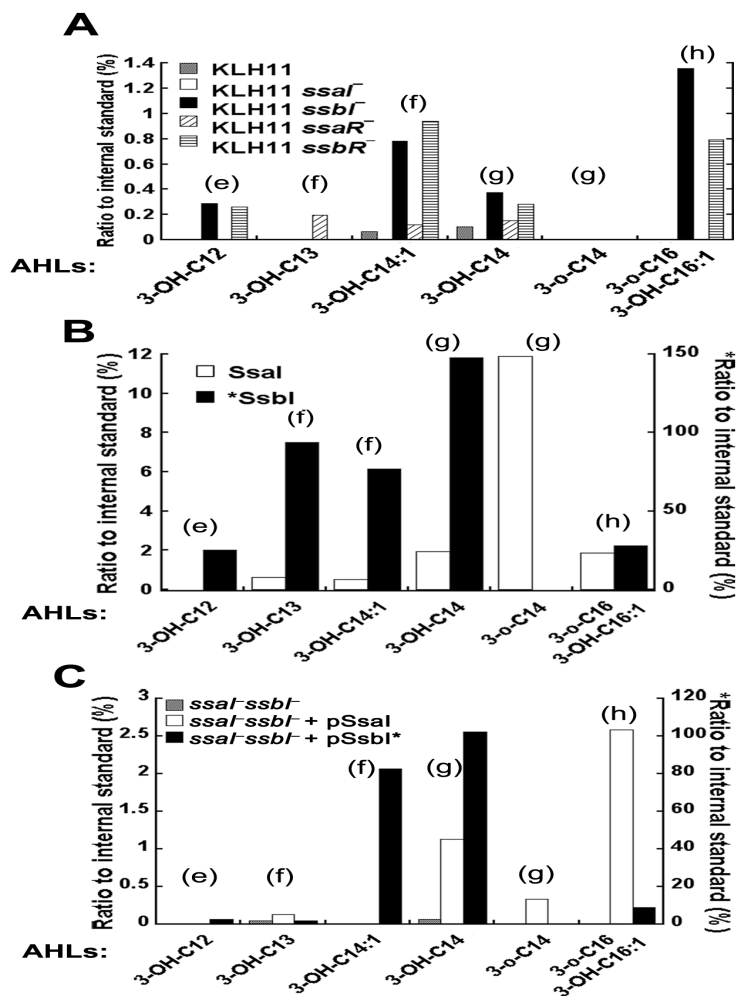
Figure 2.3. Comparative analysis of AHLs. The relative amounts of the known AHLs in (A and C) KLH11 derivatives and (B) *E. coli* MC4100 are shown in bar graphs, with the peak label above each set of bars. The plot shows the ratio of the area of the transition for each AHL to m/z 102.1 to the same transition for the internal standard D3-C6-HSL. Analysis of AHL samples was performed in

positive-ion mode with the third quadrupole set to monitor m/z 102.1 as

described in Experimental Procedures. The relative amounts of the known AHLs

in each strain are shown in the bar graph, and the data were analyzed as in

Figures 2.2 and 2.5. (e), (f), (g), and (h) referring to detection of 3-OH-C12-HSL,

3-OH-C13-HSL/3-OH-C14:1-HSL, 3-OH-C14-HSL/3-oxoC14-HSL, and 3-oxo-

C16-HSL/3-OH-C16:1-HSL respectively. In panels B and C, the SsaI y-axis is on

the left and the SsbI y axis (with an asterisk) is on the right. Figures are provided

by Clay Fuqua.
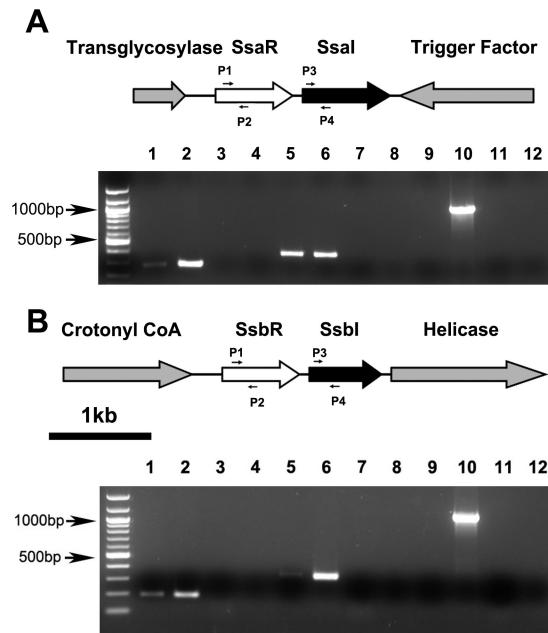
Figure 2.4. Gene maps of KLH11 *ssaR/ssaI* and *ssbR/ssbI* loci. Arrows

represent genes. A) SsaR (ZP_05123091) and SsaI (ZP_05123801) and are

predicted to be 233 and 284 amino acids, respectively. Genbank accession

numbers are ZP_05124568 for transglycosylase and ZP_05123238 for trigger

factor. Primers used to test whether *ssaRI* are in the same operon are indicated

as P1, P2, P3 and P4 (see Table 2.2, aP1, aP2, aP3, aP4). Lanes 1-4 were

PCR results using primers P1 and P2, lanes 5-8 used primers P3 and P4, and

lanes 9-12 used primers P1 and P4. Lanes 1, 5 and 9 used cDNA as template,

lanes 2, 6 and 10 used genomic DNA as template, lanes 3, 7 and 11 used RNA

as templates. Lanes 4, 8 and 12 were negative controls comprising the primer

sets. B) SsbR (ZP_05123460) SsbI (ZP_05121795) are predicted to be 239 and

212 amino acids. Genbank accession numbers are ZP_05124465 for crotonyl

CoA reductase and ZP_05122236 for helicase. Primers used to test whether

ssbRI are in the same operon are indicated as P1, P2, P3 and P4 (see Table 2.2, primers bP1, bP2, bP3, bP4). The lanes are organized the same as in panel A.

2.4.2 Ssal and Ssbl synthesize long chain–length AHLs when expressed in *E. coli*

Each presumptive AHL synthase gene was expressed from the P_{lac} promoter carried on a low copy number plasmid in *E. coli* MC4100 for mass spectrometry analysis. *E. coli* does not produce AHLs and thus those produced will generally reflect the intrinsic specificity of the enzyme in the *E. coli* background (Fig. 2.5, Table 2.3). Trimethylsilylation and methoximation of these samples (Clay and Murphy, 1979; Maclouf *et al*., 1987) revealed that Ssal produces 3-oxo-AHLs, the most abundant species of which is 3-oxo-C14-HSL (Table 2.3, Fig. 2.3B), but also including a C16 derivative, that was shown by methoximation to be a 3-oxo-C16 derivative. No 3-oxo-HSLs were observed for *E. coli* expressing Ssbl, but rather 3-hydroxy-HSLs were identified, with predominant 3-OH-C14-HSL, 3-OH-C14:1-HSL and 3-OH-C13-HSL (Figs. 2.3B and 2.5B, Table 2.3). For both AHL synthases, putative AHL derivatives were also detected, but their identity was not confirmed due to a lack of reference standards (Table 2.3). Overall, Ssal and Ssbl drive the synthesis of long chain (lc) AHLs that differ in their modification at the 3-position of the acyl chain, 3-oxo and 3-OH, respectively.

2.4.3 Mutational analysis of *ssaRI* and *ssbRI* in KLH11

Campbell-type plasmid insertions were generated in the *ssaI*, *ssaR*, *ssbI* and *ssbR* genes in KLH11 using pVIK112, which generates *lacZ* transcriptional

fusions to the disrupted gene (Kalogeraki and Winans, 1997). Mutation of *ssaI* resulted in complete loss of AHLs using the biosensor assay (Fig. 2.1 A). Consistently, mass spectrometry analyses of organic extracts from the *ssaI* mutant reveal a dramatic decrease in AHL abundance, although trace levels of 3-OH-C12-HSL were observed (Fig. 2.2B, Fig. 2.3A, Table 2.3). The loss of *ssaR* (Fig. 2.2C) did not significantly alter the pattern of AHLs observed in the wt KLH11, consistent with the bioassays (Fig. 2.1A). In contrast, chemical analysis of the *ssbI⁻* mutant surprisingly revealed an overall increase in AHL levels (Fig. 2.1B, Fig. 2.2 D), but also a shift in the spectrum of AHLs produced, including the presence of C16 derivatives (3-oxo-C16-HSL or 3-OH-C16:1-HSL, low levels precluded their distinction) as major species, but also hydroxylated derivatives, 3-OH-C14-HSL, 3-OH-C14:1-HSL, and 3-OH-C12-HSL (Fig. 2.3A, Table 2.3). Paradoxically, this indicates that although SsbI is clearly capable of driving AHL synthesis when expressed in *E. coli*, its presence in *Ruegeria* sp. KLH11 significantly repressed overall AHL production, dictating the range of AHLs synthesized. The loss of SsbR resulted in increased abundance of the AHL signals (Table 2.3, Fig. 2.2E), similar to the *ssbI⁻* mutant, although this was not clear from the bioassays (Fig. 2.1B).

2.4.4 Ectopic expression of AHL synthases in a KLH11 QS mutant

A double mutant with in-frame deletions of both *ssaI* and *ssbI* was generated in KLH11. In contrast to the *ssaI⁻* mutant, this double mutant surprisingly retained low-level synthesis of several AHLs (Fig. 2.5C), suggesting the

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presence of an additional unidentified AHL synthase. Indeed, we now know that

a third LuxI- gene is still present in this strain and is likely to be responsible (See

Chapter 3). The introduction of P_{lac}-*ssaI* or P_{lac}-*ssbI* plasmids into the Δ*ssaI*

Δ*ssbI* mutant (Fig. 2.5D and 2.5E) was consistent with the *E. coli* experiments in

that both enzymes drove synthesis of several different long chain signals. SsaI

directed synthesis of C16 derivatives 3-oxo-C16-HSL (or possibly 3-OH-C16:1-

HSL, again too low level to distinguish) and SsbI directed synthesis of 3-OH-

C14:1-HSL and 3-OH-C14-HSL (Fig. 2.3C, Table 2.3). The mutant expressing

ssaI produced longer chain length AHLs with greater hydrophobicity than it did

with *ssbI*, but as in *E. coli* the P_{lac}-*ssbI* plasmid resulted in much greater overall

amounts of AHLs.

Alignment of SsaI and SsbI sequences with several other LuxI homologues

(Fig. 2.6) shows good conservation of their N-terminal halves (Watson *et al.*,

2002; Gould *et al.*, 2004). Previous studies have found that a threonine residue

at position 143 (LuxI numbering) correlates well with the production of 3-oxo-

HSLs (Watson *et al*. 2002). Interestingly, SsaI has a threonine at the equivalent

position (SsaI 145), whereas SsbI has a glycine (SsbI 136) at this position (Fig.

2.6), consistent with the observed AHL profiles (Table 2.3). The SsaI C-terminal

half is significantly longer than typical for LuxI-type proteins. Additionally, SsbI

and SsaI vary considerably in a conserved sequence block (126-157, LuxI

numbering), as well as more C-terminal to this block. This region of the enzyme

is important for acyl-chain recognition and both SsaI and SsbI clearly deviate

here from other better-studied AHL synthases (Watson *et al*., 2002; Gould *et al*.,

2004).
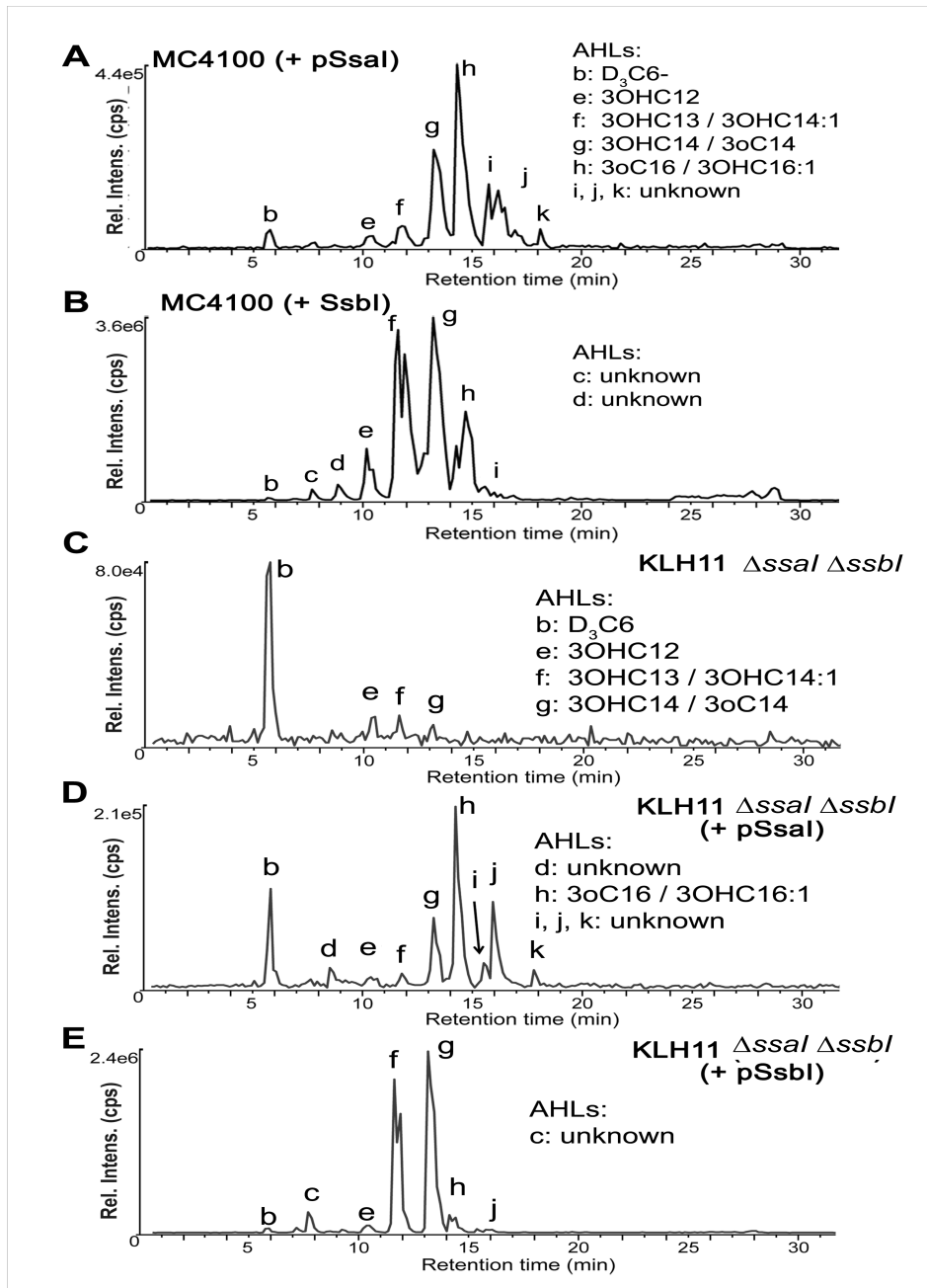
Figure 2.**5. Mass spectrometric analysis of plasmid-expressed SsaI and SsbI-directed AHLs from *E. coli* and KLH11.** Cultures of *E. coli* MC4100 and a KLH11 derivative with in-frame deletions of *ssaI* and *ssbI* expressing plasmid-borne *ssaI* or *ssbI* were extracted and subjected to reverse-phase chromatographic separation prior to tandem MS analysis using the precursor ion-

scanning mode (transitions were monitored for precursor [M + H]$^+$-> m/z 102.1) for (A) *E.coli* MC4100 + pSsaI (*P_{lac}-ssaI*). (B) *E.coli* MC4100 + pSsbI (*P_{lac}-ssbI*) (C) KLH11 Δ*ssaI* Δ*ssbI*, (D) KLH11 Δ*ssaI* Δ*ssbI* + pSsaI (*P_{lac}-ssaI*), (E) KLH11 Δ*ssaI* Δ*ssbI* + pSsbI (*P_{lac}-ssbI*). The peaks in the chromatograms are labeled with lowercase lettering and include species defined in Table 2.3 and the AHLs noted. Figures are provided by Mair Churchill.

```
        1
LuxI    MTIMIKKSDFLAIPSEEYKGILSLRYQVFKQRLEWDLVVENNLES---DEYDNSNAEYIYACDDTEN--VSGCWRLLPTTGDYMLKSVFPELLGQQSA--PKDPNIVELSRF
EsaI    MLELFDVSYEELQTTRSEELYKLRKKTFSDRLGWEVICSQGMES---DEFDGPGTRYILGICEGE---LVCSVRFTSLDRPNMITHTFQHCFSDV----TLPAYGTESSRF
LasI     MIVQIGRREEFDKKLLGEMHKLRAQVFKERKGWDVSVIDEMEI---DGYDALSPYYMLIQEDTPEAQVFGCWRILDTTGPYMLKNTFPELLHGKEA--PCSPHIWELSRF
RhlI    MIELLSESLEGLSAAMIAELGRYRHQVFIEKLGWDVVSTSRVRDQEFDQFDHPQTRYIVAMSRQG---ICGCARLLPTTDAYLLKDVFAYLCSETP---PSDPSVWELSRY
SsaI    MILVVDGLNRHLFTEVLDEMFELRARVFGGRLGWDVNIEDGKEI---DQFDHLDPAYVIGLDDEGN--VSAAVRALQTTGPHMLSDVFSDILCGEAP--MRSATMWESTRF
SsbI    MLRYLYADELHKFPVLAEGMFRDRADQFKTRLGWDVKVNEDGEER--DQYDDLNPLYVIWEEADGS--HGGSMRVLPTTGPVMVNDIFGHLTGGSP---ICSPRIWEVTRF


        106                                      ▼
LuxI    AVGKNSSKINNS---ASEITMKLFEAIYKHAVSQGITEYVTVTSTAIERFLKRIKVPCHRIGDKEIHVLGDTKSVVLSMPINEQFKKAVLN
EsaI    FVDKARARALLG--EHYPISQVLFLAMVNWAQNNAYGNIYTLVSRAMLKILTRSGWQIKVIK--EAFLTEKERIYLLTLPAGQDDKQQLGGDVVSRTGCPPVAVTTWPLTLPV
LasI    AINSGQKGSLG--FSDCTLE--AMRALARYSLQNDIQTLVTVTTVGVEKMMIRAGLDVSRFG--PHLKIGIERAVALRIELNAKTQIALYGGVLVEQRLAVS
RhlI    AASAADD-------PQLAMK-IFWSSLQCAWYLGASSVVAVTTTAMERYFVRNGVILQRLG--PPQKVKGETLVAISFPAYQERGLEMLLRYHPEWLQGVPLSMAV
SsaI    CVDTQRLTRGKEKNSVSYATCELMIGSLEYCRNAGIEDIITVIDPVMNRVLKRSNCAPYDYVGETVPMGKVPAMAALLDCSEERISGLREFAGIHHDVFVEEEQALEMFEAKKA...(284)
SsbI    CLSRTASAHT---------AGAIMLSGGEMMEGFGLTHIAGVFDARMIRIYRMIGSSPVVLGAEGTGRDRISVGLWPYSADDCNRVAARAGIPRELSRLWFNTAFGNGMRHRFALSA
```

Figure 2.6. Sequence alignment of AHL synthases. The grey shaded regions are the most conserved sequence blocks within the AHL synthase family. Residues are colored red to indicate acidic or hydrophilic, blue for basic, and orange for other. Shaded residues are absolutely conserved and the boxed residues are the most similar regions within the family. The black inverted triangle indicates the threonine at 143 of LuxI from *V. fischeri*. The numbers on top of the sequences refer to the numbering of LuxI and the number 284 indicates the length of SsaI. Figure is provided by Mair Churchill.

Table 2.3. AHLs: Retention times, identification and relative abundances based on tandem mass spectrometry.

| Peak Label | Retention time ± 0.6 (min) | [M+H] +m/z | [M+NH4] +m/z | AHL[a] | Ratio to internal standard (%) | | | | | | | | | |
| | | | | | KLH11 | | | | | E. coli | | KLH11 ΔssaI ΔssbI | | |
					WT	ssaI-	ssaR-	ssbI-	ssbR-	pSsaI	pSsbI	-	pSsaI	pSsbI
a	3.0			unk	+									
b	5.6	203	220	D3C6-HSL	‡	‡	‡	‡	‡	‡	‡	‡	‡	‡
c	7.6			unk							+			+
d	8.9			unk				+			+		+	
e	10.4	300	317	OH-C12-HSL*	<0.01	<0.01	<0.01	0.29	0.26	<0.01	25.39	<0.01	<0.01	2.6
f	11.8	314	331	OH-C13-HSL*			0.20			0.61	94.0	0.05	0.13	1.75
		326	343	OH-C14:1-HSL*	0.07		0.12	0.78	0.94	0.53	77.0			82.8
g	13.5	328	345	OH-C14-HSL*	0.10		0.15	0.37	0.28	1.94	147.6	0.07	1.13	148.0
		326	343	oxo-C14-HSL§						11.88			0.33	
h	14.5	354	371	oxo-C16-HSL§				1.4	0.80	1.87			2.60	
				OH-C16:1-HSL*							28.1			9.07†
i	15.8			unk						+			+	
j	16.4			unk						+			+	
k	18.3			unk						+			+	

[a] all confirmed AHLs modified at the 3 position, 3-oxo or 3-OH. Unk corresponds to an unknown component that generated m/z 102 upon collisional activation but could not be assigned a specific AHL structure.
+ detected, but could not be quantitated
* confirmed by silylation for *E. coli* samples
§ confirmed by methoxymation for *E. coli* samples
‡ internal standard
† oxo-C16 and OH-C16 could not be distinguished from KLH11, but the value is included in this row based on the trend for *E. coli* MC4100 in which they can be distinguished

2.4.5 Expression of *ssaI* is stimulated in response to KLH11 AHLs

LuxI-type genes often are regulated in response to their own cognate AHL(s) (Fuqua and Greenberg, 2002). Cultures of each of the KLH11 Campbell insertion mutations for *ssaI* and *ssbI* which created *lacZ* fusions to the disrupted genes were assayed for β-galactosidase activity grown in the presence and in the absence of exogenous KLH11 culture extracts. KLH11 has no significant endogenous β-galactosidase activity (Miller Unit <1). The *ssaI-lacZ* fusion exhibited close to 16-fold induction (Table 2.4) when cultures were incubated in the presence of KLH11 extracts (2.5% v/v). Given that the dominant SsaI-directed are C16-HSLs, we also tested *ssaI* expression of the mutant with synthetic 3-oxo-C16:1 Δ11-HSL. In the presence of the synthetic AHL at 2 μM, the *ssaI-lacZ* fusion was induced roughly 40 fold (P <0.001, Table 2.4). In contrast, the expression of the *ssbI-lacZ* fusion was low and was not increased by addition of culture extracts or by 20 μM 3-OH-C14-HSL (P>0.05), the dominant long chain hydroxylated AHL produced via SsbI. However, the *ssaI-lacZ* fusion was induced roughly 30% (P<0.01) by addition of 20 μM 3-OH-C14-HSL, suggesting limited cross-recognition of this AHL. The *ssbI-lacZ* fusion was not activated by addition of 2 μM 3-oxo-c16:1 Δ11-HSL.

We also created similar Campbell insertion mutants of the *ssaR* and *ssbR* genes. For each gene one derivative was created in which the plasmid was integrated to generate the *lacZ* fusion with the wild type coding sequence intact (JZ1 and JZ2), and a second derivative in which the gene was disrupted by

integration of the plasmid (EC4 and EC5). Although *ssaR* was expressed more strongly than *ssbR* (based on β-galactosidase activity), there was no effect of crude culture extracts or synthetic AHLs on the expression of these genes in the wild type or the null mutant background (Table 2.4). Disruption of the *ssaR* gene expression resulted in an approximately 4-fold decrease of *ssaR* expression, but this was independent of AHL.

Table 2.4. **QS regulator expression in KLH11 null mutants.**

Mutants	Genotype	No extract	2.5%	No AHL	+AHL[2]
			β -galactosidase activity[1]		
EC2	*ssaI-lacZ*, null	6 (1.7)	95 (10.6)	6.3 (0.7)	213.3 (9.2)
EC3	*ssbI-lacZ*, null	1 (<1)	<1 (0.1)	1.1 (0.3)	1.1 (<0.1)
JZ1	*ssaR-lacZ*, WT	107.1(0.8)	105.0(4.4)	93.7 (8.3)	99.3 (2.8)
EC4	*ssaR-lacZ*, null	23 (3)	19 (1.4)	26.7 1.3)	28.6 (2.4)
JZ2	*ssbR-lacZ*,WT	11.6 (2.8)	10.8 (3.1)	12.0 (.5)	12.6 (0.5)
EC5	*ssbR-lacZ*, null	15 (<1)	10 (4.6)	27.6 (4.5)	26.0 (4.2)

[1]Specific activity in Miller units, averages of assays in triplicate (standard deviation).

[2] 2 μM 3-oxo-c16:1 Δ11-HSL was added for *ssaRI* and 20 μM 3-OH-C14-HSL was added for *ssbRI*.

Elisha M. Cicirelli contributed the data for EC2, EC3, EC4 and EC5 with organic extract.

2.4.6 SsaR activates expression of its cognate AHL synthase gene *ssaI*

It was unclear whether AHL-activated *ssaI* expression also required the SsaR protein and whether the Ssa system might directly influence *ssbI* expression.

Plasmid-borne copies of each *luxR*-type protein paired with compatible plasmids carrying either a *P$_{ssaI}$-lacZ* fusion or a *P$_{ssbI}$-lacZ* fusion, were introduced into the AHL⁻, plasmidless derivative *A. tumefaciens* NTL4. Cultures of these *A. tumefaciens* derivatives were grown in the presence or absence of 2 μM 3-oxo-C16:1 Δ11-HSL. The presence of *ssaR* activates *ssaI* in the absence of exogenous AHLs (~7-fold induction; P<0.001) (Table 2.5). This SsaR-dependent activation was stimulated a total of 30-fold (P<0.001) by the addition of 2 μM 3-oxo-C16:1 Δ11-HSL (Table 2.5). Several C14-HSLs were also tested, but only weakly influenced *ssaI* expression (Fig. 2.7). Activation of the *ssaI-lacZ* fusion by SsaR exhibited a dose-dependent response to 3-oxo-C16:1 Δ11 -HSL that paralleled the response to crude culture extracts (Fig. 2.7). In contrast, the *P$_{ssbI}$-lacZ* (pEC121) was not activated by *P$_{lac}$-ssbR* (pEC123) irrespective of the presence of synthetic AHLs (<1 Miller Unit, P>0.05) or crude KLH11 extracts (Table 2.5, E. Cicirelli and C. Fuqua, unpublished.). Likewise, SsaR failed to activate *ssbI* and SsbR failed to activate *ssaI* in the presence of culture extracts and synthetic AHLs (Table 2.6).

Table 2.5. Expression of KLH11 P_{ssaI} and P_{ssbI} promoters in an AHL$^-$ host[1]

Expression plasmid	Fusion plasmid	β -galactosidase activity[2]	
		No AHL	+AHL[3]
Vector (pBBR1-MCS5)	ssaI-lacZ (pEC116)	51 (5)	52(5)
P_{lac}-ssaR (pEC112)	ssaI-lacZ (pEC116)	342(15)	1435(67)
Vector (pBBR1-MCS5)	ssbI-lacZ (pEC121)	<1 (<0.1)	<1 (<0.1)
P_{lac}-ssbR (pEC123)	ssbI-lacZ (pEC121)	<1 (<0.1)	<1 (<0.1)

[1]All strains derived from Ti-plasmidless *A. tumefaciens* NTL4.

[2]Specific activity in Miller Units, averages of assays in triplicate (standard deviation).

[3] 2 µM 3-oxo-C16:1 Δ11-HSL was added for *ssaI* and 20 µM 3-OH-C14-HSL was added for *ssbI.*

Table 2.6. Cross-regulation experiments for SsaR and SsbR in an AHL⁻ host[1].

Expression plasmid	Fusion plasmid	β -galactosidase activity[2]	
		No AHL	+AHL[3]
Vector (pBBR1-MCS5)	*ssbl-lacZ* (pEC121)	0.7 (0.1)	0.8 (0.1)
P_{lac}-ssaR (pEC112)	*ssbl-lacZ* (pEC121)	0.9 (0.1)	0.9 (0.2)
Vector (pBBR1-MCS5)	*ssal-lacZ* (pEC112)	57.9 (0.9)	58.5 (3.6)
P_{lac}-ssbR (pEC123)	*ssal-lacZ* (pEC112)	50.6 (3.1)	44.3 (2.6)

[1]All strains derived from Ti-plasmidless *A. tumefaciens* NTL4.

[2]Specific activities in Miller Units, averages of assays in triplicate (standard deviation).

[3] 2 µM 3-oxo-C16:1 Δ11-HSL was added for *P_{lac}-ssaR* and 20 µM 3-OH-C14-HSL was added for *ssbl.*
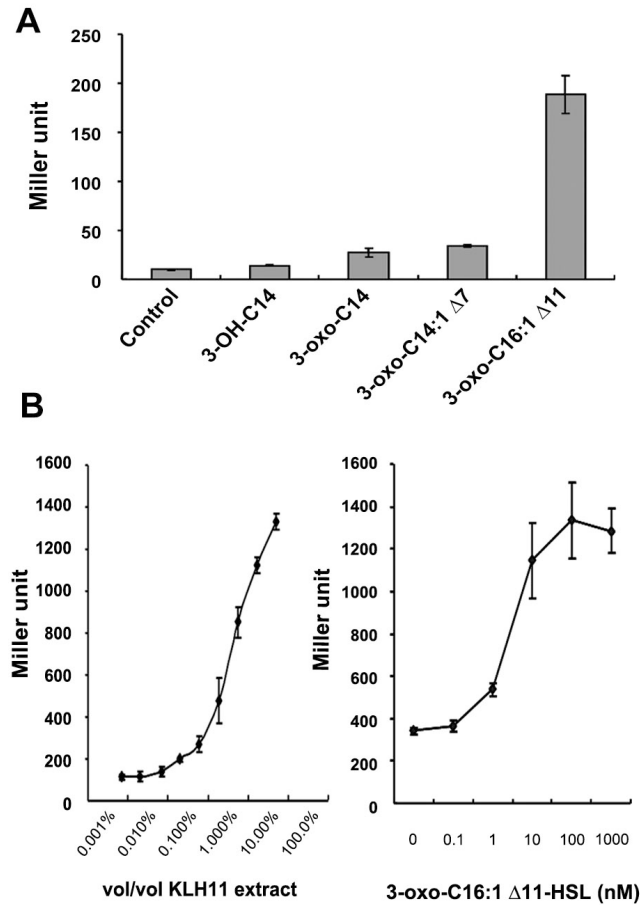
Figure 2.7. Activation of ssaI in response to synthetic AHLs. (A) Activation of *ssaI-lacZ* fusion with synthetic long chain AHLs. KLH11 *ssaI⁻* carrying the integrated *ssaI-lacZ* fusion. The final concentration of 3-OH-C14-HSL used was 20 μM and the other 3 AHLs were 2 μM. (B) Dose responsive activation of *ssaI-lacZ* by SsaR (*Plac-ssaR*) in A*. tumefaciens* NTL4 background. Different concentrations of the crude organic extract of KLH11 cultures (%, v/v) and 3-oxo-C16:1 Δ11-HSL (nM) were added at the time of culture inoculation. Values are averages of assays performed in triplicate and error bars are standard deviations. Elisha M. Cicirelli provided the dose response curve with extract.

2.4.7 Conserved sequences upstream of *ssaI* are required for activation by SsaR

LuxR homologs often recognize conserved sequence elements, called *lux*-type boxes located upstream of target promoters, including those of *luxI* homologues (Devine *et al*., 1989; Egland and Greenberg, 1999). The *ssaI* gene is downstream of the *ssaR* gene in a tandem arrangement, with an intergenic region of 118 bp but reverse transcription (RT)-PCR assays demonstrated that *ssaI* and *ssaR* are not in the same operon (Fig. 2.4A; similarly, *ssbI* and *ssbR* are also in separate operons, Fig. 2.4B). Inspection of the sequence upstream of *ssaI* revealed no inverted repeats and no motifs with primary sequence similarity to bona fide *lux*-type boxes. Comparison of this region between *ssaR* and *ssaI* and the homologous region between *silR1* and *silI1* from *R. pomeroyi* DSS-3 (Moran *et al*., 2004) revealed only 60% identity, except for a 19 bp fully conserved segment (TACGGGAAACCCCCAATAG), located 60 bp upstream of the *ssaI* start codon (Fig. 2.8A). Although it is not an inverted repeat and shares limited primary sequence similarity with known *lux*-type boxes, we reasoned the sequence might be a regulatory element given its appropriate size and location, and tentatively designated this a *ssa* box. Deletions were generated in the AHL and SsaR-responsive P_{ssaI}-*lacZ* plasmid (pEC116), one to just upstream of the *ssa* box (pEC124) and a larger deletion (pEC127) that almost completely removes the element (Fig. 2.8B). In *A. tumefaciens* NTL4 the deletion construct retaining the *ssa* box (pEC124) was inducible by *ssaR* and 3-oxo-C16:1 Δ11-HSL to the same extent as the plasmid with the complete intergenic region. Deletion

of the *ssa* box abolished this induction, although it increased basal expression

levels (Fig. 2.8C). These results reveal a role for the presumptive *ssa* box in

AHL-dependent activation of the *ssaI* gene.
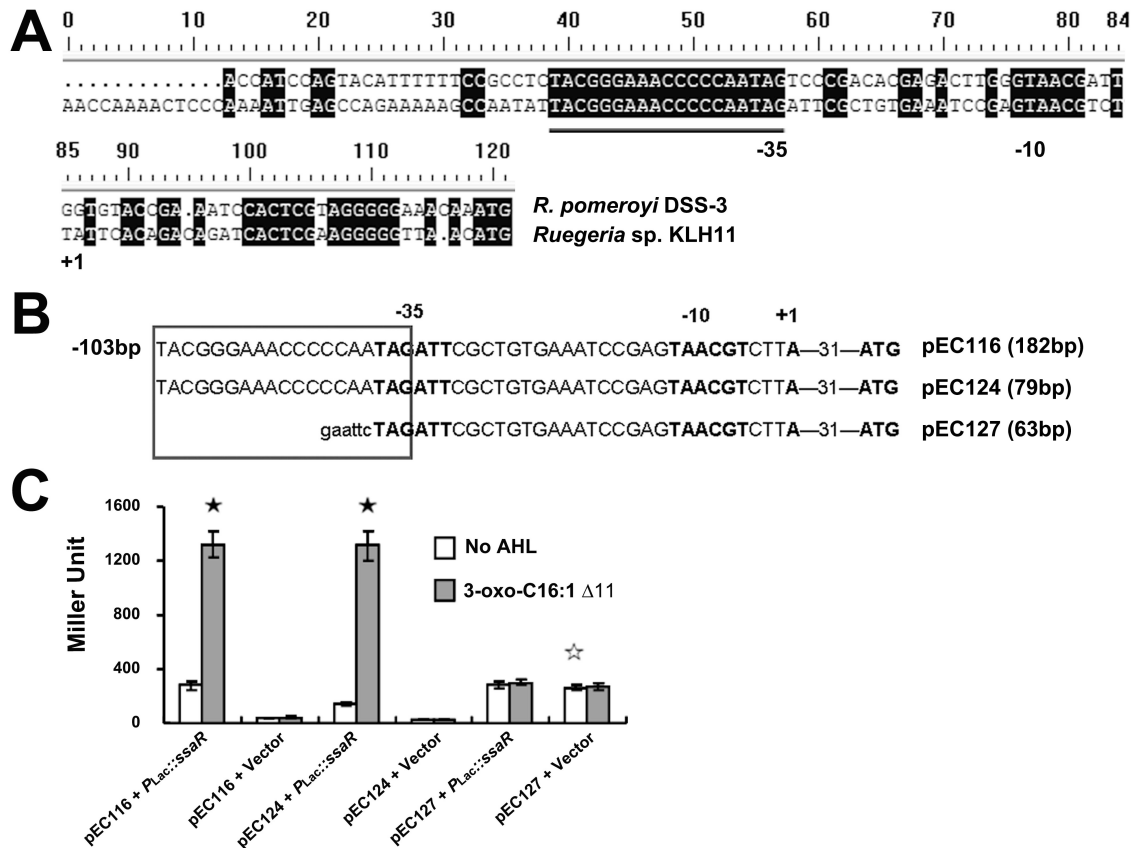
Figure 2.8. Deletion analysis of the *ssaI* promoter. (A) Putative promoter elements are indicated in boldface and putative -35 and -10 regions are indicated below the sequences; +1 indicates the predicted transcription start site. Presumptive Ssa box is underlined. (B) The presumptive *ssa* box is indicated within the rectangle. Plasmid name and size of insert are indicated adjacent to the translational start site. For the plasmid pEC116, it also included 103 bp upstream of the Ssa box. Lower case sequence indicates an *Eco*RI restriction site. (C) β-galactosidase activity of the three deletion constructs fused with the *lacZ* reporter. All strains derived from Ti-plasmidless *A. tumefaciens* NTL4. Bars represent the average of three biological replicates and the error bars are

standard deviation. The filled asterisks show the statistical significance between

samples, with and without 3-oxo-C16:1 Δ11-HSL, in the strains that have SsaR

(P<0.001). The unfilled asterisk shows the statistical significance of the basal

expression levels between pEC127 and each of pEC116 and pEC124 (P<0.001).

The result presented is a representative of several independent experiments

each with three biological replicate. Elisha M. Cicirelli provided A and B.

2.4.8 SsaRI controls swimming motility and flagellar

biosynthesis

For several different bacteria, QS regulates bacterial motility and flagellar

synthesis (Kim *et al*., 2007; Ng and Bassler, 2009). KLH11 swims under

laboratory conditions but does not exhibit swarming (E. Cicirelli and C. Fuqua,

unpublished). All four KLH11 mutants (Δ*ssaI*, *ssbI*, *ssaR⁻*, and *ssbR⁻*) were

tested for their motility on MB 2216 swim agar plates (Fig. 2.9A). The Δ*ssaI* and

ssaR⁻ mutants did not migrate from the site of inoculation, whereas the *ssbI-* and

ssbR⁻ mutants migrated through the motility agar similar to wild type. The

swimming deficiency of the Δ*ssaI* mutant was fully complemented with a plasmid-

borne copy of the gene, and the *ssaR⁻*, mutant was partially complemented by

similar provision of *ssaR*. Addition of 2 μM 3-oxo-c16:1 Δ11-HSL into swim agar

can partially restore motility in the Δ*ssaI* strain, albeit less efficiently than through

complementation (Fig. 2.9 A). As expected, motility was not restored in the

ssaR⁻ mutant in the media with 2 μM 3-oxo-C16:1 Δ11-HSL (E. Cicirelli and C.

Fuqua, unpublished). The observed differences in migration through swim agar

were not likely due to growth effects as the *ssaI*⁻ and *ssaR*⁻ mutants grow at the same rate as wild type (Fig. 2.10).

In order to visualize the presence of flagella, the wild type and mutant strains were observed by phase contrast microscopy using both wet mounts and flagellar stains. Interestingly, early stage wild type cultures did not have flagella, and no swimming cells were observed, whereas in late stage cultures flagella were clearly assembled (Fig. 2.11) and cells were visibly motile. Both the *ssaI*⁻ and the *ssaR*⁻ mutants lacked flagella and were never observed to swim, irrespective of culture stage. The cells also appeared to clump more readily. The *ssbI*⁻ and *ssbR*⁻ mutants were as motile as wild type and had abundant flagella in late stage cultures (Fig. 2.11).

The presence of flagellar proteins in KLH11 cultures was examined using immunoblotting with antisera raised against whole flagella from *Caulobacter crescentus*, a related alpha-proteobacterium. In western blots from KLH11 late stage cultures, supernatants contained a protein of approximately 43 kDa (Fig. 2.9B) that was also present from pelleted cells. This protein matches the predicted 41.5 kDa size of the only flagellin homologue in KLH11, *fliC* gene product (Zan *et al*., 2011a)(See Appendix 1). A site-specific disruption of the KLH11 *fliC* homologue abolished swimming motility (unpublished results) and caused loss of the 43 kDa protein, the same presumptive flagellin protein was also absent from the Δ*ssaI* mutant (Fig. 2.9).

To determine when flagellar biosynthesis occurs during culture growth, samples were harvested along the growth curve at four different time points; mid-

exponential, early stationary, mid-stationary, and late stationary phase from the parent strain and the *ssaI-* mutant. At an OD_{600} of 0.5, the parent strain had no detectable flagellin in either the whole cell or supernatant fraction, but as the culture density increased (OD_{600} >1.3), flagellin was detected in the whole culture fractions and weakly in the culture supernatant and continued to increase as the culture grew (Fig. 2.9C). Flagellin was never detected in samples of the *ssaI⁻*, mutant, irrespective of culture density (E. Cicirelli and C. Fuqua, unpublished).

To determine if the SsaRI system regulates the transcription of the flagellin gene (*fliC*), quantitative RT- PCR (qRT-PCR) was used to measure *fliC* expression. Late stage cultures grown to an OD_{600} at which KLH11 produces visible flagella have approximately 2 orders of magnitude higher *fliC* expression in wt KLH11 compared to the Δ*ssaI* mutant (Fig. 6D, P<0.001). The P_{lac}-*ssaI* plasmid (pEC108) complemented *fliC* expression compared to the Δ*ssaI* mutant (P<0.05), although not to full wild type levels (Fig. 2.9D). *Silicibacter* sp. TM1040 is well studied for motility (Belas *et al.*, 2009), and *flaA* is a required motor-associated protein in a putative Class II flagellar operon. Examination of the KLH11 *flaA* homologue by qRT-PCR revealed a 10-fold decrease of *flaA* expression in the *ssaI* deletion mutant (P<0.001). A plasmid-borne *ssaI* copy was able to partially restore *flaA* expression (Fig. 2.9D, P<0.05). These results suggest that an intact SsaRI system is required for swimming through regulation of expression of flagellar genes.
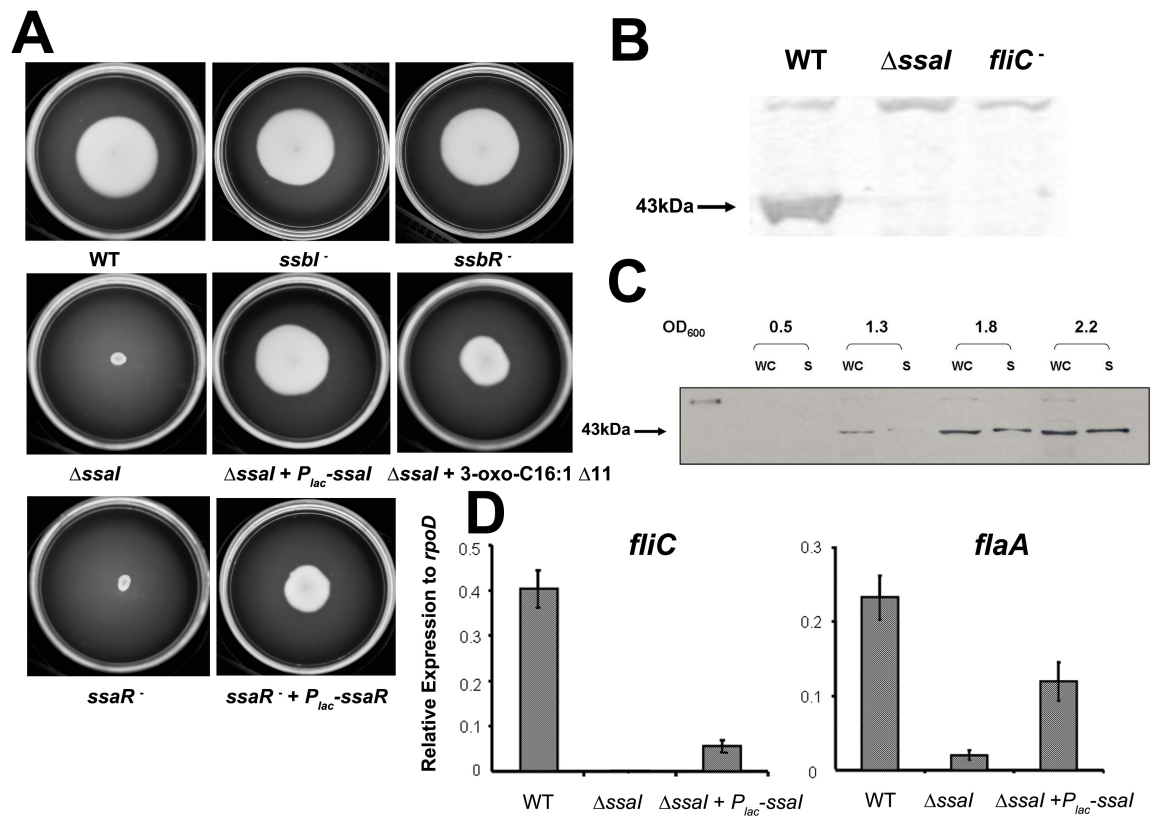
Figure 2.9. Regulation of swimming motility and flagellar biosynthesis by the SsaRI QS system. (A) Swimming motility assays for QS mutants on 0.25% Marine Agar 2216 after 8 days at 28°C. 2 µM 3-oxo-C16:1 Δ11–HSL was added for the Δ*ssaI* mutant and pEC108 (*P*$_{lac}$-*ssaI*) and pEC112 (*P*$_{lac}$-*ssaR*) were used to complement Δ*ssaI* and *ssaR⁻*, mutants, respectively. (B) Antiserum raised against whole flagella from *C. crescentus* also recognizes KLH11 flagella. Estimated size of KLH11 flagellin is 43 kDa. (C) Flagellin synthesis during late culture stages requires *ssaI*. Wild type cultures were harvested at various time points along the growth curve. WC, whole culture; S, supernatant. (D) qRT-PCR results of genes *fliC* and *flaA* in wt, the Δ*ssaI* strain and complementation strains. Error bars are the standard deviations and results are representative of

two independent experiments with triplicates. Okhee Choi provided Fig. 2.9 B

and Elisha M. Cicirelli provided Fig. 2.9C
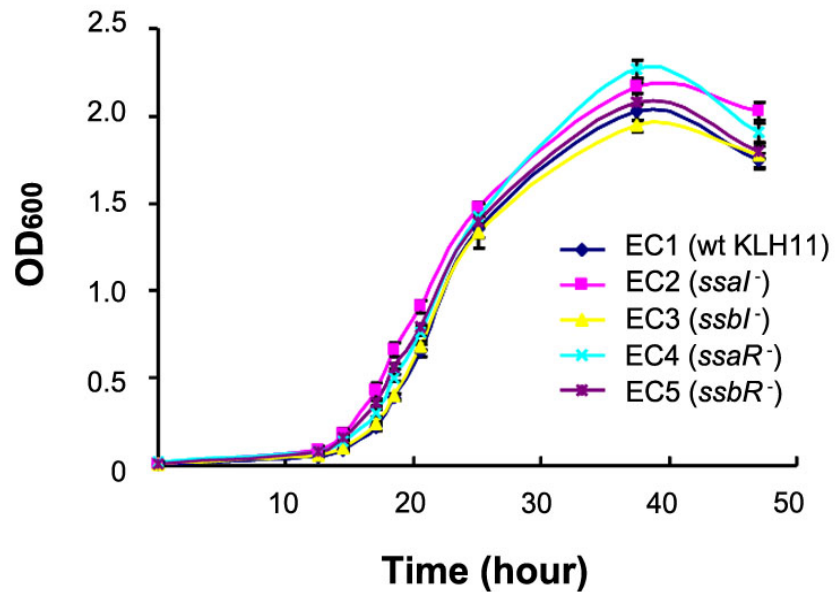
Figure 2.10. Growth curves of KLH11 and different QS mutants.   Average

OD_{600} of 5 ml cultures in MB2216. Cultures were grown in triplicate. Figure was

maded based on the data provided by Elisha M. Cicirelli.
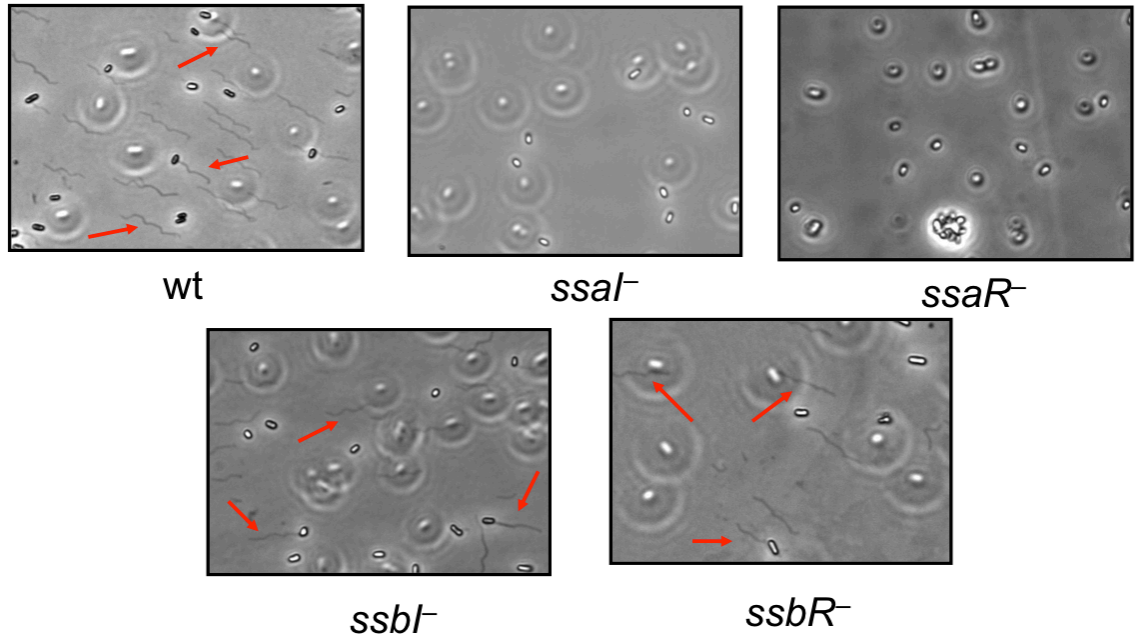
Figure 2.11. Flagellar staining of KLH11 quorum sensing mutants. Stained cells from late stage cultures were viewed under phase contrast microscopy with 100X lens. Wild type (EC1), *ssaI⁻* (EC2), *ssbi⁻* (EC3), *ssaR⁻* (EC4) and *ssbR⁻* (EC5). Red arrows indicate stained flagella. Figure is provided by Elisha M. Cicirelli and Clay Fuqua.

2.4.9 SsaRI mutants exhibit increased biofilm formation

QS can also regulate bacterial biofilm formation (Hammer and Bassler, 2003; Shrout *et al.*, 2006). Given the importance of the SsaRI system to swimming motility, we questioned if QS might influence KLH11 biofilm formation. A static coverslip biofilm assay was performed for the *ssa* and *ssb* mutants compared to the wild type. Crystal violet stained biofilms on PVC coverslips were solubilized in 33% acetic acid, and the absorbance at 600 nm (A_{600}) was measured. Relative to wild type, the Δ*ssaI* and *ssaR⁻* mutants clearly have increased biofilm

formation, most pronounced by 48 h post-inoculation (Fig. 2.12A, P<0.01). It was

plausible that increased biofilm formation was due to the loss of motility in these

mutants, limiting emigration of bacteria from biofilms. Indeed, biofilm formation in

the non-motile *fliC⁻*, mutant was also modestly increased compared to wild type

(Fig. 2.12A, P<0.01). However, biofilm formation in the Δ*ssaI fliC⁻*, mutant was

even stronger than the non-motile *fliC* mutant (P<0.05). This suggests that the

increased biofilm formation in the Δ*ssaI* mutant is not entirely due to the lack of

motility. The *ssbI* and *ssbR* mutants formed biofilms that were indistinguishable

from wild type KLH11 (Fig. 2.12A, P>0.05).

Addition of KLH11 late stage culture fluids (40% v/v) into biofilm assays with

the Δ*ssaI* mutant reduced biofilm formation to 60% the level of untreated cultures

(P<0.05) whereas planktonic growth is unaffected (Fig. 2.12B, P>0.05). This

inhibition effect is not due to nutrient depletion because 1X MB2216 was

provided in addition to the nutrients remaining in the supernatant. The inhibition

is also not due to pH changes as there was <0.1 pH unit difference between

normal MB2216 and MB2216 conditioned with wt KLH11 culture fluid**.**
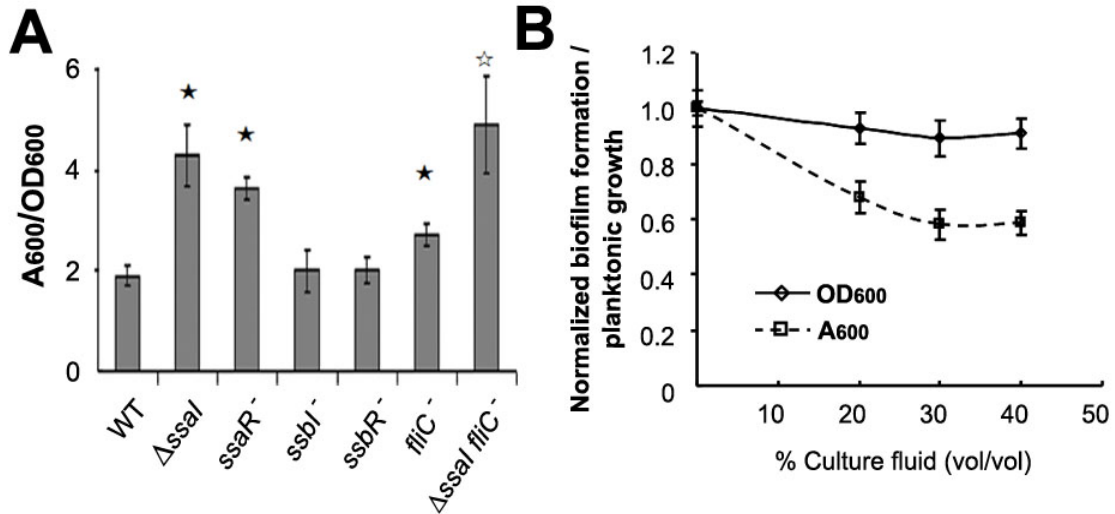
Figure 2.12. Increased biofilm formation in *ssaI* and *ssaR* mutants. (A)

Standard 48 hour coverslip biofilm assays measuring A_{600} of solubilized crystal

violet normalized to the culture density (OD_{600}) for the different KLH11 strains

were performed. Filled asterisks indicate statistical significance between wt

KLH11 and indicated strains (P<0.01). Open asterisk indicates statistical

significance between *fliC*⁻ and Δ*ssaI fliC*⁻ mutants (P<0.05). Error bars are

standard deviation of three biological replicates. (B) Inhibition of biofilm

formation for D*ssaI* mutant by wt KLH11 culture fluids. Different % (v/v) wt

KLH11 culture fluids were added into Δ*ssaI* culture at the time of inoculation.

Solubilized crystal violet stain of adherent biomass (A_{600}) and the optical density

of the cultures (OD_{600}) were measured as a function of percent culture fluid

addition. Data were normalized to the cultures with no added wt KLH11 culture

fluids.

2.5. Discussion

There is limited understanding of QS mechanisms in the diverse and
abundant bacteria living in the marine environment (Cicirelli *et al*., 2008). Our
studies on sponge symbionts initiated with the use of AHL responsive bioassays
(Mohamed *et al.,* 2008c). We have developed *Ruegeria* sp. KLH11, a member of
the *Silicibacter–Ruegeria* group of the Roseobacter clade, as a quorum sensing
model. KLH11 AHLs are readily detectable using the *A. tumefaciens* bioreporter,
ranging from non-polar, long chain AHLs to more polar, short chain AHLs (Fig.
2.1; Mohamed *et al.,* 2008c). However, even the broadly responsive *A.
tumefaciens* system is biased towards AHLs structurally similar to its cognate 3-
oxo-C8-HSL, and often cannot distinguish between related AHLs. We therefore
employed mass spectrometric analysis to provide unbiased AHL identification
that also provides information on relative abundance for at least a subset of
AHLs. This approach detected AHLs with acyl chains greater than C12 from
KLH11, but AHLs with shorter acyl chains revealed in bioassays were below the
effective detection threshold for our technique. This finding highlights the
strengths and weakness of each detection system; the AHL-responsive
bioassays are extremely sensitive, but difficult to quantitate, whereas direct
chemical detection can identify and quantitate AHLs, but at lower sensitivity. In
the end, the combined approach provided the best insights into this complex
system.

KLH11 AHLs were predominantly 12-16 carbons, some with unsaturated
bonds, with either 3-OH or 3-oxo substituents. Although SsaI-directed oxo-AHLs

were not detectable in wild type KLH11 extracts by the mass spectrometric approach (Fig. 2.2; Table 3.1), they are clearly present in the TLC assays (Fig. 2.1). The relatively abundant 3-OH-C14-HSL and 3-OH-C14:1-HSL likely originate from SsbI or the closely related SscI. The hydroxy-, oxo- and double bonded character of the long chain AHLs would be beneficial for maintaining their solubility, and longer acyl chains are more stable in moderately alkaline marine environments (Riebesell *et al.*, 2000; Yates *et al.*, 2002), consistent with other studies from marine systems (Wagner-Döbler *et al*., 2005).

The pattern of AHLs specified by SsaI and SsbI, 3-oxo-HSLs and 3-OH-HSLs, respectively, vary in their chain length depending on the bacterial species in which they are expressed. For example, in *E. coli* harboring the P_{lac}-*ssaI* plasmid, 3-oxo-C14-HSL was the most abundant AHL, and 3-oxo-C16 –HSL was present at 15% (Table 2.3). This relationship reversed when the same plasmid was expressed in the KLH11Δ*ssaI*Δ*ssbI* mutant, resulting in high relative levels of 3-oxo-C16-HSL, with 3-oxo-C14 at less than 4% of its level in *E. coli*. Likewise, *E. coli* harboring the P_{lac}-*ssbI* plasmid resulted in high levels of 3-OH-C12-HSL and detectable 3-OH-C13-HSL among the other OH-AHLs, whereas these AHLs were much less abundant when the same plasmid was expressed in the KLH11Δ*ssaI* Δ*ssbI* mutant (Table 2.3). This shift toward increased acyl-chain lengths in KLH11 relative to those in *E. coli* may be explained by the differences in acyl-ACP substrate pools available in the different strains. Indeed, this has been observed in cases of AHL synthase over-expression in *E. coli*, where unusual AHLs including those with odd-chain lengths have been observed

(Gould *et al.*, 2006). Differences in growth temperature may also influence the levels and distribution of AHLs (Yates *et al.*, 2002). However, expression in *E. coli* enables analysis of the intrinsic specificity of each AHL synthase and was important in some cases for AHL identification.

Bacteria that have multiple AHL-based QS systems can organize these systems in interconnected regulatory networks (Atkinson *et al.*, 2008). The results of our AHL chemistry, genetic analysis, and gene expression experiments reveal a complex network of signal production and regulation in KLH11 (Fig. 2.13). The *ssaI* null mutant loses the majority of detectable AHLs, as evaluated by bioassays and mass spectrometry (Figs 2.1and 2. 2, Table 2.3). The AHLs produced by wild type KLH11 are dominated by OH-AHLs, those synthesized by SsbI (and perhaps SscI), but the *ssaI* mutant phenotype suggests that it influences production of these AHLs. Surprisingly, and in contrast to the *ssaI* null phenotype, mutation of *ssbI* increases the overall AHL activity. Mass spectrometry reveals a large increase in a 3-oxo-C16-HSL, correlated with SsaI activity, which is lost in the Δ*ssaI* Δ*ssbI* mutant (Table 2.3). These findings suggest that SsbI exerts a suppressive effect on the Ssa system, and this is relieved by mutation of *ssbI* (and perhaps also *ssbR*).

Several lines of evidence suggest that the connections between the Ssa and Ssb pathways are not directly through transcriptional control. Neither system appears to directly influence expression of the other AHL synthase genes. In fact, although SsbI-directed AHLs are lost in the *ssaI⁻* mutant, the *ssaR⁻* mutant does not affect their level, indicating that SsbI control is independent of SsaR. In

contrast, both SsaR and the SsaI-specified AHLs are required to autoregulate *ssaI* expression, activate motility and inhibit biofilm formation. It is plausible that an unidentified LuxR-type protein regulates the Ssb system in response to SsaI-produced AHLs, similar to the *P. aeruginosa* QscR protein, a LuxR-type transcription factor that responds to AHLs produced via the LasI-LasR system to control its target genes (Fuqua, 2006; Lequette *et al.*, 2006). The genome sequences of KLH11 and *R. pomeroyi* DSS-3 reveal several additional solo-type LuxR-type proteins that might be functioning in response to SsaI-directed AHLs to influence the Ssb pathway.

Inhibition of Ssa activity by the Ssb system is potentially due to competition for common substrates. Our findings demonstrate that both AHL synthases catalyze production of long chain AHLs and may be in competition for long chain acyl-acyl carrier protein conjugates (Churchill and Chen, 2011). In *P. aeruginosa*, AHLs derived from LasI can directly block the activation of another LuxR homologue RhlR by its own cognate AHL (Pesci *et al.*, 1997). It was plausible that Ssb-derived AHLs may have had an inhibitory effect on SsaI activity, however addition of 3-OH-C14-HSL to KLH11 resulted in a modest activation of *ssaI* expression, and thus this is not likely.

Our experiments implicate a non-symmetrical sequence element immediately upstream of *ssaI* that is required for SsaR activation (Fig. 2.8C). LuxR homologs have been shown to bind to symmetric (Zhang *et al.*, 2002) as well as asymmetric R-boxes (Schuster *et al.*, 2004). In contrast, there is no indication of AHL-responsive autoregulation for *ssbI* (Table 2.4) nor when SsbR and the *ssbI-*

lacZ fusion are tested in *A. tumefaciens* (Table 2.6). The *ssbI* gene joins a small list of LuxI homologues that are not positively autoregulated (von Bodman and Farrand, 1995).

A growing number of bacteria including *Rhizobium etli*, *Serratia liquefaciens* and *P. aeruginosa* are recognized to control motility via quorum sensing, including swarming, twitching and swimming (Eberl *et al.*, 1996; Daniels *et al.*, 2004; Shrout *et al.*, 2006). Many of these systems are inhibited by AHLs, whereas a smaller number are activated. In *S. meliloti* QS inhibits swimming motility, whereas for the β-proteobacteria *Burkholderia glumae* QS activates flagellar biosynthesis and thereby swimming and swarming motility (Kim *et al.*, 2007). In KLH11 flagellar biosynthesis is under tight, positive SsaRI control (Fig. 2.9). Despite their low abundance, the SsaI-derived oxo-AHLs are clearly important to activate swimming in late stage cultures, mutation of *ssaI* leads to loss of swimming motility, and provision of AHLs alone can rescue the mutant's swimming deficiency. Clearly, the SsaI-directed AHLs function below our mass spectrometry detection threshold. Some AHL QS systems are tuned to exceptionally low AHL levels, such as the ExpR system from *S. meliloti* (Pellock *et al.*, 2002). Reconstructing SsaR-dependent gene regulation in *A. tumefaciens* suggests that as little as 10 nM 3-oxo-C16:1 Δ11-HSL is saturating (Fig.2.8).

Our observations suggest that the influence of the SsaRI system on motility may be needed to limit aggregation. Interestingly, although the KLH11 and *R. pomeroyi* DSS-3 genomes encode flagellar motility functions, neither have genes for chemotaxis, including Che regulatory proteins and methyl-dependent

chemotaxis proteins (Moran *et al*., 2004; Zan *et al*., 2011a)(See Appendix 1).

For KLH11, and perhaps *R. pomeroyi* DSS-3, motility may function to promote

dispersal from aggregates instead of chemotaxis, employing QS control to

provide a population density response. A similar function was proposed for QS

in the photosynthetic microbe *Rhodobacter sphaeroides* (Puskas *et al*., 1997).

How does SsaR control flagellar assembly and function in KLH11? Both *flaA,*

and *fliC*, class II and class III flagellar genes, respectively, require SsaI for

significant expression (Fig. 2.9D), which suggests that in KLH11 QS controls

motility at an early step in flagellar gene expression. In most flagellated bacteria

there is a primary regulator that initiates expression of the flagellar gene cascade

(Macnab, 1996). Although it is conceivable that SsaR could be this master

regulator in KLH11, it is more likely that it controls expression of another

regulator. In *E. coli* and several other bacteria FlhDC proteins serve as the

primary regulators of flagellar assembly (Soutourina and Bertin, 2003), but there

are no FlhDC homologues in the KLH11 or *R. pomeroyi* DSS-3 genomes (Moran

et al., 2004; Zan *et al*., 2011a)(See Appendix 1).. For *C. crescentus*, the

essential cell cycle master regulator CtrA initiates flagellar assembly (Muir and

Gober, 2004). KLH11 encodes a *ctrA* homologue, and in its relative *Silicibacter*

sp. TM1040, this gene is also required for flagellar activity (Belas *et al*., 2009).

It is well established that bacterial motility can have a profound impact on

surface adherent biofilm formation (O'Toole and Kolter, 1998; Merritt *et al.*,

2007). In *P. aeruginosa*, QS promotes biofilm maturation and QS mutants can

attach but do not differentiate into mature biofilm structures (Davies *et al.*, 1998).

In contrast, in *V. cholerae* QS inhibits biofilm formation by decreasing the expression of exopolysaccharide (EPS) genes, and may also promote dispersal from biofilms and in late infection stages (Hammer and Bassler, 2003; Waters *et al.*, 2008; Krasteva *et al.*, 2010).

Accumulation of bacteria on a surface is the net sum of attachment, growth, and emigration. Decreased motility may reduce biofilm formation by limiting initial surface contact, but it also reduces migration from biofilms, thereby increasing biofilm formation. For KLH11, the *ssaI* and *ssaR* mutants are non-motile, and have increased biofilm formation (Fig. 2.12A), which is clearly due to QS as KLH11 supernatants antagonize biofilm formation in a dose-dependent manner (Fig.2.12B). The Δ*ssaI* mutant has a much more pronounced deficiency than the equally non-motile *fliC* (flagellin) mutant and the Δ*ssaI fliC⁻* double mutant has a biofilm phenotype indistinguishable from Δ*ssaI* itself. These findings suggest that the increased biofilm formation in *ssaI* and *ssaR* mutants is not only due to a loss of motility. Any additional relevant SsaRI target(s) remains to be determined but might include genes involved in surfactant synthesis or modulation of internal signaling molecules such as cyclic di-guanosine monophosphate (c-di-GMP) (Davey *et al.*, 2003; Hengge, 2009). Searches of the KLH11 genome for matches to the *ssa* box identified here have failed to yield potential targets.

Our study has revealed the coordinated regulation of motility and biofilm formation by QS in a roseobacterial sponge symbiont. From the larger ecological perspective, the regulatory pattern we have observed and the dispersal model

we have proposed makes sense. In nature, sponges actively pump large

volumes of the surrounding seawater. Microbial symbionts obtained from

seawater at this stage likely do not require flagella to be introduced into the

sponge host. Once KLH11-type bacteria colonize the sponge and are provided a

nutrient rich environment, they begin to grow to high density, perhaps even

beginning to aggregate. QS activation of motility and adherence inhibition may

facilitate dispersal from these crowded and potentially limiting

microenvironments, and to promote more uniform colonization of the host tissue

or even stimulate release back into the water column. By coordinating motility

and biofilm formation, motile KLH11 cells can readily escape from their own

aggregates. Experiments examining the colonization and distribution of KLH11

in live sponges, and tracking marked KLH11 QS mutants, may be the most direct

approach to test these ecological hypotheses, and provide further insights into to

SsaRI and SsbRI systems.
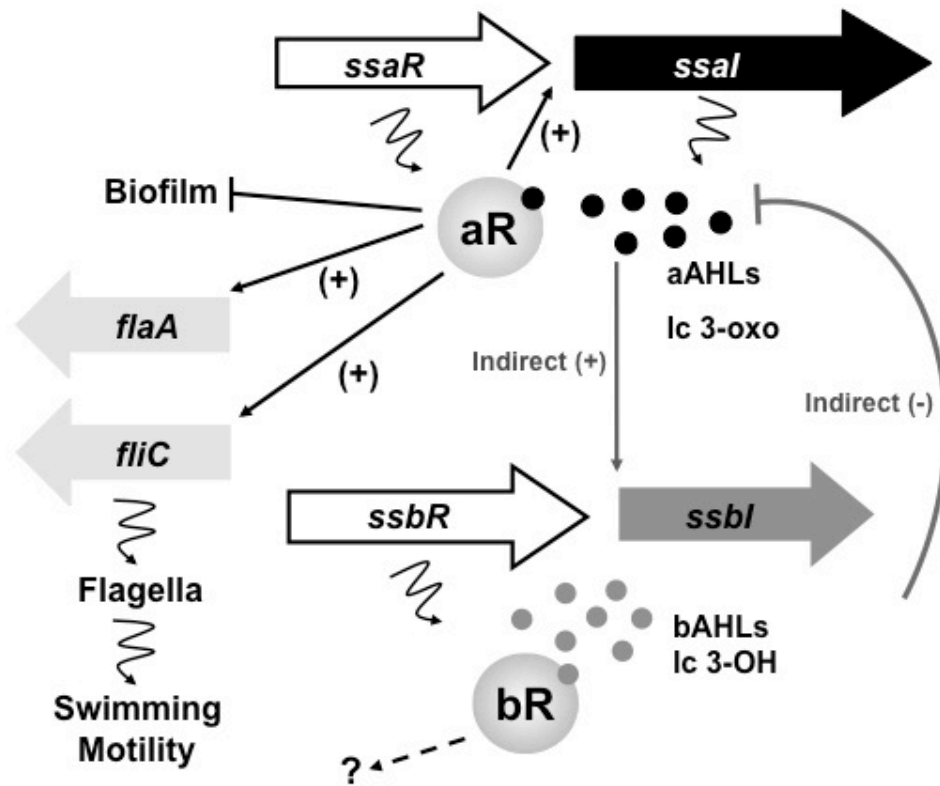
Figure 2.13. A model for the complex regulatory control of QS circuits in KLH11. The *ssaRI*, *ssbR*, *and sscI* genes are drawn according to scale while *fliC* and *flaA* are not. The black dots represent the AHLs synthesized by SsaI, mainly long chain (lc) 3-oxo-HSL. The grey dots represent the AHLs synthesized by SsbI, mainly long chain (lc) 3-OH-HSL. The lines with bars indicate inhibition while the arrows indicate activation. Squiggly lines indicate translation of genes or products of enzyme action. Dr. Clay Fuqua and I together made this figure.

Chapter 3. A LuxI "solo" synthesizes long chain acyl homoserine lactones and is involved in a reciprocal regulatory quorum sensing system in marine sponge symbiont *Ruegeria* sp. KLH11

3.1. Abstract

Marine sponges harbor abundant and diverse bacterial communities, providing an ideal environment for bacterial cell-density dependent cell-cell signaling, termed quorum sensing. *Ruegeria* sp. KLH11, isolated from the marine sponge *Mycale laxissima*, produces mainly long chain acyl-homoserine lactones (AHLs) and has been developed as a quorum-sensing model for roseobacterial sponge symbionts. Two pairs of *luxR/I* homologues were identified by genetic screening and were designated *ssaRI* and *ssbRI* (sponge-associated symbiont locus A or B, *luxRI* homologue) (Chapter 2). However, a double deletion mutant of the AHL synthase genes *ssaI ssbI* produced lower amounts of AHLs, suggesting that a third (or more) AHL synthase gene must be present. In this study, annotation of the KLH11 genome identified a third AHL synthase gene, named *sscI*. A triple mutant of the three AHL synthase genes showed no AHL production detectable by mass spectrometry, indicating that there are no additional AHL synthases in KLH11. Interestingly, *sscI* does not have a cognate LuxR homologue present in an adjacent locus and thus *sscI* is an AHL synthase solo. Expression of *sscI* in *E. coli* MC4100 shows that it produces mainly long chain 3-OH-AHLs, dominated by 3-OH-C14-HSL and 3-OH-C14:1-HSL. A genetic study showed that both *ssaI* and *ssbI* positively regulate *sscI* and that this regulation is indirect. SscI-specified AHL stimulated *ssaI* expression and has a moderate effect on the KLH11 swimming motility phenotype. Furthermore, KLH11 likely encodes a novel enzyme responsible for the synthesis of an aryl-HSL, *p*-coumaroyl-HSL (*p*C-HSL).

3.2. Introduction

In many bacteria, a *luxR*-type gene without a cognate *luxI*-type gene has been discovered and these *luxR*-type genes are termed *luxR* solos. These solos occur both in bacteria that have a complete QS system and bacteria that do not (Subramoni and Venturi, 2009). LuxR solos can regulate expression of a set of genes by binding to AHL produced by other *luxI* homologues in the bacterium such as in the case of QscR in *Pseudomonas aeruginosa* or by binding to AHLs produced by other bacteria (Lequette *et al.*, 2006). Furthermore, LuxR-type proteins can regulate gene expression independent of ligand binding (Subramoni and Venturi, 2009).

Although progress has been made in understanding LuxR solos, there is little information about LuxI solos. These are proteins encoded by *luxI* homologues without cognate *luxR* homologoues. Our previous studies have genetically identified two pairs of genes encoding LuxI-LuxR QS systems, SsaRI and SsbRI, in the marine sponge symbiont *Ruegeria* sp. KLH11 (Chapter 2). However, the double deletion mutant of both AHL synthase genes is still able to produce some AHLs, such as 3-OH-C14-HSL, indicating that a third or more AHL synthases must be present. By mining the genome sequence of KLH11, we were able to identify a third *luxI*-type gene, *sscI* (sponge-associated symbiont locus C, *luxI* homologue), which does not have a cognate *luxR* homologue. Here, we report the chemical profile of AHLs synthesized by SscI and the involvement of *sscI* in the complex regulatory QS system in KLH11. Furthermore, we also try to test whether KLH11 can produce the novel *p*-coumaroyl-HSL (*p*C-HSL) molecule that

was originally discovered in *R. palustris,* which has a ring structure in the fatty acid chain and requires the substrate *p*-courmarate produced by plants for the synthesis by the LuxI homologue RpaI (Schaefer *et al*., 2008).

3.3. Experimental procedures

3.3.1 Bacterial strains, oligonucleotides and growth conditions

Bacterial strains and plasmids used in this study are listed in Table 3.1 and oligonucleotides were obtained from Integrated DNA Technologies (Coralville, IA). Unless stated otherwise *E. coli* strains were grown in Luria-Bertani (LB) broth at 37°C with aeration, *Ruegeria* sp. KLH11 strains were grown in Marine Broth 2216 at 28°C (MB2216) (BD, Franklin Lakes, NJ) and *A. tumefaciens* strains were grown in AT minimal salt medium supplemented with 0.5% glucose and 15 mM $(NH4)_2SO_4$ (ATGN) (Tempé *et al.*, 1977). Antibiotics were used at the following final concentrations ($\mu g\ ml^{-1}$): (i) *E. coli* (ampicillin, Ap, 100; gentamicin, Gm, 25; kanamycin, Km, 25; spectinomycin, Sp, 100), (ii) KLH11 (Km, 100; rifampicin, Rif, 200; Gm 25, Sp, 100) (iii), *A. tumefaciens* (Gm, 300; Sp, 200).

3.3.2 Plasmid construction for null mutation, expression of *sscI* and *lacZ*-fusion

The method used to construct the *sscI* Campbell insertion mutant was similar to that described in Chapter 2. Briefly, an internal fragment of the *sscI* gene was amplified using forward primer: 5'-<u>GAATC</u>CATGTTTCGCGATCGAGCAGAT-3' (the *Eco*RI recognition site is underlined) and reverse primer: 5'-<u>GGTACC</u>TCTTGATACTCCCGCTC-3' (the *Kpn*I recognition site is underlined). The PCR amplicon was gel-purified and cloned into pCR 2.1-TOPO vector (Invitrogen, Grand Island, NY) to create pOKC1 and the insert was confirmed by

sequencing. For recombinational mutagenesis, pOKC1 was digested with *Eco*RI and *Kpn*I, and the resulting *sscI* fragment was ligated to a similarly digested R6K replicon, the pVIK112 suicide vector (Kalogeraki and Winans, 1997), creating pOKC2. pOKC2 was conjugated into KLH11 and transconjugants resistant to kanamycin (Km) were selected and confirmed by sequencing. To construct double and triple AHL synthase gene mutants, pOCK2 was conjugated into Δ*ssaI,* Δ*ssbI* and Δ*ssaI*Δ*ssbI* strains, respectively, and the transconjugants were selected and confirmed as described for the *sscI* single mutant. A controlled expression construct of *sscI* was generated by PCR amplification of the coding regions using the forward primer:

TCTAGAC**TGA**AAC*AGGAAA*CAGCT**ATG**CTCCGTTATGTTTTTGCA (the *Xba*I recognition site is underlined, the stop codon TGA and the start codon ATG are in bold and the *E. coli lacZ* ribosomal biding sites are in italics) and the reverse primer: CTCGAGT**CA**AGCGGTTCTTTGAAACTT (the stop codon is in bold and the *Xho*I recognition site is underlined). The PCR products were ligated into pCR®2.1-TOPO® vector (Invitrogen) to created pOKC3 and confirmed by sequencing. pOKC3 was digested by *Xba*I and *Xho*I and the insert was subcloned into the vector pSRKTc (Khan *et al.*, 2008) to create pOKC4. The insert carried by the construct was confirmed by sequencing. The forward primer: 5' GAATTCGCCGAGATGAACTGTTCAAAGAAC-3' (the *Eco*RI recognition site is underlined) and the reverse primer:

GGATCCGAGCATTTTTAACCTCTTGTTCAC (the *Bam*HI recognition site is underlined) annealing 255 bp upstream and 3 bp downstream of the *sscI*

translational start site were used to amplify its promoter. The PCR products were

cloned into pCR2.1-TOPO vector and the inserts confirmed by DNA sequencing.

The pCR2.1-TOPO derivatives were digested with *Eco*RI and *Pst*I and the

resulting fragments were ligated with pRA301 vector digested with the same

restriction enzymes (Akakura and Winans, 2002) to create pOKC8.

3.3.3 Preparation of AHL samples and analysis by RP-HPLC and ESI mass spectrometry

The method used was as described in Chapter 2 Section 2.3.2.

3.3.4 Qualitative analysis and estimation of AHL quantities

The method used was as described in Chapter Section 2.3.3.

3.3.5 Preparation of log phase cell concentrates and β-galactosidase assays

The method used was as described in Chapter 2 Section 2.3.7. The only

difference is that *p*-courmarate was added to MB2216 at the concentration of 0.5

mM when the supernatant of KLH11 and derivatives were assayed for *p*C-HSL-

like activity using *R. palustris* CGA814 (*rpaI-lacZ*) as the reporter strain (Schaefer

et al., 2008).

3.3.6 Motility assay

Bacterial swim assays were performed using MB2216 with 0.25 % (w/v) agar.

Plates were inoculated at the center with freshly isolated KLH11 colonies.

KLH11 crude organic extract (0.5% v/v) was added to MB 2216 agar. Plates

were placed in an air-tight container with a beaker containing 15 ml of K_2SO_4 to maintain constant humidity, and incubated 5-7 days at 28°C. Photos were taken by using a Nikon D90 camera.

Table 3.1. Strains and plasmids used in Chapter 3.

Bacteria/Plasmids	Relevant feature[a]	Reference
E. coli DH5α/λpir	Strain for propagating R6K suicide plasmids	Lab collection
E. coli S17-1/λpir	IncP conjugal donor	(Kalogeraki and Winans, 1997)
E.coli XL-1 Blue	Standard alpha-complementation strain	Lab collection
E. coli MC4100	K-12 derivative, Δ*lacZ*	(Casadaban, 1976)
A. tumefaciens NTL4	Ti plasmidless derivative, nopaline chromosomal background	(Zhu *et al*., 1998)
KLH11	wild type	(Mohamed *et al*., 2008c)
KLH11-EC1	Rif^R	Zan *et al*., 2012
KLH11-EC2	*ssaI-lacZ*, Rif^R, Km^R	Zan *et al*., 2012
KLH11-SK01	Δ*ssaI*, Rif^R	Zan *et al*., 2012
KLH11-EC3	*ssbI-lacZ*, Rif^R, Km^R	Zan *et al*., 2012
KLH11-SK02	Δ*ssaI* Δ*ssbI*, Rif^R	Zan *et al*., 2012
KLH11-OKC2	*sscI-lacZ*, Rif^R, Km^R	This study
KLH11-OKC3	Δ*ssbI*, Rif^R	This study
KLH11-OKC5	Δ*ssbI sscI-lacZ*, Rif^R, Km^R	This study
KLH11-OKC6	Δ*ssaI* Δ*ssbI ssc-lacZ*, Rif^R, Km^R	This study
KLH11-OKC7	Δ*ssaI sscI-lacZ*, Rif^R, Km^R	This study
CGA814	*Rhodopseudomonas palustris*; *rpaI-lacZ*, Km^R	Schaefer *et al*., 2008
pCR2.1-TOPO	PCR fragment cloning vector, Ap/Km^R	Invitrogen
pBBR1-MCS5	BHR P_{lac} expression vector, Gm^R	(Kovach *et al*., 1995)
pSRKTc	BHR expression vector containing *lac* promoter and *lacI^q*, Tc^R	(Khan *et al*., 2008)
pVIK112	R6K-based lacZ transcriptional fusion, KmR	(Kalogeraki and Winans, 1997)
pRA301	BHR *lacZ* translational fusion vector	(Akakura and Winans, 2002)

pEC108	pBBR1-MCS5 derivative carrying full length P_{lac}-*ssaI*, Gm^R	Zan *et al*., 2012
pEC109	pBBR1-MCS5 derivation carrying full length P_{lac}-*ssbI*, from pEC110, Gm^R	Zan *et al*., 2012
pEC112	pBBR1-MCS5 derivative, carrying full length P_{lac}-*ssaR*, from pEC106, Gm^R	Zan *et al*., 2012
pEC116	pRA301 derivation, P_{ssaI}-*lacZ*, Sp^R	Zan *et al*., 2012
pEC121	pRA301 derivative, P_{ssbI}-*lacZ*, Sp/Sm^R	Zan *et al*., 2012
pEC123	pBBR1-MCS5 derivative, carrying full length P_{lac}-*ssbR*, Gm^R	This study
pOKC1	pCR$^®$2.1-TOPO$^®$, carring internal fragment of *sscI*, Km^R	This study
pOKC2	pVIK112 derivative, carrying internal fragment of *sscI*. Km^R	This study
pOKC3	pCR2.1-TOPO$^®$, carrying full length of *sscI*, Km^R	This study
pOKC4	pSRKTC derivative, carrying full length P_{lac}-*sscI*, Tc^R	This study
pOKC8	pRA301 derivation, P_{sscI}-*lacZ*, Sp^R	This study

[a]Ap=ampicillin, Gm=gentamicin, Km=kanamycin, Rif=rifampicin, Sp=spectinomycin. Tc=tetracycline.

3.4. Results

3.4.1 Identification of *sscI*

Previous studies showed that the KLH11 strain deleted for both *ssaI* and *ssbI* still produced small amounts of AHL signals (Zan *et al*., 2012). This result indicated that a third or more AHL synthases must be present in KLH11. Analysis of the complete genome sequence of strain KLH11 revealed a third AHL synthase gene, designated *sscI* (sponge-symbiont associated locus C, *LuxI* homologue). It has 214 amino acids (aa) and does not have a cognate *luxR* gene. The upstream and downstream genes are predicted to be a putative transposase and a hypothetical protein, respectively (Fig. 3.1). Mass spectrometry analysis of the AHL profile of Δ*ssaI* Δ*ssbI* *sscI*⁻ mutant showed no AHL production (Fig. 3.2A), confirming that there are likely three and not more AHL synthase genes in KLH11.
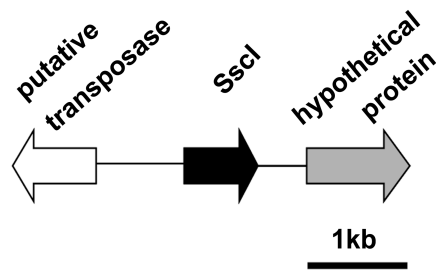
Figure 3.1. Gene maps of KLH11 *sscI* locus. Arrows represent genes. SscI is predicted to be 214 amino acids. The putative transposase is predicated to be 287 amino acids. The scale bar represents 1 kb.

3.4.2 *sscI* encodes a protein for synthesis of long chain length AHLs and its expression is not stimulated by the KLH11 AHLs

The AHL profile given by *sscI* expression in *E. coli* MC4100 was determined by the Churchill Laboratory and is shown in Fig. 3.2B. AHLs were confirmed by mass fragmentation to m/z = 102.1, retention time, as well as trimethylsilylation and methoximation of the samples (Clay and Murphy, 1979; Maclouf *et al.*, 1987) indicated that *sscI* encodes production of 3-hydroxy-HSLs, dominated by 3-OH-C14-HSL and 3-OH-C14:1-HSL. Comparison of the AHLs encoded by *sscI* to those previously identified for *ssaI* and *ssbI* (Zan *et al.*, 2012), showed that the profile of AHL production is strikingly similar to that specified by SsbI (Fig. 3.2C).

Sequence alignment showed that SscI shares 81% identity with SsbI but shares only 32% identity with SsaI on the amino acid level (Fig. 3.3), which is

consistent with the similar profiles of AHL production derived from *ssbI* and *sscI*.

The eight well-conserved amino acid residues identified in other LuxI

homologues (Churchill and Chen, 2011) can also be identified in SscI (Fig. 3.3).

Taken together, *ssbI* and *sscI* are presumably homologues while *sscI* and *ssaI*

are paralogues.

A Campbell insertion of *sscI* created a transcriptional *lacZ* fusion to the *sscI*

gene. We were able to test whether addition of KLH11 AHLs would be able to

increase the expression of *sscI*. Results showed the *sscI* was expressed (ca.

300 Miller unit) but did not respond to addition of crude organic extract of KLH11

culture ($P>0.05$) (Fig.3.4). We did a similar experiment by adding 3-OH-C14-

HSL to the final concentration of 20 μM, and no induction for *sscI* expression was

observed either (Miller units were 65.9 ± 3.9 with no AHL and 67.5 ± 3 7.4 with

AHL).
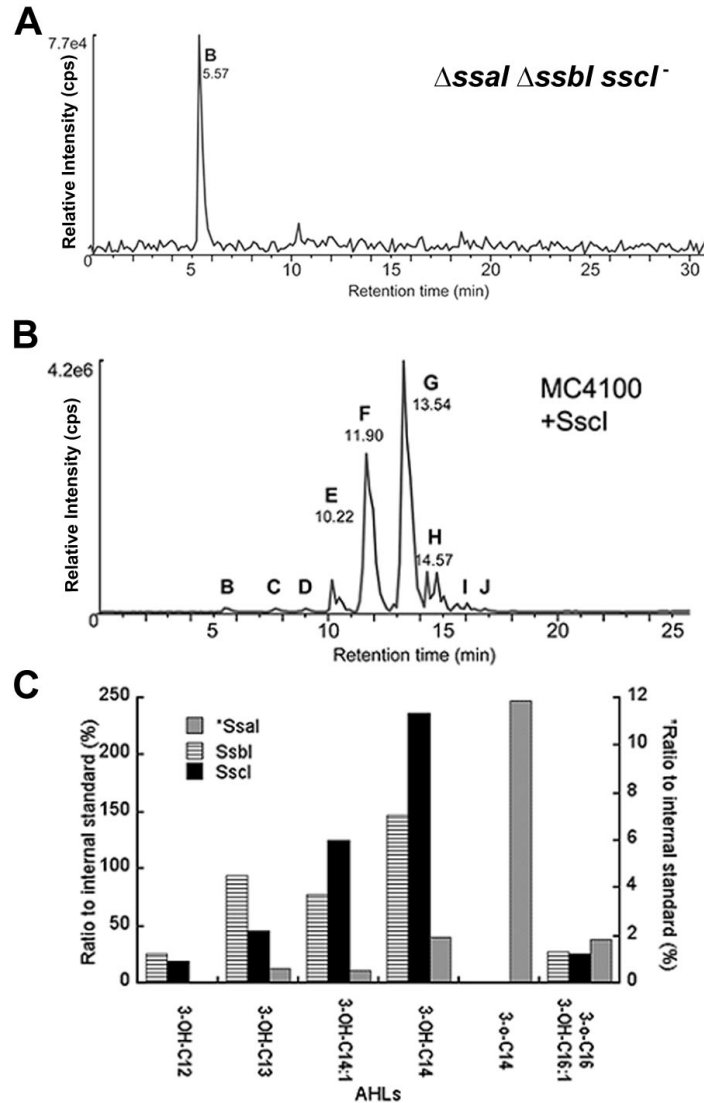
Figure 3.2. Mass spectrometry analysis of purified samples from triple mutant of Δ*ssaI* Δ*ssbI* *sscI*⁻ (A) and from SscI expressed in *E. coli* MC4100 (B). The products of reverse-phase chromatographic separation of AHLs extracted and purified from different strains were examined using the precursor ion-scanning mode (transitions were monitored for precursor -> m/z 102.1) for MC4100 + P_{lac}-*sscI*. (C) Comparative analysis of AHLs. The relative amounts of the known AHLs in SscI compared to SsaI and SsbI from Chapter 2 shown in a bar graph. The plot shows the ratio of the area of the transition for each AHL to

m/z 102.1 to the same transition for the internal standard D3-C6-HSL. The SsbI

and SscI y-axis is on the left and the SsaI y-axis is on the right. Analysis of AHL

samples was performed in positive-ion mode with Q3 set to monitor m/z 102.1 as

described in Experimental procedures in Chapter 2. The identities of each peak

indicated by letters are indicated in Table 2.3. This figure is provided by Mair

Churchill.

```
       1
LuxI  MTIMIKKSDFLAIPSEEYKGILSLRYQVFKQRLEWDLVVENNLES---DEYDNSNAEYIYACDDTEN--VSGCWRLLPTTGDYMLKSVFPELLGQQSA--PKDPNIVELSRF
EsaI  MLELFDVSYEELQTTRSEELYKLRKKTFSDRLGWEVICSQGMES---DEFDGPGTRYILGICEGE---LVCSVRFTSLDRPNMITHTFQHCFSDV----TLPAYGTESSRF
LasI   MIVQIGRREEFDKKLLGEMHKLRAQVFKERKGWDVSVIDEMEI---DGYDALSPYYMLIQEDTPEAQVFGCWRILDTTGPYMLKNTFPELLHGKEA--PCSPHIWELSRF
RhlI  MIELLSESLEGLSAAMIAELGRYRHQVFIEKLGWDVVSTSRVRDQEFDQFDHPQTRYIVAMSRQG---ICGCARLLPTTDAYLLKDVFAYLCSETP---PSDPSVWELSRY
SsaI  MILVVDGLNRHLFTEVLDEMFELRARVFGGRLGWDVNIEDGKEI---DQFDHLDPAYVIGLDDEGN--VSAAVRALQTTGPHMLSDVFSDILCGEAP--MRSATMWESTRF
SsbI  MLRYLYADELHKFPVLAEGMFRDRADQFKTRLGWDVKVNEDGEER--DQYDDLNPLYVIWEEADGS--HGGSMRVLPTTGPVMVNDIFGHLTGGSP---ICSPRIWEVTRF
SscI  MLRYVFADELMKYPSLAKGMFRDRADQFKTRLGWDVHVNAEGEER--DQYDQLNPLYVIWEESDGS--HGGSMRILPTTGPVMVNEVFGHLMGGKL---ISSPRIWEVTRF

       106
LuxI  AVGKNSSKINNS---ASEITMKLFEAIYKHAVSQGITEYVTVTSTAIERFLKRIKVPCHRIGDKEIHVLGDTKSVVLSMPINEQFKKAVLN
EsaI  FVDKARARALLG--EHYPISQVLFLAMVNWAQNNAYGNIYTIVSRAMLKILTRSGWQIKVIK--EAFLTEKERIYLLTLPAGQDDKQQLGGDVVSRTGCPPVAVTTWPLTLPV
LasI  AINSGQKGSLG--FSDCTLE--AMRALARYSLQNDIQTLVTVTTVGVEKMMIRAGLDVSRFG--PHLKIGIERAVALRIELNAKTQIALYGGVLVEQRLAVS
RhlI  AASAADD--------PQLAMK-IFWSSLQCAWYLGASSVVAVTTTAMERYFVRNGVILQRLG--PPQKVKGETLVAISFPAYQERGLEMLLRYHPEWLQGVPLSMAV
SsaI  CVDTQRLTRGKEKNSVSYATCELMIGSLEYCRNAGIEDIITVIDPVMNRVLKRSNCAPYDYVGETVPMGKVPAMAALLDCSEERISGLREFAGIHHDVFVEEEQALEMFEAKKA..(284)
SsbI  CLSRTASAHT---------AGAIMLSGGEMMEGFGLTHIAGVFDARMIRIYRMIGSSPVVLGAEGTGRDRISVGLWPYSADDCNRVAARAGIPRELSRLWFNTAFGN-GMRH-RFALSA
SscI  CLSRNASPHT---------AGAIMLSGGELMEKYKLTHIAGVFDERMIRIYRMIGSSPQVLGSEGAGRSRISVGLWPYSSDDCDRVAERAGVSRELSRLWLNISLMRYGQDHPKFQRTA
```

Figure 3.3. Alignment of SscI amino acid sequences to other AHL synthases. The grey shaded regions are the most conserved sequence blocks within the AHL synthase family. Residues are colored red to indicate acidic or hydrophilic, blue for basic, and orange for other. Shaded residues are absolutely conserved and the boxed residues are the most similar regions within the family. The numbers on top of the sequences refer to the numbering of LuxI and the number 284 indicates the length of SsaI. This figure is provided by Mair Churchill

3.4.3 *sscI* is positively regulated by *ssaI* and *ssbI*

Our previous study showed that the QS pathways in KLH11 are interconnected. When *ssaI* is knocked out, the AHL production is severely decreased (Zan *et al*., 2012). The sscI expression level was detected in different QS gene mutant backgrounds. β-galactosidase assay results showed that there was about 3-fold decrease in the expression of *sscI* in the Δ*ssaI* strain or Δ*ssbI* strain compared to that of the wild type but there was no additive effect in the Δ*ssaI* Δ*ssbI* strain on *sscI* expression (Fig. 3.4). However, the addition of KLH11 crude organic extracts or provision of plasmid-borne SsaI or SsbI did not complement *sscI* expression at all. Even provision of SsaR and SsbR with KLH11 AHLs did not give complementation of the expression of *sscI* (Fig. 3.4). The plasmid pEC112, carrying a copy of SsaR, and plasmid pOKC8, carrying P_{sscI}-*lacZ*, were transformed into the heterologous host *A. tumefaciens* NTL4. Different amounts of KLH11 AHLs were added but the *sscI* expression was not affected. Likewise, when SsbR was provided instead of SsaR, the expression of *sscI* was not affected either (O. Choi and C. Fuqua, unpublished).
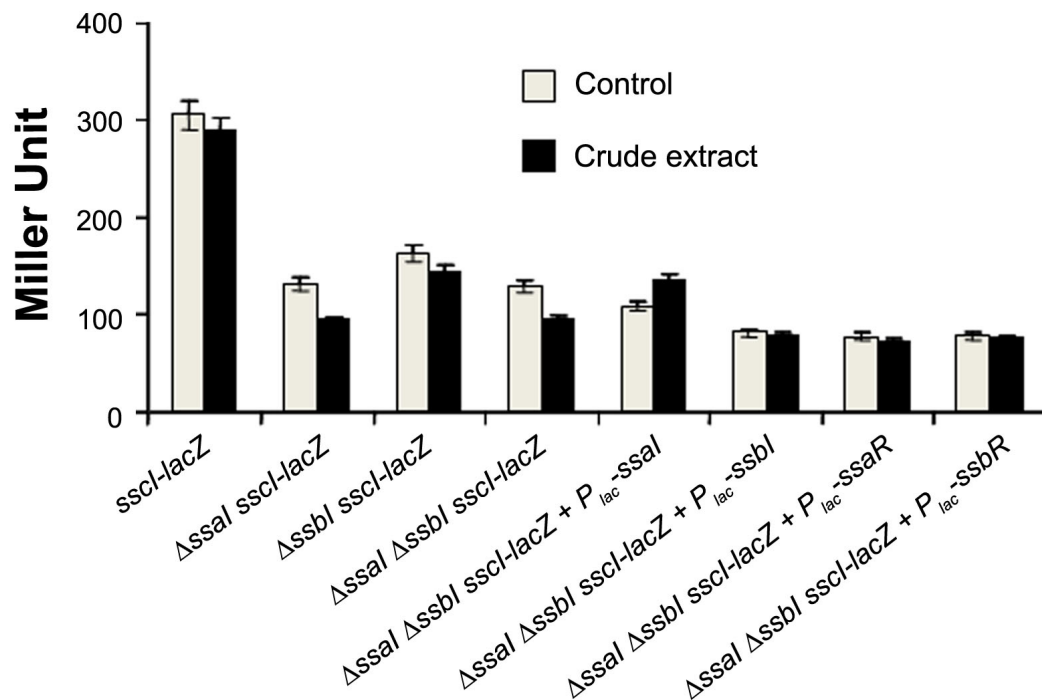
Figure 3.4. **β-galactosidase activity *of lacZ* transcriptional fusion with *sscI* in different mutant backgrounds.** Plasmids carrying *ssaI* (pEC108), *ssbI* (pEC109), *ssaR* (pEC112) and *ssbR* (pEC123) were conjugated into the Δ*ssaI* Δ*ssbI sscI⁻* mutant, respectively, to try to restore *sscI* expression. Error bars stand for the standard deviation of triplicates. The result presented is a representative of several independent experiments with three biological replicates. Figure is provided by Okhee Choi.

3.4.4 SscI-derived AHL stimulates SsaR-dependent activation of *ssaI*

To test whether SscI-derived AHLs can regulate *ssaI* expression, a plasmid-borne copy of *ssaR* paired with a compatible plasmid carrying the P_{ssaI}-*lacZ* fusion were introduced into an AHL⁻, plasmidless derivative of *A. tumefaciens* NTL4 to reconstruct the regulatory system. Cultures of the *A. tumefaciens* derivatives were grown with 2.5% (v/v) organic extract of SscI- derived AHL in *A. tumefaciens* NTL4 host with no culture amendments. The expression of *ssaI* was monitored by β-galactosidase assay. Results showed that SscI-derived AHLs increased *ssaI* expression about 4-fold compared to the negative control ($P<0.01$) (Table 3.2). This stimulation of *ssaI* expression is dependent on SsaR because *sscI*-derived AHLs did not increase *ssaI* expression when only the vector pBBR1-MCS5 was present (Table 3.2).

Table 3.2. SsaR-dependent activation of *ssaI* was stimulated by SscI-derived AHL in an AHL⁻ host[1]

		β-galactosidase Sp. Act.[2]	
Expression plasmid	**Fusion plasmid**	**+2.5%[3]**	**No extract**
pBBR1-MCS5	*ssaI-lacZ* (pEC116)	85(1)	99(6)
pEC112 (*P_{lac}-ssaR*)	*ssaI-lacZ* (pEC116)	2335(75)	644(25)

[1] All strains derived from Ti-plasmidless *A. tumefaciens* NTL4.

[2] Specific activities in Miller Units, averages of assays in triplicate (standard deviation).

[3] 2.5% (v/v) organic extract of *A. tumefaciens* NTL4 carrying *P_{lac}-sscI* (pOKC4).

3.4.5 The *sscI* mutant shows reduced swimming motility.

The *ssaRI* system positively controls KLH11 swimming motility and biosynthesis of flagella while the *ssbRI* system does not (Zan *et al*., 2012). We were interested in detecting whether *sscI* affects swimming motility. The *sscI* null mutant was tested for motility on MB2216 supplemented with 0.25% agar (w/v). There was ca. 20% decrease in the diameter of zones compared to wild type KLH11 (P<0.05) and plasmid-borne *sscI* was able to complement the swimming defects (Fig. 3.5).
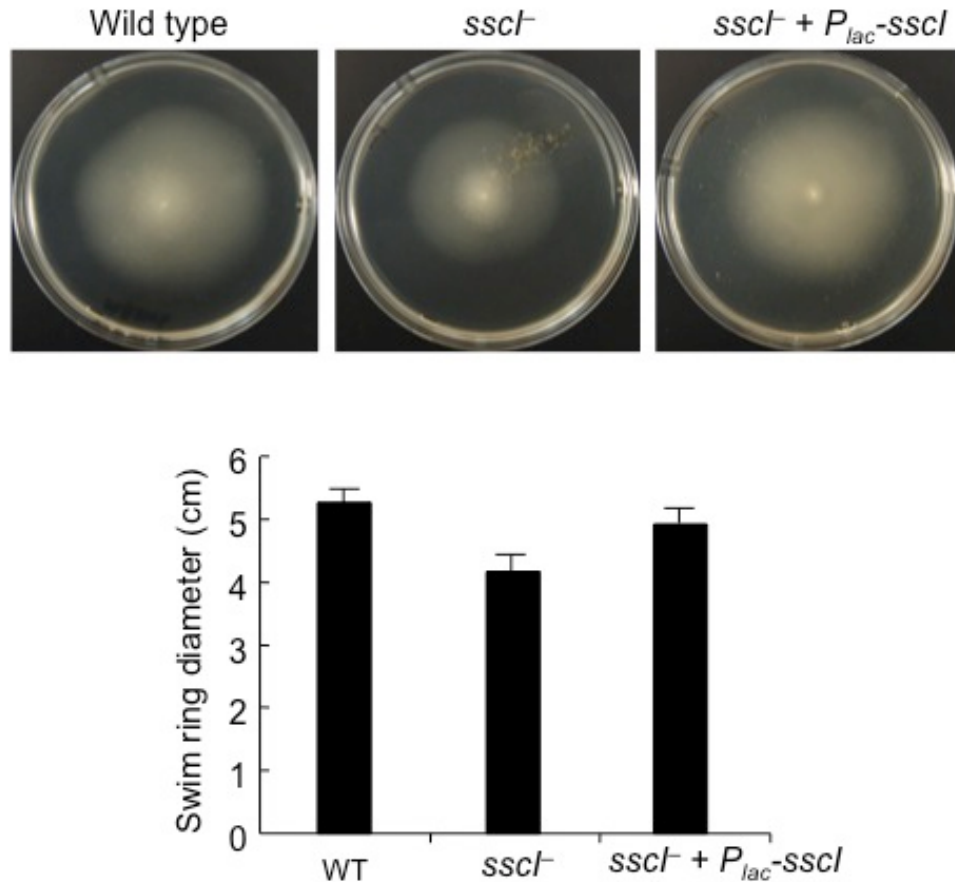
Figure 3.5 SscI affects KLH11 swimming motility moderately. Swimming motility assays on MB 2216 supplemented with 0.25% (w/v) agar after a week at 28°C. This represents one biological replicate of each strain of one experiment with three biological replicates. The diameter of swimming ring was measured. The results presented are representative of several independent experiments each with three biological replicates. Figure is provided by Clay Fuqua.

3.4.6 KLH11 contains a novel enzyme responsible for the synthesis of *p*-HSL-like molecule.

We used *R. palustris* CGA814 that has *rpaI-lacZ* fusion as the reporter strain to detect whether KLH11 produces the novel molecule *p*C-HSL (Schaefer *et al*.,

2008). Results showed that mutation of any of the three *luxI* genes did not affect

the ability of the culture supernatant to increase the expression of the *p*C-HSL

synthesis gene *rpaI*, of which the expression is stimulated by *p*C-HSL (Schaefer,

et al., 2008). Furthermore, the supernatant from the double mutant Δ*ssbI sscI⁻*

and triple mutant Δ*ssaI* Δ*ssbI sscI⁻* can still stimulate the expression of *rpaI*

(Fig.3.6).
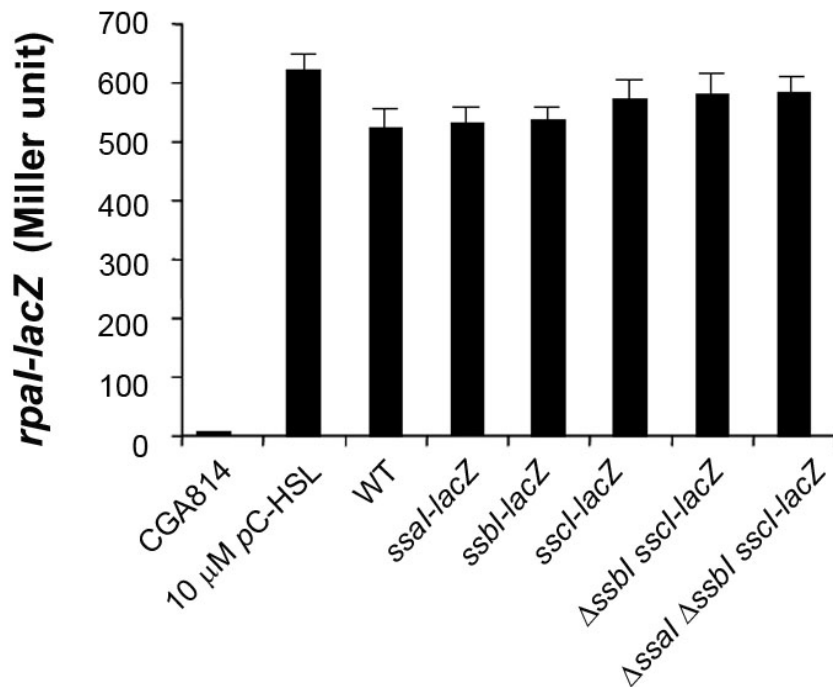
Figure 3.6. β-galactosidase assay of the expression of *rpal-lacZ* fusion.

R. palustris CGA814 was used as the reporter strains. WT = wild type KLH11

(EC1). Supernatant from CGA814 was used as negative control and *p*C-HSL

was used as a positive control. All the KLH11 derived strains were grown in

MB2216 supplemented with 0.5 mM *p*-coumarate. Bars represent the average of

three biological replicates and the error bars stand for the standard deviation of

triplicates. Figure is provided by Okhee Choi.

3.5. Discussion

Two pairs of LuxI-LuxR type QS genes (*ssaRI* and *ssbRI*) were previously identified in the sponge-associated bacterium *Ruegeria* sp. KLH11, a representative of the *Silicibacter-Ruegeria* group in the *Roseobacter* clade. Here we described the finding of *sscI*, a solo of *luxI* homologue that was not detected by the genetic screening. We reason that this could be due to several factors: 1) not enough colonies were screened; 2) the restriction enzymes used to cut the genomic DNA to prepare the screen library did not have recognition sites in the regions surrounding *sscI*, noting that the average size of fragments ligated into the pBBR1-MCS5 vector was 4 kb; or 3) *sscI* with its native promoters is not expressed in the heterologous *A. tumefaciens* system. It is highly likely that the genome annotation in which s*scI* was detected caught the only missing LuxI-type AHL synthase gene not found by the genetic screen because the triple knock out mutant of the three AHL synthase genes in KLH11 resulted in a mutant in which AHLs are undetectable by mass spectrometry. Typically, *luxI* homologues are arranged in tandem with a cognate *luxR* homologue. However, in this case a *luxI* solo, *sscI,* without a cognate *luxR* homologue was identified in KLH11. This arrangement has been reported in a roseobacterial strain that is a symbiont of the alga *Dinoroseobacter shibae* DFL12^{T}. This strain also has two sets of LuxI-LuxR QS systems and one *luxI*-solo, named *luxI*3 (Wagner-Döbler *et al.*, 2010). However, it is unknown whether LuxI3 synthesizes AHLs.

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SscI shares 81% identity to SsbI on the amino acid level and also encodes a putative protein of similar size (214 aa versus 212 aa). These two genes result in synthesis of very similar AHL profiles when expressed in *E. coli* (Fig. 3.2C). Furthermore, both *ssbI* and *sscI* lack a LuxI-type box in their promoter regions and do not respond to exogenously added KLH11 AHLs. Taken together, this suggests that *ssbI* and *sscI* are homologues. Similarly, the dominance in the *sscI*-derived AHL profile of long chain length AHLs is consistent with the types of AHLs seen in a large number of marine bacteria isolated from a variety of different sources, which might reflect adaptation to the marine environment (Wagner-Döbler *et al.*, 2005).

A transcriptional fusion of *lacZ* to *sscI* allowed monitoring its expression in different genetic backgrounds and studying how other QS genes regulate *sscI*. Generally, bacteria that have multiple QS systems tend to have a complex regulatory network (Fuqua and Greenberg, 2002). Here, results show that *sscI* is transcriptionally positively regulated by *ssaI* and *ssbI*. Also, both the *ssaI* and *ssbI* regulation on *sscI* are indirect, because in the *A. tumefaciens* NTL4 background SsaR or SsbR do not affect *sscI* transcription by addition of exogenous KLH11 AHLs. One unexplained finding is that the decrease of *sscI* expression in Δ*ssaI*, Δ*ssbI* or Δ*ssaI* Δ*ssbI* mutant strains cannot be complemented with crude extract of KLH1 or provision of SsaI or SsbI.

Our results also show that *sscI*-derived AHLs provide a feedback on *ssaI* expression via *ssaR* (Table 4.2). We know that *ssbI* expression is not affected by addition of exogenous AHLs (Zan *et al.*, 2012). In this study we did not detect

an effect of *sscI*-derived AHLs on *sscI* expression. Many LuxI-LuxR systems

tend to stay inactive when in the inactive state despite environmental

perturbations and vice versa (Tsai and Winans, 2011). In the case of the *ssaRI*

system, this results from the positive autoregulation of *ssaI* by SsaR–AHL

complexes. *sscI* contributes to this positive regulation because SsaR can also

respond to AHL synthesized by SscI, especially given the fact that the main AHL

molecule detected in the wild type KLH11 culture is 3-OH-C14-HSL and 3-OH-

C14:1–HSL (Zan *et al*., 2012). Moreover, the moderate effect of *sscI* on

swimming motility is probably due to this contribution of *sscI* to *ssaI* expression

because the *ssaRI* system positively controls KLH11 swimming motility.

LuxR solos are quite commonly found in many bacterial genomes. They

could be used for interspecies signaling or for "eavesdropping" on other bacteria

(Subramoni and Venturi, 2009). But what is the function of the *luxI* solo in the

natural environment? Are they commonly distributed in bacteria? What is the

evolutionary history of the *luxI* solo? All these questions remain to be answered.

Recent years, several novel types of AHL molecules have been reported

(Schaefer *et al*., 2008, Ahlgren *et al*., 2011; Lindemann *et al*., 2011). The *p*C-

HSL has a ring structure in the fatty acid chain and was first discovered in *R.

palustris* (Schaefer *et al*., 2008). A close relative of KLH11, *R. pomeroyi* DSS-3

was also found to produce this type of molecule. We used β-galactosidase

assay to test whether KLH11 produces similar molecules that can stimulate the

expression of *rpaI*. Surprisingly, our results show that KLH11 can also produce

similar molecules and this was independent of any of the three known LuxI-type

enzymes but we do not know what the identity of the molecule(s) in the KLH11 culture is that can stimulate the expression of the *rpaI-lacZ* reporter, suggesting the existence of novel enzyme(s) in KLH11 responsible for the synthesis. Research also suggests that *Phaeobacter gallaeciensis* BS107 (also known as DSM 17395) can respond to the presence of *p*-coumarate produced by the microalga *Emiliania huxleyi* potentially via *p*C-HSL (Seyedsayamdost *et al*., 2012). Furthermore, several different roseobacterial species can produce the novel quorum-sensing molecule TDA originally described in *Silicibacter* sp. TM1040 (Geng and Belas, 2010). Taken together, this suggests that Roseobacters represent an underexplored resource for discovery of novel quorum sensing molecules.

Chapter 4. The *cckA-chpT-ctrA* phosphorelay system is regulated by quorum sensing and controls flagellar motility in the marine sponge symbiont *Ruegeria* sp. KLH11

4.1. Abstract

Bacteria respond to their environment via signal transduction pathways, often two-component type systems that function through phosphotransfer to control expression of specific genes. Phosphorelays are derived from two-component systems but are comprised of additional components. The essential *cckA-chpT-ctrA* phosphorelay in *Caulobacter crescentus* has been well studied and is important in orchestrating the cell cycle, polar development and flagellar biogenesis. Although *cckA*, *chpT* and *ctrA* homologues are widespread among the Alphaproteobacteria, little is known about their function in the large and ecologically significant Roseobacter clade of the Rhodobacterales. In this study the *cckA-chpT-ctrA* system of the marine sponge symbiont *Ruegeria* sp. KLH11 was investigated. Our results reveal that the c*ckA, chpT* and *ctrA* genes positively control flagellar biosynthesis. In contrast to *C. crescentus*, the *cckA*, *chpT* and *ctrA* genes in *Ruegeria* sp. KLH11 are non-essential and do not affect bacterial growth. Gene fusion and transcript analyses provide evidence for *ctrA* autoregulation and the control of motility-related genes. In KLH11, flagellar motility is controlled by the SsaRI system and acylhomoserine lactone (AHL) quorum sensing. SsaR and long chain AHLs are required for *cckA*, *chpT* and *ctrA* gene expression, providing a regulatory link between flagellar locomotion and population density in KLH11.

4.2. Introduction

Roseobacters represent an abundant and important marine bacterial group in the *Alphaproteobacteria*. Members from this group can mediate key biogeochemical processes and account for up to 30% of bacterioplankton cells in some coastal environments (González and Moran, 1997). Many Roseobacters have been experimentally shown to exhibit flagellar motility, an important trait for their physical associations with eukaryotic cell surfaces or organic particles (Slightom and Buchan, 2009). For example, in *Silicibacter* sp. TM1040 flagellar mutants are defective in attaching to and forming biofilms on the dinoflagellates with which this bacterium is associated (Miller and Belas, 2006). *Alphaproteobacteria*, including Roseobacters are also found in association with marine sponges (Mohamed *et al.*, 2008b). *Ruegeria* sp. KLH11 is a sponge symbiont within the roseobacterial *Silicibacter-Ruegeria* subgroup which is consistently and specifically isolated from soft-bodied marine sponges such as species of *Mycale* and *Ircinia* (Mohamed *et al.*, 2008c). KLH11 has been developed as a model for studying the interactions of bacteria with sponge hosts.

Two-component type phosphorelay signal transduction pathways, comprised of two or more proteins, are some of the most prevalent means by which bacteria sense, respond, and adapt to changes in their environment or their intracellular state. In their simplest form two-component systems consist of a sensor histidine kinase and a cognate response regulator, through which phosphotransfer controls the regulatory output (Laub and Goulian, 2007). More complex systems can have multiple components, and individual regulators can have multiple

phosphotransfer activities. In the alphaproteobacterial developmental model system *Caulobacter crescentus* the response regulator CtrA acts to control the cell cycle and is essential for viability. CtrA is phosphorylated on a conserved asparate residue (D51), via a phosphorelay pathway that initiates with the histidine kinase CckA. When active, CckA undergoes an intramolecular phosphotransfer between a conserved histidine and an aspartate in its receiver domain at the carboxy terminal end of the protein. Active CckA subsequently phosphorylates the ChpT histidine phosphotransferase (Hpt). ChpT can transfer phosphate to either of two response regulators, CpdR or CtrA (Quon *et al.*, 1996; Biondi *et al.*, 2006). CpdR normally inhibits CtrA, but is inactive when it is phosphorylated. Phospho-CtrA, relieved of CpdR inhibition, is an active transcriptional regulator that controls about 26% (144/553) of the genes involved in cell cycle progression and also controls flagellar motility in *C. crescentus* (Laub *et al.*, 2000). Members of the *Rhodobacterales* such as KLH11 encode *cckA*, *chpT*, and *ctrA* homologues (Zan *et al.*, 2011a)(See Appendix 1), but generally no *cpdR* homologue (Brilli *et al.*, 2010). In *Silicibacter* sp. TM1040, a relative of *Ruegeria* sp. KLH11, the *cckA*, *chpT* and *ctrA* genes are required for flagellar motility, but in contrast to *C. crescentus* these genes are non-essential (Belas *et al.*, 2009).

Bacterial flagellar motility displays a critical role in many microbial processes, such as chemotaxis, colonization of hosts, and biofilm formation (Smith and Hoover, 2009). The biosynthesis of flagella is an ordered process that requires the coordinated and temporal regulation of many different genes via a very

complex regulatory hierarchy. For bacteria in which flagellar assembly has been well studied there is generally a primary regulator that initiates expression of the flagellar gene hierarchy and is referred as the master regulator. Several different types of master regulators, including CtrA from *C. crescentus*, have been identified. FlhDC is the most extensively studied master regulator in both *Escherichia coli* and *Salmonella typhimurium* (Kutsukake *et al.*, 1990; Liu and Matsumura, 1994). FleQ and FlrA are the master regulators in *Pseudomonas aeruginosa* and *Vibrio cholerae* (Arora *et al.*, 1997; Klose and Mekalanos, 1998), respectively. Although flagellar motility is common among the Roseobacters (Slightom and Buchan, 2009), little is known about its regulation.

We recently reported that the sponge symbiont *Ruegeria* sp. KLH11 utilizes two distinct but interconnected quorum sensing (QS) systems, with the LuxR-LuxI homologues SsaRI and SsbRI, that rely upon an overlapping set of long chain acylhomoserine lactone (AHL) signal molecules (Zan *et al.*, 2012). Many bacteria use intercellular signals such as AHLs to monitor their population density and accordingly regulate the expression of specific gene sets in crowded conditions. The SsaRI system is required for flagellar assembly and flagellar gene expression in KLH11 whereas the SsbRI system has no influence on motility. KLH11 specifically switches into a motile phase at high population densities, and this requires SsaRI (Zan *et al.*, 2012). Although it is possible that SsaR functions as the primary regulator of motility, it is more likely that it controls expression of a downstream regulator specific for the flagellar genes. For example, in *Burkholderia glumae* the *tofRI* QS system regulates the expression

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of *flhDC*, which in turn directly controls motility (Kim *et al.*, 2007). Although the

FlhDC and FleQ/FlrA homologues are not present in *Ruegeria pomeroyi* DSS-3

or in KLH11 genome sequences (Moran *et al.*, 2004; Zan *et al.*, 2011a)(See

Appendix 1), it is possible that the *cckA-chpT-ctrA* pathway acts in this capacity.

In this study we examined whether 1) *cckA*, *chpT* and *ctrA* genes are essential

for the viability of KLH11; 2) they can control flagellar motility; 3) they are

influenced by the SsaRI system. Our results show clearly that *cckA*, *chpT* and

ctrA are non-essential, are tightly regulated by QS, and act downstream of QS in

controlling flagellar motility.

4.3. Experimental procedures

4.3.1 Strains, growth conditions and plasmid transformation

Bacterial strains and plasmids used in this study are listed in Table 4.1. *Ruegeria* sp. KLH11 and KLH11-EC1 derivatives were grown in Marine Broth 2216 (MB2216) (BD, Franklin Lakes, NJ) at 28°C. *E. coli* strains were grown at 37°C in Luria-Bertani (LB) broth. *A. tumefaciens* strains were grown in AT minimal medium supplemented with 0.5% glucose and 15 mM $(NH_4)_2SO_4$ (ATGN) (Tempé *et al.*, 1977). Antibiotics were used at the following concentrations ($\mu g\ ml^{-1}$): (i) *E. coli* (gentamicin, Gm, 25; kanamycin, Km, 50; spectinomycin, Sp, 100), (ii) KLH11 (Km, 100; rifampicin, Rif, 200; Gm, 25; Sp, 100) (iii), *A. tumefaciens* (Gm, 300; Sp, 200).

Plasmids were introduced into KLH11 and derivatives using either electroporation or conjugation (Zan *et al.*, 2012) and into *E. coli* using standard methods of transformation and into *A. tumefaciens* using a standard electroporation method (Mersereau *et al.*, 1990).

4.3.2 Deletion of *ssaR* and generation of *cckA, chpT* and *ctrA* null mutants

DNA manipulations were performed using standard techniques or per manufacturers' specifications (Sambrook *et al.*, 1989). Restriction enzymes and Phusion^{TM} High-Fidelity DNA Polymerase were obtained from New England Biolabs (Ipswich, MA). Oligonucleotides, listed in Table 4.2, were obtained from Integrated DNA Technologies (Coralville, IA). DNA sequencing was performed

on an ABI3700 automated sequencer by the BioAnalytical Services Laboratory at

the Institute of Marine and Environmental Technology (Baltimore, MD). To

generate an in-frame, markerless deletion of the *ssaR* gene, splicing by overlap

extension (SOE) polymerase chain reaction (PCR) was used (Warrens *et al.*,

1997). An approximately 500-bp fragment upstream of and including the first

three codons of the *ssaR* coding sequence was amplified using primers ssaR D1

and ssaR D2. An approximately 500-bp fragment downstream of and including

the *ssaR* stop codon was amplified using primers ssaR D3 and ssaR D4.

Primers ssaR D2 and ssaR D3 were designed to contain an 18 bp

complementary sequence at the 5' end (the "overlap") to facilitate the SOEing

reaction (Merritt *et al.*, 2007). Following initial amplification the two fragments

were gel purified and used as template in a second round of PCR with primers

ssaR D1 and ssaR D4 generating an approximately 1 kb SOE fragment

containing a fusion of the upstream and downstream regions of the *ssaR* locus.

Primers ssaR D1 and ssaR D4 were designed to allow direct cloning using the

In-Fusion Clone Kit. The final SOE fragment was gel purified and cloned into the

sacB counter-selectable vector pNPTS138 that had been previously digested

with *Eco*RI. The resulting plasmid, pJZ014, was confirmed by sequencing and

conjugated into *Ruegeria* sp. KLH11-EC1 (Rif^R). The suicide vector pNPTS138

is a ColE1 plasmid carrying kanamycin resistance and is unable to replicate in

Ruegeria sp. KLH11 (Zan *et al.*, 2012). Transconjugants were plated onto

Marine Agar 2216 (MA2216) (BD, Franklin Lakes, NJ) plates supplemented with

both Rif and Km to select for Rif^R Km^R plasmid integrants. Presumptive

integrants were tested for sucrose sensitivity, verifying introduction of the *sacB*

counter-selectable marker on pNPTS138, by plating on MA2216 plates

supplemented with Rif, Km, and 5% (w/v) sucrose. Rif^R Km^R Suc^S colonies were

subcultured in MB2216 without Km and plated on 5% sucrose MA2216 plates

without Km to select for Suc^R allelic replacement candidates. Candidates were

verified to be Km^S by patching onto MA2216 plates supplemented with Km.

Deletion of the targeted *ssaR* locus was confirmed by PCR using primers ssaR

D1 and ssaR D4 and the Δ*ssaR* strain was designated JZ03.

    Null mutations in the *Ruegeria* sp. KLH11 *cckA*, *chpT*, and *ctrA* homologues

were generated using Campbell-type recombinational mutagenesis. Internal

gene fragments were generated by PCR using primers cckA P1/cckA P2, chpT

P1/chpT P2, and ctrA P1/ctrA P2, respectively, using KLH11 genomic DNA as

template. The partial *cckA*, *chpT*, and *ctrA* fragments were cloned directly into

the pCR2.1-TOPO vector (Invitrogen, Grand Island, NY) and then subcloned into

pVIK112, a suicide vector with an R6K conditional replication origin (Kalogeraki

and Winans, 1997), creating plasmids pJZ003 (truncated at codon 513), pJZ004

(truncated at codon 160), and pJZ005 (truncated at codon 173), respectively.

These plasmids were then conjugated into KLH11-EC1. Presumptive Km^R

transconjugants were selected and confirmed by PCR amplification using the

primer 3 designated for each of the three genes that is located upstream of the

recombined fragments and the primer 112R that is located downstream of the

KpnI recognition site on the plasmid pVIK112 (Kalogeraki and Winans, 1997) and

the amplicons were sequenced. The *cckA⁻*, *chpT⁻*, and *ctrA⁻* mutants were

designated JZ04, JZ05, and JZ06, respectively. To create strains JZ07-JZ12

plasmids pJZ003, pJZ004, and pJZ005 were conjugated into Δ*ssaI* strain (SK01)

and Δ*ssaR* strain (JZ03), respectively. The Km^R recombinants were selected

and confirmed as for strains JZ04-JZ06.

A transcriptional fusion of *E. coli lacZ* immediately downstream of the KLH11

cckA homologue at its native genomic location was generated by PCR amplifying

a 3' fragment of the *cckA* gene, ending at the stop codon, using primers

cckAintactF and cckAintactR. The PCR amplicon was cloned into pCR2.1-TOPO

and then subcloned into pVIK112, creating pJZ012. This plasmid was

conjugated into KLH11 and transconjugants were selected and confirmed as

described above. Campbell-type recombination results in *lacZ* fused to the 3'

end of the native *cckA* locus, keeping the *cckA* gene-coding region intact.

4.3.3 Cloning of phosphorelay components and promoter fusion constructs

Complementation constructs of *Ruegeria* sp. KLH11 homologues of *cckA*

(pJZ006), *chpT* (pJZ007), and *ctrA* (pJZ008) were generated by PCR

amplification of the coding regions of each gene using primers designated as P3

and P4 for each specific gene and KLH11 genomic DNA as template. An *E. coli*

lacZ ribosomal binding site was engineered into the 5' primer of each gene to

allow for efficient translation. PCR products were cloned directly into the broad-

host range vector pSRKGm that had been previously cut with *Spe*I (Khan *et al.*,

2008) using the In-Fusion HD directional cloning system (Clontech, Mountain

View, CA). The resulting expression plasmids carry each gene under the control

of an IPTG-inducible P_{lac} promoter. The insert carried by each construct was confirmed by sequencing.

The expression construct for the *A. tumefaciens cckA* homologue was created in the pSRKGm plasmid as described (Kim *et al*, submitted). Expression constructs for the *A. tumefaciens chpT* and *ctrA* homologues were generated by PCR amplification with Phusion High-Fidelity DNA polymerase using purified wild-type *A. tumefaciens* C58 genomic DNA as template. Primers JEH48 and JEH53 were used to amplify the *chpT* locus and JEH50 and JEH54 were used for the *ctrA* locus (Table 4.2). Amplicons were cloned into vector pGEM-T Easy and sequenced. Each gene was then sub-cloned into pSRKGm using engineered *Nde*I and *Nhe*I restriction sites.

Fusions of the probable promoter regions for the KLH11 homologues of *cckA*, *chpT*, and *ctrA* to a promoterless *E. coli lacZ* β-galactosidase gene were created in plasmid pRA301 (Akakura and Winans, 2002). The intergenic region upstream of the *cckA* coding sequence was PCR amplified using primers cckA P5 and cckA P6. The upstream and downstream primers anneal 145 upstream, and 69 bp downstream, of the predicted *cckA* translational start site, respectively. The PCR product was fused with pCR2.1-TOPO and the insert was confirmed by DNA sequencing. The pCR2.1-TOPO derivative was digested with *Eco*RI and *Pst*I, and the resulting fragment was ligated into similarly digested pRA301 creating pJZ009 which was confirmed by sequencing. Similarly, the intergenic regions upstream of the *chpT* and *ctrA* coding sequence were PCR amplified using primers chpT P5 and chpT P6 or ctrA P5 and ctrA P6, fused with pCR2.1-

TOPO and then subcloned into pRA301, creating plasmids pJZ010 or pJZ011, respectively. Plasmids pEC112 (*P~lac~-ssaR)* and either pJZ009 (*P~cckA~-lacZ*), or pJZ010 (*P~chpT~-lacZ*), or pJZ011 (*P~ctrA~-lacZ*) were electroporated into *A. tumefacien*s NTL4. Plasmids pBBR1-MCS5 (Kovach *et al.*, 1995) and either pJZ009, pJZ010 or pJZ011 were electroporated into *A. tumefacien*s NTL4 to serve as negative controls.

4.3.4 Evaluation of flagellar-based motility and presence of flagella

Bacterial swimming motility assays were performed using MB2216 with 0.25% (w/v) agar supplemented with 200 µM IPTG. Swim plates were inoculated with mid-log phase cultures of the relevant KLH11 strains using an inoculation needle. Plates were wrapped tightly with plastic film and incubated at 28°C. Swim ring diameters were measured and pictures taken after 8 days with a Nikon D90 digital camera.

Relative levels of flagellin in the wildtype*, cckA^-^, chpT^-^,* and *ctrA^-^* KLH11 strains were determined from culture supernatants followed by immunoblotting. Flagellin was enriched as described (Kanbe *et al.*, 2007). Strains were grown to mid-log phase in MB2216, supplemented with antibiotics when necessary, and then back-diluted to an OD_{600} ~ 0.01 in 3 ml MB2216. Samples were collected at stationary phase and OD_{600} was measured. The samples were vigorously vortexed for 30 sec and then centrifuged (5 min, 10,000 x g) at 4°C. The resulting supernatant was transferred to a new centrifuge tube and polyethylene glycol added to a final concentration of 2%. Following vortexing and 100 min

incubation on ice, the mixtures were centrifuged (15 min, 17,400 x g). The

resulting precipitate was resuspended in 100 µl 1 X SDS lysis buffer and boiled

at 100°C for 5-10 min. The denatured samples were separated on a 15% SDS-

PAGE gel at 90 V for 4 h and then were transferred to a nitrocellulose membrane

(Amersham Biosciences, Seattle, WA). Immunoblotting was performed with

polyclonal antibody raised against whole flagella from *C. crescentus* (a gift from

the laboratory of Y.V. Brun) at a dilution of 1:20,000 as described by Zan *et al.*

(2012).

Staining of flagella on intact cells used a two-component stain modified from

Mayfield and Inniss (1977). The first component contained equal volumes of

saturated $AlK(SO_4)_2 \cdot 12H_2O$ and 5% phenol in 10% tannic acid while the second

component contained 12% crystal violet in 100% ethanol. Ten ml of a 10:1

mixture of the two components was applied to the edge of a coverslip on a 3 ml

wet mount for each strain. Flagella were observed within 5 min of staining on a

Zeiss Axioskop 40 microscope equipped with an AxioCam MRm monochrome

digital camera using a 100X oil immersion objective and bright field illumination.

4.3.5 Quantification of phosphorelay component promoter activity

Promoter activities were quantified using *lacZ* translational and transcriptional

fusions as indicated. β-galactosidase specific activity was measured as

described previously, expressed in Miller Units, using *o*-nitrophenyl-b-galactoside

(ONPG) as substrate (Zan *et al*., 2012). *Ruegeria* sp. KLH11 was grown in

MB2216 supplemented with antibiotics as required overnight. Cultures were

diluted approximately 100-fold to obtain an OD_{600} ~0.01 in 3 ml MB2216 without antibiotics and incubated at 28°C. Mid-log phase KLH11 cultures were sampled and assayed for β-galactosidase activity immediately. Similarly, mid-log phase cultures of *A. tumefaciens* strain NTL4 were diluted at 1:100 dilution to an OD_{600}~ 0.01 in 3 ml ATGN media and incubated at 28°C with shaking at 200 rpm to an OD_{600} ~ 0.4.   Mid-log phase cultures were measured for OD_{600} and frozen at - 80°C and used for subsequent β-galactosidase assays. Exogenous AHL was added to each culture where indicated to a final concentration of 2 μM 3-oxo-C16:1 Δ11cis-(L)-HSL was purchased from Cayman Chemical (Ann Arbor, Michigan).

4.3.6 Analysis of KLH11 CtrA-dependent gene expression

Expression of motility- and cell cycle-related genes was measured using qRT-PCR with specific primers (Table S5). KLH11 and derivatives were grown in MB2216 to stationary phase and 0.5 ml culture was collected and stored in 1 ml RNAprotect BacteriaReagent (Qiagen, Valencia, CA). The mixtures were centrifuged (10 min, 5100 x g) and the cell pellets were stored at -80°C for subsequent RNA extraction. Total RNA was isolated using an RNeasy miniprep kit (Qiagen, Valencia, CA), with genomic DNA removed by TURBO DNase (Ambion, Austin, TX), per manufacturers' supplied protocols. cDNA was synthesized using qScript cDNA SuperMix according to the manufacturer's instructions (Quanta BioSciences, Gaithersburg, MD). RT-PCR was performed with Power SYBR Green Master Mix (Invitrogen, Grand Island, NY) on an ABI 7500 Fast Real-Time PCR system using the following cycling parameters: 2 min

at 95°C for initial denaturation, 40 cycles consisting of 10 s at 95°C, and 1 min at 60°C for primer annealing and extension. Melt curves were performed to confirm the specificity of primers and the absence of primer dimers. Expression levels were normalized to the housekeeping *rpoD* gene encoding s[70].

4.3.7 Multiple sequence alignment and phylogenetic analysis of the *ctrA* gene

Sequences of *ctrA* homologues from selected *Alphaproteobacteria* were downloaded from GenBank and aligned using ClustalW2 (http://www.ebi.ac.uk/Tools/msa/clustalw2/). The phylogenetic tree was constructed using software MEGA 4.0 (http://www.megasoftware.net/). BOXSHADE was used to determine the degree of residue shading (www.ch.embnet.org/software/BOX_form.html).

Table 4.1. Strains and plasmids used in Chapter 4

Bacteria/Plasmids	Relevant feature[a]	Reference
E. coli TOP 10 F´	Standard alpha-complementation strain, *lacI*^{Q}	Qiagen
E. coli DH5α/λpir	Strain for propagating R6K suicide plasmids	Lab collection
E. coli S17-1/λpir	IncP conjugal donor	(Kalogeraki and Winans, 1997)
A. tumefaciens NTL4	Ti plasmidless derivative, nopaline chromosomal background	(Zhu *et al.*, 1998)
KLH11	wild type	(Mohamed *et al.*, 2008c)
KLH11-EC1	Rif^R	(Zan *et al.*, 2012b)
KLH11-SK01	Δ*ssaI,* Rif^R	(Zan *et al.*, 2012b)
KLH11-OKC8	*fliC:*:pOKC12, Km^R	(Zan *et al.*, 2012b)
KLH11-JZ03	Δ*ssaR,* Rif^R	This study
KLH11-JZ04	*cckA*::pJZ003*,* Rif^R, Km^R	This study
KLH11-JZ05	*chpT*::pJZ004*,* Rif^R, Km^R	This study
KLH11-JZ06	*ctrA*::pJZ005, Rif^R, Km^R	This study
KLH11-JZ07	Δ*ssaI cckA*::pJZ003, Rif^R, Km^R	This study
KLH11-JZ08	Δ*ssaI chpT*::pJZ004*,* Rif^R, Km^R	This study
KLH11-JZ09	Δ*ssaI ctrA*::pJZ005, Rif^R, Km^R	This study
KLH11-JZ10	Δ*ssaR cckA*::pJZ003*,* Rif^R, Km^R	This study
KLH11-JZ11	Δ*ssaR chpT*::pJZ004*,* Rif^R, Km^R	This study
KLH11-JZ12	Δ*ssaR ctrA*::pJZ005, Rif^R, Km^R	This study
KLH11-JZ13	*cckA*::pJZ012, wild type *cckA*, Rif^R, Km^R	This study
pCR 2.1-TOPO®	PCR fragment cloning vector, Ap/Km^R	Invitrogen
pBBR1-MCS5	Plac expression vector, GmR	(Kovach *et al.*, 1995)1995)
pNPTS138	colE1 origin, sacB, Km^R	gift of M. Alley
pSRKGm	pBBR1MCS-5-derived expression vector containing *lac* promoter *lacI*^{q}, Gm^R	(Khan *et al.*, 2008)
pVIK112	R6K-based *lacZ* transcriptional fusion, integration vector, Km^R	(Kalogeraki and Winans, 1997)
pRA301	*lacZ* translational fusion vector	(Akakura and Winans, 2002)

pEC108	pBBR1-MCS5 derivative carrying full length P_{lac}-$ssaI$, Gm^R	(Zan *et al*., 2012)
pEC112	pBBR1-MCS5 derivative, carrying full length P_{lac}-$ssaR$, Gm^R	(Zan *et al*., 2012)
pOKC12	pVIK112 derivative carrying truncated *fliC*, Km^R	Choi and Fuqua, unpublished
pJZ003	pVIK112 derivative carrying truncated *cckA* gene, Km^R	This study
pJZ004	pVIK112 derivative carrying truncated *chpT* gene, Km^R	This study
pJZ005	pVIK112 derivative carrying truncated *ctrA* gene, Km^R	This study
pJZ006	pSRKGm derivative carrying full length P_{lac}-$cckA$, Gm^R	This study
pJZ007	pSRKGm derivative carrying full length P_{lac}-$chpT$, Gm^R	This study
pJZ008	pSRKGm derivative carrying full length P_{lac}-$ctrA$, Gm^R	This study
pJZ009	pRA301 derivative, P_{cckA}-$lacZ$, Sp^R	This study
pJZ010	pRA301 derivative, P_{chpT}-$lacZ$, Sp^R	This study
pJZ011	pRA301 derivative, P_{ctrA}-$lacZ$, Sp^R	This study
pJZ012	pVIK112 derivative, *cckA* gene with 5' truncation, to retain wt *cckA*, Km^R	This study
pJZ014	pNPTS138 carrying *ssaR* deletion fragment, Km^R	This study
pJEH010	pSRKGm derivative carrying full length P_{lac}-$cckA$ of *A. tumefaciens*, Gm^R	Kim *et al*., submitted
pJEH027	pSRKGm derivative carrying full length P_{lac}-$chpT$ of *A. tumefaciens*, Gm^R	This study
pJEH028	pSRKGm derivative carrying full length P_{lac}-$ctrA$ of *A. tumefaciens*, Gm^R	This study

[a]Ap=ampicillin, Gm=gentamicin, Km=kanamycin, Rif=rifampicin, Sp=spectinomycin.

Table 4.2. Primers used in Chapter 4.

Primer name	Sequences[b] (5'-3')	Restriction enzyme
cckA P1	GAATTCCGCGCAAGGACAAAGAGATA	*Eco*RI
cckA P2	GGTACCCGACAAGGTTCATCAACACC	*Kpn*I
cckA P3	CCGCTCTAGAACTAGTGTGAAAC*AGGAAA*CAGCT**ATG**TCCAGTGTT TCTGAATC	*Spe*I
cckA P4	CGGGGGATCCACTAGT**CTA**GTTGAGTTGCTGGAAC	*Spe*I
cckA P5	GAATTCCGGAACCGATGGATTTTACA	*Eco*RI
cckA P6	CTGCAGCGACACATAGCGGCCGTGGTC	*Pst*I
chpT P1	GAATTCCGCATCAGACGTCAACCTT	*Eco*RI
chpT P2	GGTACCCTTTCCAGAGGCCGCTATC	*Kpn*I
chpT P3	CCGCTCTAGAACTAGTG**TGA**AAC*AGGAAA*CAGCT**ATG**CAGCAGGA GGTACGCATG	*Spe*I
chpT P4	CGGGGGATCCACTAGT**CTA**AAAGCGCAGCGTTACAC	*Spe*I
chpT P5	GAATTCGGATCAAAGACCGGCATCAG	*Eco*RI
chpT P6	CTGCAGCTG**CAT**CTGCCGCAGGAGCCG	*Pst*I
ctrA P1	GAATTCGATGCTGACACATGCCAATC	*Eco*RI
ctrA P2	GGTACCATCTCTTTCGTCAGCGTGGT	*Kpn*I
ctrA P3	CCGCTCTAGAACTAGTG**TGA**AAC*AGGAAA*CAGCT**ATG**CGAATACT TCTCGTCGA	*Spe*I
ctrA P4	CGGGGGATCCACTAGT**TCA**GGCGCCGACCGCCA	*Spe*I
ctrA P5	GAATTCGGCGGAACATGGCGTCGA	*Eco*RI
ctrA P6	CTGCAGTCG**CAT**TCAACTGCTCCAAT	*Pst*I
ssaR D1	CTGGATCCACGAATTCGGTAAACCGCCCCTATTACGG	*Eco*RI
ssaR D2	**AAGCTTGGTACCGAATTC**AATATC**CAT**CGGTAACGACCA	NA
ssaR D3	**GAATTCGGTACCAAGCTT**CCAGGT**TAA**AACCAAAACTCC	NA
ssaR D4	CGAAGCTAGCGAATTCGTCGCATAGGACACCGAGTTC	*Eco*RI
JEH48	gaagaaCAT**ATG**ACGAGCAAACTCAATATCACGC	*Nde*I
JEH53	gaagaaGCTAGC**TCA**CTCGGCAACCGTCTTTGC	*Nhe*I

JEH50	gaagaa<u>CAT**ATG**</u>CGGGTTCTACTGATTGAAGACGA	*Nde*I
JEH54	gaagaa<u>GCTAGC**TCA**</u>GGCGGTTTCGAGGAA	*Nhe*I
rpoDRT1	GACGCCTATCGCGGCCGT	NA
rpoDRT2	GCCGACCTGCGCCATATCGT	NA
RT-FliFF	GCGCGGTGTTGCCTATGAGAT	NA
RT-FliFR	GATGCCGCGAATCGCTGG T	NA
RT-FlhAF	CGGGCTTCTGATCACGCTCCT	NA
RT-FlhAR	GCGTTGTCAGTGGCACCTTGT	NA
RT-FlgBF	TACGCAATGGCAACCCATGCT	NA
RT-FlgBR	GCGTCCGAAATGCCATGCAGAT	NA
RT-motBF	GTGACGGCCATGATGGCGTT	NA
RT-motBR	CCTTGTGCATCCACGCCTGT	NA
RT-FlgJF	GCTTTGGTTCCACAACAGCTAA	NA
RT-FlgJR	CTGTTGCTGCACGAGGAAAG	NA
fliCRT1	CGCAGAACCTGTCGACCGGT	NA
fliCRT2	GATGCCGCGAATCGCTGGT	NA
RT-ftsZF	GCAGCTGGACGGCGTTGAAT	NA
RT-ftsZR	CCGCCAGATGATCCACGATCTGT	NA
RT-ccrMF	GTCGACGCGGTCGATGATCACT	NA
RT-ccrMR	GTTCGACTTGCGCCACACAACAT	NA
cckAintactF	<u>GAATTC</u>ACAGGTCTGGGTCTGTCCAC	*Eco*RI
cckAintactR	<u>GGTACC**CTA**</u>GTTGAGTTGCTGGAAC	*Kpn*I
112R	GGCTGCAGGTCGACCATGGTC	NA

[b] Engineered restriction sequences are underlined. Complementary sequences for PCR-SOEing are shown in bold and

are also underlined. Start and stop codons are in bold. *E. coli lacZ* ribosomal binding sites are in italics and bold.

Protection nucleotides are in lower case. NA= not applicable.

4.4. Results

4.4.1 The KLH11 *cckA, chpT* and *ctrA* genes are non-essential and control flagellar motility

Annotation of the KLH11 genome revealed that KLH11 has homologues to each of the *cckA*, *chpT* and *ctrA* genes (Zan *et al*., 2011a)(See Appendix 1). The putative KLH11 *cckA* gene (GenBank No. ZP_05124558) encodes a 763 amino acid (aa) protein which shares 48% identity at 52% coverage over its C-terminus (367-763) to the *cckA* gene in *C. crescentus*. The N terminus of KLH11 CckA (1-366 aa) has no similarity to that of the *Caulobacter* CckA and had two transmembrane regions predicted by http://www.sbc.su.se/~miklos/DAS/. Domain scans using http://www.ebi.ac.uk/Tools/pfa/iprscan/ suggest that the KLH11 CckA protein has a sensory box (273-383 aa), a HisKA domain (394-457 aa), HATPase_c domain (500-620 aa) and REC domain (645-758 aa) (Fig. 4.1A). The domain organization of KLH11 CcKA is very similar to that of *C. crescentus* CckA (Jacobs *et al.*, 1999). Furthermore, the histidine residue at position 402 and the aspartate residue at position 697 correspond to the conserved phosphorylation sites, histidine 322 and aspartate 623 of *Caulobacter* CckA.

The ChpT Hpt homologue in KLH11 (GenBank No. ZP_05124304) encodes 204 aa and shares 34% identity at 58% coverage (13-131 aa) with *C. crescentus* ChpT, including a histidine at position 24 corresponding to the conserved histidine at position 61 of *Caulobacter* ChpT (Biondi *et al.*, 2006). It has a

hypothetical domain DUF2328 (30-204 aa) conserved in bacteria. The CtrA homologue from KLH11 (GenBank No. ZP_05124475) encodes 238 aa and shares 74% identity across its length with that of *C. crescentus*. It has a receiver domain in the N-terminus (3-112 aa) and a DNA binding domain (145-221 aa) in the C-terminus (Fig. 4.1A). The phosphorylation site aspartate in KLH11 is also conserved compared to other *ctrA* homologues (Fig. 4.2).
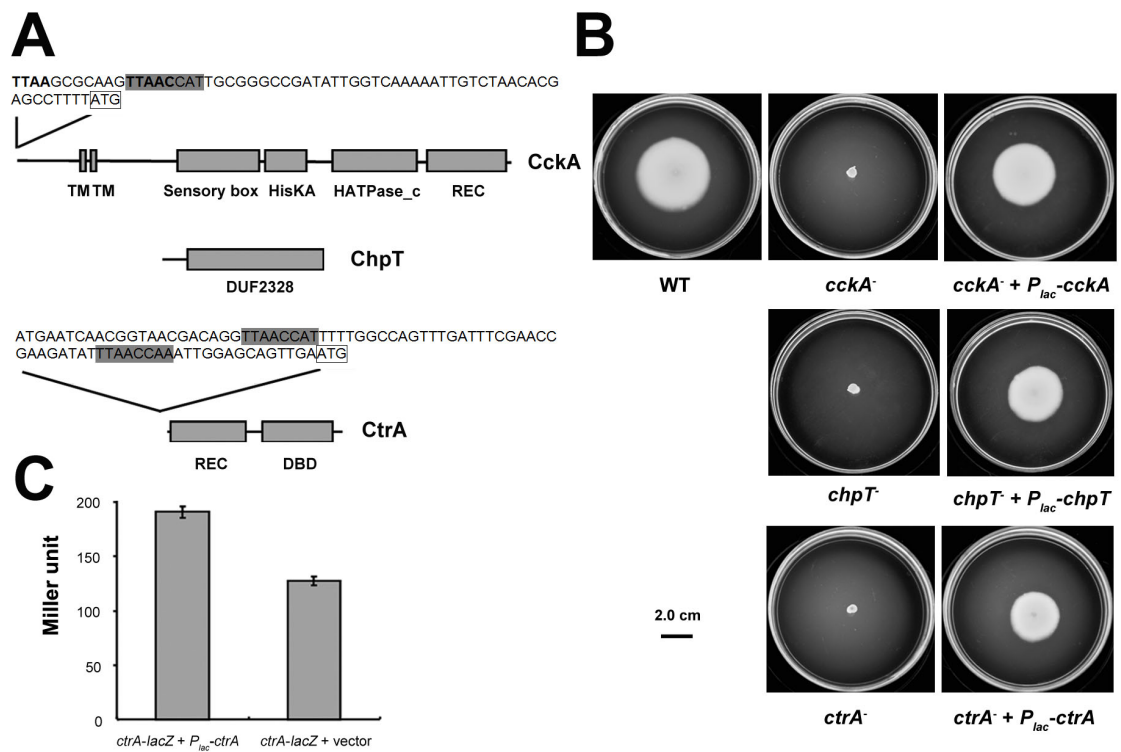
Figure 4.1. The *cckA-chpT-ctrA* pathway controls motility. A) Diagram of the predicted CckA protein, ChpT protein and CtrA protein. The N-terminus is shown at the left and the C-terminus is shown at the right. TM = transmembrane domain, HisKA = histidine kinase A dimerization/phosphoacceptor domain, HATPase_c = histidine kinase-like ATPase domain, REC = signal receiver domain. DUF2328 is a Pfam domain with unknown function. DBD= DNA binding

domain. The length of line was drawn according to scale. The partial promoter

regions of the *cckA* and *ctrA* genes are shown on top of the lines. CtrA full

recognition site (TTAAN7TTAAC) is in bold and both the CtrA half recognition

site (TTAACCAT) and the region that has one mismatch are in grey. The start

codon is boxed. **B).** Swimming motility assays. Wild-type KLH11 and derivatives

were inoculated on MB2216 (supplemented with 0.25% agar) swim agar plates

for 8 days at 28°C. 200 μM IPTG was added to the media. The results are

representative of several independent experiments each with three biological

replicates. The bar represents 2 cm. **C).** CtrA autoregulates its own expression**.**

P_{lac}-***ctrA*** plasmid (pJZ008) was conjugated into the *ctrA*‾ mutant (JZ06) and the

expression of *ctrA-lacZ* was monitored by β-galactosidase assay. The pSRKGm

was conjugated into the *ctrA*‾ mutant as a negative control. Representative

results of several independent experiments each with three biological replicates

are presented. Values are averages of assays performed in triplicate and error

bars are standard deviations.

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                              ★
KLH11                1  MRILLVEDDPTTSKSIELMITHANLNVYATDLGEEGIDLAKLYDYDLILLDLNLPDMNGHEVLRQLRIARVETPILILSG
Silicibacter sp. TM1040  1  MRILLVEDDPTTAKSIELMITHANLNVYTTDLGEEGIDLAKLYDYDLILLDLGLPDMNGHEVLRQLRLSRIETPILILSG
C. crescentus        1  MRVLLIEDDSATAQTIELMIKSEGFNVYTTDLGEEGVDLGKIYDYDLILLDLNLPDMSGIDVLRTLRVAKINTPIMILSG
A. tumefaciens       1  MRVLLIEDDSATAQSIELMIKSESFNVYTTDLGEEGVDLGKLYDYDIILLDLNLPDMSGYEVLRTLRLSKVKTPILILSG
Rhodobacter capsulatus  1  MRILLVEDDPTTSRSIELMITHANLNVYCTDLGEEGIDLAKLYDYDLILLDLNLPDMNGLEVLRQLRLARVDTPILILTG
M. magneticum        1  MRVLVVEDDQLMSRAIESMIKAEGVVVDTTALGEDGLEIGKLYDYDIILLDLMLPDMDGYEVLRRLRSARIDTPVLILSG

KLH11               81  ADDTESKIKGFGFGADDYLTKPFHREELVARIHAIIRRSKGHSQSVIHTGQVAVNLDAKTVEVDGKTVHLTGKEYQMLEL
Silicibacter sp. TM1040 81  ADDTENKIKGFGFGADDYLTKPFHREELVARIHAIIRRSKGHSQSIIKTGKISVNLDAKTVDVEGKSVHLTGKEYQMLEL
C. crescentus       81  SSEILTKVKTFAGGADDYMTKPFHKDEMIARIHAVVRRSKGHAQSVIKTGDIVVNLDAKTVEVNGNRVHLTGKEYQMLEL
A. tumefaciens      81  MAGIEDKVRGLGFGADDYMTKPFHKDELVARIHAIVRRSKGHAQSVISTGELIVNLDAKTVEVGGQRVHLTGKEYQMLEL
Rhodobacter capsulatus 81  ADDTENKIKGFGFGADDYMTKPFHREELVARIHAIIRRSKGHSQSIIRTGKISVNLDAKTVEVGGKPVHLTGKEYQMLEL
M. magneticum       81  LSEPDKKIKGLGSGADDYLTKPFDKGELVARIQAIVRRSKGHAQSVIDTGKLSVNLDARTVAVTGDPLHLTAKEYGILEL

KLH11              161  LSLRKGTTLTKEMFLNHLYGGMDEPELKIIDVFICKLRKKLSNATAGENYIETVWGRGYVLRDPQSNG-MSEPRLAVGA
Silicibacter sp. TM1040 161 LSLRKGTTLTKEMFLNHLYGGMDEPELKIIDVFICKLRKKLSTATDGENYIETVWGRGYVLRDPQEDLSAGGPRMAVGA
C. crescentus      161  LSLRKGTTLTKEMFLNHLYGGMDEPELKIIDVFICKLRKKLAASAHGKHHIETVWGRGYVLRDPNEQVNAA--------
A. tumefaciens     161  LSLRKGTTLTKEMFLNHLYGGMDEPELKIIDVFICKLRKKLANAAGGANYIETVWGRGYVLREPDGAAEFLETA-----
Rhodobacter capsulatus 161 LSLRKGTTLTKEMFLNHLYGGMDEPELKIIDVFICKLRKKLAEVTGGENYIETVWGRGYVLRDPDQGDL--DRRMVVGA
M. magneticum      161  LSLRKGQTLTKEQFLNHLYGGIDEPELKIIDVFICKLRKKLATATGGESYIETVWGRGYVLRDPDMPAVNSNKAGAAE-
```

Figure 4.2. Alignment of KLH11 CtrA amino acid sequence to selected CtrA homologues. The degree of shading is determined by using software BOXSHADE. The helix–turn–helix DNA-binding motif is boxed with a dashed line. The conserved Asparate residue is indicated with an asterisk above. Amino acid numbers for each CtrA protein are shown on the left. The GenBank accession numbers for sequences used in this alignment are shown in Fig. 4.5.

To test whether *cckA, chpT and ctrA* genes are essential, we attempted to generate Campbell insertions to disrupt the KLH11 *cckA*, *chpT* and *ctrA* genes using the pVIK112 suicide plasmid (Kalogeraki and Winans, 1997) carrying truncated, internal fragments of each of the three genes (nt 1031-1537 of the *cckA* gene, nt 24-478 of the *chpT* gene, nt 55-518 of the *ctrA* gene). Presumptive kanamycin resistant (Km[R]) recombinants were readily isolated for all *cckA, chpT and ctrA* genes and the integration of the mutagenic plasmids was confirmed by PCR amplification and sequencing. Strikingly, in contrast to their essential role in *C. crescentus*, growth curves of the *cckA*⁻, *chpT*⁻ and *ctrA*⁻ null

mutants were similar to that of wild type KLH11 (Fig. 4.3). Taken together, these results show conclusively that the *cckA*, *chpT* and *ctrA* genes in *Ruegeria* sp. KLH11 are non-essential and do not affect bacterial growth under laboratory conditions.

We tested the c*ckA⁻*, *chpT⁻* and *ctrA⁻* null mutants on MB2216 (supplemented with 0.25% agar) swim plates and found that these three null mutants cannot migrate from the inoculation site, unlike the wild-type *Ruegeria* sp. KLH11 that demonstrates motility under these test conditions (Fig. 4.1B). Provision of plasmid-borne *cckA*, *chpT* and *ctrA* genes expressed from the *lac* promoter (P_{lac}-*cckA*, pJZ006; P_{lac}-*chpT*, pJZ007; P_{lac}-*ctrA*, pJZ008) was able to partially restore motility in the corresponding *cckA⁻, chpT⁻* and *ctrA⁻* null mutants in the presence of 200 µM isopropyl-b-D-1-thiogalactopyranoside (IPTG) to induce P_{lac}. Microscopic examination of these liquid cultures also revealed no detectable motility for these three mutants (data not shown). Results of a flagellar stain of stationary cultures showed that these three mutants did not synthesize any flagella, in contrast to the wild type (Fig. 4.4A). Antiserum raised against whole flagella from *C. crescentus*, a related alpha-proteobacterium, was able to recognize KLH11 flagellin protein encoded by the *fliC* gene, of approximately 41.5 kDa in size (Zan *et al*., 2011a; Zan *et al*., 2012)(See Appendix 1) and was used in the western blot assay. Results showed that none of the three null mutants produced any detectable flagellin protein (Fig. 4.4B).
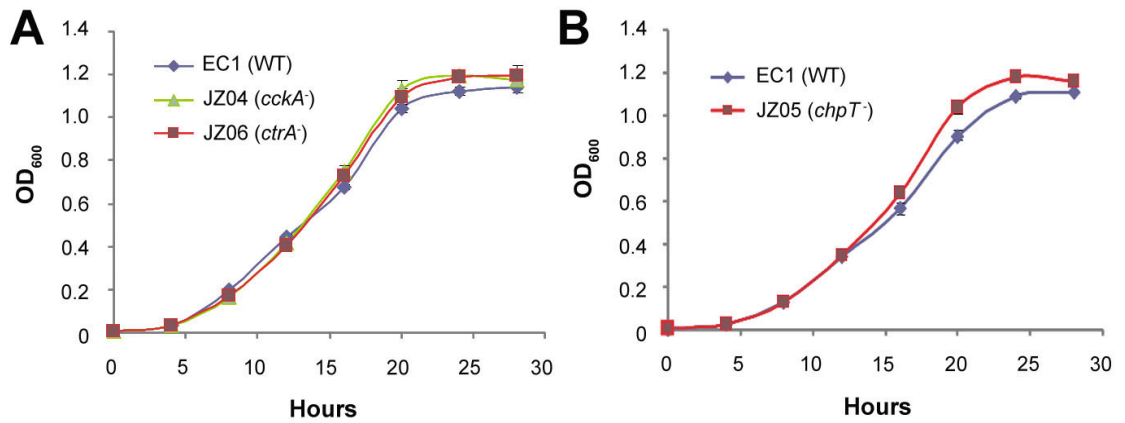
Figure 4.3. Growth curves of wild-type KLH11 (EC1) and derivatives. Values are average (standard deviation) of triplicate samples. Growth curves in (A) and (B) were examined separately. Strains were inoculated in 50 ml MB2216 in a 250 ml flask. At indicated time point, 200 µl was sampled and OD_{600} was measured on a SpectrMax M5 microplate reader (Molecular Devices, Sunnyvale, CA).
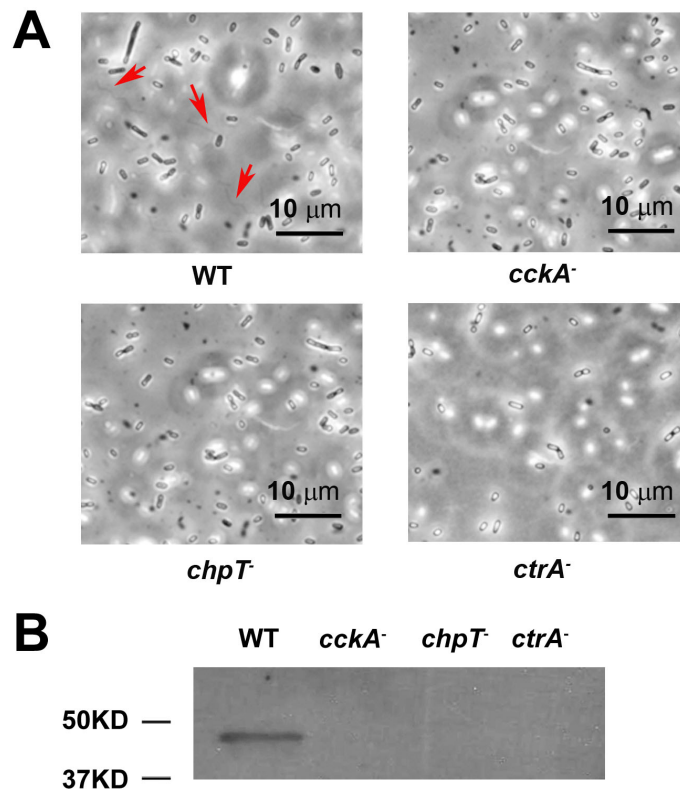
Figure 4.4. **Detection of flagella and flagellin. A)** Flagellar stain of wild type KLH11, *cckA⁻* , *chpT⁻* and *ctrA⁻* null mutants. Stained cells from late stage cultures were viewed under phase contrast microscopy with 100X lens. Wild type (EC1), *cckA⁻* (JZ04), *chpT⁻* (JZ05), *ctrA⁻* (JZ06), red arrows indicate stained flagella. The bar indicates 10 µm**. B)** Detection of flagellin in wild type KLH11, *cckA⁻* , *chpT⁻* and *ctrA⁻* null mutants. Antibody raised against *C. crescentus* whole flagella was used to probe for flagellin. Samples were collected at stationary phase. Flagellin was extracted from 3 ml late stage culture culture from each of the 4 strains with similar OD_{600}. The extraction was dissolved in 100 µl 1X sample buffer and boiled for 5 min. 30 µl was loaded onto each lane. Estimated size of KLH11 flagellin is 43 kDa. Figure 4.4A provided by Jason E. Heindl.

4.4.2 CtrA regulates motility-related gene expression but not cell cycle-related genes.

CtrA regulates expression of a wide range of genes involved in different cellular processes in several bacterial species (Laub *et al.*, 2002; Mercer *et al.*, 2010). We used quantitative reverse transcription-PCR (qRT-PCR) to detect the expression differences of motility-related genes between wild type KLH11 and *ctrA*⁻ mutant. Five genes: *motB, fliL, flgB, flgJ* and *fliG,* which are the first genes in their predicted motility-related operons, and the *flhA* gene, which is the second gene in its operon (the presumptive first gene is not homologous to any known motility genes), were chosen for analysis. The flagellin gene (*fliC*) was also selected for testing. All the predicted motility-related genes we tested were significantly decreased in the *ctrA*⁻ mutant, ranging from 9- to 93- fold differences between wild type KLH11 and the *ctrA*⁻ mutant (Table 4.3). Provision of plasmid-borne KLH11 CtrA (*P$_{lac}$-ctrA*) into the *ctrA*⁻ mutant restored the expression levels almost to those in wild type KLH11. We similarly tested CtrA regulation of the cell cycle related genes *ftsZ* (GenBank No. ZP_05121748) and *ccrM* (GenBank No. ZP_05124520), orthologues of which are CtrA-controlled in *C. crescentus*. It is clear that under the conditions we examined, CtrA does not regulate the expression of the *ftsZ* or *ccrM* genes (Table 4.4).

Table 4.3. Quantification of motility-related gene expression by qRT-PCR.

Gene name	Putative gene class	Wild-type [1,2]	*ctrA*[-1]	Plasmid-borne CtrA[1,2]
fliL	2	456 (91)	12 (3)	535 (255)
fliF	2	323 (65)	5 (1)	299 (75)
flgB	2	746 (219)	8 (3)	602 (176)
flhA	2	9 (3)	1 (1)	7 (1)
flgJ	3	154 (28)	10 (1)	174 (67)
fliC	3	93 (28)	7 (<1)	51 (16)
motB	3	285 (56)	16 (2)	323 (103)

[1]Value relative to the *rpoD* gene. Average of three biological replicates (standard deviation). The values are multiplied by 1000.

[2]All P values are < 0.05 when compared the indicated column to the *ctrA*[-] column.

Table 4.4. Quantification of *ftsZ* and *ccrM* expression by qRT-PCR

Gene name	Wild-type [1]	*ctrA*[-1]
ftsZ	9.9 (3.9)	10.9 (3.2)
ccrM	1.3 (0.4)	1.5 (0.3)

[1] Value relative to the housekeeping gene *rpoD*. Mean (standard deviation). The results presented are representative of two independent experiments each with three biological replicates.

4.4.3 CtrA autoregulates its own transcription but not that of the

cckA gene

KLH11 CtrA has an identical amino acid sequences in the putative helix-turn-

helix DNA sequence recognition region to that of *C. crescentus* (Fig. 4.2). The

DNA sequences with which this CtrA protein interacts, have been well

characterized as TTAA-N7-TTAAC (full site) and TTAACCAT (half-site) in *C.*

crescentus. However, it is clear that the CtrA protein can also bind to more

degenerate sequences that appear to share only the TTAA sequences (Laub *et*

al., 2002). Examination of the sequences upstream of the predicted *ctrA*

translation start site revealed a putative half site (62 bp upstream of the predicted

translational start, Fig. 4.1A) and thus we tested whether CtrA autoregulates its

own expression. The plasmid integration used to disrupt the *ctrA* gene (pJZ005

derived from pVIK112) simultaneously generates a transcriptional fusion to the

disrupted gene (Kalogeraki and Winans, 1997). The P_{lac}-*ctrA* plasmid (pJZ008)

and a vector control (pSRKGm) were conjugated in parallel into strain JZ06 (*ctrA-*

lacZ). Under the 200 µM IPTG induction of the P_{lac}-*ctrA* plasmid, there was a

statistically significant, yet modest ~50% increase of *ctrA* expression (P<0.05)

compared to the vector control (Fig. 4.1C).

Inspection of the *cckA* upstream sequences for CtrA binding sites identified

one putative CtrA full recognition site (62 bp upstream of the predicted

translational start) and one half site (51 bp upstream of the predicted translational

start), although these two sites overlap (Fig. 4.1A). We used a similar approach

to that described above to test whether CtrA affects *cckA* expression. However,

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we reasoned that the *cckA* gene might be required to generate the

phosphorylated CtrA capable of regulating the *cckA* promoter. Therefore instead

of using the strain JZ04 with a disrupted *cckA* gene fused to *lacZ* on the

integrated plasmid, we created strain JZ13 in which the wild type *cckA* gene is

retained, but transcriptionally fused to *lacZ* carried on the integrated plasmid (see

Experimental procedures). Introduction of the P_{lac}-*ctrA* plasmid into JZ13 did not

alter *cckA* expression (Table 4.5). Inspection of the *chpT* upstream region for the

CtrA binding sites did not identify sequences similar to either the full site or the

half site.

Table 4.5. Regulation of *cckA* by *ctrA*.

Strain, Genotype	Expression plasmid	β-galactosidase Sp. Act[1]
cckA-lacZ, WT *cckA* (JZ13)	Vector (pSRKGm)	14.5 (0.9)
cckA-lacZ, WT *cckA* (JZ13)	P_{lac}-*ctrA* (pJZ008)	13.0 (0.1)

[1] Specific activity in Miller units, averages of assays in triplicate (standard

deviation) and representative results of two independent experiments each with

three biological replicate. IPTG concentration was 200 μM.

4.4.4 Cross complementation between KLH11 and *A.*

tumefaciens homologues

Phylogenetic analysis showed that the *ctrA* gene of KLH11 falls into the non-

essential group (Fig. 4.5A) of the two proposed by Greene *et al*. (2012). In

contrast, the *ctrA* homologue in *A. tumefaciens* is within the predicted essential

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group, and disruption of this gene is not possible unless a second copy of *ctrA* is also provided (J.E. Heindl and C. Fuqua, unpublished). We introduced an expression plasmid that carries the full-length *ctrA* gene from *A. tumefaciens* expressed from the P_{lac} promoter (pJEH028) into the *Ruegeria* sp. KLH11 *ctrA*⁻ mutant (JZ06) and determined if this *A. tumefaciens* CtrA protein can restore motility. Strikingly, provision of the *A. tumefaciens* CtrA can restore motility in the *ctrA*⁻ mutant to the same extent as the KLH11 *ctrA* gene (P>0.05) (Fig. 4.5B). Similarly, the plasmids that carry the full-length *cckA* gene (pJEH010) and *chpT* gene (pJEH027) of *A. tumefaciens* were introduced into the KLH11 *cckA*⁻ (JZ04) and *chpT*⁻ (JZ05) mutants, respectively, and also partially restored motility at levels slightly lower than the KLH11 *cckA* and *chpT* genes can (P<0.05) (Fig. 4.5B). However, the *A. tumefaciens cckA* plasmid failed to restore the motility in KLH11 *cckA*⁻ mutant roughly in 30% of our experiments suggesting there may be additional variables that we are not currently controlling (data not shown).
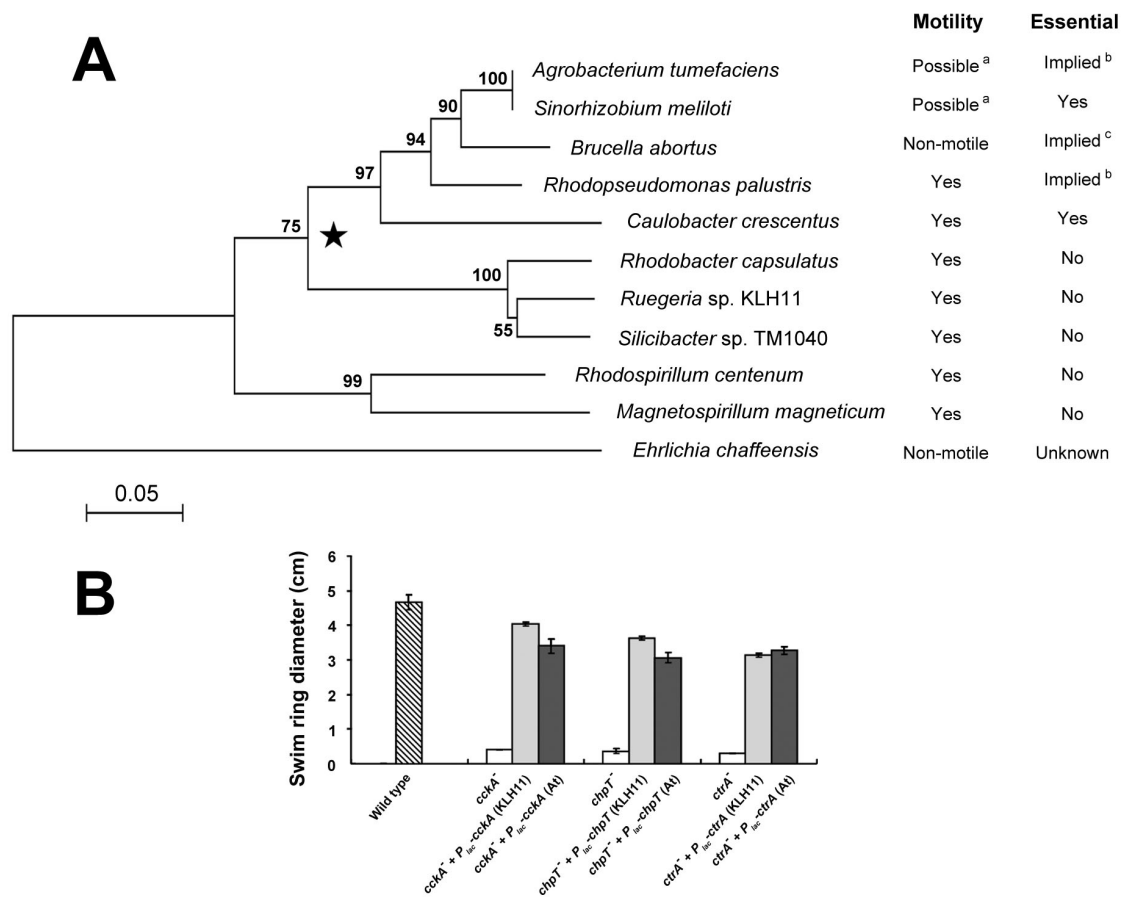
Figure 4.5. **Comparative analysis of KLH11 CtrA.** **A)** Phylogenetic analyses

of CtrA from members of *Alphaproteobacteria*. CtrA sequences from bacterial

species in which CtrA has been studied were chosen for phylogenetic anaylsis.

The sequences used were *A. tumefaciens* C58 (GenBank Accession No.

NP_355385), *B. abortus* (AAL86376), *C. crescentus* (NP_421829), *E.*

chaffeensis (YP_507798), *Magnetospirillum magneticum* AMB-1 (YP_419992),

Rhodopseudomonas palustris (NP_946978), *Rhodobacter capsulatus*

(AAF13177), *Rhodospirillum centenum* (YP_002297962), *Ruegeria* sp. KLH11

(ZP_05124475), *Silicibacter* sp. TM1040 (YP_613394) and *Sinorhizobium*

meliloti (NP_386824). The star indicates the divergence between organisms in

which CtrA is essential or implied to be essential and in which CtrA does affect viability, which was originally proposed by Green *et al.* (2012). a=motility-related genes are enriched in putative CtrA binding sites (Brilli *et al.*, 2010); b=unable to obtain a ctrA deletion mutant without providing an extra copy of the *ctrA* gene (Heindl and Fuqua, unpublished results; Mercer, *et al.*, 2010); c=gene target (*ccrM*) of CtrA is essential (Robertson *et al.*, 2000). The scale bar indicates the number of amino acid substitutions per site. **B)** Cross complementation of motility between KLH11 and *A. tumefaciens* homologues. Wild-type KLH11 (EC1) and derivatives were inoculated on MB2216 (supplemented with 0.25% agar) swim agar plates for about 8 days at 28°C. 200 µM IPTG was added to the media. The diameter of the swim ring was measured. Parentheses indicate from which species the relevant homologue is used (At stands for *A. tumefaciens*). Values are averages of assays performed in triplicate and error bars are standard deviations.

4.4.5 The SsaRI quorum sensing system regulates the transcription of *ctrA, chpT* and *cckA* genes

In KLH11, the QS circuit *ssaRI* controls flagellar motility (Zan *et al.*, 2012). We therefore tested whether *ssaRI* regulates the expression of the *ctrA*, *chpT* and *cckA* genes. Campbell-type insertions in the *ctrA*, *chpT* and *cckA* genes using the suicide vector pVIK112 with internal fragments of each gene created null mutants and simultaneously generated *lacZ* transcriptional fusions to the disrupted gene (Kalogeraki and Winans, 1997). We used β-galactosidase assays to compare the expression of *ctrA*, *chpT* and *cckA* genes in Δ*ssaI* and

Δ*ssaR* deletion mutants, respectively, from cultures grown to an $OD_{600} \sim 0.6$.

Expression of the *ctrA-lacZ* fusion was decreased approximately 25-fold in both

the Δ*ssaI* and Δ*ssaR* mutants (Fig. 4.6A; P<0.01). The *chpT-lacZ* (Fig. 4.6B) and

cckA-lacZ (Fig. 4.6C) fusions were also decreased significantly for the Δ*ssaI* and

Δ*ssaR* mutants, but less dramatically for *chpT* (2 fold for both mutants; both with

P<0.05) and 2-6 fold for *cckA* (Δ*ssaI,* 2-fold, P<0.05; Δ*ssaR*, 6-fold, P<0.05)*.*

Ectopic expression of plasmid-borne P_{lac}-*ssaI* and P_{lac}-*ssaR* restored the

expression of *cckA, chpT and ctrA* genes in the corresponding *ssaI* and *ssaR*

mutants to levels closer to wild type.

 AHL synthase gene mutants can usually be rescued by exogenous addition of

the appropriate AHL. The KLH11 *ssaI* mutant motility defect can be partially

restored with exogenous addition of synthetic 3-oxo-C16:1 Δ11-HSL, an AHL

similar to that specified by SsaI (Zan *et al*., 2012). We therefore tested whether

this AHL could rescue *ctrA*, *chpT* and *cckA* expression in the corresponding

mutants. Surprisingly, addition of this AHL failed to restore the expression of the

ctrA, *chpT* or *cckA lacZ* fusions in the Δ*ssaI* mutant (P>0.05, Fig. 4.6A-C). In all

of these Campbell insertions, generation of the fusion also disrupts the gene. To

examine QS-dependent expression of these genes in an otherwise wild type

background, we introduced the following plasmid-borne fusions into the Δ*ssaI*

strain: P_{ctrA}-*lacZ* (pJZ011), P_{chpT}-*lacZ* (pJZ010) and P_{cckA}-*lacZ* (pJZ009). The

expression of these *lacZ* fusions was monitored by β-galactosidase assays in the

presence and absence of 2 μM 3-oxo-C16:1 Δ11-HSL. A 2.5-fold increase for

P~ctrA~-lacZ (P<0.05) and 4-fold increase for *P~cckA~-lacZ* (P<0.05) was observed in

the presence of 2 μM 3-oxo-C16:1 Δ11-HSL (Table 4.6).
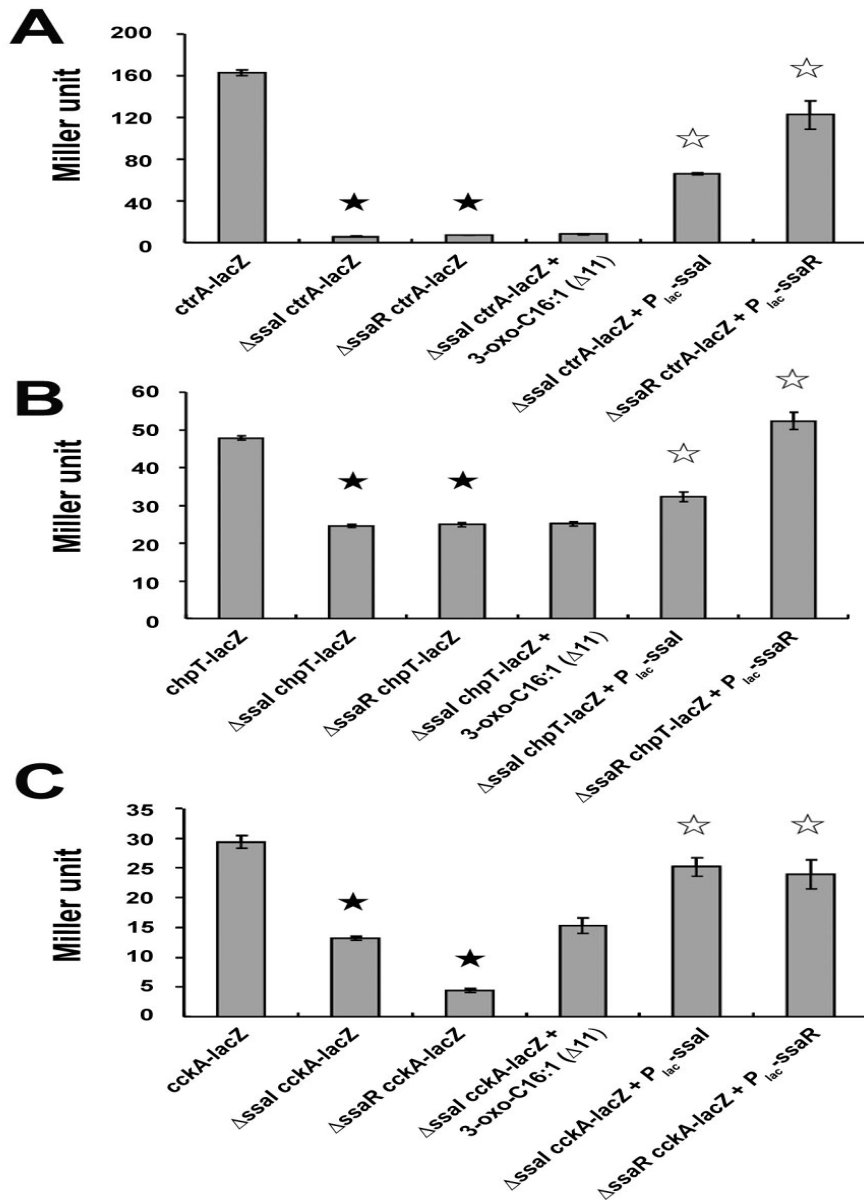
Figure 4.6. Regulation of *cckA, chpT* and *ctrA* gene expression by the *ssaRI* system. Results of β-galactosidase assays in detecting the expression *of ctrA–lacZ* (A), *chpT-lacZ* (B) and *cckA-lacZ* (C) in Δ*ssaI* and Δ*ssaR* mutants. Plasmids P_{lac}-*ssaI* (pEC108) and P_{lac}-*ssaR* (pEC112) were conjugated into the Δ*ssaI* and Δ*ssaR* mutants, respectively, to restore the expression of the *ctrA*, *chpT* and *cckA* genes. 2 μM 3-oxo-C16:1 Δ11-HSL was added into Δ*ssaI ctrA-lacZ,* Δ*ssaI chpT-lacZ and* Δ*ssaI cckA-lacZ* strains, respectively. Filled asterisks indicated statistically significant differences between the indicated strain and

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wild-type quorum sensing strain. Unfilled asterisks indicated statistically significant differences between the quorum sensing complemented strains and quorum-sensing mutants for the expression of the *ctrA*, *chpT* and *cckA* genes. Representative results of several independent experiments each with three biological replicates are presented. Addition of exogenous AHLs failed to restore expression of *ctrA, chpT* and *cckA* in Δ*ssaI* backgroud (P>0.05). Values are averages of assays performed in triplicate and error bars are standard deviations.

Table 4.6. Complementation of P_{cckA}, P_{ctrA} expression by exogenous AHL.

Fusions	Wild type[1]		Δ*ssaI*[1]	
	No AHL	**+AHL**[2]	**No AHL**	**+AHL**[2]
P_{cckA}-*lacZ* (pJZ009)	48 (2.3)	48.5 (2.6)	1.6 (0.2)	8 (0.3)[3]
P_{ctrA}-*lacZ* (pJZ011)	182.6(15.7)	182.6 (15.6)	2.9 (0.6)	7.1 (0.4)[3]

[a] β-Galactosidase activity was expressed in Miller units.

[b] 3-oxo-C16:1 Δ11-HSL (2 μM) was added.

[c] P <0.05 when compared to the expression level in Δ*ssaI* strain without AHL.

4.4.6 SsaRI regulate *ctrA*, *chpT* and *cckA* expression indirectly

The gene expression experiments in KLH11 did not allow us to distinguish direct or indirect QS regulation of the CckA-ChpT-CtrA pathway. We therefore electroporated plasmids carrying P_{ctrA}-*lacZ* (pJZ011) and P_{lac}-*ssaR* (pEC112) into the AHL⁻ *A. tumefaciens* NTL4 (Ti-plasmidless) derivative to test whether the QS-dependent expression of *ctrA* was due to SsaR-dependent activation of the *ctrA* promoter. In this same background, SsaR and 3-oxo-C16:1 Δ11-HSL strongly activate the expression of the P_{ssaI} promoter (Zan *et al*, 2012). *A. tumefaciens* NTL4 harboring P_{ctrA}-*lacZ* (pJZ011) plus a vector (pBBR1-MCS5) was used as a negative control. Expression of the P_{ctrA}-*lacZ* fusion was unaffected by addition of 2 μM 3-oxo-C16:1 Δ11-HSL (Table 4.7). These results indicate that SsaR indirectly regulates the expression of P_{ctrA}-*lacZ* and that an intermediary regulator(s) must exist. We used the same approach to test the regulation of *chpT* (P_{chpT}-*lacZ,* pJZ010) and *cckA* (P_{cckA}-*lacZ,* pJZ009) by SsaR with 2 μM 3-oxo-C16:1 Δ11-HSL. These findings suggest that SsaR and 3-oxo-C16:1 Δ11-HSL do not directly activate the expression of *ctrA, chpT* and *cckA* genes (Table 4.7).

Table 4.7. Expression of KLH11 P_{cckA}, P_{chpT} and P_{ctrA} promoters in an AHL⁻ host[1] .

		β-Galactosidase Sp. Act.[2]	
Expression plasmid	**Fusion**	**No AHL**	**+AHL[3]**
Vector (pBBR1-MCS5)	cckA-lacZ (pJZ009)	299.4 (38.0)	326.9 (33.4)
P_{lac}-ssaR(pEC112)	cckA-lacZ (pJZ009)	295.0 (26.6)	328.7 (12.4)
Vector (pBBR1-MCS5)	chpT-lacZ (pJZ010)	33.4 (1.2)	32.5 (1.2)
P_{lac}-ssaR(pEC112)	chpT-lacZ (pJZ010)	24.3 (0.5)	23.3 (1.9)
Vector (pBBR1-MCS5)	ctrA-lacZ (pJZ011)	123.8 (20.2)	139.4 (2.8)
P_{lac}-ssaR(pEC112)	ctrA-lacZ (pJ011)	138.6 (18.6)	137.7 (10.1)

[1] All strains derived from Ti- plasmidless *A. tumefaciens* NTL4.

[2] Specific activity in Miller units, averages of assays in triplicate (standard deviation) and representative results of two independent experiments each with three biological replicates.

[3] 3-oxo-C16:1 Δ11-HSL (2 µM) was added.

4.4.7 Ectopic expression of *ctrA* restores motility to the QS deletion mutant

Provision of either the plasmid-borne P_{lac}-ssaI (pEC108) or P_{lac}-ssaR (pEC112) to the corresponding mutants (Δssal cckA⁻, Δssal chpT,⁻ Δssal ctrA⁻ and ΔssaR cckA⁻, ΔssaR chpT⁻, ΔssaR ctrA⁻) does not restore motility (Fig. 4.7), although they did restore nearly wild type expression levels for each *lacZ* fusion (Fig. 4.6). This is due to the disruption of the targeted gene by the Campbell

insertions. Consistent with our previous studies however (Zan *et al*., 2012) the

P~lac~-*ssaI* (pEC108) or *P~lac~*-*ssaR* (pEC112) plasmids effectively complement

motility in the Δs*saI* and Δ*ssaR* mutants, respectively (Fig. 4.8). This suggests

that *cckA*, *chpT* and *ctrA* are required for motility and act downstream of the

ssaRI system. Accordingly, IPTG-induced expression of the *P~lac~*-*ctrA* (pJZ008) in

Δs*saI* and Δ*ssaR* did however restore motility (Fig. 4.8). Controls with the vector

alone did not correct the motility defect in any of these derivatives (data not

shown). Interestingly, the *P~lac~*-*ctrA* plasmid could not restore motility in the Δ*ssaI*

cckA⁻, Δ*ssaI chpT*⁻, Δ*ssaR cckA*⁻ or Δ*ssaR chpT*⁻ mutants, respectively (Fig. 4.9).

Thus, *cckA* and *chpT* genes are required for the suppression of the Δ*ssaI* or

Δ*ssaR* mutant phenotypes by CtrA.

$\Delta ssaI\ cckA^-$ + P_{lac}-ssaI $\Delta ssaI\ chpT^-$ + P_{lac}-ssaI $\Delta ssaI\ ctrA^-$ + P_{lac}-ssaI

$\Delta ssaR\ cckA^-$ + P_{lac}-ssaR $\Delta ssaR\ chpT^-$ + P_{lac}-ssaR $\Delta ssaR\ ctrA^-$ + P_{lac}-ssaR
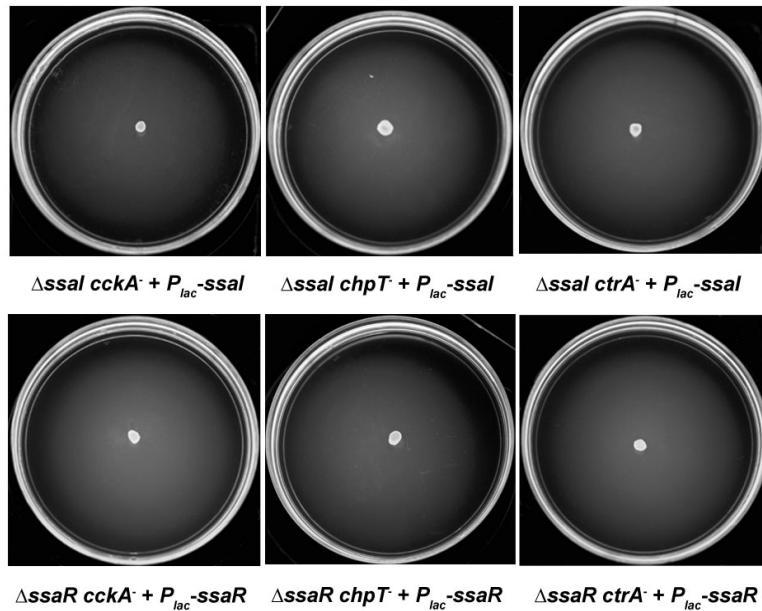
Figure 4.7. The *cckA-chpT-ctrA* phosphorely system is required for the *ssaRI* system to control motility. *P_{lac}-ssaI* (pEC108) was conjugated into $\Delta ssaI\ cckA^-$, $\Delta ssaI\ cphT^-$ and $\Delta ssaI\ ctrA^-$ double mutants and *P_{lac}-ssaR* (pEC112) was conjugated into $\Delta ssaR\ cckA^-$, $\Delta ssaR\ cphT^-$ and $\Delta ssaR\ ctrA^-$ double mutants, respectively. Strains were inoculated on MB2216 (supplemented with 0.25% agar) swim agar plates for about 8 days at 28°C. The results were representatives of several independent experiments each with three biological replicates.
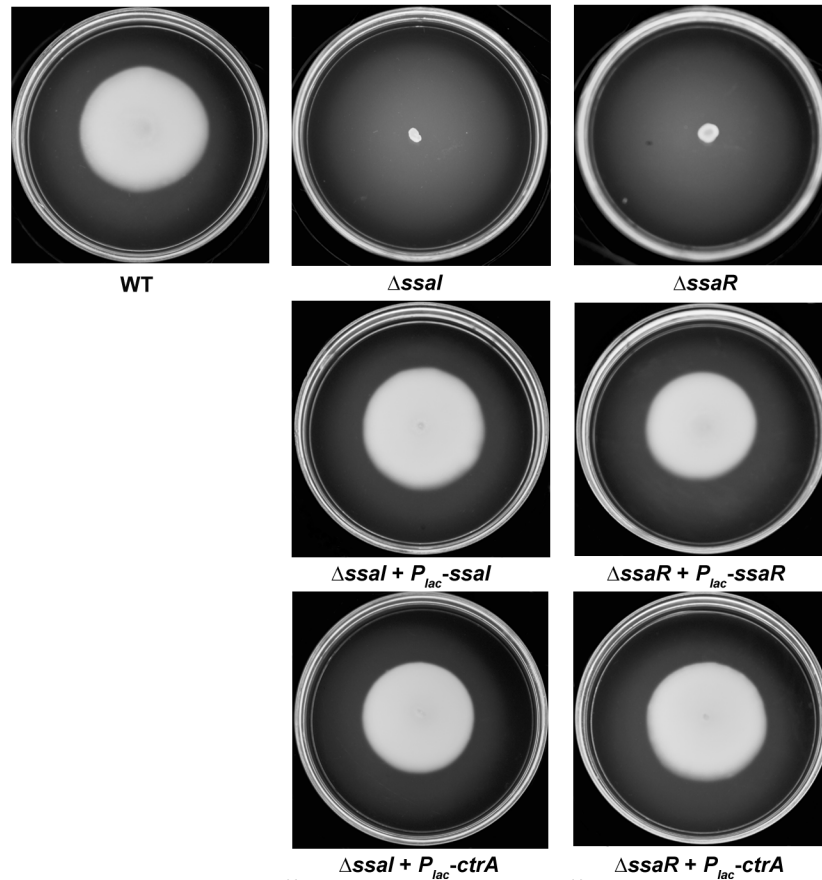
Figure 4.8. **Suppression of motility defects in Δ*ssaI* and Δ*ssaR* mutants by CtrA.** *P$_{lac}$-ctrA* plasmid (pJZ008) was conjugated into Δ*ssaI* and Δ*ssaR* mutants, respectively. The conjugants were selected and inoculated on swim agar plates for 8 days at 28 °C. 200 µM IPTG was added to the media. The Δ*ssaI* mutant complemented with *P$_{lac}$-ssaI* (pEC108) and the Δ*ssaR* mutant with *P$_{lac}$-ssaR* (pEC112) were used as positive controls. Wild type KLH11 (EC1) was used as a positive and the Δ*ssaI* and Δ*ssaR* strains were used as negative control. The results were representatives of several independent experiments each with three biological replicates.
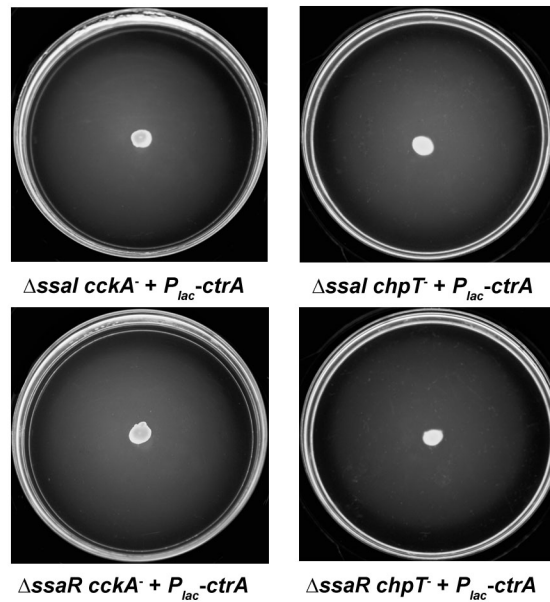
Figure 4.9.The *cckA* and *chpT* genes are required for the function of CtrA.

P~lac~-ctrA was conjugated into Δ*ssaI cckA⁻*, Δ*ssaI chpT⁻,* Δ*ssaR cckA⁻*, and Δ*ssaR chpT⁻* respectively. The conjugants were selected and inoculated for swim motility assay as described above. The results were representatives of several independent experiments each with three biological replicates.

4.5. Discussion

The *cckA-chpT-ctrA* phosphorelay system has been well characterized in *C. crescentus* in which the expression levels of 144 genes are affected due to the loss of the *ctrA* gene (Laub *et al.*, 2000). However, little is known about this phosphorelay system in the highly abundant marine *Roseobacter* clade. In several alphaproteobacterial systems, *cckA* and *ctrA* are essential, although little is known about the essentiality of the *chpT* gene other than in *C. crescentus*, *R. capsulatus* and *Silicibacter* sp. TM1040 (Biondi *et al.*, 2006; Belas *et al.*, 2009; Mercer *et al.*, 2012). However, our results clearly reveal that all the *cckA⁻*, *chpT⁻* and *ctrA⁻* mutants have growth rates nearly identical to that of wild type KLH11, demonstrating that they are non-essential under laboratory conditions. This observation is consistent with the function of *cckA* and *ctrA* in *R. capsulatus* (Lang and Beatty, 2000), *Silicibacter* sp. TM1040 (Belas *et al.*, 2009), and *R. centenum* (Bird and MacKrell, 2011). The *ctrA* gene is not essential in *Magnetospirillum magneticum* AMB-1 (Greene *et al.*, 2012) but the essentiality of *cckA* has not been examined. Moreover, qRT-PCR revealed that KLH11 CtrA did not regulate the expression of the *ftsZ* or *ccrM* genes in KLH11 (Table 4.4). In *C. crescentus* CtrA directly regulates the expression of genes involved in cell division such as *ftsZ*, encoding the primary division protein, and *ccrM*, a DNA methyltransferase that modifies sequences at the replication origin to coordinate the timing of genome replication with the cell division cycle (Laub *et al.*, 2002). Taken together, the non-essentiality of *ctrA* and the lack of regulation for *ftsZ* and

ccrM indicate that CtrA probably does not play a role in cell cycle control in KLH11.

Our findings provide evidence that CckA, ChpT and CtrA activate swimming motility and control the biosynthesis of flagella (Figs 4.1 and 4.4), similar to a portion of their roles in *C. crescentus*, although the intimate relationship between the cell cycle and flagellation confounds this role (Quon *et al*., 1996, Laub *et al*., 2000). Furthermore, qRT-PCR revealed *ctrA* to regulate all the motility-related genes that were checked. We searched for both full and half *ctrA* binding sites using the motifs as determined from Laub *et al*. (2002) in the upstream regions of these motility-related genes, but no sequences with high similarity were identified. This is similar to the situation in *R. capsulatus*, in which expression of motility related genes is decreased in a Δ*ctrA* strain but none of these genes have clear *ctrA* binding motifs (Mercer *et al*., 2010).

Identification of presumptive CtrA recognition sites in the upstream of *ctrA* suggested feedback on its own transcription. Our results indeed showed the positive feedback on the *ctrA* expression. This is consistent with findings in *C. crescentus*, although in that bacterium CtrA negatively regulates one promoter (P1) and positively regulates a second (P2) (Domian *et al.*, 1999). It is unclear whether or not the KLH11 *ctrA* gene has multiple promoters. In *S. meliloti*, CtrA~P can bind proximal to its two *ctrA* promoters, presumably regulating the transcription of the *ctrA* gene (Barnett *et al.*, 2001). Similarly, in the obligatory intracellular pathogen *Ehrlichia chaffeensis*, the CtrA protein can also bind proximal to its own promoter (Cheng *et al.*, 2011). In contrast, for *R. capsulatus*,

ctrA-binding site was identified in the *ctrA* promoter region (Lang and Beatty, 2000, but CtrA does not affect its own transcription (Leung *et al*., 2013), suggesting that this autoregulatory loop is not conserved in all bacteria with CtrA. A search of the promoter region of the *cckA* gene also identified presumptive *ctrA* binding sites (Fig. 4.1A), suggesting that CtrA could potentially regulate the expression of the *cckA* gene. However, provision of *ctrA* in the KLH11 derivative with the integrated *cckA-lacZ* that maintains an intact copy of *cckA* (JZ13) does not affect *cckA* gene expression (Table 4.6). This is similar to *R. capsulatus*, in which the expression of *cckA* gene is not affected in a Δ*ctrA* strain, although interestingly, loss of the *ctrA* gene leads to a decrease of CckA protein levels (Mercer *et al*., 2010). We do not know whether the loss of the *ctrA* gene would affect the amount of CckA protein in KLH11. This also emphasizes that the presence of upstream sequences with similarity to CtrA binding sites does not necessarily mean that the associated gene (in this example *cckA*) is regulated by CtrA. Taken together, this likely reflects the limits on current understanding of what comprises a CtrA binding site outside of *C. crescentus*.

Greene *et al*. (2012) proposed two groups of CtrA in the *Alphaproteobacteria*: in one group *ctrA* is essential and in the other it is non-essential, but in both groups it exerts control over motility. KLH11 *ctrA* clearly falls into the non-essential group by sequence comparisons (Fig. 4.5A). Interestingly, although the *cckA-chpT-ctrA* pathway is essential in *A. tumefaciens* (Kim *et al*., submitted), plasmid-borne expression of each of these *A. tumefaciens* genes can cross-complement the corresponding mutants in KLH11 for their impact on motility.

This cross-complementation suggests that the functionality of this pathway is well conserved and its role in controlling motility is ancestral among the *Alphaproteobacteria*. These proteins have retained their basic activities, even though the influence of this pathway can be expanded to include essential functions. It remains unclear whether the pathway's essentiality is derived or ancestral among the *Alphaproteobacteria*. The CckA protein from *A. tumefaciens* shows inconsistent complementation in the KLH11 *cckA*[-] mutant, which hints at an additional signal(s) that may impact the activity of the *A. tumefaciens* CckA protein.

Our results clearly show that the *cckA-chpT-ctrA* phosphorelay system is indirectly transcriptionally regulated by the SsaRI quorum sensing circuit. In *C. crescentus*, the transcription of the *cckA* gene is cell cycle dependent, but not affected by CtrA (Laub *et al.*, 2000; Laub *et al.*, 2002). Moreover, the level of the CckA protein is constant during the cell cycle whereas the phosphorylation of CckA is subject to temporal and spatial regulation (Jacobs *et al.*, 1999; Jacobs *et al.*, 2003). We do not know what signal(s) it is to which the CckA protein responds. However, it is plausible that the CckA protein may sense population density-associated signals and thus it can coordinate the activation of motility with the cell density. Of note, the Δ*ssaR* mutant exhibits a more profound deficiency in the *cckA* expression than the Δ*ssaI* mutant (Fig. 4.6C). One explanation is that SsaR is able to respond to the AHL levels synthesized in the Δ*ssaI* mutant, in which the *ssbRI* system remains genetically intact (Zan *et al.*, 2012). Meanwhile, the activity of CtrA, a key factor in driving the cell cycle, is

tightly regulated at the levels of transcription, phosphorylation, degradation, and protein-protein interaction (Gora *et al.*, 2010). On the transcriptional level, the *C. crescentus ctrA* gene is activated by the two-component type response regulator GcrA (Holtzendorff *et al.*, 2004). In *R. capsulatus*, it was found that the LuxR-holomogue GtaR indirectly represses the transcription of *ctrA* while the AHL synthesized by GtaI derepresses its transcription (Leung *et al.*, 2013). However, it was unclear whether QS affects the transcription of *cckA* and *chpT* genes in this bacterium.

Interestingly, we can complement the expression of *ctrA*, *chpT* and *cckA–lacZ* fusions as Campbell insertions in the Δ*ssaI* background by providing the *ssaI* gene in trans but were not able to restore their expression by addition of exogenous synthetic 3-oxo-C16:1 Δ11-HSL (Figs. 4.6A-C). It is known that addition of AHL into Δ*ssaI* is able to partially restore motility (Zan *et al.*, 2012) and data in this study clearly show that CtrA acts downstream of the *ssaRI* system to control flagellar assembly and motility. We reason that two factors can contribute to this observation: 1) The long chain AHL we added might not be able to partition into the cell from exterior efficiently due to its hydrophobicity. It has been shown that the long chain AHLs preferentially associate with the cell rather than being released extracellularly and that AHLs that partition into the cell membrane may not function as signals (Schaefer *et al.*, 2002). Addition of the same AHL can stimulate the expression of the *ssaI* gene (Zan *et al.*, 2012); however, the stimulatory effect of AHL on *ssaI* might not be propagated onto the *cckA-chpT-ctrA* effectively because of the indirect regulatory link between *ssaRI*

and *cckA-chpT-ctrA*. 2) There might be some positive feedback on the *ssaRI* system by this pathway. In the Campbell insertion, the gene is disrupted. It is possible that an intact copy of this pathway is required for optimal expression. Indeed, the significant, yet weak activation of the plasmid borne fusions by the addition of AHL in the Δ*ssaI* strain in which *cckA-chpT-ctrA* are intact supports this speculation (Table 4.6).

Ectopic expression of *ctrA* in either the Δ*ssaI* or Δ*ssaR* mutant can restore motility. However, failure to restore motility in either Δ*ssaI cckA⁻* or Δ*ssaR cckA⁻* by provision of *ctrA* suggests that phosphorylation by CckA is required for the function of CtrA, although it is possible that CckA can also phosphorylate other regulator (s), which is also required for motility. Furthermore, *chpT* is also required for the function of CtrA since providing *ctrA* into Δ*ssaI chpT⁻* or Δ*ssaR chpT⁻* mutants does not restore motility. It was plausible that high levels of CtrA expression from the *P$_{lac}$-ctrA* plasmid might mask the absence of the upstream components of the pathway, CckA and ChpT, via phosphorylation-independent CtrA activity. *C. crescentus* CtrA can bind to some sites in a phosphorylation-independent manner *in vivo* (Spencer *et al.*, 2009) and several different response regulators exhibit phosphorylation-independent activity (Ma *et al.*, 1998; Schär *et al.*, 2005). CtrA might crosstalk with other phospho-donors such as other kinases or acetyl phosphate. However, it is clear that the intact CckA-ChpT phosphorelay is required for CtrA motility control. Taken together, our data support the model shown in Figure 4.10. SsaRI acts upstream of the *cckA-chpT-ctrA* phosphorelay system and indirectly regulates the transcription of all the

three genes, most dramatically through *ctrA* expression. CckA and ChpT are required via presumptive phosphotransfer to CtrA, which positively feeds back on its own expression, and controls flagellar assembly and motility. CtrA could thus be the potential flagellar master regulator in KLH11. This is similar to the model reported in the rice pathogen *B. glumae*, in which the *tofRI* QS pathway controls the regulator *qsmR* which in turn directly controls the flagellar master regulator *flhDC* (Kim *et al*., 2007). However, the presumptive regulator that links SsaRI to *ctrA* remains to be identified in KLH11. Our data also suggests that there may be feedback from the CckA-ChpT-CtrA pathway on the SsaRI system.

Roseobacters are a key marine bacterial group with biogeochemical relevance and are also commonly found as symbionts of marine invertebrates, including sponges. Roseobacters are often highly abundant in phytoplankton blooms or near macroalgae and associated with organic particles (Slightom and Buchan, 2009). Motility is likely to be critical in many of these interactions. Our previous work contributed to understanding the role of quorum sensing in activating motility specifically at high cell density in the sponge-associated Roseobacter, *Ruegeria* sp. KLH11 (Zan *et al*. 2012), raising the possibility that this is a widespread mechanism in Roseobacters**.** In KLH11, flagellar motility is controlled by the SsaRI system and AHL quorum sensing. Surprisingly, the KLH11 genome and that of its relative *R. pomeroyi* DSS-3 lack any recognizable chemotaxis genes (Moran *et al.,* 2004; Zan *et al.,* 2011a)(See Appendix 1). Therefore QS regulation of motility is not simply augmenting the process, but appears to be its primary control mechanism. SsaR and long chain AHLs are

required for *cckA, chpT* and *ctrA* gene expression, revealing at least a portion of

this central control pathway, although additional environmental signals may also

function through the CckA-ChpT-CtrA cascade. The work reported here provides

a discrete regulatory link between flagellar locomotion and population density in
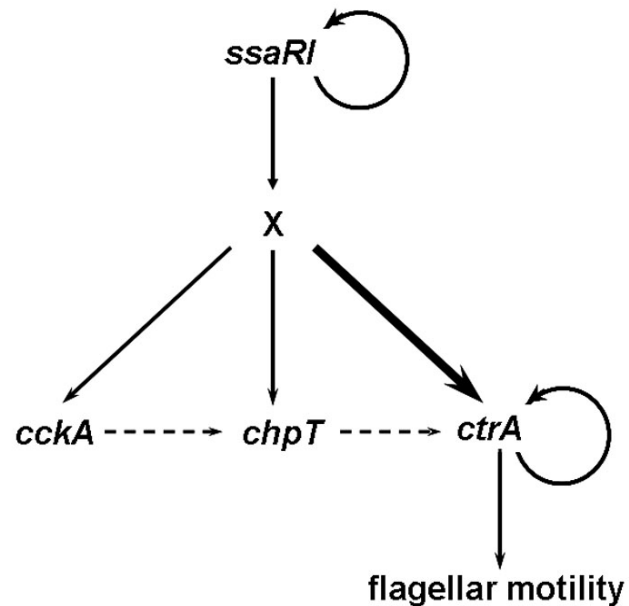
KLH11.

Figure 4.10. **A tentative model for the *ssaRI* to *cckA-chpT-ctrA* regulatory**

circuit to control KLH11 flagellar motility. The solid lines with the arrows

indicate activation. The dashed line with arrows indicates the potential

phosphate flow from CckA to CtrA via ChpT. The curved lines with arrows

around CtrA and SsaRI indicate positive feedback loops. The "X" indicates the

unknown regulator (s). The thicker line from X to *ctrA* indicates stronger

regulatory effect.

Chapter 5. Isolation and screening of AHL- and AI-2-producing bacteria and development of *Ruegeria* sp. KLH11 as a model to study bacterial colonization of sponges

5.1. Abstract

Previous studies have revealed that the sponge symbionts belonging to the roseobacterial *Silicibacter-Ruegeria* (SR) subgroup can produce acylhomoserine lactone (AHL) signal molecules but only a small number of isolates was screened (Mohamed *et al*., 2008c). To confirm and extend this finding, I screened 420 bacterial isolates from *M. laxissima* and *I. strobilina* collected in October 2008, March 2010 and June 2011 for AHL production. The AHL-producing bacteria were identified by 16S rRNA gene sequence analysis. Results showed that 44% of these isolates are able to produce AHLs and 77% belongs to the *SR* subgroup of *Roseobacter* clade. Moreover, these SR isolates were isolated consistently from these two sponges, suggesting the potential importance of these bacteria to their marine sponge hosts. In addition, degenerate primers designed based on known *luxS* sequences from the genus *Vibrio* were able to amplify the *luxS* genes from all the *Vibrio* strains. A biological reporter *V. harveyi* TL-26 was used to detect the activity of AI-2 (synthesized by LuxS) and results showed that all the *Vibrio* isolates were able to turn on light production in the TL-26 biological reporter.

Results of reverse transcription (RT)-PCR showed that the AHL synthase gene *ssaI* is actively expressed in the sponge microbial community and the results of TLC assay coupled with AHL biological reporter assay revealed the presence of AHL molecules in the sponge tissues. Genetically modified KLH11 derivatives were used to investigate the role of QS in the process of bacterial colonization of the sponge *M. laxissima*. An interesting pattern was observed

that the *ssaR* mutant seems to better colonize the sponge compared with the

wildtype.

5.2. Introduction

Quorum sensing can fine tune gene expression in responding to cell density and is commonly found in symbiotic or pathogenic bacteria associated with eukaryotic hosts (Gurich and González, 2009). It was first described in the non-pathogenic marine luminescent symbiotic bacterium *Vibrio fischeri* (Nealson *et al.*, 1970). Current QS research mainly focuses on bacterial pathogens, such as *V. cholerae*, *P. aeruginosa* and *A. tumefaciens*. Cicirelli *et al*. (2008) suggested that Roseobacters actually might be the dominant AHL producers in the marine environment. For example, the AHL-producing bacteria isolated from the marine sponges *M. laxissima* and *I. strobilina* mainly belong to the *Silicibacter-Ruegeria* subgroup of the *Roseobacter* clade; furthermore, sponges harbor a higher proportion of AHL producers in their culturable bacterial communities compared with the surrounding seawater, although only a small set of bacterial isolates are tested (Mohamed *et al*., 2008c). Marine sponges harbor a high abundance of bacteria in their tissue and thus provide an ideal environment for bacterial quorum sensing. However, prior to this study it was unknown whether the association of AHL-producing bacteria with these sponges is consistent and whether AHL molecules are present in the sponge tissues.

Autoinducer-2 (AI-2) is another well-known molecular cue in bacterial signaling and has been extensively analyzed in *V. harveyi* and *V. cholerae*, where it is involved in regulation of bioluminescence and virulence-associated traits (Ng and Bassler, 2009). The activated methyl cycle is a crucial metabolic pathway to recycle homocysteine from the major methyl donor *S*-adenosyl

methionine (SAM). LuxS, a S-ribosylhomocysteinase, catalyzes part of the cycle and functions to convert S-ribosylhomocysteine to homocysteine; meanwhile, it can also, as a side reaction, synthesize 4,5 dihydroxy-2, 3-pentanedione (DPD), the precursor of AI-2. DPD can spontaneously give rise to several furanone derivatives, collectively referred to as AI-2. The dual roles of *luxS* in metabolism and in AI-2 formation have led to controversy regarding its function in quorum sensing (Rezzonico and Duffy, 2008). The *luxS* gene is broadly distributed in gram-negative and gram-positive bacteria, although it is absent in all of the *Alphaproteobacteria*. Thus, it is interesting to study the *luxS* genes in the bacteria associated with marine sponges, especially *Vibrio* sp., since they represent the best-studied models.

Symbiosis refers to two or more different organisms living closely together and is a widespread phenomenon. Within the wide range of different symbioses, the microbe-eukaryote symbioses have received tremendous attention. Sponge-microbe symbiosis has been developed as a primary model for studying marine symbiosis (Taylor *et al*., 2007). Marine sponges harbor highly abundant and diverse microorganisms that are different from these of the surrounding planktonic communities. Significant progress has been made in understanding the composition and even the dynamics of the sponge communities (Taylor *et al*., 2007; Webster and Taylor, 2012). However, fundamental questions remain unanswered, such as: How is the symbiosis established and maintained? How do the host and microbe select and recognize each other? Does quorum sensing play roles in the symbiotic relationships? Insights have been provided in

several other symbiotic systems. For example, one well-established model is the

V. fischeri-squid symbiosis. Genetic studies have shown that flagellar motility is

required for bacteria to colonize the light organ and nonmotilte strains failed to

colonize the light organ (Graf *et al.*, 1994). The bioluminescence controlled by

QS is also critical for the maintenance of symbiosis (Visick *et al.*, 2000).

Furthermore, it has also been shown that a single bacterial regulatory gene can

determine the host specificity (Mandel *et al.*, 2009). On the other hand, inhibition

of motility by quorum sensing is essential for effective nodule invasion by the

alfalfa symbiont *Sinorhizobium meliloti* and the AHL synthase SinI and the

receptor ExpR seem to play opposite roles in the nodule invasion process

(Gurich and González, 2009). In KLH11, motility is under positive control of

ssaRI QS system although KLH11 does not encode the chemotaxis system in its

genome (Zan *et al.*, 2011a)(See Appendix 1). Importantly, a suite of genetic

tools can be applied to KLH11, which allow us to readily manipulate the genome

and ask important questions (See Chapters 2 - 4). Therefore, we developed

KLH11 as a model strain to study whether QS in KLH11 plays a role in

colonization of its sponge host by this bacterium.

In this study, I aimed at establishing that SR isolates are the important players

for AHL production in marine sponges by screening a large number of bacterial

strains isolated from sponges collected over several different years and then tried

to connect our laboratory findings with the native environment by showing the

presence of AHL molecules in the sponge tissues and expression of the AHL

synthase *ssaI* gene in situ within sponges. Finally efforts were directed towards

building a model for studying the bacterial colonization process.

5.3. Experimental procedures

5.3.1 Sponge collection, bacterial isolation and identification, and AHL screening

Three individuals of each of the marine sponges *M. laxissima* and *I. strobilina* were collected by SCUBA diving at Conch Reef, Key Largo, FL, USA at a depth of *ca.* 20 m in late October 2008, late March 2010 and June 2011. Water samples were collected within 1 m of the sponges at a depth of *ca.* 20 m in a sterile 20-L container. The water salinity was measured to be 36 ppt using a portable refractometer at all three times of collection and the water temperature was *ca.* 28ºC, 24ºC and 30ºC in October 2008, March 2010 and June 2011, respectively. Sponges were rinsed with sterile artificial seawater three times to remove transiently associated bacteria and then processed for isolation of bacteria. Sponge tissue (1 cm^3) was ground in artificial seawater using a sterile mortal and pestle and 10-fold serial dilutions were plated on Marine Agar 2216 (BD Biosciences, Franklin Lakes, NJ). Plates were incubated at 30ºC for 1 week. Approximately 80 colonies were randomly picked and screened for AHL production. The method used for AHL screening was described by Mohamed *et al*. (2008c). Briefly, four strains were arrayed onto one MA2216 plates and were incubated at 30ºC for a week. The reporter *A. tumefaciens* KYC55 was grown to mid-exponential phase in ATGN medium. Cells were harvested by centrifugation at 5000 x g for 10 min and the pellet was washed and resuspended in 30% glycerol to adjust the OD$_{600}$ to ~ 12.0. Then one ml of the concentrated reporter

was added into 100 ml of ATGN with 0.6% agar (W/V) and 20 µg ml^{-1} X-gal.

Twenty -thirty ml of this mixture was overlaid with MA2216 plates and were

incubated at 30ºC for 24-48 h. Serial dilutions of water samples were processed

similarly for bacterial isolation and screening of isolates for AHL production.

The AHL producers were identified by 16S rRNA gene sequencing. Bacterial

genomic DNA from AHL-producing bacteria was extracted from isolates using the

UltraClean Microbial DNA isolation kit (MO BIO Laboratories, Inc. Carlsbad, CA)

following the manufacturer's manual. Almost full-length 16S rRNA gene

fragments were PCR-amplified using universal primers 27F and1492R as

described by Enticknap *et al*. (2006). PCR products were sequenced using an

ABI PRISM 3130xl genetic analyzer and primers 27F and 1492R. Sequences

were assembled using online software CAP3 (http:// pbil.univ-lyon1.fr/cap3.php)

with a manual check. Chimeric sequences were identified by using the

CHECK_CHIMERA program of the Ribosomal Database Project (Maidak *et al.*,

1999) and the sequences were analyzed initially by using the BLASTn tool at the

National Center for Biotechnology Information website (NCBI). Partial 16S rRNA

gene sequences of all the AHL positive bacteria were aligned using Clustal X

2.0.12 (http://www. clustal.org/) and a phylogenetic tree was constructed using

software MEGA 4.0 (http://www.megasoftware.net/).

5.3.2 *luxS* gene amplification from the vibrios

Degenerate primers based on an alignment of available *Vibrio luxS* gene

sequences were designed for amplification of *luxS* gene fragments using online

primer design software consensus-degenerate hybrid oligonucleotide primers

(Rose *et al.*, 2003). The primers were designated VluxsF (5'

TGCTGGACTCCTTCACCGTNGAYCAYAC-3') and VluxsR (5'-

TGCATGGCGGCGGTNCCRCAYTGTT-3'). PCR mixtures consisted of 50 µl

containing two units Platinum Taq polymerase (Invitrogen Life Technologies,

Carlsbad, CA), 1X PCR Buffer, 2 mM $MgCl_2$, 200 mM dNTPs (Fermentas, Glen

Burnie, MD), 0.2 µM of each primer and 10–20 ng of genomic DNA or distilled

water as a negative control. PCR cycling conditions for *Vibrio luxS* gene

amplification consisted of 94°C for 5 min followed by 30 cycles of 1 min at 94°C,

1 min at 52°C, and 1 min at 72°C. A final 10 min extension step was done at

72°C. PCR reactions were performed in a PTC-200 cycling system (Bio-Rad,

Hercules, CA). The PCR products with expected size ~ 400 bp were purified

using QIAquick gel extraction kit (Qiagen, Valencia, CA) and sequenced as

described above for 16S rRNA gene fragments using primer VluxsF.

5.3.3 Measurement of AI-2 activity

AI-2 activity was detected by using the reporter strain *V. harveyi* TL-26

generously provided by Drs. Long and Bassler, in which all three signaling

pathways are disrupted. Receptor genes for AHL and CAI-1 are mutated as well

as the *luxS* gene. Thus, TL-26 can respond only to exogenously added AI-2 (not

AHLs or CAI) and cannot produce AI-2 (Long and Bassler; personal

communication). All the *Vibrio* isolates and *V. harveyi* TL-26 were grown

overnight in Luria–Bertani medium with 2% NaCl. All the tested strains were

grown to similar OD_{600}. Culture supernatants of test strains were filter sterilized

(0.22 µm) and 60 µl of sterile supernatant was added to 140 µl AB medium

(Greenberg *et al.*, 1979) containing *V. harveyi* TL-26 (inoculated 1:5000 from an overnight culture). *V. mimicus* ATCC 33653 was used as a positive control. The culture was incubated at 30ºC with shaking at 180 rpm for 16 h and light production was measured by using a FLUOstar OPTIMA fluorescence microplate reader (BMGlabtech, Cary, NC). The induction of luminescence by each tested supernatant was expressed relative to that in a negative control comprising sterile Luria–Bertani medium with 2% NaCl instead of culture supernatant, similar to the method described by Zhu and Mekalanos (2003).

5.3.4 Organic extraction of sponge tissues and TLC overlay assay

AHLs were extracted from sponge tissues using a modified Bligh Dyer procedure (Bligh and Dyer, 1959). In brief, frozen tissues were ground to powder in liquid nitrogen. Ten grams of ground sponge were re-suspended in 10 ml of methanol. The mixture was homogenized by vigorous vortexing and then was left overnight at room temperature. Five ml of chloroform was added to the mixture followed by sonication four times at full power. To completely homogenize the mixture, 5 ml of chloroform and 2.5 ml water were added after sonication. This mixture was further vigorously vortexed and shaken followed by centrifugation at 2,000 rpm for 1 min. The bottom layer was carefully transferred to a glass test tube using 9" disposable glass Pasteur pipettes. To increase the extraction efficiency, 5 ml of chloroform was added to the top layer followed by vigorous vortex for several times and then centrifugation at 2,000 rpm for 1 min. Again, the bottom layer was transferred to the test tubes and was evaporated to

complete dryness. The residue was resuspended in 6 ml of 1:1 isooctane: ethyl

ether for solid phase extraction, as described previously (Gould *et al.*, 2006).

Organic extract from *ca*. 10.0 g tissues of each of three sponge individuals

were dissolved in 40 µl methanol, and 20 µl was loaded onto a C18 RP-TLC

plate (Mallinckrodt Baker, Phillipsburg, NJ). AHLs were detected using the *A.*

tumefaciens ultrasensitive reporter (Zhu *et al.*, 2003) as described in Chapter 2

Section 2.3.8.

5.3.5 RNA extraction and RT-PCR from sponge tissue.

Freshly collected sponge samples were rinsed three times with sterile artificial

seawater (ASW) and were stored in RNA*later* solution (Qiagen, Valencia, CA)

immediately for later RNA extraction. Total RNA was extracted from sponge

samples using the TissueLyser system (Qiagen, Valencia, CA) and an RNeasy

Mini spin column provided by the AllPrep DNA/RNA mini kit (Qiagen, Valencia,

CA) per manufacturer's instruction. RNAase-free DNAase (Qiagen, Valencia,

CA) was added to RNAeasy mini columns in order to totally remove any DNA.

Reverse transcription (RT) reactions were performed by using *ssaI* specific

primer RtaR (below) and ThermoScript RT-PCR system (Invitrogen, Grand

Island, NY). The conditions recommended by the manufacturer were used for

the reverse transcription reaction. Primers set RtaF (5'-

AAGTACTTGACGAAATGTTCG AACTG-3') and RtaR (5'-

GGTCGATCACGGTAATGATGTCTTC -3') were used to amplify the *ssaI* gene

from *M. laxissima* sponge cDNA. RNA samples without the RT step were used

as negative controls to test for contaminating DNA.

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5.3.6 Colonization of sponge cell aggregates

A derivative of wild type KLH11 resistant to the antibiotic rifampcin (Rif) was selected and was further modified to have resistance to the antibiotic tetracycline (Tc) by conjugating the pSRKTc plasmid into it (Khan *et al.*, 2008). The *ssaI* insertional mutant EC2 has a kanamycin (Km) resistance marker carried on the plasmid pVIK112 (Kalogeraki and Winans, 1997) and the plasmid pJZ402 (From S.C. Winans, Cornell University) that carries a spectinomycin (Sp) resistance marker was electroporated into EC2. These strains were used to study colonization of sponge cell aggregates. Roughly 1 cm^3 of sponge tissue from sponges that were maintained in tanks at the Aquaculture Research Center (ARC) in the Institute of Marine and Environmental Technology (IMET) was ground in 50 ml artificial seawater (Instant Ocean, Aquarium Systems, Pearl City, HI) and squeezed through a 100 µm mesh net. Two ml of the sponge cell homogenates were distributed into small petri dishes (60mm X 15 mm) and these cells spontaneously aggregated. Wild type KLH11 (labeled with Rif and Tc resistant markers) and the *ssaI* mutant (labeled with Km and Sp resistant markers) were grown up to stationary phase and inoculated into these cell aggregates at 10^6 cells.ml^{-1}; furthermore, cells of these two strains were mixed in a 1:1 ratio, also at a concentration of 10^6 cells ml^{-1} for each strain. This bacterial cell mixture was used in competition experiments. Artificial seawater was used as a negative control. The sponge cell aggregates were sampled at three different time points after inoculation (0 h, 6 h, 24 h) and were filtered through 5 µm membranes (Waterman, Boca Raton, FL). The cells that were retained on

the membranes were plated on plates with Rif (200 µg ml^{-1}) + Tc (20 µg ml^{-1}) and plates with Km (100 µg ml^{-1}) +Sp (µg ml^{-1}). The plates were incubated at 30ºC for 4 days and the number of colonies on each plate was counted.

5.3.7 Colonization of whole sponges

Nine individual of *M. laxissima* (each ca. 6 cm in diameter) were collected off Key Largo, FL on November 14[th] 2012 and were shipped to IMET overnight. Sponges were maintained in tanks filled with circulating artificial seawater (ASW, 80 L per tank) at ARC facility located in IMET (described in detail in Mohamed *et al*., 2008b). The water temperature was ca. 21ºC. After acclimation to the laboratory environment for ca. 10 days, these nine sponges were transferred to three new tanks, each containing three randomly chosen sponge individuals. The *ssaR* insertional mutant EC4 has a kanamycin (Km) resistance marker carried on the plasmid pVIK112 (Kalogeraki and Winans, 1997) and the plasmid pJZ402 that carries a spectinomycin (Sp) resistance marker was electroporated into this strain EC4. The pJZ402 plasmid also carries a gene encoding dsRed fluorescence (gift from S.C. Winans). The Rif and Tc resistant derivative of wild type KLH11 strain described above and the strain EC4 labelled with Km and Sp were used to colonize the whole sponges. Both of these two strains were grown in MB2216 at 28ºC with shaking at 200 rpm up to stationary phase. We used three different treatments to colonize the whole sponges. The Rif and Tc resistant derivative of wild type KLH11 was added to one tank containing three sponges; EC4 was added to another tank with three sponges; and wild type KLH11 and EC4 strains were added at 1:1 ratio to a third tank with three

sponges. Each of the two strains was added at a final concentration rougly 1.0 X

10^6 CFU ml$^{-1.}$ After 14 hours, each of the three sets of sponges was rinsed with

ASW and transferred to a new tank filled with ASW. All sponges were processed

for bacterial counts 48 h after this transfer. In brief, 1.5 g of sponge tissue was

cut from each sponge, ground in 10 ml sterile ASW and serial dilutions of the

sponge tissue were plated on MA2216 supplemented with Rif (200 µg ml^{-1}) + Tc

(20 µg ml^{-1}) and /or supplemented with Km (100 µg ml^{-1}) + Sp (100 µg ml^{-1}). The

plates were incubated at 30ºC for 4 days and the number of colonies on each

plate was counted.

5.4. Results

5.4.1 Screening of AHL-producing bacteria from marine sponges

In total, 420 strains isolated from *M. laxissima* and *I. strobilina* collected in three different seasons over three years from Key Largo, FL, USA were screened for AHL production. Results showed that 109 out of 225 strains (48 ± 12%) from *M. laxissima* and 75 out of 195 strains (38 ± 8%) from *I. strobilina* could activate the AHL biological reporter *A. tumefaciens* KYC55 strain. Furthermore, 4 out of 86 strains (5%) isolated from the surrounding seawater could also activate the reporter. Results of 16S rRNA sequences analyses showed that 86 out of 109 AHL producing strains from *M. laxissima* and 55 out of 75 AHL producing strains from *I. strobilina* belong to the *Silicibacter-Ruegeria* (SR) subclade of the *Roseobacter* clade in *Alphaproteobacteria*. The phylogenetic analysis based on the 16S rRNA sequences of all the SR AHL producers is shown in Figure 5.1. All the SR isolates share >98% identity on the near full length (ca. 1300 bp) 16S rRNA gene sequence level. These isolates can be largely divided into nine different groups, each supported by a bootstrap value >50%. Group 1 contains isolates only from *M. laxissima*, including our model bacterium *Ruegeria* sp. KLH1, while groups 5 and 6 contain isolates from both *M. laxissima* and *I. strobilina*. All the other groups (2, 3, 4, 7, 8 and 9) contain isolates only from *I. strobilina*. Furthermore, group 1 contains isolates from four different collection seasons, including those collected in 2004 by Mohamed *et al*. (2008c), and is dominated by those collected in June 2011. Groups 2, 3,7, 8 and 9 contain

201

isolates from only one collection season. Group 5 contains isolates from *I. strobilina* collected over three different seasons in this study while it is dominated by those from *M. laxisssima* collected in March 2010. In contrast, group 6 is dominated by isolates from *I. strobilina* collected in June 2011.

A
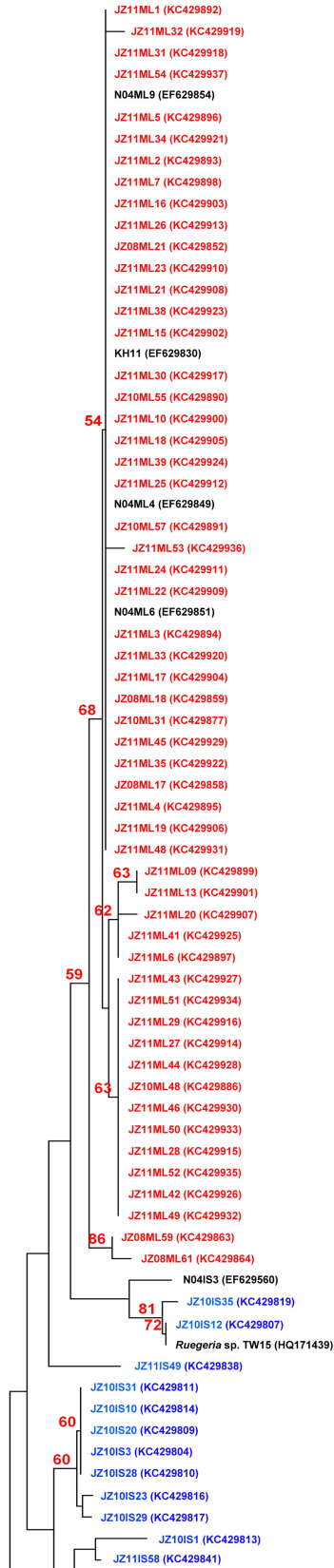
Group 1

Group 2

Group 3

0.002

Figure 5.1. Phylogenetic tree using neighbor-joining method of 16S rRNA genes (ca. 1300 bp) from SR AHL-producing bacteria. Sequences obtained from *M. laxissima* (JZ08ML, JZ10ML, JZ11ML and colored in red) and *I. strobilina* (JZ08IS, JZ10IS, JZ11IS and colored in blue) are included. Sequences obtained from Mohamed *et al*. (2008c) are labeled with N04ML, N05ML or N05IS. KLH11 was isolated from *M. laxissima* in the same study. The accession numbers are listed in parenthesis. Bootstrap values > 50% are shown at nodes. The bar indicates the number of nucleotide substitutions per site**.**

The other non-SR AHL producers can be categorized into two groups: *Alphaproteobacteria* and *Gammaproteobacteria*. The three alphaproteobacterial genera *Paracoccus*, *Pseudovibrio*, and *Stappia* also belong to the *Roseobacter* clade (Figure 5.2). Among the gammaproteobacterial AHL producers, 25 strains belong to the *Vibrio* genus. These Vibrios were mainly isolated from the sponges collected in 2008. Strains that belong to the genera of *Alteromonas*, *Pseudomonas* and *Shewanella* were also obtained although at low numbers. AHL producers from surrounding seawater belong to the genera *Vibrio* and *Alteromonas* and not to the SR group*.*

JZ08IS63 (HM117113)
JZ08IS68 (HM117114)
V. harveyi (GQ487488)
JZ08ML22 (HM117131)
JZ08ML70 (HM117130)
75
JZ08IS36 (HM117109)
JZ08IS78 (HM117116)
JZ08IS52 (HM117111)
61
JZ08IS34 (HM117107)
JZ08IS35 (HM117108)
Vibrio sp. N05IS8 (EF620869)
JZ08ML71 (HM117128)
89 85
JZ08IS48 (HM117110)
JZ08IS49 (HM117112)
JZ08ML60 (HM117127)
63
JZ08ML69 (HM117119)
58
JZ11IS17 (KC429940)
JZ08ML58 (HM117126)
Vibrio sp. PaH2.24a (GQ406770)
100
JZ08ML48 (HM117123)
V. aestuarianus (FJ161294)
JZ08ML73 (HM117132)
74
70
JZ08ML25 (HM117120)
98
Vibrio sp. N05IS5 (EF620872)
Vibrio sp. S2719 (FJ457471)
65
Vibrio sp. S1396 (FJ457399)
51
JZ08SW2 (HM117117)
94
JZ08SW6 (HM117118)
Vibrio sp. N05ML4 (EF620863)
V. agarivorans (AJ310648)
89
JZ08ML49 (HM117124)
JZ08IS72 (HM117112)
96
100
JZ08ML63 (HM117129)
JZ08ML43 (HM117121)
JZ08ML44 (HM117122)
65
JZ08ML52 (HM117125)
Vibrio sp. HB-1 (FJ465010)
JZ08SW55 (KC429942)
JZ08SW61 (KC429943)
100
Alteromonas sp. S163 (FJ457262)
66
JZ11IS72 (KC429939)
98
JZ08IS24 (KC429793)
100
JZ11IS74 (KC429938)
Shewanella sp. 3tb16 (FJ952791)
Pseudomonas stutezri (GQ470400)
100
JZ08ML3 (KC429849)
JZ08ML7 (KC429850)
JZ08ML51 (KC453996)
100
JZ08IS64 (KC429801)
Paracoccus yeei (GU083584)
50
JZ08ML26 (KC429855)
100
100
Pseudovibrio sp. N05ML11 (EF620867)
95
JZ08IS8 (KC429790)
99
JZ08IS43 (KC429796)
91
JZ10ML41 (KC429941)
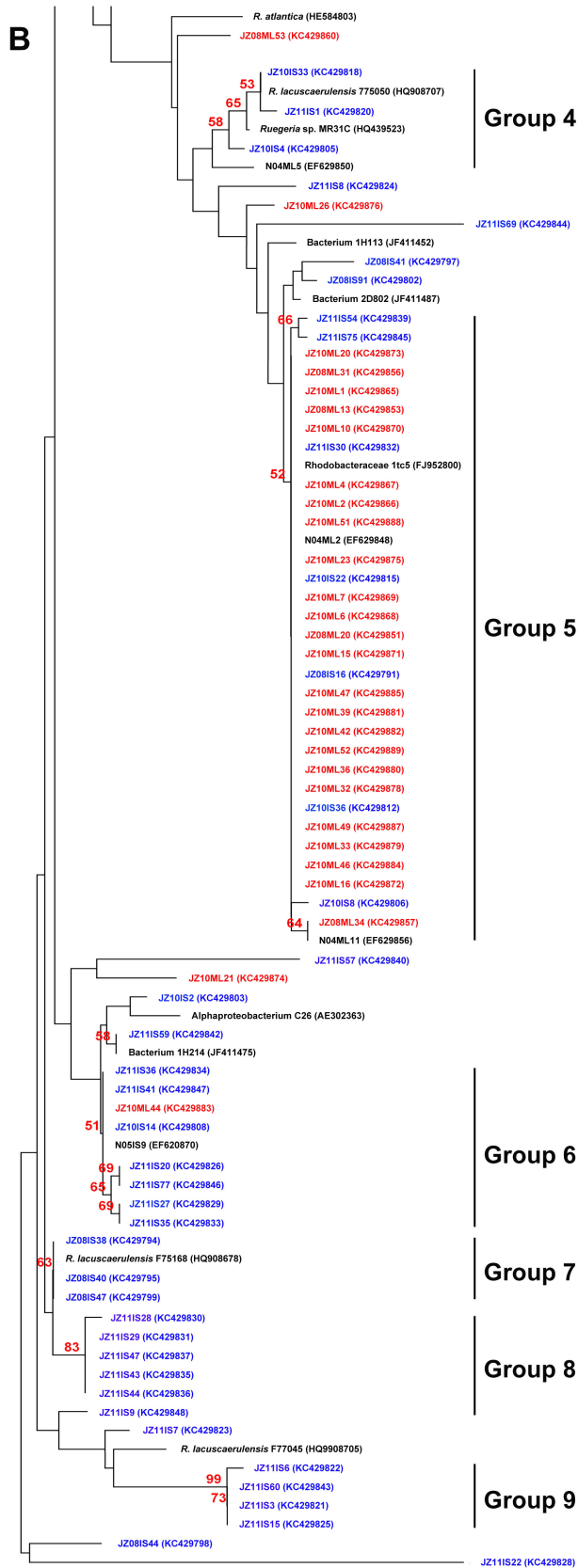*Stappia* sp. M8 (AY307927)

0.02

206

Figure 5.2. Phylogenetic tree using neighbor-joining method of 16S rRNA genes (ca. 700 bp) from all the non-SR AHL-producing bacteria. Sequences of bacteria isolated from *M. laxissima* (JZ08ML, JZ10ML, JZ11ML, in red), *I. strobilina* (JZ08IS, JZ10IS, JZ11IS, in blue) and seawater (JZ08SW) in this study are included. Sequences obtained from Mohamed *et al*. (2008c) are labeled with the genus name followed by N05IS or N05ML. The accession numbers are listed in parentheses. Bootstrap values > 50% are shown at nodes. The scale indicates the number of nucleotide substitutions per site.

5.4.2 *luxS* genes from Vibrios in sponges

Degenerate primers VluxsF and VluxR based on *Vibrio luxS* sequences were able to amplify partial *luxS* gene sequences from all the *Vibrio* strains isolated from *M. laxissima* and *I. strobilina* collected in October 2008. A phylogenetic tree of the *luxS* gene sequences from these vibrios is shown in Fig. 5.3. The majority of *luxS* genes from these sponge-derived *Vibrio* isolates are closely related to that of *V. harveyi,* which is consistent with the close relationship between these isolates and *V. harveyi* shown by 16S rRNA phylogeny (Fig. 5.2). A novel cluster (named Cluster MI) of *luxS* gene sequences, comprising those from isolates JZ08ML43, JZ08ML44, JZ08ML48, JZ08ML49, JZ08ML63, JZ08IS73, shared only 88%-90% identity to their closest relative in GenBank (boxed in Fig. 5.3). All the isolates were evaluated for AI-2 activity using reporter strain *V. harveyi* TL-26. All isolates were able to induce light production (Fig. 5.4), showing that all the *Vibrio* isolates, all of which have *luxS* genes, are able to synthesize AI-2-type molecules.
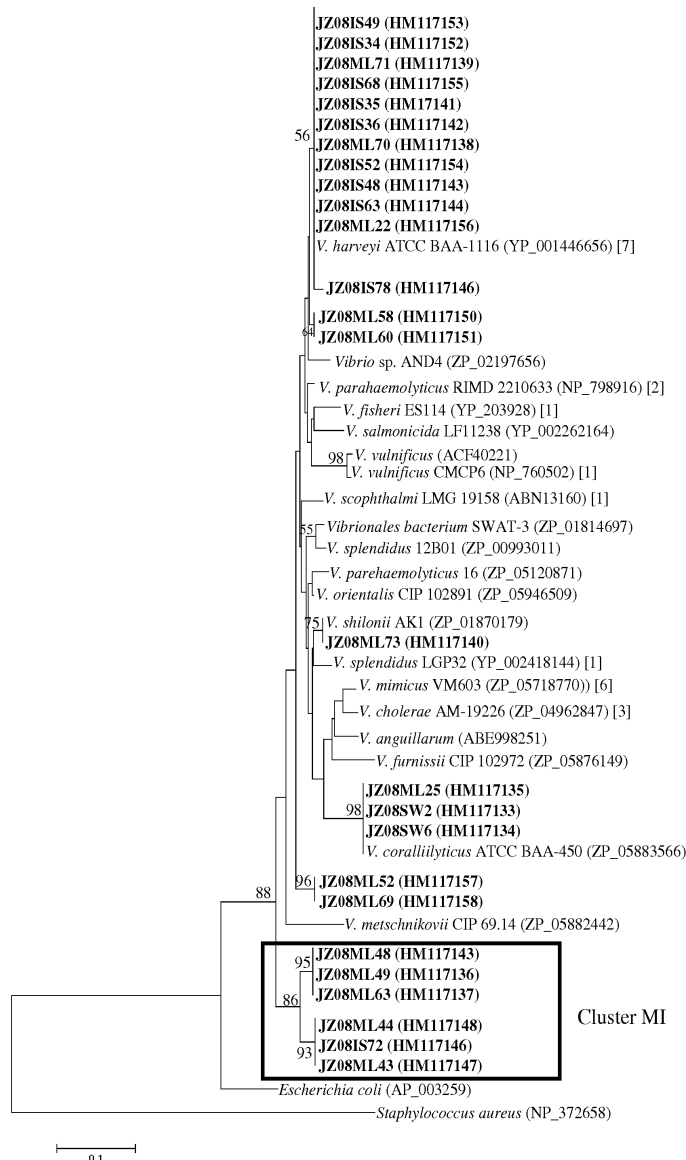
.**Figure 5.3 Phylogenetic tree using neighbor-joining method based on the predicted 96 aa residues encoded by *luxS* genes from *Vibrio* isolates**.

Sequences isolated from *M. laxissima* (JZ08ML), *I. strobilina* (JZ08IS) and seawater (JZ08SW) in this study are in bold and the accession number of each of these *luxS* sequences is listed after the isolate name. The novel *luxS* cluster is named Cluster MI. Bootstrap values > 50% are shown at nodes. The scale indicates the number of amino acid substitutions per site.
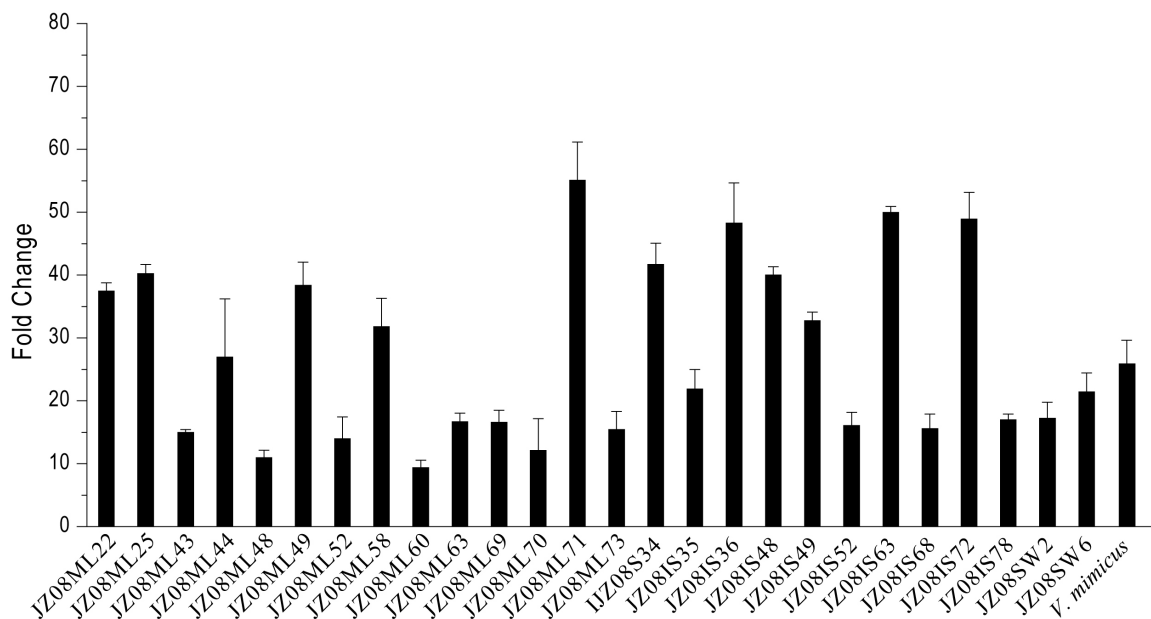
Figure 5.4. AI-2 activities of all *Vibrio* isolated from sponges detected by reporter strain *V. harveyi* TL-26. *V. mimicus* ATCC 33653 was used as positive control. Bars are average of three biological replicates. Error bars represented the standard deviations.

5.4.3 Sponge tissues contain AHLs and detectable levels of *ssaI* transcripts.

Sponge tissues were extracted with a modified Bligh-Dyer procedure (Bligh and Dyer, 1959) and these samples were fractionated by reverse phase TLC. TLC plates were overlaid with the highly sensitive AHL biological reporter *A. tumefaciens* KYC55 (Zhu *et al*., 2003) and this revealed the presence of AHL-type compounds in the extracts (Fig. 5.5A), one with migration similar to octanoyl-HSL (C8-HSL). In addition, two of three extracts also had an activity (somewhat obscured due to co-extracted pigments) that barely migrated from the

point of application (Fig. 5.5A, Lanes 4 and 5), similarly to a synthetic 3-oxo-C16:1 Δ11-HSL standard.

 M. laxissima tissues were used to extract RNA from which cDNA was synthesized, and RT-PCR revealed that *ssaI* was actively expressed in these tissues (Fig. 5.5B). No amplification was detected in the control RNA sample of *M. laxissima* without the RT reaction step, suggesting that the RNA sample is free of DNA contamination. The PCR amplicons were sequenced and of 13 clones sequenced all were greater than 98.5% identical to the KLH11 *ssaI* gene on the nucleotide level.
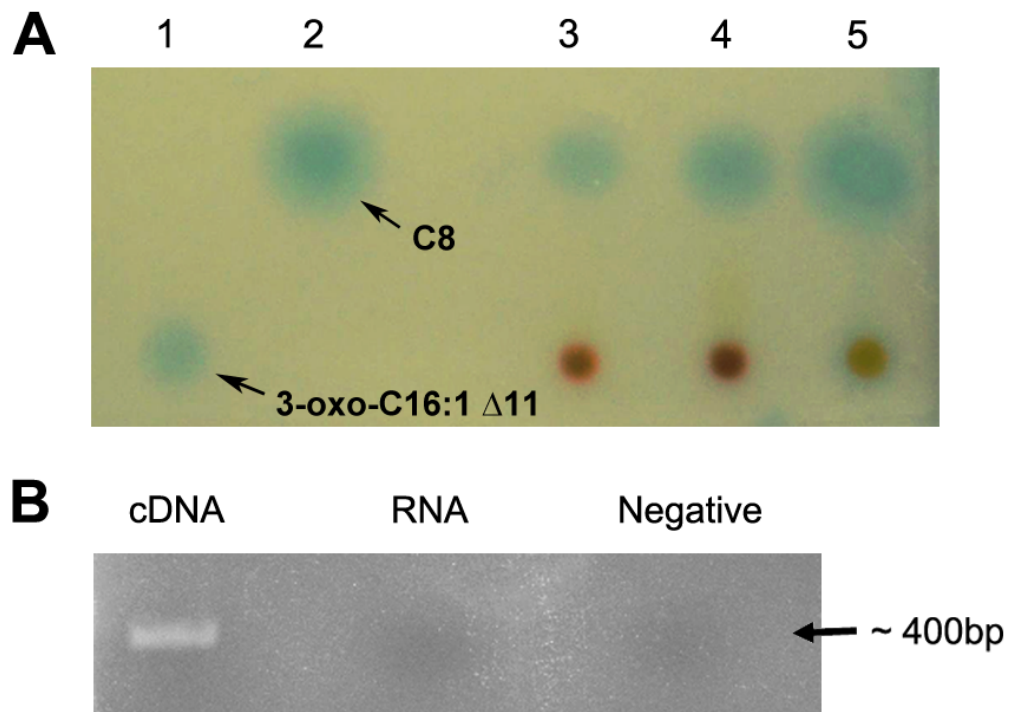
Figure 5.5. Detection of *ssaI* gene expression and AHLs in sponge tissue**.**

A) Reverse phase C18 thin layer chromatography plates overlaid with *A. tumefaciens* AHL reporter for detecting AHLs from sponge tissues. Lane 1: 3-oxo-C16:1 Δ11-HSL, lane 2: C8-HSL, lanes 3-5: *M. laxissima* individuals 1-3. B) RT-PCR detection of expression of *ssaI* gene in sponge tissue. The PCR amplicon is about 400 bp. The first lane used cDNA as template, the second lane used RNA as template to test for DNA contamination (RNA control) and the last lane was a no template negative control.

5.4.4 Colonization of sponge cell aggregates

The results of sponge cell aggregate colonization experiments (Fig. 5.6) show that the combination of antibiotic markers used in this experiment selected very well against bacteria present in sponges. However, there was no consistent trend of higher or lower rates of colonization of *M. laxissima* cell aggregates by the *ssaI* mutant at the time points we examined (0 h, 6 h, and 24 h).
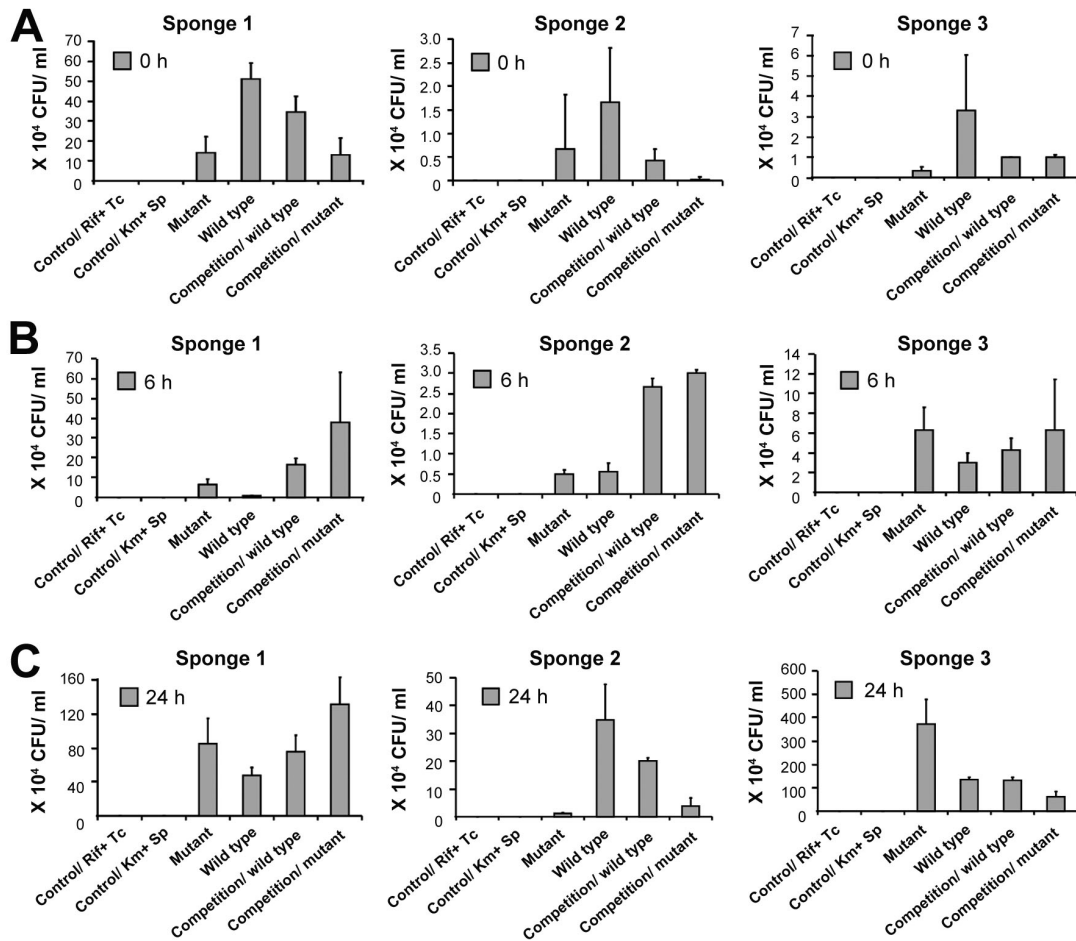
Figure 5.6. Bacterial colonization of *M. laxissima* sponge cells at different timepoints after inoculation (0 h, 6 h, and 24 h). The first two columns are the results from the negative control, which only had sponge cell aggregates. The "mutant" column is the result from the cell aggregate with *ssaI* mutant inoculation only and the "wild type" column is with wt KLH11 only. The competition/wild type and competition/mutant represented the numbers of wt KLH11 and *ssaI* mutant from sponge cells aggregates inoculated with both types of strains at a 1:1 ratio. Rif=rifampicin, Tc=tetracycline, Km=kanamycin, Sp=spectinomycin.

5.4.5 Colonization of whole sponges

We used the whole sponge individuals maintained in tanks with circulating

water to study the bacterial colonization process. The antibiotic resistance

markers that were proven to select very well against bacteria present in sponges

in the sponge cell aggregate colonization experiments were used to label the wild

type and *ssaR*⁻ mutant. The results showed that the combination of Rif and Tc

still worked well while the combination of Km+Sp did not select well against

bacteria present in the sponges as several different bacterial morphotypes grew

on the plates. However, we were able to count the *ssaR*⁻ mutant as its colony

displays a yellow-green color because of the dsRed fluorescence carried on the

plasmid pJZ402. The results presented in Fig. 5.7 indicate that the *ssaR*⁻ mutant

colonized the sponges better than the KLH11 wild-type derivative and the

presence of wild type strains reduced the colonization efficiency compared to the

addition of the *ssaR* mutant only ($P<0.05$).
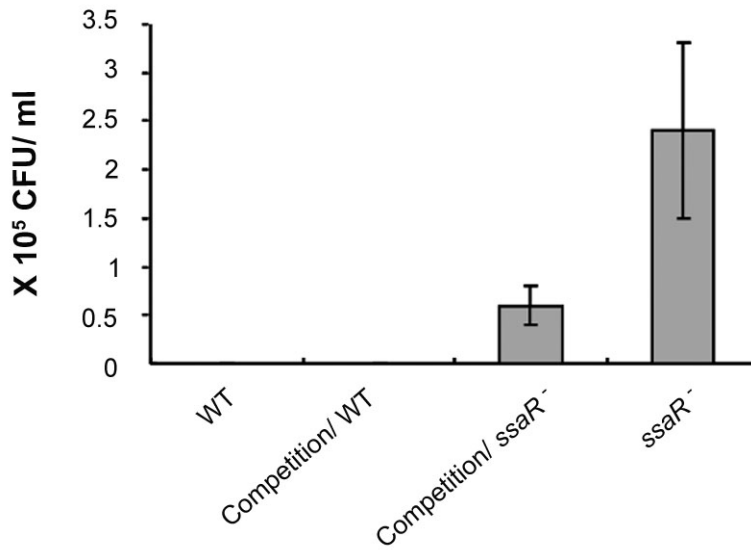
Figure 5.7. Bacterial colonization of *M. laxissima* whole sponges. The "WT"

column is the result from whole sponge with wt KLH11 only and the *ssaR*⁻ column

is with *ssaR*⁻ mutant inoculation only. The competition/ WT and competition/

ssaR⁻ represented the numbers of wt KLH11 and *ssaR*⁻ mutants from whole

sponges inoculated with both types of strains at a 1:1 ratio.

5.5. Discussion

Marine sponges harbor highly diverse and dense microbial communities and provide an ideal environment for bacterial quorum sensing (Mohamed *et al.*, 2008c; Zan *et al.*, 2011b). Our finding that a high proportion (40%-50%) of the 420 strains isolated from *M. laxissima* and *I. strobilina* can produce AHL molecules provides strong evidence to support this. However, it is unclear what proportion of bacteria that associate with marine sponges can produce AHLs as the majority of bacteria in sponges are resistant to cultivation (Webster and Taylor, 2012). All the AHL producers identified in this study belong to the phylum *Proteobacteria*, in which more than 100 different species have been shown to be capable of AHL production (Algren *et al.*, 2011). The result that there is a higher proportion of AHL producers among isolated strains from sponges than from the surrounding seawater is consistent with what was previously found in these two sponges with roughly 40 strains screened (Mohamed *et al.*, 2008c) and therefore confirms and extends this prior study. More importantly, these AHL producers are dominated by bacterial strains that belong to the SR group, which is also consistent with the findings of Mohamed *et al.* (2008c). Wagner-Döbler *et al.* (2005) showed that the majority of AHL producers among the marine isolates collected from various marine habitats including seawater are dominated by *Roseobacter* although no strains belonging to the SR group were identified. Taylor *et al.* (2004) were also able to isolate one AHL[+] SR strain closely related to *Ruegeria atlantica* (99% identity on the 16S rRNA gene level) from an Australian sponge *Cymbastela concentrica*. Taken together, this suggests that

Roseobacter is an important group of AHL producers in the marine environment generally and in association with marine sponges.

The consistent isolation of members in group 1 from four different seasons and only from *M. laxissima* suggest that they are specific to this sponge and also true sponge symbionts based on the definition used in Taylor *et al*. (2007). Thus *Ruegeria* sp. KLH11 represents an excellent model for studying quorum sensing in sponge symbionts as it falls into this group. Likewise, some isolates could only be isolated from *I. strobilina*. Although all our SR AHL producers from the two sponges share >98% identity on the near full length 16S rRNA gene sequence level, it is clear that they are not identical SR bacteria. This is similar to findings of Montalvo and Hill (2011), in which sequences of bacteria isolated from each of two closely related but geographically distant sponges *Xestosongia muta* and *X. testudinaria* cluster tightly according to which sponges they are derived. Both *M. laxissima* and *I. strobilina* were collected from the same geographic locations but they are only distantly related sponges and presumably provide different physiological environments. Thus it is possible that the last common ancestor of these two different sponges hosted tightly associated SR bacteria and as the sponges evolved, the SR bacteria diverged in the different sponges. Another possibility is that these SR bacteria are horizontally acquired from a pool of SR bacteria present at very low concentrations in the surrounding seawater and the different environmental conditions and/or active selection by the sponges results in the sponge specific SR grouping that we observed. Our study provides evidence that there might be sponge-specific bacteria as previously proposed

(Henstchel *et al*., 2002; Taylor *et al*., 2007), for example, six groups identified in this study can only be isolated from sponges.

Mohamed *et al*. (2008c) showed that these closely related SR AHL producers (> 99% identity on the 16S rRNA level) produced diverse AHL profiles. It is possible that the same trend remains for the SR bacteria obtained in this tudy. Generally, little is known about the function of sponge symbionts in their hosts (Webster and Blackall, 2009). However, some functions can be speculated. For instance, some rosoebacterial strains have been shown to produce antibiotics and show potential for secondary metabolite production (Buchan *et al*., 2005; Martens *et al*., 2007). Furthermore, particle-associated Roseobacters were 10 times more likely to produce antibacterial compounds than their planktonic relatives (Long and Azam, 2001; Slightom and Buchan, 2009). It is possible that SR bacteria obtained in this study produce secondary metabolites and thus provide the host sponge with defensive tools against pathogenic bacteria and help to structure the bacterial community associated with the sponges. It would be interesting to explore this possibility and also to test whether QS mediated pathways play a role in controlling secondary metabolite production, including antibiotic production. Furthermore, these SR bacteria might play a role in sulfur metabolism, such as in the degradation of dimethylsulfoniopropionate (DMSP). Our model bacterium *Ruegeria* sp. KLH11 encodes one key enzyme involved in the enzymatic cleavage of DMSP (Curson *et al*., 2011; Zan *et al*., 2011a)(See Appendix 1).

We also observed some slight differences compared to the study of Mohamed *et al*. (2008c). For example, we did not obtain any AHL producers that belong to the genera *Thalassomonas* and *Spongiobacter*. Furthermore, the AHL producers isolated in this study that belong to the genera: *Alteromonas*, *Pseudomonas*, *Paracocuss* and *Stappia* were not reported in Mohamed *et al*. (2008c). This might reflect the variability of the bacteria associated with sponges that can be cultured or subtle shifts in the bacterial communities associated with the sponges.

AHL is considered to be an intraspecies signaling molecule while AI-2 is an interspecies signaling molecule (Ng and Bassler, 2009). We took advantage of the fact that a relatively large amount of *Vibrio* strains were obtained from the collection of 2008 and designed degenerate primers to amplify the *luxS* gene, the synthase of AI-2, from these *Vibrio* strains. Not surprisingly, all the Vibrios contain *luxS* genes and were also able to trigger the AI-2 biological reporter, suggesting that AI-2 molecules are synthesized in all these strain. Our result also revealed a novel group of *luxS* sequence, thus extending the diversity of available *luxS* genes. However, it is unclear what the ecological significance of AI-2 mediating pathways in these Vibrios and this remains an interesting area for further investigation.

In this study we not only showed the isolation of AHL-producing bacteria but also demonstrated the production of AHLs from tissues of the soft-bodied, shallow water sponge *M. laxissima*, similar to those produced by abundant cultivatable, AHL+ sponge-associated bacteria (Mohamed *et al*., 2008c). The

chemical identities of the molecules present in the *M. laxissima* need to be further confirmed. Likewise we also show expression of the *ssaI* AHL synthase gene directly in total RNA from the sponge, effectively connecting our laboratory findings on QS in these symbiotic bacteria with their native host environment. Similarly, Gardères *et al*. (2012) identified the main AHL molecules present in the tissues of *Suberites domuncula* as 3-oxo-C12-HSL, the same compound that is produced by the cultivated isolates from this sponge. Taylor *et al*. (2004) showed that organic extracts of 24 out of 31 marine sponge species could activate the AHL reporter *Chromobacterium violaceum* (CV026), suggesting the presence of AHL molecules in the tissues. Taken together, these findings suggest that AHL-mediated signaling processes are important within sponge hosts.

We invested efforts for the first time to study the role that QS plays in the colonization process*. Ruegeria* sp. KLH11 was used as a model as the *ssaI* gene in this bacterium is actively expressed in situ within sponge tissue. Wild type KLH11 strain and QS mutant strain were labeled with different antibiotic resistance markers to colonize the sponge cell aggregates. However, we did not observe a consistent trend in the role that QS plays in the colonization process in sponge aggregates. The approach we used has proven to be effective in several other systems (Visick *et al*., 2000; Gurich and González, 2009). We reasoned that several factors can contribute to the inconsistent results that we obtained: 1) there might be variation in the three sponge individuals, such as the age, weight and health status. 2) Sponge cell aggregates might not be a suitable experimental system in which to study the interaction between host and microbes

occurring naturally in intact and healthy sponges. 3) The *ssaR* receptor mutant would be a better strain to use since the sponge tissue contains bacteria that produce similar molecules to those encoded by *ssaI*, which could compensate for loss of production of AHLs in an *ssaI* mutant. 4) It is possible that within the time frame (24 h) we tested, a stable relationship is not yet established.

We refined the design of colonization assay based on the lessons learned from the sponge cell aggregate colonization experiment. The wild type KLH11 and *ssaR* mutant strains were used to colonize whole sponges under laboratory conditions. We observed an interesting pattern in which the *ssaR* mutant strain colonized the sponges better than the labeled wild type KLH11 derivative and the presence of the wild type KLH11 strain seemed to reduce the colonization efficiency. KLH11 is motile but lacks chemotaxis machinery (Zan *et al*., 2011a) (See Appendix 1) and thus motility might function as a mechanism of random dispersion as previously proposed (Badger *et al*., 2006). This might explain why we failed to detect the wild type strain in sponges since the sponges used are relatively small and have a small surface area. The SsaR mutant cannot swim and also forms aggregates in culture and these characteristics might have facilitated attachment to the sponges. This does not explain why the colonization efficiency of *ssaR* mutant decreased when wild type strain was present. Another possibility is that QS affects the survival rate as reported in several other species (Joelsson *et al*., 2007; Goo *et al*., 2012) but this still does not explain why in the competition assay, the *ssaR* mutant colonized less efficiently in the presence of the wildtype.

Thus far, no clear conclusion can be made about how QS affects colonization process. To better understand this, a few additional aspects need to be explored: 1) How does QS affect the survival of *Ruegeria* sp. KLH11 in seawater? 2) How is *Ruegeria* sp. KLH11 transmitted? 3) Do adult sponges with established microbial communities actively acquire symbionts from surrounding environments besides through the process of filtering water for food? 4) It would be ideal to conduct colonization experiments in the field, such as enclosing the sponges with bags, rather than on sponges held in captivity.

Chapter 6. Conclusions and future directions

My research focused on the bacterial signaling in the two marine sponges,
Mycale laxissima and *Ircinia strobilina* collected off Key Largo, Florida. I began
my research project by isolating and screening approximately 420 bacterial
strains from these two marine sponges (*M. laxissima* and *I. strobilina*) for
acylhomoserine lactone (AHL) production. Previous studies in our laboratory and
in collaboration with Dr. Clay Fuqua's group from Indiana University had shown
that bacteria belonging to the *Silicibacte*r-*Ruegeria* (SR) subclade of the
ecologically abundant and important *Roseobacter* clade are the main AHL
producers in these two sponges (Mohamed *et al.*, 2008c), although a relatively
small number of isolates were screened. My work significantly expanded the
number of isolates screened and showed that roughly 40% of the cultured
isolates from these two sponges are able to produce AHL molecules and isolates
from the SR group are the dominant and consistent AHL-producers, which
strongly supports the findings by Mohamed *et al*. (2008c). Furthermore, the
repeated isolation of the same AHL-producing SR bacteria regardless of the
collection times and the failure to isolate them from the surrounding seawater
suggests that these SR bacteria may be true sponge symbionts. We follow the
definition of symbiosis as used by Taylor *et al*. (2007) and in the original sense of
deBary (1879), in which symbiosis refers simply to two or more different
organisms that live together over a long period of time without any judgment
made regarding benefits to either partner. It is unclear what functions these SR
bacteria can play to benefit their sponge hosts. However, it can be speculated
that SR bacteria may participate in the defense mechanisms of the sponges since

many roseobacters have been shown to produce antibiotics. For instance, some rosoebacterial strains have been shown to produce antibiotics and show potential for secondary metabolite production (Buchan *et al.*, 2005; Martens *et al.*, 2007). Furthermore, particle-associated Roseobacters were found to be 10 times more likely to produce antibacterial compounds than their planktonic relatives (Long and Azam, 2001; Slightom and Buchan, 2009). It is possible that SR bacteria obtained in this study produce secondary metabolites and thus provide the host sponge defensive tools against pathogenic bacteria and help to structure the bacterial community associated with the sponges. It would be interesting to explore this possibility and also to test whether QS mediated pathways play a role in controlling secondary metabolite production, including antibiotic production. We recently sequenced 17 SR genomes, which provide a great opportunity to explore whether they have the potential to produce secondary metabolites and this analysis is in progress.

Another interesting question to study is how broadly these AHL-producers are distributed. Many studies have supported the idea that sponges seem to have uniform sponge-specific microbial communities (Hentschel *et al.*, 2002; Taylor *et al.*, 2007). Do sponges harbor highly diverse AHL producers or are these SR bacteria the dominant AHL producers in sponges? Generally, sponges can be categorized as high microbial abundance or low microbial abundance sponges (Taylor *et al.*, 2007). Is it possible that only these high microbial abundance sponges harbor AHL producers since quorum sensing is a cell-density related process? Bacteria isolated from different marine sponges, collected from different

geographic locations and belonging to different species, need to be screened for AHL production to answer these questions.

What is the ecological significance of these QS pathways in the SR bacteria in the context of their native sponge host environment? This is the fundamental question that drove my dissertation research. In collaboration with Dr. Clay Fuqua from Indiana University and Dr. Mair Churchill from the University of Colorado Denver, we chose *Ruegeria* sp. KLH11 that was originally isolated from the sponge *M. laxissima* (Mohamed *et al*., 2008c) as our model. Genetic screening combined with genome sequencing identified two sets of *luxR-luxI* type QS pathways: *ssaRI* and s*sbRI*, and one *luxI* solo *sscI*. The results of mass spectrometry conducted in the laboratory of our collaborator Dr. Mair Churchill showed that all the three LuxI enzymes are able to synthesize long-chain AHLs, with SsbI and SscI showing similar profiles of AHLs and much stronger activity than SsaI. Furthermore, we now know the complex interconnected arrangement among all the three systems through a series of genetic studies (Chapters 2 and 3). However, it is still unclear what the exact mechanisms are for *ssaI* to indirectly affect *ssbI* and likewise how *ssbI* affects *ssaI*. It is possible that the proteins encoded by these genes compete for the substrate pool and thereby creates the regulatory network affecting each other's activity. Furthermore experimental evidence is required to investigate this possibility, such as the expression of all three genes in a heterologous system and examination of the profile of the AHLs synthesized to provide support for the hypothesis that they compete for the substrate pool. Nevertheless, the architecture of the QS system in KLH11 is

indeed consistent with many other well-studied bacterial species, in which multiple QS pathways exist (Fuqua and Greenberg, 2002).

Phenotypic studies showed that SsaRI activates flagellar motility and inhibits biofilm formation but SsbRI does not affect flagellar motility or biofilm formation. Furthermore, the SscI has only a very minor effect on motility. It is perplexing that KLH11 produces mainly the AHLs synthesized by SsbI and SscI in culture but these genes do not seem to play a significant role in regulating the two important phenotypes of motility and biofilm formation whereas SsaI that is key in these phenotypes produces much lower amounts of AHLs. Metatranscriptomic or microarray analyses where the gene expression profile of wild type KLH11 is compared with that of *ssbR*, *ssbI* and *sscI* mutants would be likely to provide clear insights into the genes or pathways that are affected by the *ssb* system and *sscI*. This would also provide clues about what is controlled by the systems similar to the *ssb* system in other Roseobacters. It has been shown by genomic analysis that the *ssb*-like system is more prevalent than the *ssa* system in Roseobacters (Cicirelli *et al.*, 2008). Such future studies may provide new insights into why a single bacterium needs to encode multiple quorum sensing systems.

Motility has been suggested to play a critical role in mediating interaction between roseobacters and the environment (Slightom and Buchan, 2009). We show that KLH11 exhibits flagellar motility and also established methods to study the interaction between sponges and KLH11. We do not yet know how general this system is among all the AHL-producing SR strains that we isolated. Do all of them exhibit motility? Does quorum sensing control the motility in all of these SR

bacteria? What is the distribution of the *ssa* and *ssb* systems and *sscI* that we have identified in *Ruegeria* sp. KLH11? The work accomplished in this study has established the basis to test all of these questions in the SR strains that we obtained in this study.

The transcription data show that *ssaI* is expressed at a very high level but SsaI seems to have quite weak catalytic activity for AHL synthesis (Chapter 2). It is possible that SsaI is only translated at a very low level and the quantification at the protein level is required to test this hypothesis. It is also possible that the SsaI protein is unstable and subject to rapid proteolysis. A third possibility is that the protein has intrinsically low activity, which might be caused by the extra long C-terminus. It would be useful to perform a series of mutagenesis studies on the protein and then test its activity in synthesis of AHLs. Furthermore, we showed that SsaR complexed with AHLs could stimulate the expression of *ssaI* in a heterologous system. However, it is unclear if this is also true in the native KLH11 background. Two approaches could be used to test this: 1) since a *ssaR* deletion strain has now been constructed, the expression of P_{ssaI}-*lacZ* could be compared in the wild-type strain and in the ∆*ssaR* strain. The expression could also be examined in the absence and in the presence of cognate AHL molecules to test whether SsaR in the native KLH11 background requires AHL to stimulate *ssaI* expression. 2) Campbell insertion could also be used. One additional strain (∆*ssaR ssaI-lacZ)* would need to be constructed. The *ssaI-lacZ* expression could then be compared to ∆*ssaR ssaI-lacZ* expression. The advantage of this approach over the first approach is that the *ssaI* promoter is in its native location.

Our data also show that SsaR very likely binds to the *ssaI* promoter region and the *ssa* box we identified is critical for this potential binding. However, experiments such as gel mobility shift assays are required to provide direct evidence to confirm this. Also, a more detailed understanding of the *ssa* box, such as what defines the minimal nucleotide set for SsaR binding, is needed. For instance, six critical nucleotides in the *lux* box define the LuxR-binding site (Egland and Greenberg, 2009). Understanding of the *ssa* box would be useful to predict what genes or pathways can be affected by the SsaR and also provide a baseline for defining this type of box in other closely related roseobacters.

Our results show that the expression of the *ssaI* gene is greatly induced by the long chain AHL, 3-oxo-C16:1 Δ11-HSL. Currently, several biological reporters have been developed to detect the long chain AHLs (C12-C18), such as *Sinorhizobium meliloti* 1021 that can respond to AHLs with chain lengths of C16-C20 (Llamas *et al*., 2004). However, it elicits a weak response (Mohamed *et al*., 2008c). Thus it would be useful to develop a sensitive KLH11-derived reporter strain based on the fact that *ssaI-lacZ* fusion shows a very sensitive response to long chain AHLs, presumably via SsaR.

The coordination of biofilm formation and motility control by the SsaRI system led us to hypothesize that quorum sensing is a key factor in the successful symbiosis between sponges and their SR alphaproteobacterial symbionts. One possibility is that quorum sensing is a determining factor whereby sponges select their symbionts from the huge bacterial load to which sponges are exposed through filtration of large volumes of water. We were able to show the presence

of AHLs and the expression of *ssaI* transcripts in the sponge tissues, which effectively connects what we found under laboratory conditions with its native host and thus makes KLH11 an excellent model system for understanding the complex symbiotic interactions with its host. The pattern observed from the whole sponge colonization experiment was very interesting but also very confusing. We do not yet have a good understanding of the role of quorum sensing in colonization of sponges by bacteria. Further efforts need to be directed towards the following to better study this process: 1) Ideally, experiments investigating the role of QS in colonization should be done under *in situ* conditions where the sponge individuals are covered with some kind of enclosure and wild type KLH11 and quorum sensing mutants labeled with the appropriate antibiotic resistance markers are released in these closed environments. The outcome of colonization of these strains could be determined at different timepoints. 2) Fluorescently tagged KLH11 and derivatives can be used in sponge colonization experiments. Advanced microscopy could be employed to monitor how these strains interact with the sponge tissues. 3) Several basic questions as listed in Chapter 5 need to be answered: i) How does QS affect the survival of bacteria in the seawater? ii) How is KLH11 transmitted? iii) Do adult sponges with established microbial community actively acquire symbionts from surrounding environments besides filtering for food?

   Another interesting aspect is to understand whether sponges can respond to AHL molecules i.e. whether inter-domain signaling occurs between the SR bacteria and sponge cells. Zoospores of the macroalga *Ulva* can "probe" the

nature of surfaces for potential attachment by sensing the presence of AHLs in the biolfilm on the surface (Joint *et al.*, 2002; Tait *et al.*, 2009). It is possible that sponge cells can respond to AHLs to monitor the presence of bacteria. Proteomic approaches could be utilized to provide useful insights about the potential inter-domain signaling.

The observation that QS inhibits biofilm formation indeed resembles the situation found in *V. cholerae*. At low cell density, *V. cholerae* forms biofilms while at high cell density, QS inhibits the biofilm formation, which can promote the dispersion of bacterial cells away from the biofilm and release into the environment (Hammer and Bassler, 2003; Zhu and Mekalanos, 2003; Higgins *et al.*, 2007). It is possible that the same dispersal mechanism is at play in KLH11 to promote a uniform distribution of this symbiont in the sponge tissues or even to facilitate release back into a planktonic form in the surrounding seawater for colonizing other sponges. When KLH11 grows to high cell density, it would be beneficial for the motile cells to swim rather than attach to surfaces and in this situation, biofilm formation is inhibited. It is however unclear how this coordination works. One possibility is that QS regulates the concentration of a second messenger molecule called cyclic di-GMP (c-di-GMP). C-di-GMP is synthesized by diguanylate cylase enzymes that contain a GGDEF domain and degraded by phosphodiesterase enzymes that contain either an EAL or HD-GYP domain (Waters *et al.*, 2008). It has been shown in several bacterial species that high concentrations of c-di-GMP promote biofilm formation and repress motility, facilitating transition from motile phase to sessile phase (Massie *et al.*, 2012;

Purcell *et al.*, 2012). In *V. cholerae*, QS has been shown to affect the expression

of the genes that encode these related enzymes with the overall effect of reducing

the concentration of c-di-GMP (Waters *et al.*, 2008). It is worthwhile examining

the relationship between QS and c-di-GMP in KLH11 to have a better

understanding of the mechanisms to regulate the transition between sessile and

motile phases.

My research on the *cckA-chpT-ctrA* phosphorelay system makes *Ruegeria* sp.

KLH11 an excellent model for understanding this pathway outside of the group in

which this pathway is essential (Greene *et al.*, 2012). The findings of the

regulatory link between a QS pathway and a two-component system is not

surprising *per se* since previous studies have shown that the GacS/GacA two-

component system positively controls the quorum sensing pathways in

Pseudomonas aeruginosa (Kay *et al.*, 2006) and the quorum sensing SmaR

protein in *Serratia* sp. ATCC 39006 can repress the response regulator PigQ

(Williamson *et al.*, 2008). The novelty is that we identified the SsaRI system as

the regulator for the *cckA-chpT-ctrA* system outside of the model bacterium

Caulobacter crescentus, in which this system is essential. The *ctrA* gene has

been extensively studied in *C. crescentus* in the control of the cell cycle, polar

development, and flagella biogenesis. Furthermore, in several species of the

Alphaproteobacteria, the involvement of this phosphorelay system in flagellar

motility has been well characterized, especially for the *ctrA* gene (Mercer *et al.*,

2010; Greene *et al.*, 2012). However, little is known about the regulation of the

ctrA gene in these bacterial species. My study for the very first time identified QS

as the regulator for CtrA in *Ruegeria* sp. KLH11 and opens a new avenue for the

regulation of the *ctrA* gene in the bacteria in which this gene is not essential.

Issues remaining to be addressed include: 1) what is the nature of the

phosphorelay between the *cckA-chpT-ctrA* ? 2) what component(s) exists in

between the *ssaRI* and the *ctrA* genes to explain my results that show indirect

transcriptional regulation of SsaRI on the *ctrA* gene? I optimized the protocol of

using the mariner transposon pFD1 (Rubin *et al.*, 1999) to create a large random

pool of mutants in KLH11. The Δ*ssaI* mutant that has the P_{ctrA}-*lacZ* fusion shows

very light blue color on the plates with X-Gal since the basal expression level of

this fusion is very low. Therefore it would be possible to identify mutants that

show very strong blue color on the plates. The mutants that might be identified by

this approach could then be characterized and the target components might be

revealed. 3) The difficulty in restoring the expression of the *cckA-chpT-ctrA*

genes in the Δ*ssaI* mutant by exogenous addition of AHL seems to be related to

the poor entry of the long-chain AHL into the cell. However, further evidence is

required to support this explanation. One way to test this is to label the AHL

molecules with fluorescence markers and then monitor the movement of these

molecules and also localize their location. It would be technically challenging to

label the small AHL molecule but this approach would provide very strong

evidence. 4) Does quorum-sensing coordinate the expression and

phosphorylation of the histidine kinase CckA? We showed that SsaRI

transcriptionally regulates the expression of *cckA* while little is known about the

phosphorylation of CckA. One way to test this is to quantify the level of

phosphorylated CckA in the wild type strain and also in the quorum sensing

mutant strain at different stages of growth. If CckA can only be phosphorylated in

late stage growth of the wild type KLH11, this would suggest that the signal(s) that

CckA can recognize and respond to are associated with cell-density.

The paradigm of the structure of AHLs as a fatty acyl chain attached to a

homoserine lactone via an amide bond changed when the *p*-coumaroyl-HSL was

discovered, in which the fatty acid chain has a ring structure. More interestingly,

this molecule is synthesized by a LuxI homologue RpaI, which uses the nutrient

coumaric acid as a precursor (Schaefer *et al.*, 2008). It seems that KLH11 can

also synthesize similar molecules detected by a biological reporter and this

production is independent of the three LuxI homologues although the identity of

the molecule is unknown (Chapter 3). It is reasonable to speculate that a novel

type of enzyme exists in KLH11, although it is possible that an unknown *luxI*

homologue that has eluded genome sequencing is responsible for the synthesis

of *p*-coumaroyl-HSL. Genetic screening of the genomic library that was used to

identify *ssaI* and *ssbI* genes could be applied to search for this potential enzyme.

Identification of this enzyme in *Ruegeria* sp. KLH11 has broad significance as

several bacterial species have been shown to respond to the addition of *p*-

coumarate, the substrate for the synthesis of *p*C-HSL. For example, *R. pomeroyi*

DSS-3, a close relative of KLH11, can produce a molecule biologically and

chemically similar to *p*C-HSL (Schaefer *et al.*, 2008). In *Silicibacter* sp. TM1040,

a strain that does not encode LuxI or LuxM homologues, the compound named

RMI can be induced by addition of *p*-coumarate (Sule and Belas, 2012).

Phaeobacter gallaeciensis BS107 (also known as DSM 17395), a member of the *Roseobacter* clade, responds to the presence of *p*-coumarate produced by the microalga *Emiliania huxleyi* to control the production of algaecides and thus convert itself into an opportunistic pathogen of the host microalga (Seyedsayamdost *et al.*, 2011). Taken together, dissection of this pathway in these roseobacters represents an exciting area for further exploration.

Marine sponges harbor diverse and highly abundant bacteria in their tissues. Do these bacteria living in the same environment interact with each other? Do they coordinate as a group or compete with each other for nutrients and resources? For example, it has been shown that antagonism among coral bacterial symbionts is common and can potentially structure the microbial communities associated with corals and might also be a protective mechanism against pathogens (Rypien *et al.*, 2010). Quorum sensing might provide an entry point to study the interactions. Coordination of group behavior via chemical languages is advantageous. Thus the ability to disrupt this conversation, which is generally termed as quorum quenching (Dong and Zhang, 2005), might be an effective approach for other bacteria that compete with some of the AHL-producing sponge symbionts. Marine sponges are well known for being rich resources of bioactive natural compounds (Hill, 2004). Do they also produce quorum-quenching compounds? Our model organism KLH11 provides an excellent model for exploring these questions. We can use the motility phenotype as the readout to screen for compounds that can inhibit motility in the wild type, potentially through quorum quenching. We can also use the *ssaI* nonmotile

mutant to screen for interspecies signaling. These aspects will provide further insights into the interactions between bacteria living in the same host.

Overall, my dissertation work combined with our collaborators' work established that KLH11 is an excellent model organism to study the complex quorum sensing regulatory circuits, motility control and biofilm formation in sponge symbionts and also other related roseobacterial relatives. Furthermore, approaches and tools have been established to further examine the interactions between the microbes and hosts. Our findings thus lay the groundwork for studies of this process and provide an important foundation for elucidating these pervasive and influential symbiotic relationships.

Appendix 1: Genome sequence of *Ruegeria* sp. Strain KLH11, an *N*-acylhomoserine lactone-producing bacterium isolated from the marine sponge *Mycale laxissim*a.

Genome Sequence of *Ruegeria* sp. Strain KLH11, an *N*-Acylhomoserine Lactone-Producing Bacterium Isolated from the Marine Sponge *Mycale laxissima*

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Ruegeria sp. strain KLH11, isolated from the marine sponge *Mycale laxissima*, produces a complex profile of *N*-acylhomoserine lactone quorum-sensing (QS) molecules. The genome sequence provides insights into the genetic potential of KLH11 to maintain complex QS systems, and this is the first genome report of a cultivated symbiont from a marine sponge.

Sponges can harbor diverse assemblages of microbes, which in some cases can comprise up to 60% of the total biomass of the holobiont (6). This dense microbial community associated with sponges provides conditions in which quorum sensing (QS) may be important (3, 8). QS is a process by which bacteria coordinate group activities such as bioluminescence, antibiotic

production, and virulence at high cell density via signal molecules, typified by N-acylhomoserine lactone (AHL) in Proteobacteria (2). We isolated a *Ruegeria* sp. strain, KLH11 (99% 16S rRNA gene se- quence similarity with Ruegeria lacuscaerulensis [GenBank accession no. HQ908678]), which can produce a complex profile of long-chain AHLs (3). The *Silicibacter-Ruegeria* subgroup belongs to the *Roseobacter* clade, which is widespread and numerous in surface waters of the oceans and includes organisms that are involved in global sulfur cycling (1). Many roseobacters have been fully sequenced and annotated (5). *Ruegeria* sp. strain KLH11 is the first readily culturable sponge symbiont to have its genome sequenced. This microorganism has been isolated from several samples of the sponge *Mycale laxissima* and is termed a symbiont using the term as defined by Taylor et al. (6).

The genome was sequenced by a combination of Roche/454 and Sanger whole-genome shotgun sequencing. Sequences were assembled with a Celera assembler (4), and genes were predicted and annotated using the automated CloVR-Microbe pipeline (7).

The draft genome has a total assembly size of ca. 4.5 Mb, with an average G+C content of 57.3 mol%, and is predicted to have 4,493 coding sequences (CDS), of which 3,243 (ca. 72.0%) were functionally annotated. This assembly is comprised of 13 scaffolds, of which the largest is ca. 3.1 Mb, having 3,193 CDS. One scaffold of 81 kbp shows similarity to the megaplasmid identified in *Ruegeria pomeroyi* DSS-3 (accession number CP000032) and could indicate the presence of extrachromosomal elements in the genome. Approximately 82.0% of all the

predicted CDS have ATG sequences at the presumptive start position, and 17.8% may use GTG or TTG. One complete rRNA operon, one partial 16S rRNA gene, two 5S rRNA genes, and 52 tRNAs were identified. About 34 genes were found to be related to the synthesis of bacterial flagella and regulation of bacterial motility. No candidates for chemotaxis functions were observed in the genome. Two sets of quorum-sensing circuits were identified, implying the potential for a complex quorum-sensing mechanism. Roughly 44 genes were predicted to function in the biosynthesis and degradation of polysaccharides.

Comparison of KLH11 genome sequences to closely related free-living bacteria, such as *R. pomeroyi* DSS-3, provides an opportunity to define the unique set of genes important for host-symbiont interactions and the ecological roles and bio- geochemical functions. Complex quorum-sensing circuits in KLH11 make it a perfect model for studying the ecological role of QS in the complex symbiosis between sponges and their associated bacteria.

Nucleotide sequence accession number. The data of the genome sequence of KLH11 were deposited in GenBank under the accession number ACCW00000000.

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