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A Complex LuxR-LuxI Type Quorum Sensing Network in a Roseobacterial Marine Sponge Symbiont Activates Flagellar Motility and Inhibits Biofilm Formation

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Summary

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 and one solo *luxI* homologue were identified and designated *ssaRI*, *ssbRI*, and *sscI* (sponge-associated symbiont locus A, B, and C, *luxRI* or *luxI* homologue). *SsaI* produced predominantly long-chain 3-oxo-AHLs and both *SsbI* and *SscI* specified 3-OH-AHLs. Addition of exogenous AHLs to KLH11 increased the expression of *ssaI* but not *ssaR*, *ssbI* or *ssbR*, 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 only at high culture density. Conversely, *ssaI* and *ssaR* mutants formed more robust biofilms than wild type KLH11. AHLs and transcript of the *ssaI* gene were detected in *M. laxissima* extracts suggesting that AHL signaling contributes to the decision between motility and sessility and that it also may facilitate acclimation to different environments including the sponge host.

Keywords

quorum sensing; acyl-homoserine lactones; LuxI-LuxR type regulation; sponge symbionts; motility; biofilm

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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; Rusch *et al.*, 2007), including in association with marine sponges (Webster and Hill, 2001; Rusch *et al.*, 2007; Mohamed *et al.*, 2008a). More specifically, the *Roseobacter* clade, is estimated to account for 20-30% of the bacterial 16S rDNA 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). Within the densely colonized sponge, there is also ample opportunity for both inter-species and intra-species signaling (Zan *et al.*, 2011b).

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. The acyl-homoserine lactones (AHLs), that are widespread among *Proteobacteria*, are most often synthesized by enzymes of the LuxI family (Churchill and Chen, 2011). In the AHL-based QS model initially described for control of *Vibrio fischeri* bioluminescence (*lux*) gene expression, each cell produces a small amount of a signal that can be dissipated by passive diffusion and is therefore incapable of eliciting a response. As the population density and AHL concentration reaches a threshold concentration within the cell, AHL receptors, usually LuxR homologues, bind to the AHLs. The LuxR-AHL complexes typically activate a specific suite of target genes, although a small number of LuxR-type proteins act to repress target gene expression and are inhibited by AHLs (Fuqua and Greenberg, 2002; Minogue *et al.*, 2002). Many LuxR type proteins recognize an 18-20 bp “*lux*-type” box (often an inverted repeat), which, in the case of the *V. fischeri lux* operon, is centered -42.5 base pairs (bp) upstream of the transcriptional start site of the *lux* operon, overlapping with the presumptive -35 element (Devine *et al.*, 1989; Eglund and Greenberg, 1999). Liganded LuxR-type proteins typically activate transcription, via interactions with RNA polymerase. Activated genes often include the gene encoding the cognate LuxI-type AHL synthase, thereby creating a positive feedback loop (Eglund and Greenberg, 1999).

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.*, 2008b). *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.*, 2008b). 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 detection of AHL synthase expression and AHL bioactivity from native sponge extracts, and (iv) 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.

Results

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. 1; Mohamed *et al.*, 2008b). 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. 2A, Fig. S1A, Table 1). The shorter chain AHLs detectable in bioassays (Fig. 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 *HindIII* or *SaI*, 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.*, 2008b). 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 isolated and sequenced, identifying two separate loci with similarity to *luxI* from *V. fischeri*, each adjacent and in tandem arrangement downstream of a *luxR* homologue (Fig. S2A 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.*, 2011a) and 100% identical loci were identified; thus we refer to these Genbank Accession numbers for *ssaRI*, *ssbRI* and flanking genes (Fig. S2).

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 *sscI*. Although *sscI* had escaped our genetic screen, it shares 81% identity to *SsbI* on the amino acid level (Fig S3), is of similar size and is likely to be a recent gene duplication. The *sscI* gene is not genetically linked to a *luxR* homologue, and is absent in the related *R. pomeroyi* DSS-3 genome.

SsaI and SsbI 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. 3A, Table 1, Fig. S1B). Trimethylsilylation and methoximation of these samples (Clay and Murphy, 1979; Maclouf *et al.*, 1987) revealed that SsaI produces 3-oxo-AHLs, the most abundant species of which is 3-oxo-C14-HSL (Table 1, Fig. S1B), but also including a C16 derivative, that was shown by methoximation to be a 3-oxo-C16 derivative. No 3-oxo-AHLs were observed for *E. coli* expressing SsbI, but rather 3-hydroxy-HSLs were identified, with predominant 3-OH-C14-HSL, 3-OH-C14:1-HSL and 3-OH-C13-HSL (Fig. 3B, Fig. S1B, Table 1). For both AHL synthases, putative AHL derivatives were also detected, but their identity was not confirmed due to a lack of reference standards (Table 1). Overall, SsaI and SsbI 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. Expression of *sscI* in *E. coli* revealed the same pattern of AHLs as did *ssbI* (compare Figures 2B, S1B, S4A and S4B).

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. 1A). 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. 2B, Fig. S1A, Table 1). The loss of *ssaR* (Fig. 2C) did not significantly alter the pattern of AHLs observed in the wt KLH11, consistent with the bioassays (Fig. 1A). In contrast, chemical analysis of the *ssbI* mutant surprisingly revealed an overall increase in AHL levels (Fig. 1B, Fig. 2D), 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, low levels precluded their distinction) as major species, but also hydroxylated derivatives, 3-OH-C14, 3-OH-C14:1, and 3-OH-C12 (Table 1, Fig. S1A). 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 1, Fig. 2E), similar to the *ssbI* mutant, although this was not clear from the bioassays (Fig. 1B).

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. 3C), suggesting the presence of an additional unidentified AHL synthase. Indeed, we now know that the *sscI* gene is still present in this strain and is likely to be responsible. The introduction of P_{lac} -*ssaI* or P_{lac} -*ssbI* plasmids into the *ssaI* *ssbI* double mutant (Fig. 3D and 3E) 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 (or possibly 3-OH-C16:1-HSL, again too low level to distinguish) and SsbI directed synthesis of 3-OH-C14:1 and 3-OH-C14-HSL (Table 1, Fig. S1C). 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. S3) 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-AHLs (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. S3), consistent with the observed AHL profiles (Fig. 3, Table 1). 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).

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 *A. tumefaciens* AHL biological reporter (Zhu *et al.*, 2003) and this revealed the presence of AHL-type compounds in the extracts (Fig. 4A), 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. 4A, Lanes 4 and 5), similarly to a synthetic 3-oxo-C16:1 Δ 11 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. 4B). No amplification was detected in the control RNA sample of *M. laxissima* without the RT reaction step. 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. The same approach failed to detect *ssbI* gene expression in sponge tissue.

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 Units <1). The *ssaI-lacZ* fusion exhibited close to 16-fold induction (Table 2) when cultures were incubated in the presence of KLH11 extracts (2.5% v/v). Given that the dominant SsaI-directed are C16-AHLs, we also tested *ssaI* expression of the mutant with synthetic 3-oxo-C16:1 Δ 11. In the presence of the synthetic AHL at 2 μ M, the *ssaI-lacZ* fusion was induced 40 fold (P <0.001, Table 2). In contrast, the expression of the *ssbI-lacZ* fusion in the *ssbI* null mutant was low and was not increased by addition of culture extracts or by 20 μ M 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 μ M 3-OH-C14-HSL, suggesting limited cross-recognition of this AHL. The *ssbI-lacZ* fusion was not activated by addition of 2 μ M 3-oxo-c16:1 Δ 11.

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). Disruption of the *ssaR* gene expression resulted in an approximately 4-fold decrease of *ssaR* expression, but this was independent of AHL.

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_{ssaR}-lacZ* fusion or a *P-lacZ* fusion, were introduced into the AHL⁻ *ssbI*, 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 3). 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 3). Several C14-HSLs were also tested, but only weakly influenced *ssaI* expression (Fig. S5A). 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. S5B). 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, Table S1). Likewise, SsaR failed to activate *ssbI* and SsbR failed to activate *ssaI* in the presence of culture extracts and synthetic AHLs (Table S1).

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; Eglund 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 transcriptase (RT)-PCR assays demonstrated that *ssaI* and *ssaR* are not in the same operon (Fig. S2A; similarly, *ssbI* and *ssbR* are also in separate operons, Fig. S2B). 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 *silR3* from *R. pomeroyi* DSS-3 (Moran *et al.*, 2004) revealed only 60% identity, except for a 19 bp fully conserved segment (TACGGGAAACCCCAATAG), located 60 bp upstream of the *ssaI* start codon (Fig. 5A). 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_{ssaR}-lacZ* plasmid (pEC116), one to just upstream of the *ssa* box (pEC124) and a larger deletion (pEC127) that almost completely removes the element (Fig. 5B). 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. These results reveal a role for the presumptive *ssa* box in AHL-dependent activation of *ssaI*.

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 (data not shown). All four KLH11 mutants (Δ*ssaI*, *ssbI*⁻, *ssaR*⁻, and *ssbR*⁻) were tested for their motility on MB 2216 swim agar plates (Fig. 6A). 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. 6A). As expected, motility was not restored in the *ssaR*-mutant in the media with 2 μM 3-oxo-c16:1 Δ11-HSL (unpublished

results). 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. S6).

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. S7) 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. S7). 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. 6B) 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). 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. 6B).

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 OD₆₀₀ of 0.5, the parent strain had no detectable flagellin in either the whole cell or supernatant fraction, but as the culture density increased (OD₆₀₀ >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. 6C). Flagellin was never detected in samples of the *ssaI*-mutant, irrespective of culture density.

To determine if the SsaRI system regulates the transcription of the flagellin gene (*fliC*), quantitative Real-Time PCR (qRT-PCR) was used to measure *fliC* expression. Late stage cultures grown to an OD₆₀₀ 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. 6D). *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. 6D, P<0.05). These results suggest that an intact SsaRI system is required for swimming through regulation expression of flagellar genes.

SsaRI mutants exhibit increased biofilm formation

QS can also regulate bacterial biofilm formation (Hammer and Bassler, 2003; Shrouf *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 (A₆₀₀) 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. 7A, 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. 7A, P<0.01).

However, biofilm formation in the $\Delta ssaI fliC$ -mutant was even stronger than the non-motile *fliC* mutant ($P < 0.05$). This suggests that the increased biofilm formation in the $\Delta ssaI$ 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. 7A, $P > 0.05$).

Addition of KLH11 late stage culture fluids (40% v/v) into biofilm assays with the $\Delta ssaI$ mutant reduced biofilm formation to 60% the level of untreated cultures ($P < 0.05$) whereas planktonic growth is unaffected (Fig. 7B, $P > 0.05$). 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 fluids.

Discussion

There is limited understanding of QS mechanisms in the diverse and abundant bacteria living in the marine environment (Cicirelli *et al.*, 2008). In this study we have 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. Likewise we also detect 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.

Our studies on sponge symbionts initiated with the use of AHL responsive bioassays (Mohamed *et al.*, 2008b). 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. 1; Mohamed *et al.*, 2008b). 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; Table 1), they are clearly present in the TLC assays (Fig. 1). The relatively abundant 3-OH-C14-HSL and 3-OH-C14:1-HSLs 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-Dobler *et al.*, 2005).

The pattern of AHLs specified by *SsaI* and *SsbI*, 3-oxo-AHLs and 3-OH-AHLs, 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 was the most

abundant AHL, and 3-oxo-C16 was present at 15% (Table 1). This relationship reversed when the same plasmid was expressed in the KLH11 $\Delta ssaI\Delta ssbI$ mutant, resulting in high relative levels of 3-oxo-C16:1, with 3-oxo-C14 at roughly 4% those levels. 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 KLH11 $\Delta ssaI\Delta ssbI$ mutant (Table 1). 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. 8). The *ssaI* null mutant loses the majority of detectable AHLs, as evaluated by bioassays and mass spectrometry (Figs. 1 and 2, Table 1). The AHLs produced by wild type KLH11 are dominated by OH-AHLs, those synthesized by SsbI (and perhaps SsaI), 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 an oxo-C16 AHL, correlated with SsaI activity, which is lost in the *ssbI ssaI* double mutant (Table 1). 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*-mutation does not affect their level, indicating that Ssb 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. pomeroiyi* 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 RhIR 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. 5C). 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) nor

when SsbR and an *ssbI-lacZ* fusion are tested in *A. tumefaciens* (Table 3). The *ssbI* gene joins a small list of LuxI homologues that are not positively autoregulated (Beck 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; Honag *et al.*, 2008). In KLH11 flagellar biosynthesis is under tight, positive SsaRI control (Fig. 6). 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 *Sinorhizobium meliloti* (Pellock *et al.*, 2002). Reconstructing SsaR-dependent gene regulation in *A. tumefaciens* suggests that as little as 10 nM 3-oxo-C16:1 Δ 11 is saturating (Fig S5B), and this may be even lower at native *ssaR* expression levels.

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). 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. 6D), 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). 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 *et al.*, 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. 7A), which is clearly due to QS as KLH11 supernatants antagonize biofilm formation in a dose-dependent manner (Fig. 7B). 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. Overall, the QS activation of motility and inhibition of adherence in KLH11 dictates the transition between free-living and sessile modes of growth and may limit aggregation within the sponge.

Experimental procedures

Reagents, strains, plasmids, growth conditions and DNA introduction

All strains and plasmids used in this study are listed in Table S2 and all the primers used are listed in Table S3. Antibiotics were obtained from Sigma Chemical Co. (St. Louis, MO). Standards used for AHL analysis; D₃C₆-HSL (N-[(3S)-tetrahydro-2-oxo-3-furanyl]-hexanamide-6,6,6-d₃ 99% deuterated product), 3-oxo-C₁₄-HSL and 3-oxo-C₁₆:1 Δ 11cis-(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 (trimethylsilyl) trifluoroacetamide (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 (Waters WAT020520).

DNA manipulations were performed by standard techniques (Sambrook *et al.*, 1989) and restriction enzymes and PhusionTM 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 at the Indiana Molecular Biology Institute (Bloomington, IN). 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)₂SO₄ (ATGN) (Tempé *et al.*, 1977). Antibiotics were used at the following concentrations (μg ml⁻¹): (i) *E. coli* (ampicillin, Ap, 100; gentamycin, Gm, 25; kanamycin, Km 25; spectinomycin, Sp, 100), (ii) KLH11 (Km, 100; Rifampicin, Rif, 200; Gm 25, Sp, 100) (iii), *A. tumefaciens* (Gm, 3000; 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).

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 (OD₆₀₀ ~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 OD₆₀₀ 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 D₃-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-NH₄ pH 5.7) and 2 µl solvent B (methanol). This solution was injected on to a 50 × 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* 102.3 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.

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 [M+NH₄]⁺ 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 (See Supplementary Methods). 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.

Genomic library screen of KLH11 QS genes

KLH11 genomic DNA was obtained using the Bactozol™ DNA Isolation Kit from Molecular Research Center Inc. (Cincinnati, OH). The DNA was independently digested to completion by restriction enzymes *HindIII* and *SaI* 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 *HindIII* and *SaI*

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 $\mu\text{g ml}^{-1}$). 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 *HindIII* genomic library. However, only 62 bp of the *ssbR* sequence was carried on pECH101, due to a *HindIII* 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 presumptively flanking *ssbR* was generated, and used to PCR amplify the complete *ssbR* sequence from KLH11.

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 *EcoRI* and *KpnI*, 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 *ssaR*intact F and *ssaR*intact R. The PCR amplicon was cloned into pGEM®-T Easy (Promega, Madison, WI) first and then subcloned into pVIK112, creating pJZ001. The pJZ001 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* D1 and *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 S3). 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 *SpeI* and *SphI*. 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 S3). 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.

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 *PstI*, and the resulting fragments were ligated with 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.

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 Δ 11-HSL. The cell suspension was thoroughly mixed and then aliquoted into 15 ml test tubes, incubated at 28°C for 24 h to an OD₆₀₀~0.4. Mid-log phase cultures were measured for OD₆₀₀ 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 and 3-oxo-C16:1 Δ 11 were added at the concentration of 20 μ M and 2 μ M, respectively. Mid-log phase of KLH11 cultures was sampled and β -galactosidase assays were performed immediately.

AHL detection using an ultrasensitive *A. tumefaciens* reporter for KLH11 derivatives and sponge tissues

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.*, 2008b; see Supplementary Methods). Whole *M. laxissima* individuals were harvested in Key Largo and immediately frozen on dry ice

upon surfacing from collection dives. AHLs were extracted using a modified Bligh Dyer procedure (Bligh and Dyer, 1959). Frozen tissue was ground to a powder in liquid nitrogen. To 10 grams of the powder-like tissue, 10 ml of methanol was added and the tube vortexed vigorously prior to overnight incubation. The sample was then vortexed with each addition of 5 ml of chloroform followed by 2.5 ml water and finally 5 ml of chloroform. The chloroform layer was separated and evaporated to 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 μ l methanol, and 20 μ l was loaded onto a C18 RP-TLC plate (Mallinckrodt Baker, Phillipsburg, NJ, USA). AHLs were again detected using the *A. tumefaciens* ultrasensitive reporter (Zhu *et al.*, 2003).

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 Δ 11-HSL was added to MB 2216 agar to 2 μ M. Plates were placed in an air-tight container with a beaker containing 15 ml of K₂SO₄ 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 OD₆₀₀ of 0.6, and portions of these were centrifuged at 716 \times 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 \times 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 on 15% SDS-PAGE gels transferred to nitrocellulose membranes by electroblotting (see Supplementary Methods). 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 OD₆₀₀ of 0.5, 1.3, 1.8, and 2.2; strain EC2 (*ssaI*) OD₆₀₀ 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.

RNA extraction and quantitative reverse transcriptase PCR (qRT-PCR) analysis

Expression levels of *fliC* and *flaA* were measured using q-RT-PCR with specific primers (Table S3). KLH11 and derivatives were grown in MB2216 to an OD₆₀₀ of 1.8 and 0.5 ml culture was collected and stored in 1 ml RNA Protect Bacterial Reagent (Qiagen, Valencia, CA) 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 60°C for primer annealing, and a 30 s extension at 72°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*).

RNA extraction and RT-PCR from sponge tissue

Samples of *M. laxissima* were collected by SCUBA diving at Conch Reef, Key Largo, Florida in late October 2008 at a depth of *ca.* 20 m as described by Zan *et al.*, (2011). Sponge samples were rinsed 3x with sterile artificial seawater (ASW) and were stored in RNAlater solution (Qiagen, Valencia, CA, USA) immediately for RNA extraction. Total RNA was extracted from sponge samples using the TissueLyser system (Qiagen,) and the AllPrep DNA/RNA mini kit (Qiagen). RNAase-free DNAase (Qiagen) 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 (Table S3) and ThermoScript RT-PCR system (Invitrogen, Grand Island, NY, USA). The conditions recommended by the manufacturer were used for the reverse transcription reaction. Primer set RtaF and RtaR 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.

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 OD₆₀₀~0.06 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 (A₆₀₀) was measured. These values were normalized by the OD₆₀₀ 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 × 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 Δ *ssaI* mutant was grown in MB2216 overnight and then diluted to an OD₆₀₀ ~ 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.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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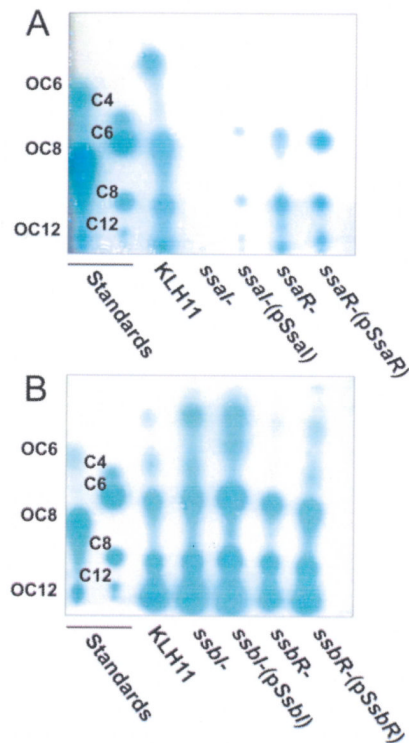


Figure 1. RP-TLC analysis of AHLs from KLH11 and quorum sensing mutants

TLC plates were overlaid with the *A. tumefaciens* ultrasensitive AHL reporter strain (Zan 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 μ M; C8, 50 nM; C10, 125 μ M. 3-oxo derivatives, 3-oxo-C6, 50 nM; 3-oxo-C8, 42 nM; 3-oxo-C12, 68 μ M.

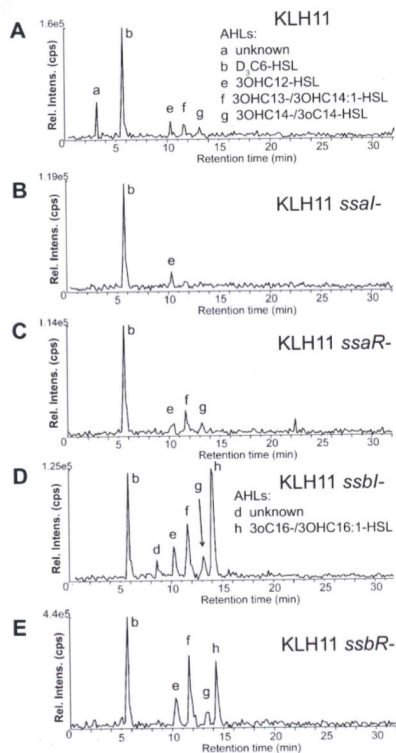


Figure 2. Mass spectrometry analysis of purified samples from KLH11 derivatives

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 $[M + H]^+ \rightarrow m/z 102$). 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 1 and the AHLs noted.

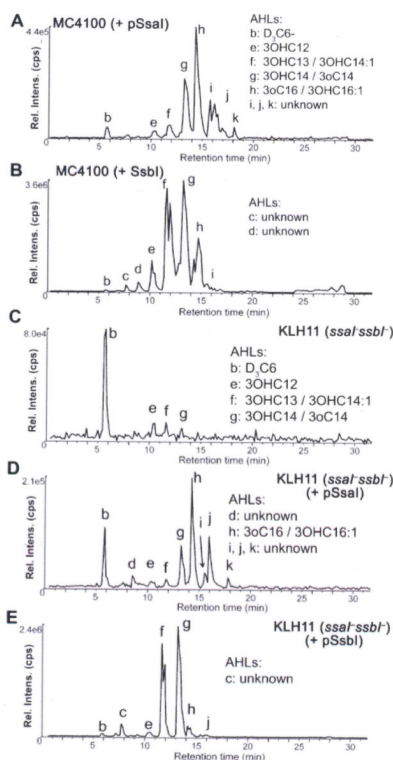


Figure 3. Mass spectrometric analysis of plasmid-expressed SsaI and SsbI-directed AHLs from *E. coli* and KLH11

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 $[M + H]^+ \rightarrow m/z 102$) for (A) MC4100 + SsaI, (B) MC4100 + SsbI, (C) KLH11 *ssaI-ssbI-*, (D) KLH11 *ssaI-ssbI-* + $P_{lac-ssaI}$, (E) KLH11 *ssaI-ssbI-* + $P_{lac-ssbI}$. The peaks in the chromatograms are labeled with lowercase lettering and include species defined in Table 1 and the AHLs noted.

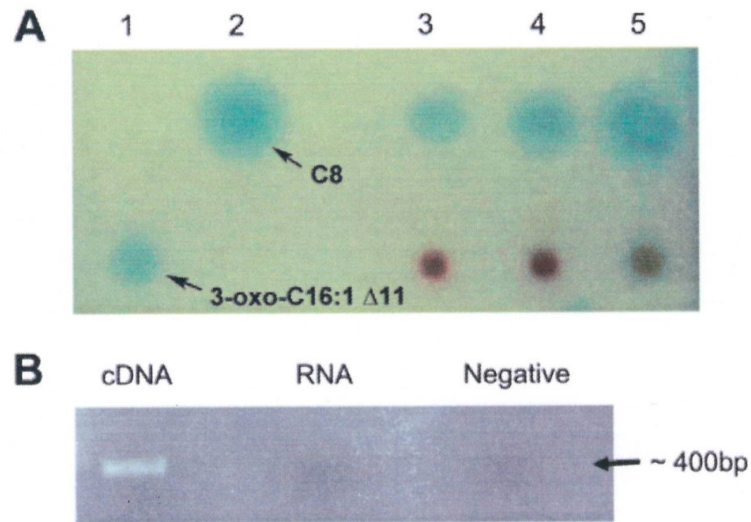


Figure 4. Detection of *ssaI* gene expression and AHLs in sponge tissue

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 Δ 11, 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.

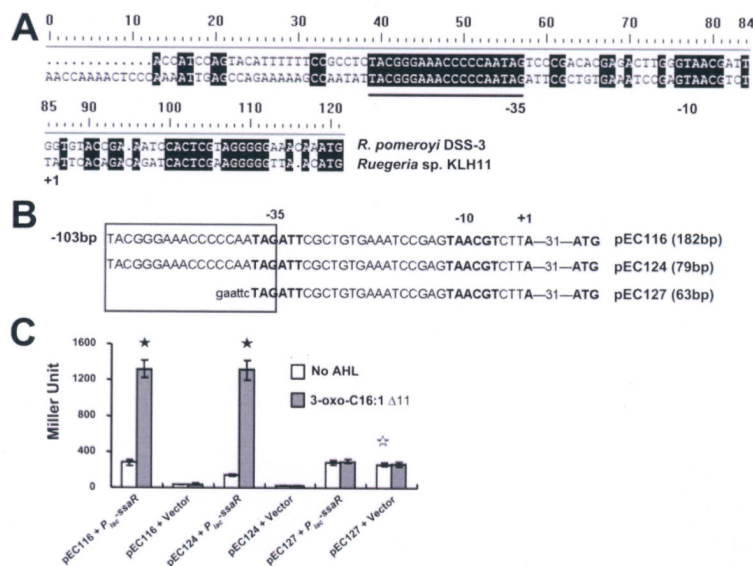


Figure 5. Deletion analysis of the *ssaI* promoter

(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 *EcoRI* 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 ($\Delta 11$), 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 pEC127 ($P < 0.001$). The result presented is a representative of several independent experiments each with three biological replicates.

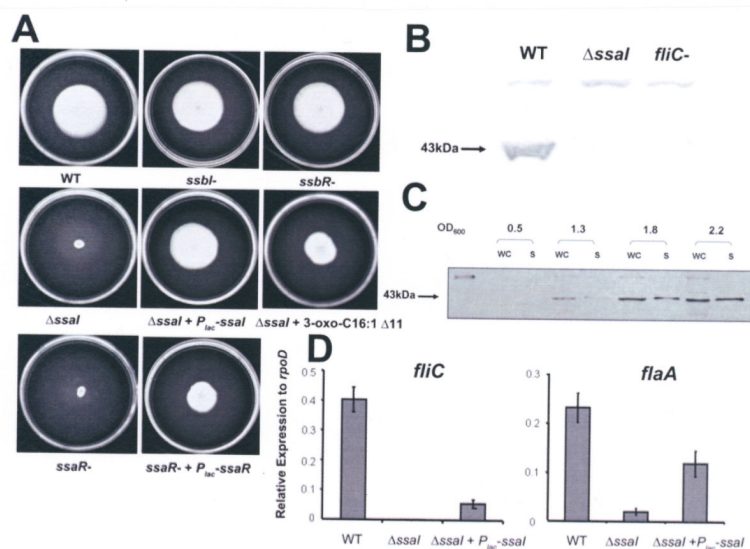


Figure 6. Regulation of swimming motility and flagellar biosynthesis by the SsaRI QS system (A) Swimming motility assays for QS mutants on 0.25% Marine Agar 2216 after 8 days at 28°C. 2 μ M 3-oxo-C16:1 Δ 11 was added for the Δ *ssaI* mutant and pEC108 (P_{lac} -*ssaI*) and pEC112 (P_{lac} -*ssaR*) were 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 *ssaI* deletion strain and complementation strains. Error bars are the standard deviations and results are representative of two independent experiments with triplicates.

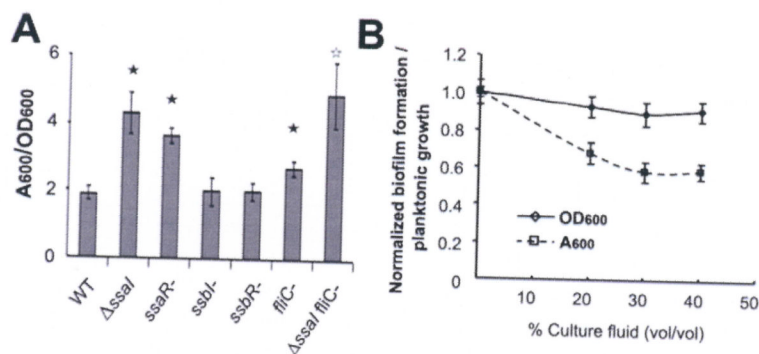


Figure 7. Increased biofilm formation by KLH11 in *ssaI* and *ssaR* mutants

(A) Standard 48 hr 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 $\Delta ssaI fliC$ ⁻ mutants ($P < 0.05$). Error bars are standard deviation of three biological replicates. (B) Inhibition of biofilm formation for $\Delta ssaI$ mutant by wt KLH11 culture fluids. Different % (v/v) wt KLH11 culture fluids were added into $\Delta ssaI$ 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. Values are averages of assays performed in triplicate and error bars are standard deviations.

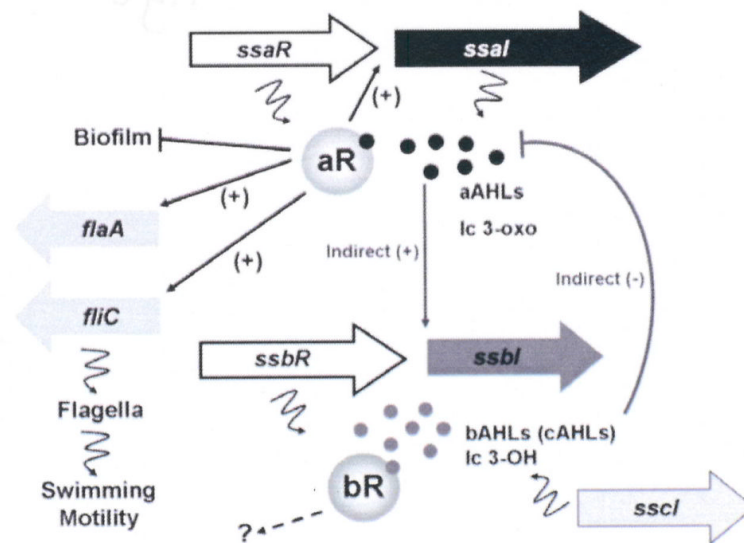


Figure 8. A simplified model for the complex regulatory control of QS circuits in KLH11
 Model depicts a presumptive role of QS in KLH11 dispersal during the interaction between sponge host and symbiont. The *ssaRI*, *ssbR*, and *sscI* genes are drawn according to scale while *fliC* and *flaA* are not. 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.

Table 1

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

Peak Label	Retention time \pm 0.6 (min)	[M+H] ⁺ m/z	[M+NH ₄] ⁺ m/z	AHL ^a	Ratio to internal standard (%)											
					WT			KLH11			E. coli			KLH11ssaI-ssbI-		
					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	0.29	0.26	<0.01	25.39	<0.01	<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		
			343	oxo-C14-HSL [§]					11.88			0.33				
h	14.5	354	371	oxo-C16-HSL [§]		1.4	0.80	1.87		28.1		2.60		9.07 [‡]		
				OH-C16:1-HSL [*]												
i	15.8			unk					+					+		
j	16.4			unk					+					+		
k	18.3			unk					+					+		

Peak Label-Reversed phase LC/MS/MS separation and AHL detection based on precursors of m/z 102.1 as labeled in Figures 2,3 and supplemental Figure 3.

^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

Table 2

QS regulator expression in KLH11 null mutants

Mutants ²	Relevant Genotype	β -Galactosidase Sp. Act. ¹		
		KLH11 Extracts	Synthetic AHLs	
		No Extract	2.5%	No AHL + AHL ³
EC2	<i>ssaI-lacZ, null</i>	6 (1.7)	95 (10.6)	6.3 (0.7) 213.3 (9.2)
EC3	<i>ssbI-lacZ, null</i>	1 (<1)	<1 (<0.1)	1.1 (0.3) 1.1 (<0.1)
JZ1	<i>ssaR-lacZ, WT</i>	107.1(0.8)	105.0 (4.4)	93.7 (8.3) 99.3 (2.8)
EC4	<i>ssaR-lacZ, null</i>	23 (3)	19 (1.4)	26.7 (1.3) 28.6 (2.4)
JZ2	<i>ssbR-lacZ, WT</i>	11.6 (2.8)	10.8 (3.1)	12.0 (0.5) 12.6 (0.5)
EC5	<i>ssbR-lacZ, null</i>	15 (<)	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 All strains are derived from EC1, spontaneous Rif^r mutant. Strains EC2, EC3, JZ1 and JZ2 carry Campbell insertions and lacZ transcriptional fusions;

³ 2 μ M 3-oxo-c[6:1 Δ 11 was added for *ssaIR* and 20 μ M 3-OH-C14 was added for *ssbIR*.

Table 3Expression of KLH11 PssaI and PssbI promoters in an AHL- host¹

Expression plasmid	Fusion plasmid	β -Galactosidase Sp. Act. ²	
		No AHL	+ AHL ³
Vector (pBBR1-MCS5)	<i>ssaI-lacZ</i> (pEC116)	51 (5)	52 (5)
<i>P</i> _{lac} -ssaR (pEC112)	<i>ssaI-lacZ</i> (pEC116)	341 (15)	1435 (67)
Vector (pBBR1-MCS5)	<i>ssbI-lacZ</i> (pEC121)	<1 (<0.1)	<1 (<0.1)
<i>P</i> _{lac} -ssbR (pEC123)	<i>ssbI-lacZ</i> (pEC121)	<1 (<0.1)	<1 (<0.1)

¹All strains derived from Ti-plasmidless *A. tumefaciens* NTL4²Specific activity in Miller Units, averages of assays in triplicate (standard deviation)³2 μ M 3-oxo-C16:1 Δ 11 was added for *ssaI* and 20 μ M 3-OH-C14 was added for *ssbI*