A solo *luxI*-type gene directs acylhomoserine lactone synthesis and contributes to motility control in the marine sponge symbiont *Ruegeria* sp. KLH11

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Marine sponges harbour abundant and diverse bacterial communities, providing an ideal environment for bacterial cell-density-dependent cell–cell signalling, termed quorum sensing. The marine sponge symbiont *Ruegeria* sp. KLH11 produces mainly long chain acylhomoserine 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, *luxR/luxI* homologue). In this study, we identified a third *luxI*-type gene, named *sscI*. The *sscI* gene does not have a cognate *luxR* homologue present at an adjacent locus and thus *sscI* is an AHL synthase solo. The *sscI* gene is required for production of long-chain hydroxylated AHLs, contributes to AHL pools and modestly influences flagellar motility in KLH11. A triple mutant for all *luxI*-type genes cannot produce AHLs, but still synthesizes *para*-coumaroyl-homoserine lactone.

INTRODUCTION

Marine sponges harbour highly diverse and dense microbial communities and in some cases up to 30–40 % of the sponge biomass is from the associated bacteria (Taylor *et al.*, 2007). These sponges provide a highly conducive environment for bacterial quorum sensing (QS), a process by which bacteria use chemical signals to communicate with each other and coordinate group behaviours, such as bioluminescence, antibiotic production and virulence at high cell density (Ahlgren *et al.*, 2011; Fuqua & Greenberg, 2002). Acylhomoserine lactone (AHL)-based QS was first discovered in the marine squid symbiont *Vibrio fisheri* about 40 years ago and since then it has been reported in over 100 different bacterial species, although mainly restricted to the phylum *Proteobacteria* (Ahlgren *et al.*, 2011; Fuqua & Greenberg, 2002).

Typically, each QS circuit has a *luxI*-type gene, responsible for AHL synthesis, and a *luxR*-type gene, encoding a protein that binds and provides a response to AHL(s). These two genes are often genetically linked, arranged in tandem or convergently expressed (Fuqua & Greenberg, 2002). However, in many different bacteria, *luxR*-type genes without a linked *luxI*-type gene have been discovered and these *luxR*-type genes are termed *luxR* solos. These solos occur both in bacteria that have complete LuxR-LuxI-type QS systems and bacteria that do not (Subramoni & Venturi, 2009). LuxR solos can regulate gene expression by binding to AHLs produced by other *luxI* genes encoded elsewhere in the same bacterial genome, such as in the cases of QscR in *Pseudomonas aeruginosa* (Lequette *et al.*, 2006) and ExpR in *Sinorhizobium meliloti* (McIntosh *et al.*, 2008), or by binding to AHLs produced by other bacteria, such as in the case of SdiA in *Escherichia coli* and *Salmonella enterica* (Ahmer, 2004; Yao *et al.*, 2006). Furthermore, some LuxR-type proteins can regulate gene expression in response to non-AHL signals or independently of ligand

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Abbreviations: AHL, acylhomoserine lactone; Ap, ampicillin; Gm, gentamicin; HSL, homoserine lactone; Km, kanamycin; *p*C, *para*-coumaroyl; QS, quorum sensing; Rif, rifampicin; Sp, spectinomycin.

binding (Subramoni & Venturi, 2009). Although progress has been made in understanding these *luxR* solos, there is little information about the less common scenario in which functional *luxI* genes are not linked to *luxR*-type genes, which we designate as *luxI* solos.

We have found that members of the *Silicibacter-Ruegeria* (SR) subgroup of the ecologically important *Roseobacter* clade are the primary AHL producers among cultivatable bacterial isolates from the marine sponges *Mycale laxissima* and *Ircinia strobilina* (Mohamed *et al.*, 2008) and over 80 % of available roseobacterial genomes encode at least one *luxI* homologue (Zan *et al.*, 2014). We have developed *Ruegeria* sp. KLH11 (hereafter referred to as KLH11) as a model to study QS in marine sponge symbionts. We previously reported detailed analyses of two *luxRI* systems: *ssaRI* and *ssbRI* in KLH11 (Zan *et al.*, 2012). SsaI and SsbI direct the synthesis of long chain AHLs ranging from C_{12}- to C_{16}-homoserine lactone (HSL), dominated by 3-oxo and 3-hydroxy moieties at the beta-position in the acyl chain, respectively. The SsaRI system provides QS-dependent control of flagellar motility in KLH11, functioning through the CtrA master regulator (Zan *et al.*, 2013). Analysis of whole sponge tissues revealed the presence of *ssaI* transcripts and AHLs (Zan *et al.*, 2012). The function of the SsbRI system remains unclear, but it is indirectly regulated by SsaRI. We have also presented preliminary evidence for a solo *luxI*-type gene, *sscI*, in KLH11, which is not genetically linked to a *luxR* homologue (Zan *et al.*, 2012). In the current study, we have analysed the functional characteristics of the *sscI* solo in KLH11. Furthermore, we tested whether KLH11 can produce the novel *para*-coumaroyl-HSL (*p*C-HSL) molecule that was originally discovered in *Rhodopseudomonas palustris* and requires the substrate *para*-courmarate for synthesis by the LuxI homologue RpaI (Schaefer *et al.*, 2008).

METHODS

Bacterial strains, oligonucleotides and growth conditions. Bacterial strains and plasmids used in this study are listed in Table 1. Oligonucleotides were obtained from Integrated DNA Technologies (Coralville, IA, USA). 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 (MB 2216; BD) and *Agrobacterium tumefaciens* strains were grown in AT minimal salt medium supplemented with 0.5 % (w/v) glucose and 15 mM $(NH4)_2SO_4$ (ATGN; Tempé *et al.*, 1977). *Rhodopseudomonas palustris* CGA814 was grown in LB broth. Antibiotics were used at the following final concentrations (μg ml^{-1}): (i) *E. coli* (ampicillin, Ap, 100; gentamicin, Gm, 25; kanamycin, Km, 25; spectinomycin, Sp, 100; tetracycline, Tc, 5). (ii) KLH11 (Km, 100; rifampicin, Rif, 200; Gm 25; Sp, 100; Tc, 5). (iii) *A. tumefaciens* (Gm, 300; Sp, 200). (iv) *Rh. palustris* CGA814 (Km, 50).

Plasmid construction for *sscI* null mutation, expression and *lacZ*-fusion. Several regions around the *sscI* gene were isolated by PCR amplification from KLH11 genomic DNA. The method used to construct the *sscI* Campbell insertion mutant was similar to that described by Zan *et al.* (2012). Briefly, an internal fragment of the *sscI* gene was amplified using forward primer 5′-GAATTCATGTTTCG-CGATCGAGCAGAT-3′ (the *Eco*RI recognition site is underlined) and reverse primer 5′-GGTACCTCTTGATACTCCCGCTC-3′ (the *Kpn*I recognition site is underlined). The PCR amplicon was gel-purified and cloned into pCR 2.1-TOPO vector (Invitrogen) 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 & Winans, 1997), creating pOKC2. pOKC2 was conjugated into KLH11 and Km-resistant (Km^R) transconjugants were selected and confirmed by sequencing using the forward primer 5′-ATTAACCATAATCAAGCATCTCTT-3′. To construct double and triple AHL synthase gene mutants, pOCK2 was conjugated into Δ*ssaI* and 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 5′-TCTAGAC**TGA**AAC*AGGAAA*CAGCT**ATG**CTCCGTTATGTTTTTG-CA-3′ (the *Xba*I recognition site is underlined, the stop codon TGA and the start codon ATG are in bold type and the *E. coli lacZ* ribosome-binding site is in italics) and the reverse primer 5′-CTCGAG**TCA**AGCGGTTCTTTGAAACTT-3′ (the stop codon is in bold type and the *Xho*I recognition site is underlined). The PCR products were ligated into pCR2.1-TOPO vector (Invitrogen) to create 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.

In order to generate a plasmid-borne P_{sscI}-*lacZ* fusion, the presumptive promoter sequences were PCR amplified. The forward primer 5′-GAATTCGCCGAGATGAACTGTTCAAAGAAC-3′ (the *Eco*RI recognition site is underlined) and the reverse primer 5′-GGATCCGAGC-ATTTTTAACCTCTTGTTCAC-3′ (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 were 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 & Winans, 2002) to create pOKC8 with the P_{sscI}-*lacZ* translational fusion.

Organic extraction, TLC and MS analysis of AHLs. As described previously, organic extraction of KLH11-derivative cultures, followed by reverse-phase TLC of organic extracts and AHL bioassay analysis with an ultrasensitive AHL bioreporter derived from *A. tumefaciens* was used to characterize the AHLs specified by SscI (Zan *et al.*, 2012; Zhu *et al.*, 2003).

Identification of SscI AHLs by LC-MS-MS was also performed. KLH11 derivatives were grown in MB 2216 with appropriate antibiotics (and 0.5 mM IPTG to induce the P_{lac} promoter) at 28 °C to stationary phase (OD_{600}~2.0) in the presence of 5 g Amberlite XAD 16 resin l^{-1} for 36 h. Cells and resin were separated by centrifugation and extracted with 50 ml methanol and dried to 2 ml. Three nanomoles of D_3-C_6-HSL (D_3 indicates that there are three deuterium atoms at the terminal position of the acyl chain in the AHL molecule) was added to each sample as an internal standard and a volume of 0.2 ml of each extract was purified using solid phase extraction methods as described previously (Gould *et al.*, 2006).

Extracts were dried down and resuspended in 38 μl solvent A (8.3 mM ammonium acetate, pH 5.7) and 2 μl solvent B (methanol). This solution was injected on to a 50 × 3.00 mm 2.6 μ 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

Table 1. Bacterial strains and plasmids used in this study

Bacteria/plasmid	Relevant feature	Reference
E. coli DH5α/λpir	Strain for propagating R6K suicide plasmids	Lab collection
E. coli S17-1/λpir	IncP conjugal donor	Kalogeraki & Winans (1997)
E. coli XL-1 Blue	Standard alpha-complementation strain	Lab collection
E. coli MC4100	K-12 derivative, Δ*lacZ*	Lab collection
A. tumefaciens NTL4	Ti plasmid-less derivative, nopaline chromosomal background	Zhu *et al.* (1998)
KLH11	WT	Mohamed *et al.* (2008)
KLH11-EC1	Rif^R	Zan *et al.* (2012)
KLH11-SK01	Δ*ssaI*, Rif^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-OKC6	Δ*ssaI* Δ*ssbI ssc-lacZ*, Rif^R Km^R	This study
CGA814	*Rh. 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, Km^R	Kalogeraki & Winans (1997)
pRA301	BHR *lacZ* translational fusion vector	Akakura & 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)
pOKC1	pCR2.1-TOPO, carrying 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 *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

Sm, Streptomycin; Tc, tetracycline.

was interfaced to the electrospray source of a triple quadrupole mass spectrometer (Sciex API2000, PE Sciex). 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.

Preparation of extracts and bioassay for *p*C-HSL. KLH11 and derivative strains were grown to stationary phase in MB 2216 supplemented with or without 1 mM *p*-coumaric acid. The cultures (acidified by 0.01 % acetic acid) were extracted twice with an equal amount of acetyl acetate and the extracts were dried in a rotary evaporator under vacuum. Each extract was concentrated 1000-fold, dissolving in 50 % (v/v) methanol. Fifty microlitres of the enriched extract was added to 1 ml of *Rh. palustris* CGA814 culture grown in LB. β-Galactosidase assays were performed as described previously (Zan *et al.*, 2013).

Motility assays. Bacterial swim assays were performed using MB 2216 with 0.25 % (w/v) agar. Plates were inoculated at the centre with freshly isolated KLH11 colonies. KLH11 crude organic extract (0.5 %, v/v) was added to MB 2216 agar. Plates were placed in an airtight container with a beaker containing 15 ml K_2SO_4 to maintain constant humidity, and incubated for 5–7 days at 28 °C. Photos were taken by using a Nikon D90 camera.

RESULTS AND DISCUSSION

Identification of *sscI*

Genome sequencing of KLH11 revealed the presence of a *luxI* homologue, designated *sscI*, encoded on a large assembled sequence scaffold (>700 kb) that contains neither the *ssaRI* nor the *ssbRI* operons (Zan *et al.*, 2011). The *sscI* and *ssbI* genes and their translation products are highly similar (70 % and 76 % nucleotide and amino acid identity, respectively) (Fig. 1), and much less similar to those of *ssaI* (52 % and 27 %, respectively). The sequence conservation between the *ssbI* and *sscI* genes is strikingly confined to the coding sequences, without significant similarity in their flanking regions (Fig. 1). This strongly suggests that *sscI* arose from a gene duplication event with *ssbI*. The closely related *Ruegeria pomeroyi* DSS-3 genome encodes *ssaRI* and *ssbRI* regions that are highly homologous and syntenic to KLH11, but does not encode an *sscI* gene (Moran *et al.*, 2004). This indicates that *sscI* was either generated by duplication in KLH11 or, conversely, lost in DSS-3 since the time of their most recent common ancestor in the *Ruegeria* lineage. The region downstream of *sscI* in KLH11 is conserved with *Ru. pomeroyi* DSS-3, particularly linkage to a large gene encoding

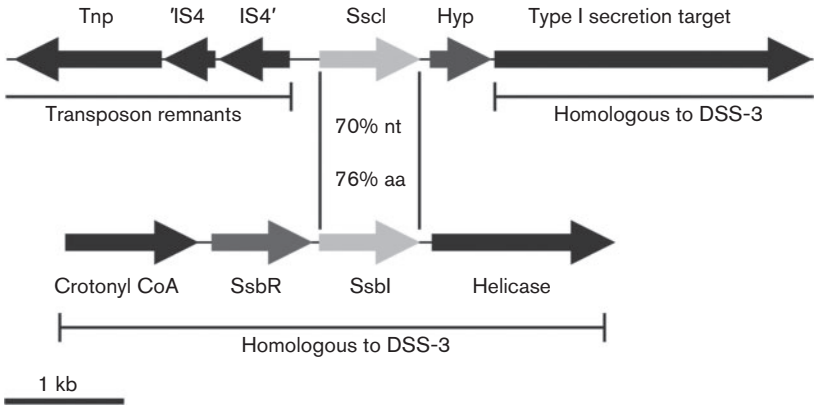

Fig. 1. Genetic map of KLH11 *sscI* region and comparison with the *ssbR-ssbI* region. The *sscI* region shows homology to an analogous region of *Ru. pomeroyi* DSS-3 on the downstream side of the gene, and upstream of the gene there is a similar yet not identical area with several transposase and phage integrase partial gene fragments.

a predicted type I secretion target repeat protein (SPO2401). Upstream of *sscI* both genomes are chequered with several transposase and phage integrase gene remnants (Fig. 1) with a large number of frame-shift mutations, suggesting a high level of chromosomal rearrangement.

Solo *luxR*-type genes are common in bacterial genome sequences, but there are very few reported intact *luxI*-type solos. One exception is in another roseobacter, *Dinoroseobacter shibae* DFL 12^{T}, an algal symbiont that also has two sets of *luxR-luxI* QS systems and one *luxI* solo, designated *luxI3* (Wagner-Döbler *et al.*, 2010). The genomic location of this *luxI*-type solo is not recognizably similar to that of *sscI* in KLH11 and the LuxI3 protein is not particularly similar to SsbI or SscI (~30 % identity). The *luxI3* solo in *D. shibae* is therefore distinct from *sscI* in KLH11.

SscI-derived AHL production

A targeted *sscI* mutation using a 514 bp internal fragment of *sscI* and the pVIK112 suicide plasmid (Kalogeraki & Winans, 1997) was made, generating a *sscI* null mutant (OKC2), with a *sscI-lacZ* transcriptional fusion. β-Galactosidase assays of OKC2 revealed significant levels of *sscI-lacZ* expression that were unaffected by the addition of KLH11 whole culture extracts containing AHLs (Miller units: 189.6 ± 20.2 and 199.8 ± 7.9; $P > 0.05$; unpaired Student's *t*-test) that strongly activate expression of *ssaI*. This level of expression, although AHL-independent, was ~200-fold higher than the expression of an *ssbI-lacZ* fusion generated in an analogous manner with the pVIK112 plasmid (Zan *et al.*, 2012). This difference in expression probably reflects the lack of identity in the regions immediately upstream of these coding sequences. It is worth noting that neither a Lux box nor the previously defined *ssa* box (Zan *et al.*, 2012) was found in either the *ssbI* or *sscI* promoter regions (data not shown).

A triple mutant, Δ*ssaI* Δ*ssbI* *sscI*$^{-}$ (OKC6, with *sscI* disrupted using the pVIK112 derivative), was analysed for AHL production in whole-cell extracts using TLC overlaid with agar containing an *A. tumefaciens* AHL reporter as described by Zan *et al.* (2012). No AHL production was observed for this mutant (Fig. 2a). Quantitative MS, as previously described (Gould *et al.*, 2006; Zan *et al.*, 2012), also failed to detect AHLs in this mutant (Fig. 2b). Provision of plasmid-borne copies of each AHL synthase gene individually in this triple mutant resulted in AHL synthesis (Fig. 2a); the *ssaI* plasmid is weakly active and its AHLs were difficult to detect by this bioassay, but clearly detected by MS (Zan *et al.*, 2012). MS analysis of the *sscI*-expressing derivative revealed high-level synthesis of several hydroxylated AHLs (Fig. 2c), consistent with our findings on its expression in *E. coli* (Zan *et al.*, 2012). The high levels of AHL driven by *sscI* suggest that it encodes a highly active enzyme.

SscI-derived AHLs are involved in *ssaI* expression and influence swimming motility

SsaR responds to SsaI-directed 3-oxo-HSL derivatives, and also, but more weakly, to those synthesized by SsbI (Zan *et al.*, 2012). To test whether SsaR can also respond to SscI-derived AHLs, a plasmid-borne copy of *ssaR* (pEC112) or a vector control were paired with a compatible plasmid carrying the P_{ssaI}-*lacZ* fusion (pEC116), in an AHL^{-}, plasmid-less derivative of *A. tumefaciens* NTL4. Cultures of *A. tumefaciens* derivatives were grown with 2.5 % (v/v) culture extracts containing SscI-derived AHLs (whole culture dichloromethane extracts from an *A. tumefaciens* NTL4 derivative grown with IPTG to induce expression of the P_{lac}-*sscI* plasmid). Expression of *ssaI* increased ~four-fold in response to the extracts compared with the negative control ($P < 0.01$) (Fig. 3a; as in prior studies, the P_{lac}-ssaR plasmid modestly stimulates AHL-independent *ssaI* expression). This response of SsaR to SscI-directed AHLs adds another layer of complexity to the QS network in KLH11.

Flagellar motility is strictly dependent on activation by the *ssaRI* system through the CckA-ChpT-CtrA motility regulators (Zan *et al.*, 2012, 2013). The *sscI* null mutant (OKC2) consistently showed a 20 % decrease in swim ring diameter relative to WT KLH11 ($P < 0.05$) and plasmid-borne *sscI* complemented this defect (Fig. 3b). We hypothesize that this mild effect on motility is most likely due to the impact of SscI-derived AHLs on the *ssaI* gene expression through SsaR.

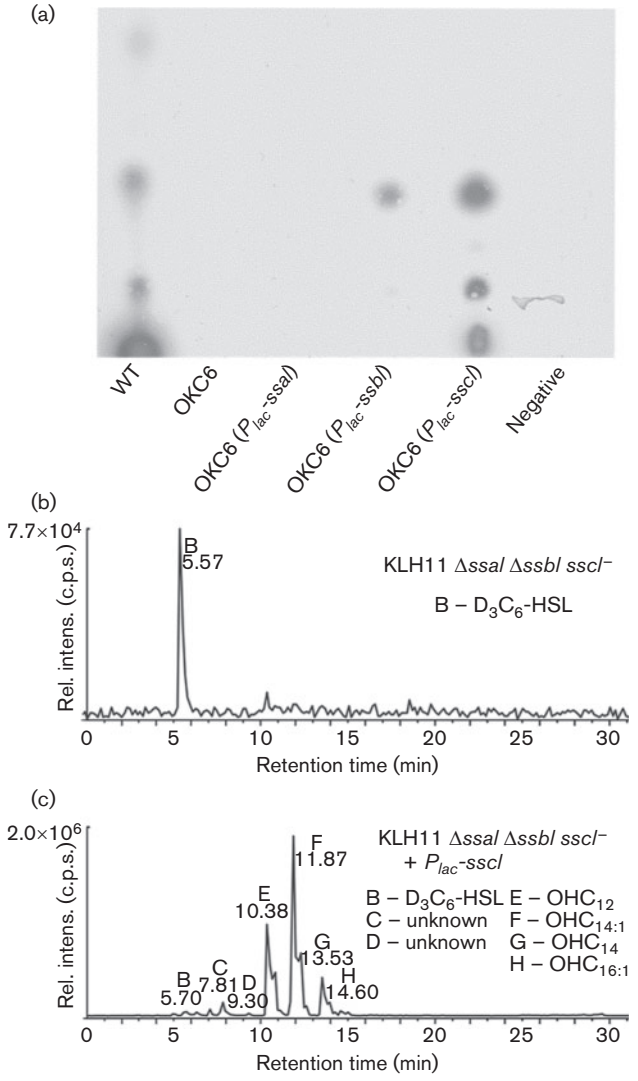

Fig. 2. (a) Reverse-phase (RP)-TLC analysis of AHLs from KLH11 and QS mutants. TLC plate was overlaid with the *A. tumefaciens* ultrasensitive AHL reporter strain (Zhu *et al.*, 2003). Organic extracts of cultures of strain OKC6 containing vector pSRKTc (Khan *et al.*, 2008) were used as negative control. The concentration of X-Gal in the agar was 40 μg ml^{-1}. (b, c) MS analysis of purified samples. The products of reverse-phase chromatographic separation of AHLs extracted and purified from (b) OKC6 (Δ*ssaI* Δ*ssbI sscI*$^-$) and (c) OKC6 (Δ*ssaI* Δ*ssbI sscI*$^-$/P_{lac}-*sscI*) were examined using the precursor ion-scanning mode (transitions were monitored for precursor $[M+H]^+$, $\rightarrow m/z$ 102.1). The peaks in the chromatograms are labelled with upper-case lettering and include the AHLs noted and as described by Zan *et al.* (2012).

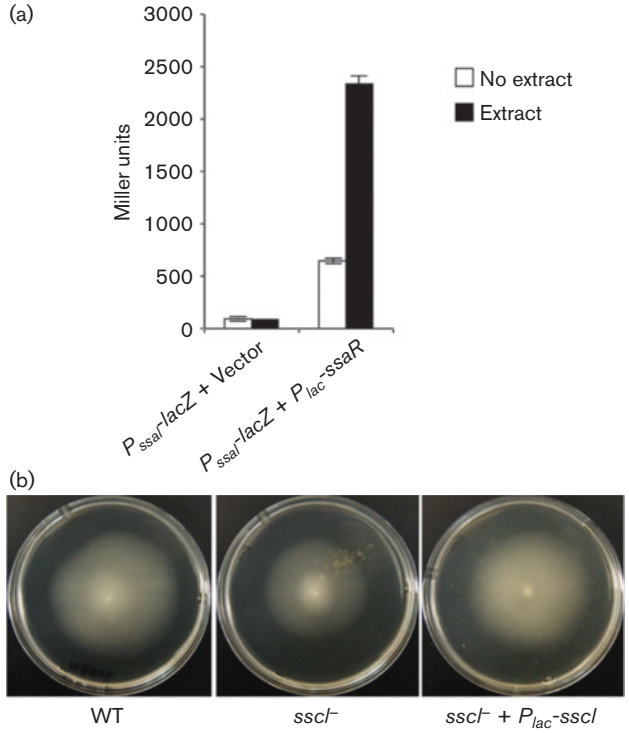

Fig. 3. (a) SscI-derived AHLs stimulate *ssaI* expression and the *sscI* mutant is decreased for motility. Promoter fusion of *ssaI* with *lacZ* reporter (pEC116) paired with P_{lac}-*ssaR* (pEC116) (Zan *et al.*, 2012) or empty vector (pBBR1-MCS5; Kovach *et al.*, 1995) were transformed into *A. tumefaciens* NTL4. β-Galactosidase assay was used to monitor the *lacZ* activity with and without organic extracts. Error bars represent the standard deviation from three replicates. (b) *sscI* is involved in motility control. WT KLH11 and derivatives were inoculated on MB 2216 (supplemented with 0.25 %, w/v, agar) swim agar plates for 1 week at 28 °C. The results shown are representatives of several independent experiments each with three biological replicates.

Production of *p*C-HSL independently from LuxI homologues

Several novel types of AHL molecules have been reported recently (Ahlgren *et al.*, 2011; Lindemann *et al.*, 2011; Schaefer *et al.*, 2008). *Rh. palustris* produces *p*C-HSL, which is an arylhomoserine lactone incorporating a coumaroyl group rather than an acyl chain. Remarkably, *p*C-HSL is only produced in the presence of *para*-coumarate, a compound synthesized by plants and certain algae, and directly incorporated into the signal molecule via the LuxI-type protein RpaI. In the absence of *para*-coumarate, RpaI does not synthesize a product. *Ru. pomeroyi* DSS-3, a relative of KLH11, was also found to produce *p*C-HSL in cultures grown with *para*-coumarate (Schaefer *et al.*, 2008). We used the *p*C-HSL reporter strain *Rh. palustris* CGA814 that cannot synthesize *p*C-HSL but directs RpaR-dependent expression of a target *rpaI-lacZ* fusion (Hirakawa *et al.*, 2011) to examine KLH11 culture extracts. KLH11 grown in the presence of 1 mM *para*-coumarate can activate the expression of *rpaI-lacZ* to a level equivalent to 1 μM *p*C-HSL, suggesting the presence of *p*C-HSL or a structurally similar molecule. Surprisingly, a KLH11 mutant disrupted for all three *luxI*-type genes (*ssaI*, *ssbI* and *sscI)* retained this activity (Fig. 4), suggesting the existence of a novel enzyme(s) in KLH11 responsible for its synthesis.

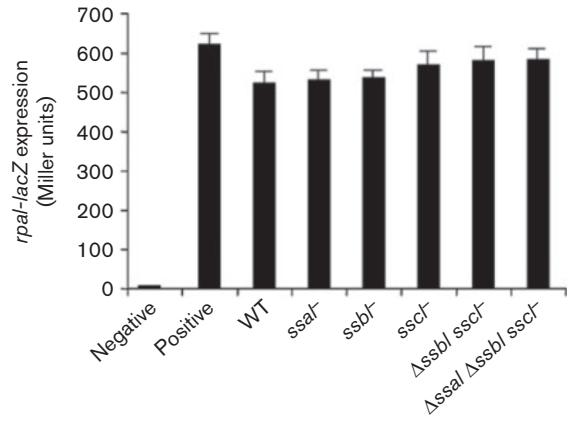

Fig. 4. β-Galactosidase assay of the expression of an *rpaI-lacZ* fusion. *Rh. palustris* CGA814 was used as the reporter strain. Organic extracts of KLH11 strains were prepared from cultures grown in MB 2216 supplemented with 1 mM *para*-coumarate. An extract of MB 2216 plus *para*-coumarate was used as the negative control and 1 μM *p*C-HSL was used as the positive control. Bars represent the mean of three biological replicates and the error bars represent the SD of triplicates.

The roseobacter *Phaeobacter gallaeciensis* BS107 can respond to the presence of *para*-coumarate produced by the microalga *Emiliania huxleyi* potentially via *p*C-HSL (Seyedsayamdost *et al.*, 2011). Novel signal molecules are synthesized by other roseobacters, including *Silicibacter* sp. TM1040, which does not encode *luxI* homologues or *luxM* (Cao & Meighen, 1989; Ng & Bassler, 2009), the gene encoding an alternative AHL synthase that directs the synthesis of 3-OH-C_4 HSL in *Vibrio harveyi* and exists in several *Vibrio* species, but rather produces the Roseobacter Motility Inducer (RMI) that can be induced by addition of *para*-coumarate (Sule & Belas, 2013). Several roseobacters produce the antibiotic and novel QS molecule tropodithietic acid (TDA), which regulates its own synthesis. Our findings contribute to the emerging impression that the roseobacter group may be an underexplored and rich source of novel signalling molecules.

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