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
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 luxl 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 luxI systems: ssaRI and ssbRI in KLH11 (Zan et al., 2012). SsaI and SsbI direct the synthesis of long chain AHLs ranging from C12- to C16- 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 (pC-HSL) molecule that was originally discovered in Rhodopseudomonas palustris and requires the substrate para-coumarate for synthesis by the LuxI homologue Rpal (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 (Coralville, IA, USA). Unless stated otherwise, bacterial strains, oligonucleotides and growth conditions.

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'-GGTACCTCTTGTATCCGCTCTC-3' (the KpnI recognition site is underlined) and reverse primer 5'-GGTACCTCTTGTATCCGCTCTC-3' (the KpnI 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 recombinatorial mutagenesis, pOKC1 was digested with EcoRI and KpnI, 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 (KmA) transconjugants were selected and confirmed by sequencing using the forward primer 5'-ATTACACATAATCAAGCGATCTCTTC-3'. To construct double and triple AHL synthase gene mutants, pOKC2 was conjugated into Assal and Assbl, and Assal Assbl 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'-TCTAGACTGAAACAGGAAACAGCTATGCTCGGTTATGGTTTGG-CA-3' (the Xhol recognition site is underlined, the stop codon TGA and the start codon ATG are in bold type and the reverse primer 5'-CTCAGATCGAAACAGGTTCTTGAACAT-3' (the reverse primer 5'-CTCAGATCGAAACAGGTTCTTGAACAT-3' (the 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 (pC-HSL) molecule that was originally discovered in Rhodopseudomonas palustris and requires the substrate para-coumarate for synthesis by the LuxI homologue Rpal (Schaefer et al., 2008).

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 ultrasonisful 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 LuxI recognition site is underlined, the reverse primer 5'-GGATCCGAGCAGATCGGGATCCGGAGCGAGTATACCTTTGATGGTTTGGCA-3' (the EcoRI recognition site is underlined) and the start codon TGA and the stop codon ATG are in bold type and the reverse primer 5'-CTCAGATCGAAACAGGTTCTTGAACAT-3' (the reverse primer 5'-CTCAGATCGAAACAGGTTCTTGAACAT-3' (the 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 (pC-HSL) molecule that was originally discovered in Rhodopseudomonas palustris and requires the substrate para-coumarate for synthesis by the LuxI homologue Rpal (Schaefer et al., 2008).

In order to generate a plasmid-borne PsaI-lacZ fusion, the presumptive promoter sequences were PCR amplified. The forward primer 5'-GAATTCGAGCAGATCGGGATCCGGAGCGAGTATACCTTTGATGGTTTGGCA-3' (the EcoRI recognition site is underlined) and the reverse primer 5'-GGATCCGAGCAGATCGGGATCCGGAGCGAGTATACCTTTGATGGTTTGGCA-3' (the 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 (pC-HSL) molecule that was originally discovered in Rhodopseudomonas palustris and requires the substrate para-coumarate for synthesis by the LuxI homologue Rpal (Schaefer et al., 2008).

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 x 3.00 mm 2.6 μ C18 Kinexet (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). 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, 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, scan a mass range of 170 to 700 over 9 s.

### 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 *Ruegeria* DSS-3, particularly linkage to a large gene encoding...
a predicted Type I secretion target repeat protein (SPO2401). Upstream of ssc1 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 127, an algal symbiont that also has two sets of luxR-luxI QS systems and one lux solo, designated luxI3 (Wagner-Döbler et al., 2010). The genomic location of this luxI-type solo is not recognizably similar to that of ssc1 in KLH11 and the LuxI3 protein is not particularly similar to SsBI or Ssc1 (~30% identity). The LuxI3 solo in D. shibae is therefore distinct from ssc1 in KLH11.

### Ssc1-derived AHL production

A targeted ssc1 mutation using a 514 bp internal fragment of ssc1 and the pVIK112 suicide plasmid (Kalogeraki & Winans, 1997) was made, generating a ssc1 null mutant (OKC2), with a ssc1-lacZ transcriptional fusion. β-Galactosidase assays of OKC2 revealed significant levels of ssc1-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 sbbI-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 sbbI or ssc1 promoter regions (data not shown).

A triple mutant, ΔssaI ΔsbbI ssc1 (OKC6, with ssc1 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 ssc1-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 ssc1 suggest that it encodes a highly active enzyme.

### Ssc1-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 Ssc1-derived AHls, a plasmid-borne copy of ssaI (pEC112) or a vector control were paired with a compatible plasmid carrying the pssaI-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 Ssc1-derived AHls (whole culture dichloromethane extracts from an A. tumefaciens NTL4 derivative grown with IPTG to induce expression of the P<sub>lac</sub>-ssc1 plasmid). Expression of ssaI increased ~fourfold in response to the extracts compared with the negative control (P < 0.01) (Fig. 3a; as in prior studies, the P<sub>lac</sub>-ssaR plasmid modestly stimulates AHL-independent ssaI expression). This response of SsaR to Ssc1-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 ssc1 null mutant (OKC2) consistently showed a 20% decrease in swim ring diameter relative to WT KLH11 (P < 0.05) and plasmid-borne ssc1 complemented this defect (Fig. 3b). We hypothesize that this mild effect on motility is most likely due to the impact of Ssc1-derived AHls on the ssaI gene expression through SsaR.
Production of pC-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 pC-HSL, which is an arylhomoserine lactone incorporating a coumaroyl group rather than an acyl chain. Remarkably, pC-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 pC-HSL in cultures grown with para-coumarate (Schaefer et al., 2008). We used the pC-HSL reporter strain Rh. palustris CGA814 that cannot synthesize pC-HSL but directs RpaR-dependent expression of a target rpa-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 rpa-lacZ to a level equivalent to 1 μM pC-HSL, suggesting the presence of pC-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. 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 pSRK5c (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 (ΔssaI ΔssbI ΔsscI⁻) and (c) OKC6 (ΔssaI ΔssbI ΔsscI⁺/ Pₜₜₜ-sscI) were examined using the precursor ion-scanning mode (transitions were monitored for precursor [M+H]⁺, ∼ m/z 102.1). The peaks in the chromatograms are labelled with uppercase lettering and include the AHLS noted and as described by Zan et al. (2012).

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The roseobacter *Phaeobacter gallaeciensis* BS107 can respond to the presence of *para*-coumarate produced by the microalga *Emiliania huxleyi* potentially via *pC*-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-C4 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 tropidicoetic 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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