



Quorum Sensing Interference and Structural Variation of Quorum Sensing Mimics in Australian Soft Coral

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Bacterial Quorum Sensing (QS), the indirect regulation of gene expression through production and detection of small diffusible molecules, has emerged as a point of interaction between eukaryotic host organisms and their associated microbial communities. The extracellular nature of QS molecules enables interference in QS systems, in many cases via mimicry. This study targeted QS induction and inhibition in soft coral holobionts, as many soft coral species commonly contain compounds with structural similarities to the well-studied bacterial QS molecules acyl homoserine lactones. Screening with two bacterial biosensors, *Agrobacterium tumefaciens* A136 and *Chromobacterium violaceum* CV026, demonstrated that QS interference differed between the two biosensor strains and extended across the soft coral families *Alcyoniidae*, *Clavulariidae*, *Nephtheidae*, and *Xeniidae*. Bioassay-guided fractionation revealed chemical activity patterns, particularly in the induction of QS. Cembranoid diterpenes from active fractions were purified and tested for QS interference activity. Interestingly, the type of QS activity (induction or inhibition) in *A. tumefaciens* A136 correlated with structural variability of the secondary oxygen ring; cembranoid diterpenes with a furan ring or five-membered lactone induced QS, while compounds with larger (six or seven membered) lactone rings inhibited QS. Addition of the dominant cembranoid diterpene in the soft coral *Lobophytum compactum*, isolobophytolide, to bacterial culture media increased the number and morphological diversity of bacteria recovered from the mucosal layer of this soft coral, demonstrating a selective effect on certain members of the soft coral bacterial community. The identity and QS activity of recovered isolates differed between the mucosal layers of *L. compactum* and *Sinularia flexibilis*. In conclusion, this study provides information on the complexity of the interaction between soft corals and their associated bacteria, as well as, a structural understanding of how QS mimic compounds are able to interfere with a bacterial communication system.

Keywords: quorum sensing, soft coral, bacterial isolates, cembrenolide, quorum sensing mimic

INTRODUCTION

Quorum Sensing (QS) is one form of cell to cell signaling employed by bacteria to coordinate gene expression across entire populations through release and detection of extracellular signal molecules (Miller and Bassler, 2001). The aspects of multicellularity gained through QS, enable bacteria to perform many important ecological functions such as the ability to interact with their physical and biological environment (Miller and Bassler, 2001), form biofilms (Rice et al., 2005), and secrete virulence factors (Zhu and Mekalanos, 2003). The extracellular nature of QS signaling molecules facilitates their disruption and mimicry (Chhabra et al., 2005). Consequently, many bacteria possess the ability to detect and respond to QS signals of other species (Joint et al., 2007). Indeed, QS systems are more prevalent amongst bacteria associated with mixed bacterial biofilms and macro-organisms, suggesting that possession of QS systems confers an advantage in these habitats (Dudler and Eberl, 2006).

QS mimics are extrinsic signals that can interfere directly with QS gene expression (Bauer and Robinson, 2002). To be effective, QS mimic compounds must specifically interfere with the target QS system. Multiple QS systems have been discovered; in Gram-negative bacteria (Papenfert and Bassler, 2016) the most well studied system is the Auto Inducer One system (AI-1), which utilizes acyl homoserine lactones (AHLs) as signal molecules. Studies into the structure and functions of AHLs suggest that the γ -lactone ring is required for QS activity and that the length and functionality of the acyl side chain provides specificity (Parsek and Greenberg, 2000; Watson et al., 2002; Geske et al., 2008). For this reason, it has been hypothesized that AI-1 QS mimics would also contain a γ lactone ring or homologous functionality such as the furanones of the red alga *Delisea pulchra*. The furanones are one of the few QS inhibitors to have been structurally elucidated and the presence of an oxygenated ring was demonstrated to be essential to their activity (Manefield et al., 1999).

Disruption and mimicry of QS signals are increasingly recognized as mechanisms that are commonly employed by macro-organisms to regulate and manipulate their associated microbial communities (Bauer and Robinson, 2002; González and Keshavan, 2006). QS interference by host organisms can confer the ability to respond to the presence of certain pathogenic or mutualistic bacteria quickly and reliably (Kjelleberg et al., 1997; Mathesius et al., 2003), render a pathogenic species of bacteria non-pathogenic (Dong et al., 2007; Swem et al., 2009), and enable manipulation of the abundance and composition of its associated bacterial assemblies (Givskov et al., 1996). A host's microbiota can be a first line of defense against pathogen invasion (McFall-Ngai et al., 2013), therefore manipulation of QS could strengthen the resilience of the holobiont (González and Keshavan, 2006; Teplitski and Ritchie, 2009).

The ability of some bacterial species to detect non-native QS molecules has allowed the development of bacterial biosensor strains. QS bacterial biosensor strains are genetically modified bacterial isolates that require external addition of QS signal molecules. Their expression of QS-regulated genes is linked to reporter genes, which typically produce a pigment or bioluminescence (Steindler and Venturi, 2007). Two of the most commonly used AI-1 bacterial biosensor strains are based

on the species *Chromobacterium violaceum* and *Agrobacterium tumefaciens*. In *C. violaceum*, QS regulates the production of the secondary metabolite violacein, which is purple in color (McClellan et al., 1997). Bacterial biosensors based on this species utilize the LuxI/LuxR homolog genes Civi/CivR and are sensitive to AHLs with C4–C8 carbon chains as well as 3-oxo-C6 and -C8 carbon chains (McClellan et al., 1997; Steindler and Venturi, 2007). *A. tumefaciens* QS biosensors have a genetically modified QS plasmid such that QS by these strains results in an enzymatic breakdown of X-gal and the formation of an indigo colored product (Zhu et al., 1998; Farrand et al., 2002; Zhu and Mekalanos, 2003). *A. tumefaciens* QS biosensors often utilize the TraI/TraR genes that provide sensitivity to AHLs with C6–C14 acyl side chains as well their equivalent 3-oxo-acyl side chains and C6–C10 hydroxy acyl side chains (Zhu et al., 1998; Farrand et al., 2002; Zhu and Mekalanos, 2003; Steindler and Venturi, 2007). Despite the partly overlapping acyl chain lengths detected by these two sensors, previous studies have observed differences in their responses during screening of isolates (Chong et al., 2012).

Widespread QS inhibitory activity has been observed to occur in the marine benthos, particularly in sponges and soft corals (Taylor et al., 2004; Skindersoe et al., 2008; Hunt et al., 2012). Soft corals contain a number of secondary metabolites with the structural potential to mimic QS, including furanocembrenes (cembranoid diterpenes with fused 5-membered ether rings) and cembranolides (cembranoids that possess a fused second ring in the form of a lactone). Cembranoid diterpenes are most commonly, but not exclusively, found within the family *Alcyoniidae*. Furthermore, cembranoid diterpenes are inherently variable in the presence, position and size of oxygenated ring systems (Wahlberg and Eklund, 1992). Variation of substituents, direction of cyclisation and the corresponding position of the isoprenoid double bonds of different diterpenes from different species of soft coral are also encountered (Wahlberg and Eklund, 1992) making cembranoid diterpenes a natural pool of compounds to investigate QS mimic structure-activity relationships.

Soft corals have a high incidence of QS interference; however, so far all screened soft corals have been from a single family, *Alcyoniidae*, and the presence of QS induction has not yet been investigated. While QS and QS interference have been implicated in the regulation of mixed bacterial communities including those found in the surface mucosal layer (SML) of hard corals (Tait et al., 2010; Golberg et al., 2011, 2013), it is unknown whether these communities may be regulated by QS mimics. Currently very little known about bacterial communities that associate with soft corals. In hard corals, bacteria in the SML, the first and largest point of interaction between a coral and the environment, are considered important to the health and resilience of the holobiont (Reshef et al., 2006; Rosenberg et al., 2007; Zilber-Rosenberg and Rosenberg, 2008; Shnit-Orland and Kushmaro, 2009).

This study aims to assess the potential of soft corals to interact with their associated microbes via QS, with a focus on their associated secondary metabolites such as cembranolides and furanocembrenes. Crude extracts of 15 soft coral species, representing 4 families of soft corals, were tested for their

ability to induce or inhibit QS in 2 bacterial biosensors, *A. tumefaciens* A136 and *C. violaceum* CV026. Cembranolides and furanocembrenes were isolated from soft corals and the effect of their structural variability on QS interference was assessed. To gain a better understanding of the role that QS can play in regulating eukaryote associated bacterial communities, bacterial strains isolated from the surface mucosal layer (SML) of two soft corals, *Sinularia flexibilis* and *Lobophytum compactum* were similarly evaluated for QS activity. The possible role that isolobophytolide, the major secondary metabolite of *L. compactum*, might play in bacterial selection within the soft corals mucus was also assessed by supplementing culture media with this secondary metabolite.

MATERIALS AND METHODS

Soft Coral Collection

Twenty four specimens of soft coral, representing 15 species (Supplementary Tables 1–3), were collected at a depth of 1–3 m near Orpheus Island (Great Barrier Reef, Australia; latitude 18°36.878'S; longitude 146°29.990'E). All specimens except *Cespitularia* sp. were photographed (Supplementary Figure 1) and sampled underwater, with samples placed directly into plastic bags filled with seawater. Samples were frozen (−80°C) within 1 h of collection and stored until freeze drying. Additional colonies of *L. compactum*, *S. flexibilis*, and *Pachyclavularia violacea* were collected for fraction analysis and isolation of pure compounds (Supplementary Tables 2,3). ¹H NMR spectra of the crude extracts of all soft coral samples were compared between collections and with reference spectra from the Bowden laboratory to ensure consistency in the metabolites and species tested.

Soft Coral Extract Preparation

Dried soft coral tissue was weighed and homogenized before extraction. Three extracts of different polarity were generated for each soft coral sample. Solvents used for extraction were, in order, dichloromethane (DCM), methanol (MeOH), and water (H₂O). Each extract was the result of three successive applications of solvent. Extracts were concentrated by rotary evaporation before being dried under a stream of nitrogen (N₂) and stored at −20°C until analysis. The DCM and MeOH extracts were dissolved in ethanol and the aqueous extracts in H₂O to a concentration of 20 mg/ml. All soft coral extracts were tested at two concentrations (4 μg/well and 40 μg/well) three times for the presence of QS induction and QS inhibition activity in *A. tumefaciens* A136 and *C. violaceum* CV026 in at least two independent experiments.

Soft Coral Extract Fractionation

To further investigate the patterns of QS activity observed in crude extracts, nine soft coral species from five genera (three families) were chosen for further fractionation (Supplementary Table 2). The chosen species displayed five patterns of crude extract activity (Supplementary Table 4). In addition to four species with strong crude extract activities, five species were selected to investigate observed within genera variation in

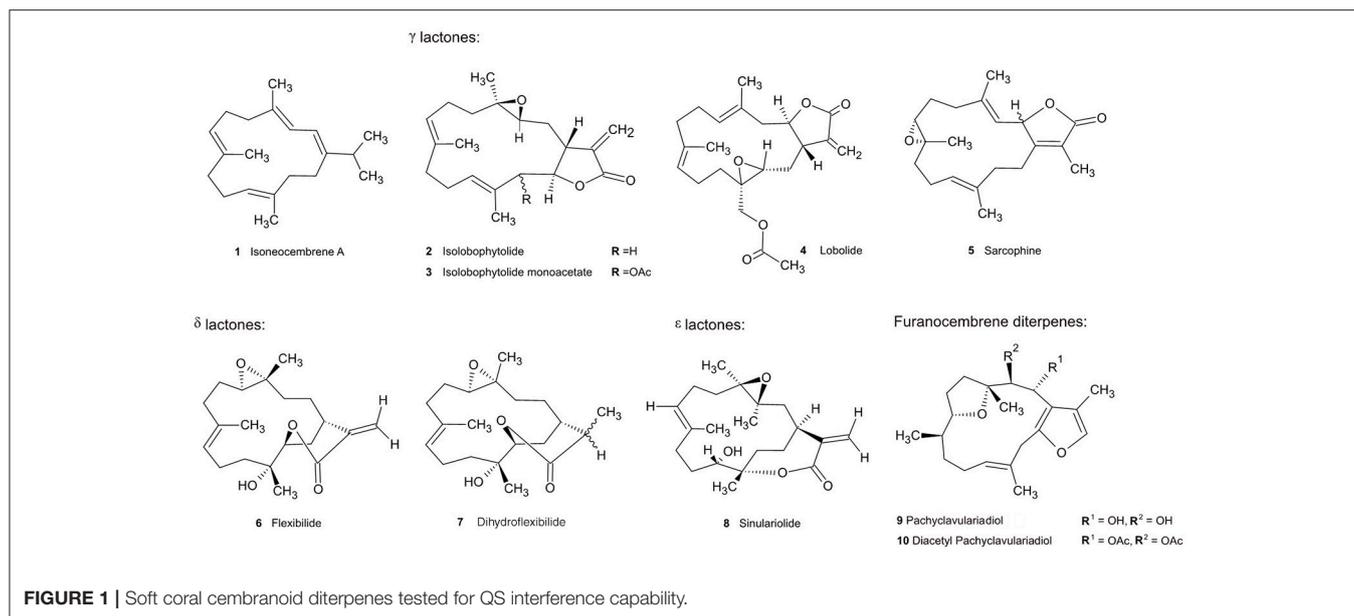
activity patterns or because they represent common genera on the Great Barrier Reef (GBR). Ten fractions of decreasing polarity were generated from the dichloromethane extracts of the chosen soft corals. Extracts were fractionated using flash column chromatography on RP-C18 silica cartridges (Phenomenex Strata C18-E 55 μm 70 Å, 1,000 mg) eluted with a stepwise 20–100% MeOH: H₂O gradient followed by a 1:1 DCM: MeOH wash. The resulting fractions were concentrated to dryness under a stream of N₂ gas and re-dissolved in ethanol for QS screening as described above. ¹H-NMR spectra (600 MHz) of active fractions were recorded with a Bruker 600 Avance spectrometer in deuterated chloroform (CDCl₃).

Isolation of Purified Metabolites From the Corals

Ten cembranoid diterpenes with variable secondary ring structures were assessed in this study (Figure 1, Table 1). Isonocembrene A (1) represents the base cembrene backbone, without an additional ring (Figure 1, Table 1). Compounds 2–4 are γ-lactones (5-membered rings). Two δ-lactones (6-membered rings) and one ε-lactone (7-membered ring) are also included in compounds 6–8 respectively (Figure 1, Table 1). The final two compounds are furanocembrenes (Figure 1, Table 1). Pure samples of isoneocembrene A (1), lobolide (4) and sarcophine (5) were acquired from the Bowden Laboratory, Townsville Australia. Isolobophytolide (2), and isolobophytolide monoacetates (3) were isolated from freshly collected and extracted *L. compactum* (see below). Flexibilide (6), dihydroflexibilide (7) and sinulariide (8) were isolated from freshly collected and extracted *S. flexibilis* (see below). The furanocembrenes, pachyclavulariadiol (9) and pachyclavulariadiol diacetate (10), were isolated from freshly collected and extracted *P. violacea*. Extracts of each species were generated as described previously (section Soft Coral Extract Preparation).

For the *L. compactum* extract, vacuum liquid chromatography of active crude DCM extract (2 g) was performed over reverse phase C18 silica gel (Phenomenex Luna 10 mm C18 silica gel) and 10 fractions (200 ml) were collected using MeOH: H₂O 0–100% stepwise gradient for each extract. Activity was identified in the 80% MeOH fraction and this fraction was subjected to RP-HPLC 60–100% MeOH gradient over 30 min (Phenomenex Gemini 3 μm NX-C18 110 Å, LC Column 30 × 4.6 mm). Isolobophytolide (2) eluted at 15 min and the two isomers of isolobophytolide monoacetate (3) eluted at 17 min.

For the *S. flexibilis* extract, flexibilide (6) and dihydroflexibilide (7) were isolated as reported by Kazlauskas et al. (1978). In brief, the DCM extract was subjected to normal phase flash column chromatography with combinations of hexane, DCM and EtOAc. Flexibilide was eluted by 6:1 DCM: EtOAc, 4:1 DCM: EtOAc yielded a mixture of 6 and 7 and 3:1 DCM: EtOAc afforded pure dihydroflexibilide. Sinulariide (8) was isolated separately by a method adapted from Tursch et al. (1975) by direct crystallization of the DCM extract after dissolution in diethyl ether. Purification of compounds was performed by HPLC as described above.

**TABLE 1** | Functional group analysis of tested soft coral metabolites.

#	Cembranoid diterpene	Secondary ring type	Secondary ring position	Other oxygenated functional groups	Double bonds	Exo methylene	-OH	Oxygenated side chain	Acetate
1	Isonocembrene A	Absent	Absent	Absent	C4-C5, C8-C9, C12-C13, C14-C1	Absent	Absent	Absent	Absent
2	Isolobophytolide	γ lactone	C1-C2	C12-C13 (Epoxide)	C4-C5, C8-C9	Present	Absent	Absent	Absent
3	Isolobophytolide monoacetate	γ lactone	C1-C2	C12-C13 (Epoxide)	C4-C5, C8-C9	Present	Absent	Absent	Present
4	Lobolide	γ lactone	C1-C14	C3-C4 (Epoxide)	C7-C8, C11-C12	Present	Absent	On C4	C20
5	Sarcophine	α, β unsaturated γ lactone	C1-C14	C8-C9 (Epoxide)	C4-C5, C12-C13	Present	Absent	Absent	Absent
6	Flexibilide	δ -lactone	C1-C3	C11-C12 (Epoxide)	C7-C8	Present	C4	Absent	Absent
7	Dihydroflexibilide	δ -lactone	C1-C3	C11-C12 (Epoxide)	C7-C8	Absent	C4	Absent	Absent
8	Sinulariolide	ϵ -lactone	C1-C4	C12-C13 (Epoxide)	C8-C9	Present	C5	Absent	Absent
9	Pachyclavariadiol	furan	C1-C2	C9-C12 (Ether)	C1-C2, C14-C15, C15-C16	Absent	Absent	Absent	Absent
10	Diacetyl Pachyclavariadiol	Furan	C1-C2	C9-C12 (Ether)	C1-C2, C14-C15, C15-C16	Absent	Absent	Absent	C13, C14

Due to instability of the furanocembranes from *P. violacea*, the pure compounds were generated semi-synthetically as per Bowden et al. (1979). In brief, the DCM extract was prepared at 4°C then partitioned between hexane and 10% aqueous MeOH. After removal of the solvent, the aqueous MeOH fraction was subjected to normal phase flash chromatography with a hexane: EtOAc gradient. All fractions containing (by TLC and ¹H NMR) pachyclavariadiol, diacetyl pachyclavariadiol and the two monoacetyl pachyclavariadiols were combined and hydrolyzed to pachyclavariadiol (**9**) by incubation for 24 h at room temperature with MeOH containing 1% (w/v) potassium hydroxide. Methanol was removed under vacuum and the residue was partitioned between diethyl ether and water. The ether fraction was evaporated and the residue

was dissolved in hexane. Diacetyl pachyclavariadiol (**10**) was acquired by acetylation of half of the obtained pachyclavariadiol (**9**). Acetylation was affected by incubation for 24 h with 1:1 acetic anhydride in pyridine before evaporation of the solvent and retrieval via liquid a partition between hexane and water. The semi-synthesis of monoacetyl pachyclavariadiols was not performed as both **9** and **10** exhibited similar activity, so it was considered unlikely that the activities of monoacetyl pachyclavariadiols would be different.

Structure and purity of each extracted compound was confirmed by 1D and 2D NMR and comparison with literature values. ¹H-NMR (600 MHz) and ¹³C-NMR (150 MHz) spectra were recorded with a Bruker 600 Avance spectrometer in CDCl₃, with tetramethylsilane (TMS) as internal standard. High

resolution mass spectra were collected using an unmodified Bruker BioAPEX 47e mass spectrometer equipped with an Analytica model 103426 (Branford, CT) electrospray ionization (ESI) source. Analytical thin layer chromatography (TLC) was performed on Merck Kieselgel 60. Spots were visualized by UV light or by spraying with a 1% vanillin in acidified ethanol solution. Pure compounds were re-solubilized in ethanol and serially diluted to generate five different concentration solutions (1×10^2 mM to 1×10^{-2} mM) for each compound.

Collection, Culture and Extraction of Bacterial Isolates From *S. flexibilis* and *L. compactum* Surface Mucosal Layer

Surface mucosal layer samples (SML) were collected from three healthy replicate colonies of *S. flexibilis* and *L. compactum* from the same depth and location. SML samples were collected underwater from the mid-capitulum region of the coral colony using 50 ml needleless sterile syringes. Samples were maintained at ambient temperatures and processed within 3 h of collection. At the same time as SML samples were retrieved, tissue samples of each replicate were collected and their metabolite ^1H NMR spectral profiles were compared with samples from *S. flexibilis* and *L. compactum* collected previously to ensure correct species identification.

SML samples were serially diluted (10^{-2} , 10^{-3} , 10^{-4}) using autoclaved artificial seawater (Instant Ocean; Spectrum Brands, Madison, WI, USA). One hundred microliters of each dilution were spread plated in triplicate on two types of media commonly used for studies of marine bacteria: 50% Marine Agar (50MA; BD) and Glycerol Artificial Seawater (GASW) agar (Smith and Hayasaka, 1982). Additionally, Thiosulfate Citrate Bile Salts (TCBS; BD) agar, which specifically selects for members of the family *Vibrionaceae*, was included. *L. compactum* SML dilutions were additionally plated onto 50MA and GASW agar supplemented with *L. compactum*'s major secondary metabolite, isolobophytolide. All plates were incubated at 28°C and sampled after 48 h, 72 h, 1, and 2 weeks. Representatives of each colony morphotype from each plate were sub-cultured to purity for identification.

Where possible, two representatives of each morphotype were selected for QS activity screening. Where bacteria had initially been isolated using media embedded with isolobophytolide, growth was attempted on the equivalent medium without isolobophytolide. Strains that could not be cultured without isolobophytolide were not included in the screening. Screening was performed on acidified ethyl acetate (EtOAc) extracts of the cell free supernatant of soft coral isolates. These were acquired by transferring single colonies from 50MA plates to liquid culture (10 ml 50% Marine broth culture, 28°C at 170 rpm) and grown to late exponential phase. Cultures were centrifuged for 10 min at 4°C at 10,000 g to obtain the cell free supernatant (CFS). Each CFS was subjected to exhaustive extraction with acidified EtOAc (1% acetic acid) and concentrated to dryness under a stream of N_2 gas. Extracts were then dissolved and diluted to a concentration of 20 mg/l with ethanol.

Bacterial QS Biosensor Strains and Culture Medium

The biosensor strains *A. tumefaciens* A136 (Fuqua and Winans, 1996) and *C. violaceum* CV026 (McClellan et al., 1997) were used for detection of QS induction and inhibition in soft coral extracts. *A. tumefaciens* A136 was grown on ABt media (Clarrk and Maaloe, 1967) and *C. violaceum* CV026 was grown on Luria Bertani (LB) media (Bertani, 1951). In order to ensure that the QS plasmid was intact and functional, QS biosensor strains were grown in the presence of the appropriate antibiotic (Ravn et al., 2001). *A. tumefaciens* A136 was grown on media supplemented with 4.5 µg/ml of tetracycline and 50 µg/ml of spectinomycin, whereas, *C. violaceum* CV026 was grown on media supplemented with 20 µg/ml of kanamycin (Ravn et al., 2001).

QS Screening Assays

The presence of AHL type QS induction activity in soft coral extracts, fractions and pure compounds was detected by performing an agar diffusion assay as described in detail by Ravn et al. (2001). The QS biosensor strain, either *A. tumefaciens* A136 or *C. violaceum* CV026, was embedded within the agar and the sample being tested was added to a well cut or formed in the agar. For induction of QS, N-hexanoyl homoserine lactone was used as a positive control and extraction solvents were used as negative controls. Positive results were read as a blue coloration surrounding the wells of *A. tumefaciens* A136 and a purple coloration surrounding the wells of *C. violaceum* CV026 (see above and Supplementary Figure 2). The intensity of the response was measured as the diameter of the colored zone and normalized to the response of the positive control.

The agar diffusion assays described above were modified in order to detect QS inhibition. Briefly, as *A. tumefaciens* A136 and *C. violaceum* CV026 are not able to QS without the exogenous addition of AHLs, 8.5 µmol n-hexanoyl homoserine lactone was added into the agar embedded with the biosensor strain in order to test for QS inhibition. Two positive controls were chosen based on their previously reported ability to inhibit QS: n-dodecanoyl-DL-homoserine lactone (McClellan et al., 1997) and vanillin (Choo et al., 2006). These controls proved effective for both biosensors. The extraction solvents were once again used as negative controls. Positive results in the inhibition assay were read as inhibition of blue or purple coloration of the plates containing *A. tumefaciens* A136 and *C. violaceum* CV026, respectively (Supplementary Figure 2). The intensity of the response was measured as the width of the inhibition zone surrounding the well and normalized to the positive control.

To generate dose response curves for pure compounds, agar containing the respective biosensor was poured into custom built molds with 28 preformed wells 4 mm in diameter. After solidification of the agar, 20 µl of sample was added to each well. Pure compounds were only tested with the *A. tumefaciens* A136 strain as it was the only strain to have both QS induction and inhibition activity.

Bacterial DNA Extraction, PCR and Sequencing

Genomic DNA of bacterial isolates was extracted using the Promega Wizard Genomic DNA Isolation kit (Promega, Madison WI USA) according to the manufacturer's directions. PCR amplification of 16S rRNA gene fragments was performed using the primers 27F and 1492R (Lane, 1991). The PCR reactions contained the following reagents: 0.4 mM of each primer, 1x MyTAQ buffer (Bioline, Australia), 1.25 U MyTAQ (Bioline, Australia), 1 μ L DNA extract (final volume of 50 μ L). Cycling conditions were an initial denaturing step of 94°C for 5 min, followed by 30 cycles at 95°C for 1 min, 56°C for 45 s, 72°C for 60 s, and a final elongation step at 72°C for 10 min. PCR products were verified by agarose gel electrophoresis and purified for sequencing using the Qiaquick PCR purification Kit (Qiagen, Valencia, CA) according to company supplied directions. Sanger sequencing was carried out at Macrogen (Seoul, South Korea) using both 27f and 1492R as sequencing primers.

Phylogenetic Analysis of Bacterial Isolates

Sequence fragments were assembled using Sequencher (Version 5, Gene Codes, Ann Arbor, USA). For each isolate, the 16S rRNA gene sequence was aligned with sequences in the nr and Ref_Seq database at the NCBI using the megablast tool (RRID:SCR_001598; Altschul et al., 1990) to identify closely related database sequences. Sequences of isolates and database matches were imported into MEGA6 (MEGA Software, RRID:SCR_000667) and aligned using ClustalW (Larkin et al., 2007). A Maximum Likelihood-based phylogenetic tree was constructed using the Maximum Parsimony algorithm for the starting tree, the Tamura-Nei model for nucleotide substitution, and 500 bootstrap replicates (Supplementary Figure 3). The 16S rRNA gene sequences for the 72 bacterial isolates were deposited into the NCBI Genbank database, under accession numbers KM360403-KM360473. Quantification and statistical analysis of CFUs and isolate morphotypes.

Colony forming unit (CFU) concentrations were estimated based on dilutions yielding between 30 and 300 colonies per plate. Differences in the number of CFUs between samples were determined based on three replicates for the corresponding dilution and media type. Statistical differences were determined using the non-parametric Kruskal-Wallis statistic, as the data violated both normality and homogeneity of variances required for ANOVA. Colony morphotype profile analysis was conducted on the variables color, size and texture and were compared using a nonmetric Multidimensional Scaling (nMDS) analysis in the vegan package (version 2.5-1; Oksanen et al., 2018) in R. The nMDS was chosen based on its suitability for spatial representation of complex data sets containing multiple variables, large numbers of zeroes and non-normal distributions (Rabinowitz, 1975). The statistical analyses were performed using Graphpad PRISM (GraphPad Prism, RRID:SCR_015807).

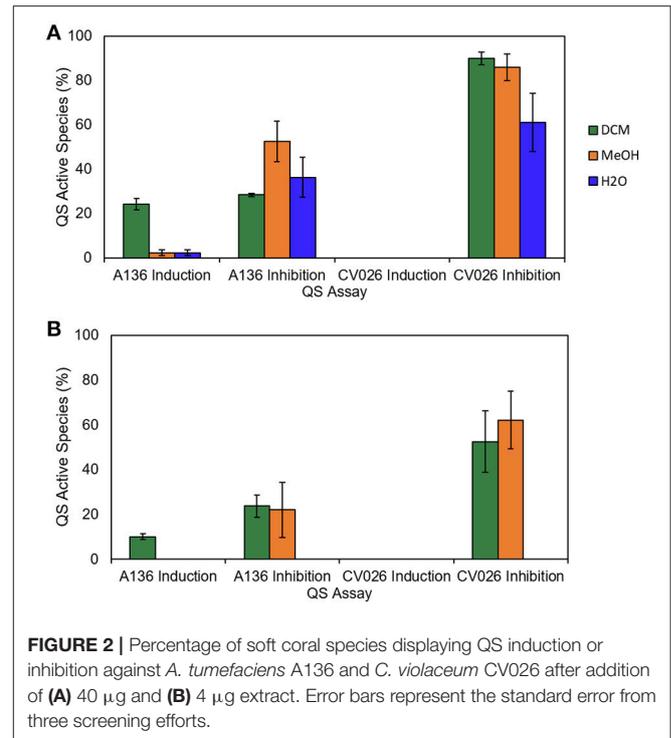


FIGURE 2 | Percentage of soft coral species displaying QS induction or inhibition against *A. tumefaciens* A136 and *C. violaceum* CV026 after addition of (A) 40 μ g and (B) 4 μ g extract. Error bars represent the standard error from three screening efforts.

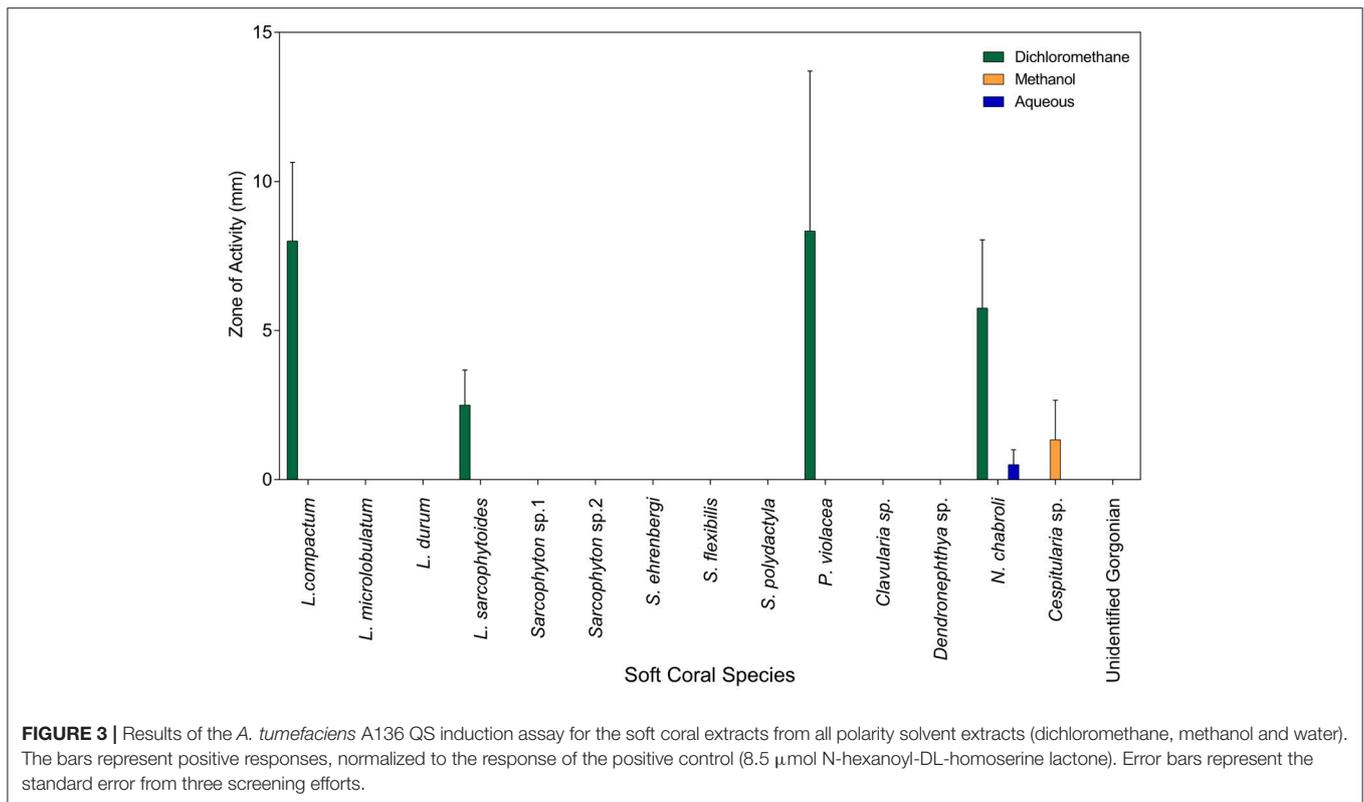
RESULTS

Quorum Sensing Activities of Soft Coral Crude Extracts

Crude soft coral extracts demonstrated the ability to both induce and inhibit QS in the biosensors tested. While both induction and inhibition of QS were observed for the biosensor *A. tumefaciens* A136, none of the extracts, regardless of source coral species, polarity, or concentration, were able to induce QS in *C. violaceum* CV026 (Figure 2A). Inhibition of QS was more prevalent than induction across all polarity extracts (Figure 2A). For both biosensors, the number of active soft coral extracts was reduced at lower extract concentration, with none of the aqueous extracts retaining their activity at the lower dosage (Figure 2B).

Crude extracts of five species induced QS in *A. tumefaciens* A136; two species from the family *Alcyoniidae* (*L. compactum*, *Lobophytum sarcophytoides*), one from the family *Nephtheidae* (*Nephthea chabroli*), one from the family *Clavulariidae* (*P. violacea*), and one from the family *Xeniidae* (*Cespitularia* sp.) (Figure 3). The highest incidence and strength of induction activity was seen for DCM crude extracts, with the largest haloes of coloration produced by DCM extracts of *L. compactum* and *P. violacea* (Figure 3).

Low level QS inhibition of *A. tumefaciens* A136 was demonstrated for DCM crude extracts of most species, with the only exceptions being *L. microlobulatum*, *Sinularia polydactyla*, *P. violacea*, *Clavularia* sp., and *N. chabroli* (Figure 4A). The same trend was seen for MeOH crude extracts, with the additional exception of *Sarcophyton* sp. 2 (Figure 4A). Inhibition of QS in *C. violaceum* CV026 was present in DCM crude extracts of



all species except *P. violacea* and *Clavularia* sp. (Figure 4B). Of the five species that induced QS in *A. tumefaciens* A136, three (*L. compactum*, *L. sarcophytoides* and *Cespitularia* sp.) also inhibited QS in both biosensors (Figure 4). This contrasted with the other two species capable of QS induction; *N. chabroli* extracts inhibited QS only in *C. violaceum* CV026 (Figure 4) while *P. violacea* demonstrated no QS inhibitory activity with either biosensor (Figure 4).

Quorum Sensing Activities of Soft Coral Fractions

For all fractionated species, at least two fractions induced QS in *A. tumefaciens* A136 (Figure 5), regardless of whether their corresponding crude extracts were active (Figure 4). All species produced fractions that induced QS and inhibited QS in at least one biosensor strain. The only exception was *P. violacea*, which did not inhibit QS in either strain (Figure 5) consistent with crude extract results (Figure 4). The two largest inductive haloes were observed for *N. chabroli* and *L. compactum*, which also retained their activity at the higher (1:10) dilution level (Figure 4). The two major bands of induction activity seen in the *A. tumefaciens* A136 bioassay occurred for fractions eluted at 60% MeOH and at 80–90% MeOH (Figure 5). Many of the latter fractions (80–90% MeOH) also showed activity in the corresponding QS inhibition bioassay (Figure 5). The distinct patterns of QS activity observed for *A. tumefaciens* A136 contrasted strongly with the broad *C. violaceum* CV026 inhibition activity (Figure 5). The presence

of cembrene diterpenes correlated well with the QS active fractions (data not shown).

Quorum Sensing Activities of Pure Compounds

The response to pure compounds also depended on the biosensor strain utilized. None of the tested cembranoid diterpenes induced QS in *C. violaceum* CV026. In contrast, both QS induction and inhibition activity was observed against *A. tumefaciens* A136 over three to four orders of magnitude, with a loss of activity at higher concentrations (Figure 6). No QS interference was observed for isoneocembrene A (Compound 1; Figure 6). The strongest induction of QS in *A. tumefaciens* A136 was observed for pachyclavulariadiol and diacetyl pachyclavulariadiol (Compounds 9 and 10; Figure 6). Induction was also observed in isolobophytolide (Compounds 2 and 3), lololide (Compound 4), and sarcophine (Compound 5). QS inhibition was strongest in the ϵ -lactone ring of sinulariolide (Figure 6). Peak QS interference for all compounds occurred at approximately 1×10^{-5} mM (or 3 ppm; Figure 6).

Culturable Bacteria

A significantly higher number of colony forming units (CFUs) were estimated for mucus of *S. flexibilis* as compared to *L. compactum* (Kruskal-Wallis, $H = 7.200$, 2 d.f., $P = 0.0036$; Supplementary Figure 4). Interestingly, when growth media for *L. compactum* were amended with isolobophytolide, the estimated number of CFUs increased for this species but

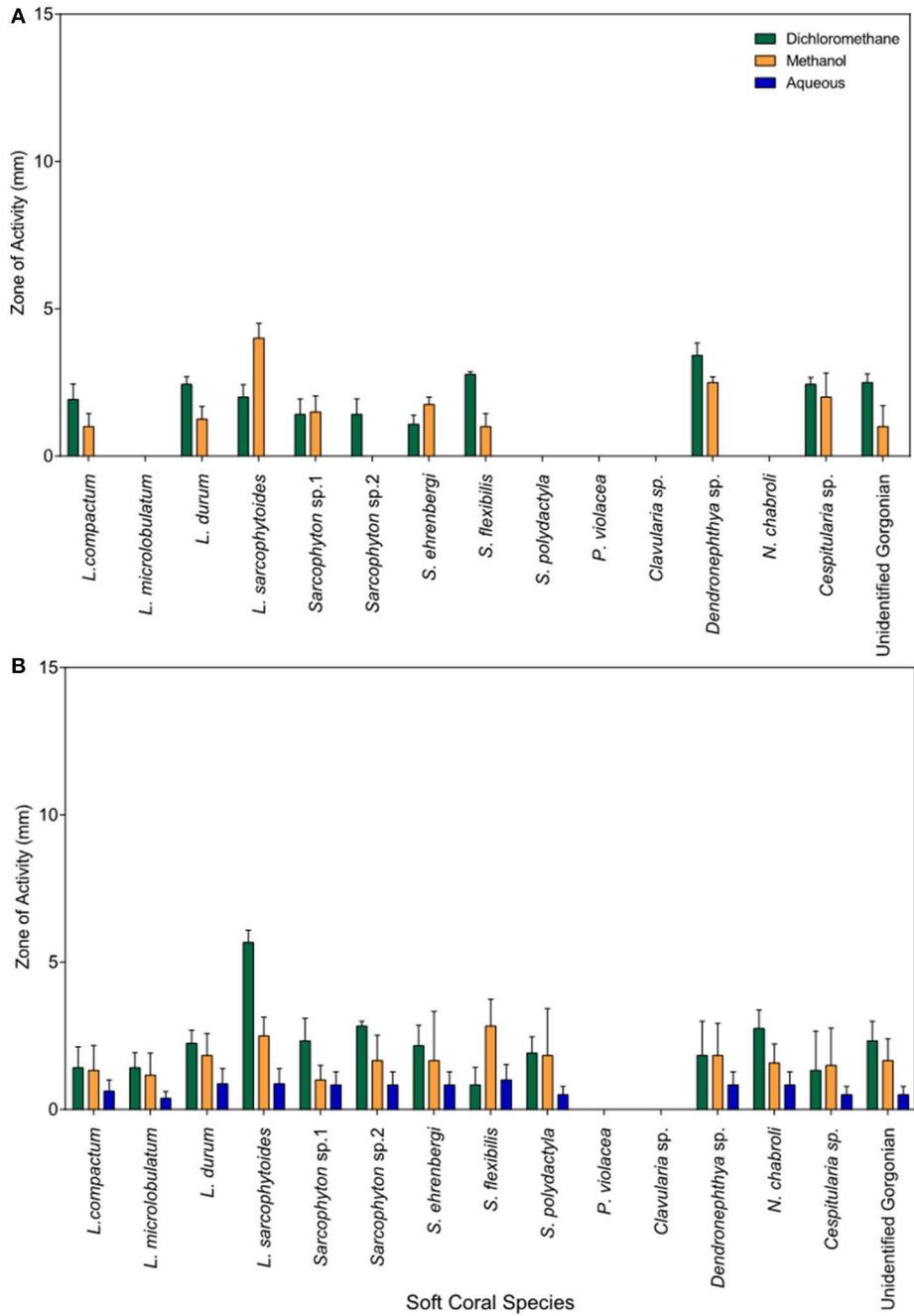


FIGURE 4 | Results of the QS inhibition assay for the soft coral extracts from all polarity solvent extracts (dichloromethane, methanol and water) **(A)** *A. tumefaciens* A136 and **(B)** *C. violaceum* CV026 QS inhibition assay for the soft coral extracts from all polarity solvent extracts (dichloromethane, methanol and water). The bars represent positive responses, normalized to the response of the positive control (vanillin). Error bars represent the standard error from three screening efforts.

remained lower than the estimates for *S. flexibilis* (Supplementary Figure 4). The number and type of colony morphotypes also differed between *L. compactum* and *S. flexibilis* (Figure 7). *S. flexibilis* showed little variation in the morphotype profiles of GASW or 50MA media, forming a tight cluster on the nMDS biplot (Figure 7). In comparison, the morphotype profiles generated from *L. compactum* showed higher variation in the

same culture media. This trend was also consistent when isolobophytolide was added as a selection agent (Figure 7).

***Sinularia flexibilis* Bacterial Isolates**

In total, 20 bacterial isolates from *S. flexibilis* were identified through 16S rRNA gene sequencing followed by BLAST searches and construction of phylogenetic trees (Supplementary

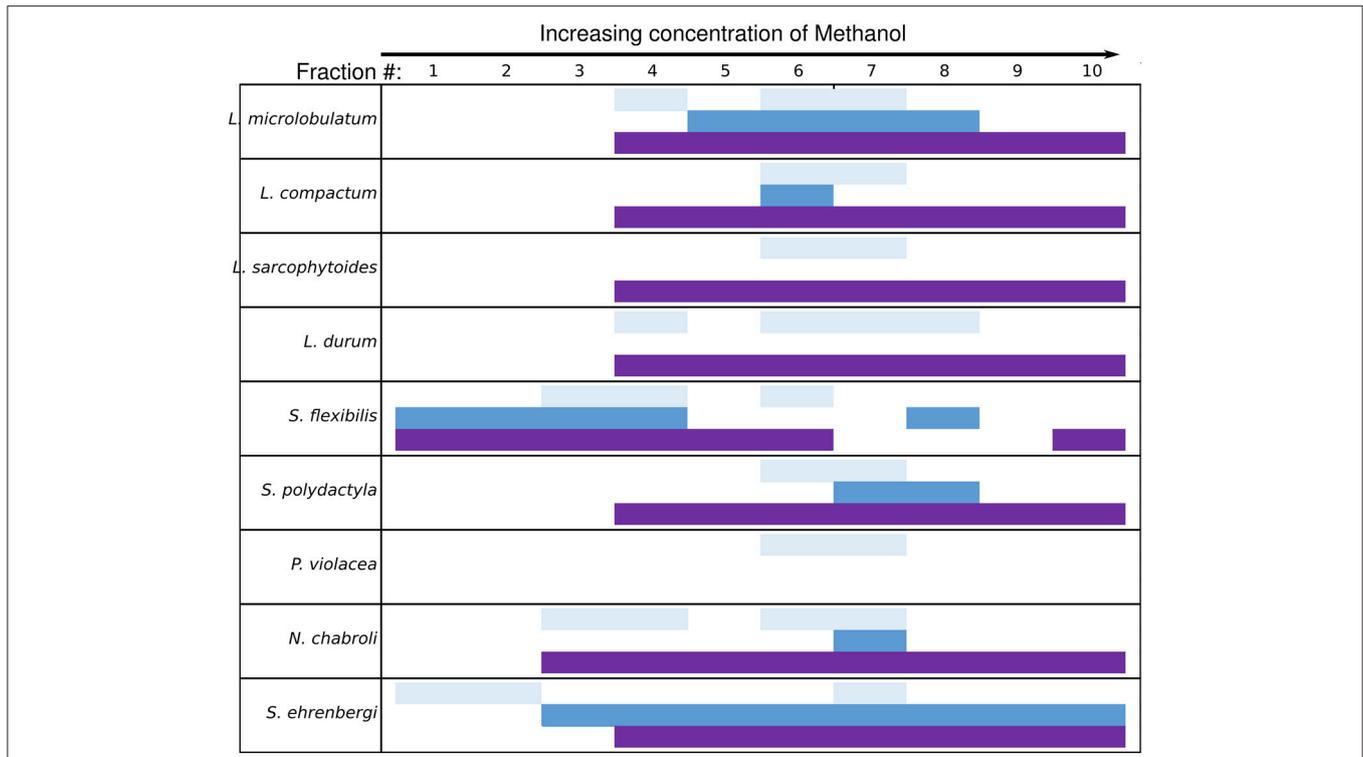


FIGURE 5 | Active soft coral fractions by QS biosensor assay. Fractions were generated using a C18 flash column with a stepwise MeOH:H₂O gradient. Fraction numbers reflect elution order with increasing MeOH percentage. Light blue bars represent the *A. tumefaciens* A136 induction assay, dark blue bars represent the *A. tumefaciens* A136 inhibition assay and the purple bars represent the *C. violaceum* CV026 inhibition assay.

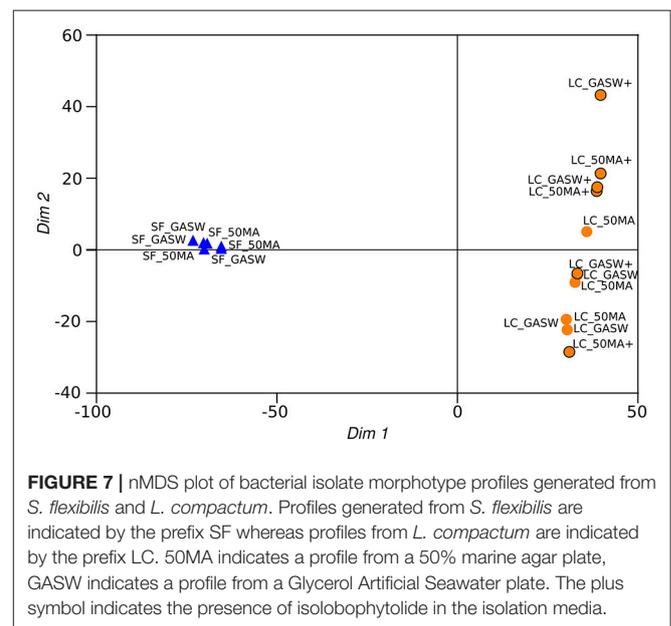
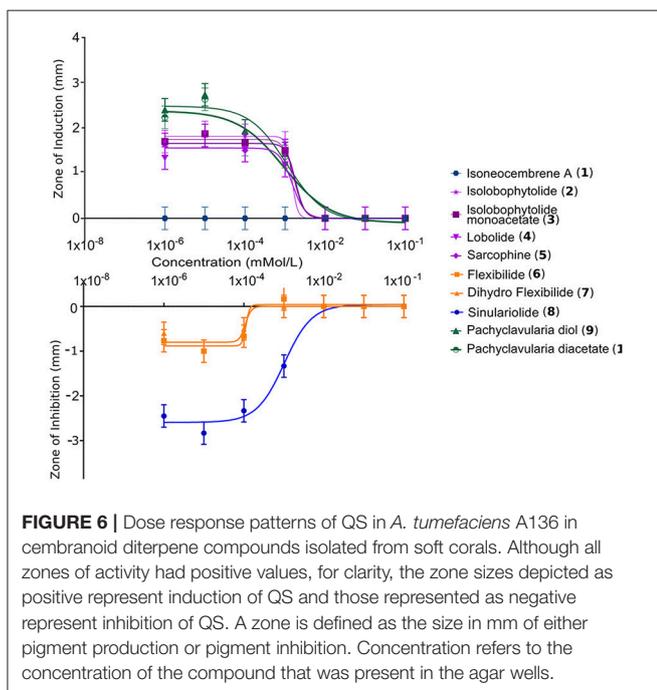


Table 5, **Figures 8, 9**). The isolates were dominated by Gammaproteobacteria belonging to the family *Vibrionaceae* both for the non-*Vibrionaceae* targeted media (GASW and

50MA) as well as the *Vibrionaceae* targeted medium (TCBS). Other Gammaproteobacteria included two isolates whose closest relative was “*Spongiobacter nickelotolerans*” (hereafter referred to

as *Endozoicomonas*, see below); and three *Alteromonas*-related strains. Finally, one isolate was identified with 99% sequence identity to *Bacillus megaterium* and *Bacillus aryabhatai* (phylum *Firmicutes*).

The potential of soft coral isolates from *S. flexibilis* to participate in AHL-type QS communication systems was investigated using the same reporter bioassays as used for coral extracts. Quorum sensing activity under the used test conditions was demonstrated for 52.4% of the tested *S. flexibilis* isolates. Both tested *Alteromonas* strains exhibited QS induction activity. The *Alteromonas* SFB10_2 strain triggered QS induction in both sensors strains, whereas, the *Alteromonas* YSF strain only triggered QS induction in *C. violaceum* CV026 (Figure 8). Both *Endozoicomonas*-related strains triggered QS induction in *C. violaceum* CV026 only. In addition, both strains triggered QS inhibition in *A. tumefaciens* A136, while QS inhibition in *C. violaceum* CV026 was only triggered by strain SF102 (Figure 8). We note that out of the 62 bacterial strains screened in this study, the two *Endozoicomonas*-related strains from *S. flexibilis* were the only strains with both induction and inhibition QS activity. Of the tested *Vibrionaceae* strains, 6 of 14 strains showed QS activity and this was evenly split between induction (3 strains) and inhibition (3 strains) activity.

Lobophytum compactum Bacterial Isolates

The isolates cultured from *L. compactum* demonstrated a number of similarities to the bacteria isolated from *S. flexibilis* (Supplementary Table 5, Figures 10, 11). Firstly, the majority of *L. compactum* isolates were gammaproteobacteria of the genus *Vibrionaceae* (30/51). Secondly, strains related to the genera *Endozoicomonas* and *Bacillus*, and the order *Alteromonadales*, were isolated also from this soft coral species. In this instance, however, the diversity of *Alteromonadales*-related strains was higher with strains related not only to the genus *Alteromonas* (seven strains) but also to the genera *Pseudoalteromonas* (six strains), *Paramoritella*, *Ferrimonas*, and *Shewanella*. In contrast to the *S. flexibilis* isolates, the *L. compactum* isolates also included strains belonging to the genera *Psychrobacter* (class *Gammaproteobacteria*), *Erythrobacter* (class *Alphaproteobacteria*), and *Micrococcus* (class *Actinobacteria*).

The potential of soft coral isolates from *L. compactum* to participate in AHL-type QS communication systems was also investigated (Figure 10). Of the tested isolates from *L. compactum*, 47.5% demonstrated QS activity at the growth conditions tested and activity was mixed between induction and inhibition. Three of the tested *Vibrio* strains (LC111, LC103, and LC105) showed inhibitory activity against both biosensors. Of the strains that were initially isolated with media containing isolobophytolide, 41% were unable to be cultured in the absence of this compound and consequently were not tested for QS activity.

DISCUSSION

Soft Coral Extracts

This study has demonstrated that QS interference extends across at least four soft coral families (*Alcyoniidae*, *Clavulariidae*,

Nephtheidae, and *Xeniidae*). Further, it was shown that both induction and inhibition QS activity extends across both polar and non-polar fractions, indicating that QS interference capability is widespread in soft corals from the central Great Barrier Reef, Australia.

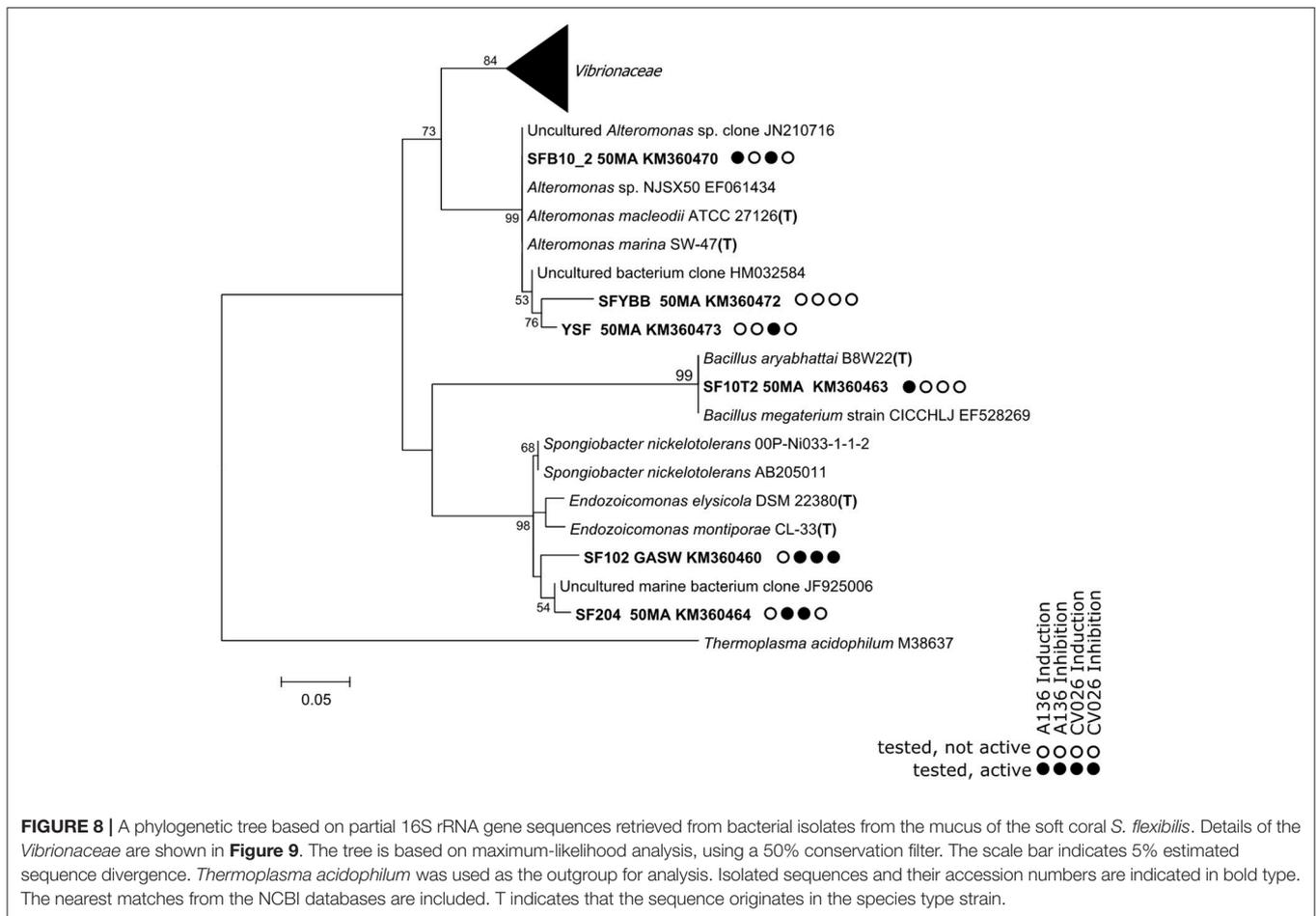
Widespread activity, across not only species that are known to contain different metabolite types but also across a range of polarities (indicated by the activities of extracts obtained by use of solvents with different polarities), is probably indicative of active compounds of more than one structural type. This is further supported by the finding that most (except *P. violacea*) of the soft coral species that induced QS in *A. tumefaciens* A136, also inhibited both biosensors. The widespread prevalence of QS inhibition as well as the presence of QS induction in the soft corals screened here is consistent with QS activity found across a range of marine invertebrates (Taylor et al., 2004; Skindersoe et al., 2008; Hunt et al., 2012). The dual presence of induction and inhibition of QS is similar to that found previously in gorgonian coral extracts (Hunt et al., 2012) but contrasts with the sole QS inhibition activity that was identified in *D. pulchra* (Kjelleberg et al., 1997). QS induction was also established in extracts of marine sponges and sponge associated bacteria (Taylor et al., 2004). Results from this study highlight the need to examine both induction and inhibition of QS to generate a realistic understanding of the complexity of ecological interactions between a host organism and its associated bacteria.

Soft Coral Fractions

In contrast with the initial crude extract testing, all the fractionated soft corals displayed at least one active fraction in the *A. tumefaciens* A136 QS induction assay. This may reflect an inherent increase in concentration of the active components or a decrease in complexity of the samples being tested. Soft coral extracts and fractions may be highly complex mixtures of compounds with contrasting QS regulatory activities. The potential for activity masking within extracts, a phenomenon previously observed in the QS screening of marine sponge extracts (Taylor et al., 2004), is high. This is particularly true if only one concentration or level of complexity is tested.

The QS induction pattern of the soft coral fractions was generally limited to one or two active fractions (80 and 90% methanol elution) for each species suggests that the inductive capability may be due to the presence of structurally similar compounds. Cembranoid diterpenes are well documented in eleven of the soft coral species tested (MarinLit Database, 2013) and correlated well to QS induction therefore could be responsible for the observed QS activity in these species. The same diterpene scaffolds, however, are not commonly known in the genera *Nephthea* (Amir et al., 2012a,b) *Cespitularia* (Elshamy et al., 2016) or in gorgonian corals (Changyun et al., 2008), so cembranoid diterpenes cannot fully explain the observed QS interference in these species. Isolation of cembranoid diterpenes was therefore required to understand the relative importance of these secondary metabolites to act as QS mimics.

A similarly discrete pattern was not reflected in the QS inhibition profile for these fractions. The broad QS inhibition profile of these fractions, might be due to multiple compounds,



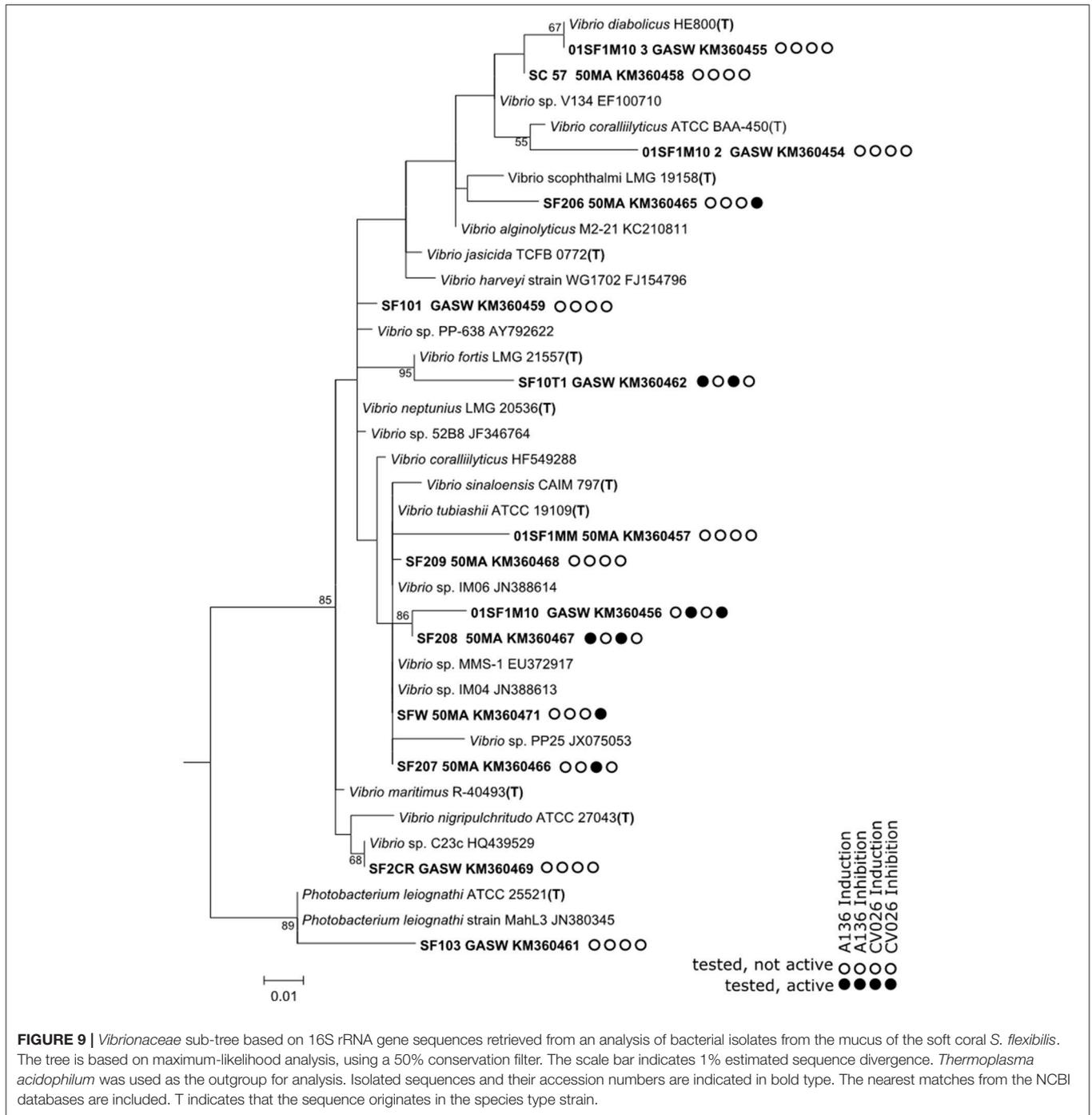
or, the compound(s) responsible may not be suited to the method of fractionation used and the same compounds could be spread across several fractions. The presence of multiple QS compounds within a single holobiont would potentially enable a larger number of interactions with different bacterial strains and / or trigger different QS responses. This complexity may also reflect the capability of some bacteria to possess multiple QS systems (Reshef et al., 2006), with each system regulating a different process or interaction.

Pure Compounds

The strength and type of QS interference by cebranoid diterpenes was observed to correlate with the size of the oxygenated ring. Those cebranoid diterpenes that contained either a five membered furan or lactone ring were capable of inducing QS in *A. tumefaciens* A136, whereas the cebranoid diterpenes with larger lactone rings (six or seven membered) were seen to inhibit QS in *A. tumefaciens* A136. The type of oxygenated functional group also appears to impact the strength these QS mimics, with the furans tested (Compounds **9** and **10**) having higher activities than the γ lactones (Compounds 2-5). In keeping with our current understanding of the QS mechanism, QS interference was only observed for cebranoid diterpenes possessing secondary oxygen rings

(Fuqua et al., 2001; Watson et al., 2002; Geske et al., 2008). The presence of other minor functional groups (epoxides, acetates or level of saturation) had minimal discernible impact on the strength of QS interference observed and no obvious effect on the type of activity observed with respect to *A. tumefaciens* A136. QS mimics have previously been isolated that possess a γ lactone, however, these mimics (such as the furanones of *D. pulchra*) are often associated with QS inhibition rather than the inductive activity demonstrated here (Givskov et al., 1996; Defoirdt et al., 2013). In the case of the furanones from *D. pulchra*, bromine substituents are also present and may be influencing the type of activity. The presence of these metabolites in soft corals is strongly correlated to their taxonomy and may represent different strategies of interaction between species.

A common feature of QS mimic compounds previously identified from eukaryotic extracts is multiple forms of biological activity (Davies, 2006; Yim et al., 2007; Defoirdt et al., 2013). Cebranoid diterpenes appear to be no different with a number, including those identified in the current study, having previously been reported to demonstrate antibiotic (Aceret et al., 1995), cytotoxic (Maida et al., 1993) and algacidal properties. The antimicrobial activity identified in flexibilide (**7**) by Aceret et al. (1995), however, was exhibited at concentrations at least one order of magnitude higher than those that produced QS



interference in this study. The peak QS active concentration occurred 1×10^{-5} mM (or 3 ppm), reflecting the concentrations of flexilide and sarcophytoxide in the mucous and water column surrounding *S. flexibilis* and *S. crassocaula* detected by Coll et al. (1982). Rather than being incompatible, the contrasting activities could be evidence of a hormetic response. Hormetic relationships have been previously observed in the QS mimics from garlic (Persson et al., 2005) and some antibiotic compounds, whereby growth stimulation or cell signaling properties are

exhibited at concentrations below their minimum growth inhibitory concentration (Davies, 2006; Yim et al., 2007). A hormetic response could be relevant in soft corals with loosely packed sclerites, where uptake or release of water from the tissue can lead to large changes in volume over a matter of hours (Freckelton, 2015). As a result, associated metabolites will show a correspondingly dramatic change in concentration in the tissues on a volumetric basis over the same time period. More research is required to understand the potential of hormetic relationships

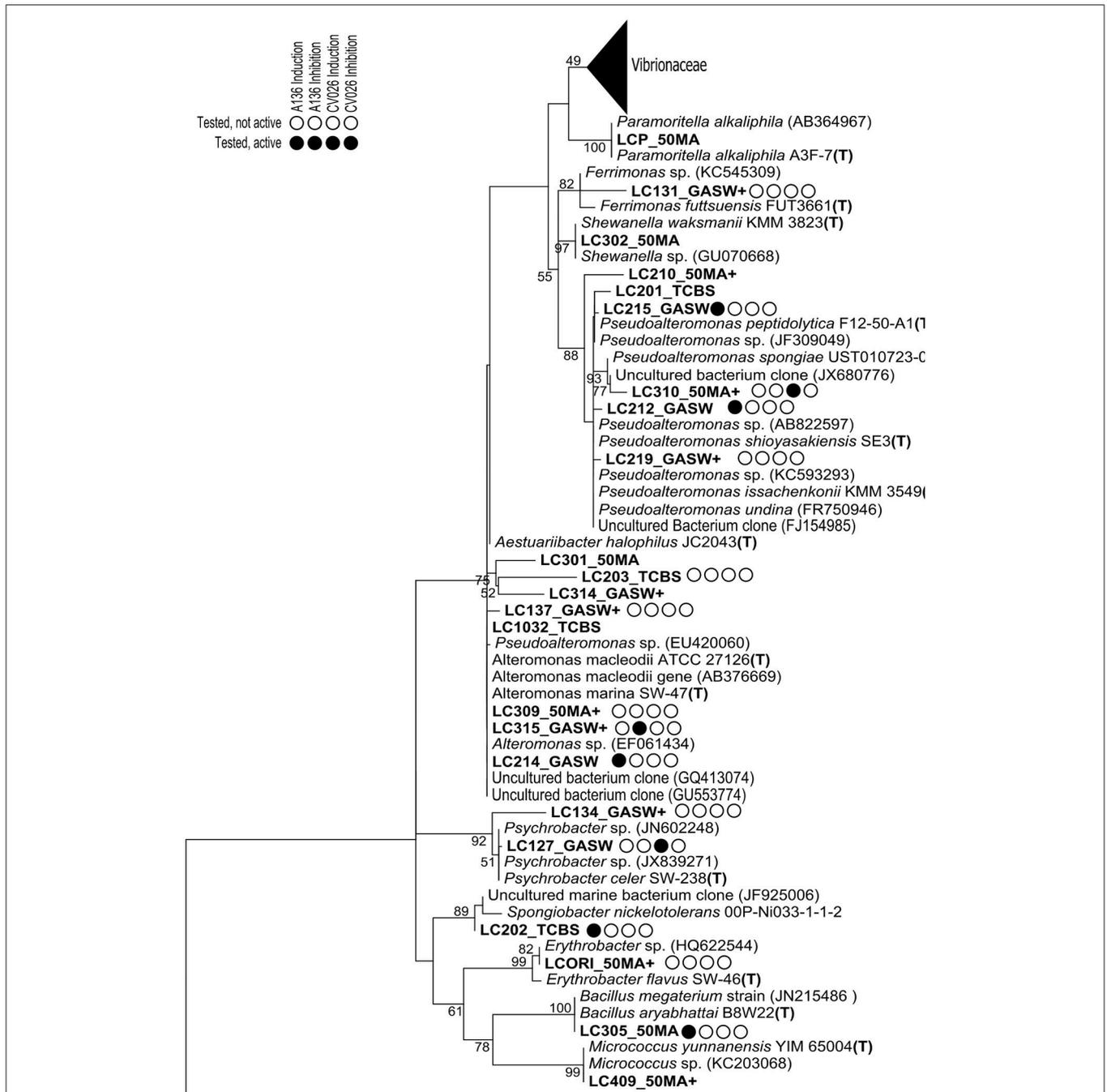


FIGURE 10 | A phylogenetic tree based on 16S rRNA gene sequences retrieved from an analysis of bacterial isolates from the mucus of the soft coral *L. compactum*. Details of the *Vibrionaceae* are shown in **Figure 11**. The tree is based on maximum-likelihood analysis, using a 50% conservation filter. The scale bar indicates 10% estimated sequence divergence. *Thermoplasma acidophilum* was used as the outgroup for analysis. Isolated sequences and their accession numbers are indicated in bold type. The nearest matches from the NCBI databases are included. T indicates that the sequence originates in the species type strain.

in QS mimics and how such concentration changes could be manipulated in the defense of the coral.

Strong evidence for the ecological role of cembranolides and furanocembranoid diterpenes as QS mimics is further exhibited in the strong differences in the ability of the two biosensor strains to respond to QS mimics in the soft corals. The QS

inductive compounds present within the soft corals were more readily detected by the *A. tumefaciens* A136 strain. In contrast, QS inhibition was observed more frequently for *C. violaceum* CV026. This could suggest that *C. violaceum* CV026 can be inhibited by a broader range of compounds or that it is more sensitive to a broader range of compound concentrations. This

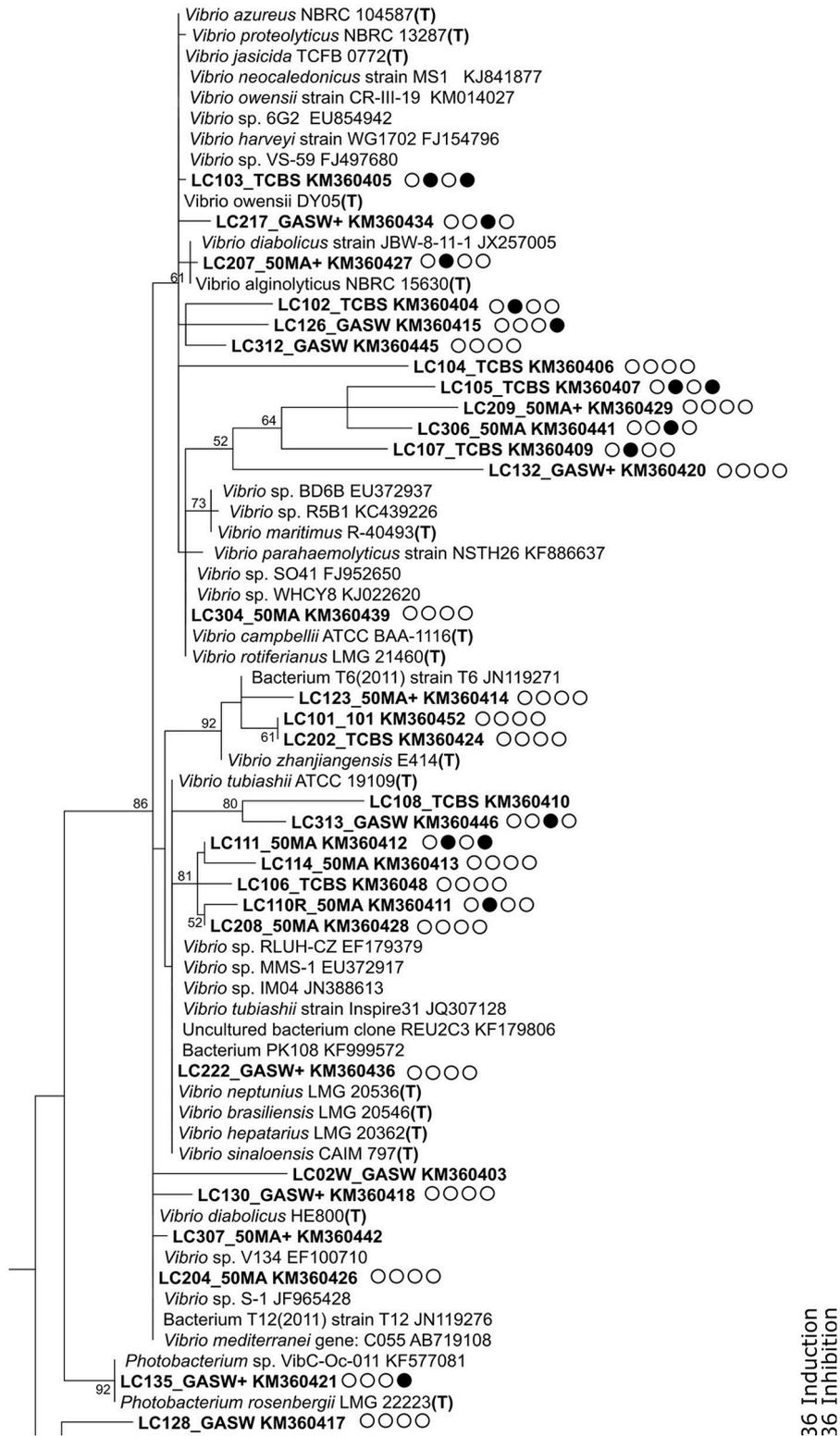


FIGURE 11 | *Vibrionaceae* sub-tree tree (part of tree presented in **Figure 10**) based on 16S rRNA gene sequences retrieved from an analysis of bacterial isolates from the mucus of the soft coral *L. compactum*. The tree is based on maximum-likelihood analysis, using a 50% conservation filter. The scale bar indicates 10% estimated sequence divergence. *Thermoplasma acidophilum* was used as the outgroup for analysis. Isolated sequences and their accession numbers are indicated in bold type. The nearest matches from the NCBI databases are included. T indicates that the sequence originates in the species type strain.

difference in sensitivity is despite an overlap of AHL acyl chain length detection by the two biosensor strains (Steindler and Venturi, 2007). *A. tumefaciens* A136 utilizes the TraR QS response regulator system and responds to a broad range of acyl chain lengths in AHL molecules (Steindler and Venturi, 2007). *C. violaceum* responds to a shorter range of acyl chain lengths in AHL molecules and utilizes the CivR QS response regulator (Steindler and Venturi, 2007). The differential responses of these two biosensors highlights the advantage of using multiple biosensors when screening for QS mimics where chain length sensitivities may have little applicability.

Isolated Bacteria

This study strongly suggests that isolobopytolide, the major secondary metabolite in *L. compactum*, is an important selection factor regulating the microbial community of this soft coral. Firstly, we demonstrated that isolobophytolide can interfere with the QS activity of sensor strains, and secondly we demonstrated that addition of isolobophytolide to culture media increased the number and morphological variation of colonies produced from *L. compactum*. Moreover, the latter result suggests that the inclusion of secondary metabolites in growth media can improve the success of culturing soft coral associated bacterial isolates.

Most isolates generated in this study had high sequence identity with bacterial sequences sourced from the marine environment, including marine invertebrate hosts (Supplementary Table 5). Many of the recovered genera have also previously been isolated from coral mucus samples including *Alteromonas*, *Bacillus*, *Endozoicomonas*, *Erythrobacter*, *Micrococcus*, *Pseudoalteromonas*, *Shewanella*, and *Vibrio* (Lampert et al., 2006; Nithyanand and Pandian, 2009; Pootakham et al., 2017). The isolates were dominated by gammaproteobacteria belonging to the family *Vibrionaceae*, a result that is consistent with previous observations in scleractinian corals (Kvennefors et al., 2010, 2012). For both coral species, several *Vibrio* strains were isolated whose sequences clustered together and separately from the most closely related database sequences and hence may represent novel species. Scleractinian corals have previously been recognized as harboring a number of novel bacterial taxa (Rohwer et al., 2002; Sunagawa et al., 2010). This situation still remains, with amplicon-based studies of coral microbiomes returning many unassigned OTUs (Blackall et al., 2015). The bacteria of alcyonacean corals are less well studied and it is reasonable to assume that a similar situation could exist.

Multiple strains capable of inducing and/or disrupting QS in the bacterial biosensors were isolated from *L. compactum* and *S. flexibilis* (57.5 and 57.8% of tested strains, respectively). The genera of all the bacteria isolated, regardless of activity detected in this study, have previously been reported to possess or interact with QS systems (Ansaldi et al., 2002; Long et al., 2003; Waters and Bassler, 2005; Case et al., 2008; Tait et al., 2009, 2010; Nithya et al., 2010; Albuquerque and Casadevall, 2012; Lade et al., 2014), providing support for the hypothesis that QS is one of the mechanisms regulating coral associated microbial communities.

There was no clear taxonomic pattern of QS activity within the *Vibrionaceae*, which is consistent with a previous study that assessed QS activity in 29 *Vibrionaceae* strains (Tait et al., 2010). It is well recognized that QS in *Vibrio* spp. is tightly regulated by environmental conditions including host-released cues and nutritional status (Waters and Bassler, 2005). *Vibrio* spp. are ubiquitous in the marine environment (Urakawa and Rivera, 2006), however many *Vibrio* strains have been implicated in disease either as primary or opportunistic pathogens (Urakawa and Rivera, 2006). Given that QS is involved in the regulation of a number of the genes involved in pathogenicity (de Kievit and Iglewski, 2000; LaSarre and Federle, 2013), the presence of a wide range of *Vibrio* spp. with QS capabilities in otherwise healthy corals warrants further investigation to elucidate which genes are under QS control in these species.

“*Spongiobacter*,” now recognized as belonging to the genus *Endozoicomonas* (Neave et al., 2016), was originally recovered from a marine sponge (Pike et al., 2013) but is also present in many gorgonian (Sunagawa et al., 2010; La Rivière et al., 2013) and scleractinian corals (Raina et al., 2009; Blackall et al., 2015; Bourne et al., 2016). “*Spongiobacter*” strains have been attributed a number of ecological roles; “*Spongiobacter*” strains from *A. millepora* demonstrated a dependence on DMSP and consequently a role in the biogeochemical sulfur cycle was postulated (Raina et al., 2009), whereas, “*Spongiobacter*” strains from the sponge *Suberites carnosus* demonstrated antibacterial activity (Flemer et al., 2012). Of greatest interest to this study is the QS activity detected in “*Spongiobacter*” strains from the sponges *Mycale laxissima* and *Ircinia strobilina* (Mohamed et al., 2008). The *Endozoicomonas*-related strains SF102 and SF204 from *S. flexibilis* that were tested in this study induced QS activity in *C. violaceum* CV026 and not in *A. tumefaciens* A136, whereas Mohamed and coworkers found the opposite response (positive in *A. tumefaciens* and negative in *C. violaceum*).

In this study, QS activities were assessed for the culturable fraction of bacteria associated with the mucus of two soft coral species. In future, functional gene analysis and gene expression analysis may allow a more complete assessment of the genes that are responsible for and regulated by QS in these bacteria. Moreover, new -omics techniques will allow investigations of quorum sensing genes and their expression also in bacteria that cannot easily be cultured with standard methods. A combination of culture-independent studies and manipulative experiments using isolates holds great promise for further elucidation of QS mechanisms in soft coral holobiomes.

CONCLUSION

This research establishes a framework for the importance of QS and the identity of potential QS mimics within the soft coral holobiont, highlighting the potential value of soft corals as a model system for both structural and ecological investigations of QS mimics. The results presented here

clearly show that cembranolides and furanocembrenes are partially responsible for previously observed QS interference in soft coral extracts. Their QS interference translates to a potentially new structural backbone for QS mimic compounds. The size of the oxygenated ring had more bearing on the activity expressed than the presence or position of epoxides, double bonds or acetate groups, an observation which extends the structural understanding of QS mimics. QS interference extended however also to soft coral species not known to contain cembranolides and furanocembrenes, suggesting that new structural backbones with QS activity remain to be elucidated. The presence of both QS metabolites and QS bacteria within soft corals supports the role of QS as a way of mediating soft coral associated microbial communities. If the active compounds in these extracts are indeed produced by the soft coral, this interaction with QS could be important to the health and resilience of the host organism and may reflect a more widespread strategy of sessile marine invertebrates.

ETHICS STATEMENT

Soft coral samples were legally collected between 2009 and 2015 under Great Barrier Reef Marine Park Authority (GBRMPA) permits (G09/30327.1, G12/35236.1).

DATA AVAILABILITY STATEMENT

The raw data supporting the conclusions of this manuscript will be made available by the authors, without undue reservation, to any qualified researcher.

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AUTHOR CONTRIBUTIONS

MF experimental design, sample collection, lab work, data analysis and write-up. LH experimental design, data analysis and write-up. BB experimental design, sample collection, data analysis and write-up.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: <https://www.frontiersin.org/articles/10.3389/fmars.2018.00198/full#supplementary-material>

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Conflict of Interest Statement: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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