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Diversity and Bioactivity of Marine Bacteria Associated with the Sponges *Candidaspongia flabellata* and *Rhopaloeides odorabile* from the Great Barrier Reef in Australia

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Abstract: Sponges and their associated microbial communities have sparked much interest in recent decades due on the abundant production of chemically diverse metabolites that in nature serve as functional compounds required by the marine sponge host. These compounds were found to carry therapeutic importance for medicinal applications. In the presented study, 123 bacterial isolates from the culture collection of the Australian Institute of Marine Science (AIMS) previously isolated from two different sponge species, namely *Candidaspongia flabellata* and *Rhopaloeides odorabile*, originating from different locations on the Great Barrier Reef in Queensland, Australia, were thus studied for their bioactivity. The symbiotic bacterial isolates were first identified using 16S rRNA gene analysis and they were found to belong to five different dominating classes of Domain Bacteria, namely Alphaproteobacteria, Gammaproteobacteria, Flavobacteria, Bacilli and Actinobacteria. Following their taxonomical categorization, the isolates were screened for their antimicrobial activity against human pathogenic microbial reference strains: *Escherichia coli* (ATCC® BAA-196™), *E. coli* (ATCC® 13706™), *E. coli* (ATCC® 25922™), *Klebsiella pneumoniae* (ATCC® BAA-1705™), *Enterococcus faecalis* (ATCC® 51575™), *Bacillus subtilis* (ATCC® 19659™), *Staphylococcus aureus* (ATCC® 29247™), *Candida albicans* (ATCC® 10231™) and *Aspergillus niger* (ATCC® 16888™). Over 50% of the isolates displayed antimicrobial activity against one or more of the reference strains tested. The subset of these bioactive bacterial isolates was further investigated to identify their biosynthetic genes such as polyketide synthase (PKS) type I and non-ribosomal peptide synthetase (NRPS) genes. This was done using polymerase chain reaction (PCR) with degenerate primers that have been previously used to amplify PKS-I and NRPS genes. These specific genes have been reported to be possibly involved in bacterial secondary metabolite production. In 47% of the bacterial isolates investigated, the PKS and NRPS genes were located. Some of the bacterial isolates were found to possess both gene types, which agrees with the previous reported biosynthetic ability of certain sponge-symbiotic bacteria such as the Actinobacteria or Gammaproteobacteria to produce secondary metabolites with antimicrobial activity. All these reported activities further confirm that sponge-symbiotic bacteria hold significant bioactivity with medicinal and biotechnological importance.

Keywords: sponge-associated bacteria; *Candidaspongia flabellata*; *Rhopaloeides odorabile*; biodiscovery; antibiotics; bioactive compounds; PKS and NRPS genes

1. Introduction

The marine environment has become an important source for natural product discovery due to the uniqueness and complexity of marine-derived metabolites that may contribute significantly towards discovery of novel and potent antibiotics [1]. Extremity of the environmental factors present in these marine environments (e.g., varying salt concentration, hydrostatic pressure, the range of available marine nutrients present in these environments) and the symbiosis with marine macro-organisms (e.g., the invertebrates) [2] would, in turn, induce changes in microbial metabolism, resulting in the production of chemically diverse compounds [3].

Sponges, of the phylum Porifera, have been the largest source of bioactive compounds providing a greater number of novel metabolites than any other marine taxon each year, contributing nearly 30% of all of the natural marine compounds discovered [4–11].

Sponges are among the oldest and most stable metazoans known to inhabit this planet [12] and appear in most tropical and temperate aquatic habitats. They exist in various shapes, sizes and colours [13] and are highly evolved and successfully adapted organisms [14–16].

Microorganisms are associated with marine sponges either transiently and come into contact through the host's filter feeding mechanism or live symbiotically within the host and are responsible for a range of functional metabolic activities aiding the survival of the host including the production of defense metabolites [5,12,17]. These sponge-associated microorganisms can make up 35–60% of the total sponge mass [5,7,11,18,19]. Previously, compounds isolated from sponges were thought to be produced by the sponge itself however, further research identified abundant evidence that microorganisms living within the sponge are in most cases responsible for the production of most of these metabolites of interest [4,5,20,21]. Examples include the production of the glycoglycerolipid with anti-tumour properties by the sponge *Halichondria panacea* as well as from a *Microbacterium* species found within the sponge. Several quinolones with cytotoxic and antimicrobial properties were also isolated from the sponge *Homophymia* sp. as well as from a pseudomonad species isolated from the same sponge [5].

Microorganisms, including the sponge-associated bacteria, may produce biologically active compounds which requires the activation of specific gene clusters encoding multi-modular enzymes. These gene clusters may be in the form of non-ribosomal peptide synthetases (NRPS) and polyketide synthases (PKS), however, other gene clusters may also be responsible for biological activity. The variations of these enzymatic modules (PKS and NRPS) results in the production of numerous biologically active compounds [8,22,23]. In order to produce these types of secondary metabolites, a set of domains are required within the bacterial biosynthetic pathways; these include ketosynthase (KS), acyltransferase (AT) and acyl carrier proteins in PKSs and adenylation (A), condensation (C) and peptidyl carrier proteins (PCPs) for peptide elongation in NRPSs [8]. Furthermore, antibiotic compounds such as tetracycline and erythromycin, anticancer agent, e.g., bleomycin and the immunosuppressive agent, e.g., rapamycin [24,25] were reported to be produced from PKS and NRPS pathways. Accordingly, complex polyketides isolated from sponges and their associated microorganisms are claimed to be the most promising biologically active compounds with therapeutic applications [8,26]. Examples of these important polyketides include swinholide from the sponge *Theonella swinhoei* [27,28] and mayamycin produced by *Streptomyces* sp. HB202 from the sponge *Haliclona simulans* and *H. panicea* [29,30] that have potent antibiotic activities. Halichondrin B isolated from the sponge *Halichondria okadai* also displays potent anticancer activity [31,32].

The sponge species *Candidaspongia flabellata* is a rare and biochemically active Dictyoceratida sponge [7], noteworthy for the diversity and potency of its homosesterterpene and bishomosclarane secondary metabolite production. *Rhopaloeides odorabile* is a common Dictyoceratida sponge species to the Great Barrier Reef and is known to house an uncommon group of C20 diterpenes [33]. Both these sponge species are known to house a variety of bacterial genera [7,34]. Previously, the full cultivatable heterotrophic bacterial community associated with *C. flabellata* was identified by Burja and Hill [7] and the main bioactive compound isolated from this sponge species was fanolide, which has been shown to inhibit the growth of some tumour cells [7]. Phylogenetic analysis of the cultivatable community

of *R. odorabile* has also been carried out by Webster et al. [33] which was found to be dominated by a single bacterial strain of the class alphaproteobacteria [33] with bioactivity. Therefore, by isolating and fermenting in conditions conducive to triggering biosynthetic gene pathways (e.g., PKS and NRPS) involved in metabolite production in these marine sponge bacteria, novel compounds with antimicrobial activity may be recovered.

In light of the information presented above, the aims of this study were (1) molecular level identification of previously isolated sponge-associated bacteria by the Australian Institute of Marine Science (AIMS); (2) examination of their potential to produce antimicrobial compounds active against human pathogenic and antibiotic-resistant bacteria; and (3) detection of their biosynthetic genes potentially involved in the production of some of these biologically-active metabolites.

2. Materials and Methods

2.1. Sponge Collection and Isolation of Bacteria

A set of sponge associated bacteria from the marine microbial culture collection of the Australian Institute of Marine Science (AIMS, http://aims.gov.au/) was supplied to the University of the Sunshine Coast (USC) for further analysis. Sponge samples were collected by the AIMS by SCUBA as described by Webster and Hill [35]. Sections of the sponge were removed using sterile scalpel blades and the tissue transferred directly into a plastic sampling bags that contained seawater. Sponge tissue were processed within 15 min of collection. A 1 cm^3 portion of the sponge was excised and rinsed briefly in 70% ethanol and quickly transferred to sterile artificial seawater (ASW). The sponge tissue was then removed from the ASW and cut into sections using a sterile scalpel and finely ground using a mortar and pestle [35]. Isolates (n = 105) collected from 12 different sponge samples from *Candidaspongia flabellata* and 18 isolates from the four different sponge samples of *Rhopaloeides odorabile* (n = 123) were collected from the Great Barrier Reef (see Appendix A, Tables 1 and 2 for locations and numbers of sponge samples) over a two-year period. These bacterial isolates were grown on Marine agar 2216 (BD Difco™) at temperatures of 22 °C, 26 °C and 27 °C, respectively (under similar conditions to their tropical marine environment), for 2 to 21 days (Appendix A, Tables A1 and A2). Bacterial isolates were then stored at 80 °C in a cryoprotectant solution (tryptone soy broth and 30% glycerol) and used for further testing as needed.

2.2. Molecular Identification of the Bacterial Isolates

2.2.1. DNA Extraction and 16S rRNA Gene Sequencing

Firstly, DNA extraction for Gram-negative isolates was carried out using the FastPrep®-24 Instrument and FastDNA® kit (MP Biomedicals, Irvine, CA, USA), according to the FastDNA® kit instruction manual protocol for bacterial DNA extraction. Gram-positive isolates were extracted using the DNeasy Blood and Tissue kit (Qiagen Inc., Frederick, MD, USA) according to the manual for the extraction of DNA from Gram-positive isolates. Isolates were tested for their cell wall types using the Gram-staining technique. DNA extracts were run on a 1% agarose gel (1 h at 110 V) to ensure successful DNA extraction and stored at −20 °C for extended periods and 4 °C until use.

All bacterial isolates were identified using Polymerase Chain Reaction (PCR) amplification of 16S rRNA genes using the HotstarTaq *Plus* Master Mix kit (Qiagen Inc.) following the instruction manual: PCR Using HotStarTaq *Plus* Master Mix. Universal primers B27F (5′-AGAGTTTGATCCTGGCTCAG-3′) and U1492R (5′-GGTTACCTTGTTACGACTT-3′) were used to obtain an amplicon length of 1500 base pairs (bp). Cycling parameters for the amplification of the genes were carried out per the HotstarTaq *Plus* Master Mix instruction manual. Negative controls using sterile distilled water were used to ensure the amplified gene was not a result of contamination. PCR was performed in a T100 Thermal Cycler (BioRad) and the PCR products were viewed on a 1% agarose gel in Gel Doc™ XR+ Imager (BioRad). Amplified PCR products were sent to Macrogen Inc. (Seoul, Korea) for sequencing and the same universal primers were used.

2.2.2. Sequence Alignment and Phylogenetic Analysis

The 16S rDNA sequences were prepared using CLC genomic work bench (Qiagen Inc.) and were aligned using the standard nucleotide Basic Local Alignment Search Tool (BLAST) analysis with the National Centre for Biotechnology Information (NCBI) database (http://www.ncbi.nlm.nih.gov/) [36].

The ARB program [37] was used for sequence alignment and phylogenetic analyses. Only sequences >1300 (bp) were used. Phylogenetic trees were constructed using the maximum likelihood algorithm, with bootstrap analysis using 1000 data re-samplings. Phylogenetic trees were used to determine the relatedness of the isolates to their nearest relative. The sequences from bacterial isolates in this study were deposited to GenBank (See Appendix A, Tables A1 and A2 for GenBank accession numbers).

2.3. Antimicrobial Assay

All bacterial extracts were tested for antimicrobial activity. Isolates were first grown in 20 mL of Marine broth (Benton Dickinson Difco™) at 28 °C on a floor-shaker (Bioline Global) at 150 rpm to obtain seed cultures. An amount of 5 mL of each bacterial sample was transferred into flasks containing 50 mL Marine broth (BD Difco™) and incubated at 28 °C on a floor-shaker (Bioline Global) at 150 rpm for 7 days [38]. The liquid fermentation medium was then centrifuged at 7000× *g* for 20 min [18] to obtain the cell-free supernatant (CFS). The CFSs were then extracted twice with ethyl acetate (EtOAc, Honeywell Research Chemicals) [18,38] and the organic phase was evaporated to dryness on a rotary evaporator (BÜCHI™ Rotavapor R-205) at 37 °C to obtain a crude extract [11,39–41]. In this instance, bioactivity was tested using the method by Dashti, et al. [42]. Crude extracts were weighed and dissolved in known volumes (500 µL) of dimethyl sulfoxide (DMSO, Sigma-Aldrich, St. Louis, MO, USA). 50 µL of a 500–1000 µg/mL solution of extract was loaded onto sterile commercial blank discs (Oxoid).

The reference strains were: American Type Culture Collection (ATCC) reference strains, *Escherichia coli* (ATCC® BAA-196™), *E. coli* (ATCC® 13706™), *E. coli* (ATCC® 25922™), *Klebsiella pneumoniae* (ATCC® BAA-1705™), *Enterococcus faecalis* (ATCC® 51575™), *Bacillus subtilis* (ATCC® 19659™), *Staphylococcus aureus* (ATCC® 29247™), *Candida albicans* (ATCC® 10231™) and *Aspergillus niger* (ATCC® 16888™).

Reference strains and the yeast *C. albicans* were grown for 18 to 24 h in Mueller–Hinton (MH) broth at 37 °C and their optical density (OD) measured at 600 nm to obtain an OD reading of 0.063 corresponding to a McFarland tube density of 0.5 [43]. An amount of 200 µL of each reference strain was then spread onto MH agar plates into which wells (6 mm in diameter) were made and the CFS from the different isolates inoculated. Whereas, for the antifungal activity testing, the fungal inoculum was prepared on a potato-dextrose agar (PDA) plate. Following full growth, an agar plug was taken and placed in the center of a new PDA plate to allow the growth in a concentric ring fashion. After 48 h of growth on the new plate, wells were made on the PDA plate for the inoculation of the CFSs. Discs loaded with crude extracts were also placed evenly apart on the MH agar inoculated with the ATCC pathogenic reference strains. Plates were incubated for 24 to 48 h at 37 °C for bacteria and yeast [44] and for 48 to 96 h at 28 °C for the fungal strain. Marine broth and dimethyl sulfoxide (DMSO) were used as negative controls. Vancomycin (Oxoid) and gentamicin (Oxoid) were used as the positive controls against the different bacteria and cycloheximide (Oxoid) against fungi. Following incubation at the above given conditions, the plates were examined for zones of inhibition.

2.4. Amplification of Type 1 Polyketide Synthase and Non-Ribosomal Peptide Synthetase Genes

The occurrence of specific genes involved in the reported production of secondary metabolites [45] was screened in all isolates. For all the heterotrophic bacteria (except the actinobacteria), degenerate primers MDPQQRf (5′-RTRGAYCCNCAGCAICG-3′) and HGTGTr (5′-VGTNCCNGTGCCRTG-3′) [45] were used to amplify the B-ketosynthase (KS) domain fragment within the Type I polyketide synthase genes (PKS-I). Amplification of the non-ribosomal peptide

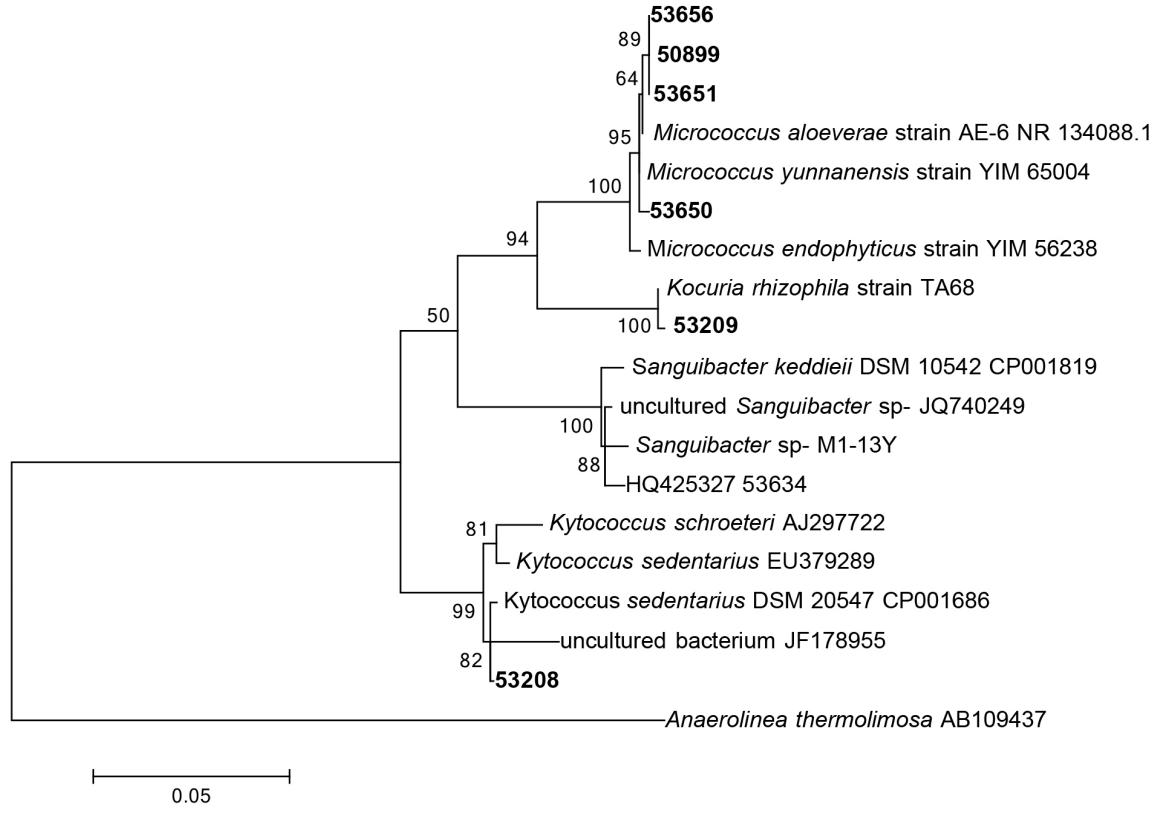
synthetase (NRPS) gene was carried out using primers MTF (5′-CCNCGDATYTTNACYTG-3′) and MTR (5′-GCNGGYGGYGCNTAYGTNCC-3′) to amplify the conserved A domain [46]. The actinobacteria required a different set of primers and K1 (5′-TSAAGTCSAACATCGGBCA-3′) and M6R (5′-CGCAGGTTSCSGTACCAGTA-3′) were used to amplify PKS-I ketosynthase and methyl-malonyl-CoA transferase sequences and A3F (5′-GCSTACSYSATSTACACSTCSGG-3′) and A7R (5′-SASGTCVCCSGTSCGGTAS-3′) were used to amplify NRPS adenylation sequences [47,48]. An amount of 10% DMSO was also added to each reaction for the actinobacteria [49]. Amplification was carried out using the HotStarTaq *Plus* Master Mix Kit (Qiagen) as per cycling conditions given in the HotStarTaq *Plus* Manual. An annealing temperature of 56 °C was used for amplification of genes for the actinobacteria. PCR was performed as described above for Section 2.2.1.

3. Results

3.1. Molecular Identification of the Isolates

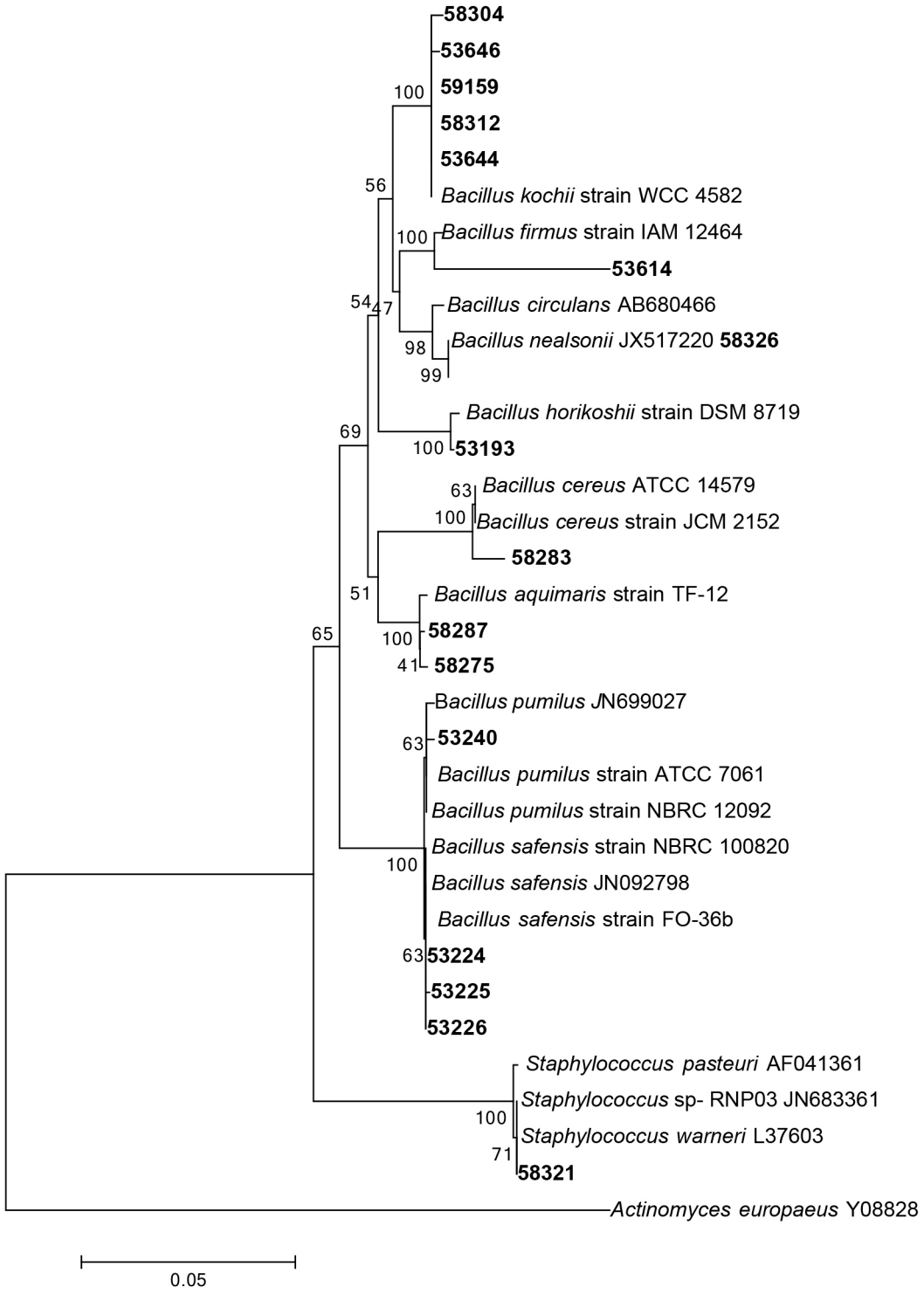
3.1.1. Phylogenetic Analysis Based on 16S rRNA Gene Sequencing

Following 16S rRNA gene sequencing, the isolates were found to belong to five major taxonomic classes of Domain Bacteria that were namely Gammaproteobacteria, Alphaproteobacteria, Bacilli, Actinobacteria and Flavobacteria. Analysis of the 16S rRNA gene sequences revealed the taxonomic position of each isolate in relation to their closest relative strains and the phylogenetic trees (Figure 1) were constructed according to these major classes. However, there was only one isolate belonging to the class Flavobacteria (isolate 58330), isolated from *C. flabellata* and its closest relative strain was *Aquimarina spongiae* strain A6 [50]. Closest relative species for each isolate as well as the percentage of similarity is given in Tables 1 and 2.



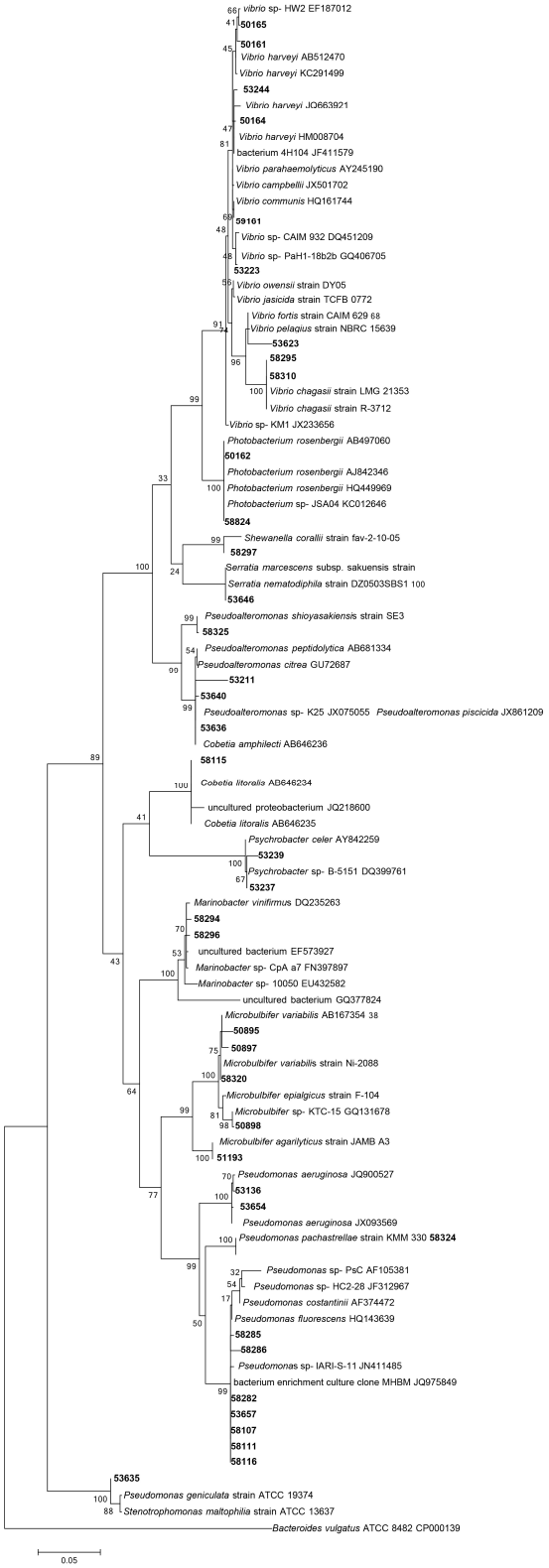
(**a**)

Figure 1. *Cont.*



(**b**)

Figure 1. *Cont.*



(**c**)

Figure 1. *Cont.*

(**d**)

Figure 1. Phylogenetic diversity of bacteria associated with sponge species *C. flabellata* and *R. odorabile* located on the Great Barrier Reef in Queensland, Australia. Phylogenetic trees were constructed using the maximum likelihood algorithm with bootstrap analysis using 1000 data re-samplings within Mega 6 [51]. Trees represent the phylogenetic diversity of the classes Actinobacteria (**a**); Bacilli (**b**); Gammaproteobacteria (**c**) and Alphaproteobacteria (**d**). Bacteria used as an out-group to root the different trees include, *Anaerolinea thermolimosa* for Actinobacteria, *Actinomyces europaeus* for Bacilli and *Bacteriodes vulgatus* for the Alpha- and Gammaproteobacteria. The scale bar represents 5% sequence divergence.

3.1.2. Distribution of Isolates in Relation to Their Sponge Hosts and Sponge Collection Locations

The distribution of the bacterial families isolated from *C. flabellata* and *R. odorabile* in relation to sponge species as well as to geographical locations of these sponges are given in Figures 2–5. The main bacterial families isolated from *Candidaspongia flabellata* belonged to Rhodobacteraceae (48%) and Pseudomonadaceae (16%, Figure 2); from *Rhopaloeides odorabile*, they were from Vibrionaceae (28%) and Alteromonadaceae (22%, Figure 3). Some of these isolates were found to be related to the members of families known to produce bioactive compounds [34,52–54].

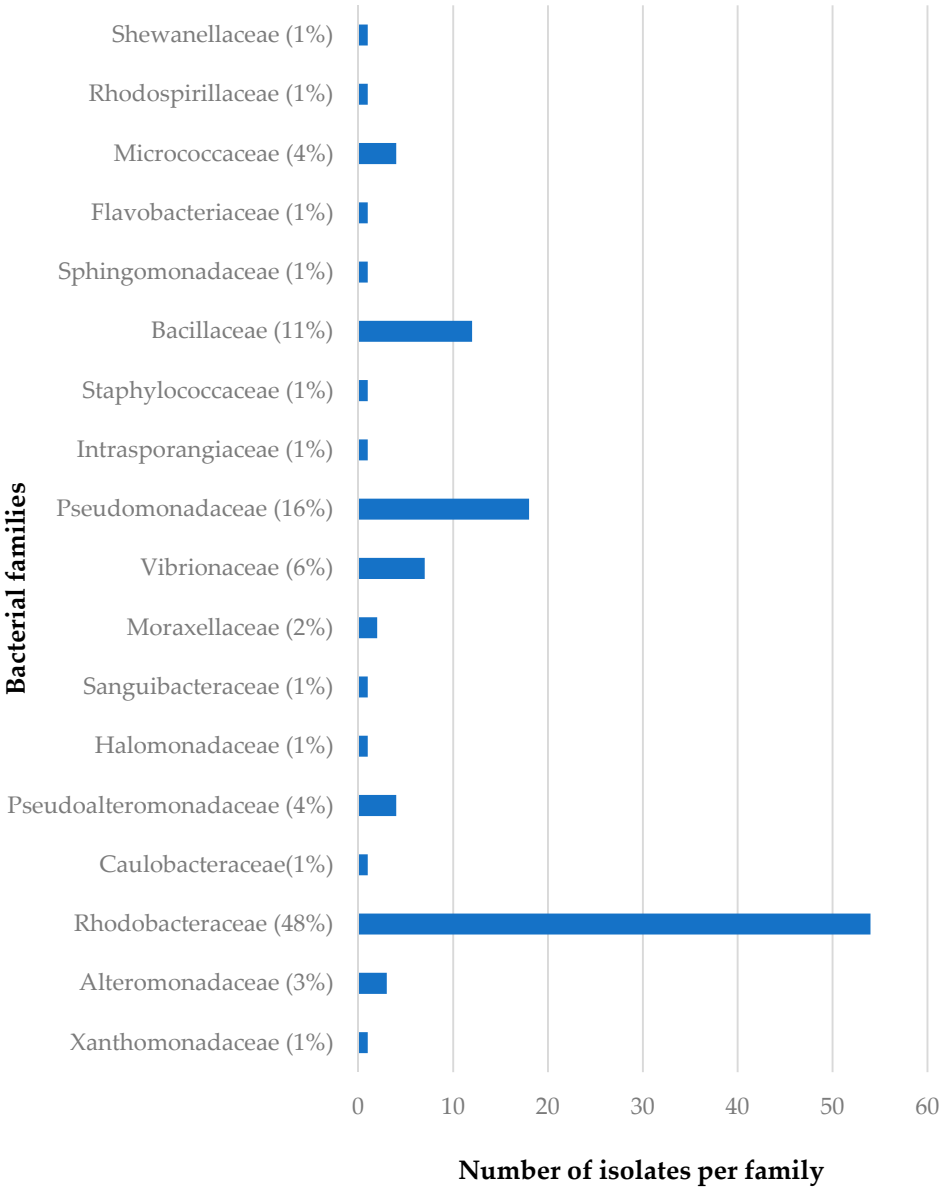


Figure 2. The diversity of the bacterial families isolated from twelve different sponge samples of *C. flabellata*.

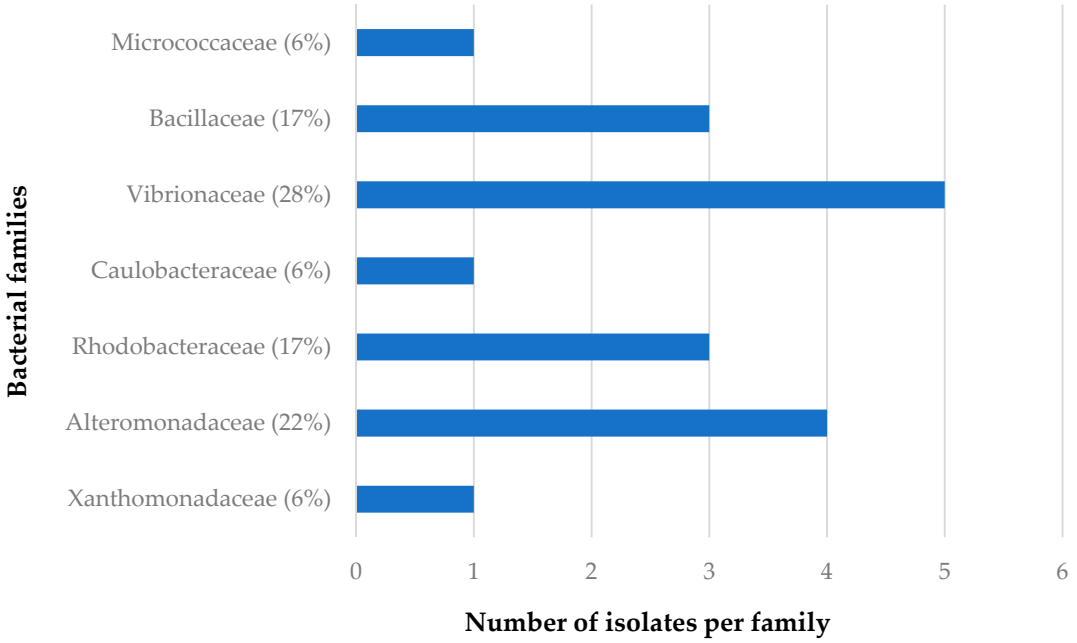
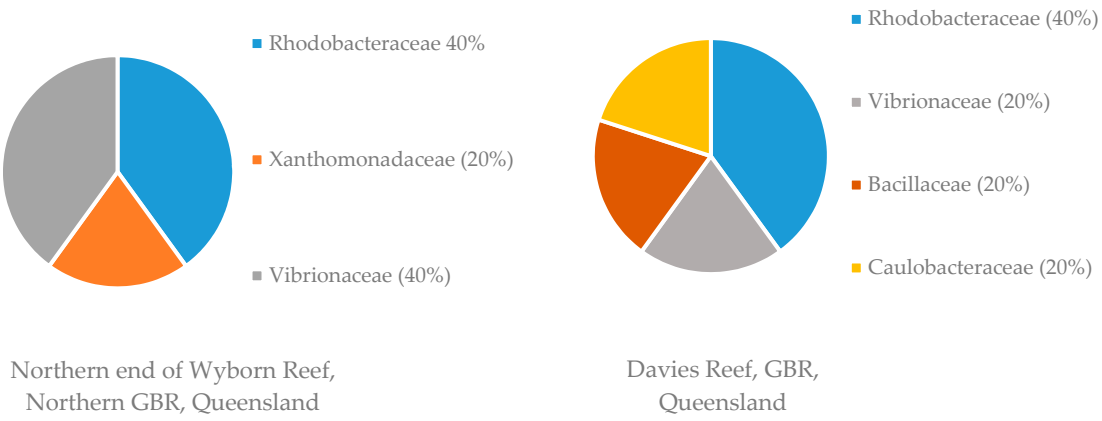


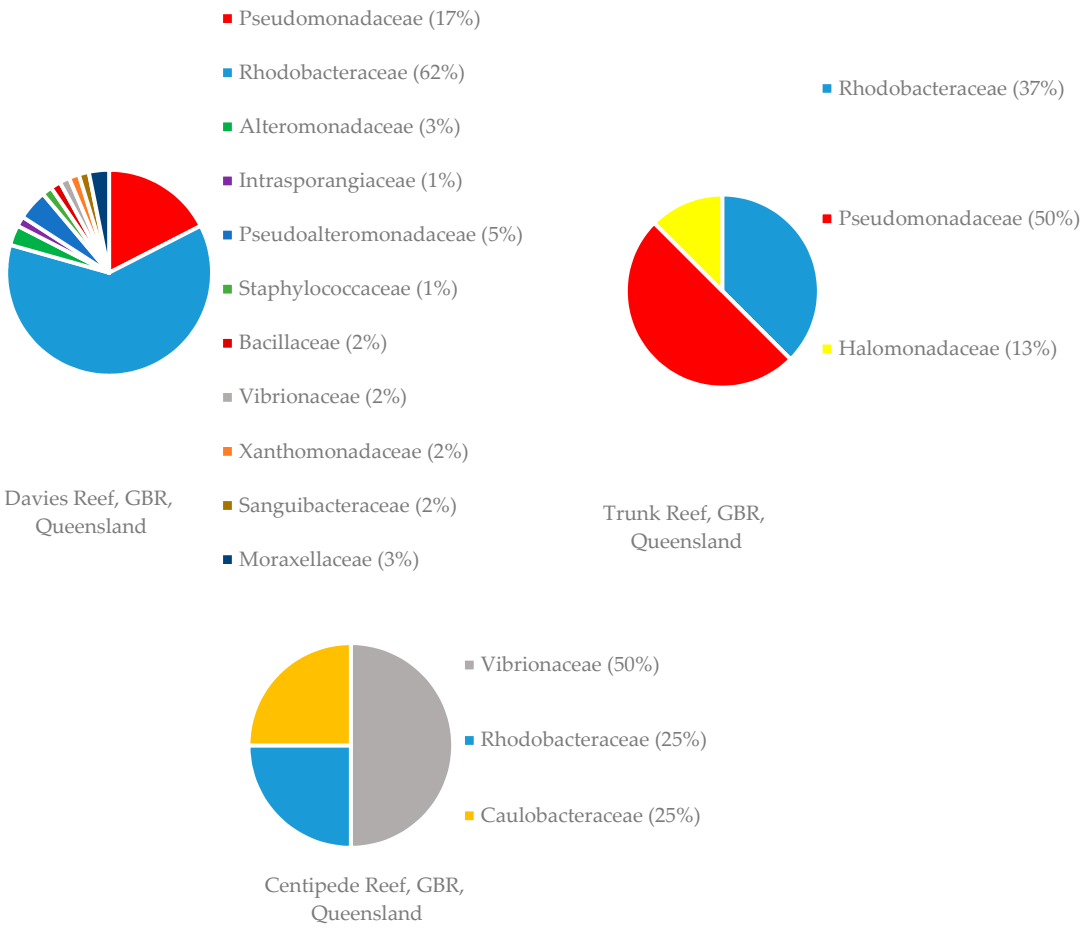
Figure 3. The diversity of the bacterial families isolated from four different sponge samples of *R. odorabile*.



Footnote: Six isolates were isolated from Northern end of Wyborn Reef and ten isolates from Davies Reef.

Figure 4. Diversity of bacterial families isolated from two sponge samples of *R. odorabile* collected at different locations on the Great Barrier Reef (GBR). **Footnote:** Six isolates were isolated from Northern end of Wyborn Reef and ten isolates from Davies Reef.

Sponge samples of *R. odorabile* were collected from three different locations on the Great Barrier Reef, one sponge from Wyborn Reef, two sponges from the Davies Reef and a sponge from North East Percy Island. The familial diversity of two of these locations is given in Figure 4. From the sponge sample taken at North East Percy Island in the North-West corner of the Great Barrier Reef, only one family (two species of Alteromonadaceae were isolated) was able to be cultivated within the laboratory. Sponge samples of *C. flabellata* were collected from three locations on the Great Barrier Reef, ten sponges from the Davies Reef, one from the Trunk Reef and one from Centipede Reef. The familial diversity of these samples collected at the different locations is given in Figure 5. The reason for the increased number of bacterial isolates isolated from *C. flabellata* compared to *R. odorabile* is due to the increased number of *C. flabellata* sponge samples collected.



Footnote: Ninety-three isolates were isolated from sponge samples from Davies Reef, nine isolates from Trunk Reef and three from Centipede Reef.

Figure 5. Diversity of bacterial families isolated from 12 sponge samples of *C. flabellata* collected at various locations on the GBR. **Footnote:** Ninety-three isolates were isolated from sponge samples from Davies Reef, nine isolates from Trunk Reef and three from Centipede Reef.

3.2. Antimicrobial Screening

Out of the total isolates from both sponges, 51% exhibited inhibitory activity against one or more of the pathogenic test strains. The comparison of antimicrobial activity exhibited by the isolates at genus level is given in Figure 6. The isolates belonging to the genus *Pseudomonas* produced extracts with the highest activity against eight out of the nine test strains followed by *Pseudovibrio* (active against seven out of the nine test strains) and *Bacillus* strains (active against three of the test strains). Isolate 53654 with its closest relative strain being *Pseudomonas aeruginosa* PAO1 strain PAO1 was active against eight of the nine strains. Isolate 58264, closest relative strain being *Pseudovibrio* sp. FO-BEG1 strain FO-BEG1 was also active against six out of the nine strains with other isolates with this closest relative strain being highly active too. Among all the isolates belonging to the class actinobacteria, isolates belonging to genera *Kocuria* and *Kytococcus* produced extracts with the highest antimicrobial activity. Isolates with their closest relative strain, similarity and activity spectrum are given in Tables 1 and 2.

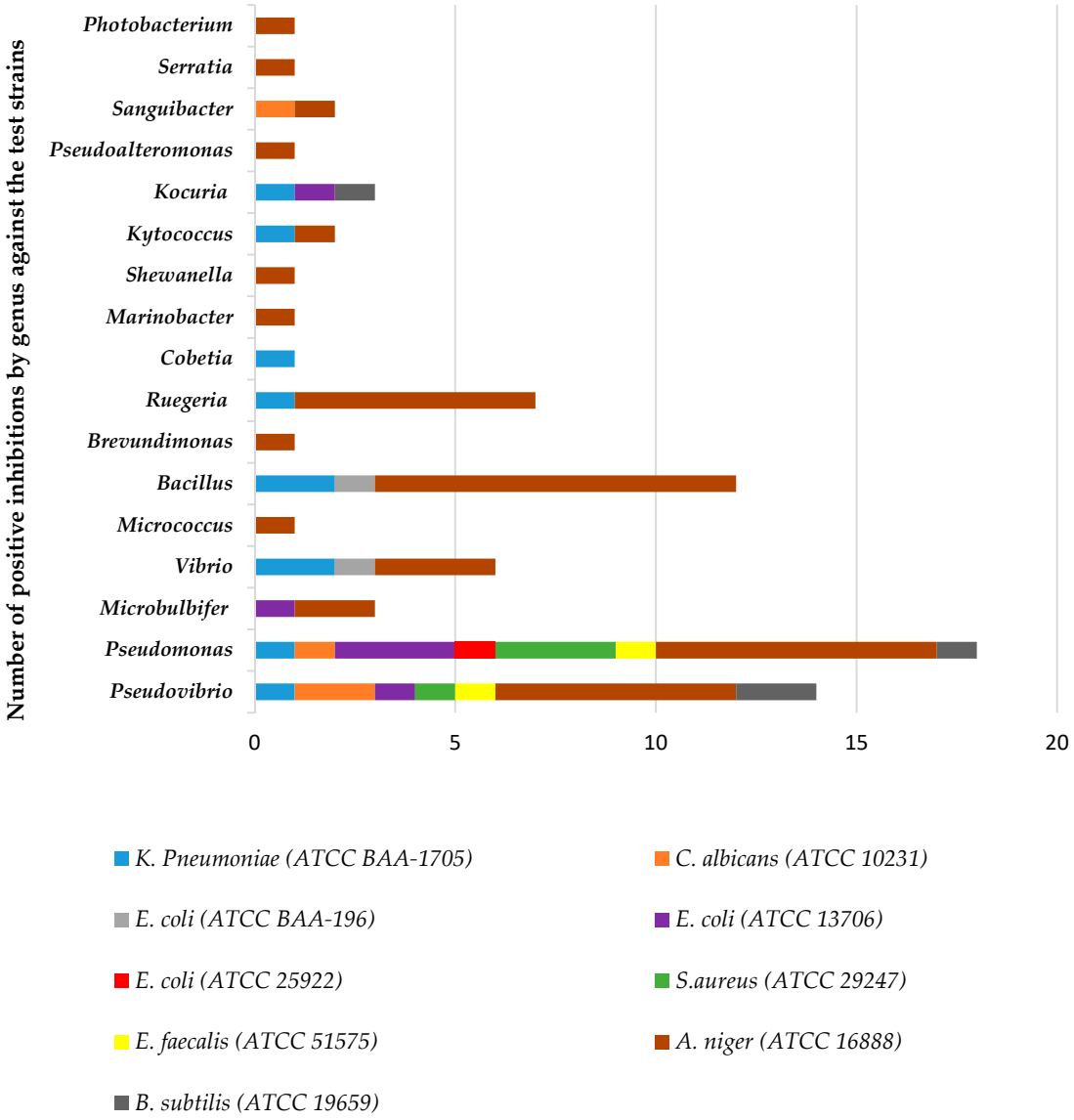


Figure 6. Number of antimicrobial hits from sponge associated-genera obtained against pathogenic test organisms used in the antimicrobial assays.

3.3. Amplification of PKS-I and NRPS Genes

Detection of PKS-I and NRPS genes in all isolates was indicated by the presence of the corresponding fragment size range: 700 bp for PKS-I and 750–1000 bp for NRPS in non-actinobacteria and 1250–1400 bp for PKS-I and 700 bp for NRPS in actinobacteria. Of all isolates (n = 123) analyzed, 47% had at least one of the gene types. In total, 22% of isolates from *R. odorabile* possessed one gene type, PKS-I. 51% of isolates from *C. flabellata* possessed at least one of the gene types with PKS-I dominating and 5% of these *C. flabellata* isolates possessed both PKS-I and NRPS genes. The presence of these genes as well as the activity spectrum and closest relative strain for all isolates is given in Tables 1 and 2.

Table 1. Presence of potential PKS-I and NRPS genes, closest relative strain and similarity in isolates from *R. odorabile* sponge samples exhibiting biological activity.

Isolate Code	Similarity %	Closest Relative Strain	Biological Activity Spectrum	Gene Type
50161	99.4	*Vibrio harveyi* strain NBRC 15634	N	ND
50162	99.5	*Photobacterium rosenbergii* strain CC1	*A. niger* (ATCC 16888)	ND
50163	99.6	*Stenotrophomonas maltophilia* strain ATCC 19861	N	ND
50164	99.7	*Vibrio campbellii* strain ATCC 25920	*K. pneumoniae* (ATCC BAA-1705)	ND
50165	99.4	*Vibrio harveyi* strain NBRC 15634	*K. pneumoniae* (ATCC BAA-1705)	ND
50166	99.0	*Pseudovibrio denitrificans* strain NBRC 100825	*K. pneumoniae* (ATCC BAA-1705)	PKS-I
50895	99.3	*Microbulbifer variabilis* strain Ni-2088	*A. niger* (ATCC 16888)	ND
50897	99.6	*Microbulbifer variabilis* strain Ni-2088	N	ND
50898	99.1	*Microbulbifer variabilis* strain Ni-2088	N	ND
50899	99.0	*Micrococcus aloeverae* strain AE-6	*A. niger* (ATCC 16888)	ND
51193	99.0	*Microbulbifer agarilyticus* strain JAMB A3	*E. coli* (ATCC 13706)	PKS-I
53189	99.4	*Pseudovibrio* sp. FO-BEG1 strain FO-BEG1	*K. pneumoniae* (ATCC BAA-1705) and *S. aureus* (ATCC 29247)	PKS-I
53190	99.8	*Pseudovibrio* sp. FO-BEG1 strain FO-BEG1	*K. pneumoniae* (ATCC BAA-1705), *E. coli* (ATCC 13706) and *S. aureus* (ATCC 29247)	PKS-I
53223	99.6	*Vibrio owensii* strain DY05	*E. coli* (ATCC BAA-196)	ND
53224	99.9	*Bacillus safensis* strain NBRC 100820	*E. coli* (ATCC BAA-196)	ND
53225	99.0	*Bacillus safensis* strain NBRC 100820	N	ND
53226	99.0	*Bacillus safensis* strain NBRC 100820	*A. niger* (ATCC 16888)	ND
53249	99.8	*Brevundimonas diminuta* strain NBRC 12697	*A. niger* (ATCC 16888)	ND

* N—no activity; ND—not detected; PKS-I—polyketide synthase type I.

Table 2. Presence of potential PKS-I and NRPS genes, closest relative strain and similarity in isolates from *C. flabellata* sponge samples exhibiting biological activity.

Isolate Code	Similarity %	Closest Relative Strain	Biological Activity Spectrum	Gene Type
53136	99.7	*Pseudomonas aeruginosa* PAO1 strain PAO1	*E. coli* (ATCC 13706), *S. aureus* (ATCC 29247), *E. coli* (ATCC 25922)	ND
53137	92.0	*Sphingopyxis alaskensis* strain RB2256	N	ND
53178	99.0	*Ruegeria arenilitoris* strain G-M8	N	ND
53179	98.9	*Ruegeria arenilitoris* strain G-M8	*A. niger* (ATCC 16888)	PKS-I
53650	99.0	*Micrococcus yunnanensis* strain YIM 65004	N	ND
53651	99.0	*Micrococcus aloeverae* strain AE-6	N	ND
53654	99.7	*Pseudomonas aeruginosa* PAO1 strain PAO1	*E. coli* (ATCC 13706), E. faecalis (ATCC 51575), *S. aureus* (ATCC 29247), *B. subtilis* (ATCC 19659), *C. albicans* (ATCC 10231)	PKS-I
53656	99.0	*Micrococcus aloeverae* strain AE-6	N	ND
53657	99.6	*Pseudomonas azotoformans* strain NBRC 12693	*E. coli* (ATCC 13706), *S. aureus* (ATCC 29247), *A. niger* (ATCC 16888)	NRPS
58101	98.3	*Ruegeria arenilitoris* strain G-M8	*K. pneumoniae* (ATCC BAA-1705)	PKS-I and NRPS
58102	99.0	*Ruegeria arenilitoris* strain G-M8	*K. pneumoniae* (ATCC BAA-1705)	NRPS
58103	99.3	*Ruegeria arenilitoris* strain G-M8	N	ND
58104	99.9	*Pseudomonas azotoformans* strain NBRC 12693	N	ND
58105	99.2	*Ruegeria arenilitoris* strain G-M8	N	ND
58107	99.8	*Pseudomonas azotoformans* strain NBRC 12693	N	ND
58111	99.9	*Pseudomonas azotoformans* strain NBRC 12693	*K. pneumoniae* (ATCC BAA-1705)	ND
58115	100	*Cobetia amphilecti* strain 46-2	*K. pneumoniae* (ATCC BAA-1705)	PKS-I and NRPS
58116	99.9	*Pseudomonas azotoformans* strain NBRC 12693	*K. pneumoniae* (ATCC BAA-1705), *A. niger* (ATCC 16888)	PKS-I
58264	100	*Pseudovibrio* sp. FO-BEG1 strain FO-BEG1	*K. pneumoniae* (ATCC BAA-1705), *C. albicans* (ATCC 10231), *A. niger* ATCC 16888)	NRPS
58265	99.1	*Pseudomonas azotoformans* strain NBRC 12693	*K. pneumoniae* (ATCC BAA-1705)	ND
58266	99.6	*Pseudomonas azotoformans* strain NBRC 12693	*K. pneumoniae* (ATCC BAA-1705)	ND
58271	99.5	*Pseudovibrio* sp. FO-BEG1 strain FO-BEG1	*A. niger* (ATCC 16888)	PKS-I
58273	99.0	*Pseudovibrio denitrificans* strain NBRC 100825	*K. pneumoniae* (ATCC BAA-1705)	ND
58274	100	*Pseudovibrio* sp. FO-BEG1 strain FO-BEG1	N	ND
58275	99.0	*Bacillus aquimaris* strain TF-12	*A. niger* (ATCC 16888)	ND
58276	99.4	*Ruegeria arenilitoris* strain G-M8	N	ND
58277	99.1	*Ruegeria arenilitoris* strain G-M8	*A. niger* (ATCC 16888)	PKS-I
58278	99.0	*Ruegeria arenilitoris* strain G-M8	*A. niger* (ATCC 16888)	PKS-I
58279	99.9	*Pseudomonas azotoformans* strain NBRC 12693	N	ND
58280	99.1	*Ruegeria arenilitoris* strain G-M8	N	ND
58281	99.6	*Pseudomonas azotoformans* strain NBRC 12693	*A. niger* (ATCC 16888)	PKS-I and NRPS
58282	99.8	*Pseudomonas azotoformans* strain NBRC 12693	*A. niger* (ATCC 16888)	ND
58283	99.0	*Bacillus cereus* ATCC 14579	*K. pneumoniae* (ATCC BAA-1705), *A. niger* (ATCC 16888)	ND
58285	99.6	*Pseudomonas azotoformans* strain NBRC 12693	N	ND
58286	99.3	*Pseudomonas azotoformans* strain NBRC 12693	*A. niger* (ATCC 16888)	NRPS
58287	99.0	*Bacillus aquimaris* strain TF-12	*K. pneumoniae* (ATCC BAA-1705), *A. niger* (ATCC 16888)	PKS-I
58289	99.1	*Ruegeria arenilitoris* strain G-M8	N	ND

Table 2. *Cont.*

Isolate Code	Similarity %	Closest Relative Strain	Biological Activity Spectrum	Gene Type
58290	99.2	*Ruegeria arenilitoris* strain G-M8	*A. niger* (ATCC 16888)	PKS-I
58291	96.0	*Ruegeria arenilitoris* strain G-M8	N	ND
58292	98.9	*Ruegeria arenilitoris* strain G-M8	N	ND
58293	99.0	*Vibrio owensii* strain DY05	*K. pneumoniae* (ATCC BAA-1705)	ND
58294	99.6	*Marinobacter vinifirmus* strain FB1	N	ND
58295	99.0	*Vibrio chagasii* strain LMG 21353	*A. niger* (ATCC 16888)	ND
58296	99.5	*Marinobacter vinifirmus* strain FB1	*A. niger* (ATCC 16888)	PKS-I
58297	98.0	*Shewanella corallii* strain fav-2-10-05	*A. niger* (ATCC 16888)	ND
58298	99.0	*Ruegeria arenilitoris* strain G-M8	N	ND
58299	98.9	*Ruegeria arenilitoris* strain G-M8	N	ND
58300	99.5	*Pseudovibrio* sp. FO-BEG1 strain FO-BEG1	*A. niger* (ATCC 16888)	ND
53193	99.0	*Bacillus horikoshii* strain DSM 8719	*A. niger* (ATCC 16888)	ND
53208	99.6	*Kytococcus sedentarius* strain DSM 20547	*K. pneumoniae* (ATCC BAA-1705), *A. niger* (ATCC 16888)	ND
53209	99.0	*Kocuria rhizophilia* strain TA68	*K. pneumoniae* (ATCC BAA-1705), *E. coli* (ATCC 13706), *C. albicans* (ATCC 10231)	NRPS
53211	98.1	*Pseudoalteromonas piscicida* strain NBRC 103038	N	ND
58301	99.7	*Pseudovibrio* sp. FO-BEG1 strain FO-BEG1	*K. pneumoniae* (ATCC BAA-1705)	ND
58302	99.9	*Pseudovibrio* sp. FO-BEG1 strain FO-BEG1	N	ND
58303	99.2	*Ruegeria atlantica* strain NBRC 15792	N	ND
58304	99.0	*Bacillus kochii* strain WCC 4582	*S. aureus* (ATCC 29247)	ND
58305	99.3	*Ruegeria arenilitoris* strain G-M8	*A. niger* (ATCC 16888)	PKS-I
58306	99.8	*Pseudovibrio* sp. FO-BEG1 strain FO-BEG1	*A. niger* (ATCC 16888)	ND
58307	98.5	*Ruegeria arenilitoris* strain G-M8	N	ND
58308	99.1	*Ruegeria arenilitoris* strain G-M8	N	ND
58310	99.0	*Vibrio chagasii* strain LMG 21353	N	ND
58312	99.0	*Bacillus kochii* strain WCC 4582	*A. niger* (ATCC 16888)	ND
58313	99.8	*Pseudovibrio* sp. FO-BEG1 strain FO-BEG1	N	ND
58315	99.8	*Pseudovibrio* sp. FO-BEG1 strain FO-BEG1	N	ND
58316	98.8	*Ruegeria arenilitoris* strain G-M8	*A. niger* (ATCC 16888)	PKS-I
58317	99.0	*Pseudovibrio denitrificans* strain NBRC 100825	N	ND
58318	98.4	*Ruegeria arenilitoris* strain G-M8	N	ND
58319	99.8	*Pseudovibrio* sp. FO-BEG1 strain FO-BEG1	*A. niger* (ATCC 16888)	ND
58320	99.0	*Microbulbifer variabilis* strain Ni-2088	*A. niger* (ATCC 16888)	ND
58321	100	*Staphylococcus warneri* SG1 strain SG1	N	ND
58322	99.0	*Thalassospira permensis* strain SMB34	N	ND
58323	99.0	*Ruegeria arenilitoris* strain G-M8	N	ND

Table 2. *Cont.*

Isolate Code	Similarity %	Closest Relative Strain	Biological Activity Spectrum	Gene Type
58324	99.0	*Pseudomonas pachastrellae* strain KMM 330	N	ND
58325	99.0	*Pseudoalteromonas shioyasakiensis* strain SE3	*A. niger* (ATCC 16888)	PKS-I
58326	99.4	*Bacillus nealsonii* strain DSM 15077	N	ND
58327	99.1	*Ruegeria arenilitoris* strain G-M8	N	ND
58328	98.8	*Ruegeria arenilitoris* strain G-M8	N	ND
58330	99.0	*Aquimarina spongiae* strain A6	N	ND
58332	99.7	*Pseudovibrio* sp. FO-BEG1 strain FO-BEG1	N	ND
58333	98.1	*Ruegeria atlantica* strain NBRC 15792	N	ND
58334	99.9	*Pseudovibrio* sp. FO-BEG1 strain FO-BEG1	*A. niger* (ATCC 16888)	PKS-I
58335	98.7	*Ruegeria arenilitoris* strain G-M8	N	ND
53244	99.7	*Vibrio campbellii* strain ATCC 25920	N	ND
53611	99.7	*Pseudovibrio* sp. FO-BEG1 strain FO-BEG1	*A. niger* (ATCC 16888)	ND
53613	99.7	*Pseudovibrio* sp. FO-BEG1 strain FO-BEG1	N	ND
53614	96.0	*Bacillus oceanisediminis* strain H2	N	ND
53616	99.6	*Pseudovibrio* sp. FO-BEG1 strain FO-BEG1	N	ND
53620	99.0	*Ruegeria arenilitoris* strain G-M8	N	ND
53622	99.7	*Pseudovibrio* sp. FO-BEG1 strain FO-BEG1	N	ND
53623	98.0	*Vibrio fortis* strain CAIM 629	*A. niger* (ATCC 16888)	PKS-I
53624	98.8	*Stenotrophomonas maltophilia* R551-3 strain R551-3	*A. niger* (ATCC 16888)	PKS-I
53625	99.0	*Ruegeria arenilitoris* strain G-M8	N	ND
53634	98.0	*Sanguibacter inulinus* strain ST50	*A. niger* (ATCC 16888), *C. albicans* (ATCC 10231)	NRPS
53635	98.0	*Pseudomonas geniculate* strain ATCC 19374	N	ND
53636	100	*Pseudoalteromonas piscicida* strain NBRC 103038	*A. niger* (ATCC 16888)	ND
53640	99.9	*Pseudoalteromonas piscicida* strain NBRC 103038	N	ND
53641	98.9	*Ruegeria arenilitoris* strain G-M8	N	ND
53240	99.0	*Bacillus safensis* strain NBRC 100820	N	ND
53237	99.3	*Psychrobacter celer* strain SW-238	N	ND
53239	98.9	*Psychrobacter celer* strain SW-238	N	ND
59159	99.0	*Bacillus kochii* strain WCC 4582	*A. niger* (ATCC 16888)	ND
59161	99.7	*Vibrio alginolyticus* strain ATCC 17749	N	ND
58824	99.6	*Photobacterium rosenbergii* strain CC1	N	ND
58284	99.2	*Ruegeria arenilitoris* strain G-M8	*A. niger* (ATCC 16888)	ND
53260	99.9	*Brevundimonas diminuta* strain NBRC 12697	N	ND

* N—no activity; ND—not detected; PKS-I—polyketide synthase type I; NRPS—nonribosomal peptide synthetase.

4. Discussion

Due to the emergence and re-emergence of multi-drug resistant microorganisms, it is vital that new antimicrobial compounds are discovered that counteract the resistant mechanisms exhibited by these microorganisms. These include methicillin-resistance exhibited by *S. aureus* and multi-drug resistance exhibited by *E. coli*, *K. pneumoniae*, *E. faecalis and C. albicans* [24,55,56]. Currently, one of the most effective ways of discovering novel therapeutic agents is the cultivation and fermentation of novel or under-studied microorganisms isolated from diverse environments such as the marine environment [1]. In the presented study, antimicrobial activity was detected when sponge-associated isolates were fermented in marine broth, indicating their potential to produce bioactive compounds under marine sponge conditions. Due to the marine broth having a high salt content and other nutrients that help simulate the sea water environment, it is a highly suitable medium for marine bacterial growth. Marine broth may possess greater levels of nutrients compared to the surrounding sea water; however, due to constant seawater filtration by the sponge, these isolates may come into contact with more nutrients within the host environment as well as having access to other nutrients that may be provided by the sponge host [57]. Under diverse fermentation conditions with different nutrients and parameters, these bacteria may have the ability to produce more than one type of active metabolite. Here, the simple and previously successful one-strain-many-active-compounds (OSMAC) approach may be used which aims to activate metabolic pathways to produce different types of metabolites in order to identify the range of compounds that may be produced by a specific bacterial isolate [58]. The use of different fermentation nutrients and parameters as well as co-culturing of competing or antagonistic microorganisms may aid in the search for important active metabolites that may be used as antimicrobial or anticancer agents [34,59]. A study carried out by Graça et al. (2015) reported that the use of liquid fermentation, the same technique used in this study, over solid media fermentation to obtain antimicrobial extracts is advantageous in its ability to be quantitative, simple, quick to carry out, reproducible, less expensive than other methods used and able to be conducted in a high throughput way [8]. For this study, liquid fermentation was chosen as the main medium type as this was less time consuming for extraction, quick and simple and less expensive than when solid-state fermentation was trialed.

Detection of bioactive actinobacterial isolates from the genera *Kocuria* and *Kytococcus* once again confirmed that actinobacteria are prolific producers of compounds with antibiotic properties [44]. Previous examples include the production of Kocurin by sponge-derived *Kocuria* and *Micrococcus* sp. active against methicillin-resistant *S. aureus* [44]. Other well known-bioactive compound producers from different genera include *Pseudoalteromonas* [8,21,60], *Bacillus* [61], *Pseudovibrio* [62] and *Pseudomonas* [8,63] which is consistent with the findings of the presented study that isolates belonging to these genera were also found to produce antibacterial activity against the pathogenic reference strains.

Previously, a strain from the genus *Pseudoalteromonas*, *Pseudoalteromonas piscida*, isolated from a sponge species from the China Sea, *Hymeniacidon perlevis*, has been identified as a producer of the wide spectrum antimicrobial metabolite, norharman, a β-carboline alkaloid [21,64]. This genus has been well studied and out of the 41 species hosted by *Pseudoalteromonas* 16 of them were illustrated to produce antimicrobial metabolites [65]. *Bacillus* species from a range of marine macroorganisms have also been found to produce metabolites with antimicrobial and antifouling properties [66]. One *Bacillus* sp. in particular isolated from the sponge *Halichondria* sp. was found to have potent activity against a range of clinically pathogenic microorganisms. This species was found to be closely related to *Bacillus licheniformis* HNL09 and produce compounds such as indole, 3-phenylpropionic acid and dimer 4,4′-oxybis [3-phenylpropionic acid] [38]. The genus *Pseudovibrio* is known as the second most prolific bacterial genus that has been isolated from sponges and produces antimicrobial activities. This genus produces the antibacterial compound tropodithietic acid [29]. *Pseudomonas* sp. from sponges have also been found to produce potent antimicrobial activity against a range of bacteria, including resistant ones. *Pseudomonas* sp. from the sponge *Callyspongia* sp. was found to produce a chomophore substance against methicillin resistant *S. aureus* [67].

The antimicrobial assays in this study allowed for the observation of a high number of bacterial extracts active against three main reference strains: the fungal strain *A. niger*, the bacterial strain *K. pneumoniae* and the yeast *C. albicans*. Furthermore, the greatest level of bioactivity detected in the presented study against *K. pneumoniae*, *S. aureus* and *A. niger* originated from the genera *Pseudomonas*, *Pseudovibrio* and *Bacillus*. These genera have also been identified as important producers of biologically active compounds from many other studies [17,34,68].

Molecular identification of the sponge-associated bacteria involved in this study indicated that isolates belonged to five different bacterial classes. The main two groups of bacteria identified were alpha- and gammaproteobacteria and this finding was consistent with a previous study carried out by Thomas et al. [12] who investigated the sponge microbiome across 81 different sponge species from 20 different countries. They also reported that the main phylum identified was Proteobacteria across all the samples studies, with the divisions' alpha- and gammaproteobacteria of this phylum being the most dominant [12]. These two bacterial classes were consistent across both sponge species (*C. flabellata* and *R. odorabile*) as being the most abundant with gammaproteobacteria dominating in *R. odoabile*. The dominant class from *C. flabellata* sponge samples was alphaproteobacteria which is comparable to the high number of isolates identified with this class from a previous study carried out on *C. flabellata* [7]. A previous study investigating the bacterial diversity on another Great Barrier Reef sponge, *Cinachyrella* sp., also found that these two classes were the most abundant [69]. Bacterial isolates identified to their closest relative that were common to both sponge species include *Pseudovibrio* sp., a *Micrococcus* sp., a *Brevundimonas* sp., a *Vibrio* sp., a *Microbulbifer* and a *Photobacterium* sp. The genera that prevailed among the sponge samples from *C. flabellata* were *Pseudovibrio*, *Ruegeria* and *Bacilli* and from *R. odorabile*, *Pseudovibrio*, *Vibrio* and *Bacilli* suggesting a similar diversity between the two Great Barrier Reef sponge species even though the cultivable community isolate numbers from *R. odorabile* was considerably lower than that of *C. flabellata*. The fact that these bacterial species have also been found to be associated with other sponge species from different reef locations around the world [8,18,70–73] suggests that these symbiotic bacterial classes may be common associates of marine sponges, even in the distantly related ones.

Amplification of specific genes, such as PKS-I and NRPS genes, within bacterial biosynthetic pathways is a valued method in the search for new bioactive metabolites as the presence of these genes might indicate the ability of bacterial isolates to produce metabolites with bioactivity of medical importance such as the antibacterial or anticancer activities if they are switched on using the right fermentation conditions [8,22,74]. PKS-I and NRPS genes were amplified in both bioactive isolates as well as isolates that did not produce antimicrobial. Of all isolates analyzed from both sponge species, 47% potentially had at least one of the gene types. The dominant bacterial genera, *Pseudovibrio*, *Bacillus* and *Ruegeria*, across all of the sponge samples tested in this study were found to carry these genes. This finding was in line with the previous studies that reported the existence of such genes in these genera, however, to the best of our knowledge, this is the first time the PKS-I gene has been potentially located in a member of the genera *Microbulbifer* isolated from *R. odorabile* but not from *C. flabellata*. Moreover, the isolates that had not displayed any activity but possessed one or both gene types may be due to inadequate activation of the gene biosynthetic pathway or the fact that not all PKS-I and NRPS genes might result in antimicrobial activity but may produce other activity such as anticancer activity [48]. Isolates may possess these biosynthetic genes, however, the required parameters and nutrients to induce activation [75,76] may have not met with the marine broth fermentation alone. Therefore, further fermentation trialing a range of parameters and nutrients is required. One way to trigger these silent biosynthetic gene pathways may be the use of rare earth elements (REEs) in fermentation media. These REEs consist of 17 elements which include scandium, yttrium and the lanthanides (15 elements from lanthanum to lutetium) and their use in fermentation media has been shown to cause the overproduction of antibiotics and the activation of silent genes within bacteria. An example includes the addition of scandium added to different Streptomyces (*S. coelicolor*, *S. griseus*, *S. antibioticus* and *S. lividans*) cultures that enhanced the antibiotic production by 2–25-fold [76,77].

The use of these elements also eliminates the need to use gene engineering technology or strain genomic information due to them being scattered throughout the global environment which suggests that microorganisms may have acquired the ability to respond to low levels of REEs as a way of adapting to the surrounding environment [76].

5. Conclusions

The isolation and molecular identification of these cultivatable bacteria from the two different sponge species, *C. falbaellata* and *R. odorabile*, revealed broad diversity of taxa. The results also illustrate the potential of bacteria associated with these two different sponge species to produce bioactive metabolites against pathogenic microorganisms which indicate that these bacterial strains might be of greater value if further studies are conducted on them, such as, genome mining, different fermentation conditions, silent gene activation and so on, that in turn may result in the discovery of novel metabolites of therapeutic value. Chemical dereplication of extracts will also be conducted to evaluate the number of known and novel compounds.

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Author Contributions: Candice M. Brinkmann and D. İpek Kurtböke designed and conceived the experiments. Candice M. Brinkmann performed the experiments, carried out the antimicrobial assays, conducted the molecular work and analysed the microbiological and molecular data. Philip S. Kearns and Elizabeth Evans-Illidge supplied the bacterial samples that were collected by AIMS. Candice M. Brinkmann wrote the manuscript. D. İpek Kurtböke and Philip S. Kearns supervised Candice M. Brinkmann's PhD project and provided feedback on the manuscript.

Conflicts of Interest: The authors declare no conflict of interest.

Appendix A

Table A1. Incubation conditions, Genbank accession numbers and location of *R. odorabile* collection for bacterial isolates.

Isolate Code	Closest Relative Strain	Sponge Sample Number	Purification Temperature (°C)	Days Grown	GenBank Accession Number	Latitude	Longitude	Location of Sponge Samples at the Great Barrier Reef (GBR), Queensland, Australia
50161	*Vibrio harveyi* strain NBRC 15634	16595	22	14	KX418475	−10.823	142.743	NORTHERN END OF WYBORN REEF, NORTHERN GBR
50162	*Photobacterium rosenbergii* strain CC1	16595	22	14	KX418476	−10.823	142.743	NORTHERN END OF WYBORN REEF, NORTHERN GBR
50163	*Stenotrophomonas maltophilia* strain ATCC 19861	16595	22	14	KX418463	−10.823	142.743	NORTHERN END OF WYBORN REEF, NORTHERN GBR
50164	*Vibrio campbellii* strain ATCC 25920	16595	22	14	KX418477	−10.823	142.743	NORTHERN END OF WYBORN REEF, NORTHERN GBR
50165	*Vibrio harveyi* strain NBRC 15634	16595	22	14	KX418478	−10.823	142.743	NORTHERN END OF WYBORN REEF, NORTHERN GBR
50166	*Pseudovibrio denitrificans* strain NBRC 100825	16595	22	14	KX418552	−10.823	142.743	NORTHERN END OF WYBORN REEF, NORTHERN GBR
50895	*Microbulbifer variabilis* strain Ni-2088	17643	27	14	KX418479	−18.826	147.64	DAVIES REEF
50897	*Microbulbifer variabilis* strain Ni-2088	17643	27	14	KX418480	−18.826	147.64	DAVIES REEF
50898	*Microbulbifer variabilis* strain Ni-2088	17643	27	14	KX418464	−18.826	147.64	DAVIES REEF
50899	*Micrococcus aloeverae* strain AE-6	17643	27	14	KX418589	−18.826	147.64	DAVIES REEF
51193	*Microbulbifer agarilyticus* strain JAMB A3	17766	27	14	KX418502	−21.659	150.324	DAVIES REEF
53189	*Pseudovibrio* sp. FO-BEG1 strain FO-BEG1	19500	27	3	KX418515	−18.833	147.617	NE-PERCY IS., NW CORNER
53190	*Pseudovibrio* sp. FO-BEG1 strain FO-BEG1	19500	27	3	KX418516	−18.833	147.617	NE-PERCY IS., NW CORNER
53223	*Vibrio owensii* strain DY05	19500	30	2	KX418481	−18.833	147.617	DAVIES REEF, GBR
53224	*Bacillus safensis* strain NBRC 100820	19500	30	2	KX418582	−18.833	147.617	DAVIES REEF, GBR
53225	*Bacillus safensis* strain NBRC 100820	19500	30	2	KX418572	−18.833	147.617	DAVIES REEF, GBR
53226	*Bacillus safensis* strain NBRC 100820	19500	30	2	KX418577	−18.833	147.617	DAVIES REEF, GBR
53249	*Brevundimonas diminuta* strain NBRC 12697	19500	30	6	KX418517	−18.833	147.617	DAVIES REEF, GBR

Table A2. Incubation conditions, Genbank accession numbers and location of *C. flabellata* collection for bacterial isolates.

Isolate Code	Closest Relative Strain	Sponge Sample Number	Purification Temperature (°C)	Days Grown	GenBank Accession Number	Latitude	Longitude	Location of Sponge Samples at the Great Barrier Reef (GBR), Queensland, Australia
53136	*Pseudomonas aeruginosa* PAO1 strain PAO1	19496	27	10	KX418482	−18.820	147.63	DAVIES REEF, GBR
53137	*Sphingopyxis alaskensis* strain RB2256	19496	27	6	KX418562	−18.820	147.63	DAVIES REEF, GBR
53178	*Ruegeria arenilitoris* strain G-M8	19497	27	5	KX418522	−18.846	147.63	DAVIES REEF, GBR
53179	*Ruegeria arenilitoris* strain G-M8	19497	27	5	KX418523	−18.846	147.63	DAVIES REEF, GBR
53651	*Micrococcus aloeverae* strain AE-6	21250	26	19	KX418483	−18.838	147.642	DAVIES REEF, GBR
53654	*Pseudomonas aeruginosa* PAO1 strain PAO1	21250	26	19	KX418585	−18.838	147.642	DAVIES REEF, GBR
53656	*Micrococcus aloeverae* strain AE-6	21250	26	19	KX418588	−18.838	147.642	DAVIES REEF, GBR
53657	*Pseudomonas azotoformans* strain NBRC 12693	21250	26	19	KX418490	−18.838	147.642	DAVIES REEF, GBR
58101	*Ruegeria arenilitoris* strain G-M8	21556	26	21	KX418524	−18.838	147.642	TRUNK REEF, GBR
58102	*Ruegeria arenilitoris* strain G-M8	21556	26	21	KX418555	−18.332	146.829	TRUNK REEF, GBR
58103	*Ruegeria arenilitoris* strain G-M8	21556	26	21	KX418508	−18.332	146.829	TRUNK REEF, GBR
58104	*Pseudomonas azotoformans* strain NBRC 12693	21556	26	21	KX418491	−18.332	146.829	TRUNK REEF, GBR
58105	*Ruegeria arenilitoris* strain G-M8	21556	26	21	KX418509	−18.332	146.829	TRUNK REEF, GBR
58107	*Pseudomonas azotoformans* strain NBRC 12693	21556	26	21	KX418492	−18.332	146.829	TRUNK REEF, GBR
58111	*Pseudomonas azotoformans* strain NBRC 12693	21556	26	21	KX418493	−18.332	146.829	TRUNK REEF, GBR
58115	*Cobetia amphilecti* strain 46-2	21556	26	21	KX418494	−18.332	146.829	TRUNK REEF, GBR

Table A2. *Cont.*

Isolate Code	Closest Relative Strain	Sponge Sample Number	Purification Temperature (°C)	Days Grown	GenBank Accession Number	Latitude	Longitude	Location of Sponge Samples at the Great Barrier Reef (GBR), Queensland, Australia
58116	*Pseudomonas azotoformans* strain NBRC 12693	21556	26	21	KX418495	−18.332	146.829	TRUNK REEF, GBR
58264	*Pseudovibrio* sp. FO-BEG1 strain FO-BEG1	21717	26	5	KX418510	−18.833	147.626	DAVIES REEF, GBR
58265	*Pseudomonas azotoformans* strain NBRC 12693	21717	26	5	KX418465	−18.833	147.626	DAVIES REEF, GBR
58266	*Pseudomonas azotoformans* strain NBRC 12693	21717	26	5	KX418496	−18.833	147.626	DAVIES REEF, GBR
58271	*Pseudovibrio* sp. FO-BEG1 strain FO-BEG1	21717	26	5	KX418511	−18.833	147.626	DAVIES REEF, GBR
58273	*Pseudovibrio* sp. FO-BEG1 strain FO-BEG1	21717	26	10	KX418565	−18.833	147.626	DAVIES REEF, GBR
58274	*Pseudovibrio* sp. FO-BEG1 strain FO-BEG1	21717	26	10	KX418512	−18.833	147.626	DAVIES REEF, GBR
58275	*Bacillus aquimaris* strain TF-12	21717	26	5	KX418579	−18.833	147.626	DAVIES REEF, GBR
58276	*Ruegeria arenilitoris* strain G-M8	21717	26	5	KX418513	−18.833	147.626	DAVIES REEF, GBR
58277	*Ruegeria arenilitoris* strain G-M8	21717	26	5	KX418514	−18.833	147.626	DAVIES REEF, GBR
58278	*Ruegeria arenilitoris* strain G-M8	21717	26	5	KX418557	−18.833	147.626	DAVIES REEF, GBR
58279	*Pseudomonas azotoformans* strain NBRC 12693	21717	26	5	KX418497	−18.833	147.626	DAVIES REEF, GBR
58280	*Ruegeria arenilitoris* strain G-M8	21722	26	5	KX418518	−18.833	147.626	DAVIES REEF, GBR
58281	*Pseudomonas azotoformans* strain NBRC 12693	21722	26	5	KX418466	−18.833	147.626	DAVIES REEF, GBR
58282	*Pseudomonas azotoformans* strain NBRC 12693	21722	26	5	KX418467	−18.833	147.626	DAVIES REEF, GBR
58283	*Bacillus cereus* ATCC 14579	21722	26	5	KX418567	−18.833	147.626	DAVIES REEF, GBR
58285	*Pseudomonas azotoformans* strain NBRC 12693	21722	26	5	KX418468	−18.833	147.626	DAVIES REEF, GBR
58286	*Pseudomonas azotoformans* strain NBRC 12693	21722	26	5	KX418469	−18.833	147.626	DAVIES REEF, GBR
58287	*Bacillus aquimaris* strain TF-12	21722	26	5	KX418568	−18.833	147.626	DAVIES REEF, GBR
58289	*Ruegeria arenilitoris* strain G-M8	21722	26	5	KX418525	−18.833	147.626	DAVIES REEF, GBR
58290	*Ruegeria arenilitoris* strain G-M8	21722	26	5	KX418526	−18.833	147.626	DAVIES REEF, GBR
58291	*Ruegeria arenilitoris* strain G-M8	21722	26	5	KX418566	−18.833	147.626	DAVIES REEF, GBR
58292	*Ruegeria arenilitoris* strain G-M8	21722	26	5	KX418519	−18.833	147.626	DAVIES REEF, GBR
58293	*Vibrio owensii* strain DY05	21717	26	5	KX418505	−18.833	147.626	DAVIES REEF, GBR
58294	*Marinobacter vinifirmus* strain FB1	21722	26	5	KX418470	−18.833	147.626	DAVIES REEF, GBR
58295	*Vibrio chagasii* strain LMG 21353	21722	26	5	KX418498	−18.833	147.626	DAVIES REEF, GBR
58296	*Marinobacter vinifirmus* strain FB1	21722	26	5	KX418471	−18.833	147.626	DAVIES REEF, GBR
58297	*Shewanella corallii* strain fav-2-10-05	21722	26	5	KX418503	−18.833	147.626	DAVIES REEF, GBR
58298	*Ruegeria arenilitoris* strain G-M8	21722	26	5	KX418560	−18.833	147.626	DAVIES REEF, GBR
58299	*Ruegeria arenilitoris* strain G-M8	21722	26	5	KX418527	−18.833	147.626	DAVIES REEF, GBR
58300	*Pseudovibrio* sp. FO-BEG1 strain FO-BEG1	21722	26	5	KX418528	−18.833	147.626	DAVIES REEF, GBR
53193	*Bacillus horikoshii* strain DSM 8719	19496	27	4	KX418578	−18.820	147.630	DAVIES REEF, GBR
53208	*Kytococcus sedentarius* strain DSM 20547	19496	27	4	KX418583	−18.820	147.630	DAVIES REEF, GBR
53209	*Kocuria rhizophilia* strain TA68	19496	27	4	KX418587	−18.820	147.630	DAVIES REEF, GBR
53211	*Pseudoalteromonas piscicida* strain NBRC 103038	19496	27	4	KX418484	−18.820	147.630	DAVIES REEF, GBR
58301	*Pseudovibrio* sp. FO-BEG1 strain FO-BEG1	21722	26	5	KX418529	−18.833	147.626	DAVIES REEF, GBR
58302	*Pseudovibrio* sp. FO-BEG1 strain FO-BEG1	21722	26	5	KX418530	−18.833	147.626	DAVIES REEF, GBR
58303	*Ruegeria atlantica* strain NBRC 15792	21722	26	5	KX418531	−18.833	147.626	DAVIES REEF, GBR
58304	*Bacillus kochii* strain WCC 4582	21722	26	5	KX418569	−18.833	147.626	DAVIES REEF, GBR
58305	*Ruegeria arenilitoris* strain G-M8	21722	26	5	KX418532	−18.833	147.626	DAVIES REEF, GBR
58306	*Pseudovibrio* sp. FO-BEG1 strain FO-BEG1	21722	26	5	KX418533	−18.833	147.626	DAVIES REEF, GBR
58307	*Ruegeria arenilitoris* strain G-M8	21722	26	5	KX418564	−18.833	147.626	DAVIES REEF, GBR
58308	*Ruegeria arenilitoris* strain G-M8	21722	26	5	KX418534	−18.833	147.626	DAVIES REEF, GBR
58310	*Vibrio chagasii* strain LMG 21353	21722	26	5	KX418507	−18.833	147.626	DAVIES REEF, GBR
58312	*Bacillus kochii* strain WCC 4582	21722	26	5	KX418574	−18.833	147.626	DAVIES REEF, GBR
58313	*Pseudovibrio* sp. FO-BEG1 strain FO-BEG1	21722	26	5	KX418535	−18.833	147.626	DAVIES REEF, GBR

Table A2. *Cont.*

Isolate Code	Closest Relative Strain	Sponge Sample Number	Purification Temperature (°C)	Days Grown	GenBank Accession Number	Latitude	Longitude	Location of Sponge Samples at the Great Barrier Reef (GBR), Queensland, Australia
58315	*Pseudovibrio* sp. FO-BEG1 strain FO-BEG1	21722	26	5	KX418536	−18.833	147.626	DAVIES REEF, GBR
58316	*Ruegeria arenilitoris* strain G-M8	21722	26	5	KX418537	−18.833	147.626	DAVIES REEF, GBR
58317	*Pseudovibrio* sp. FO-BEG1 strain FO-BEG1	21727	26	5	KX418558	−18.833	147.626	DAVIES REEF, GBR
58318	*Ruegeria arenilitoris* strain G-M8	21727	26	5	KX418538	−18.833	147.626	DAVIES REEF, GBR
58319	*Pseudovibrio* sp. FO-BEG1 strain FO-BEG1	21727	26	5	KX418539	−18.833	147.626	DAVIES REEF, GBR
58320	*Microbulbifer variabilis* strain Ni-2088	21727	26	5	KX418501	−18.833	147.626	DAVIES REEF, GBR
58321	*Staphylococcus warneri* SG1 strain SG1	21727	26	5	KX418580	−18.833	147.626	DAVIES REEF, GBR
58322	*Thalassospira permensis* strain SMB34	21727	26	5	KX418554	−18.833	147.626	DAVIES REEF, GBR
58323	*Ruegeria arenilitoris* strain G-M8	21727	26	5	KX418556	−18.833	147.626	DAVIES REEF, GBR
58324	*Pseudomonas pachastrellae* strain KMM 330	21727	26	5	KX418499	−18.833	147.626	DAVIES REEF, GBR
58325	*Pseudoalteromonas shioyasakiensis* strain SE3	21727	26	5	KX418500	−18.833	147.626	DAVIES REEF, GBR
58326	*Bacillus nealsonii* strain DSM 15077	21727	26	5	KX418581	−18.833	147.626	DAVIES REEF, GBR
58327	*Ruegeria arenilitoris* strain G-M8	21727	26	5	KX418540	−18.833	147.626	DAVIES REEF, GBR
58328	*Ruegeria arenilitoris* strain G-M8	21727	26	5	KX418541	−18.833	147.626	DAVIES REEF, GBR
58330	*Aquimarina spongiae* strain A6	21727	26	5	KX418462	−18.833	147.626	DAVIES REEF, GBR
58332	*Pseudovibrio* sp. FO-BEG1 strain FO-BEG1	21727	26	5	KX418520	−18.833	147.626	DAVIES REEF, GBR
58333	*Ruegeria atlantica* strain NBRC 15792	21727	26	5	KX418542	−18.833	147.626	DAVIES REEF, GBR
58334	*Pseudovibrio* sp. FO-BEG1 strain FO-BEG1	21727	26	5	KX418543	−18.833	147.626	DAVIES REEF, GBR
58335	*Ruegeria arenilitoris* strain G-M8	21727	26	5	KX418544	−18.833	147.626	DAVIES REEF, GBR
53244	*Vibrio campbellii* strain ATCC 25920	19496	30	2	KX418485	−18.82	147.63	DAVIES REEF, GBR
53611	*Pseudovibrio* sp. FO-BEG1 strain FO-BEG1	21241	26	18	KX418545	−18.838	147.642	DAVIES REEF, GBR
53613	*Pseudovibrio* sp. FO-BEG1 strain FO-BEG1	21241	26	18	KX418546	−18.838	147.642	DAVIES REEF, GBR
53614	*Bacillus oceanisediminis* strain H2	21241	26	18	KX418576	−18.838	147.642	DAVIES REEF, GBR
53616	*Pseudovibrio* sp. FO-BEG1 strain FO-BEG1	21241	26	18	KX418547	−18.838	147.642	DAVIES REEF, GBR
53620	*Ruegeria arenilitoris* strain G-M8	21241	26	18	KX418548	−18.838	147.642	DAVIES REEF, GBR
53622	*Pseudovibrio* sp. FO-BEG1 strain FO-BEG1	21241	26	18	KX418549	−18.838	147.642	DAVIES REEF, GBR
53623	*Vibrio fortis* strain CAIM 629	21241	26	18	KX418504	−18.838	147.642	DAVIES REEF, GBR
53624	*Stenotrophomonas maltophilia* R551-3 strain R551-3	21241	26	18	KX418472	−18.838	147.642	DAVIES REEF, GBR
53625	*Ruegeria arenilitoris* strain G-M8	21241	26	18	KX418561	−18.838	147.642	DAVIES REEF, GBR
53634	*Sanguibacter inulinus* strain ST50	21241	26	19	KX418584	−18.838	147.642	DAVIES REEF, GBR
53635	*Pseudomonas geniculate* strain ATCC 19374	21241	26	19	KX418506	−18.838	147.642	DAVIES REEF, GBR
53636	*Pseudoalteromonas piscicida* strain NBRC 103038	21250	26	19	KX418486	−18.838	147.642	DAVIES REEF, GBR
53640	*Pseudoalteromonas piscicida* strain NBRC 103038	21250	26	19	KX418487	−18.838	147.642	DAVIES REEF, GBR
53641	*Ruegeria arenilitoris* strain G-M8	21250	26	19	KX418550	−18.838	147.642	DAVIES REEF, GBR
53650	*Micrococcus yunnanensis* strain YIM 65004	21250	26	19	KX418586	−18.838	147.642	DAVIES REEF, GBR
53240	*Bacillus safensis* strain NBRC 100820	19496	30	2	KX418571	−18.820	147.630	DAVIES REEF, GBR
53237	*Psychrobacter celer* strain SW-238	19496	30	2	KX418473	−18.820	147.630	DAVIES REEF, GBR
53239	*Psychrobacter celer* strain SW-238	19496	30	2	KX418474	−18.820	147.630	DAVIES REEF, GBR
59159	*Bacillus kochii* strain WCC 4582	22821	26	14	KX418575	−18.732	147.519	CENTIPEDE REEF : BACK BOMMIE, GBR
59161	*Vibrio alginolyticus* strain ATCC 17749	22821	26	14	KX418488	−18.732	147.519	CENTIPEDE REEF : BACK BOMMIE, GBR
58824	*Photobacterium rosenbergii* strain CC1	22821	28	15	KX418489	−18.732	147.519	CENTIPEDE REEF : BACK BOMMIE, GBR
58284	*Ruegeria arenilitoris* strain G-M8	21722	26	5	KX418551	−18.833	147.626	DAVIES REEF, GBR
53260	*Brevundimonas diminuta* strain NBRC 12697	19496	30	5	KX418521	−18.820	147.630	DAVIES REEF, GBR

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