Investigation of the Microbial Communities Associated with the Octocorals *Erythropodium caribaeorum* and *Antillogorgia elisabethae,* and Identification of Secondary Metabolites Produced by Octocoral Associated Cultivated Bacteria.

By

Erin Patricia Barbara McCauley

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Russell Kerr (Supervisor)

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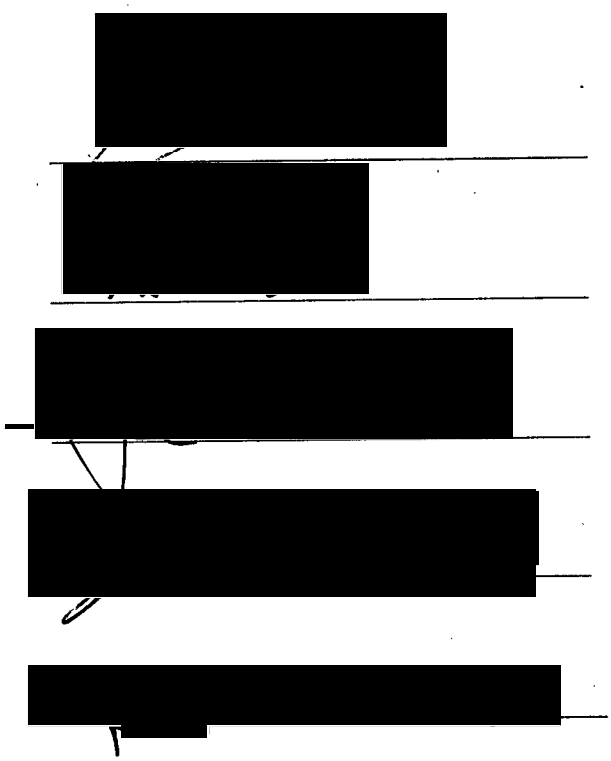

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ABSTRACTS

Octocorals are important members of coral reef ecosystems. They are also a prolific source of bioactive secondary metabolites. Over the years there has been a growing body of evidence that suggests that secondary metabolites detected in marine macroorganisms, such as octocorals, are in fact biosynthesised by associated microorganisms. Therefore this research characterized the microbiomes associated with the octocorals *Erythropodium caribaeorum* and *Antillogorgia elisabethae.* In addition to the secondary metabolites produced by the cultivatable bacteria associated with these octocorals, as these octocorals are associated with the bioactive secondary metabolites desmethyleleutherobin and pseudopterosins, respectively.

Culture–independent studies utilizing 16S small subunit rRNA gene amplicon pyrosequencing were used to characterize the microbiome of *E. caribaeorum* collected from Florida, USA and San Salvador, The Bahamas at multiple time points. As well as the microbiome of *A. elisabethae* collected from San Salvador, The Bahamas, and the microbial communities associated with the dinoflagellates and larvae of this octocoral.

E. caribaeorum was found to have a very high microbial richness with an average Chao1 estimated richness of 1464 ± 707 operational taxonomic units (OTUs) and average Shannon diversity index of 4.26 ± 1.65. The taxonomic class *Gammaproteobacteria* was a dominant member in all samples and the genus *Endozoicomonas* accounted for an average of 37.7% ± 30.0% of the total sequence reads. One *Endozoicomonas* sp. was found to be a stable member of all *E. caribaeorum* sequence libraries regardless of location or time of collection and accounted for 30.1% of all sequence reads.

The microbiome of *A. elisabethae* from San Salvador was found to have low microbial richness with an average Chao1 estimated richness of 245 ± 81 and Shannon diversity index of 2.63 ± 0.43. A stable association with the genera *Endozoicomonas*, *Vibrio*, and *Pseudoalteromonas* was observed for the samples used in this research. However when compared to previously reported *A. elisabethae* microbiomes no stable taxonomic genera were detected. The dinoflagellates and larvae had a different microbial community structure than the overall microbiome, and no stable OTUs were detected across all *A. elisabethae* sample types.

A total of 143 different species of bacteria that spanned across six different taxonomic classes were cultivated from *A. elisabethae* samples. Three were putatively novel species, two putatively novel genera, and one (RKEM 611) was determined to be a member of a novel family within the order *Bdellovibrionales*. The names *Pseudobacteriovoracaceae* for the family and *Pseudobacteriovorax antillogorgiicola* for the genus and species were formally assigned for this isolate. These cultivated bacteria produced a wide range of previously reported secondary metabolites. In addition to one new metabolite, a long-chain *N*-acyl L-leucine, that had mild antibiotic activity against MRSA, VRE, and *Staphylococcus warneri.*

This research provided valuable information about the microbial communities associated with these Caribbean octocorals. Furthermore it revealed that these octocorals are an excellent source of unique cultivatable bacteria, as well as known and new secondary metabolites.

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DEDICATION

To my Father, Mother,
and
Wendy

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LIST OF ABBREVIATIONS

^{13}C = Carbon NMR
^{1}H = Proton NMR
ACN = Acetonitrile
AMOVA = Analysis of molecular variance
ANOVA = Analysis of variance
ATCC = American type culture collection
BALO = Bdellovibrio-and-like organisms
BFM = Bacterial fermentation media
Bim = Bimini
BLAST = Basic local alignment search tool
bp = Base pair
Col = Colombia
COSY = Correlation spectroscopy
D = Dinoflagellates
d = Doublet
dd = Doublet of doublets
ddd = Doublet of doublet of doublets
DMSO = Dimethyl sulfoxide
DMSP = Dimethylsulfoniopropionate
DNA = Deoxyribonucleic acid
dR2A = dilute Reasoner's 2A
DSMZ (or DSM) = Deutsche Sammlung von Mikroorganismen und Zellkulturen GmbH
dt = Doublet of triplets
E = Shannon equitability index
EC = Erythropodium caribaeorum
EDTA = Ethylenediaminetetraacetic acid
Ele = Eleuthera
ELSD = Evaporative-light scattering detector
EPS = Exopolyssacharides
ESI = Electrospray ionization
EtOAc = Ethyl acetate
EtOH = Ethanol
FAME = Fatty acid methyl ester
FDA = Food and drug administration
FL = Florida
G+C mol% = Guanine and cytosine molecular percentage
GB = Grand Bahama
GBR = Great Barrier Reef

GGPP = Geranylgeranyl pyrophosphate
H = Holobiont
H' = Shannon diversity index
HCl = Hydrochloric acid
HMBC = Heteronuclear multiple bond correlation
HPLC = High performance liquid chromatography
HRMS = High resolution mass spectrometry
HSQC = Heteronuclear single quantum correlation
Hz = Hertz
IC_{50} = Half maximal inhibitory concentration
ID = Identity
L = Larvae
m = multiplet
m/z = Mass-to-charge ratio
MA = Marine agar
MALDI-TOF MS = Matrix-assisted laser desorption/ionization-time of flight mass spectrometry
MB = Marine broth
ME = Minimum evolution
MEGA = Molecular evolutionary genetics analysis
MeOH = Methanol
MIS = Microbial Identification System
mM = Millimolar
MNP = Marine natural products
MP = Maximum-parsimony
MRSA = Methicilin-resistant *Staphylococcus aureus*
MS-MS = Tandem mass spectrometry
Na_2HPO_4 = Disodium phosphate
NaCl = Sodium chloride
NaOH = Sodium hydroxide
NCBI = National center for biotechnology information
NCCB = Netheralands culture collection of bacteria
NGS = Next generation sequencing
NJ = Neighbor-joining methods
NMR = Nuclear magnetic resonance
NP = Natural products
OTUs = Operational taxonomic units
PCA = Principal component analysis
PCR = Polymerase chain reaction
PDA = Photodiode array
ppm = parts per million
q = quartet
RDP = Ribosomal database project

REF = Reference
Rnase = Ribonuclease
RP = Reverse-phase
rpm = Revolutions per minute
rRNA = Ribosomal ribonucleic acids
RT = Retention time
s = Singlet
SCUBA = Self-contained underwater breathing apparatus
SDS = Sodium dodecyl sulphate
Sest = Chao1 estimated species richness
SFSW = Sterile filtered seawater
Sobs = Observed species richness
SS = San Salvador
t = triplet
TEM = Transmission electron microscopy
TOCSY = Total correlated spectroscopy
T-RFLP = Terminal restriction fragment length polymorphism
UHPLC = Ultra high performance liquid chromatgraphy
UPGMA = Unweighted paired group method with arithmetic mean
UV = Ultraviolet
V = 16S rRNA gene hypervariable region (Chapter 2)
V = Volts
v/v = Volume per volume
VRE = Vancomycin-resistant *Enterococcus faecium*
W = Seawater
w/v = Weight per volume

Chapter 1: Introduction and Objectives of Thesis Research

1.1 Octocoral Holobiont

Octocorallia is a subclass in the phylum *Cnidaria* and the class *Anthrozoa* that represent a diverse group of organisms including over 3000 species.[1] They are sedentary, colonial soft corals whose polyps contain eight tentacles, eight septa with filaments, one siphonoglyph, and their skeletons consist of calcareous spicules with a calcified horny central axis.[2] They are abundant in both Pacific and Atlantic reefs, and account for ~40% of the known fauna in the Caribbean. Like many marine invertebrates, octocorals are associated with a wide variety of microorganisms including symbiotic algal dinoflagellates, bacteria, archaea, and fungi. Collectively, these along with the coral colonies are referred to as the coral holobiont.[3] The microbial component of the holobiont can contribute to the overall coral health via any of the following mechanisms: (1) Cycling of nutrients such as nitrogen, carbon, or sulphur;[4,5] (2) protection from pathogenic infection via spatial exclusion,[6-8] and/or through the production of bioactive antimicrobial metabolites;[8-12] (3) removal of metabolic waste generated by the coral and/or the *Symbiodinium* spp.;[4,5] and (4) production of quorum sensing molecules that regulate phenotypic behaviours between organisms within the holobiont.[13-16] These functions can be so vital that the 'coral probiotic hypothesis' suggests that corals can adapt to changing environmental conditions by altering their microbial community to maximize the health of the overall holobiont.[17]

1.2 Investigations of Coral Microbiomes

Traditionally, investigation of coral microbiomes was through culture dependent based techniques. However, a vast majority of the microbes present in coral microbiomes are not cultivatable and it has been suggested that less than 1% of the taxonomic diversity is detectable using these techniques.[18] Therefore, over the years culture-independent techniques such as

denaturing gradient gel electrophoresis, terminal restriction fragment length polymorphism, and clone libraries were used to identify the microbiomes of corals.[7,19-26] However, with the advancement of next generation sequencing (NGS) technologies, such as 454 pyrosequencing, researchers have been able to identify the 'long tail' of low abundance taxa in these microbiomes that were not previously obtainable.[27,28] This allowed for in-depth analysis of coral microbiomes that has enabled the following broad conclusions: (1) Coral microbiomes are distinctly different from that of the surrounding seawater;[29,30] (2) coral microbiomes are rich in microbial abundance as well as taxonomic diversity;[30,31] and (3) even though coral microbiomes are taxonomically diverse, many maintain a core microbiome, a set of specific microbial taxa that form a stable association with a particular coral species.[32,33] While overall microbiome membership varies with geographic location or changing environmental factors, the core microbiome remains intact as long as the coral remains healthy.[34] Table 1.1 gives an overview of all coral microbiomes that have been investigated using 454 pyrosequencing to date.

Table 1.1. Summary of richness and alpha diversity measurements for all reported coral microbiomes investigated using 454 pyrosequencing. All values reported below are from healthy corals. Corals that belong to the subclass *Octocorallia* (Octocorals) are in bold.

Ref.	Corals (Sample number)	#OTUs (S_{obs})	Chao1 (S_{est}) (mean ± standard deviation)	*H'*	*E'*	Location	Primers; 16S rDNA Variable Region
35	*Montastraea annularis* (n=4)	214 ± 39	321 ± 38	3.63 ± 0.73	-	Water Factory site, Island of Curacao	27F & 534R; V1 - V4
29	***Eunicella cavolini* (n=9)**	**299 ± 270**	**474 ± 430**	**2.12 ± 1.37**	**0.37 ± 0.19**	French Mediterranean	27F & 338R; V1 - V2
32	*Styliphora pistillata* (n=5)	289 ± 150	585 ± 291	1.44 ± 0.56	-	Southern Red Sea	784F & 1061R; V5 - V6
36	*Pocillopora damicornis* (n=3)	471 ± 60	-	-	-	Great Barrier Reef, Australia	63F & 533R; V1 - V3
	Acropora millipora (n=3)	427 ± 104	-	-	-		
	Seriatopora hystrix (n=3)	192 ± 42	-	-	-		
	Sinularia flexibilis (n=3)	181 ± 41	-	-	-		
	Sarcophyton sp. (n=3)	80 ± 13	-	-	-		
	Nephyta sp. (n=1)	377	-	-	-		
37	*Diploria strigosa* (n=5)	378 ± 13	800 ± 153	5.4 ± 0.1	-	Aguja Island, Columbia	784F & 1061R; V5 - V6
	Siderasrea siderea (n=5)	256 ± 7	513 ± 123	4.5 ± 0.1	-		
38	*Acropora tenuis* (n=6)	234 ± 81	253 ± 90.1	-	-	Ningaloo Reef, Western Australia	63F & 533R; V1 - V3
	Posillopora damicornis (n=3)	81 ± 40	142 ± 90	-	-		
	Tubastrea faulkneri (n=4)	319 ± 121	331 ± 136	-	-		
39	***Antillogorgia elisabethae* (n=3)**	**184 ± 126**	**291 ± 159**	**2.13 ± 0.19**	**0.46 ± 0.03**	El Planchon, Providencia Island, Colombia	27F & 534R; V1 – V3
40	*Acropora hemprichii* (n=9)	159 ±30	427 ±140	-	-	Saudi Arabia, Central Red Sea	784F & 1061R; V5 - V6

41	*Pocillopora verrucosa* (n=3)	334 ± 257	545 ± 328	4.54 ± 0.45	-	Red Sea, coast of Saudi Arabia	341F & 685R; V3 - V4
	Astereopora myriophthalma (n=2)	207 ± 17	372 ± 31	3.71 ± 0.74	-		
	Stylophora pistillata (n=1)	230	374	5.23	-		
	***Sarcophyton* spp. (n=3)**	**419 ± 73**	**735 ± 285**	**5.48 ± 0.89**	-		
42	*Acropora millepora* (n=20)	37 ± 18	59 ± 33	-	-	Cattle Bay, Trunk Reef, GBR	28F & 519R; V1 – V2
30	*Porites lutes* (n=3)	1623 ± 748	3848 ± 1500	6.29 ± 0.66	-	South China Sea	27F & 534R; V1 – V3
	Galaxea fascicularis (n=3)	1181 ± 463	3537 ± 1687	5.84 ± 1.45	-		
	Acropora millepora (n=3)	523 ± 185	1143 ± 325	5.52 ± 0.29	-		
43	*Montastraea faveolata* (n=5)	50 to 145	76 to 402	5.5 ± 0.5	-	Caribbean; US Virgin Islands, Florida Keys, FL, Belize	27F & 518R; V1 – V2
	Porites asteroides (n=6)	11 to 85	26 to 225	3.1 ± 1.1	-		
34	*Ctenactis echinata* (n=54)	48.9 ± 8.5	-	2.14 ± 0.02	-	Central Red Sea	784F & 1061R; V5 - V6
31	*Montastraea faveolata* (n=1)	1553	2925	-	-	Bocas del Toro, Panama	967F & 1046R; V6
	Montastraea franksi (n=1)	2050	4026	-	-		
	Diploria strigosa (n=1)	1759	3801	-	-		
	Acropora palmata (n=1)	1671	2576	-	-		
	Acropora cervicornis (n=1)	1616	2602	-	-		
	Porites astreoides (n=1)	1340	3106	-	-		
	***Gorgonia ventalina* (n=1)**	**1143**	**2177**	-	-		
44	***Pamamuricea clavata* (n=5)**	**1069 to 2501**	-	**3.2 ± 1.6**	-	Mediterranean Sea	967F & 1046R; V6
45	*Platygyra carnosus* (n=4)	-	350 ± 107	5.9 ± 0.4	-	Hoi Ha Wan Marine Park, Hong Kong	341F & 926R; V3 - V5

46.	***Palythoa australiae* (n=3)**	-	-	-	-	South China Sea	27F & 533R; V1 – V3
	***Eunicea Fusca* (n=9)**	**45 ± 18**	**84 ± 31**	**1.02 ± 0.29**	**0.27 ± 0.07**	Florida, The Bahamas	
47	***Eunicea sp.* (n=1)**	**31**	**71**	**0.98**	**0.29**	The Bahamas	27F & 519R; V1 – V2
	***Plexaura* sp. (n=2)**	**14**	**36**	**0.29**	**0.11**		
48	***Antillogorgia elisabethae* (n=14)**	**113 ± 78**	**298 ± 193**	**2.48 ± 1.25**	**0.53 ± 0.19**	The Bahamas	27F & 519R; V1 – V2
Chapter 3	***Antillogorgia elisabethae* (n=5)**	**137 ± 34**	**245 ± 81**	**2.63 ± 0.43**	**0.54 ± 0.03**	The Bahamas	515F & 806R; V4

Abbreviations: GBR = Great Barrier Reef; OTUs = Operational taxonomic units; Chao1 = Species richness estimator; H' = Shannon diversity index; E = Shannon equitability index.

1.3 Natural Products

Natural products (NP) are secondary metabolites produced by an organism that are not required for the basic sustenance of life, but offer an evolutionary advantage to the producing organism.[49,50] The ecological roles of these secondary metabolites are often enigmatic but can range from chemical defense against predation, to acting as an antifouling or antibiotic agent, to signalling compounds involved in the organism's survival and fecundity.[49,51-53] In addition to their ecological roles, plant-based NPs have been a part of traditional medicine for thousands of years.[54,55] This continues today, as NPs are an essential part of the current healthcare system. In fact, approximately 64% of all approved therapeutic drugs are either naturally derived or inspired.[56-58] The success of NPs and their derivatives as therapeutic agents is largely due to their structural diversity and highly specific biological activity.[59] Owing to the high metabolic cost of biosynthesising NPs, these chemicals presumably increase the overall fitness of the organism through evolutionary selection, and therefore have coevolved with some receptor binding capacity that could be put to anthropogenic use.[60] Their wide range of pharmacophores with a high degree of stereochemistry makes NPs great candidates not only for pharmaceutical uses, but also cosmeceutical, nutraceutical, and agrochemical applications.

1.4 Marine Natural Products

Secondary metabolites derived from marine organisms, or marine natural products (MNPs) do not have the same significant history or prevalence in the pharmaceutical world as their terrestrial counter parts. This is simply due to the fact that the investigation of the marine environment for bioactive compounds is relatively new compared to the terrestrial environment. Systematic investigation of the marine environment for novel secondary metabolites only began

in the 1970s with the advent of SCUBA equipment.[61] However, once MNP research began the marine environment proved to be a rich source of bioactive compounds as from 1977 to 1987 approximately 2,500 new metabolites were reported from a variety of marine origins.[57] The number of novel chemical structures continued to grow dramatically, by 2014 there were over 9,200 publications reporting over 24,660 new compounds.[62-66] However, despite the number of reported MNPs the oceans are still a relatively untapped resource. They cover over 70% of the Earth's surface and are predicted to contain as many as 2.2 million different eukaryotic marine species,[67] to say nothing of the vast prokaryotic diversity. This high level of biodiversity encompasses a large amount of genetic diversity, which in turn leads to high chemical diversity and the potential for novel MNPs, making the oceans a rich environment for MNP discovery.[68,69]

Although the field of MNP research is relatively young compared to its terrestrial counterpart, there have been seven therapeutic agents approved for clinical use (Table 1.2), six (**1-6**) of which are in current use today (Figure 1.1). In addition to the clinically approved MNPs there is one over-the-counter MNP as well as a number more that have entered clinical trials, ten of which are currently progressing through the clinical pipeline (Table 1.3).[70,71] While this is promising for the field of MNP research and drug discovery, some of these clinical trials have been discontinued over the last few years due to a limited supply of the desired MNP. This "supply issue" is also a common reason that many promising MNPs never enter into the clinical pipeline. Often MNPs from marine invertebrates account for a small percentage of the overall biomass of the organism.[72,73] Therefore large-scale harvesting of the organism proves to be inefficient, not to mention the ecological and environmental impacts that such harvesting would have on the marine ecosystem. The low yield of MNPs within organisms often means that aquiculture of the

organism does not always prove to be a cost effective way to obtain the desired compound.[74] Total- and semi-synthesis have proven to be an effective means to obtain MNPs in some cases.[75] However, in many cases the structural complexity of the desired MNP can be challenging and generating yields high enough to create a stable source of these compounds can be difficult.[76-78] Often an alternative method of obtaining a sustainable supply of certain MNPs is required for these compounds to proceed through clinical trials. There is a growing body of evidence that in many cases the MNP isolated from marine invertebrates are in fact biosynthesised by an associated symbiotic microorganism.[79-84] This will be discussed in greater detail in a later section (1.6.1). In cases where microbes are the true producers, the desired MNP could be obtained through fermentation of the producing organism.

While MNPs are a promising source of pharmaceuticals, they have also proved to be an excellent source of cosmeceuticals. For example, Abyssine® (Unipex, New York, USA) which consists of exopolysaccharides (EPS) obtained from a marine *Alteromonas* sp. extract that has been shown to reduce the expression of a skin stress marker involved in skin reactivity (ICAM-1), and is marketed as a "protectant for reducing irritation in sensitive skin" by "increasing resistance to mechanical and sun aggression."[85-88] Seacode® (Lipotec, Barcelona, Spain) which consists of EPS and glycoproteins obtained from a marine *Pseudoalteromonas* sp. extract is marketed as a "marine eraser for aging lines" that "enhances the synthesis of dermal proteins, such as collagen I, visibly reducing aging signs in the short and long term."[89] Dermochlorella D® (CODIF, Britany, France) which consists of oligopeptides obtained from a marine *Chlorella* sp. extract is marketed to "stimulate synthesis of components of the dermis and dermal-epidermal junction....after 28 days, the skin is firmer and more toned."[90]

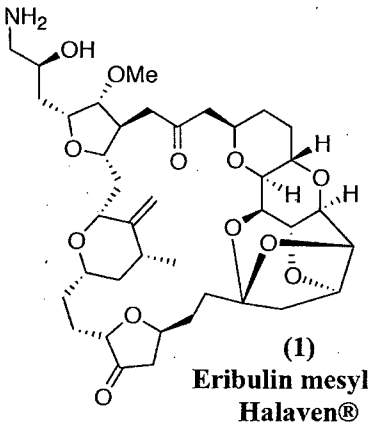
Resilience® (Estee Lauder, New York, USA) which consists of pseudopterosins (**10-13**) obtained from the extract of the coral *Antillogorgia elisabethae,* has potent anti-inflammatory activity and will be discussed in greater detail below (1.5.2).[71,91] These examples only scratch the surface, as there are thousands of different commercially available cosmetic products that contain MNPs obtained from various marine sources.[71,92]

Table 1.2. Clinically approved marine natural products and marine natural product derivatives.

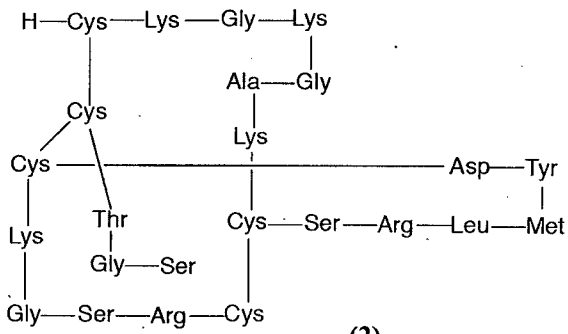
Ref.	Compound (Trademark)	MNP or D*	Collected Source Organism	Predicted Bio-synthetic Source	Biosynthetic Class	Therapeutic Area	Molecular Target	Clinical Status
93-95	Eribulin mesylate (Halaven®)	D	Sponge *Halichodria okadai*	B	Polyketide	Cancer	Microtubules	FDA/EMEA Approved
96-98	Ziconotide (Prial®)	MNP	Marine snail *Conus magus*	-	Cysteine Knot Peptide	Neuropathic Pain	N-type Ca channel	FDA/EMEA Approved
99-101	Brentuximab vedotin (SGN-35) (Adcetris®)	D	Sea hare *Dolabella auricularia*	CB	Cyclic Depsipeptide	Cancer	CD30 and microtubules	FDA/EMEA Approved
102-105	Cytarabine (Cytosar-U®; Depocyt ®)	D	Sponge *Cryptotethya crypta*	B	Nucleoside	Cancer	DNA polymerase	FDA/EMEA Approved
104,106	Vidarabine (Vira-A®)*	D	Sponge *Cryptotethya crypta*	B	Nucleoside	Anti-viral	Viral DNA polymerase I	Discontinued (Previously FDA/EMEA Approved)
75,79, 107-109	Trabectedein (Yondelis®)	MNP	Tunicate *Ecteinascidia turbinata*	B	NRPS-derived Alkaloid	Cancer	Minor groove of DNA	EMEA Approved
110-112	Omega-3-acid ethylesters (Lovaza®)	D	Fish	MA	Omega-3 fatty acids	Hypertri-glyceridemia	Triglyceride synthesizing enzymes	FDA/EMEA Approved

Abbreviations: MNP = Marine Natural Product; D = Natural product derivative; B = Bacteria; MA = Microalgae; CB = Cyanobacteria

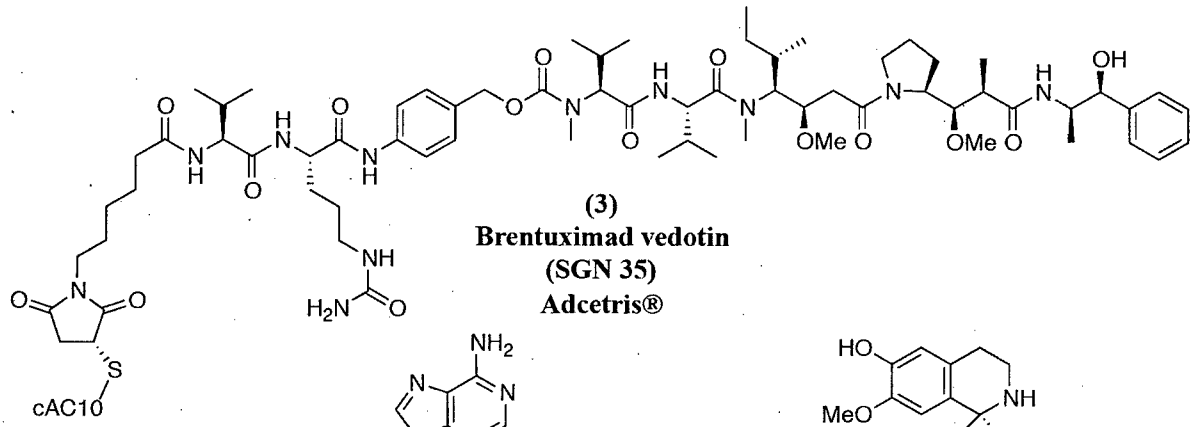
*Vidaradine was discontinued as an antiviral drug in 2001, but is still used in the EU for ophthalmological applications



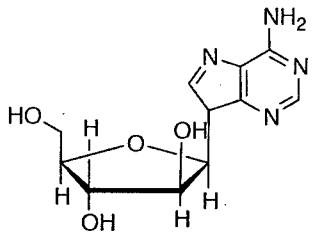
(1)
Eribulin mesylate
Halaven®



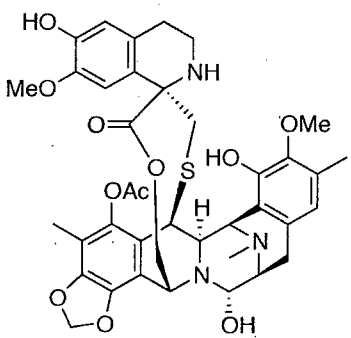
(2)
Ziconotide
Prialt®



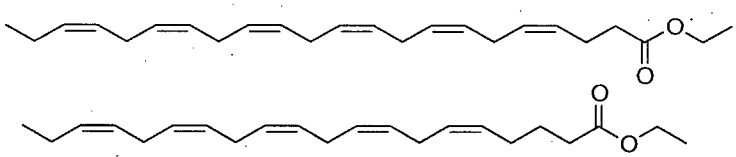
(3)
Brentuximad vedotin
(SGN 35)
Adcetris®



(4)
Cytarabine
Cytosar-U®; Depocyt®



(5)
Trabectedein
Yondelis®



(6)
Omega-3-acid ethylesters
Lovaza®

Figure 1.1. Chemical structures of marine natural product inspired drugs currently in use as therapeutic agents.

Table 1.3. Marine natural products and derivatives that have entered into the pharmaceutical clinical trial pipeline. Table derived from data summarized by Gerwick *et al.*[70] and Martins *et al.*[71].

Compound (Trademark)	MNP or D*	Collected Source Organism	Predicted Biosynthetic Source	Therapeutic Area	Clinical Status
Iota-carrageenan (Carragelose®)	MNP	Red algae *Rhodophyceae*	-	Cancer	Over the counter drug
Plilditepsin (Aplidin®)	MNP	Ascidian *Aplidium albicans*	Bacterium	Cancer	Phase III
PM00104 (Zalypsis ®)	D	Sea slug *Joruna funebris*	Bacterium	Cancer	Phase II
DMXBA GTS-21	D	Worm *Paranemertes peregrina*	Worm	Alzheimer's	Phase II
Lurbinectedin	D	Tunicate *Ecteinascidia turbinata*	Bacterium	Cancer	Phase II
Glembatumumab vedotin CDX-011	D	Sea hare *Dolabella auricularia*	Cyanobacterium	Cancer	Phase II
SGN-75	D	Sea hare *Dolabella auricularia*	Cyanobacterium	Cancer	Phase I
PM060184	MNP	Sponge *Lithoplocamia lithistoides*	-	Cancer	Phase I
Salinosporamide A Marizomib	MNP	Actinomycetes *Salinispora tropica*	-	Cancer	Phase I
ASG-5ME	D	Sea hare *Dolabella auricularia*	Cyanobacterium	Cancer	Phase I
Bryostatin I	MNP	Bryozoan *Bugula neritina*	Bacterium	Cancer/ Alzheimer's	Phase I/II
Soblidotin	D	Sea hare *Dolabella auricularia*	Cyanobacterium	Cancer	Discontinued (Phase III)
Synthadotin	D	Sea hare *Dolabella auricularia*	Cyanobacterium	Cancer	Discontinued (Phase II)
Methopterosins	NP	Octocoral *Antillogorgia elisabethae*	Bacterium	Wound Healing	Discontinued (Phase II)
Elisidepsin (Irvalec®)	D	Sea slug *Elysia rufescens*	Bacterium	Cancer	Discontinued (Phase II)
Plinabulin NPI-2358	D	Maine fungus *Aspergillus sp.*	-	Cancer	Discontinued (Phase II)
Tasidotin ILX-651	D	Sea hare *Dolabella auricularia*	Cyanobacterium	Cancer	Discontinued (Phase II)

Hemiasterlin	MNP	Sponge *Hemiastrella minor*	Bacterium	Cancer	Discontinued (Phase II)
Kahalalide F	MNP	Sea slug *Elysia rufescens*	-	Cancer	Discontinued (Phase II)
Squalamine	MNP	Dogphish shark *Squalus acanthias*	-	Cancer	Discontinued (Phase II)
Hemiasterlin HTI-286	D	Sponge *Hemiastrella minor*	Bacterium	Cancer	Discontinued (Phase II)
Discodermolide	MNP	Sponge *Discodermia dissouta*	-	Cancer	Discontinued (Phase I)
E7389	D	Sponge *Halichondria okadai*	Bacterium	Cancer	Discontinued (Phase I)
Spisulosine ES-285	MNP	Marine clam *Spisula polynyma*	-	Cancer	Discontinued (Phase I)
Agelasphins KRN-7000	D	Sponge *Agelas mauritianus*	-	Cancer	Discontinued (Phase I)
AE-941 (Neovastat®)	MNP	Shark cartilage	-	Cancer	Discontinued (Phase I)
Psammaplin A NVP-LAQ824	D	Sponge *Aplysinella rhax*	-	Cancer	Discontinued (Phase I)
Conotoxin G CGX-1160	MNP	Marine snail *Conus geographus*	-	Pain	Discontinued (Phase I)

Abbreviations: MNP = Marine Natural Product; D = Marine natural product derivative

1.5 Marine Natural Products from Octocorals

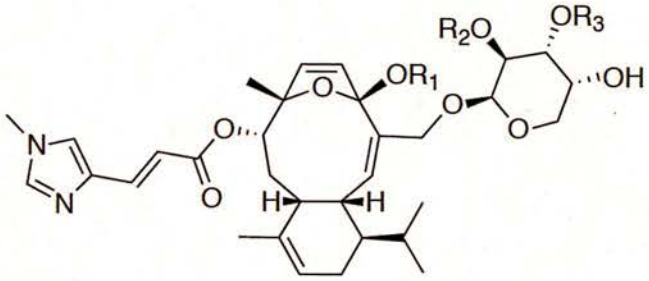
Octocorals are also known as 'soft corals' as they lack the calcareous exoskeleton the reef building scleractinian or 'stony corals' have for physical protection.[113,114] Octocorals tend to rely on chemical defenses for protection making them a wonderful source of bioactive MNPs that have a wide range of biological activity ranging from anticancer, antibiotic, anti-inflammatory, antiviral, to antioxidant.[115,116] The octocorals that are the driving force behind this thesis research are *Erythropodium caribaeorum* and *Antillogorgia elisabethae*, which are associated with the bioactive MNPs the eleutherobins and pseudopterosins, respectively.

1.5.1 *Erythropodium caribaeorum* and the Eleutherobins

Erythropodium caribaeorum is an octocoral in the suborder *Scleraxonia* and the family *Anthothelidae* that is found in the Caribbean Sea from southern Florida to the Virgin Islands. The colonies grow in a thin firm purplish-grey encrusting morphology over their substrate. The polyps appear hair-like along the surface of the coral when they are extended, and when they are retracted slightly projected star-shaped apertures are visible.[2] They are a source of a family cytotoxic diterpene glycosides known as the eleutherobins.[117,118] Eleutherobin (Figure 1.2) was initially isolated from a Western Australian *Eleutherobia* sp. by Lindel *et al.*[117] Then work by Andersen and co-workers used a cell based antimitotic assay coupled with bioassay-guided fractionations to isolate an additional six analogs.[118,119] It was later determined that eleutherobin was not a MNP but an isolation artifact formed by reaction of desmethyleleutherobin with methanol during extraction and purification.[120] The MNPs Desmethyleleutherobin (IC_{50} 20nM), and isoeleutherobin (IC_{50} 50nM) (Figure 1.2) are of particular interest due to their antimitotic activity at nanomolar concentrations that have a mechanism of action similar to the well-known

cancer drug Taxol® and acts through microtubule stabilization.[117,118]

The eleutherobins are structurally related to other compounds isolated from *Eleutherobia* sp. and *Sarcodictyon* sp. from South Africa and the Mediterranean, bringing into question the true biosynthetic source of these compounds.[121,122] A common microorganism in the microbiomes of these corals could be the true biosynthetic source and may account for the structural similarity of these compounds.



Eleutherobin (**7**) R_1 = CH3, R_2 = Ac, R_3 = H
Desmethyleleutherobin (**8**) R_1 = R_3 = H, R_2 = Ac
Isoeleutherobin (**9**) R_1 = CH3, R_2 = H, R_3 = Ac

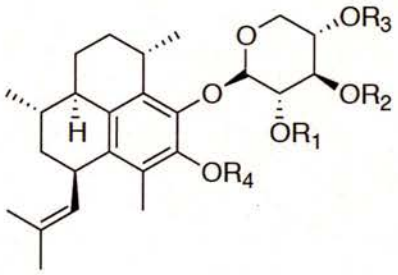
Figure 1.2. The octocoral *E. caribaeorum,* eleutherobin (7), desmethyleleutherobin (8), and isoeleutherobin (9).

1.5.2 *Antillogorgia elisabethae* and the Pseudopterosins

Antillogorgia elisabethae is an octocoral in the suborder *Holaxonia* in the family *Gorgoniidae* and is a dominant octocoral in many Caribbean reef communities.[123,124] This coral was previously classified as a member of the *Pseudopterogorgia* genus but has since been reassigned to the resurrected genus *Antillogorgia.*[125] Morphologically, they are sea plume in structure and consist of feather-like branches with a central skeleton and closely spaced branchlets that extend from the central skeleton.[2] They are the sole source of a family of diterpene glycosides known as the pseudopterosins that were first isolated in 1986 by Look *et al.* who reported the structures of pseudopterosins A-D[126,127] (Figure 1.3). Since then 30 pseudopterosins and 11 *seco*-pseudopterosins have been identified. These compounds have been identified from *A. elisabethae* collected from the Bahamas, Bermuda, the Florida Keys, and Colombia.[116,128-136] The pseudopterosins attracted commercial attention due to their anti-inflammatory activity that have been shown to be more potent than the clinically used drug indomethacin.[126,127,129,137] A simple derivative of pseudopterosin A, methopterosin (Figure 1.3) has successfully completed Phase I and II of clinical trials as a wound healing agent, but development has been discontinued due to the supply issue.[73,92,138] The total synthesis of pseudopterosin A was first reported by Broka *et al.* in 1988,[139] since then there have been numerous reported syntheses of pseudopterosins and pseudopterosin-like molecules.[133,140,141] However this has not proven an economically viable option for the sustainable production of pseudopterosins for commercial use, due to the complexity of these compounds only low quantities could be obtained. While the pseudopterosins have not progressed through clinical trials due to the supply issues "natural extracts" of *A. elisabethae* are currently used in the Resilience® line of skincare products from Estee Lauder.[71,91]

In addition to anti-inflammatory activity, pseudopterosins have been shown to exhibit a wide range of bioactivity including anticancer, antiviral, antituberculosis, and antibiotic activity against Gram positive bacteria.[129,131,134] The mechanism of action is incompletely understood, but it is believed to involve competitive binding to adenosine receptors, which belong to the G-protein coupled receptor class.[142,143]

The biosynthetic machinery that is involved in pseudopterosin biosynthesis has been detected in the larvae of *A. elisabethae* as well as the symbiotic algal dinoflagellates, which bring to question the true biosynthetic producer of these commercially relevant compounds. This will be discussed in greater detail in Chapter 3.



PsA (**10**) $R_1 = R_2 = R_3 = R_4 = H$
PsB (**11**) R_1 =Ac, $R_2 = R_3 = R_4 = H$
PsC (**12**) R_2 =Ac, $R_1 = R_3 = R_4 = H$
PsD (**13**) R_3 =Ac, $R_1 = R_2 = R_4 = H$

Methopterosin (**15**) R_4 =CH_3, $R_1 = R_2 = R_3 = H$

Figure 1.3. The octocoral *A. elisabethae*, pseudopterosins (Ps) A-D (10-13), and methopterosin (14).

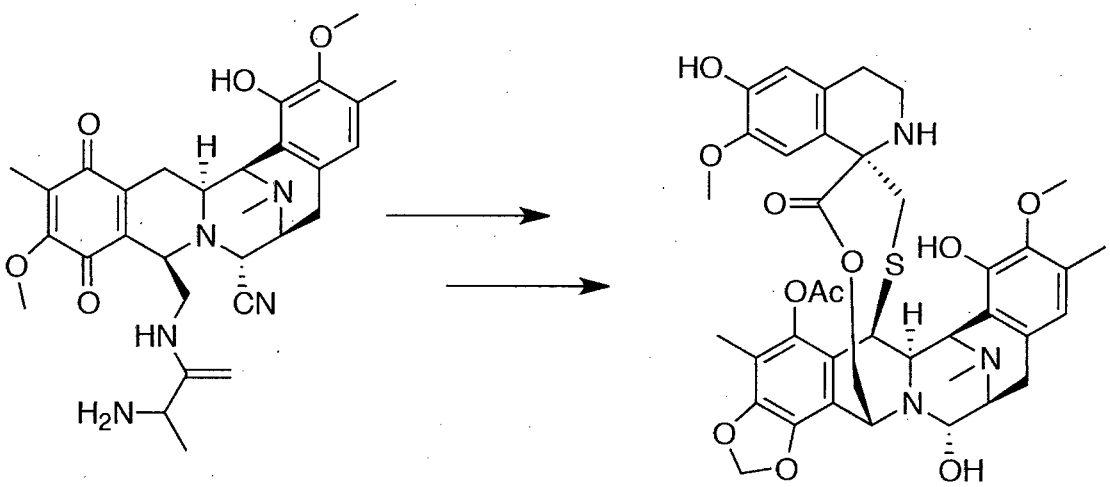
1.6 Microorganisms as a Source of Natural Products

Microorganisms from the terrestrial environment are responsible for a vast majority of the clinically approved natural products on the market to date. Of FDA approved antibacterial agents 69% of them are NPs or NP-derived, and of those 97% originate from a microbial source.[58] Although there are no FDA approved MNPs from marine microorganisms, the anti-cancer compounds Salinosporamide A isolated from the marine actinomycetes *Salinispora tropica* is currently in the clinical trial pipeline[71] (Table 1.2). Additionally, many of the bioactive MNP isolated from marine microorganisms that are approved for clinical use are believed to be biosynthesised by associated microorganisms.[80]

1.6.1 Microorganisms as Biosynthetic Producers of Marine Natural Products Isolated from Macroorganisms

Over the years it has long been suspected that many of the MNPs isolated from macroorganisms are in fact biosynthesised by associated microorganisms. Some estimate that as many as 80% of the approved and clinical trial MNPs and MNP-inspired derivatives are biosynthesised by microorganisms.[70,80] Many of the MNPs isolated from macroorganisms are structurally similar to known microbial metabolites.[81-83] For example, trabectedin which is currently used under the trademark Yondelis®, was originally isolated from the tunicate *Ecteinascidia turbinate* however it is structurally similar to the *Pseudomonas fluorescens* metabolite, cyanosafracin B. In fact due to the close structural similarity, cyanosafracin B is used as the starting point for the semi-synthesis of trabectedin for commercial use[75,109] (Figure 1.4). Other evidence that suggested microbial symbionts might be the true producers of many MNPs is the fact that many of the macroorganisms have stable core microbiomes. For example, keeping with the trabectedin

theme, the *E. turbinate* microbiome was dominated by the *Gammaproteobacteria,* Candidatus *Endoecteinascidia frumentensis* in samples collected from both the Mediterranean and the Caribbean.[144] Trabectedin biosynthesis by *E. frumentensis* was finally confirmed when Rath *et al.* used metagenomic analysis to confirm presence of 25 genes involved in trabectedin biosynthesis within the *E. frumentensis* genome.[79,145] This is not an isolated incident, microbial biosynthesis has been implicated in many approved and clinical trial MNPs that have a wide range of chemical structural diversity[70,80] (Table 1.2, 1.3).



(14) **(5)**

Figure 1.4. Chemical structure of cyanosafracin B (14) and trabectedin (5).

1.6.2 Octocorals as a Reservoir of Unique Microorganisms

Over the past few years there has been a dramatic rise in the number of novel metabolites being reported from marine microorganisms,[63,80,146] and NGS has revealed that the marine environment is a vast resource of untapped taxonomically diverse microorganisms.[31,147] Marine invertebrates, such as sponges and corals, act as a reservoir for unique microbes by providing a higher nutrient environment than that of the surrounding seawater; which is why marine invertebrates have proven to be a valuable source of novel cultivatable microorganisms.[148-153] Therefore, octocorals are potentially a great source for novel cultivatable microbial diversity. As greater biological diversity has been shown to lead to greater genetic and chemical diversity, microbes cultivated from octocorals may be an excellent source of MNPs.[68]

1.7 Objectives of Thesis Research

With the advent of NGS technologies in-depth investigation of the microbiomes associated with marine corals has become more accessible. While the microbiomes of reef-building scleractinian corals have been extensively studied using these techniques,[30-35,37,40,42,43,154-156] relatively fewer have examined the microbiomes of octocorals even though these corals are dominant members of many reef communities.[29,39,44] Therefore, understanding the microbial communities associated with these corals may provide insight into the overall health of the reef communities in which they inhabit. Additionally, octocorals have been shown to be associated with bioactive MNPs and understanding any stable association between these corals and their microbial communities may provide insight into the biosynthetic source of these MNPs. Lastly, octocorals may be a valuable source of cultivatable taxonomically diverse microorganisms that may produce known or novel MNPs.

Therefore the hypotheses of this thesis research are: (1) The octocorals *E. caribaeorum* and *A. elisabethae* have a stable microbiome, a set of bacteria that form a stable association with the octocoral regardless of geographic location; (2) *A. elisabethae* is a reservoir for unique cultivatable bacteria; and (3) bacteria cultivated from *E. caribaeorum* and *A. elisabethae* will be an excellent source of known and novel MNPs. The resulting objectives of this thesis research are as follows: (1) Characterize the culture-independent microbial communities associated with the octocorals *E. caribaeorum* and *A. elisabethae*. (2) Characterize the cultivatable bacterial communities associated with *A. elisabethae*. (3) Identify any known and novel MNPs produced by the cultivated octocoral bacteria.

Figure 1.5 gives an overview of the workflow of this thesis research. The microbiomes of *E. caribaeorum* collected at different times and locations were investigated using culture-independent pyrosequencing (Chapter 2). Samples of *A. elisabethae* were divided into coral derived fractions (holobiont, dinoflagellates, and larvae) that are involved in the health and fecundity of the coral. This will be described in greater detail in Chapter 3. Each coral derived fraction was divided and the samples were used for two different studies; one investigating the bacterial communities associated with the coral derived fractions using culture independent pyrosequencing (Chapter 3), and the other investigating the cultivatable bacteria from these coral derived fractions (Chapter 4). Additionally, an investigation into the cultivatable bacteria from *A. elisabethae* under aerobic, microaerophilic, and anaerobic conditions was conducted (Chapter 4). A formal species description of a bacterium cultivated from *A. elisabethae* that was determined to be a member of a novel taxonomic family was conducted (Chapter 5). Microorganisms cultivated from *A. elisabethae* and *E. caribaeorum* (obtained from another study by Brad Haltli –

Research Manager, Nautilus Bioscience and Adjunct Faculty, University of Prince Edward Island) were fermented and screened for the presence of known and novel MNPs (Chapter 6).

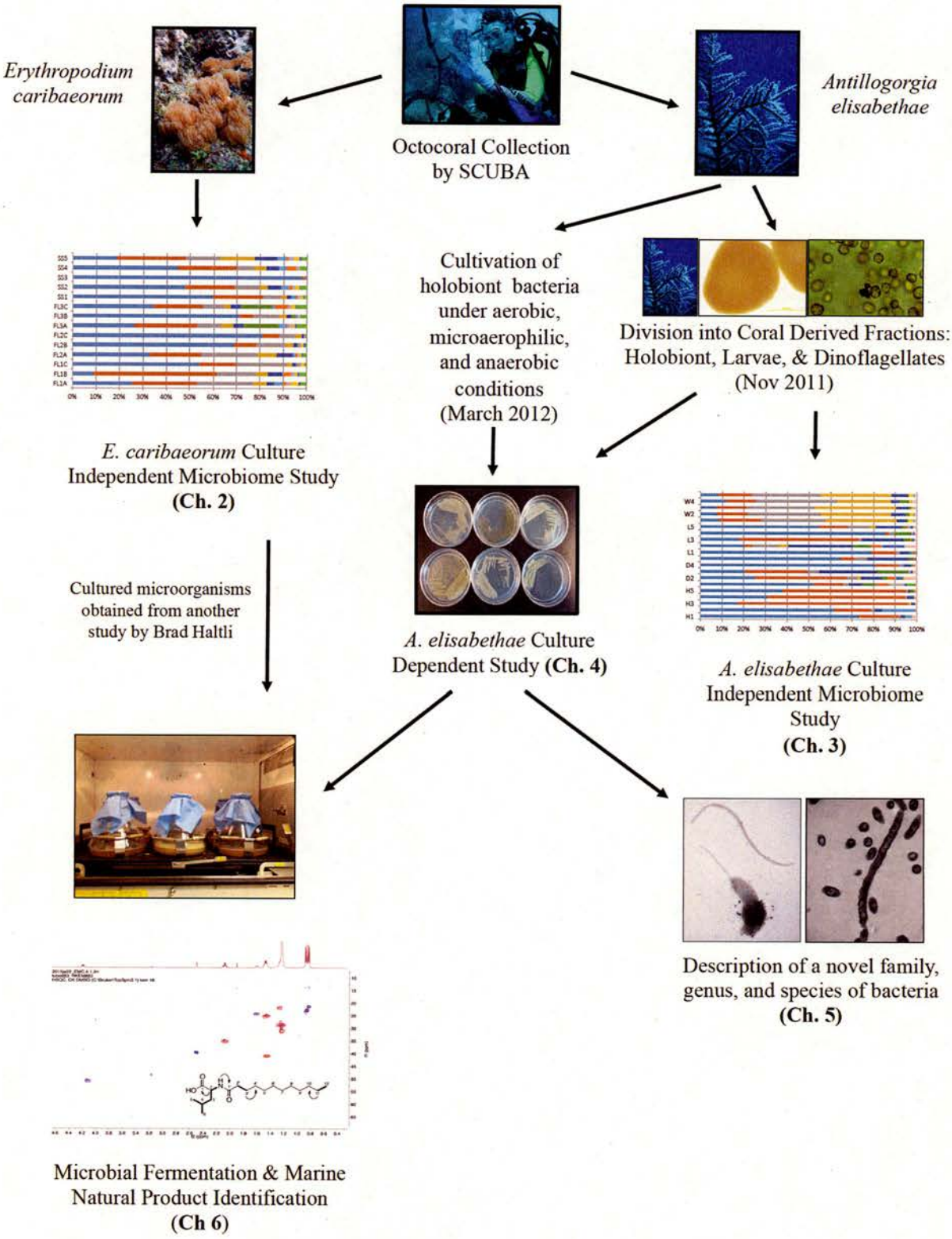


Figure 1.5. Overall workflow for thesis research.

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Chapter 2: Spatial and Temporal Investigation of the Microbiome of the Caribbean Octocoral *Erythropodium caribaeorum.*

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2.1 Introduction

The association between marine invertebrates and microorganisms has been extensively studied, however the role of these microbes is largely unknown.[1-3] Shallow water corals have a well-documented association with dinoflagellate symbionts of the genus *Symbiodinium.*[4] The colonial coral polyps, along with the *Symbiodinium* spp., and associated microbial assemblages or microbiomes, make up the coral 'holobiont.'[1] The microbial component of the holobiont can contribute to overall coral health via several mechanisms such as nutrient cycling (*e.g.* C, N, and S)[5-11] and infection prevention via spatial exclusion[6-8] or through the production of antimicrobial metabolites.[12-14] These functions can be so vital that the 'coral probiotic hypothesis' suggests that corals can adapt to changing environmental conditions by altering their microbial community to maximize the health of the overall holobiont.[15]

Advances in next generation sequencing (NGS) technology have allowed for in-depth analysis of coral microbiomes.[16] These studies have clearly established that corals host taxonomically diverse microbiomes that are distinct from their surroundings.[17,18] Even though coral microbiomes are taxonomically diverse, many species maintain a core microbiome, a set of specific microbial taxa that form a stable association with a particular coral species.[19,20] While overall microbiome membership varies with geographic location or changing environmental factors, the core microbiome remains intact as long as the coral remains healthy.[21]

The microbiomes of reef-building scleractinian corals have been extensively studied using modern NGS approaches[19,22,23] while relatively few studies have examined the microbiomes of octocorals.[17,24-28] Given that these corals are keystone members of many reef communities,

understanding the structure and dynamics of their microbiomes is important to understanding the health of the reef ecosystems which they inhabit.[29] Studies investigating octocoral microbiomes have found lower microbial richness and diversity compared to the microbiomes of scleractinian corals.[17,24,25] Bacterial diversity associated with octocorals has been shown to be sensitive to anthropogenic disturbance; resulting in increased bacterial diversity and altered community membership compared to octocorals sampled from undisturbed sites.[25] All studies that have investigated the microbiome of octocorals using NGS reveal a stable and dominant association with the bacterial class *Gammaproteobacteria*. The genus *Endozoicomonas* is frequently encountered as the most abundant *Gammaproteobacteria* in the microbiomes of healthy octocorals.[17,24,27,30] For example, *Endozoicomonas* spp. accounted for ~90% of the microbiome in healthy *Paramuricea clavata* colonies collected from pristine sites, but were supplanted by other *Gammaproteobacteria* taxa (e.g. *Vibrio* spp.) at sites subjected to anthropogenic disturbance.[25] This highlights the importance of understanding the microbiome of these ecologically important corals.

2.1.1 Rationale for the Investigation of the Microbiome of *E. caribaeorum*

This study investigates the microbiome of the octocoral *Erythropodium caribaeorum*, a coral found in the Caribbean Sea from southern Florida to the Virgin Islands.[31] *E. caribaeorum* is morphologically unique compared to most Caribbean octocorals as it exhibits an encrusting morphology, growing in a stolon or mat over the substrate; as opposed to most other octocoral species which are sea plumes or sea fans. These corals are of specific interest as they are the source of the diterpene natural product, desmethyleleutherobin.[32] Desmethyleleutherobin is a cytotoxic compound of pharmaceutical interest due to its ability to cause mitotic arrest through

microtubule stabilization at nanomolar concentrations,[33,34] a mechanism of action shared with the anticancer drug Taxol®.[35,36] The ecological role of desmethylelutherobin is unknown, but it may act as a deterrent to predation, aid *E. caribaeorum* colonization of crowded reef substrata, or prevent overgrowth of *E. caribaeorum* by other reef-colonizing organisms.[37-40] There is a growing body of evidence that suggest that many natural products associated with microorganisms are in fact biosynthesized by associated microorganisms,[3,41-43] therefore understanding the microbiome associated with these encrusting corals may provide insight into the biosynthetic source of desmethyleleutherobin.

2.1.2 Overall Objective of Study

In this study we set out to characterize the microbiome of *E. caribaeorum* utilizing 16S small subunit rRNA gene amplicon 454 pyrosequencing. Samples were collected from two locations at three different time points to investigate geographic and temporal variation of microbial communities, as well as to identify the core microbiome associated with *E. caribaeorum*. This research serves as a starting point for the further investigation of the microbiome of this pharmaceutically relevant coral.

2.2 Materials and Methods

2.2.1 Sample Collection

Individual *E. caribaeorum* specimens were collected by SCUBA off the coast of Deerfield Beach, Florida in June 2009 (26°18.736' N, 80°03.583' W, n=3, FL1A-C; 26°18.068' N, 80°04.112' W, n=3, FL2A-C) and December 2011 (26°18.736' N, 80°03.583' W, n=3, FL3A-C), and off the coast of San Salvador, The Bahamas in February 2011 (24°03.816' N, 74°

32.628' W, n=2, SS1-2; 24°03.090' N, 74°32.391' W, n=3, SS3-5). All samples were collected at depths between 20-30 m using nitrile gloves and a sterile scalpel. For each octocoral collected an approximately 5 cm x 5 cm piece was removed from the surface of the animal to the base in contact with the substratum and placed in sterile Whirl-Pak™ bags (Nasco®). To preserve tissue for DNA extractions 2 - 3 g of tissue from each sample was washed three times in 40 mL of sterile filtered seawater (SFSW) (0.2 μm polyethersulfone membrane, Nalgene Rapid Flow™) in sterile 50 mL centrifuge tubes to remove loosely associated bacteria. Washed samples were either frozen on dry ice (Florida samples; ~ -78.5°C) or stored in the vapor phase of liquid nitrogen (San Salvador samples; ≤ -150°C) in a cryogenic vapor shipper (MVE model XC20/3V, Chart Industries, Ball Ground, GA, USA) during transport to Canada. Upon arrival in Canada samples were stored at -80°C until processed.

2.2.2 DNA Extraction and Purification

DNA was extracted from the coral tissue using a modified phenol-chloroform extraction as previously described.[24] Coral samples (0.5 g) were ground to powder in liquid nitrogen. The power was suspended in DNA lysis buffer (1 mg/ml lysozyme, 50 μg/ml RNase, 9 mg/ml PVP, 0.5% sodium dodecyl sulfate (SDS), 25 mM EDTA, 25 mM Tris-HCl pH 8.0, 0.5 M NaCl) and incubated for 30 min at 37°C. Proteinase K (1 mg/ml) was added to the solution, which was incubated for 2.5 hr at 55°C. Insoluble material was removed by centrifugation (4500 x *g*, 15 min) and sodium acetate was added to a final concentration of 0.3 M. The supernatant was extracted with a 1:1 ratio of phenol/chloroform/isoamyl alcohol (25:24:1) mixture. Followed by a second extraction with a 1:1 ratio of chloroform/isoamyl alcohol (24:1) mixture. DNA was precipitated from the aqueous layer using a 0.7 volume of isopropanol and pelleted by

centrifugation (13,000 x *g*, 50 min). The DNA pellets were washed with 70% ethanol and re-suspended in buffer (0.1 mM EDTA, 10 mM Tris-HCl, pH 8.0). DNA samples were further purified using the PowerClean® DNA Clean-Up Kit according to manufacturers recommendations (MO BIO Laboratories, Inc. Carlsbad, CA).

2.2.3 Pyrosequencing and Bioinformatics

Bacterial tag-encoded FLX amplicon library construction and 454 pyrosequencing analysis using the 454 GS FLX Titanium system (Roche)[44] was performed by Research and Testing Laboratories (Lubbock, TX). Amplicons were generated using universal 16S rRNA primers 28F (5'-GAGTTTGATCNTGGCTCAG-3')[45] and 519R (5'–GAATTACCGCGGCGGCTG-3') [46], which covered V1 and V2 regions of the 16S rRNA. Initial processing of .sff files was performed using mothur v.1.33.3.[47] Sequences were quality filtered using the following conditions: minimum average quality of 30 in each 50-bp window, minimum length of 200 bp, zero ambiguous base calls, and homopolymers less than 9 bp. The resulting filtered sequencing reads were aligned using the Silva reference alignment and the alignments were filtered to remove gaps. Chimeras were identified using UCHIME[48] and removed. The chimera-free sequences were classified using the mothur Bayesian classifier (80% confidence) which uses the mothur formatted Greengenes dataset.[49] Sequences classified as chloroplasts, mitochondria, and unknown were removed from the analysis. The remaining sequences were clustered using the UCLUST module from QIIME into species level operational taxonomic units (OTUs) with a pairwise identity threshold of 97%.[50] All diversity calculations were performed using mothur on datasets subsampled to 2960 sequence reads per sample.[47] Alpha-diversity was analyzed using Shannon diversity and equitability indices. Beta-diversity was assessed by the analysis of

molecular variance (AMOVA)[51] on a Yue and Clayton distance matrix.[52] Beta-diversity trees were viewed using FigTree version 1.4.2 (Institute of Evolutional Biology, University of Edinburgh). The "get.coremicrobiome" function of mothur was used to determine the number of shared OTUs at a relative abundance level > 1%. Community composition graphs were prepared using Microsoft® Excel® for Mac 2011 version 14.5.8. Sequence data have been archived in the NCBI Short Read Archive under accession number PRJNA304248.

2.2.4 Phylogenetic analysis

Sequence alignments were prepared using MEGA version 6.[53] Evolutionary distance matrices were generated using the Jukes-Cantor model,[54] and phylogenetic histories were inferred using the neighbor-joining method.[55] Bootstrap analysis was based on 1000 resampled datasets.[56] The final dataset consisted of 287 nucleotide positions. The tree was constructed using *Hahella antartica* NBRC 102683^T (GenBank accession no. NR114177), *Kistimonas asteriae* KMD 001^T (NR116386), and *Alcanivorax balearicus* MACL04^T (NR043109) as out groups. Sequence data has been archived in the NCBI GenBank Archive under accession numbers KU179036 – KU179042

2.3 Results and Discussion

2.3.1 *Alpha*- and *Beta*-Diversity

Fourteen samples of *E. caribaeorum* were collected by SCUBA, nine were from Deerfield Beach, Florida in June 2009 (n=6, FL1A-C, FL2A-C) and December 2011 (n=3, FL3A-C) and five samples were collected off the coast of San Salvador, The Bahamas in February 2011 (n=5, SS1-5). Pyrosequencing of 16S rRNA amplicons derived from these samples yielded 66,492

sequences averaging 233 bp in length after removal of short (<200 bp), low quality, chimeric and contaminant sequences. Samples contained between 2968 and 9132 sequence reads with an average of 4749 reads per sample. Bacterial diversity calculations were performed at the species level (*i.e.* OTUs delineated using a 97% sequence identity cutoff) on data sets subsampled to 2960 sequence reads per sample to account for variability in sampling depth. The coverage for all samples ranged from 74.7% (FL1A) to 98.4% (SS3) (86.0 ± 6.7%) [(mean ± standard deviation)] (Table 2.1). These coverage estimates were supported by rarefaction analysis as rarefaction curves for most of the samples approached the plateau of the asymptote (Figure 2.1). Observed bacterial richness ranged from 64 OTUs (SS3) to 1152 OTUs (FL1A) (662 ± 308 OTUs). The Chao1 estimator was used to calculate estimated richness at the species level and ranged from 184 OTUs (SS3) to 2576 OTUs (FL1A) (1464 ± 707 OTUs) (Table 2.1). These richness values are higher than other reported richness estimates for octocorals[17,24,25] and closer to reported values for scleractinian corals.[22] However, accurate comparisons cannot be made between different corals species due to methodological differences between studies (*e.g.* analysis based on different 16S rDNA hypervariable regions and/or sequence annotation methods).[57]

The Shannon diversity (*H'*) index ranged from 0.25 (SS3) to 6.12 (FL3A) (4.26 ± 1.65), and Shannon equitability (*E*) index from 0.06 (SS3) to 0.88 (FL3A) (0.65 ± 0.22) (Table 2.1). Due to non-methodological factors a low number of OTUs were present in Sample SS3, this sample was dominated by one particular OTU (OTU 001) that made up 97.1% of all sequence reads and accounted for the low index values compared to the other samples, which were substantially higher. The high index values calculated for all other *E. caribaeorum* samples indicated a high level of bacterial diversity is harbored by this octocoral.

With the exception of sample SS3, measures of bacterial diversity in *E. caribaeorum* are higher than those reported for other octocorals[17,24,25] but similar to those reported for scleractinian corals.[22] The greater richness and diversity observed in *E. caribaeorum* may in part be due to the encrusting morphology of this octocoral compared to other branching octocorals investigated to date.[17,24,25] Sea plumes/fans are attached to the substrate with an erected branched or fan like structure extending outwards whereas *E. caribaeorum* forms a mat over the surface.[31] This close proximity to the substratum may allow for infiltration of stratum-associated microbes into the coral tissue, as well as microbes from the surrounding water column. Conversely, non-encrusting octocorals would obtain the majority of their microbes from the surrounding water column.

To determine if there was any pattern in OTU community structure between *E. caribaeorum* samples based on time and location of collection, the Yue and Clayton distance indices were calculated. Three major clades were observed in this analysis (Figure 2.2). The top clade contained samples from Florida (both years) and San Salvador. The middle clade contained corals from Florida, which formed sub-clades corresponding to year of collection. The bottom clade consisted of a single sample from The Bahamas (SS5). The microbial communities in the top clade formed a tighter cluster than those in the clades below, indicating a high degree of similarity between the microbial communities in the top clade. Inspection of OTU abundance across all samples revealed that a single OTU (OTU1) was highly abundant (29% to 97% of reads) in all samples in the top clade and substantially less abundant in the other samples (<6%). Thus, the clustering of microbial communities from samples collected from different sample groups is largely driven by the abundance of this OTU. The distribution of this OTU will be discussed in more detail later.

To assess if there was any variation in the community structure of the samples based on location or time of collection the Yue and Clayton distance matrix was analyzed using AMOVA. Groups were delineated as follows: samples F1A-C and FL2A-C were grouped into Florida 2009 (FL09), samples FL3A-C into Florida 2011 (FL11), and samples SS1-5 into San Salvador 2011 (SS11). The employment of AMOVA analysis allowed for the determination of any differentiation in community structure between groups that is statistically significant from differentiation that would arise were the groups pooled together. When comparing the community structure between all three groups there was no statistical significance (AMOVA, $p = 0.066$) (Table 2.2). However pairwise AMOVA between groups revealed a significant difference in community structure between the FL11 and SS11 samples (AMOVA, $p \doteq 0.034$). This indicates that the taxonomic composition of these *E. caribaeorum* microbial communities varied in response to factors that vary temporally and by geographic location. However, differentiation was not observed in any other pairwise comparison (Table 2.2). Factors that may drive the structure of *E. caribaeorum* microbial communities may include changes in water quality, water temperature, or levels of anthropogenic disturbance as has been reported for other corals.[25,58] Unfortunately, detailed environmental data was not collected at the time of sampling, thus further research will be required to determine factors that significantly affect the microbiome of *E. caribaeorum.*

Table 2.1. Richness and alpha diversity measurements of *Erythropodium caribaeorum* microbial communities. OTUs were calculated using a 97% sequence identity threshold. Calculations were based on datasets subsampled to 2960 sequence reads per sample.

Sample		Richness (#OTUs)	Coverage (%)	Chao1 Estimated Richness	Shannon Diversity Index (H')	Shannon Equitability Index (E)
Florida 2009	FL1A	1152	74.7	2576	6.12	0.87
	FL1B	895	80.9	2113	5.81	0.85
	FL1C	578	88.1	1186	4.19	0.66
	FL2A	742	82.7	2022	4.75	0.72
	FL2B	414	91.9	818	3.37	0.56
	FL2C	301	92.5	985	2.20	0.39
Florida 2011	FL3A	1031	78.8	2117	6.08	0.88
	FL3B	355	93.8	569	3.49	0.59
	FL3C	956	80.0	2010	5.77	0.84
San Salvador 2011	SS1	553	88.6	1188	3.47	0.55
	SS2	632	88.6	1111	4.30	0.67
	SS3	64	98.4	184	0.25	0.06
	SS4	724	84.5	1494	4.48	0.68
	SS5	867	80.7	2127	5.31	0.78
Mean		662	86.0	1464	4.26	0.65
SD		308	6.7	707	1.65	0.22

Table 2.2. AMOVA comparisons of community structure (Yue and Clayton index). Samples were grouped by location and time of collection, FL09: Florida 2009; FL11: Florida 2011; SS11: San Salvador 2011. All data sets were subsampled to 2960 sequence reads per sample prior to calculations.

Yue & Clayton Index Community Structure (AMOVA, p-value)	
FL09-FL11-SS11[a]	0.066
FL09-FL11[b]	0.337
FL11-SS11[b]	0.034*
FL09-SS11[b]	0.062

*Statistically significant
[a]Bonferoni-corrected significance level p = 0.017
[b]Significance level p = 0.05

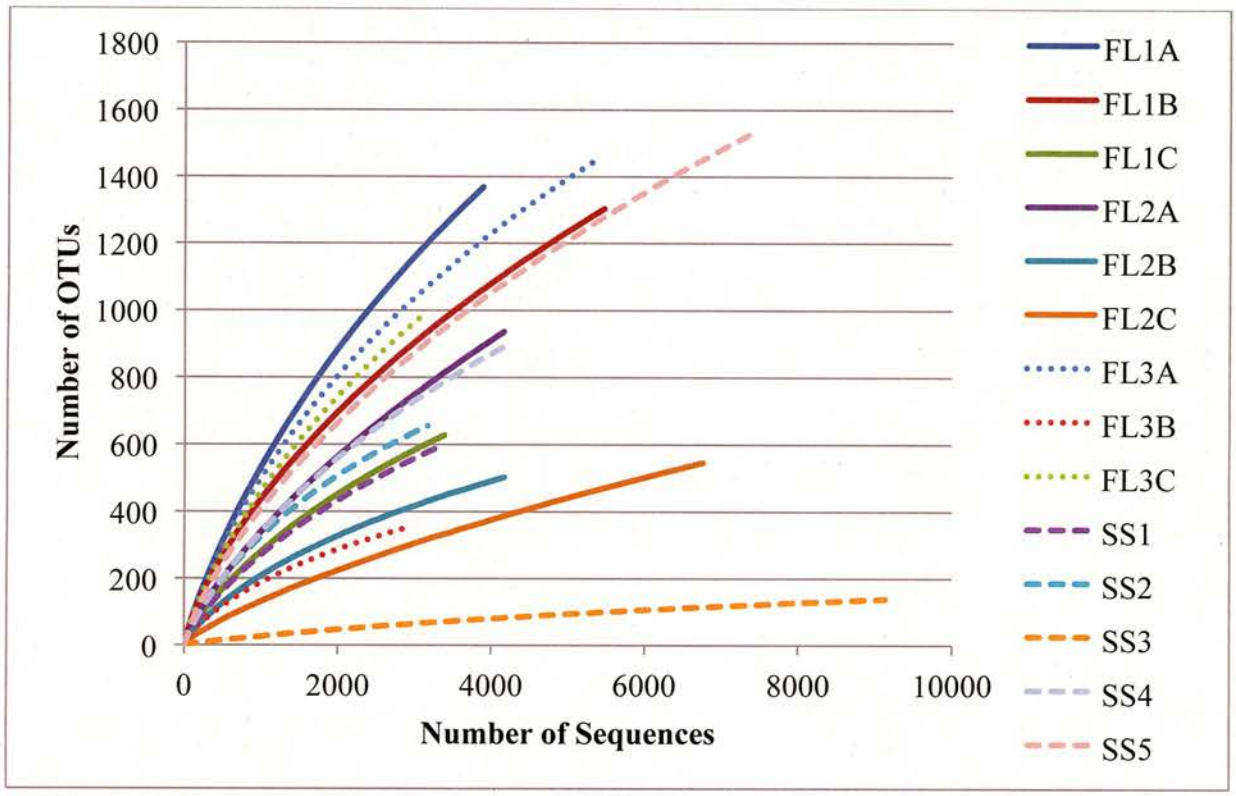

Figure 2.1. Rarefaction curves of 14 *E. caribaeorum* microbiomes. Curves prepared using OTUs identified at a 0.03 distance.

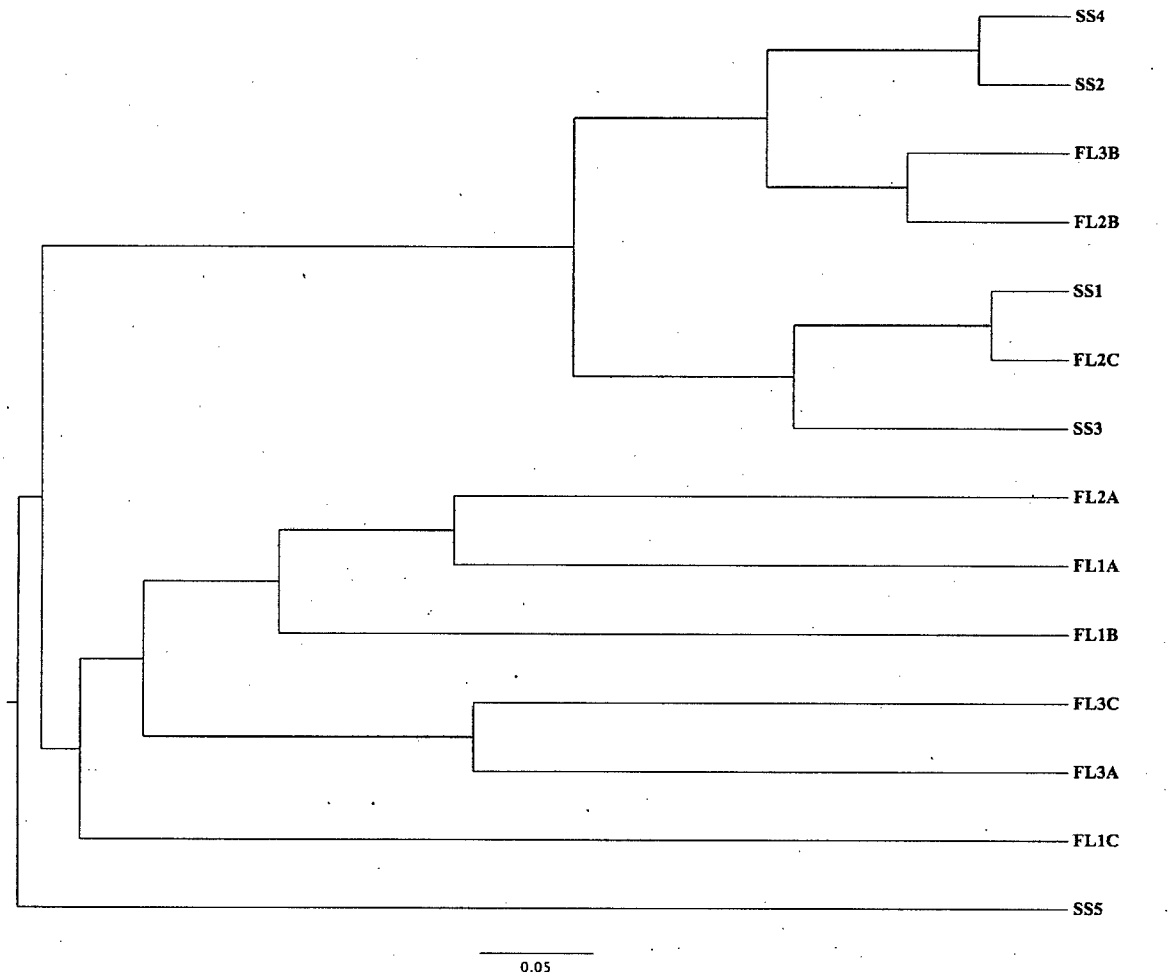

Figure 2.2. Yue and Clayton distance matrix comparison of *E. caribaeorum* microbial communities. All data sets were subsampled to 2960 sequence reads per samples prior to calculations.

2.3.2 Taxonomic Composition

The 66,492 sequence reads were classified using the Greengenes classifier with a confidence threshold of 80%. A total of 33 phyla were present across all samples with 12 to 26 phyla present in each sample. The dominant phylum in all samples was *Proteobacteria*, making up 36.7% to 98.4% of the total population. Other phyla that had a small but consistent presence in all samples were *Bacteroidetes* (0.3% to 18.7%), *Actinobacteria* (0.05% to 10.1%), *Planctomycetes* (0.2% to 4.5%), *Chloroflexi* (0.03% to 18.3%), *Firmicutes* (0.01% to 13.7%), and *Acidobacteria* (0.04% to 6.0%).

Ninety-six classes were present across all samples with 22 to 65 classes present in each sample. The most abundant class was *Gammaproteobacteria.* It accounted for greater than one-third of the sequence reads in 10 of the 14 samples and accounted for 10.5% to 97.9% of the sequences across all samples (Figure 2.3). The majority of these sequence reads belonging to the genus *Endozoicomonas* (order *Oceanospirillaceae*, family *Hahellaceae*), and ranged from 2.9% (SS5) to 97.3% (SS3) of the sequence reads in each a sample (Figure 2.4). The high abundance of this one genus in sample SS3 explains the low richness (Chao1) and diversity levels (*H'*) calculated for this sample. Other classes that were consistently present in all samples were *Alphaproteobacteria* (0.4% to 21.1%), *Flavobacteria* (0.08% to 14.7%), *Anaerolineae* (0.03% to 7.5%), *Acidimicrobiia* (0.04% to 6.0%), *Deltaproteobacteria* (0.1% to 3.8%), *Planctomycetacia* (0.08% to 3.7%), *Actinobacteria* (0.01% to 3.4%), and *Cytophagia* (0.2% to 2.3%). Unclassified OTUs, sequence reads that could not be assigned to a phylum with an 80% confidence threshold, were also prevalent in all the samples ranging from 0.3% to 17.0% of the total population (Figure 2.3 and 2.4).

The taxonomic composition of the *E. caribaeorum* microbiome is similar to that reported for other octocorals, which are also dominated by *Gammaproteobacteria,* and in particular the genus *Endozoicomonas.*[17,24,25] The predominance of *Endozoicomonas* is not unique to the microbiomes of octocorals, as this genus forms a major component of the microbiomes of scleractinian corals,[19,22,59-63] sponges,[64] and many other marine invertebrates.[30,65-71]

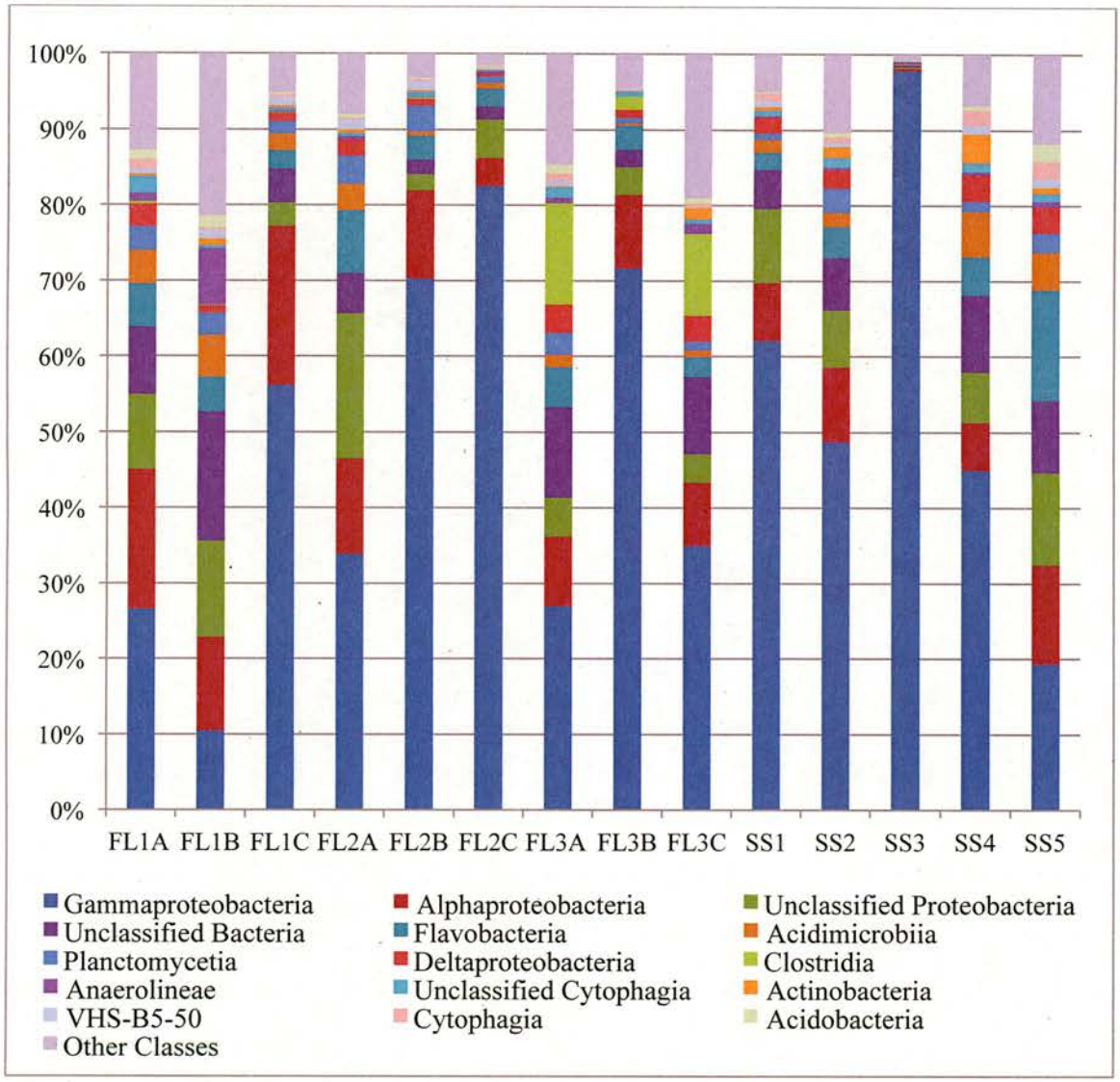

Figure 2.3. Class level composition of *E. caribaeorum* microbiomes. Sequences were classified with a minimum confidence level of 80%. The "Rare Classes" group consists of classes that comprise <1% of the total sequence reads.

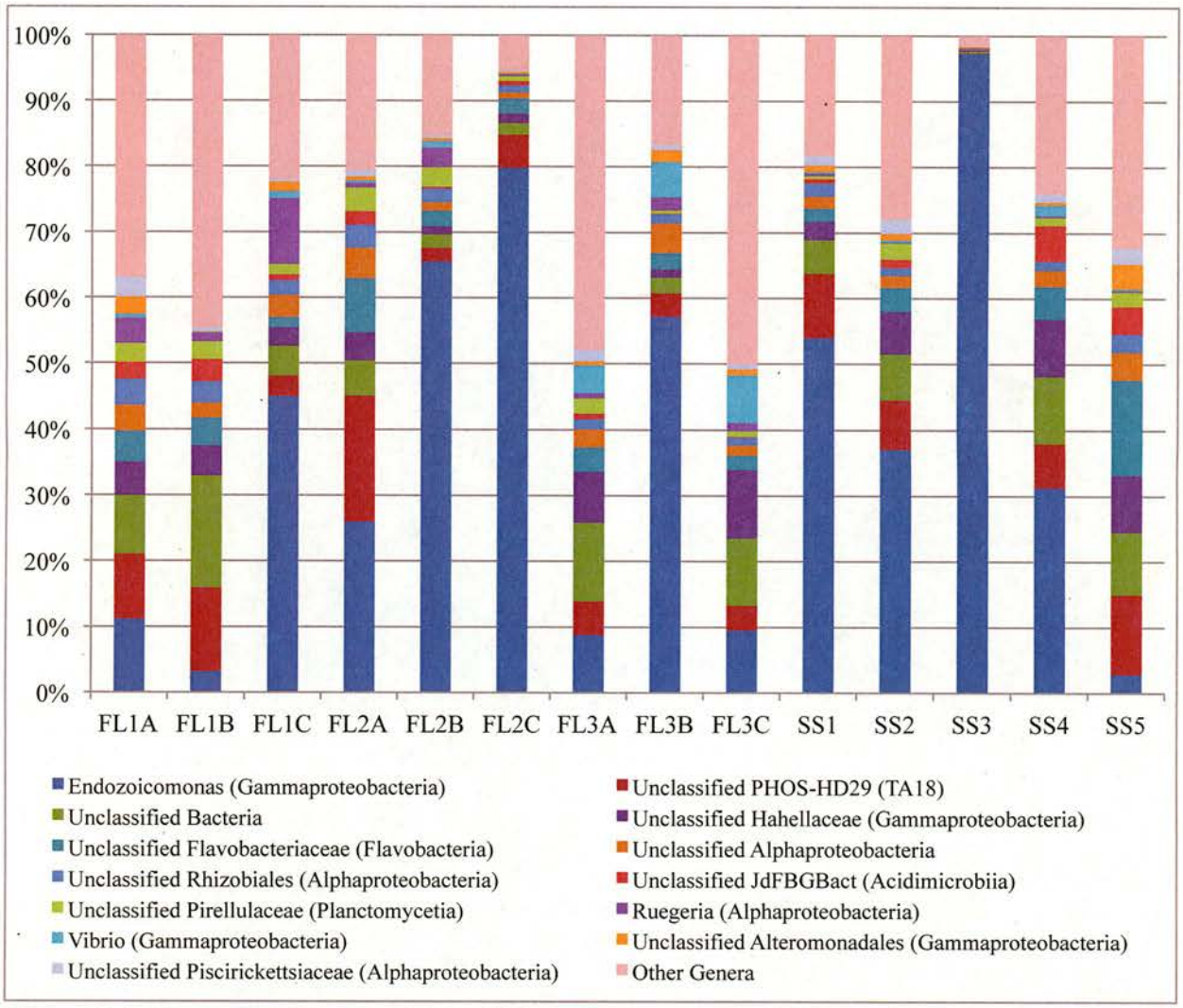

Figure 2.4. Genus level composition of *E. caribaeorum* microbiomes. Sequences were classified with a minimum confidence level of 80%. Read counts other than the 13 most abundant are summarized in the "Other" category. The class level for each taxa are shown in parentheses.

2.3.3 Putative Core Microbiome

Several marine invertebrates have been shown to contain a core microbiome, a set of taxonomically distinct microbes that are a stable presence in the microbiome of the coral regardless of geographic location or environmental factors.[17,19,21,24] The existence of core bacterial taxa in complex microbiomes suggests that these taxa carry out vital functions in the holobiont. To assess whether *E. caribaeorum* possess a core microbiome the distribution of species level OTUs ($D = 0.03$) with a relative abundance > 1% across all coral samples was determined (Table 2.3). Three OTUs were constantly found in all samples regardless of location or time of collection, but their relative abundance was variable and none were present at a relative abundance > 1% across all samples. The most abundant of the stable OTUs, *Endozoicomonas* sp. OTU 001, accounted for 30.1% of all sequence reads, but only accounted for > 1% of the sequence reads in 12 of 14 samples and ranged from 0.7% (FL1B) to 97.1% (SS3) of the total reads. The other two stable OTUs were an unclassified *Alphaproteobacteria* (OTU 007) and an unclassified *Gammaproteobacteria* (OTU 013), these OTUs accounted for 1.9% and 0.6% of the total sequences reads, respectively.

The phylogenetic relationship of these stable OTUs to type strains as well as closely related uncultured strains identified by BLAST analysis, was explored[48] (Figure 2.5). *Endozoicomonas* sp. OTU 001 formed a strongly supported clade (96% bootstrap support) with an uncultured *Spongiobacter* sp. clone (DQ889928, 99.4% identity) that was obtained from an *E. caribaeorum* collected off the coast of Florida in 2006.[72] The genus *Spongiobacter* is not a valid taxonomic classification and sequences that are attributed to this genus show a strong phylogenetic relationship to the genus *Endozoicomonas*. This clade clustered with an uncultured bacterium

clone (JQ609314) that was obtained from the octocoral *Antillogorgia elisabethae*[24] and *Endozoicomonas gorgoniicola* PS125^T (JX488685) which was isolated from an unidentified octocoral belonging to the genus *Plexaura* collected off the coast of Bimini, The Bahamas.[30] It has been suggested that the close relation between *Endozoicomonas* relatives from different octocoral habitats is the result of an evolutionarily old association between the octocoral hosts and the genus *Endozoicomonas*.[17]

While *Endozoicomonas* spp. form significant and stable associations with a variety of marine invertebrates, their role in the holobiont is still enigmatic. They are hypothesized to aid in the sulfur cycling, in particular the degradation of dimethylsulfoniopropionate (DMSP), an osmolyte that is generated in large amounts by the coral *Symbiodinium* spp. endosymbionts that inhabit shallow water corals.[9,11] *Endozoicomonas* spp. have also been implemented in the production of antimicrobial compounds, the degradation of complex organic carbon sources, and the conversion and assimilation of nitrate.[27,60,73,74] However further research will be required to identify the factors driving the association between *E. caribaeorum* and *Endozoicomonas* spp. to determine the role of this stable microbial associate.

The unclassified *Alphaproteobacteria* (OTU 007) formed a clade with strong 99% bootstrap support with two uncultured bacterial sequences (GU118148, 96.5% identity; GU118248, 96.9% identity) detected in the scleractinian coral *Diploria strigosa*.[63] The unclassified *Gammaproteobacteria* (OTU 013) clustered with 70% bootstrap support with an uncultured *Marinobacter* sp. (DQ889901, 98.1% identity), and an uncultured *Gammaproteobacterium* (DQ889884, 95.6% identity) obtained from an *E. caribaeorum* sample from Florida.[72] Stable

microbial associations with various *Alpha-* and *Gammaproteobacteria*, other than *Endozoicomonas*, have been observed in many corals.[18,23,75,76] For example, *Roseobacter* and *Marinobacter* are associated with juvenile *Porites astreoides* and transmitted vertically from parent to offspring.[77] Additionally, Alagely *et al.*[78] showed that *Alphaproteobacteria* and *Marinobacter* spp. isolated from coral mucus and cultured dinoflagellate *Symbiodinium* spp. were able to inhibit swarming and biofilm formation of the coral pathogen *Serratia marcescens*. Further supporting the hypothesis that members of the coral microbiome aid in protection from unwanted pathogens.[15]

The detection of these three phylotypes in all *E. caribaeorum* microbiomes studied regardless of time or location of collection suggest that these corals may maintain core microbiome. However, greater sampling over a larger geographic area and time range would be required to determine if these OTUs are in fact core members of the *E. caribaeorum* microbiome.

Table 2.3. Summary of OTUs (*D=0.03*) that comprise the stable OTUs of the *E. caribaeorum* microbiome. The bottom panel displays the taxonomic assignment of the OTUs based on the Greengenes classifier using a confidence threshold of 80%.

	Sample	OTU % Abundance		
		001	007	013
Florida 2009	FL1A	2.0	5.6	1.2
	FL1B	0.7	4.3	0.1
	FL1C	2.6	1.3	0.8
	FL2A	5.9	9.7	0.1
	FL2B	33.4	0.6	0.2
	FL2C	61.2	3.2	0.03
Florida 2011	FL3A	0.8	0.6	0.2
	FL3B	30.6	0.03	0.4
	FL3C	4.0	0.3	0.3
San Salvador 2001	SS1	51.6	0.1	0.03
	SS2	35.0	0.8	0.5
	SS3	97.1	0.02	0.1
	SS4	29.5	0.3	0.2
	SS5	2.9	0.6	3.0

OTU	Greengenes Taxonomic Assignment
001	*Proteobacteria*; *Gammaproteobacteria*; *Oceanospirillales*; *Hahellaceae*; *Endozoicomonas*
007	*Proteobacteria*; *Alphaproteobacteria*; unclassified
013	*Proteobacteria*; *Gammaproteobacteria*; unclassified

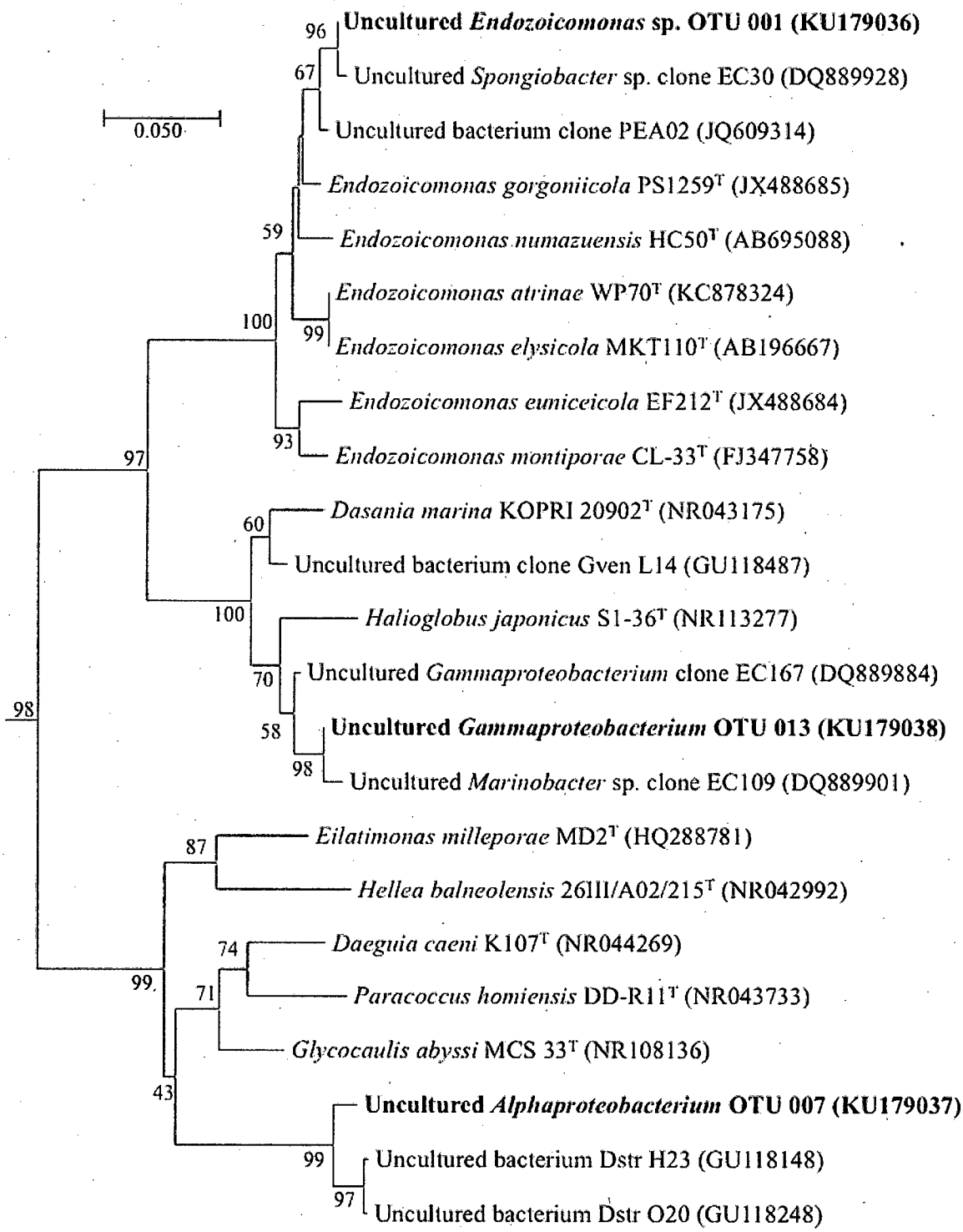

Figure 2.5. Neighbor-joining phylogenetic tree based on 16S rRNA gene sequences of stable OTUs. Evolutionary distances were computed using Jukes-Cantor method. A total of 287 nt

positions were used. Bootstrap values are expressed as a percentage of 1000 replicates; bootstrap values < 50 are not shown. The scale bar represents the number of substitutions per site. The tree was constructed using *Hahella antarctica* NBRC 102683T (GenBank accession no. NR114177), *Kistimonas asteriae* KMD 001T (NR116386), and *Alcanivorax balearicus* MACL04T (NR043109) as out groups (not shown).

2.4 Conclusions

This is the first comprehensive investigation of the microbiome of the encrusting octocoral *E. caribaeorum*. The data presented here demonstrates that these corals possess very high levels of microbial taxonomic diversity relative to other reported octocoral microbiomes. They have a dominant association with the taxonomic class *Gammaproteobacteria*, in particular the genus *Endozoicomonas*. Amid the high microbial diversity there were three stable species-specific OTUs that were present in all *E. caribaeorum* samples regardless of geographic and temporal variation. One of these, *Endozoicomonas* sp. OTU 001, was highly abundant and most likely plays an essential role in the biology of the *E. caribaeorum* holobiont. They may aid in the biogeochemical cycling of nutrients, or are potentially involved in secondary metabolite production. This data provides a valuable starting point for further investigation into the microbiome associated with *E. caribaeorum* and other octocorals in the Caribbean.

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Chapter 3: Culture-Independent Investigation into the Microbiome of *Antillogorgia elisabethae* from San Salvador, The Bahamas, and the Microbial Communities Associated with the Holobiont, Algal Dinoflagellates, and Larvae

Data from section 3.3.2 was published in Microorganism.
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Information from this publication that relates to this data is discussed in section 3.3.3.

3.1 Introduction

3.1.1 The Octocoral *Antillogorgia elisabethae*

The octocoral *Antillogorgia elisabethae* is a purple sea plume that is widely distributed throughout the Caribbean[1] and is a keystone member of many reef communities.[2] It should be noted that this coral was previously classified as *Pseudopterogorgia* spp. but has since been reassigned to the resurrected genus *Antillogorgia.*[3] Previous research investigating the microbiome associated with this coral from Providencia Island, Colombia,[4] as well as Eleuthera, Bimini, and Grand Bahama Island, The Bahamas[5] have been reported. These investigations revealed that *A. elisabethae* have moderate microbial richness and diversity compared to other reported octocorals,[6,7] and that the taxonomic diversity is highly variable across the locations from which the coral is sampled.

A. elisabethae has been of commercial interest over the last two decades as it is the sole source of the pseudopterosin family of diterpene glycoside marine natural products (MNPs).[8-19] These MNPs have attracted attention due to their anti-inflammatory activity, which has been shown to be more potent than the clinically used indomethacin.[8,9,11,20] A simple derivative of pseudopterosin A, methopterosin has successfully completed Phase II clinical trials.[21-23] Unfortunately further clinical development has been stalled due in part to the supply issue. However, their anti-inflammatory properties have made them a valuable component of topical cosmetic products, such as the Resilience® line from Estee Lauder.[24,25]

3.1.2 Algal Dinoflagellate Symbionts of *A. elisabethae*

A. elisabethae, like many shallow water octocorals, have an obligatory symbiotic relationship with algal dinoflagellates of the genus *Symbiodinium* that is mutualistic in nature.[26] The dinoflagellates reside in the gastroderm of the coral and are responsible for providing translocated photosynthates in the form of sugars and amino acids.[27] In exchange, the coral host provides a protected hospitable environment, and inorganic nutrients such as ammonia and phosphate.[28,29] Several studies have investigated the association between coral host species and specific phylotypes of *Symbiodinium* spp.[30,31] In some cases corals will host a number of different *Symbiodinium* spp.,[32-34] while other coral species will only host a specific strain.[35] *A elisabethae* conform to the latter, and only host the *Symbiodinium* clade B1/B184[35,36] which it acquires at the juvenile polyp stage of life through horizontal transmission from the surrounding environment.[37]

The association between marine corals and their algal symbionts is an area of great interest because the breakdown of this association leads to coral bleaching, the expulsion of dinoflagellates from the coral, which can lead to coral mortality.[26,38] However, the dinoflagellates associated with *A. elisabethae* are of additional interest to this study as they have been implicated in pseudopterosin biosynthesis. Work by Mydlarz *et al.*[39] found that pseudopterosins comprised ~ 5% of the lipid extract of the *A. elisabethae* holobiont, but ~11% of the lipid extract of *Symbiodinium* sp. cells purified from the holobiont. Additionally, regions of the holobiont that had a greater density of *Symbiodinium* sp. cells had a greater concentration of pseudopterosins. Furthermore, *Symbiodinium* sp. cells incubated with ^{14}C-$NaHCO_3$ or ^{3}H-geranylgeranyl pyrophosphate (GGPP), pseudopterosin biosynthetic precursors, yielded

radiolabeled pseudopterosins. This suggests that the biosynthetic machinery required for pseudopterosin biosynthesis is present within the *Symbiodinium* sp. cells.

3.1.3 Larvae of *A. elisabethae*

Marine corals reproduce through one of two modes, broadcast or brooding spawning. Broadcast spawning involves the release of a large amount of sperm and eggs into the water column, and fertilization and development occur outside of the parent coral. Brooding spawning involves fertilization within the gastrovascular cavity, followed by brooding of the embryos on the surface of the coral colony before release into the water column.[40] *A. elisabethae* reproduce via brooding spawning and spawn on a lunar cycle which takes place within days of the full moon between November and January.[41,42]

Studies investigating the microbial communities associated with the early and late life stages of corals have revealed that there is commonly a shift in the microbial composition.[43,44] For example, the larvae of *Porites astreoides* have been shown to have a selected association with *Roseobacter* spp. and *Marinobacter* spp.,[45] but *Oceanspirillaceae* dominate the adult coral colonies.[46] *Pocillopora meandrina* larvae have a selective association with *Roseobacter* spp. and *Pseudoalteromonas* spp., but only members of the *Roseobacter* clade remain as a dominant member in the adult coral colonies.[47,48] Studies revealing both vertical (from parent to offspring)[45] and horizontal (from the surrounding environment)[43,47] transmission of microorganisms to coral larvae have been reported. The ecological role these microorganisms have in larvae remains unclear, but they are hypothesized to play a part in influencing larval settlement and metamorphosis.[49-52]

The larvae of *A. elisabethae* of have been implicated in pseudopterosin biosynthesis.[53] These anti-inflammatory compounds made up ~30% of the larval lipid extract, as opposed to the ~11% observed in the *Symbiodinium* sp. cell extract, or ~5% observed in the holobiont extract. Furthermore, when the larvae were incubated with the pseudopterosin biosynthetic precursor ^{3}H-GGPP, ^{3}H-labelled pseudopterosins were produced. Suggesting that like the *Symbiodinium* sp. cells, the larvae contain the biosynthetic machinery required for pseudopterosin biosynthesis. However, the larvae do not contain any *Symbiodinium* sp. cells, as they do not acquire their dinoflagellate symbionts until the juvenile polyp stage of life. This research suggests that pseudopterosin biosynthesis may not be from the dinoflagellates or larvae but from a microbial origin that is associated with both cell types.

3.1.4 Rationale for the Investigation of the Microbiome of *A. elisabethae* from San Salvador and the Microbial Communities Associated with the Larvae and Algal Dinoflagellates Symbiont and Larvae

Investigation of the microbiomes of *A. elisabethae* from San Salvador, The Bahamas will add to the previous body of research on the microbiomes of this species from Colombia and other regions of The Bahamas. As this coral is a dominant member of many reef communities, understanding the microbiomes of this coral is important to understanding the health of the reef ecosystem in which they inhabit. Additionally, the dinoflagellates and larvae are essential to the health and fecundity of this coral; therefore understanding any microbial associations between them may provide insight into the role of those microorganisms within the coral holobiont. The holobiont, dinoflagellates, and larvae are also of specific interest as they have all been implicated

in pseudopterosin biosynthesis and understanding any stable microbial association may provide insight into the biosynthetic source of these industrially important MNPs.

3.1.5 Overall Objective of Study

The overall objective of this study was to investigate the microbiome of *A. elisabethae* from San Salvador, The Bahamas as well as the microbial communities associated with the dinoflagellates and larvae utilizing next generation 16S small subunit rRNA gene amplicon pyrosequencing.

3.2 Materials and Methods

3.2.1 Sample Collections and Initial Processing

Samples from five *A. elisabethae* colonies were collected by SUCBA off the coast of San Salvador, Bahamas in November 2011 during the spawning season from two locations, Runway10 (24°03.816' N, 74° 32.628' W; n=3) and Cable Crossing (24°03.090' N, 74°32.391' W; n=2). All samples were collected within a depth of 10 – 15m and placed into sterile Whirl-Pak™ bags (Nasco®), and at each collection site ~1 L of surrounding seawater was collected into a sterile Nalgene® polypropylene bottle (Nalge Nunc International). The holobiont samples were washed three times in ~40 mL of sterile filtered seawater (SFSW) (0.2 μm polyethersulfone membrane, Nalgene Rapid Flow™) in sterile 50 mL Falcon® centrifuge tubes (50 ml polypropylene, Corning®) to remove loosely associated bacteria. For each sample the larvae were removed from the outer surface of the coral using a sterile scalpel and washed three times with SFSW to remove any coral tissue; there were ~50-100 larvae from each sample. The algal dinoflagellates were obtained using a modified protocol to the one described by Mydlarz *et al.*[39] A portion of the holobiont was homogenized (VWR VDI 25 ULTRA-TURRAX) in SFSW and

filtered through sterile cheesecloth to remove any large coral debris. The dinoflagellates were pelleted by centrifugation (250 x g, 5 min) and further purified using a Percoll® gradient of 40% and 80%. The dinoflagellates were collected from the top of the 80% Percoll® layer, the Percoll® gradient was repeated a minimum of six times until <1% impurities were visible under light microscopy. The bacteria present in the surrounding seawater sample were collected onto 0.22 μm Cellulose Acetate filters (Corning). All samples were divided in two with half used for the culture dependent study (Chapter 4) and the other half were flash frozen in a in a cryogenic vapor shipper (MVE model XC20/3V, Chart Industries, Ball Ground, GA, USA) and transported to Canada. Upon arrival in Canada samples were stored at -80°C until processed.

3.2.2 DNA Extraction and Purification

DNA was isolated from the holobiont, larvae, and dinoflagellate samples using the PowerSoil® DNA Isolation Kit. Holobiont samples (0.5g) were ground into powder in liquid nitrogen prior to kit use. A 1ml aliquot of a SFSW blank was included in the DNA isolation procedure to account for any contaminations that may arise from the DNA isolation kit or initial processing. DNA from bacteria filtered from the surrounding seawater samples was isolated using the UltraClean® Water DNA Isolation Kit. DNA samples were further purified using the PowerClean® DNA Clean-Up Kit. All kits were used according to the manufacturers recommendations (MO BIO Laboratories, Inc. Carlsbad, CA).

3.2.3 Pyrosequencing and Bioinformatics

Bacterial tag-encoded FLX amplicon library construction and 454 pyrosequencing analysis using the 454 GS FLX Titanium system (Roche)[54] was performed by MR DNA (Shallowater, TX).

Amplicons were generated using universal 16S rRNA primers 16S515F (5'-GTGCCAGCMGCCGCGGTAA-3') and 16S806R (5'–GACTACCAGGGTATCTAATCC-3'),[55] which covered the V4 variable region of the 16S rRNA gene. Initial processing of .sff files was performed using mothur v.1.33.3.[56] Sequences were quality filtered using the following conditions: minimum average quality of 30 in each 50-bp window, minimum length of 150 bp, zero ambiguous base calls, and homopolymers less than 9 bp. The resulting filtered sequencing reads were aligned using the Silva reference alignment and the alignments were filtered to remove gaps. Chimeras were identified using UCHIME[57] and removed. The chimera-free sequences were classified using the mothur Bayesian classifier (80% confidence) which uses the mothur formatted Ribosomal Database Project (RDP) dataset.[58] Sequences classified as chloroplasts, mitochondria, and unknown were removed from the analysis. The remaining sequences were clustered using the UCLUST module from QIIME into operational taxonomic units (OTUs) with a pairwise identity threshold of 97%.[59] All diversity calculations were performed using mothur on normalized data sets subsampled to the smallest sample size.[56] *Alpha*-diversity was analyzed using Shannon diversity and equitability indices. *Beta*-diversity was assessed by the analysis of molecular variance (AMOVA)[60] on a Yue and Clayton distance matrix.[61] *Beta*-diversity trees were viewed using FigTree version 1.4.2 (Institute of Evolutionary Biology, University of Edinburgh). Student t-test, ANOVA calculations, and community composition graphs were prepared using Microsoft® Excel® for Mac 2011 version 14.5.8.

3.2.4 Phylogenetic Analysis

Sequence alignments were prepared using MEGA version 6,[62] and phylogenetic histories were inferred using the neighbor-joining methods.[63] Evolutionary distance matrices were generated

using the Jukes-Cantor model[64] in units of number of base substitutions per site. All positions containing gaps and missing data were eliminated. Bootstrap analysis was based on 1000 resampled datasets.[65]

3.3 Results and Discussion

3.3.1 Initial Processing

Samples from five *A. elisabethae* colonies were collected by SCUBA off the coast of San Salvador, The Bahamas in November 2011 during the coral's spawning season at two reef locations. Each *A. elisabethae* specimen was divided into three fractions; the holobiont (H1-H5), larvae (L1-L5), and algal dinoflagellate (D1-D5) fractions. For each collected coral, a surrounding seawater sample was collected adjacent to the coral colony (W1-W5). DNA was extracted from each of the 15 coral derived fractions and the five seawater samples. A SFSW blank was also subjected to the same DNA isolation protocol as the holobiont, dinoflagellate, and larvae samples to control for any contamination introduced during the DNA extraction procedure.

Pyrosequencing of the 16S rRNA genes from these samples generated a total of 78,766 sequences that averaged 231 bp in length after short (<150 bp) and low quality (<30) sequences were removed. Additionally, any overlapping with the SFSW blank control, which contained 40 sequences that consisted of 12 OTUs, was removed. The number of reads varied significantly between samples, ranging from 379 (D3) to 8526 reads per sample (W5) (Table 3.1). The coverage for all samples was sufficient ranging from 95.2% (L5) to 99.4% (H2); any additional coverage would not have uncovered a significant amount of additional diversity. This was further supported by rarefaction analysis in which rarefaction curves for most samples approached an

asymptote (Figure 3.1).

Table 3.1. ***A. elisabethae*** **holobiont (H1-H5), dinoflagellate (D1-D5), larvae (L1-L5), and surrounding seawater (SW1-SW5) collection location, number of sequence reads, and percent coverage for non-normalized data sets.** OTUs were calculated at a distance of 0.03.

Sample		Collection Location	Number Sequences	Coverage (%)
Holobiont	H1	Cable Crossing	3063	97.1
	H2	Runway 10	5478	99.4
	H3	Runway 10	5988	98.9
	H4	Runway 10	5168	97.9
	H5	Cable Crossing	5950	98.4
Dino	D1	Cable Crossing	6596	99.2
	D2	Runway 10	473	95.8
	D3	Runway 10	379	95.8
	D4	Runway 10	3097	98.4
	D5	Cable Crossing	4596	98.9
Larvae	L1	Cable Crossing	3704	97.9
	L2	Runway 10	1059	97.4
	L3	Runway 10	525	97.1
	L4	Runway 10	843	99.3
	L5	Cable Crossing	483	95.2
Water	W1	Cable Crossing	3345	97.7
	W2	Runway 10	4247	98.6
	W3	Runway 10	7680	98.9
	W4	Cable Crossing	7566	98.4
	W5	Cable Crossing	8526	98.9

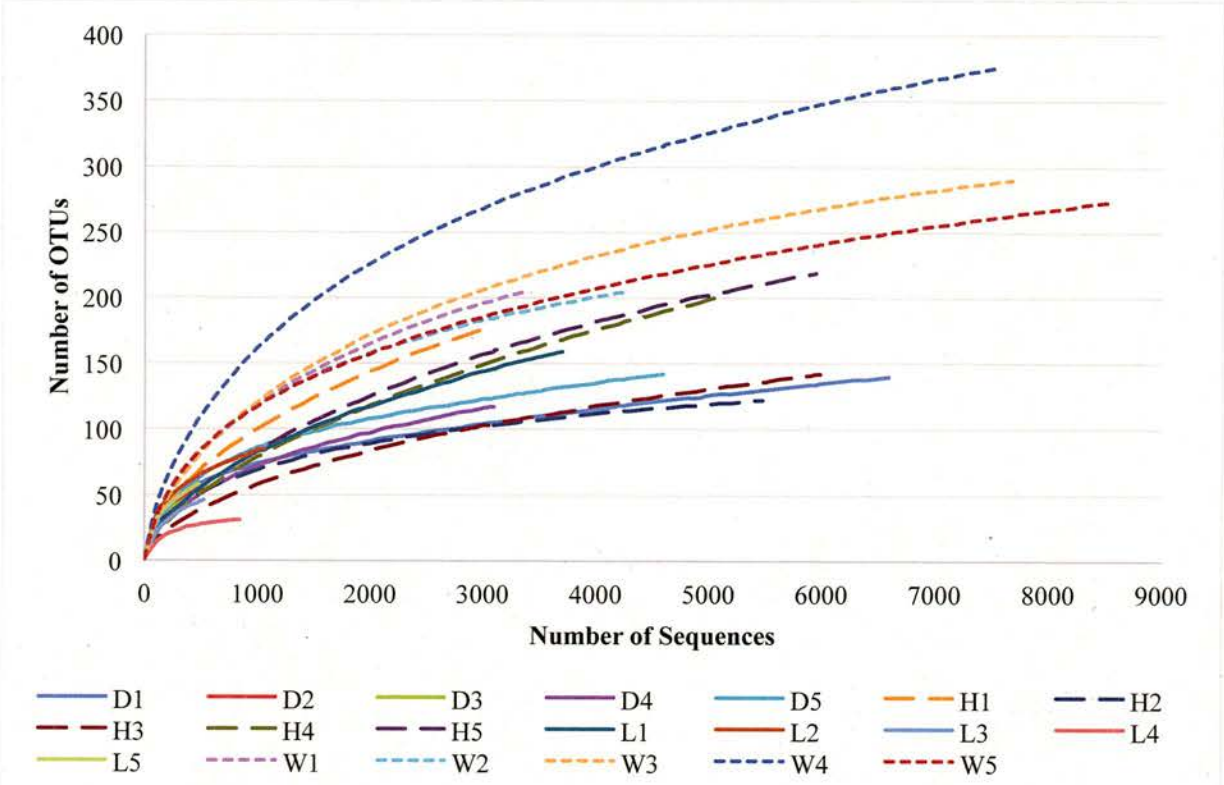

Figure 3.1. Rarefaction curve of all coral-derived and seawater datasets. Curves prepared using OTUs identified at a 0.03 distance.

3.3.2 Comparison of Microbial Communities between Holobiont and Surrounding Seawater Samples

Although it has been well documented that the microbiomes associated with corals are distinct from the surrounding environment, the microbial communities associated with the holobiont and seawater samples were compared to confirm this observation with the San Salvador *A. elisabethae* samples in this study.

3.3.2.1 *Alpha-* and *Beta*-Diversity

All holobiont and seawater sample analyses were normalized to 3000 sequence reads per sample. The normalized observed richness (S_{obs}) for the holobiont samples ranged from 102 OTUs (H2 & H3) to 176 OTUs (H1) (172 ± 49 OTUs) (mean ± standard deviation) and the Chao1 estimated richness (S_{est}) ranged from 137 OTUs (H2) to 322 OTUs (H4) (274 ± 73 OTUs). The holobiont Shannon diversity (*H'*) index ranged from 2.13 (H3) to 3.12 (H1) (2.98 ± 0.49), and Shannon equitability (*E*) index from 0.45 (H4) to 0.65 (H2) (0.58 ± 0.07) (Table 3.2). The seawater samples had slightly higher observed (S_{obs} = 208 ± 35 OTUs) and estimated richness (S_{est} = 302 ± 59 OTUs) than the holobiont samples. The greater observed richness in the water samples was statistically significant (Student's two-sample t-test, p = 0.006) while the estimated richness was not (t-test, p = 0.121). The Shannon diversity index (*H'* = 302 ± 59) and equitability index (*E* = 0.62 ± 0.03) were both slightly higher than that of the holobiont, but only the Shannon diversity index was significantly greater than in the holobiont samples (t-test, p = 0.009). These richness and diversity levels are consistent with previously reported seawater microbial communities.[5,6,66-68]

To compare the community composition between the holobiont and seawater samples the Yue and Clayton distance index was calculated. The holobiont and seawater samples formed distinct clades (Figure 3.2). Additionally, the seawater samples formed a tighter clade than the holobiont samples, indicating a greater degree of similarity in the seawater microbial communities.

To evaluate the statistical significance of the observed clades the distance matrix were assessed using AMOVA. The holobiont microbial communities differed significantly from the surrounding seawater samples (AMOVA, $p = 0.009$). This is consistent with other studies of coral microbiomes which have found corals harbor microbial communities distinct from that of the surrounding sea water.[6,67-69]

Table 3.2. Richness and alpha diversity measurements of *A. elisabethae* holobiont and surrounding seawater microbial communities. OTUs were calculated at a distance of 0.03. Calculations were based on datasets normalized to 3000 sequence reads per sample.

Sample	Richness (# OTUs, S_{obs})	Chao1 Estimated Richness (S_{est})	Shannon Diversity (H')	Shannon Equitability (E)	Coverage (%)
H1	176	306	3.12	0.60	97.1
H2	102	137	2.98	0.65	99.0
H3	102	183	2.13	0.46	98.3
H4	149	322	2.26	0.45	97.1
H5	157	279	2.64	0.52	97.2
Mean	**137**	**245**	**2.63**	**0.54**	**97.8**
SD	**34**	**81**	**0.43**	**0.09**	**0.8**
W1	196	270	3.19	0.60	97.5
W2	183	242	3.20	0.62	97.9
W3	206	311	3.09	0.58	97.0
W4	268	398	3.75	0.67	96.4
W5	185	290	3.24	0.62	97.6
Mean	**208**	**302**	**3.29**	**0.62**	**97.3**
SD	**35**	**59**	**0.26**	**0.03**	**0.6**

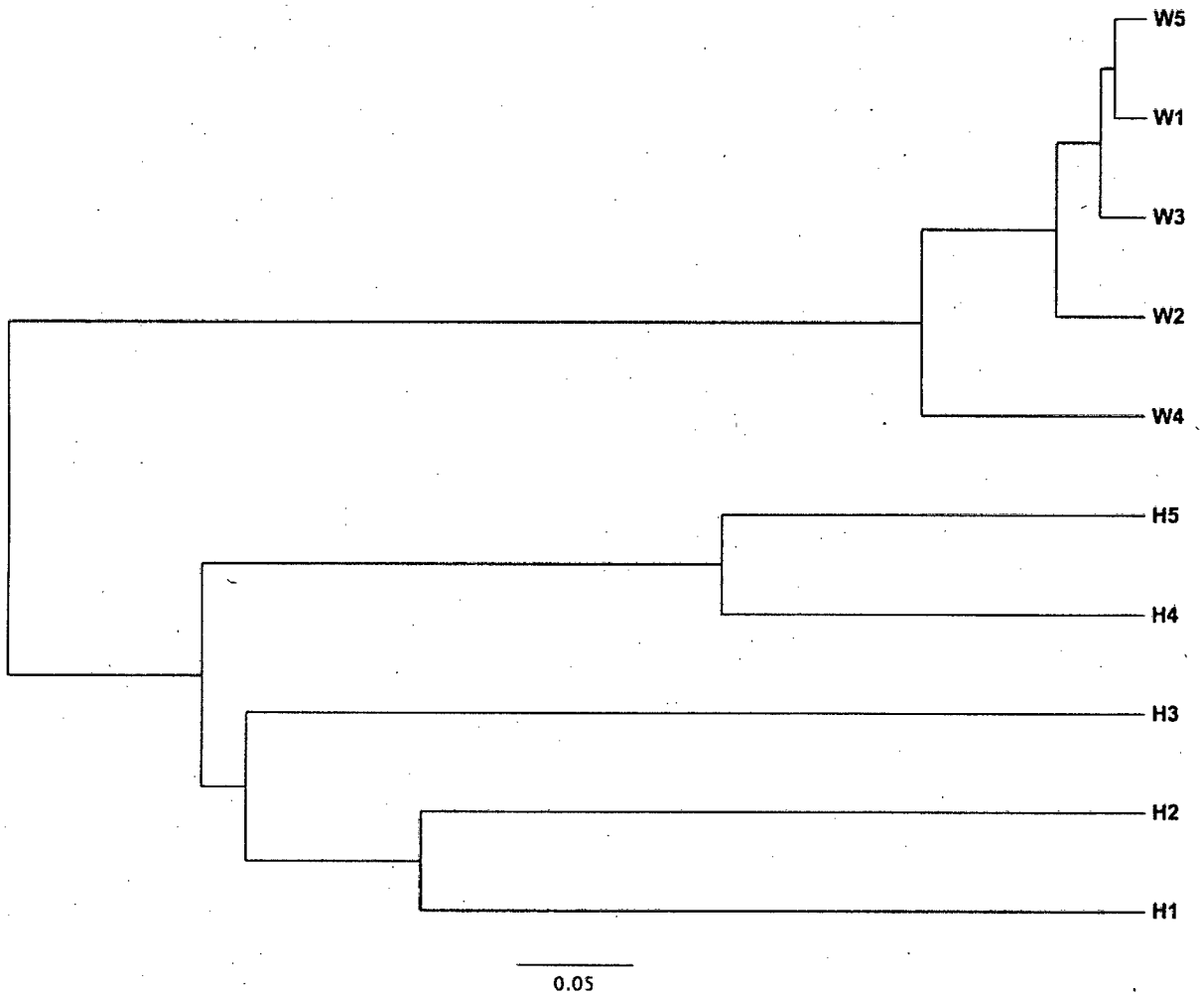

Figure 3.2. Yue and Clayton distance matrix comparison of *A. elisabethae* holobiont and surrounding seawater microbial communities. All datasets were subsampled to 3000 sequence reads per samples prior to calculations.

3.3.2.2 Taxonomic Composition

The 25,647 holobiont sequence reads were classified using the RDP classifier with a confidence threshold of 80%. A total of 13 phyla were present across all holobiont samples with 7 to 10 phyla present in each sample. The dominant phylum in all holobiont samples was *Proteobacteria*, making up 17.8% to 78.5% of the total population in each sample. Other phyla that had a small but consistent presence in all samples were *Bacteroidetes* (1.4% to 4.1%), *Actinobacteria* (0.02% to 1.7%), *Firmicutes* (0.02% to 0.9%), *Cyanobacteria* (0.03% to 1.0%), and *Planctomycetes* (0.02% to 0.1%). Twenty-one different classes were present across all holobiont samples with 12 to 17 classes present in each sample. The most abundant class was *Gammaproteobacteria*, accounting for 17.2% to 73.5% of the total holobiont sequence reads (Figure 3.3). The dominant classifiable genera within this class that were detected in all samples were *Endozoicomonas* (9.0% to 21.8%), *Vibrio* (0.3% to 34.0%), and *Pseudoalteromonas* (0.9% to 3.4%) (Figure 3.4), all of which have been implicated in the degradation of dimethylsulfoniopropionate (DMSP), an osmolyte that is generated in high abundance by the dinoflagellates in the coral.[70,71] Other classes that had a consistent presence in the holobiont samples were *Flavobacteria* (0.9% to 3.8%), *Alphaproteobacteria* (0.4% to 1.5%), *Actinobacteria* (0.3% to 1.5%), *Cyanobacteria* (0.05% to 1.0%), *Clostridia* (0.02% to 0.6%), and *Planctomycetacia* (0.02% to 0.09%). Unclassified OTUs, sequence reads that could not be assigned to a phylum with an 80% confidence threshold, were also prevalent in all samples ranging from 16.8% to 79.1%..

There were a total of 31,364 seawater sample sequence reads, a total of 13 phyla were present across all samples, with 8 to 10 samples present in each sample. The dominant phylum in all

samples was *Proteobacteria*, making up 48.8% to 57.0%. Other phyla that had a consistent presence in all water samples were *Cyanobacteria* (24.9% to 37.5%), *Bacteroidetes* (4.7% to 8.3%), *Verrucomicrobia* (1.2% to 3.1%), *Actinobacteria* (0.6% to 4.4%), *Planctomycetes* (0.4% to 0.6%), and *Firmicutes* (0.03% to 0.3%). There were 21 different classes present in all the seawater samples, with 14 to 16 classes in each sample. The two most abundant classes were *Alphaproteobacteria* (31.4% to 37.7%) and *Cyanobacteria* (24.9% to 37.5%) (Figure 3.3). The most abundant classifiable genera within *Alphaproteobacteria* were *Pelagibacter* (26.4% to 31.1%) and *Erythrobacter* (0.04% to 12.2%), while the GpIIa group were the most abundant *Cyanobacteria* (29.8% to 38.5%) (Figure 3.4). Other classes that had a consistent presence were *Gammaproteobacteria* (7.5% to 11.9%), *Flavobacteria* (3.8% to 7.3%), *Actinobacteria* (0.6% to 1.9%), *Opitutae* (0.9% to 2.7%), *Planctomycetacia* (0.4% to 0.6%), *Verrucomicrobiae* (0.07% to 0.5%), and *Sphingobacteria* (0.06% to 0.2%). This difference in taxonomic composition is consistent with the results of the *beta*-diversity calculations (Figure 3.2). This taxonomic composition was consistent with other reported seawater samples that were also dominated by GpIIa and *Pelagibacter*.[6,67,68] Unclassified OTUs were less prominent than in the holobiont samples and range from 13.7% to 25.7% of the total sequence reads per sample.

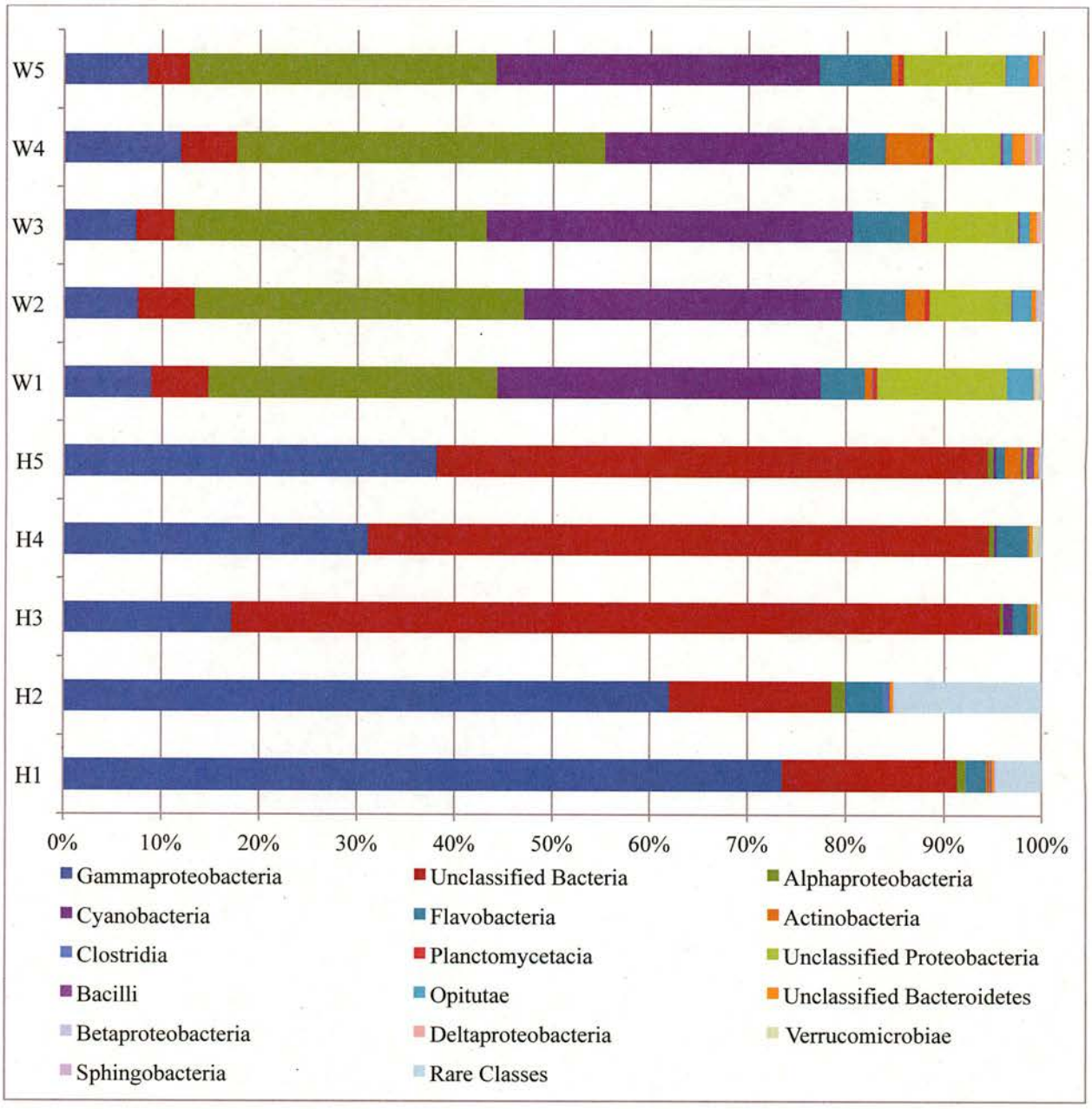

Figure 3.3. Class level microbial composition of holobiont and seawater samples. Sequences were classified with a minimum confidence level of 80%. The "Rare Classes" group consists of classes that comprise <1% of the total sequence reads

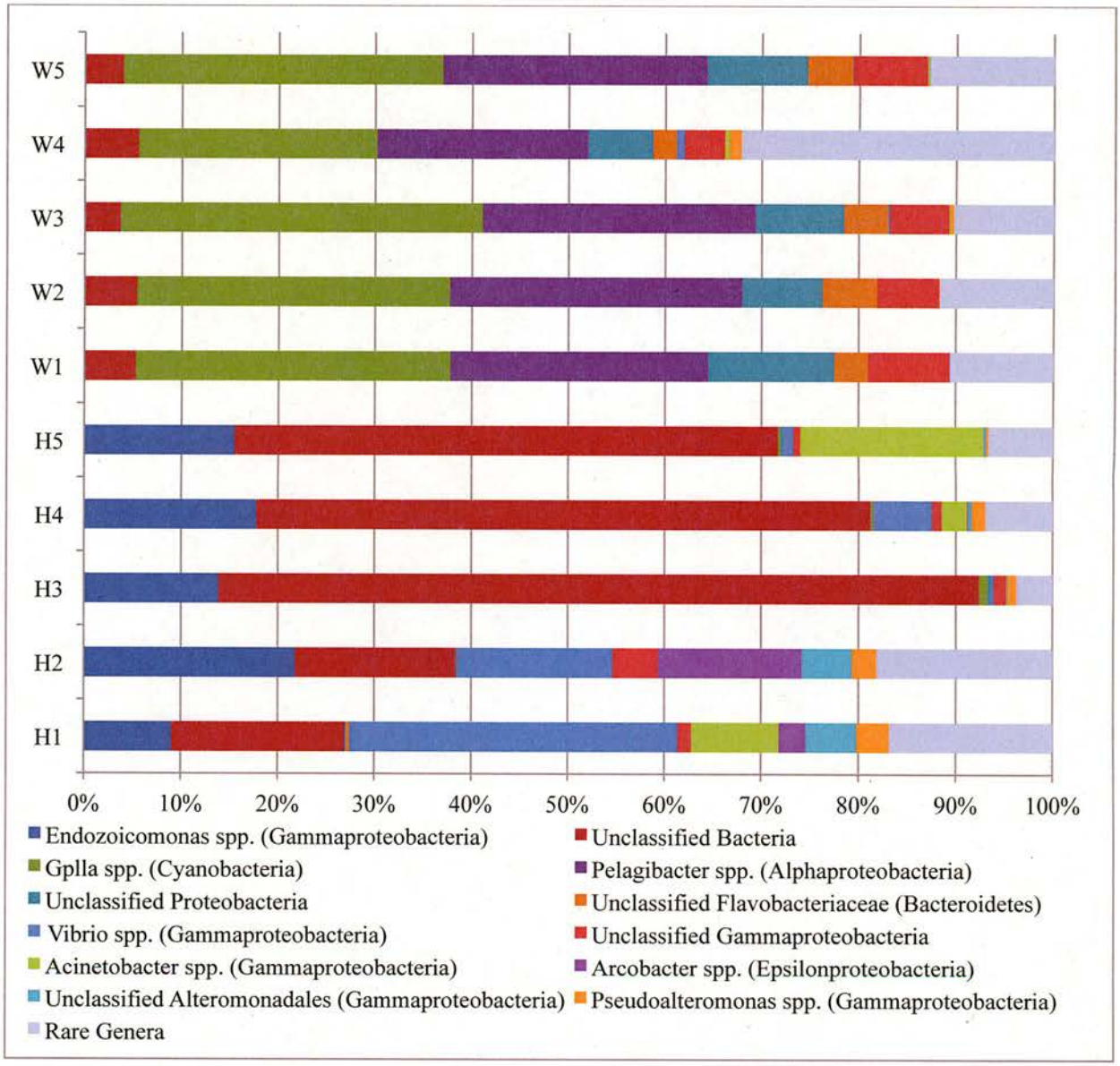

Figure 3.4. Genus level microbial composition of holobiont and seawater samples.

Sequences were classified with a minimum confidence level of 80%. Read counts other than the 12 most abundant are summarized in the "Other" category. The class level for each taxa are shown in parentheses.

3.3.3 Comparison to Other Reported *A. elisabethae* Microbiomes

In order to determine if *A. elisabethae* has a core microbiome, a set of taxonomically distinct microbes that are a stable presence in the microbiome of the coral regardless of geographic location or environmental factors.[4,6,68,72] The San Salvador holobiont microbiomes were compared to previously reported *A. elisabethae* microbiomes from Providencia Island, Colombia, as well as Eleuthera, Bimini, and Grand Bahama Island, The Bahamas have been reported[4,5,67] (Figure 3.5).

3.3.3.1 *Alpha-* and *Beta*-Diversity

The richness values reported for the San Salvador microbiomes were similar to those previously reported for *A. elisabethae* off the coast of Colombia (S_{obs} = 184 ± 126 OTUs, S_{est} = 291 ± 159 OTUs), Eleuthera (S_{obs} = 73 ± 21 OTUs, S_{est} = 189 ± 55 OTUs), Bimini (S_{obs} = 121 ± 88 OTUs, S_{est} = 337 ± 203 OTUs), and Grand Bahama Island (S_{obs} = 96 ± 59 OTUs, S_{est} = 241 ± 135 OTUs),[4,66] and appear within the range of richness values for other reported octocoral microbiomes[6,67] (Figure 3.6). However direct comparisons cannot be made between this study and previously reported studies as those microbiomes were analyzed using different 16S rRNA gene hypervariable regions. Analysis of different regions of the 16S rRNA gene can result in different outcomes in terms of diversity and taxonomic composition.[73] When observing the Shannon indices, the San Salvador microbiomes are similar to previously reported values for *A. elisabethae* collected off the coast of Colombia (H' = 2.13 ± 0.19, E = 0.43 ± 0.03), Eleuthera (H' = 2.14 ± 0.21, E = 0.50 ± 0.03), Bimini (H' = 2.41 ± 1.45, E = 0.50 ± 0.23), Grand Bahama Island (H' = 2.29 ± 1.20, E = 0.50 ± 0.20)[4,5,66] and within the range reported for other octocoral microbiomes (Figure 3.7).

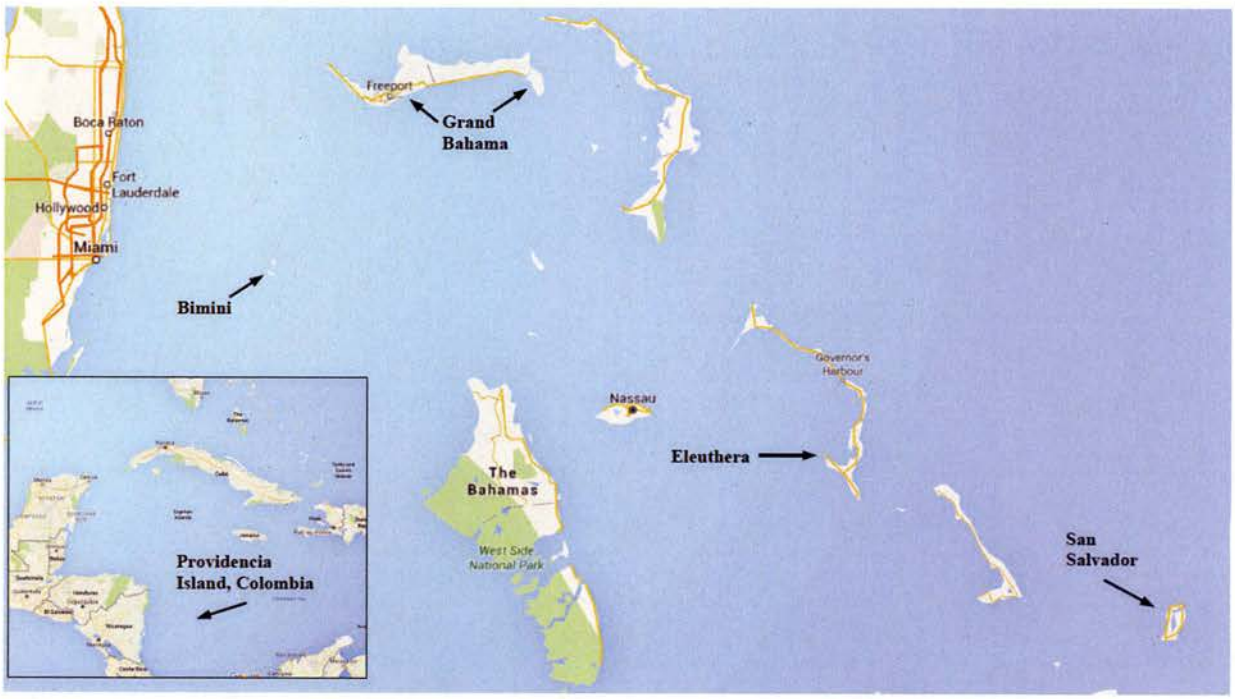

Figure 3.5. *A. elisabethae* sampling locations. Inset map shows the location of Providencia Island, approximately 1390 km from Bimini, The Bahamas. Map prepared using Google Maps™ mapping service.

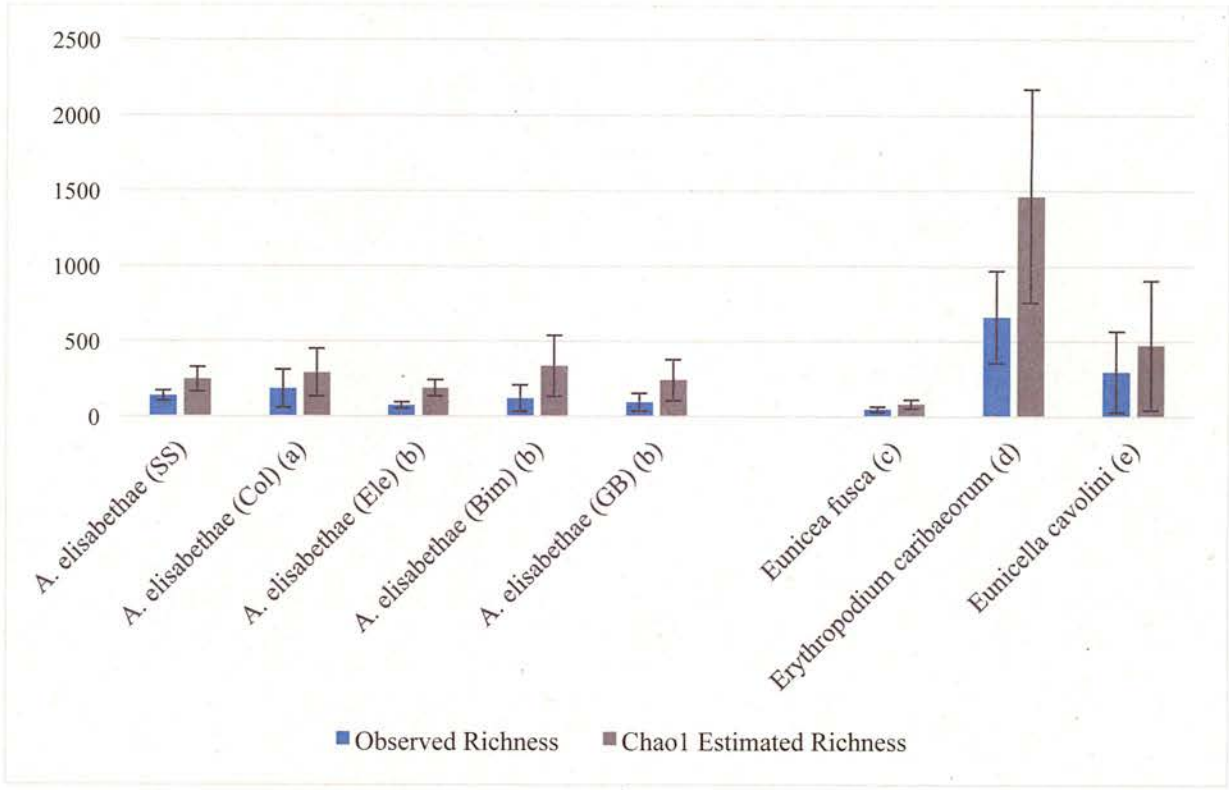

Figure 3.6. Observed and chao1 estimates richness for all reported *A. elisabethae* and octocoral microbiomes investigated using 454 pyrosequencing. SS: San Salvador; Col: Colombia; Ele: Eleuthra; GB: Grand Bahama: Bim: Bimini. Bars represent standard deviation. References: (a) Correa *et al.*[4]; (b) Robertson *et al.*[5]; (c) Pike *et al.*[67]; (d) McCauley *et al.* (Chapter 2) (e) Bayer *et al.*[6].

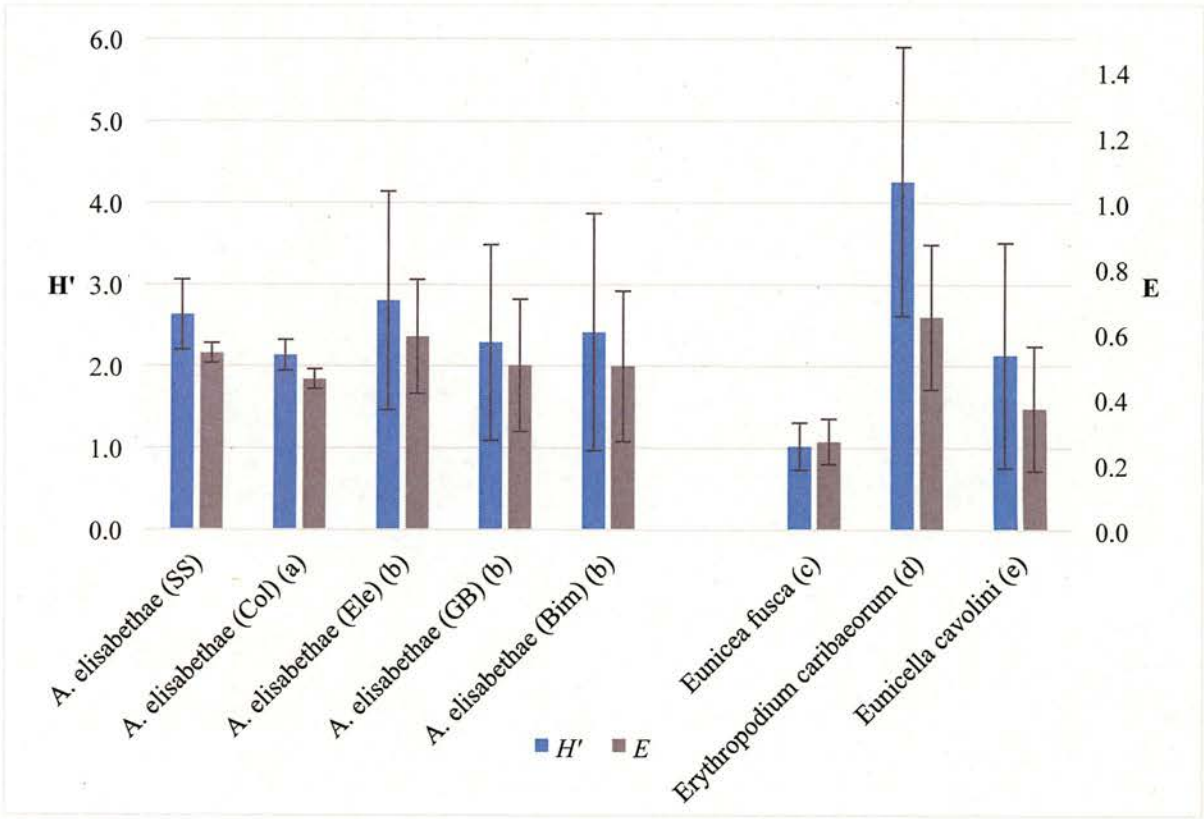

Figure 3.7. Shannon diversity and equitability indices for all reported *A. elisabethae* and octocoral microbiomes investigated using 454 pyrosequencing. SS: San Salvador; Col: Colombia; Ele: Eleuthra; GB: Grand Bahama: Bim: Bimini. Bars represent standard deviation. References: (a) Correa *et al.*[4]; (b) Robertson *et al.*[5]; (c) Pike *et al.*[67]; (d) McCauley *et al.* (Chapter 2) (e) Bayer *et al.*[6].

3.3.3.2 Taxonomic Composition

The taxonomic composition of the San Salvador microbiomes from this study had similarities at the higher taxonomic levels to those from Providencia Island, Colombia,[4] both were dominated at the class level by *Gammaproteobacteria* (73.9% to 83.4%) (Figure 3.8). However, there were variations at the lower taxonomic levels, while all *A. elisabethae* samples had a consistent *Endozoicomonas* spp. presence, the major genus in Colombian samples was *Pseudomonas* spp. (31.8% to 48.1%); a group not detected in any of the San Salvador samples. As for the genera consistently found in the San Salvador samples, *Vibrio* spp. was only detected in two of the three Colombian samples, and *Pseudoalteromonas* spp. was not detected in any samples.

Even greater taxonomic variability was observed when comparing the microbiomes associated with *A. elisabethae* collected off the coast of Eleuthera, Bimini, and Grand Bahama Island, The Bahamas.[5] Unlike the samples from San Salvador and Colombia, there were no observable taxonomic trends based on where they were collected. These microbiomes were dominated either by *Gammaproteobacteria* (0.3% to 86.0%), *Cyanobacteria* (0.6% to 54.6%), *Alphaproteobacteria* (1.1% to 50.7%), or *Flavobacteria* (0.0% to 64.3%) (Figure 3.8). At the genus level no classifiable taxa were consistent across all 14 samples. *Endozoicomonas* spp. were detected in all of the samples collected from Eleuthera, but in only three of the five samples from Bimini, and four of the five samples from Grand Bahama Island. However, closely related bacteria, unclassified bacteria within the order *Oceanospirillales*, were detected in all samples and accounted for 0.04% to 84.1% of the total sequence reads.

The high level of taxonomic heterogeneity observed among *A. elisabethae* microbiomes from specimens collected throughout the Caribbean suggests that this coral does not require a stable association with any specific bacterial species for survival. One of the major roles of microorganisms in the coral holobiont is the biogeochemical cycling of nutrients.[74] The genes involved in these processes can be found in a variety of origins and are not specific to one taxonomic group.[71,75,76] It has been hypothesised that in corals with high microbial taxonomic heterogeneity, such as *A. elisabethae,* the functional potential of the microorganisms in the holobiont is more important than the taxonomic composition. The microbes in these holobionts most likely adapted to local conditions through horizontal gene transfer.[77] Therefore *A. elisabethae* may not require any one specific taxa of microorganism as was observed in *Erythropodium caribaeorum* (Chapter 2), but instead can utilize a wide variety of microbes to accomplish the functions required of the microbiome within the holobiont.

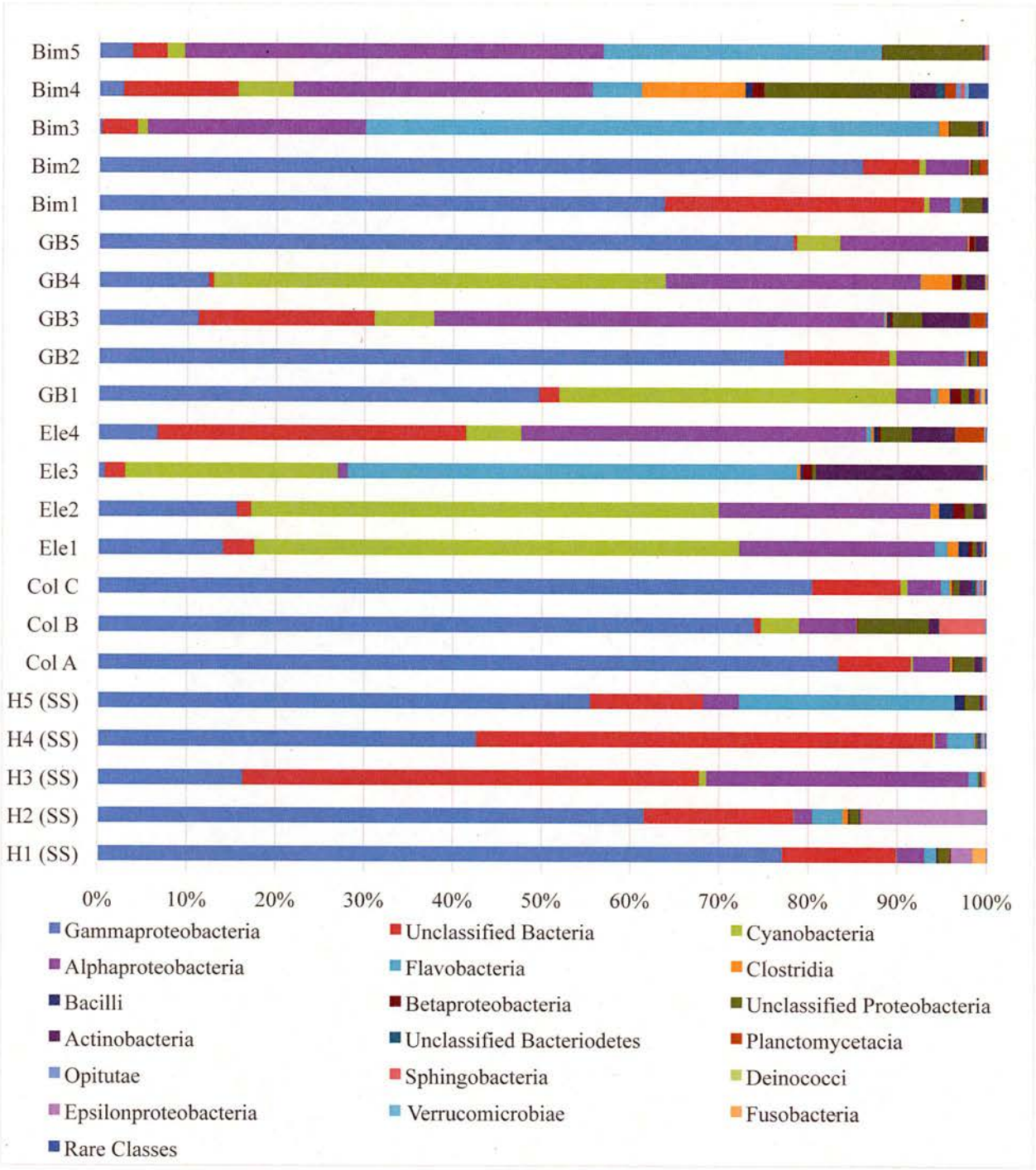

Figure 3.8 - Class level composition of all reported *A. elisabethae* microbiome. Sequences were classified with a minimum confidence level of 80%. The "Rare Classes" group consists of classes that comprise <1% of the total sequence reads. Bim: Bimini, GB: Grand Bahamas, Ele: Eleuthera[5]; Col: Colombia[4]; H: Holobiont, SS: San Salvador (this study)

3.3.4 Comparison of Holobiont, Dinoflagellate and Larvae Samples.

The holobiont, dinoflagellates, and larvae have all been implicated in pseudopterosin biosynthesis, therefore any stable microbial association between these coral derived fractions may provide insight into the true producer of these bioactive MNPs. Additionally, the dinoflagellates and larvae are vital to the overall health and fecundity of the coral colony, so understanding and stable association may provide insight into the role those microbes play in the coral holobiont.

3.3.4.1 *Alpha-* and *Beta*-Diversity

All samples were normalized to 370 sequence reads per sample for comparison to the dinoflagellate and larvae samples that had lower numbers of sequence reads (Table 3.1). This decreased the diversity calculations for the holobiont samples, as *alpha*-diversity measurements are highly sensitive to sampling size. The normalized observed richness (S_{obs}) ranged from 33 OTUs (H3) to 59 OTUs (H1) (45 ± 8 OTUs) and the Chao1 estimated richness (S_{est}) ranged from 76 OTUs (H3) to 122 OTUs (H1) (100 ± 19 OTUs). These richness values were similar to that of the dinoflagellate (S_{obs} = 50 ± 5 OTUs, S_{est} = 76 ± 13 OTUs) and larvae (S_{obs} = 45 ± 13 OTUs, S_{est} 73 ± 30 OTUs) samples (Table 3.3). There was no significance between any of the sample types for the observed (ANOVA, p = 0.640) or estimated richness (ANOVA, p = 0.147) (Figure 3.9). The holobiont Shannon indices also decreased with the smaller sampling size (H' = 2.50 ± 0.37, E = 0.66 ± 0.07) and were similar to index values calculated for the dinoflagellates (H' = 2.46 ± 0.24, E = 0.63 ± 0.06) and larvae (H' = 2.36 ± 0.86, E = 0.61 ± 0.19) (Figure 3.10). As with the calculated richness values, there was no significance between the diversity (ANOVA, p = 0.926) and equitability (ANOVA, p = 0.855) for these sample types.

The dinoflagellate and larvae are subsets of the holobiont samples, meaning the microbes detected in the holobiont samples encompass the microbes associated with the dinoflagellates and larvae samples. Therefore it would be expected that the calculated richness and Shannon indices be lower in these samples. Previously imaged octocoral bacteria were ~ 2 to 4μm in diameter, while dinoflagellates are ~ 8 to 10μm in diameter, and the larvae ~ 400-500μm in diameter.[78,79] A reduction in these values would also be expected based on the physical size of the dinoflagellates and larvae. Therefore, especially in the dinoflagellate cells, there would only be room for a few bacterial cells. If any specific associations were occurring in the dinoflagellate cells the Shannon indices would be substantially lower than that of the holobiont. Since this is not the case it may be that there is no specific microbial association with the dinoflagellate cells, and the microbes detected in the samples are trace amounts of the holobiont microbes carried over from the holobiont during the purification of the dinoflagellate cells.

To determine if the microbes detected in these samples were in fact a dilution of the holobiont microbiome the overall community compositions were compared using the Yue and Clayton indices. If the dinoflagellate or larvae samples were a dilution of the holobiont microbiomes remaining after purification of these samples, it would be expected that there would be no statistical difference in the community compositions when subsampled to the same sequencing depth.

When comparing the dinoflagellates to the holobiont, three distinct clades were observed (Figure 3.11A), the top clade contained four holobiont samples, the middle clade three dinoflagellate samples, and the bottom clade one holobiont and two dinoflagellate samples. When the distance

matrix was accessed using AMOVA the samples were significantly different from each other (AMOVA, $p = 0.03$). When comparing the community structure between the holobiont and larvae samples, two distinct clades were observed one clade contained all the larvae samples and one holobiont samples, while the other contained the remaining holobiont samples (Figure 3.11B). The groups were significantly different from each other (AMOVA, $p = 0.02$).

This data suggests that the microbial communities associated with these fractions may not be a dilution of the holobiont microbiome but distinct communities associated with the dinoflagellates and larvae.

Table 3.3. Richness and alpha diversity calculations of *A. elisabethae* holobiont, dinoflagellate, larvae, microbial communities. OTUs were calculated at a distance of 0.03. Calculations were based on datasets normalized to 370 sequence reads per sample.

	Sample	Observed Richness (# OTUs, S_{obs})	Chao1 Estimated Richness (S_{est})	Shannon Diversity (H')	Shannon Equitability (E)	Coverage (%)
Holobiont	H1	59	122	2.95	0.73	91.4
	H2	45	80	2.88	0.76	94.6
	H3	33	76	2.03	0.58	94.7
	H4	43	110	2.13	0.57	92.9
	H5	47	114	2.50	0.65	92.6
	Average	**45**	**100**	**2.50**	**0.66**	**93.2**
	SD	**8**	**19**	**0.37**	**0.07**	**1.26**
Dino	D1	53	73	2.51	0.63	94.5
	D2	50	71	2.61	0.67	94.8
	D3	47	60	2.72	0.71	95.7
	D4	44	84	2.12	0.56	93.5
	D5	55	93	2.33	0.58	92.2
	Average	**50**	**76**	**2.46**	**0.63**	**94.1**
	SD	**5**	**13**	**0.24**	**0.06**	**1.3**
Larvae	L1	47	109	2.54	0.66	92.6
	L2	58	86	3.15	0.77	94.0
	L3	40	55	2.10	0.57	95.5
	L4	25	32	1.01	0.31	97.8
	L5	52	83	3.00	0.76	94.6
	Average	**45**	**73**	**2.36**	**0.61**	**94.9**
	SD	**13**	**30**	**0.86**	**0.19**	**1.9**

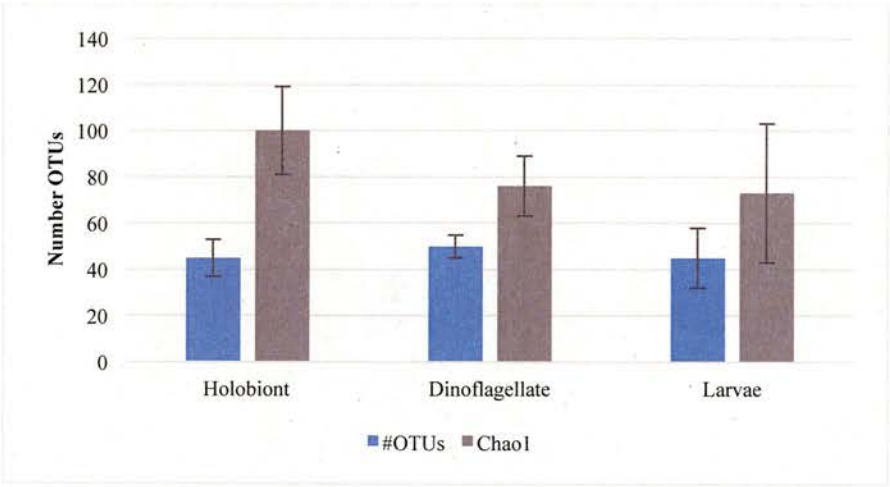

Figure 3.9. Observed and Chao1 estimated richness for holobiont, dinoflagellate, and larvae samples. OTUs were calculated at a distance of 0.03. Bars represent standard deviation. Calculations were based on datasets normalized to 370 sequence reads per sample.

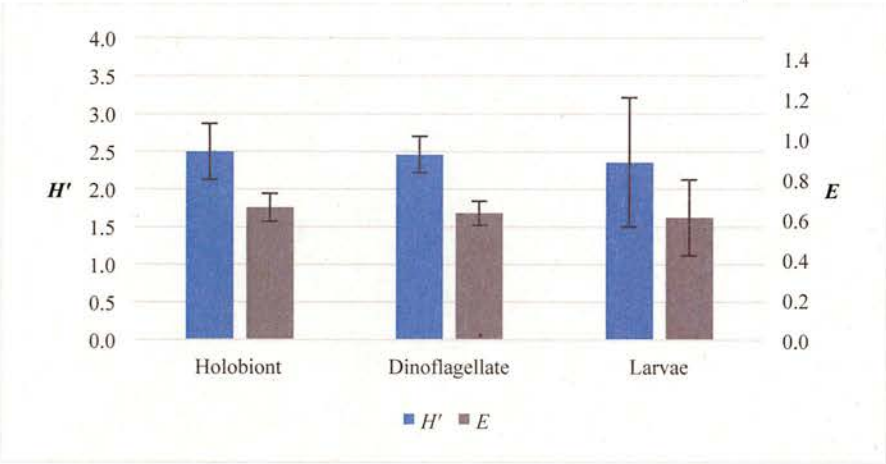

Figure 3.10. Shannon diversity (*H'*) and equitability (*E*) indices for holobiont, dinoflagellate, and larvae samples. OTUs were calculated at a distance of 0.03. Bars represent standard deviation. Calculations were based on datasets normalized to 370 sequence reads per sample.

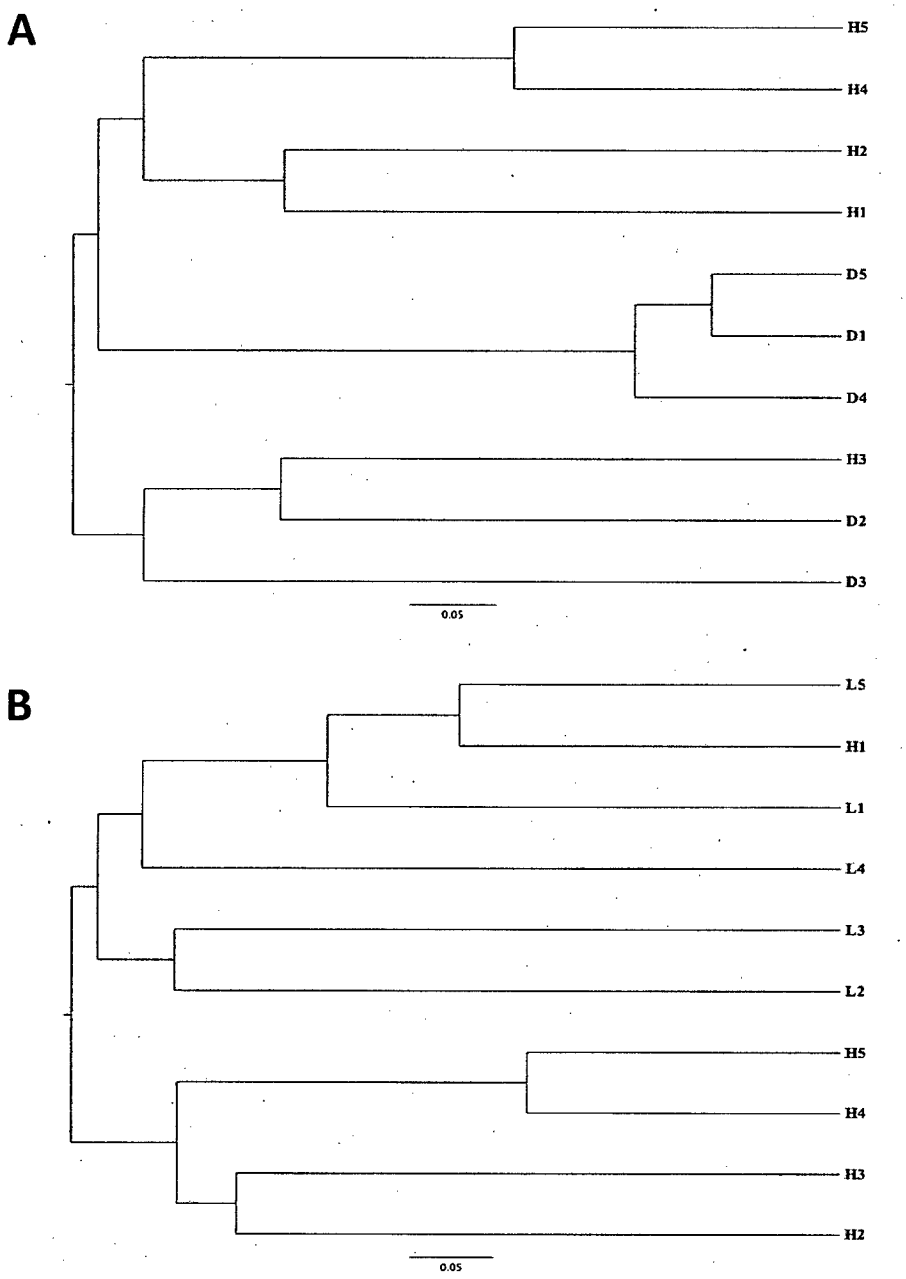

Figure 3.11. Yue and Clayton distance matrix comparison of *A. elisabethae* holobiont, dinoflagellate, and larvae microbial communities. Comparison of holobiont and dinoflagellates is shown in dendrogram A and comparison of holobiont and larvae is shown in dendrogram B.

3.3.4.2 Taxonomic Composition

There were a total of 15,141 dinoflagellate sample sequence reads, a total of eight phyla were present across all samples, with four to six present in each sample. The dominant phylum in all dinoflagellate samples was *Proteobacteria*, making up 26.9% to 83.8%. Other phyla that had a consistent presence in all dinoflagellate samples were *Bacteroidetes* (3.29% to 27.2%), *Actinobacteria* (0.8% to 14.5%), and *Firmicutes* (0.7% to 13.1%). Sixteen classes were detected across all samples with eight to twelve present in each sample. The most abundant class was *Gammaproteobacteria,* accounting for 20.8% to 80.9% of the sequence across all samples (Figure 3.12). Other classes that were consistently present were *Flavobacteria* (3.3% to 26.9%), *Actinobacteria* (0.8% to 14.5%), *Alphaproteobacteria* (2.4% to 6.0%), *Clostridia* (0.09% to 7.1%), and *Betaproteobacteria* (0.3% to 3.0%). Unclassified OTUs were prevalent and ranged from 5.4% to 25.2% of the sequence reads.

The taxonomic composition of the larvae samples was very similar to the dinoflagellate samples. There were a total of 6,614 larvae sample sequence reads, a total of six phyla were present across all samples, with four to six present in each samples. The dominant phylum in all samples was *Proteobacteria* (21.7% to 89.2%). Other phyla that were consistently present were *Bacteroidetes* (0.4% to 30.0%), *Actinobacteria* (0.6% to 8.2%), and *Firmicutes* (0.7% to 5.6%). Fifteen classes were present across all samples with seven to eleven classes present in each sample. As with the dinoflagellate samples the dominant microbial class was *Gammaproteobacteria* ranging from 18.1% to 85.5% of the sequence reads. The other classes that had a consistent presence were *Flavobacteria* (0.1% to 30.0%), *Alphaproteobacteria* (1.4% to 12.1%), *Actinobacteria* (0.6% to

8.2%), and *Betaproteobacteria* (0.2% to 10.1%) (Figure 3.12). Unclassifiable OTUs ranged from 3.0% to 54.7% of the total sequence reads.

These taxonomic classifications are similar to those observed in the holobiont samples. At the phylum level, all three are all dominated by *Proteobacteria* and have a consistent presence of *Bacteroidetes* and *Actinobacteria*. At the class level they are dominated by *Gammaproteobacteria* and have a consistent presence of *Flavobacteria, Alphaproteobacteria,* and *Actinobacteria*. The dominant classifiable genera that were consistently detected in the holobiont samples were *Endozoicomonas*, *Vibrio,* and *Pseudoalteromonas*. All of which were consistently detected in the dinoflagellate samples along with *Acinetobacter* and *Aquimarina* (Figure 3.13). There was a slight shift in composition in the larvae samples from the holobiont samples at the genus level as *Acinetobacter*, *Vibrio*, and *Aquimarina* were consistently present but *Endozoicomonas,* and *Pseudoalteromonas* were not.

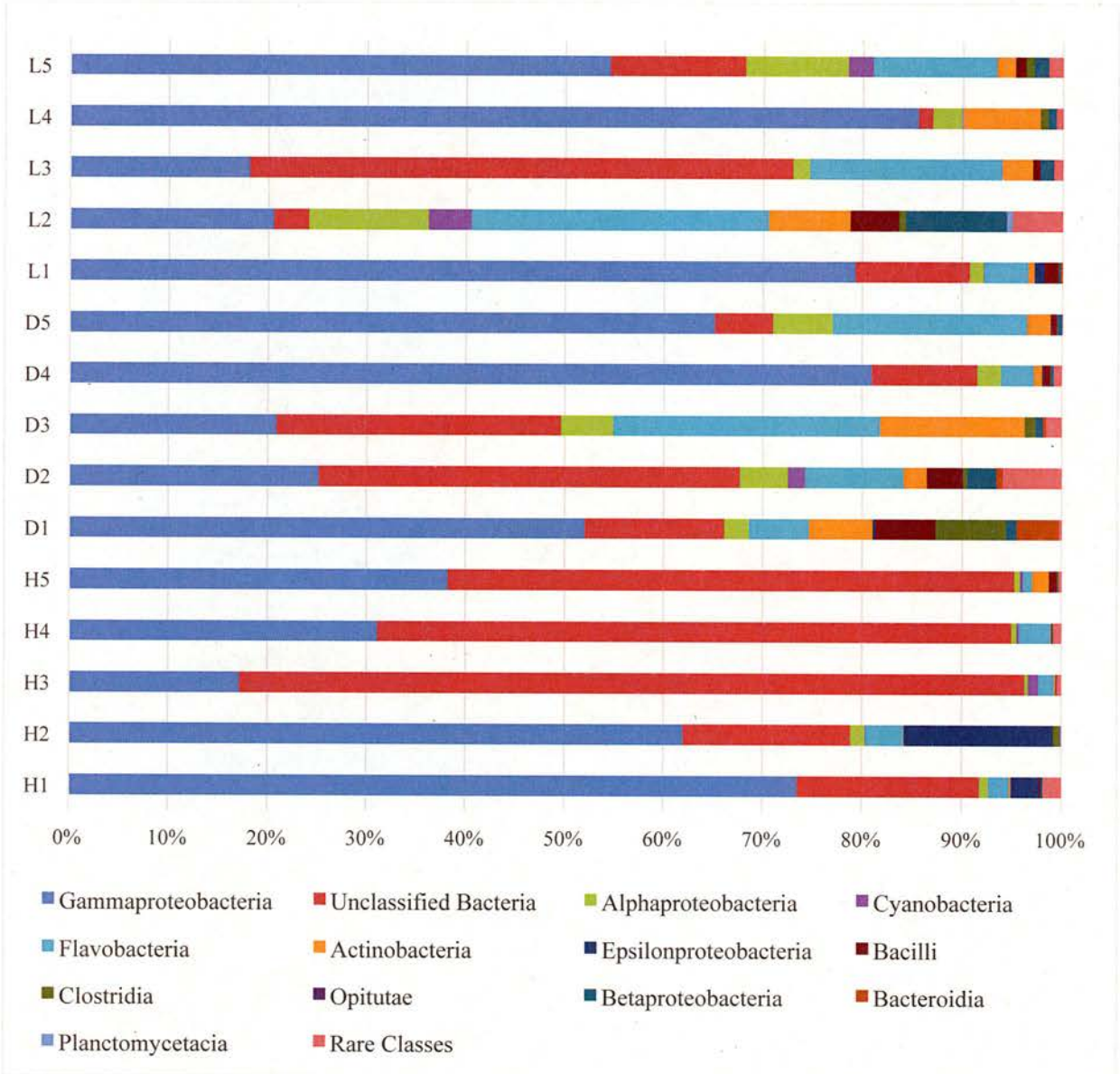

Figure 3.12. Class level microbial composition of coral-derived (holobiont, dinoflagellates, and larvae) fractions. Sequences were classified with a minimum confidence level of 80%. The "Rare Classes" group consists of classes that comprise <1% of the total sequence reads..

Figure 3.13. Genus level microbial composition of coral-derived (holobiont, dinoflagellates, and larvae) fractions. Sequences were classified with a minimum confidence level of 80%. Read counts other than the 16 most abundant are summarized in the "Other" category. The class level for each taxa are shown in parentheses.

3.3.5 San Salvador *A. elisabethae* Putative Core Microbiome

There were no consistent microbial taxa, or core microbiome associated with all reported *A. elisabethae* (Figure 3.8). However, there were operational taxonomic units (OTUs; $D = 0.03$) that were consistently found in all San Salvador microbiomes, suggesting that there may be a putative location specific core microbiome associated with these samples. Only OTUs that were present in all holobiont data sets and were absent from all seawater datasets, as well as the blank SFSW DNA extraction control, were considered. A total of seven OTUs were consistently present in all samples. The most abundant was an *Endozoicomonas* sp. (OTU 004) that accounted for 16.7% of all of the holobiont sequence reads, ranging from 9.6 % (H1) to 21.6% (H2) of each sample (Table 3.4). It was also detected in all dinoflagellate samples and four of the five larvae samples. This OTU formed a well supported (95% bootstrap support) clade with two uncultured bacterial clones obtained from marine sponges (HG423525; KF373182; 100% identity) as well as *Endozoicomonas euniceicola* EF212 (JX488684; 99% identity) which was isolated from the octocoral *Eunicea fusca*[67] (Figure 3.14). *Endozoicomonas* spp. have been detected in many marine corals and have been hypothesized to aid in the biogeochemical cycling of sulfur, in particular the degradation of DMSP.[80] *Endozoicomonas* spp. have also been implemented in the production of antimicrobial compounds, the degradation of complex organic carbon sources, and the conversion and assimilation of nitrate.[81-84]

Six other OTUs constitute the remainder of the putative San Salvador core microbiome. One of which was an uncultured *Alphaproteobacteria* (OTU 010) that accounted for 8.1% of the total holobiont sequence reads and formed a distinct clade with strong 100% bootstrap support to two uncultured bacterium clones (KP008700, KP008684; 97% identity) obtained from the octocoral

Corallium rubrum. The remaining OTUs all belong to the class *Gammaproteobacteria*; a *Psychrosphaera* sp. (OTU 015) that accounted for 1.9% of the total sequence reads and formed a distinct clade with an uncultured bacterium clone (JN694817; 99% identity) obtained from the coral *Porites astreoides,*[45] and the strain *Psychrosphaera saromensis* SA4-48T[85] (AB545807; 98% identity). A *Vibrio* sp. (OTU 016) that accounted for 3.6% of the total sequence reads and formed a distinct clade with strong 99% bootstrap support to an uncultured bacterium clone (FJ202984; 99% identity) obtained from the coral *Monastraea faveolata*[86] and *Vibrio* sp. (AB470932; 99% identity) isolated from a coral *Montipora* sp. A *Thalassomonas* sp. (OTU 044) that accounted for 0.6% of the total sequence reads and clustered with 98% bootstrap support to a *Thalassomonas* sp. (FJ463711; 100% identity) isolated from the coral *Halocordyle disticha*. A *Endozoicomonas* sp. (OTU 050) that accounted for 0.4% of the sequence reads, formed a distinct clade with 67% bootstrap support with the strain *Endozoicomonas gorgoniicola* PS125^T (JX488685; 99% identity) isolated from a *Plexaura* sp.[87] Lastly, a *Neptunomonas* sp. (OTU 0110) that accounted for 0.4% of the sequence reads, and clustered with strong 98% bootstrap support to an uncultured bacterium clone (KP008777; 100% identity) obtained from *C. rubrum*.

One OTU was detected in all dinoflagellate and larvae samples, *Aquimarina* sp. (OTU 011), it accounted for 4.5% of all the total sequence reads and clustered with 97% bootstrap support to an *Aquimarina* sp. isolated from an *A. elisabethae* collected from The Bahamas[66] (KC545299). This OTU was only detected in three of the five holobiont samples, however *Aquimarina* spp. were the dominant member of two of the previously reported *A. elisabethae* microbiomes from The Bahamas[66] and may therefore have a significant role in some holobionts. Also if the dinoflagellate and larvae samples consist of dilute amounts of the holobiont microbiome, then

the *Aquimarina* sp. OTU 014 was present in all samples and simply not detected in the holobiont samples due to being present is such low abundance compared with other dominant sequence reads. The role of *Aquimarina* spp. in marine invertebrates is unknown, but *A. salinaria,* a species isolated from the marine environment was shown to demonstrate algicidal activity in co-culture experiments.[88] Therefore these microbes may play a role in mediating interactions between the coral and their algal symbionts. The close phylogenetic relationship of all of these OTUs to other microorganisms detected or isolated from marine corals suggests that these are common members of the coral microbiomes and may play a functional role in the holobiont in which they inhabit.

Table 3.4. Summary of OTUs (*D=0.03*) that comprise the putative San Salvador *A. elisabethae* core microbiome, as well as the OTUs consistently found in the dinoflagellate and larvae samples. The bottom panel displays the taxonomic assignment of the OTUs based on the RDP classifier using a confidence threshold of 80%.

Sample	OTU % Abundance							
	0004	**0010**	**0015**	**0016**	**0044**	**0050**	**0110**	**0011**
H1	9.6	1.5	5.2	8.4	0.1	0.3	0.2	0.1
H2	21.6	0.3	5.0	11.2	2.3	0.1	0.6	-
H3	13.3	26.7	0.1	0.1	0.02	0.6	0.02	-
H4	17.9	0.4	0.3	0.02	0.02	0.2	0.1	1.5
H5	18.0	5.9	0.3	0.2	0.3	1.0	0.1	0.7
Total % Holobiont	**16.7**	**8.1**	**1.9**	**3.6**	**0.6**	**0.4**	**0.2**	**n/a**
D1	0.8	3.3	0.9	-		1.1	-	11.7
D2	1.3	-	0.4	-	2.2	-	-	7.6
D3	5.9	20.9	-	-	0.6	1.1	-	27.1
D4	0.6	-	3.1	1.0	2.1	0.2	-	4.0
D5	0.7	-	-	-	-	0.7	-	34.6
L1	0.9	-	14.7	0.5	0.1	0.1	0.2	4.3
L2	2.2	-	0.4	-	0.4	0.8	-	29.7
L3	3.8	-	-	0.2	-	0.6	-	18.2
L4	1.3	-	-	-	-	-	-	0.1
L5	-	-	-	-	-	-	-	3.2
Total % Coral Reads	**8.8**	**4.5**	**2.3**	**1.9**	**0.4**	**0.4**	**0.1**	**4.5**

OTU	Taxonomic Assignment
0004	*Proteobacteria; Gammaproteobacteria; Oceanospirillales; Hahellaceae; Endozoicomonas*
0010	*Proteobacteria; Alphaproteobacteria*; unclassified
0015	*Proteobacteria; Gammaproteobacteria; Alteromonadales; Pseudoalteromonadaceae; Psychrosphaera*
0016	*Proteobacteria; Gammaproteobacteria; Vibrionales; Vibrionaceae; Vibrio*

0044	*Proteobacteria; Gammaproteobacteria; Alteromonadales; Colwelliaceae; Thalassomonas*
0050	*Proteobacteria; Gammaproteobacteria; Oceanospirillales; Hahellaceae; Endozoicomonas*
0110	*Proteobacteria; Gammaproteobacteria; Oceanospirillales; Oceanospirillaceae; Neptunomonas*
0011	*Bacteroidetes; Flavobacteria; Flavobacteriales; Flavobacteriaceae; Aquimarina*

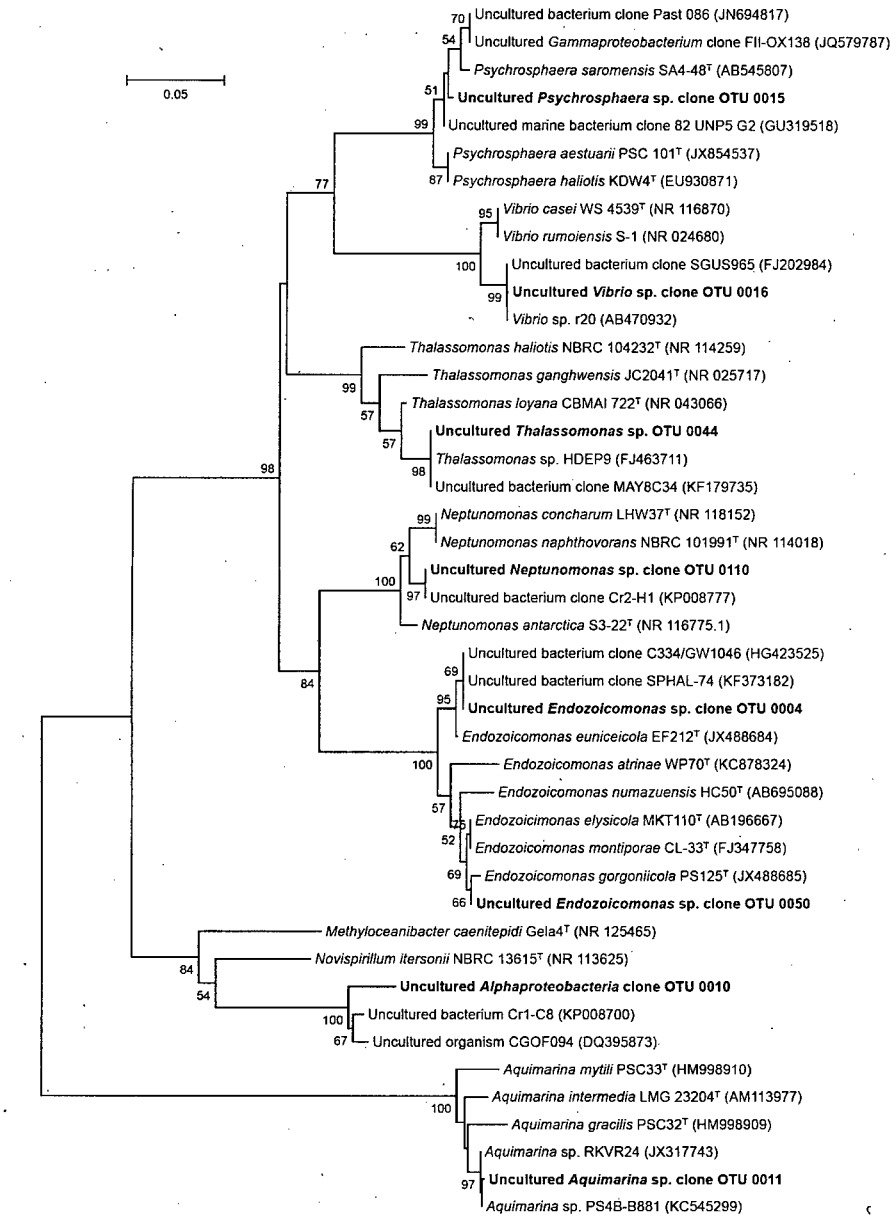

Figure 3.14 . Neighbor-joining phylogenetic tree based on 16S rRNA gene sequences of *A. elisabethae* holobiont, dinoflagellate, and larvae putative San Salvador core microbiomes. Evolutionary distances were computed using Jukes-Cantor method. A total of 235 nt positions were used. Bootstrap values are expressed as a percent of 1000 replicates; bootstrap values < 50 are not shown.

3.4 Conclusions

This study adds to the body of research on the microbiomes associated with the octocoral *A. elisabethae* and is the first report that attempted to investigate microbial communities associated with the dinoflagellates and larvae of this coral. From this research the following conclusions can be made: (1) The microbial community associated with *A. elisabethae* were distinct from the surrounding seawater; (2) the most dominant stable microbiome association observed in the San Salvador samples was with *Endozoicomonas* spp., *Vibrio* spp., and *Pseudoalteromonas* spp.; (3) there were no stable taxonomic groups at the genus level in all reported *A. elisaebethae* microbiomes, and high taxonomic variability was observed across all samples, suggesting that these corals do not require a stable association with any taxonomic groups for survival; (4) the dinoflagellates and larvae had similar richness, diversity, and taxonomic composition to the holobiont samples; and (5) Seven OTUs were consistently found in all holobiont samples suggesting a putative San Salvador specific core microbiome. However, further sampling would be required in order determine the presence of a site-specific core microbiome.

While there appears to be no microbial communities directly associated with the dinoflagellates of *A. elisabethae* there may be with their larvae. Further studies investigation the microbial communities associated with the larvae would provide insight into the role these microbes play in the fecundity of this coral. For example, investigation of the larvae before and after they have detached from the parent coral may provide insight into whether the microorganisms are acquired vertically or horizontally. This may also help to understand the true biosynthetic source of pseudopterosins, which still remains unclear.

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Chapter 4: Culture-Dependent Investigation of the Bacterial Communities Associated with *Antillogorgia elisabethae*

4.1 Introduction

Microorganisms are known to be abundant in the seawater surrounding corals and within the coral colony. Various culture-dependent and -independent techniques have been employed to investigate the microbial diversity associated with marine corals.[1-5] However the use of culture-dependent techniques has declined over the last decade, as many microorganisms are 'uncultivatable,' or at least have resisted cultivation thus far. It has been suggested that less than 1% of environmental microorganisms can be recovered using standard plating conditions,[6,7] however recoveries ranging from 0.01% to 12.5% have been reported.[8-10] With the advancement of next generation sequencing (NGS) technologies most research into the microbial communities associated with marine corals is being performed using culture-independent sequencing techniques.[11-14] While these techniques allow for a greater understanding of the microbial taxonomic diversity associated with corals, they do not provide any information about the microorganism's physiology or potential to produce secondary metabolites. Therefore the combination of both culture-dependent and -independent methodologies helps to avoid limitations associated with either technique and provides a more thorough understanding of the microorganisms associated with these marine invertebrates.

4.1.1 Rationale for the Methodology of the Culture-Dependent Study

Octocorals have been shown to be a viable source of taxonomically diverse bacteria,[11,13,15,16] however many of these can be difficult to cultivate. Therefore, in order to obtain the greatest cultivatable taxonomic diversity this study employed two different techniques. The first of which was to investigate the bacteria associated with various coral derived fractions. The rationale being that the holobiont hosts a great amount of bacterial diversity and if only the holobiont is

being investigated some of that diversity may be outnumbered and overlooked. By investigating additional coral derived fractions that are subsets of the holobiont, such as the larvae and dinoflagellates, slower growing or less abundant bacteria may be obtainable. The second technique was to investigate microaerophilic and anaerobic conditions. Like other marine invertebrates, octocorals have variations in their redox potential generating hypoxic and anoxic areas.[17-20] Any bacteria that thrive in those areas may not be cultivatable under aerobic conditions. Therefore, by cultivating bacteria from the holobiont under conditions that are conducive to microaerophilic and anaerobic bacteria, greater bacterial diversity may be obtained.[21]

A dilution-to-extinction method was used when plating the samples for bacterial cultivation. The rationale being that at the highest dilution series in which growth is still observed, some of the media wells will contain only one bacterial cell per plate. Therefore, any slow growing bacteria present will not be overgrown and live colonies will be obtainable. Additionally, two media types were used in this study, a high nutrient media targeting heterotrophic non-fastidious bacteria, as well as a low nutrient media targeting oligotrophic slow-growing bacteria.

4.1.2 Overall Objective of Study

The overall objective of this study was to isolate and identify a wide diversity of cultivatable bacteria from *A. elisabethae*. The bacterial isolates were taxonomically identified by sequencing of the 16S rRNA gene and compared to the culture-independent libraries in Chapter 3 to determine the amount of the microbiome recovered by these culture dependent techniques.

4.2 Materials and Methods

4.2.1 Collection of Bacteria from Holobiont, Dinoflagellate, and Larvae Fractions

Samples from five *A. elisabethae* colonies were collected by SUCBA off the coast of San Salvador, The Bahamas in November 2011 during the coral's spawning season from two locations, Runway10 (24°03.816' N, 74° 32.628' W; n=3) and Cable Crossing (24°03.090' N, 74°32.391' W; n=2). All samples were collected within a depth of 10 - 15 m into sterile Whirl-Pak™ bags (Nasco®) and at each collection site ~1 L of surrounding seawater was collected into a sterile Nalgene® polypropylene bottle (Nalge Nunc International). The holobiont samples were washed three times with ~40mL of sterile filtered seawater (SFSW) (0.2 μm polyethersulfone membrane, Nalgene Rapid Flow™) in sterile Falcon® centrifuge tubes (50 ml polypropylene, Corning®) to remove loosely associated bacteria. For each sample the larvae were removed from the outer surface of the coral using a sterile scalpel and washed three times to remove any coral tissue, there were ~ 50-100 larvae from each sample. The algal dinoflagellates were obtained using a modified protocol to the one described by Mydlarz *et al.*[22] A portion of the holobiont was homogenized using a homogenizer (VWR VDI 25 ULTRA-TURRAX) in SFSW and filtered through sterile cheesecloth to remove any large coral debris. The dinoflagellates were pelleted by centrifugation (250 x g, 5 min) and further purified using a Percoll® gradient of 40% and 80%. The dinoflagellates were collected from the top of the 80% Percoll® layer, the Percoll® gradient was repeated a minimum of six times until <1% impurities were visible under light microscopy. All samples were divided in half, half were used for the culture independent study (Chapter 3) and the other half was used for this study.

The holobiont samples were homogenized in SFSW and serial dilutions (10^{-2} to 10^{-5}) were prepared in SFSW. Serial dilutions (1 to 10^{-3}) of the purified dinoflagellates and surrounding seawater samples were prepared in SFSW. Aliquots (10µl) of each serial dilution were plated into 48-well plates onto the following media in triplicate: (1) Marine Agar (MA; 2216, BD Difco), a high-nutrient medium for targeting heterotrophic non-fastidious bacteria, and (2) Dilute (1/100) R2A Agar (dR2A; 218263, BD Difco), a low nutrient medium for targeting oligotrophic, slow-growing bacteria. Serial dilutions of the larvae were not made. Instead associated cultivatable bacteria were obtained by placing one larva into each well of the MA and dR2A 48-well plates. Plates were incubated at 21°C for up to four months, and any bacteria that were growing were purified as single colonies during that time. All isolated bacteria were grown in Marine Broth (MB 2216, BD Difco) and preserved at -80°C in 25% (v/v) glycerol (VWR) until needed for further processing

4.2.2 Collection of Bacteria from *A. elisabethae* under Aerobic, Microaerophilic, and Anaerobic Conditions

Samples from three *A. elisabethae* colonies were collected by SUCBA off the coast of San Salvador, The Bahamas in March 2012 from Runway 10 (24°03.816' N, 74° 32.628' W; n=3) at a depth of ~10 m. All samples were collected into sterile Whirl-Pak™ bags and at each collection site ~1 L of surrounding seawater was collected into a sterile Nalgene® polypropylene bottle. The coral samples were washed three times with ~40 mL of SFSW in sterile Falcon® centrifuge tubes to remove loosely associated bacteria. The coral samples were homogenized and serial dilutions of the homogenate (10^{-2} to 10^{-5}) and surrounding seawater (1 to 10^{-3}) were prepared in SFSW. Aliquots (10 µl) of each serial dilution were plated onto 48-well plates of

either MA or dR2A. Each media type and serial dilution was placed in triplicate under either aerobic, microaerophilic, or anaerobic conditions. The microaerophilic and anaerobic conditions were achieved using BD GasPakTM Anaerobic Systems (BD Diagnostics). Plates were incubated at 21°C for up to four months and any bacteria that were growing were purified as single colonies during that time. All isolated bacteria were grown in MB and preserved at -80°C in 25% (v/v) glycerol until needed for further processing.

4.2.3 Dereplication of Microbes using MALDI-TOF MS

Bacteria were initially dereplicated based on their protein fingerprints using Matrix-Assisted Laser Desorption/Ionization – Time of Flight Mass Spectrometry (MALDI-TOF MS) (Microflex LT, Bruker Daltonics Mass Spectrometer, Leipzig, Germany). Bacterial cells were grown from frozen glycerol stock on MA for 48 ± 2 hours at 30°C, stamped onto a stainless steel target plate, and covered with 1.5µl of matrix solution (0.5% (w/v) α-cyano-4-hydroxycinnamic acid in 50:48:2 acetonitrile:water:trifloroacetic acid solution).[23] The protein profile of each bacterium was examined using MALDI-TOF MS equipped with a 50-Hz nitrogen laser. MS analysis was performed in linear, positive ionization mode (laser power 60%; up to 200 shots fired, mass range 2,000-12,000 m/z), and MALDI-TOF MS peak profiles were generated using FlexControl software (Bruker Daltonics). Cluster analysis of the spectra was performed by BioTyper Version 2.0 software package (Bruker Daltonics) using an Unweighted Paired Group Method with Arithmetic mean (UPGMA), as this method has been shown to be suitable at grouping bacterial isolates at the species level.[24] The *Escherichia coli* strain DH15H was used as a control strain in triplicate on each target plate. The distance at which the *E. coli* DH15H spectra clustered was used as a cut-off in the constructed dendrogram. Bacteria that clustered at a distance greater than

the cut-off were deemed different species of bacteria and were taxonomically identified by sequencing of their 16S rRNA genes.

4.2.4 Genomic DNA extraction and PCR Amplification

MALDI-TOF MS dereplicated bacteria were grown in MB (48 hours, 250 rpm, 30°C). Bacterial cultures were pelleted, the media supernatant discarded, and resuspended in 300 uL of 50/20 TE buffer (50mM Tris-HCl, 20mM EDTA, pH 8.0) supplemented with lysozyme (5mg/ml; Sigma-Aldrich) for 30 min at 37°C. After which 50 uL of 10% SDS (w/v; EMD Millipore) was added followed by 85 uL of 5 M NaCl (VWR International). The lysate was extracted with an equal volume of phenol: chloroform solution (1:1, v/v, Fisher Scientific), vortexed for 30 s, then centrifuged for 10 min at 8,000 rpm on a desktop centrifuge (Sorvall Biofuge pico). The aqueous layer was retained and transferred to a fresh tube where the DNA was precipitated by adding 0.5 ml of isopropanol (Sigma-Aldrich). The DNA was pelleted by centrifugation and washed with cold 70% ethanol (Commercial Alcohols), allowed to dry, then re-suspended in deionized water.

Polymerase chain reaction (PCR) amplification of the16S rRNA gene was achieved using the universal eubacteria 16S rRNA gene primers pA (5'-AGAGTTTGATCCTGGCTCAG-3') and pH (5'-AAGGAGGTGATCCAGCC-3')[25] with the following conditions: 1X concentration EconoTag® Plus Green 2X Master Mix (Lucigen), 5% DMSO (v/v; Sigma Aldrich), 1 uM of each primer, 20 ng of template DNA. Thermal cycling parameters were as follows: an initial denaturing cycle at 95°C for 3 min, followed by 35 cycles of 95°C for 45 s, 54°C for 1 min, 72°C for 1.5 min, and a final extension of 72°C for 10 min. PCR amplicons were assessed by gel electrophoreses in a 1.0% agarose gel (Fisher Scientific) containing 0.001% ethidium bromide

(Sigma Aldrich), at 120V for 30 min (BioRad, Mississauga, ON). PCR products were visualized on a UV transilluminator (BioSpectrum®, OptiChemi HR Camera, Upland, CA) and amplicons of the correct size (~1500 bp) were sent for sequencing.

4.2.5 Sequencing and Phylogenetic Analysis

Sequencing was performed by Eurofins MWG Operon (Huntsville, AL, USA), using the 936R primer (5'-GGGGTTATGCCTGAGCAGTTTG-3')[26]. Sequences were trimmed and assembled using Vector NTI Express (Invitrogen, Life Technologies), and compared to available sequences in the GenBank database.[27] Sequence alignments were prepared using MEGA version 6.[28] Phylogenetic histories were inferred using the neighbor-joining (NJ) method.[29] Evolutionary distance matrices were generated using the Jukes-Cantor method[30] and are in units of the number of base substitutions per site. All positions containing gaps and missing data were eliminated. Bootstrap analysis is based on 1000 resampled datasets.[31]

4.2.6 Comparison of Culture-Dependent Library to Culture-Independent Library

The 16S rRNA gene sequence data from the culture-dependent libraries was compared to the 454-pyrosequencing culture-independent libraries (Chapter 3) in order to determine the overlap between the two library types. A Local BLAST (NCBI, Bethesda, MD) nucleotide database[27] containing representative sequences of each operational taxonomic unit (OTU, D=0.03) was created in BioEdit version 7.2.5. Cultured sequences were search against this local database using the Matrix BLOSUM62 with an Exception value of $1E^{-100}$. Cultures with sequence similarity $\geq$ 99% to sequences in the database were considered 'hits.'

4.3 Results and Discussion

4.3.1 Cultivatable Bacteria from *A. elisabethae* Holobiont, Dinoflagellates, and Larvae

Samples from five of *A. elisabethae* colonies were collected off the coast of San Salvador, The Bahamas in November 2011 during the coral's spawning season at two reef locations. Each *A. elisabethae* specimen was divided into three fractions: the holobiont (H1-H5), larvae (L1-L5), and dinoflagellate (D1-D5) fractions. For each collected coral, a surrounding seawater sample was collected adjacent to the coral colony (W1-W5). Serial dilutions were plated onto 48 well plates of MA and dR2A and all bacteria that grew on those plates over a four month period were isolated resulting in 921 bacterial isolates. Following dereplication using MALDI-TOF MS, with an *E. coli* strain as an internal standard, there were 273 unique bacterial 'strains'. The 16S rRNA gene of these bacteria was sequenced and they were further dereplicated using 99% sequence similarity as a cut off for unique species,[32,33] this resulted in 89 unique bacterial species. Since only 35.2% of the MALDI-TOF MS unique bacterial 'strains' were determined to be unique species based on their 16S rRNA gene sequence, the cut-off for the MALDI-TOF MS dereplication may have been too stringent. The cut-off for dereplication varied per analysis and was based on the distance level at which the *E. coli* control clustered at within the constructed dendrograms. However previous research has shown that the discriminatory power with which MALDI-TOF MS can differentiate bacteria can vary between genera.[24] In some genera it is capable of discriminating to the subspecies or strain level, while in others it can only discriminate to the species level. Since only the species level was required for this research MALDI-TOF MS proved to be an adequate dereplication tool.

The 89 sequences were taxonomically identified based on BLAST analysis[27] and the taxonomic identifications were applied to the original 921 bacterial isolates. The bacteria were grouped into libraries based on the sample type they originated from. Any overlap between the seawater library and the coral derived libraries was removed from the latter. The majority of the bacteria isolated regardless of sample origin belonged to the class *Gammaproteobacteria,* ranging from 44.3% (Seawater) to 63.7% (Larvae) of the total bacteria isolated from each sample type (Figure 4.1). At the genus level most cultivatable bacteria were *Vibrio* spp., ranging from 15.3% (Holobiont) to 49.3% (Larvae) of the total culture libraries (Figure 4.2). Other abundant genera from these libraries that were easily cultivated were *Pseudoalteromonas* spp. (3.8% to 26.7%), *Bacillus* spp. (3.1% to 6.0%), and *Alteromonas* spp. (1.1% to 10.4%). As for the seawater samples, bacteria belonging to the genera *Halomonas* spp. (9.7%), *Pseudoalteromonas* spp. (8.3%), and *Psychrobacter* spp. (7.8%) were the most cultivatable. The dominant genera in all libraries were fast growing heterotrophic bacteria that easily grow on nutrient rich media, and are therefore commonly found in coral and seawater culture dependent libraries.[4,5,34-37]

The greatest taxonomic diversity was obtained from the holobiont samples, which after removing sequences that overlapped with the seawater library had 41 different bacterial species (Table 4.1). Thirty of these were 'unique' to the holobiont samples, meaning they were not obtained from any other isolation source. While the holobiont proved to be a valuable source of taxonomic diversity, investigation of the larvae and dinoflagellates also provided unique bacterial species. There were an additional 12 species obtained from the other coral derived fractions that would have not been obtained had only the holobiont been investigated. The

seawater samples also proved to be a great source of cultivatable bacteria, providing 19 'unique' species.

Table 4.1. Number of different and unique bacterial species present in culture-dependent libraries.

Sample Origin	# Different Species	# Unique Species
Holobiont	41	30
Larvae	20	4
Dinoflagellates	26	8
Seawater	25	19

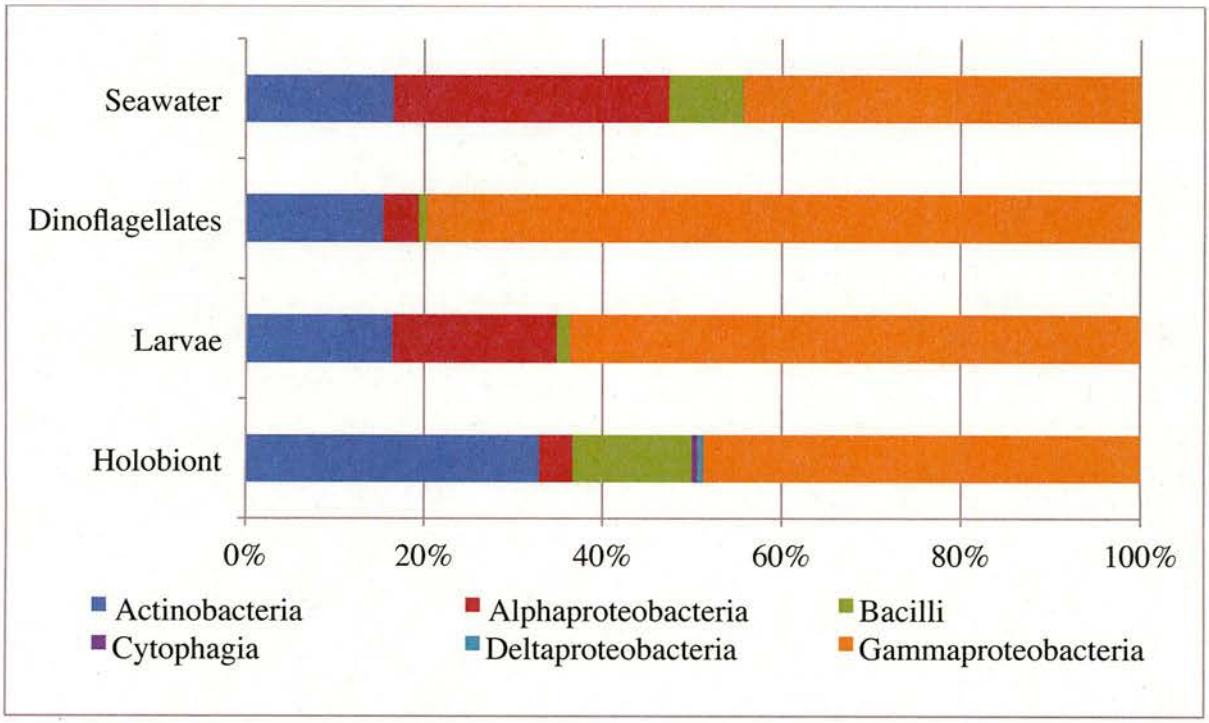


Figure 4.1. Class level bacterial composition of culture-dependent libraries. Sequences were classified based on BLAST analysis.

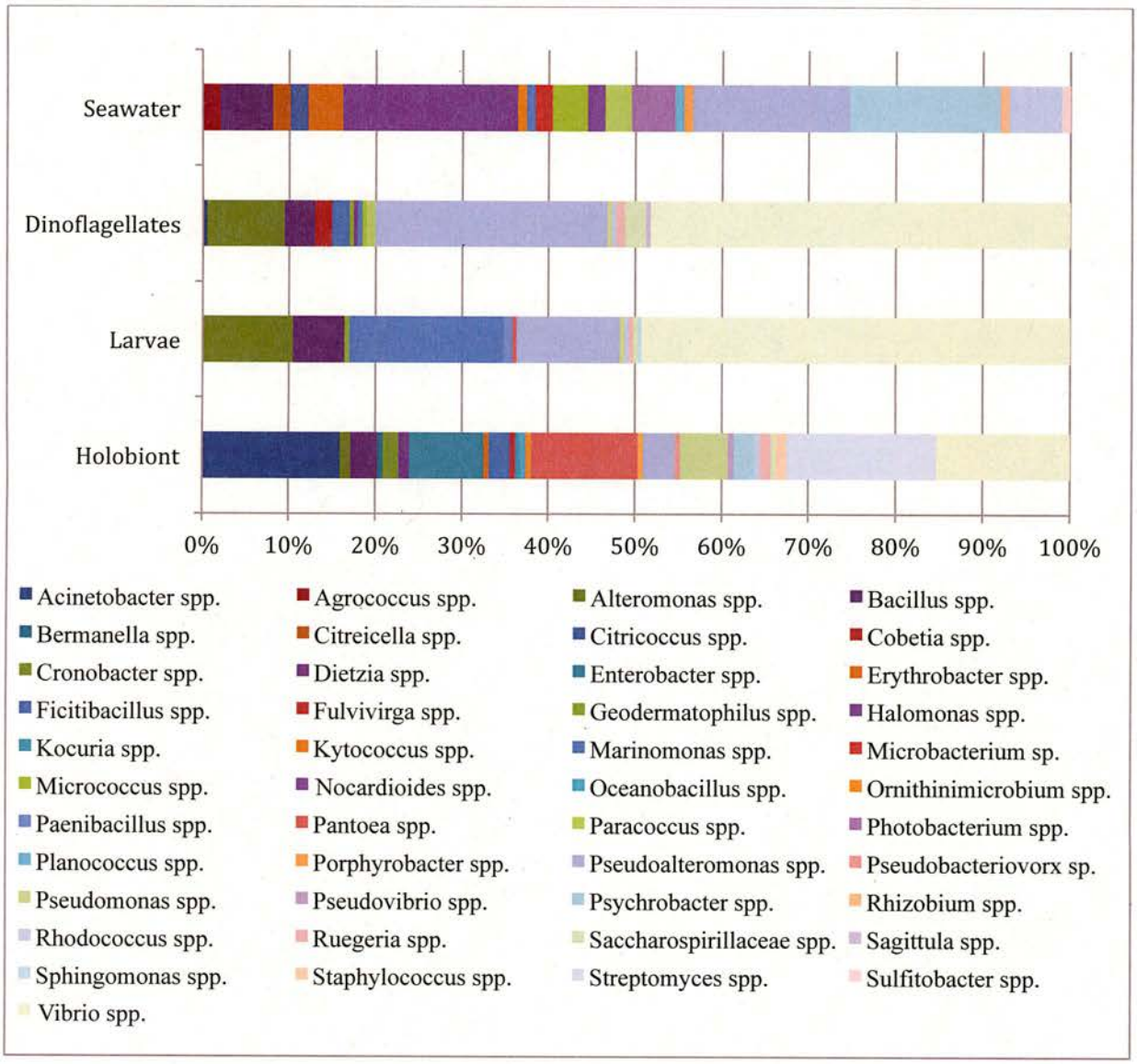


Figure 4.2. Genus level bacterial composition of culture-dependent libraries. Sequences were classified based on BLAST analysis.

4.3.2 Comparison of Cultured-Dependent and -Independent Bacterial Communities

The cultured bacterial sequences were compared to the sequences from the culture-independent study (Table 4.2) (Chapter 3). Cultured sequences with ≥ 99% sequence similarity to sequences in the culture-independent libraries were considered 'hits.' All but 21 of the cultured sequences were present in the culture-independent libraries. For both the culture-dependent and -independent data any overlap between the seawater library and the coral derived libraries was removed from the latter. A total of 3.9% of the holobiont culture independent library was recovered through cultivation (Table 4.3). The recovery rate was even higher in the larvae and dinoflagellate libraries, which were 7.5% and 10.0%, respectively. Between the higher recovery rates and the 12 unique isolates, investigation of the cultivatable bacteria from these coral derived subsets have thus proven valuable. The highest recovery was obtained from the seawater samples, from which 12.2% of the culture independent library was obtained. While these recovery rates are notable they are still only a small percentage of the actual microbial diversity of these samples. Therefore to truly understand the microbial communities associated with *A. elisabethae* both culture-dependent and -independent libraries are required.

Table 4.2. Cultured bacterial isolates and the culture-dependent and -independent libraries in which they were detected. Bacterial isolates were detected in the culture-dependent libraries either through 16S rRNA gene sequence similarity (≥ 99%), or MALDI-TOF MS protein fingerprint similarity. Bacterial isolates were detected in the culture-independent libraries based on local BLAST analysis (≥ 99%).

	Culture Dependant Library				Culture Independent Library			
	H	**L**	**D**	**W**	**H**	**L**	**D**	**W**
Acinetobacter sp. RKEM 528	X				X	X	X	
Acinetobacter sp. RKEM 532	X				X	X	X	
Acinetobacter sp. RKEM 817	X					X	X	X
Agrococcus sp. RKEM 917				X		X		
Alteromonas sp. RKEM 316		X	X		X	X	X	X
Alteromonas sp. RKEM 369		X	X		X	X	X	X
Alteromonas sp. RKEM 717			X		X	X	X	X
Alteromonas sp. RKEM 765		X	X		X	X	X	X
Arthrobacter sp. RKEM 332	X	X	X			X	X	
Bacillus sp. RKEM 266	X							
Bacillus sp. RKEM 441		X		X				
Bacillus sp. RKEM 444		X			X	X		
Bacillus sp. RKEM 513C	X				X	X		
Bacillus sp. RKEM 632			X		X	X		
Bacillus sp. RKEM 667	X	X	X		X		X	X
Bacillus sp. RKEM 720			X					
Bacillus sp. RKEM 730	X	X	X	X				
Bacillus sp. RKEM 731			X	X			X	
Bacillus sp. RKEM 755				X				
Bacillus sp. RKEM 781B		X						
Bacillus sp. RKEM 829B	X				X			
Bermanella sp. RKEM 777	X							
Citreicella sp. RKEM 868				X	X			X
Citricoccus sp. RKEM 914				X				
Cobetia sp. RKEM 646			X				X	
Cronobacter sp. RKEM 537A	X							
Cronobacter sp. RKEM 548	X				X	X	X	
Dietzia sp. RKEM 832	X							
Erythrobacter sp. RKEM 642	X							X
Erythrobacter sp. RKEM 937				X	X		X	X
Erythrobacter sp. RKEM 947				X		X	X	X
Fictibacillus sp. RKEM 566	X		X					

Fulvivirga sp. RKEM 712	X							
Geodermatophilus sp. RKEM 688		X	X					
Halomonas sp. RKEM 883				X	X	X	X	X
Halomonas sp. RKEM 901				X	X	X	X	X
Kocuria sp. RKEM 639	X				X	X	X	X
Kytococcus sp. RKEM 512A	X	X	X	X				
Marinomonas sp. RKEM 313		X	X		X			
Marinomonas sp. RKEM 715	X	X	X	X			X	
Marinomonas sp. RKEM 924	X						X	
Microbacterium sp. RKEM 922				X				
Micrococcus sp. RKEM 702	X	X	X	X		X	X	X
Nocardioides sp. RKEM 944				X				X
Oceanobacillus sp. RKEM 814	X						X	
Ornithinimicrobium sp. RKEM 638	X							X
Paenibacillus sp. RKEM 768		X						
Pantoea sp. RKEM 516	X				X	X	X	X
Pantoea sp. RKEM 552	X				X		X	
Pantoea sp. RKEM 701	X		X		X		X	X
Paracoccus sp. RKEM 656			X				X	X
Paracoccus sp. RKEM 942				X		X	X	X
Paracoccus sp. RKEM 943				X				X
Paracoccus sp. RKEM 946				X				
Planococcus sp. RKEM 918				X				
Porphyrobacter sp. RKEM 933				X				X
Pseudoalteromonas sp. RKEM 243	X	X			X	X	X	X
Pseudoalteromonas sp. RKEM 647	X		X		X	X	X	X
Pseudoalteromonas sp. RKEM 660			X		X	X	X	X
Pseudoalteromonas sp. RKEM 732		X	X		X	X	X	X
Pseudoalteromonas sp. RKEM 857	X				X	X	X	X
Pseudobacteriovorax antillogorgiicola RKEM 611	X				X			
Pseudomonas sp. RKEM 545	X					X	X	
Pseudomonas sp. RKEM 824A	X				X	X	X	X
Pseudomonas sp. RKEM 891		X	X					
Pseudovibrio sp. RKEM 536	X							X
Psychrobacter sp. RKEM 535	X							X
Psychrobacter sp. RKEM 670	X		X	X		X	X	X
Psychrobacter sp. RKEM 879				X				X
Psychrobacter sp. RKEM 927				X		X	X	X
Rhizobium sp. RKEM 870				X				
Rhodococcus sp. RKEM 42			X					
Rhodococcus sp. RKEM 450	X	X						
Rhodococcus sp. RKEM 843	X							X

Ruegeria sp. RKEM 265	X	X	X		X	X	X	X
Saccharospirillum sp. RKEM 770		X	X				X	
Sagittula sp. RKEM 724B			X		X	X	X	X
Sphingomonas sp. RKEM 364		X						
Staphylococcus sp. RKEM 613	X				X		X	X
Streptomyces sp. RKEM 774	X					X	X	X
Sulfitobacter sp. RKEM 910				X				
Vibrio sp. RKEM 201	X	X			X	X	X	
Vibrio sp. RKEM 309	X				X			
Vibrio sp. RKEM 328	X				X			
Vibrio sp. RKEM 48		X	X		X	X	X	X
Vibrio sp. RKEM 501	X		X		X	X	X	X
Vibrio sp. RKEM 562	X				X	X		X
Vibrio sp. RKEM 601	X	X	X					
Vibrio sp. RKEM 69	X		X		X	X	X	X

Abbreviations – H: holobiont; L: larvae; D: dinoflagellate; W: seawater

Table 4.3, Recoverability of culture-independent libraries.

Cultured 16S rRNA gene sequences were detected in the culture-independent libraries based on local BLAST analysis (> 99%).

Sample	Overlap between libraries (%)
Holobiont	3.9
Larvae	7.5
Dinoflagellate	10.0
Seawater	12.2
Average	**8.4**

4.3.3 Cultivatable bacteria from *A. elisabethae* under Aerobic, Microaerophilic, and Anaerobic Conditions

Samples from three *A. elisabethae* colonies were collected off the coast of San Salvador, The Bahamas in March 2012. For each sample collected, a surrounding seawater sample was collected adjacent to the coral colony. The coral was homogenized and serial dilutions of the coral and seawater were plated onto 48 well plates of MA and dR2A. The plates were placed under aerobic, microaerophilic, or anaerobic conditions. All bacteria that grew on those plates over a four-month period were isolated, resulting in 736 bacterial isolates. Using MALDI-TOF MS it was determine that there were 20 isolates that had the same protein fingerprint as 'strains' from the previous collection (Section 4.3.1) (Table 4.4), and 103 'strains' that were not detected in the previous collection. The 16S rRNA gene sequence of the new 103 isolates were sequenced and further dereplicated using 99% sequence similarity as a cut off for unique species.[32,33] This resulted in 54 unique bacterial species that had not been obtained in the previous collection. These 54 sequences as well as and the 20 sequences from the previous study were taxonomically identified based on BLAST analysis[27] and the taxonomic identifications were applied to the original 736 bacterial isolates. Any overlap between isolates cultured from the seawater samples and the holobiont samples were removed from the library of the latter.

Not surprisingly, the greatest diversity was observed under aerobic conditions and decreased with decreasing oxygen concentration. The aerobic holobiont and seawater libraries were dominated by *Gammaproteobacteria,* as was observed in the previous collection (4.3.1) (Figure 4.3). At the genus level, the holobiont microaerophilic library was dominated by *Shewanella* spp. (33.3%), a genus that was not obtained in the previous collection. The holobiont anaerobic

library only contained *Nocardioides* spp. (60.0%) and *Rhizobium* spp. (40.0%) (Figure 4.4). The seawater microaerophilic library was dominated by *Microbacterium* spp. (30.4%) and *Bacillus* spp. (26.1%), while the anaerobic library only contained *Nocardioides* spp. (17.6%), *Rhizobium* spp. (20.5%), *Aeromicrobium* spp. (14.7%), and *Photobacterium* spp. (47.1%). These results are consistent with previous research investigating cultivatable bacteria from marine invertebrates and surrounding seawater under microaerophilic and anaerobic conditions.[18,20,38]

Microaerophilic conditions proved to be a very viable source of unique bacteria from the holobiont samples. There were 18 different bacteria that grew under microaerophilic conditions and 16 of them were unique (Table 4.5). Anaerobic conditions were not as successful; only four different species grew under these conditions, none of which were unique. As for the seawater samples, microaerophilic and anaerobic only generated one and two unique sequences, respectively.

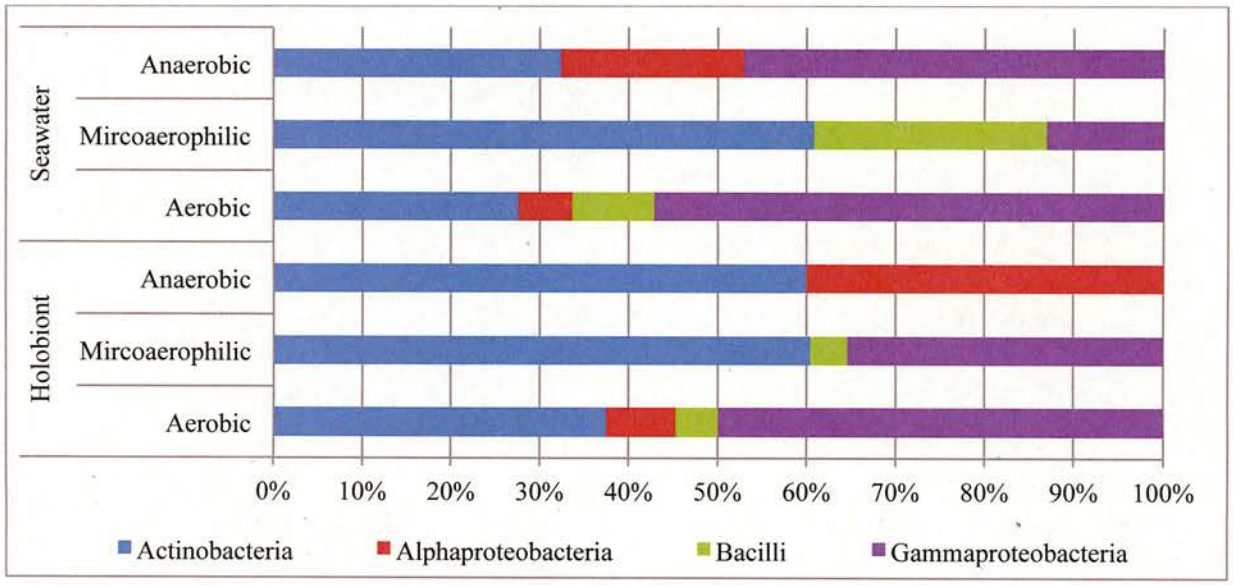


Figure 4.3. Class level bacterial composition of aerophilic, microaerophilic, and anaerobic culture-dependent libraries. Sequences were classified based on BLAST analysis.

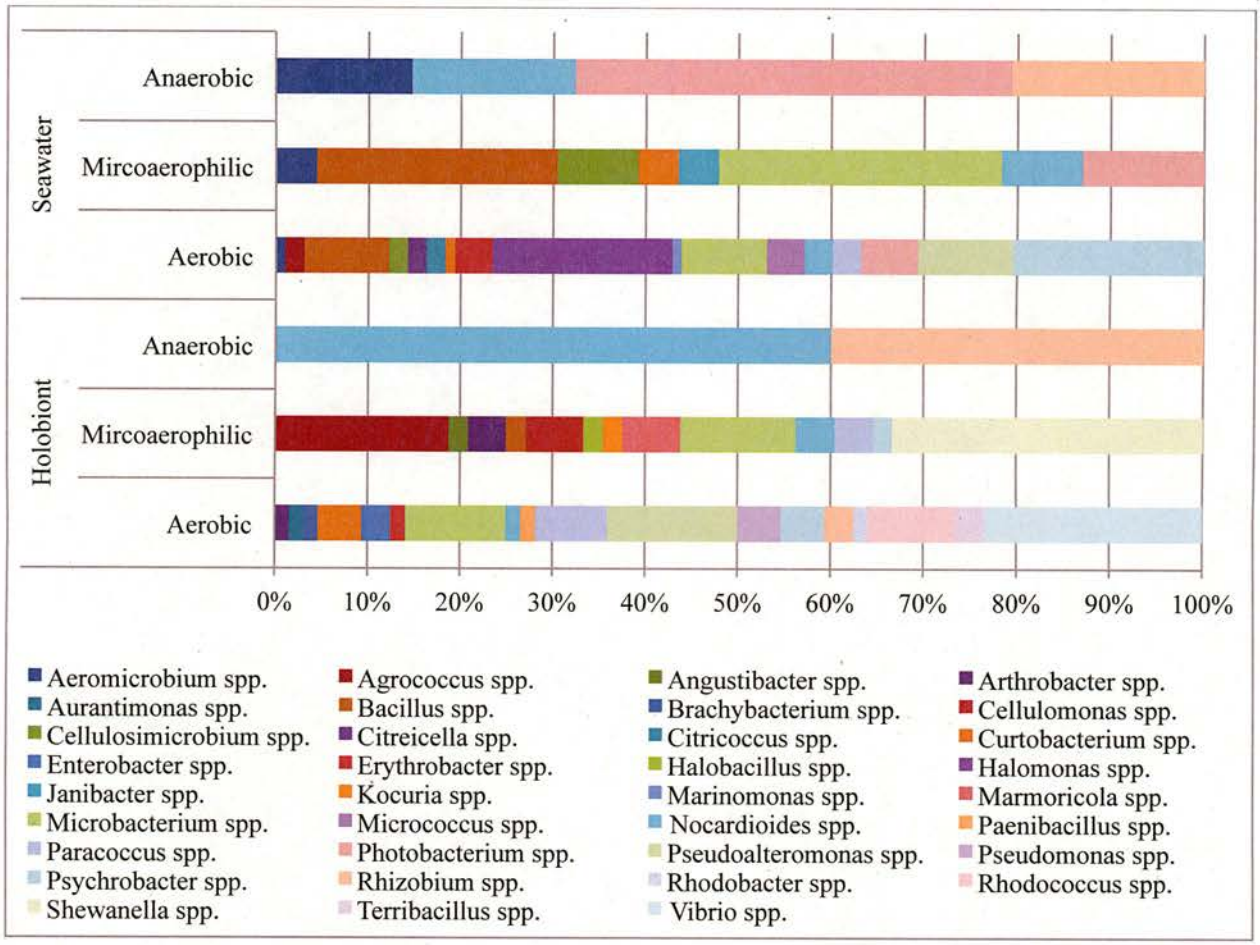


Figure 4.4. Genus level bacterial composition of aerophilic, microaerophilic, and anaerobic culture-dependent libraries. Sequences were classified based on BLAST analysis.

Table 4.4. Cultured isolates from aerobic, microaerophilic, and anaerobic conditions, and the culture-dependent and -independent libraries in which they were detected. Bacterial isolates were detected in the culture-dependent libraries either through 16S rRNA gene sequence similarity (>99%), or MALDI-TOF MS protein fingerprint similarity. Bacterial isolates were detected in the culture-independent libraries based local BLAST analysis (> 97%).

	Culture Dependent						Culture Independent			
	Holobiont			Seawater						
	AR	MA	AN	AR	MA	AN	H	L	D	W
Aeromicrobium sp. RKEM 1713		X	X	X	X	X	X	X	X	
Agrococcus sp. RKEM 1635		X						X		X
Agrococcus sp. RKEM 1685		X						X		X
Agrococcus sp. RKEM 917*				X				X		
Angustibacter sp. RKEM 1664		X								
Arthrobacter sp. RKEM 1571	X							X	X	
Arthrobacter sp. RKEM 1637		X					X	X	X	X
Aurantimonas sp. RKEM 1543	X									
Bacillus sp. RKEM 731*				X					X	
Bacillus sp. RKEM 1569	X	X		X	X		X	X		
Bacillus sp. RKEM 755*		X		X						
Bacillus sp. RKEM 1771		X					X		X	X
Bacillus sp. RKEM 829B*	X	X		X			X	X	X	X
Brachybacterium sp. RKEM 1576	X									X
Cellulomonas sp. RKEM 1775		X						X		X
Cellulosimicrobium sp. RKEM 1754		X		X	X					
Citreicella sp. RKEM 868*	X			X			X		X	X
Citricoccus sp. RKEM 914*	X			X			X		X	X
Curtobacterium sp. RKEM 1515	X							X		X
Curtobacterium sp. RKEM 1741		X		X	X			X		X
Enterobacter sp. RKEM 1504	X						X	X	X	
Erythrobacter sp. RKEM 1590	X						X		X	
Erythrobacter sp. RKEM 937*		X		X			X		X	X
Halobacillus sp. RKEM 1822		X							X	
Halomonas sp. RKEM 901*				X			X	X	X	X
Janibacter sp. RKEM 1825					X					X
Kocuria sp. RKEM 1608		X					X	X	X	X
Marinomonas sp. RKEM 715*		X		X			X		X	
Marmoricola sp. RKEM 1784		X					X	X	X	
Microbacterium sp. RKEM 1514	X							X		
Microbacterium sp. RKEM 1559	X							X		

Microbacterium sp. RKEM 1560				X	X			X		
Microbacterium sp. RKEM 1591	X							X		X
Microbacterium sp. RKEM 1651		X						X		
Microbacterium sp. RKEM 1661		X						X		X
Microbacterium sp. RKEM 1691		X						X		
Microbacterium sp. RKEM 1778		X						X		
Microbacterium sp. RKEM 1786		X						X		
Microbacterium sp. RKEM 1814				X	X			X		
Microbacterium sp. RKEM 1815		X		X	X			X		
Microbacterium sp. RKEM 922*				X				X		
Microbacterium sp. RKEM 1562	X							X		
Micrococcus sp. RKEM 702*				X				X	X	X
Nocardioides sp. RKEM 1595	X		X					X		
Nocardioides sp. RKEM 1695		X	X					X		
Nocardioides sp. RKEM 1698		X	X					X		
Nocardioides sp. RKEM 1712						X				
Nocardioides sp. RKEM 1764				X	X	X		X		X
Nocardioides sp. RKEM 1781						X		X		X
Nocardioides sp. RKEM 944*				X						X
Paenibacillus sp. RKEM 1589	X									
Paracoccus sp. RKEM 1551	X						X	X		X
Paracoccus sp. RKEM 1671		X					X	X		X
Paracoccus sp. RKEM 1682		X					X	X		X
Paracoccus sp. RKEM 942*				X				X	X	X
Paracoccus sp. RKEM 943*				X						X
Paracoccus sp. RKEM 946*				X			X	X	X	X
Photobacterium sp. RKEM 1507		X	X		X	X	X	X	X	X
Pseudoalteromonas sp. RKEM 243*	X						X	X	X	X
Pseudoalteromonas sp. RKEM 647*				X			X	X	X	X
Pseudoalteromonas sp. RKEM 660*	X						X	X	X	X
Pseudoalteromonas sp. RKEM 857*				X			X	X	X	X
Pseudomonas sp. RKEM 1577	X						X	X	X	X
Psychrobacter sp. RKEM 1523	X							X	X	X
Psychrobacter sp. RKEM 670*				X				X	X	X
Psychrobacter sp. RKEM 927*				X				X	X	X
Psychrobacter sp. RKEM 1796		X						X	X	X
Rhizobium sp. RKEM 1529	X									
Rhizobium sp. RKEM 1707			X			X				X
Rhodobacter sp. RKEM 1557	X							X	X	X
Rhodococcus sp. RKEM 1538	X						X			
Shewanella sp. RKEM 1626		X						X	X	X
Shewanella sp. RKEM 1631		X					X			
Streptomycetes sp. RKEM 1715	X						X	X	X	X
Terribacillus sp. RKEM 1564	X								X	

Vibrio sp. RKEM 562*	X		X	X
Vibrio sp. RKEM 601*	X			

*Bacteria first isolated in previous culture collection
Abbreviations – AR: aerobic; MA: microaerophilic; AN: anaerobic; H: holobiont; L: larvae; D: dinoflagellate; W: seawater

Table 4.5. Number of different and unique species present in aerobic, microaerophilic, and anaerobic libraries.

Sample Origin	# Different Species	# Unique Species
Holobiont-Aerobic	23	20
Holobiont-Microaerophilic	18	16
Holobiont-Anaerobic	4	0
Seawater-Aerobic	26	13
Seawater-Microaerophilic	10	1
Seawater-Anaerobic	6	2

4.3.4 Overall Culture-Dependent Bacterial Library

A total of 143 different species of bacteria were obtained once all culture dependent libraries were combined. They spanned six classes: *Gammaproteobacteria* (Figures 4.5, 4.6, and 4.8), *Alphaproteobacteria* (Figures 4.8 and 4.9), *Deltaproteobacteria* (Chapter 5), *Actinobacteria* (Figures 4.10, 4.11, and 4.12), *Bacilli* (Figures 4.13 and 4.14) and *Cytophagia* (Figure 4.15). The majority of these bacteria had been previously characterized, meaning the isolates had ≥97% 16S rRNA gene sequence similarity to previously characterized bacterial type species (Table 4.6).[32] However there were four bacteria with < 97% sequence similarity to characterized bacteria and were putatively novel. Three of which, *Angustibacter* sp. RKEM 1664 (Figure 4.12), *Rhizobium* sp. RKEM 1529 (Figure 4.9), and *Saccharospirillum* sp. RKEM 770 (Figure 4.5) formed distinct clades with type strains from their respective genera and therefore could only be novel at the species level. The other, *Paracoccus* sp. RKEM 946 (Figure 4.8), may be a novel genus and not actually belong to *Paracoccus*. The RKEM 946 sequence did not cluster with *Paracoccus* sp. type strains, but remained within the clade of the family *Rhodobacteraceae*, suggesting that RKEM 946 may belong to a novel genus within the family *Rhodobacteraceae.* The isolate *Pseudobacteriovorax antillogorgiicola* RKEM 611 was determined to belong to a novel family of bacteria, whose formal taxonomic description is detailed in Chapter 5.

Table 4.6. Taxonomic classification of culture-dependent library. Sequences were classified based on BLAST analysis. Sequences with < 97% identity are highlighted in yellow and < 90% identity are highlighted in red.

Strain	Accession Number	Class	Seq. Length (bp)	Closest Blast Hit	Accession Number	Query Cover	ID
Streptomyces sp. RKEM_1715	KU198832	Actino	838	*Streptomyces parvulus* NBRC 13193	NR_041119.2	99	99
Streptomyces sp. RKEM_774	KU198759	Actino	843	*Streptomyces albus* J1074	NR_102949.1	100	99
Dietzia sp. RKEM_832	KU198781	Actino	819	*Dietzia cinnamea* IMMIB	NR_042390.1	100	99
Rhodococcus sp. RKEM_1538	KU198776	Actino	820	*Rhodococcus corynebacterioides* DSM 20151	NR_119107.1	100	99
Rhodococcus sp. RKEM_42	KU198794	Actino	661	*Rhodococcus ruber* DSM 43338	NR_118602.1	100	100
Rhodococcus sp. RKEM_450	KU198737	Actino	841	*Rhodococcus corynebacterioides* DSM 20151	NR_119107.1	99	99
Rhodococcus sp. RKEM_843	KU198844	Actino	819	*Rhodococcus fascians* CF17	NR_037021.1	100	100
Geodermatophilus sp. RKEM_688	KU198754	Actino	849	*Geodermatophilus brasiliensis* Tu6233	NR_126197.1	99	99
Angustibacter sp. RKEM_1664	KU321277	Actino	1421	*Angustibacter aerolatus* 7402J-48	NR_109610.1	97	95
Cellulomonas sp. RKEM_1775	KU198768	Actino	841	*Cellulomonas pakistanensis* NCCP-11	NR_125452.1	97	99
Brachybacterium sp. RKEM_1576	KU198817	Actino	825	*Brachybacterium conglomeratum* J 1015	NR_104689.1	100	99
Kytococcus sp. RKEM_512A	KU198778	Actino	844	*Kytococcus sedentarius* DSM 20547	NR_074714.1	100	99

Janibacter sp. RKEM_1825	KU198841	Actino	828	*Janibacter melonis* CM2104	NR_025805.1	100	99
Ornithinimicrobium sp. RKEM_638	KU321282	Actino	1500	*Ornithinimicrobium kibberense* K22-20	NR_043056.1	99	99
Agrococcus sp. RKEM_1635	KU198774	Actino	833	*Agrococcus terreus* DNG5 16S	NR_116650.1	100	99
Agrococcus sp. RKEM_1685	KU198777	Actino	838	*Agrococcus jejuensis* SSW1-48	NR_042551.1	96	100
Agrococcus sp. RKEM_917	KU198788	Actino	798	*Agrococcus baldri* IAM 15147	NR_041543.1	100	99
Curtobacterium sp. RKEM_1515	KU198764	Actino	825	*Curtobacterium luteum* DSM 20542	NR_026157.1	100	99
Curtobacterium sp. RKEM_1741	KU198833	Actino	825	*Curtobacterium oceanosedimentum* ATCC 31317	NR_104839.1	100	99
Microbacterium sp. RKEM_1514	KU198766	Actino	814	*Microbacterium testaceum* StLB037	NR_074641.1	100	99
Microbacterium sp. RKEM_1559	KU198813	Actino	829	*Microbacterium yannicii* G72	NR_117001.1	100	99
Microbacterium sp. RKEM_1560	KU198814	Actino	815	*Microbacterium hatanonis* FCC-01	NR_041529.1	100	98
Microbacterium sp. RKEM_1562	KU198815	Actino	796	*Microbacterium maritypicum* DSM 12512	NR_114986.1	100	99
Microbacterium sp. RKEM_1591	KU198820	Actino	817	*Microbacterium foliorum* P 333/02	NR_025368.1	100	99
Microbacterium sp. RKEM_1651	KU198775	Actino	831	*Microbacterium arthrosphaerae* CC-VM-Y	NR_117046.1	98	100
Microbacterium sp. RKEM_1661	KU198824	Actino	815	*Microbacterium flavescens* 401	NR_029350.1	100	99
Microbacterium sp. RKEM_1691	KU198773	Actino	816	*Microbacterium flavescens* 401	NR_029350.1	100	98

Microbacterium sp. RKEM_1778	KU198780	Actino	813	*Microbacterium testaceum* StLB037	NR_074641.1	100	99
Microbacterium sp. RKEM_1786	KU198837	Actino	833	*Microbacterium yannicii* G72	NR_117001.1	100	98
Microbacterium sp. RKEM_1814	KU198839	Actino	772	*Microbacterium testaceum* StLB037	NR_074641.1	100	99
Microbacterium sp. RKEM_1815	KU198770	Actino	830	*Microbacterium testaceum* StLB037	NR_074641.1	100	99
Microbacterium sp. RKEM_922	KU198765	Actino	832	*Microbacterium oleivorans* BAS69	NR_042262.1	100	99
Arthrobacter sp. RKEM_1571	KU198816	Actino	774	*Arthrobacter phenanthrenivorans* Sphe3	NR_074770.1	99	99
Arthrobacter sp. RKEM_1637	KU198823	Actino	806	*Arthrobacter ureafaciens* NC	NR_029281.1	100	98
Arthrobacter sp. RKEM_1692	KU198827	Actino	775	*Arthrobacter ureafaciens* NC	NR_029281.1	99	99
Arthrobacter sp. RKEM_332	KU321279	Actino	1306	*Arthrobacter humicola* KV-653	NR_041546.1	99	94
Citricoccus sp. RKEM_914	KU198786	Actino	844	*Citricoccus alkalitolerans* YIM 70010	NR_025771.1	100	99
Kocuria sp. RKEM_1608	KU198821	Actino	845	*Kocuria sediminis* FCS-11	NR_118222.1	100	98
Kocuria sp. RKEM_639	KU198796	Actino	800	*Kocuria palustris* TAGA27	NR_026451.1	100	100
Micrococcus sp. RKEM_702	KU198741	Actino	841	*Micrococcus aloeverae* AE-6	NR_134088.1	100	100
Cellulosimicrobium sp. RKEM_1754	KU198779	Actino	823	*Cellulosimicrobium terreum* DS-61	NR_044070.1	100	98
Aeromicrobium sp. RKEM_1713	KU198831	Actino	640	*Aeromicrobium tamlense* SSW1-57	NR_043791.1	100	99

Marmoricola sp. RKEM_1784	KU198836	Actino	838	*Marmoricola korecus* Sco-A36	NR_116963.1	100	97
Nocardioides sp. RKEM_1695	KU198828	Actino	728	*Nocardioides ganghwensis* JC2055	NR_025776.1	100	97
Nocardioides sp. RKEM_1698	KU198829	Actino	803	*Nocardioides ganghwensis* JC2055	NR_025776.1	100	98
Nocardioides sp. RKEM_1712	KU198830	Actino	836	*Nocardioides terrigena* DS-17	NR_044185.1	99	97
Nocardioides sp. RKEM_1764	KU198834	Actino	813	*Nocardioides soli* mbc-2	NR_133797.1	100	99
Nocardioides sp. RKEM_1781	KU198835	Actino	820	*Nocardioides aestuarii* JC2056	NR_025777.1	100	99
Nocardioides sp. RKEM_944	KU198791	Actino	821	*Nocardioides salarius* J112	NR_044419.1	100	99
Erythrobacter sp. RKEM_1590	KU198819	Alpha	736	*Erythrobacter gaetbuli* SW-161	NR_025818.1	100	98
Erythrobacter sp. RKEM_642	KU321283	Alpha	1416	*Erythrobacter westpacificensis* JL2008	NR_132719.1	100	97
Erythrobacter sp. RKEM_937	KU198790	Alpha	740	*Erythrobacter citreus* RE35F/1	NR_028741.1	99	99
Erythrobacter sp. RKEM_947	KU198793	Alpha	740	*Erythrobacter vulgaris* 022 2-10	NR_043136.1	98	100
Aureimonas sp. RKEM_1543	KU198811	Alpha	589	*Aureimonas altamirensis* S21B	NR_043764.1	100	99
Rhizobium sp. RKEM_1529	KU321275	Alpha	1033	*Rhizobium cellulosilyticum* ALA10B2	NR_043985.1	90	96
Rhizobium sp. RKEM_1707	KU321278	Alpha	1399	*Rhizobium cellulosilyticum* ALA10B2	NR_043985.1	100	98
Rhizobium sp. RKEM_870	KU198846	Alpha	589	*Rhizobium skierniewicense* Ch11	NR_118035.1	100	99

Citreicella sp. RKEM_868	KU198782	Alpha	794	*Citreicella marina* CK-I3-6	NR_116507.1	100	99
Paracoccus sp. RKEM_1551	KU198769	Alpha	639	*Paracoccus caeni* MJ17	NR_108571.1	100	99
Paracoccus sp. RKEM_1671	KU198825	Alpha	757	*Paracoccus aminovorans* NBRC 16711	NR_113864.1	100	98
Paracoccus sp. RKEM_1682	KU198826	Alpha	789	*Paracoccus chinensis* NBRC 104937	NR_114276.1	99	99
Paracoccus sp. RKEM_656	KU198758	Alpha	619	*Paracoccus zeaxanthinifaciens* ATCC 21588	NR_025218.1	100	100
Paracoccus sp. RKEM_942	KU198851	Alpha	796	*Paracoccus homiensis* DD-R11	NR_043733.1	100	98
Paracoccus sp. RKEM_943	KU198852	Alpha	571	*Paracoccus stylophorae* KTW-16	NR_117275.1	100	100
Paracoccus sp. RKEM_946	KU198731	Alpha	987	*Paracoccus siganidrum* M26	NR_118463.1	100	96
Pseudovibrio sp. RKEM_536	KU321281	Alpha	1458	*Pseudovibrio denitrificans* DN34	NR_029112.1	97	100
Rhodobacter sp. RKEM_1557	KU198812	Alpha	790	*Rhodobacter sphaeroides* 2.4.1	NR_029215.1	100	98
Ruegeria sp. RKEM_265	KU198725	Alpha	755	*Ruegeria pelagia* NBRC 102038	NR_116522.1	100	100
Sagittula sp. RKEM_724B	KU198756	Alpha	731	*Sagittula marina* F028-2	NR_109096.1	99	98
Sulfitobacter sp. RKEM_910	KU198849	Alpha	789	*Sulfitobacter delicatus* KMM 3584	NR_025692.1	100	97
Erythrobacter sp. RKEM_933	KU198783	Alpha	740	*Porphyrobacter sanguineus* A91	NR_036841.1	100	97
Sphingomonas sp. RKEM_364	KU198801	Alpha	557	*Sphingomonas parapaucimobilis* NBRC 15100	NR_113729.1	100	100

Bacillus sp. RKEM_1569	KU198771	Bacilli	850	*Bacillus anthracis* Ames	NR_074453.1	100	99
Bacillus sp. RKEM_1771	KU198804	Bacilli	611	*Bacillus hwajinpoensis* SW-72	NR_025264.1	100	100
Bacillus sp. RKEM_266	KU198732	Bacilli	866	*Bacillus carboniphilus* JCM9731	NR_024690.1	98	98
Bacillus sp. RKEM_441	KU198736	Bacilli	869	*Bacillus idriensis* SMC 4352-2	NR_043268.1	97	99
Bacillus sp. RKEM_444	KU198724	Bacilli	873	*Bacillus tianshenii* YIM M13235	NR_133704.1	100	99
Bacillus sp. RKEM_513C	KU198802	Bacilli	780	*Bacillus circulans* NBRC 13626	NR_112632.1	100	99
Bacillus sp. RKEM_632	KU198792	Bacilli	788	*Bacillus simplex* LMG 11160	NR_114919.1	100	99
Bacillus sp. RKEM_667	KU198760	Bacilli	882	*Bacillus aryabhattai* B8W22	NR_115953.1	100	99
Bacillus sp. RKEM_720	KU198755	Bacilli	850	*Bacillus endophyticus* 2DT	NR_025122.1	100	99
Bacillus sp. RKEM_730	KU198750	Bacilli	880	*Bacillus pumilus* SAFR-032	NR_074977.1	100	99
Bacillus sp. RKEM_731	KU198789	Bacilli	855	*Bacillus niabensis* 4T19	NR_043334.1	100	98
Bacillus sp. RKEM_755	KU198757	Bacilli	873	*Bacillus firmus* NCIMB 9366	NR_118955.1	100	98
Bacillus sp. RKEM_781B	KU198806	Bacilli	756	*Bacillus oceanisediminis* H2	NR_117285.1	100	98
Bacillus sp. RKEM_829B	KU198808	Bacilli	835	*Bacillus drentensis* LMG 21831	NR_118438.1	100	99
Fictibacillus sp. RKEM_566	KU198749	Bacilli	851	*Fictibacillus phosphorivorans* Ca7	NR_118455.1	100	99

Halobacillus sp. RKEM_1822	KU198840	Bacilli	789	*Halobacillus litoralis* SL-4	NR_029304.1	100	98
Oceanobacillus sp. RKEM_814	KU198761	Bacilli	794	*Oceanobacillus picturae* R-5321	NR_028952.1	100	100
Terribacillus sp. RKEM_1564	KU198767	Bacilli	784	*Terribacillus saccharophilus* 002-048	NR_041356.1	100	100
Paenibacillus sp. RKEM_1589	KU198818	Bacilli	614	*Paenibacillus illinoisensis* NBRC 15959	NR_113828.1	100	99
Paenibacillus sp. RKEM_768	KU198730	Bacilli	752	*Paenibacillus pabuli* JCM 9074	NR_112164.1	100	99
Planococcus sp. RKEM_918	KU198850	Bacilli	827	*Planococcus rifietoensis* M8	NR_025553.1	100	100
Staphylococcus sp. RKEM_1626	KU198822	Bacilli	815	*Staphylococcus haemolyticus* JCSC1435	NR_074994.1	100	100
Staphylococcus sp. RKEM_613	KU198751	Bacilli	910	*Staphylococcus epidermidis* RP62A	NR_074995.1	97	99
Fulvivirga sp. RKEM_712	KU198797	Cytophagia	574	*Fulvivirga imtechensis* AK7	NR_117079.1	100	97
Alteromonas sp. RKEM_316	KU198735	Gamma	855	*Alteromonas macleodii* NBRC 102226	NR_114053.1	99	99
Alteromonas sp. RKEM_369	KU198721	Gamma	865	*Alteromonas macleodii* 107	NR_074797.1	100	99
Alteromonas sp. RKEM_717	KU198727	Gamma	854	*Alteromonas hispanica* F-32	NR_043274.1	99	99
Alteromonas sp. RKEM_765	KU198803	Gamma	852	*Alteromonas australica* H 17 1	NR_116737.1	100	99
Pseudoalteromonas sp. RKEM_243	KU198720	Gamma	864	*Pseudoalteromonas piscicida* IAM 12932	NR_040946.1	98	100
Pseudoalteromonas sp. RKEM_647	KU198753	Gamma	854	*Pseudoalteromonas agarivorans* DSM 14585	NR_025509.1	100	99

Pseudoalteromonas sp. RKEM_660	KU198752	Gamma	870	*Pseudoalteromonas arabiensis* k53	NR_113220.1	100	100
Pseudoalteromonas sp. RKEM_732	KU198729	Gamma	859	*Pseudoalteromonas shioyasakiensis* SE3	NR_125458.1	100	99
Pseudoalteromonas sp. RKEM_857	KU198845	Gamma	817	*Pseudoalteromonas lipolytica* LMEB 39	NR_116629.1	100	100
Shewanella sp. RKEM_1631	KU198763	Gamma	795	*Shewanella corallii* fav-2-10-05	NR_116537.1	98	99
Cronobacter sp. RKEM_537A	KU198745	Gamma	861	*Cronobacter zurichensis* LMG 23730	NR_104924.1	99	99
Cronobacter sp. RKEM_548	KU198739	Gamma	872	*Cronobacter massiliensis* JC163	NR_125600.1	100	98
Enterobacter sp. RKEM_1504	KU198762	Gamma	803	*Enterobacter cloacae* DSM 30054	NR_118011.1	100	99
Pantoea sp. RKEM_516	KU198742	Gamma	870	*Pantoea vagans* C9-1	NR_102966.1	100	99
Pantoea sp. RKEM_552	KU198747	Gamma	821	*Pantoea dispersa* DSM 30073	NR_116797.1	100	99
Pantoea sp. RKEM_701	KU198743	Gamma	873	*Pantoea stewartii* CIP 104006	NR_104928.1	100	98
Cobetia sp. RKEM_646	KU198728	Gamma	863	*Cobetia litoralis* KMM 3880	NR_113403.1	99	99
Halomonas sp. RKEM_883	KU321285	Gamma	789	*Halomonas nitroreducens* 11S	NR_044317.1	100	98
Halomonas sp. RKEM_901	KU198848	Gamma	841	*Halomonas axialensis* Althf1	NR_027219.1	100	99
Bermanella sp. RKEM_777	KU198805	Gamma	854	*Bermanella marisrubri* RED65	NR_042750.1	100	99
Marinomonas sp. RKEM_313	KU198723	Gamma	859	*Marinomonas communis* NBRC 102224	NR_114051.1	99	99

Marinomonas sp. RKEM_715	KU198722	Gamma	858	*Marinomonas posidonica* IVIA-Po-181	NR_074719.1	100	97
Marinomonas sp. RKEM_924	KU321286	Gamma	1308	*Marinomonas posidonica* IVIA-Po-181	NR_074719.1	100	99
Saccharospirillum sp. RKEM_770	KU321284	Gamma	1507	*Saccharospirillum impatiens* EL-105	NR_028953.1	96	96
Acinetobacter sp. RKEM_528	KU198726	Gamma	858	*Acinetobacter pittii* ATCC 19004	NR_117621.1	100	100
Acinetobacter sp. RKEM_532	KU198738	Gamma	857	*Acinetobacter lwoffii* JCM 6840	NR_113346.1	99	97
Acinetobacter sp. RKEM_817	KU198798	Gamma	858	*Acinetobacter radioresistens* NBRC 102413	NR_114074.1	99	99
Psychrobacter sp. RKEM_1523	KU198810	Gamma	856	*Psychrobacter celer* SW-238	NR_043225.1	99	99
Psychrobacter sp. RKEM_1796	KU198838	Gamma	823	*Psychrobacter piscatorii* T-3-2	NR_112807.1	100	98
Psychrobacter sp. RKEM_535	KU198795	Gamma	783	*Psychrobacter celer* SW-238	NR_043225.1	100	99
Psychrobacter sp. RKEM_670	KU198744	Gamma	860	*Psychrobacter pacificensis* NBRC 103191	NR_114238.1	99	99
Psychrobacter sp. RKEM_879	KU198847	Gamma	824	*Psychrobacter marincola* KMM 277	NR_025458.1	100	99
Psychrobacter sp. RKEM_927	KU198787	Gamma	858	*Psychrobacter nivimaris* 88/2-7	NR_028948.1	100	100
Pseudomonas sp. RKEM_1577	KU198772	Gamma	637	*Pseudomonas stutzeri* A1501	NR_074829.1	100	99
Pseudomonas sp. RKEM_545	KU198746	Gamma	847	*Pseudomonas putida* KT2440	NR_074596.1	100	99
Pseudomonas sp. RKEM_824A	KU198807	Gamma	589	*Pseudomonas indoloxydans* IPL-1	NR_115922.1	100	99

Pseudomonas sp. RKEM_891	KU198785	Gamma	661	*Pseudomonas indoloxydans* IPL-1	NR_115922.1	100	99
Photobacterium sp. RKEM_1507	KU198809	Gamma	734	*Photobacterium damselae* NBRC 15633	NR_113783.1	100	100
Vibrio sp. RKEM_201	KU198719	Gamma	868	*Vibrio pelagius* NBRC 15639	NR_113789.1	99	99
Vibrio sp. RKEM_309	KU198733	Gamma	733	*Vibrio coralliilyticus* ATCC BAA-450	NR_117892.1	99	99
Vibrio sp. RKEM_328	KU198734	Gamma	864	*Vibrio alginolyticus* NBRC 15630	NR_122059.1	100	99
Vibrio sp. RKEM_48	KU198842	Gamma	818	*Vibrio sinaloensis* CAIM 797	NR_043858.1	100	99
Vibrio sp. RKEM_501	KU198740	Gamma	883	*Vibrio xuii* R-15052	NR_025478.1	98	99
Vibrio sp. RKEM_562	KU198748	Gamma	766	*Vibrio rotiferianus* LMG 21460	NR_118091.1	100	99
Vibrio sp. RKEM_601	KU198800	Gamma	441	*Vibrio mediterranei* 50	NR_029257.1	100	99
Vibrio sp. RKEM_69	KU198843	Gamma	790	*Vibrio tubiashii* NBRC 15644	NR_113791.1	100	100
Pseudobacteriovorax antillogorgiicola RKEM 611	KJ685394	Delta	1466	*Desulfovibrio senezii* CVL	NR_024887.1	97	89

Abbreviations: Actino: *Actinobacteria*; Alpha: *Alphaproteobacteria*; Gamma: *Gammproteobacteria*; Delta: *Deltaproteobacteria*

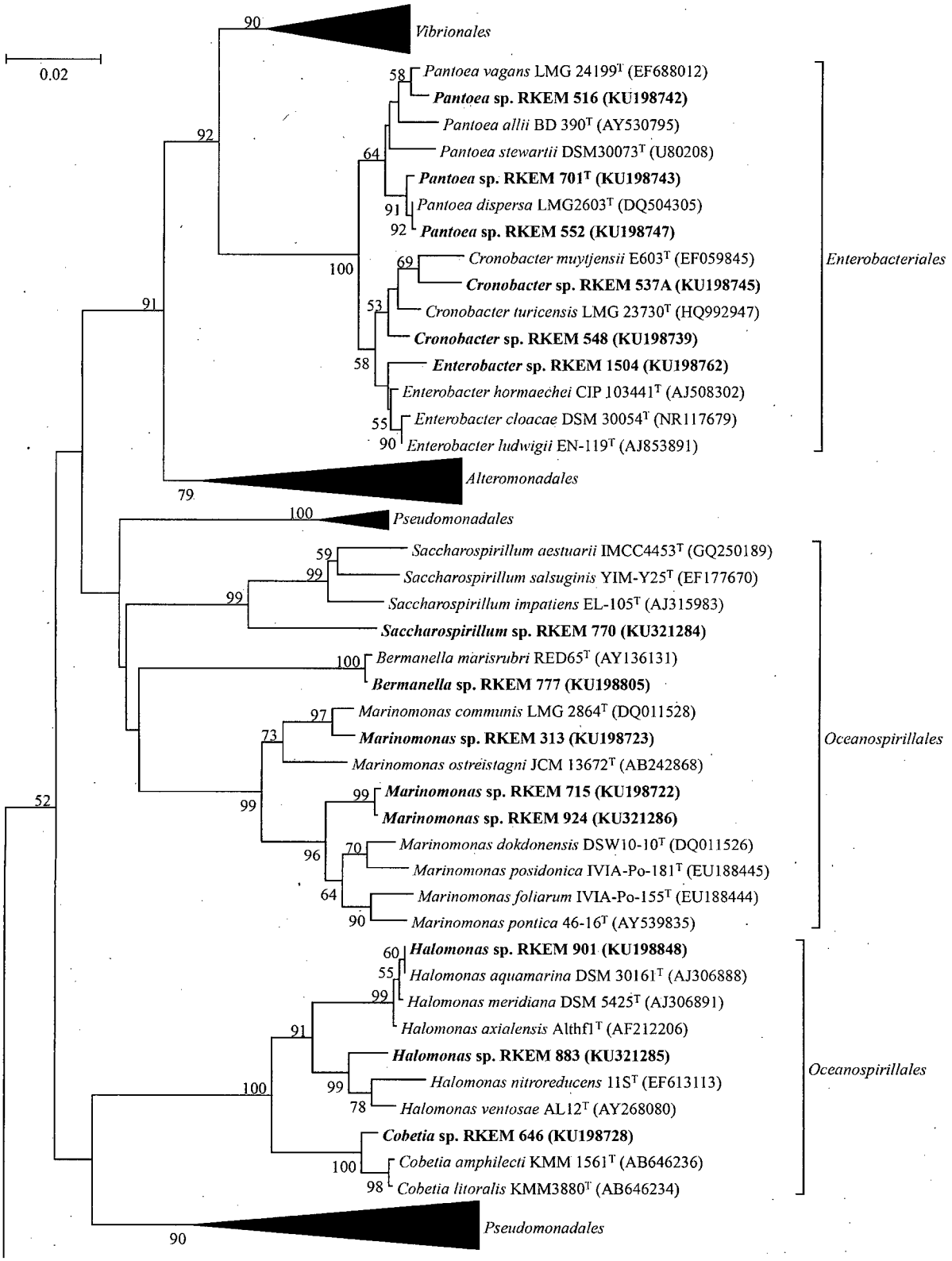

0.02
Vibrionales
Pantoea vagans LMG 24199T (EF688012)
Pantoea sp. RKEM 516 (KU198742)
Pantoea allii BD 390T (AY530795)
Pantoea stewartii DSM30073T (U80208)
Pantoea sp. RKEM 701T (KU198743)
Pantoea dispersa LMG2603T (DQ504305)
Pantoea sp. RKEM 552 (KU198747)
Cronobacter muytjensii E603T (EF059845)
Cronobacter sp. RKEM 537A (KU198745)
Cronobacter turicensis LMG 23730T (HQ992947)
Cronobacter sp. RKEM 548 (KU198739)
Enterobacter sp. RKEM 1504 (KU198762)
Enterobacter hormaechei CIP 103441T (AJ508302)
Enterobacter cloacae DSM 30054T (NR117679)
Enterobacter ludwigii EN-119T (AJ853891)
Enterobacteriales
Alteromonadales
Pseudomonadales
Saccharospirillum aestuarii IMCC4453T (GQ250189)
Saccharospirillum salsuginis YIM-Y25T (EF177670)
Saccharospirillum impatiens EL-105T (AJ315983)
Saccharospirillum sp. RKEM 770 (KU321284)
Bermanella marisrubri RED65T (AY136131)
Bermanella sp. RKEM 777 (KU198805)
Marinomonas communis LMG 2864T (DQ011528)
Marinomonas sp. RKEM 313 (KU198723)
Marinomonas ostreistagni JCM 13672T (AB242868)
Marinomonas sp. RKEM 715 (KU198722)
Marinomonas sp. RKEM 924 (KU321286)
Marinomonas dokdonensis DSW10-10T (DQ011526)
Marinomonas posidonica IVIA-Po-181T (EU188445)
Marinomonas foliarum IVIA-Po-155T (EU188444)
Marinomonas pontica 46-16T (AY539835)
Oceanospirillales
Halomonas sp. RKEM 901 (KU198848)
Halomonas aquamarina DSM 30161T (AJ306888)
Halomonas meridiana DSM 5425T (AJ306891)
Halomonas axialensis Althf1T (AF212206)
Halomonas sp. RKEM 883 (KU321285)
Halomonas nitroreducens 11ST (EF613113)
Halomonas ventosae AL12T (AY268080)
Cobetia sp. RKEM 646 (KU198728)
Cobetia amphilecti KMM 1561T (AB646236)
Cobetia litoralis KMM3880T (AB646234)
Oceanospirillales
Pseudomonadales

Figure 4.5. Neighbor-joining phylogenetic tree based on 16S rRNA gene sequences of bacteria in the orders *Enterobacteriales* and *Oceanospirillales*. The tree was constructed using all *Gammaproteobacteria* in the culture dependent library and closely related type strains; bacteria from this study are in bold. The tree was rooted using *Asticcacaulis solisilvae* CGM1-3EN^{T} (NR 109665.1), *Achromobacter aegrifaciens* LMG 26852^{T} (NR 117707.1), and *Desulfarculus baarsii* DSM 2075 (NR 074919.1) (not shown). Evolutionary distances were computed using the Jukes-Cantor method. A total of 567 nucleotide positions were used. Bootstrap values are expressed as a percentage of 1000 replicates, bootstrap values < 50 are not shown.

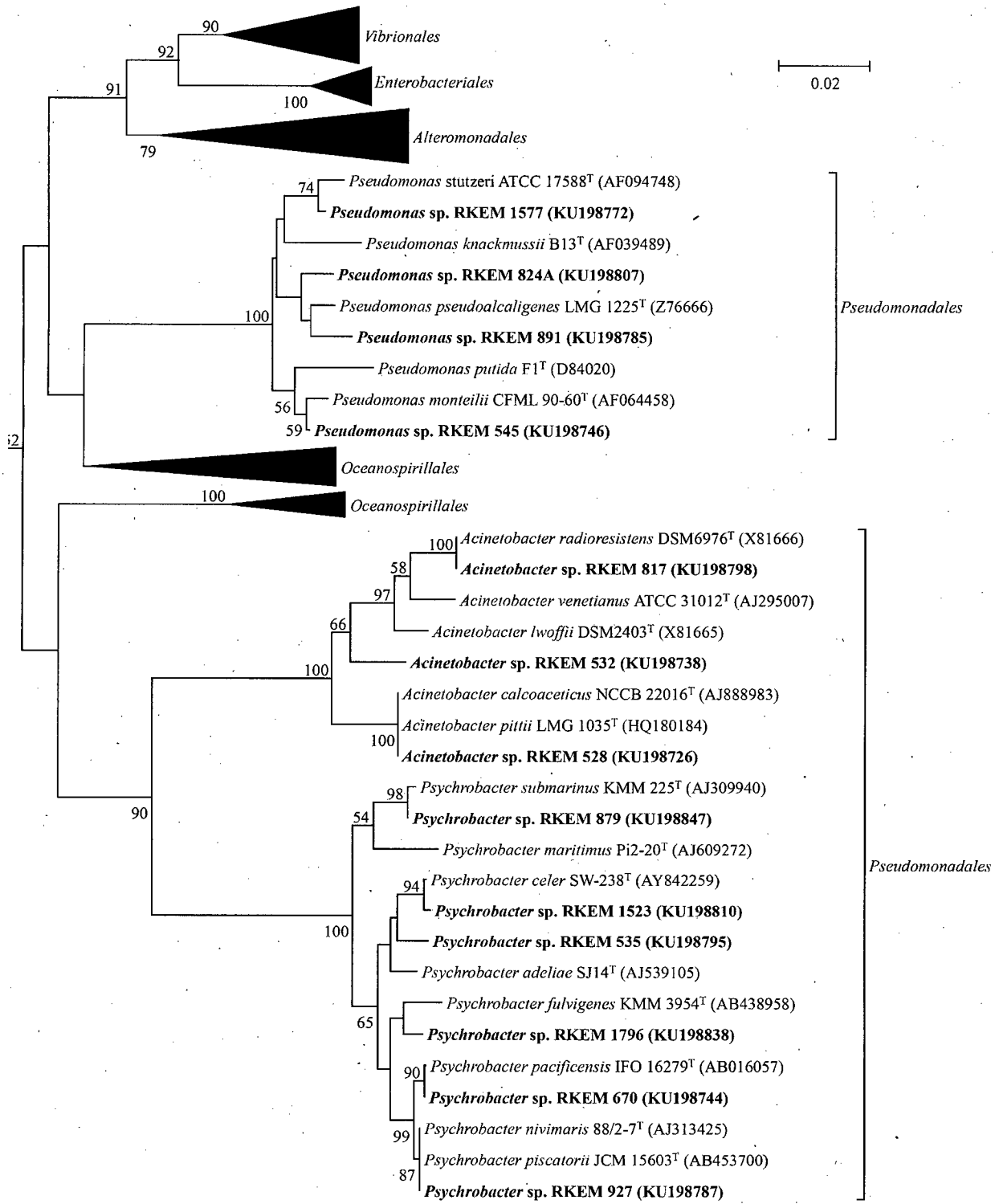


Figure 4.6. Neighbor-joining phylogenetic tree based on 16S rRNA gene sequences of bacteria in the order *Pseudomonadales*. The tree was constructed using all

Gammaproteobacteria in the culture dependent library and closely related type strains; bacteria from this study are in bold. The tree was rooted using *Asticcacaulis solisilvae* CGM1-3EN^T (NR 109665.1), *Achromobacter aegrifaciens* LMG 26852^T (NR 117707.1), and *Desulfarculus baarsii* DSM 2075 (NR 074919.1) (not shown). Evolutionary distances were computed using the Jukes-Cantor method. A total of 567 nucleotide positions were used. Bootstrap values are expressed as a percentage of 1000 replicates, bootstrap values < 50 are not shown.

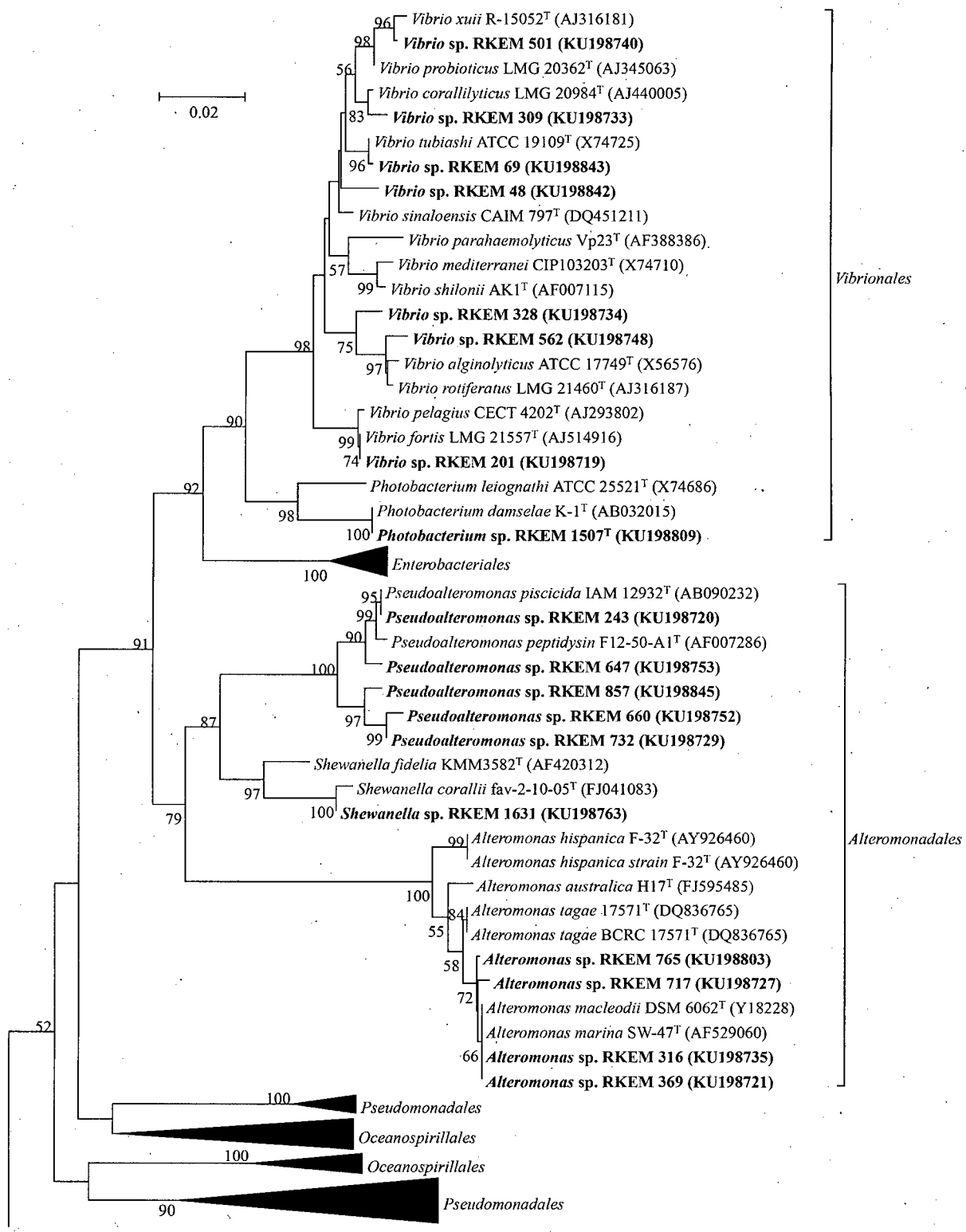


Figure 4.7. Neighbor-joining phylogenetic tree based on 16S rRNA gene sequences of bacteria in the orders *Vibrionales* and *Alteromonadales*. The tree was constructed using all

Gammaproteobacteria in the culture dependent library and closely related type strains; bacteria from this study are in bold. The tree was rooted using *Asticcacaulis solisilvae* CGM1-3EN^T (NR 109665.1), *Achromobacter aegrifaciens* LMG 26852^T (NR 117707.1), and *Desulfarculus baarsii* DSM 2075 (NR 074919.1) (not shown): Evolutionary distances were computed using the Jukes-Cantor method. A total of 567 nucleotide positions were used. Bootstrap values are expressed as a percentage of 1000 replicates, bootstrap values < 50 are not shown.

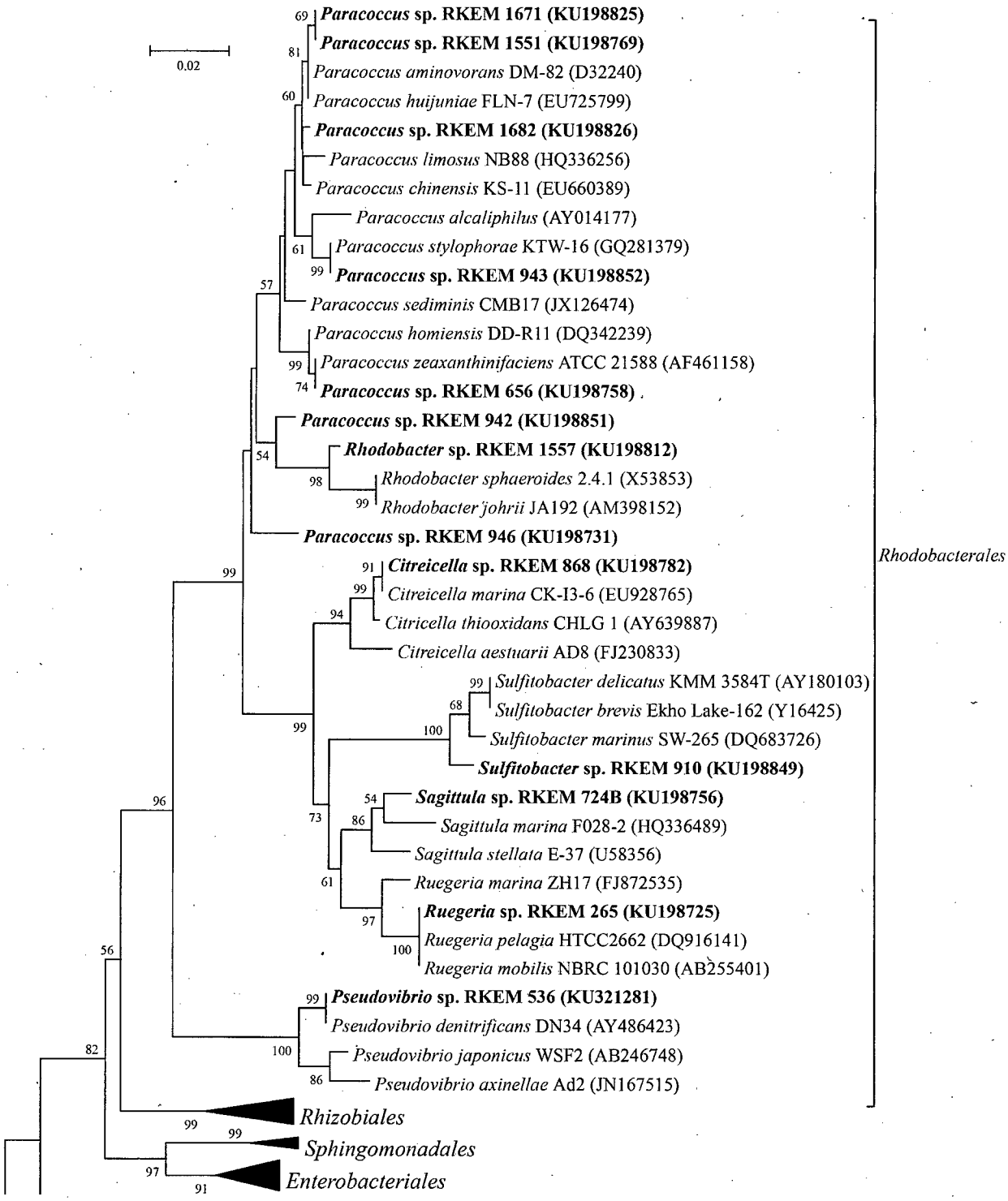


Figure 4.8. Neighbor-joining phylogenetic tree based on 16S rRNA gene sequences of bacteria in the order *Rhodobacterales*. The tree was constructed using all *Alphaproteobacteria* in the culture dependent library and closely related type strains; bacteria from this study are in

bold. The tree was rooted using *Acidiferrobacter thiooxydans* DSM2392 (NR_114870.1) *Achromobacter aegrifaciens* LMG 26852^T (NR 117707.1), and *Desulfarculus baarsii* DSM 2075 (NR 074919.1) (not shown). Evolutionary distances were computed using the Jukes-Cantor method. A total of 569 nucleotide positions were used. Bootstrap values are expressed as a percentage of 1000 replicates, bootstrap values < 50 are not shown.

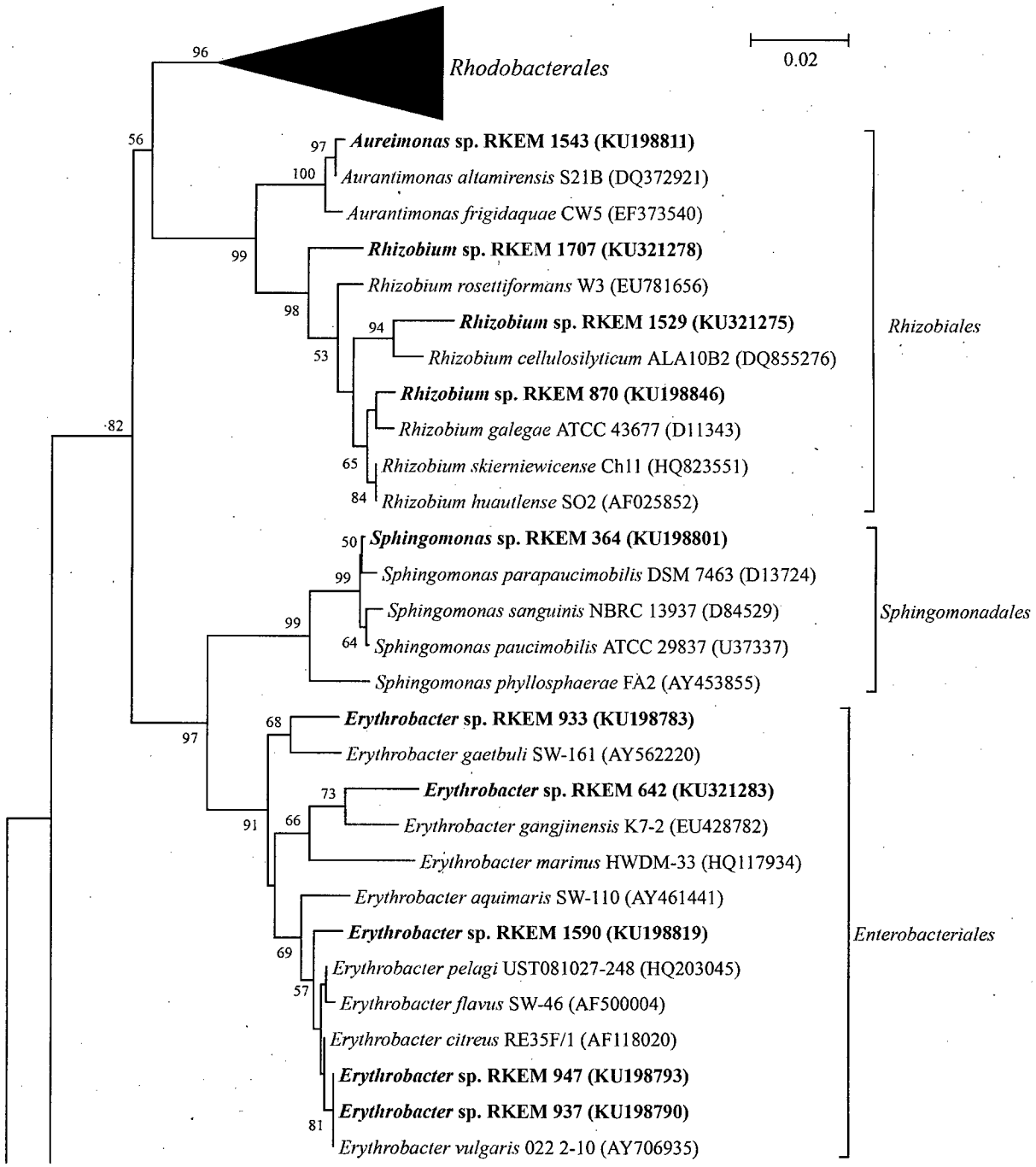


Figure 4.9. Neighbor-joining phylogenetic tree based on 16S rRNA gene sequences of bacteria in the orders *Rhizobiales, Sphingomonadales,* and *Enterobacteriales*. The tree was constructed using all *Alphaproteobacteria* in the culture dependent library and closely related type strains; bacteria from this study are in bold. The tree was rooted using *Acidiferrobacter*

thiooxydans DSM2392 (NR_114870.1) *Achromobacter aegrifaciens* LMG 26852^{T} (NR 117707.1), and *Desulfarculus baarsii* DSM 2075 (NR 074919.1) (not shown). Evolutionary distances were computed using the Jukes-Cantor method. A total of 569 nucleotide positions were used. Bootstrap values are expressed as a percentage of 1000 replicates, bootstrap values < 50 are not shown.

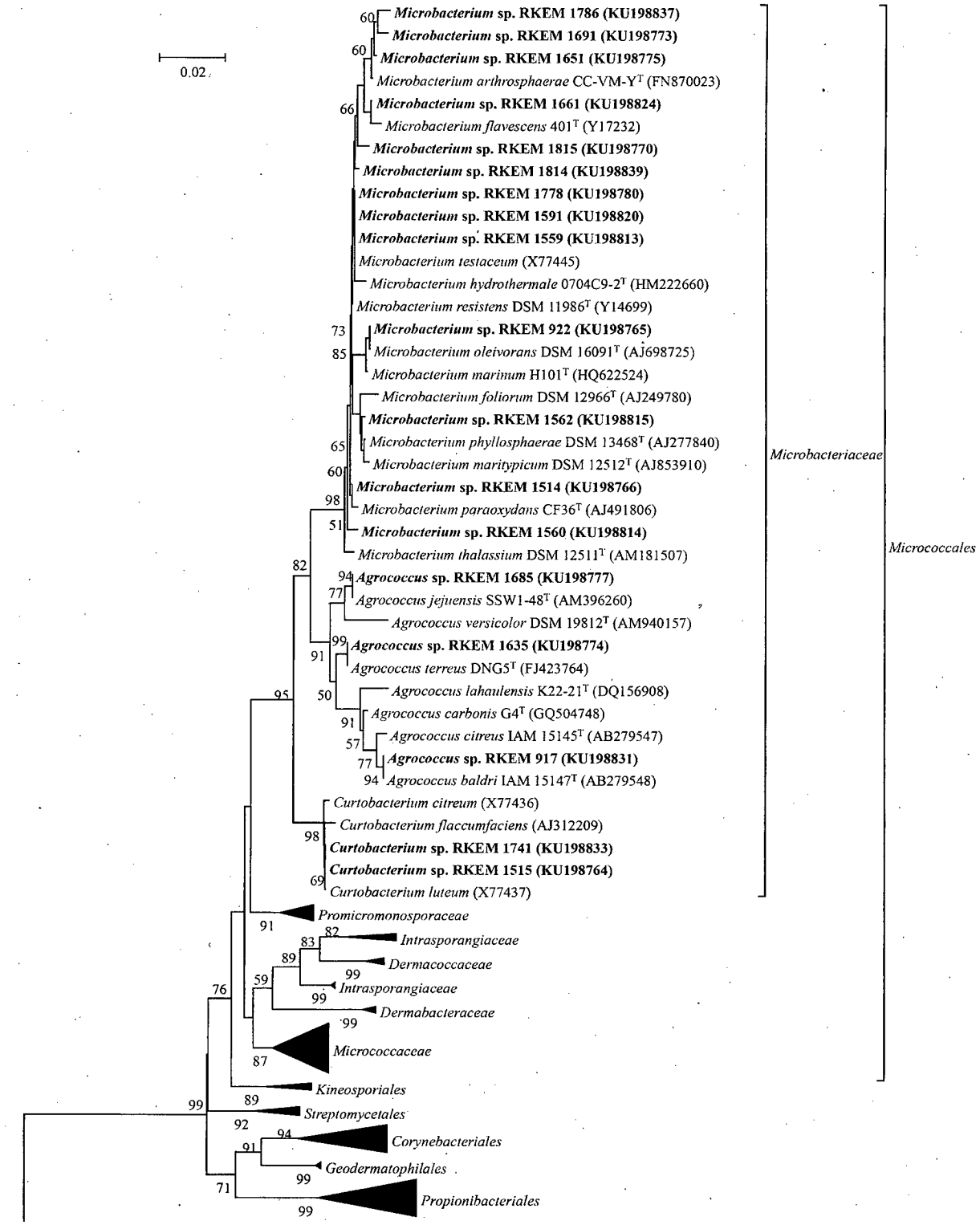
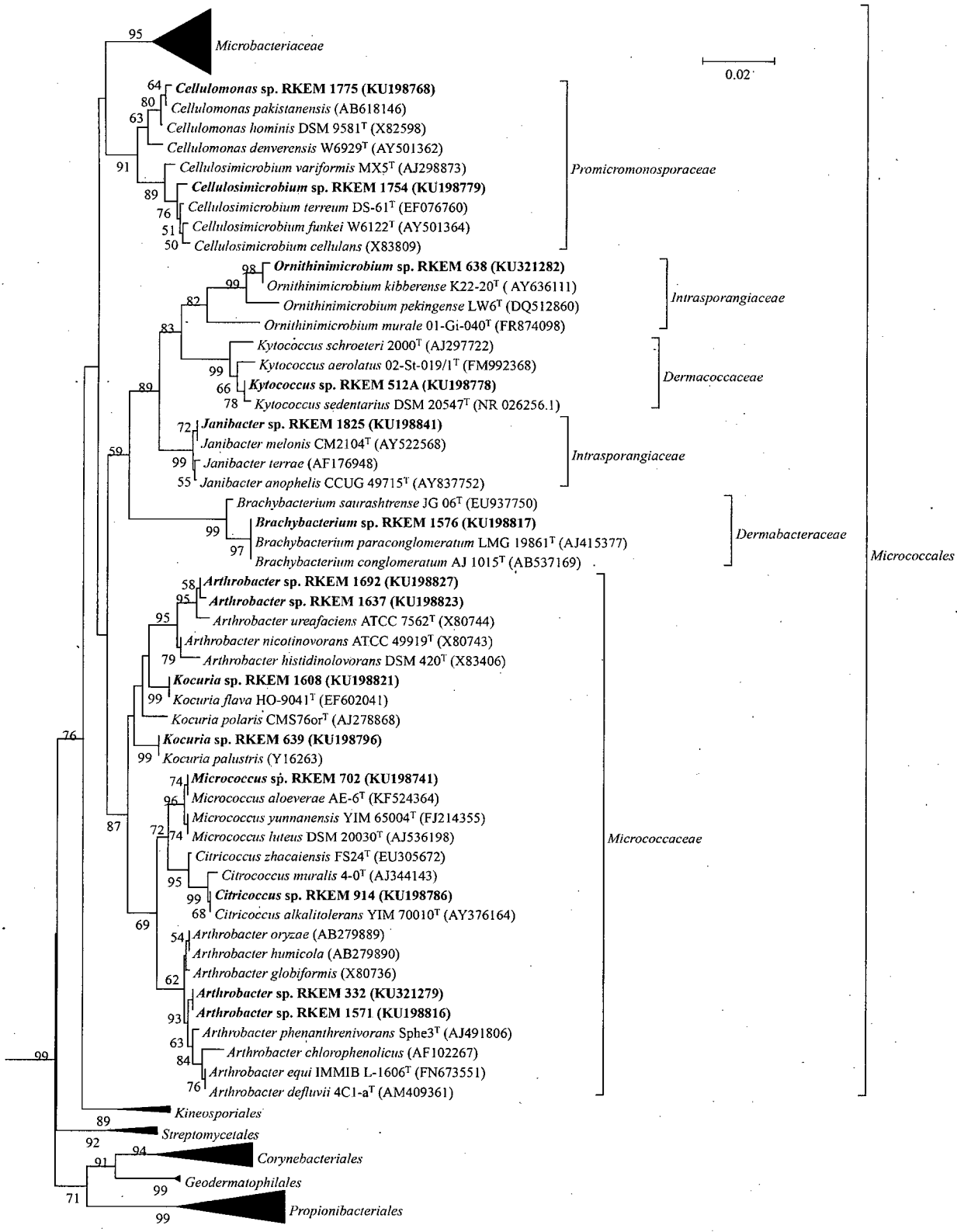


Figure 4.10. Neighbor-joining phylogenetic tree based on 16S rRNA gene sequences of bacteria in the family *Microbacteriaceae*. The tree was constructed using all *Actinobacteria* in the culture dependent library and closely related type strains; bacteria from this study are in bold.

The tree was rooted using *Asticcacaulis solisilvae* CGM1-3EN^T (NR 109665.1), *Achromobacter aegrifaciens* LMG 26852^T (NR 117707.1), and *Desulfarculus baarsii* DSM 2075 (NR 074919.1) (not shown). Evolutionary distances were computed using the Jukes-Cantor method. A total of 620 nucleotide positions were used. Bootstrap values are expressed as a percentage of 1000 replicates, bootstrap values < 50 are not shown.


Microbacteriaceae
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Cellulomonas sp. RKEM 1775 (KU198768)
Cellulomonas pakistanensis (AB618146)
Cellulomonas hominis DSM 9581T (X82598)
Cellulomonas denverensis W6929T (AY501362)
Cellulosimicrobium variformis MX5T (AJ298873)
Cellulosimicrobium sp. RKEM 1754 (KU198779)
Cellulosimicrobium terreum DS-61T (EF076760)
Cellulosimicrobium funkei W6122T (AY501364)
Cellulosimicrobium cellulans (X83809)
Promicromonosporaceae
Ornithinimicrobium sp. RKEM 638 (KU321282)
Ornithinimicrobium kibberense K22-20T (AY636111)
Ornithinimicrobium pekingense LW6T (DQ512860)
Ornithinimicrobium murale 01-Gi-040T (FR874098)
Intrasporangiaceae
Kytococcus schroeteri 2000T (AJ297722)
Kytococcus aerolatus 02-St-019/1T (FM992368)
Kytococcus sp. RKEM 512A (KU198778)
Kytococcus sedentarius DSM 20547T (NR 026256.1)
Dermacoccaceae
Janibacter sp. RKEM 1825 (KU198841)
Janibacter melonis CM2104T (AY522568)
Janibacter terrae (AF176948)
Janibacter anophelis CCUG 49715T (AY837752)
Intrasporangiaceae
Brachybacterium saurashtrense JG 06T (EU937750)
Brachybacterium sp. RKEM 1576 (KU198817)
Brachybacterium paraconglomeratum LMG 19861T (AJ415377)
Brachybacterium conglomeratum AJ 1015T (AB537169)
Dermabacteraceae
Micrococcales
Arthrobacter sp. RKEM 1692 (KU198827)
Arthrobacter sp. RKEM 1637 (KU198823)
Arthrobacter ureafaciens ATCC 7562T (X80744)
Arthrobacter nicotinovorans ATCC 49919T (X80743)
Arthrobacter histidinolovorans DSM 420T (X83406)
Kocuria sp. RKEM 1608 (KU198821)
Kocuria flava HO-9041T (EF602041)
Kocuria polaris CMS76orT (AJ278868)
Kocuria sp. RKEM 639 (KU198796)
Kocuria palustris (Y16263)
Micrococcus sp. RKEM 702 (KU198741)
Micrococcus aloeverae AE-6T (KF524364)
Micrococcus yunnanensis YIM 65004T (FJ214355)
Micrococcus luteus DSM 20030T (AJ536198)
Micrococcaceae
Citricoccus zhacaiensis FS24T (EU305672)
Citrococcus muralis 4-0T (AJ344143)
Citricoccus sp. RKEM 914 (KU198786)
Citricoccus alkalitolerans YIM 70010T (AY376164)
Arthrobacter oryzae (AB279889)
Arthrobacter humicola (AB279890)
Arthrobacter globiformis (X80736)
Arthrobacter sp. RKEM 332 (KU321279)
Arthrobacter sp. RKEM 1571 (KU198816)
Arthrobacter phenanthrenivorans Sphe3T (AJ491806)
Arthrobacter chlorophenolicus (AF102267)
Arthrobacter equi IMMIB L-1606T (FN673551)
Arthrobacter defluvii 4C1-aT (AM409361)
Kineosporiales
Streptomycetales
Corynebacteriales
Geodermatophilales
Propionibacteriales

Figure 4.11. Neighbor-joining phylogenetic tree based on 16S rRNA gene sequences of bacteria in the order *Micrococcales*. The tree was constructed using all *Actinobacteria* in the culture dependent library and closely related type strains; bacteria from this study are in bold. The tree was rooted using *Asticcacaulis solisilvae* CGM1-3EN^T (NR 109665.1), *Achromobacter aegrifaciens* LMG 26852^T (NR 117707.1), and *Desulfarculus baarsii* DSM 2075 (NR 074919.1) (not shown). Evolutionary distances were computed using the Jukes-Cantor method. A total of 620 nucleotide positions were used. Bootstrap values are expressed as a percentage of 1000 replicates, bootstrap values < 50 are not shown.

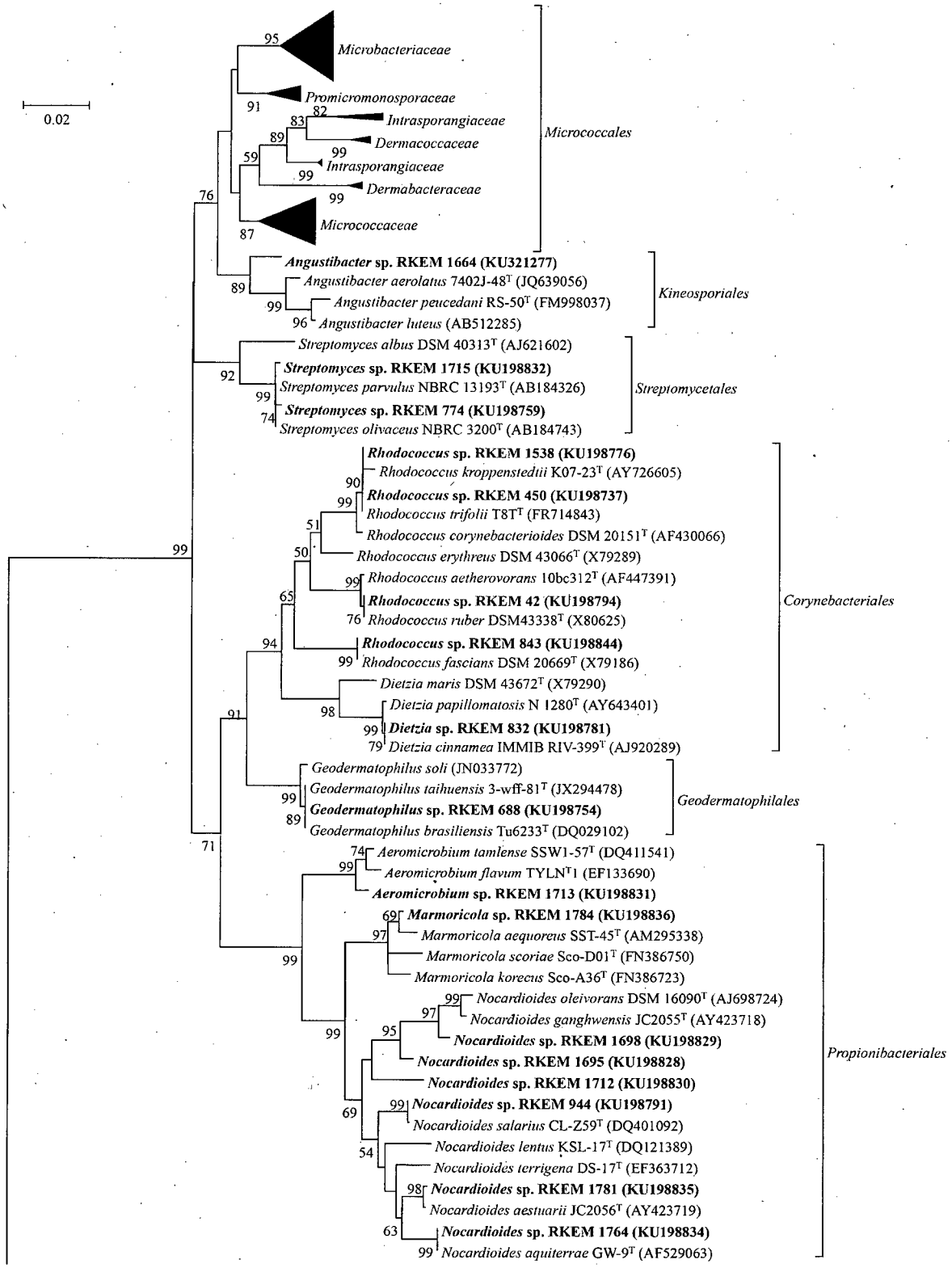

Figure 4.12. Neighbor-joining phylogenetic tree based on 16S rRNA gene sequences of bacteria in the orders *Kineosporiales, Streptomycetals, Corynebacteriales,*

Geodermatophilales,* and *Propionibacteriales. The tree was constructed using all *Actinobacteria* in the culture dependent library and closely related type strains; bacteria from this study are in bold. The tree was rooted using *Asticcacaulis solisilvae* CGM1-3EN^T (NR 109665.1), *Achromobacter aegrifaciens* LMG 26852^T (NR 117707.1), and *Desulfarculus baarsii* DSM 2075 (NR 074919.1) (not shown). Evolutionary distances were computed using the Jukes-Cantor method. A total of 620 nucleotide positions were used. Bootstrap values are expressed as a percentage of 1000 replicates, bootstrap values < 50 are not shown.

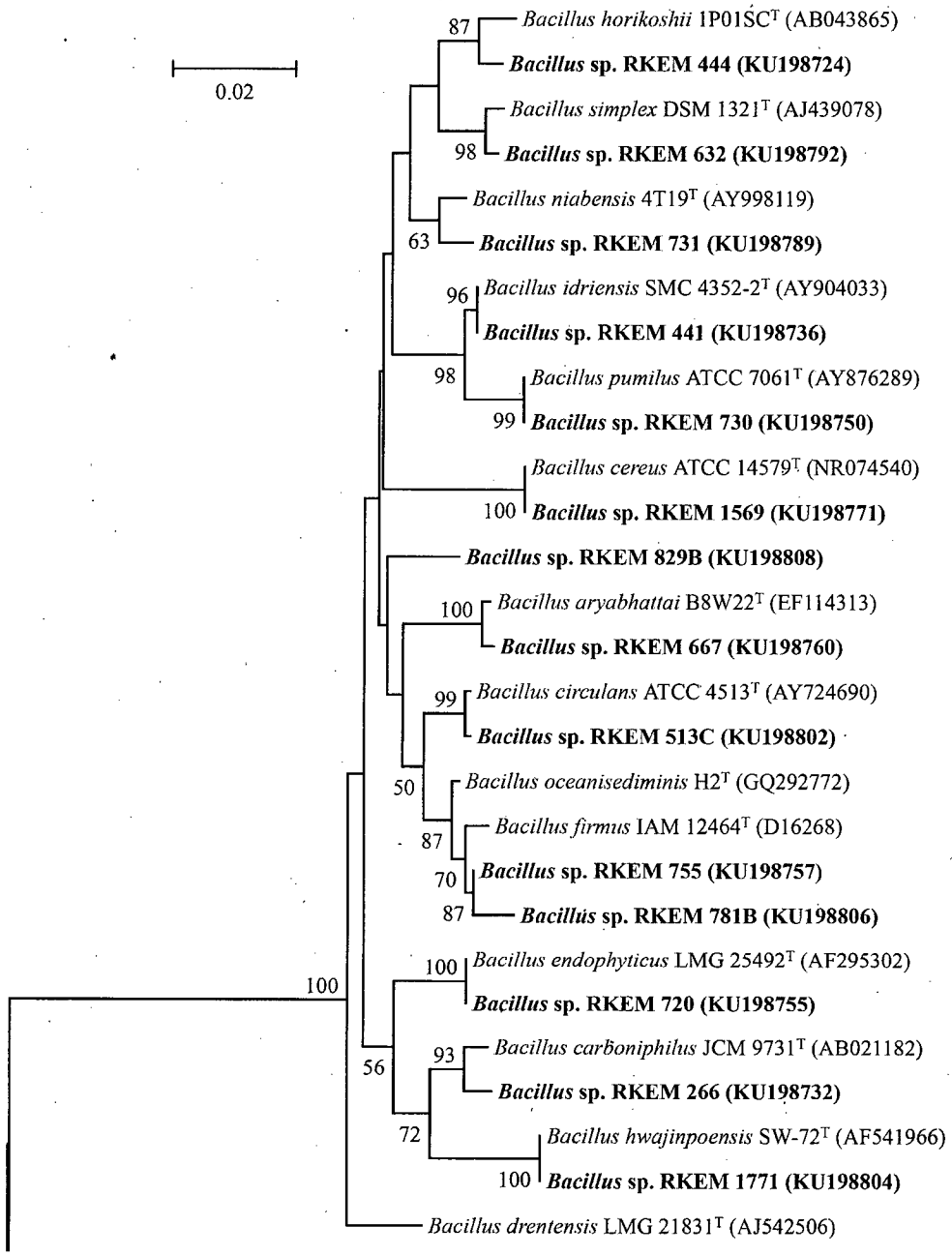

Figure 4.13. Neighbor-joining phylogenetic tree based on 16S rRNA gene sequences of bacteria in the genus *Bacillus*. The tree was constructed using all *Bacillus* spp. in the culture dependent library and closely related type strains; bacteria from this study are in bold. The tree was rooted using *Alkalibacillus almallahensis* S1LM8^T (NR 133692.1), *Allobacillus halotolerans*

B3A^T (NR 116607.1), and *Amphibacillus cookii* JWT (NR 108984.1) (not shown). Evolutionary distances were computed using the Jukes-Cantor method. A total of 609 nucleotide positions were used. Bootstrap values are expressed as a percentage of 1000 replicates, bootstrap values < 50 are not shown.

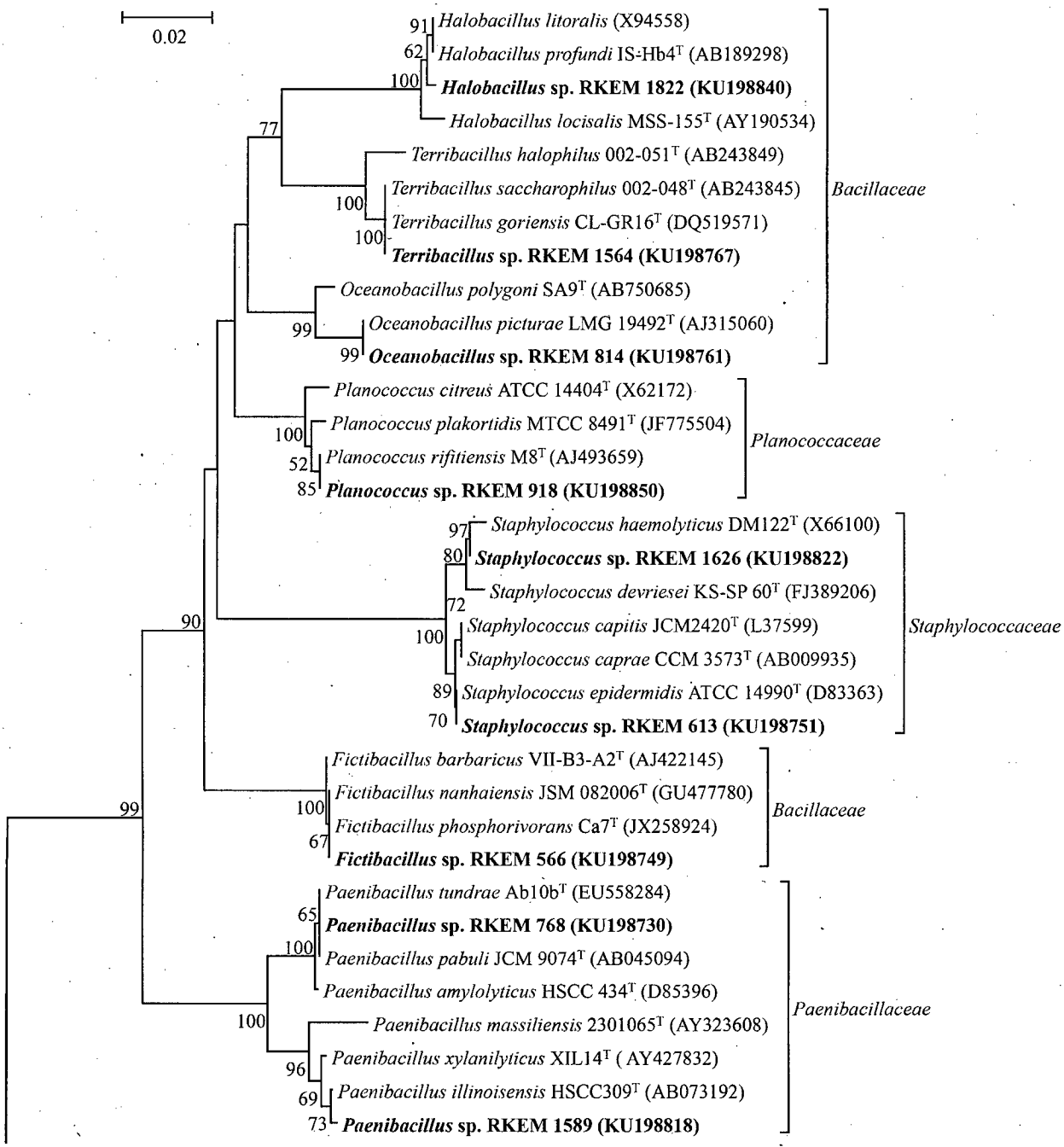

Figure 4.14. Neighbor-joining phylogenetic tree based on 16S rRNA gene sequences of bacteria in the class *Bacilli*. The tree was constructed using all *Bacilli* except the genus *Bacillus* in the culture dependent library and closely related type strains; bacteria from this study are in bold. The tree was rooted using *Asticcacaulis solisilvae* CGM1-3EN^T (NR 109665.1),

Achromobacter aegrifaciens LMG 26852^T (NR 117707.1), and *Desulfarculus baarsii* DSM 2075 (NR 074919.1) (not shown). Evolutionary distances were computed using the Jukes-Cantor method. A total of 612 nucleotide positions were used. Bootstrap values are expressed as a percentage of 1000 replicates, bootstrap values < 50 are not shown.

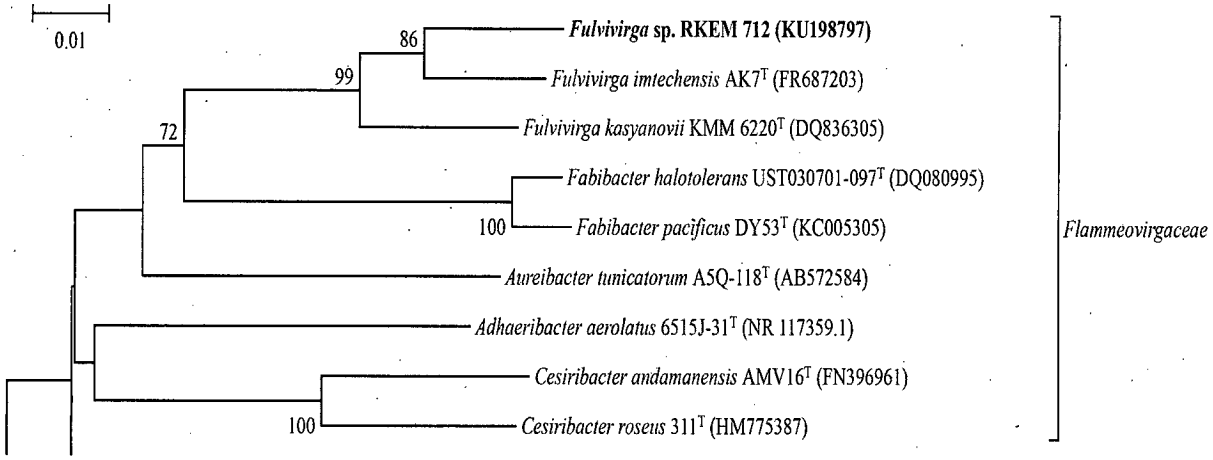

Figure 4.15. Neighbor-joining phylogenetic tree based on 16S rRNA gene sequences of bacteria in the family *Flammeovirgaceae*. The tree was constructed using type strains in the family *Flammeovirgaceae*, the bacterium from this study is in bold. The tree was rooted using *Algoriphagus alkaliphilus AC-74*^{T} (NR 042278.1), *Flexithrix dorotheae* IFO 15987^T (AB078077), and *Mooreia alkaloidigena* CNX-216^T (NR 126230.1) (not shown). Evolutionary distances were computed using the Jukes-Cantor method. A total of 580 nucleotide positions were used. Bootstrap values are expressed as a percentage of 1000 replicates, bootstrap values < 50 are not shown.

4.4 Conclusions

Although only a small percentage of the *A. elisabethae* microbiome is obtainable under standard plating conditions, different techniques were employed in this study to increase the recoverability. From this research the following conclusions can be made: (1) Unique bacterial isolates can be obtained by investigation of coral derived fractions other than the holobiont, namely the larvae and dinoflagellates. By determining the cultivatable bacteria associated with the larvae and dinoflagellates, an additional 12 unique bacteria were obtained that had not been obtained from the holobiont. (2) Dilution-to-extinction was an effective method for obtaining cultivatable bacteria as high recoverability was observed in all libraries, ranging from 3.9% to 12.2% of the total culture independent libraries. (3) Unique bacterial isolates can be obtained by culturing *A. elisabethae* samples under microaerophilic and anaerobic conditions. By employing these conditions an additional 54 bacteria were added to the culture dependent library. (4) *A. elisabethae* is a valuable source of novel cultivatable bacteria. From this study three putatively novel species, one putatively novel genus, and one member of a novel family were obtained. Based on these results, the octocoral *A. elisabethae* has proven to be an excellent source of cultivatable taxonomically diverse and novel bacteria.

4.4 References

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Chapter 5: Description of *Pseudobacteriovorax antillogorgiicola* gen. nov., sp. nov., a bacterium isolated from the gorgonian octocoral *Antillogorgia elisabethae*, belonging to a novel bacterial family, *Pseudobacteriovoraceae* fam. nov., within the order *Bdellovibrionales*

McCauley E.P., Haltli B., Kerr R.G., *Int. J. Syst. Evol. Microbiol.* **2014**, *65*, (2), 522-530.

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5.1 Introduction

5.1.1 Bdellovibrio-and-like organisms

Bdellovibrio-and-like organisms (BALO) are Gram-negative bacteria that prey upon other Gram-negative bacteria. They exhibit a biphasic morphology consisting of a predatory phase and non-predatory phase. During the predatory phase a vibriod morphology with a polar flagellum is observed, and during the non-predatory phase the cellular morphology is highly variable.[1,2] These organisms are classified under the order *Bdellovibrionales,*[3] and apart from their predatory abilities they exhibit few common characteristics. A wide range of isolation sources, NaCl tolerance, cellular fatty acids, DNA G+C content, and antibiotic susceptibility have been observed. To date six species assigned to four genera and three families have been described. The family *Bdellovibrionaceae* is comprised of *Bdellovibrio bacteriovorus* and *Bdellovibrio exovorus*. These bacteria were isolated from fresh water and exhibit a DNA G+C content ranging from 46.1 - 51.5 mol%.[4-6] The family *Bacteriovoracaceae* includes *Bacteriovorax marinus* and *Bacteriovorax litoralis*. *Bacteriovorax* spp. differ from *Bdellovibrio* spp. in a variety of characteristics.[7] The most notable phenotypic difference between the *Bacteriovoracaceae* and *Bdellovibrionaceae* is the requirement of NaCl for growth by *Bacteriovorax* spp.[8] The most notable genotypic differences are that *Bacteriovorax* spp. have a lower DNA G+C content (37.7 - 38.3 mol%), and two key variations in their 16S rRNA secondary structure. Helices formed starting at nucleotide positions 195 and 451 (according to *Escherichia coli* numbering) are substantially longer in *Bacteriovorax* spp. than the *Bdellovibrio* spp.[9,10] The most recently described family, *Peredibacteraceae,* contains two species, *Peredibacter starrii*, and *Bacteriolyticum stolpii.*[10,11] Based on phylogenetic analysis of the 16S rRNA gene this family of bacteria demonstrates a closer association with *Bacteriovorax* spp. than *Bdellovibrio* spp.;

however, these bacteria do not require NaCl for growth, and have a higher DNA G+C content (41.8 - 43.5 mol%) than *Bacteriovorax* spp.

5.1.2 Overall Objective of Study

The overall objective of this study was to compare the available phenotypic, genotypic, and chemotaxonomic data on characterized BALO and propose the addition of a novel family, *Pseudobacteriovoraceae,* to accommodate the novel genus and species *Pseudobacteriovorax antillogorgiicola* for the strain RKEM611.

5.2 Materials and Methods

5.2.1 Collection and Bacterial Isolation

Samples of *Antillogorgia elisabethae* were collected aseptically at a depth of ~10 m by SCUBA off the coast of San Salvador, The Bahamas in November 2011. The samples were treated as described in Chapter 4 Section 4.2.1. Plates were incubated at 21°C and strain RKEM611 was isolated within one week of initial plating. The strain was grown in Marine Broth 2216 (MB; BD, Difco) for 24 h at 30°C and 250 rpm, and preserved in 25% (v/v) glycerol at -80°C.

5.2.2 Phenotypic Experiments

The Gram reaction was determined using a Gram stain kit (BD Difco). Cell morphology was examined using a phase-contrast microscope (Leica DME; EC3 Microsystems), and transmission electron microscopy (TEM) (Hitachi BioTEM 7500; Nissei-Sangyo). For TEM, cells were fixed with 3% (v/v) glutaraldehyde in SFSW then stained with either 5% (w/v) uranyl acetate in SFSW or 1% (w/v) sodium phosphotungstate in distilled water. Samples were viewed on a Hitachi

H7500 BIO-Transmission Electron Microscope (Nissei-Sangyo, Rexdale, Ontario) at an acceleration voltage of 80kV. Images were obtained using an ATM XR40 side mount digital camera with Image Capturing Engine Software, Version 600.149 (Advanced Microscopy Techniques, Danvers, MA, USA).

The optimal pH, salinity, and temperature growth ranges were determined by measuring turbidity (OD_{600}) of broth cultures using a NanoDrop spectrophotometer (ND-1000; NanoDrop Technologies). All growth studies were conducted in broth culture medium at 250 rpm for 3 days and performed in triplicate. The optimal pH range was determined in MB using the following buffers: pH 3.0-4.0, glycine/HCl; pH 4.0-8.0, citrate/Na_2HPO_4; pH 6.0-8.0, phosphate buffer; pH 9.0-11, glycine/NaOH. Growth was tested at intervals of 1.0 pH unit. The pH was adjusted prior to sterilization, and then verified after sterilization prior to inoculation, and again after the growth experiment. The optimal salinity range was determined in NaCl-free MB prepared according to the manufactures recipe (BD, Difco), NaCl was then supplemented into the media at 0, 0.5, and 1-10% (w/v) in increments of 1%. Both optimal salinity and pH growth ranges were determined at 30°C. The optimal temperature was determined in MB at 4, 15, 22, 30, 37, and 45°C. All media used to determine optimal growth conditions were filtered prior to inoculation to remove particulates (0.2 μm polyethersulfone membrane, Nalgene Rapid Flow™). A comparison of optimal growth conditions for all characterized BALO is provided in Table 5.1. Anaerobic and microaerophilic cultivation was tested on MA at 30°C using a gas generating and pouch system (BD GasPak EZ).

5.2.3 Chemotaxonomic Experiments

Fatty acid methyl esters (FAME) and respiratory quinones was carried out by the Identification Services of the DSMZ (Braunschweig, Germany). Biomass for analysis was obtained from cells in the stationary phase of growth after 48h in MB at 30°C and 250 rpm. DNA G+C content was determined by HPLC using the method described by Mesbah *et al.*[12] Predominate FAMEs were determined by saponification, methylation, and extraction of the biomass using the method by Miller[13] with minor modifications. The FAME mixtures were separated using a Sherlock Microbial Identification System (MIS) (MIDI, Microbial ID).[14] A comparison of DNA G+C and FAME content of characterized BALO is provided in Table 5.1. Respiratory quinones were extracted with methanol:hexane, followed by a phase extraction into hexane as described by Tindall.[15,16]

Oxidase and catalase activity was determined using commercially available reagent stains and droppers (BD, Difco). Enzymatic activity and carbon utilization was determined using API ZYM (bioMérieux), API NE 20 (bioMérieux), GN2 MicroPlates (Biolog), and API CH (bioMérieux) kits. The API ZYM strips were read after 4 h at 37°C, the API NE 20 strips and GN2 MicroPlates were read after 24 h and 48 h at 30°C. For API CH the CHB/E Medium was used and the strips were read after 24 h and 48 h at 30°C. The tests were performed according to the manufacturer's recommendations with the exception that the final NaCl concentration of the suspension media for the API NE 20, MicroPlate GN2, and API CH kits were adjusted to 2% (w/v) NaCl.

Antibiotic susceptibility was determined using a disk diffusion method.[17] RKEM611^T was grown in MB for at 30°C and 250 rpm for 24 h after which the optical density of the culture was standardized to a 0.5 McFarland standard and spread onto MA plates. Disks containing the

following antibiotics were tested: methicillin (5 μg), ampicillin (20 μg), kanamycin (30 μg), novobiocin (30 μg), penicillin G (10 μg), streptomycin (10 μg), nalidixic acid (5 μg), vancomycin (30 μg), neomycin (30 μg), and gentamycin (10 μg). Inhibition zones were observed after 48 h at 30°C and zones ≥ 2 mm were scored as susceptible. A comparison of the antibiotic profiles of characterized BALO is provided in Table 5.1.

Predatory behavior of RKEM611^T was assessed using the double-layer agar plaque-forming assay with slight variations to previously described methods.[4,18,19] Gram-negative bacteria that were isolated from the same octocoral as RKEM611^T, and that belonged to a taxonomic genus that had been previously shown to be capable of infection were used as potential prey.[20] All isolates were grown in MB at 30°C and 250 rpm for 24 h. Culture turbidity was measured on a NanoDrop spectrophotometer (ND-1000; NanoDrop Technologies) and adjusted to an OD_{600} between 0.04 – 0.06 (1 mm path length) prior to plating. Three plating techniques were performed all used MA as the bottom layer and saline soft agar as the top layer (0.7% (w/v) agar, 2% (w/v) NaCl). All plating techniques were performed in triplicate. Samples were plated with either potential prey bacteria on the bottom layer and RKEM611^T in the top layer, RKEM611^T on the bottom layer and potential prey bacteria in the top layer, or equal amount of both RKEM611^T and potential prey bacterial in the top layer. Plates were incubated at 30°C for 14 days and plaque formation assessed every 24 h.

5.2.4 Genotypic Experiments

Analysis of DNA G+C content was carried out by the Identification Services of the DSMZ. For phylogenetic analysis, genomic DNA was extracted using the UltraClean Microbial DNA Isolation Kit (MoBio Laboratories, Inc.) from cells grown in MB at 30°C and 250 rpm for 2

days. All PCR reaction mixtures consisted of a 1X concentration of EconoTaq PLUS GREEN 2X master mix (Lucigen), 1.0 μM of each primer, 5% (v/v) DMSO, and 40 ng of template DNA. PCR amplification of the 16S rRNA gene was achieved using the universal eubacterial 16S rRNA gene primers pA (5'-AGAGTTTGATCCTGGCTCAG-3') and pH (5'-AAGGAGGTGATCCAGCC-3').[21] These primers, in addition to 530R (5'-GTATTACCGCGGCTGCTG-3')[22], 514F (5'-GTGCCAGCASCCGCGG-3'), 936R (5'-GGGGTTATGCCTGAGCAGTTTG-3'),[23] and 1114F (5'-GCAACGAGCGCAACCC-3')[24] were used to sequence the nearly full-length 16S rRNA gene. PCR amplification of a region of the *β*-subunit of the RNA polymerase (*rpoβ*) from nucleotide 2073 - 3315 (according to *Bacteriovorax marinus* SJ numbering) was carried out as previously described.[10] Both the forward and reverse primers were used to sequence the *rpoβ* amplicon. Sequencing was performed by Eurofins MWG Operon (Huntsville, AL, USA). Sequences were trimmed and assembled using Vector NTI Express (Invitrogen, Life Technologies) (16S rRNA, 1466bp; *rboβ*, 1135bp), and compared to available sequences in the GenBank database.[25] Multiple sequence alignments were prepared using MEGA version 6.[26] Phylogenetic histories were inferred using maximum-parsimony (MP),[27] minimum evolution (ME),[28] and neighbor-joining (NJ) methods.[29] The topology for all phylogenetic trees generated using the 16S rRNA and *rpoβ* sequences were consistent (Appendix A). Evolutionary distance matrices were generated using the Jukes-Cantor method[30] and are in units of the number of base substitutions per site. All positions containing gaps and missing data were eliminated. Bootstrap analysis is based on 1000 resampled datasets.[31] The MP trees were obtained using the Subtree-Pruning-Regrafting algorithm with search level 1 in which the initial trees were obtained by the random addition of sequences. The ME trees were searched using the Close-Neighbor-

Interchange algorithm at a search level of 1.[32] The final dataset of the 16S rRNA and *rpoβ* sequences consisted of 1150 and 973 nucleotide positions, respectively.

Analysis of 16S rRNA secondary structures were achieved through alignment of BALO 16S rDNA genes to *Escherichia coli* (J01695.2) using MEGA version 6.[26] Computational investigation of mRNA folding was achieved using MFOLD.[33]

5.3 Results and Discussion

5.3.1 Phenotypic and Chemotaxonomic Analysis

Phenotypic analysis of RKEM611^T revealed characteristics consistent with those of other BALO[1,2] The strain was Gram-negative, an obligate aerobe, and exhibited a biphasic morphology. Cells were either vibriod with a single polar flagella (0.4-0.6 μm in diameter and 1.0-2.0 μm in length), or filamentous with no flagella (0.4-0.6 μm in diameter and 1.0-25.0 μm in length) (Figure 5.1). Predatory behavior was observed via the formation of plaques in confluent lawns of *Pseudoalteromonas* sp. RKEM680 (KJ719255). This activity was observed when RKEM611^T was plated on the bottom layer of the double-layer agar and *Pseudoalteromonas* sp. RKEM680 on the top layer. However after subsequent transfers on solid media RKEM611^T appeared to lose predatory activity against *Pseudoalteromonas* sp. RMEK680.[1,18] No predatory behavior was observed against any of the other bacteria isolates that were used as potential prey. While these phenotypic characteristics are common to all BALO, the requirement of NaCl for growth of RKEM611^T aligned this bacterium most closely with the family *Bacteriovoracaceae.*

Table 5.1. Differential characteristics of strains representing the order *Bdellovibrionales.*

Type strains: **1**, *Pseudobacteriovorax antillogorgiicola* RKEM611; **2**, *Bacteriovorax marinus* SJ^{T};[8] **3**, *Bacteriovorax litoralis* JS5^{T};[8] **4**, *Bacteriolyticum stolpii* UKi2^{T};[7,11] **5**, *Peredibacter starrii* A3.12^{T};[7,11] **6**, *Bdellovibrio bacteriovorus* 109J^{T}.[1,5,8] +, Positive; −, negative; W, weak positive; V, variable; ND, no data available; R, resistant; S, susceptible. All results are for the prey independent derivatives of each strain.

Characteristic	1	2	3	4	5	6
Na^{+} required for growth	+	+	+	−	−	−
Optimal growth salinity (%,w/v)	1-2	2-3	5	ND	ND	ND
Optimum growth temperature (°C)	30-37	15-30	15-35	15-35	20-30	15-35
DNA G+C content (mol%)	46.3	37.7	37.8	41.8	43.5	51.5
Major fatty acids	$C_{16:1}\omega5c$ $C_{16:0}$	ND	ND	$C_{15:1}\omega8c$C $_{13:0}$ $C_{13:0}$iso	$C_{16:1}\omega9c$ $C_{13:0}$iso $C_{13:0}$ $C_{15:1}\omega8c$	ND
Enzyme Activities Tested:						
Alkaline phosphate	+	+	+	ND	ND	+
Esterase (C4)	W	+	+	ND	ND	+
Esterase lipase (C8)	W	+	+	ND	ND	+
Lipase (C4)	−	V	−	ND	ND	−
Leucine aminopeptidase	+	+	+	ND	ND	+
Valine aminopeptidase	W	+	+	ND	ND	−
Cystine aminopeptidase	W	+	V	ND	ND	−
Trypsin	+	+	−	ND	ND	+
α-chymotrypsin	−	−	−	ND	ND	+
Acid phosphate	+	+	+	ND	ND	+
Phosphoamidase	+	+	+	ND	ND	+
α-Galactosidase	−	−	−	ND	ND	−
β-Galactosidase	−	−	−	ND	ND	−
β-Glucuronidase	−	−	−	ND	ND	−
α-Glucosidase	−	−	−	ND	ND	−
β-Glucosidase	−	−	−	ND	ND	−
N-Acetyl-β-glucosaminidase	−	V	V	ND	ND	−
α-Mannosidase	−	−	−	ND	ND	−
α-Fucosidase	−	−	−	ND	ND	−
Antibiotic Sensitivity:						
Methicillin (5 μg)	R	R	R	S	S	S
Ampicillin (20 μg)	R	R*	R*	ND	ND	S*
Kanamycin (30 μg)	S	S	R	S	S	S
Novobiocin (30μg)	R	ND	ND	ND	ND	S
Penicillin G (10 μg)	R	ND	ND	S	S	R
Streptomycin (10 μg)	R	ND	ND	S	S	ND

Nalidixic Acid (5 μg)	R	R	V	S	R	R
Vancomycin (30 μg)	R	R	R	S	S	V
Neomycin (30 μg)	S	ND	ND	S	S	S
Gentamycin (10μg)	S	ND	ND	S	S	S

*Antibiotic tested against was ampicillin/sublactam (20 μg)

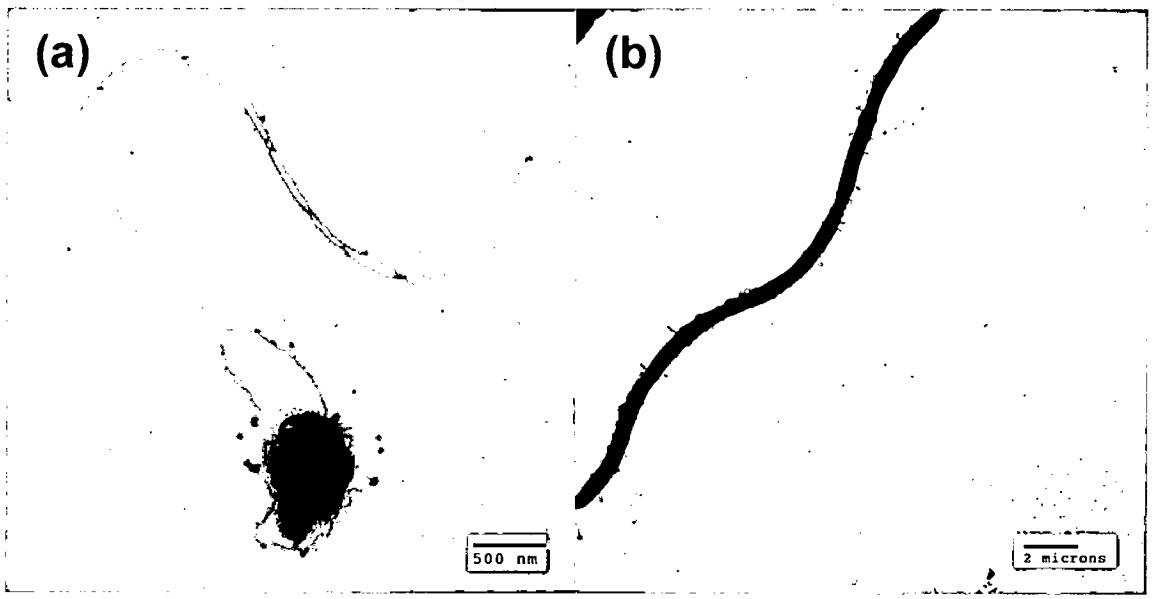

Figure 5.1. Transmission Electron Micrograph Images of RKEM611.

5.3.2 Genotypic Analysis

BlastN analysis of the 16S rRNA gene revealed low sequence identity to any taxa within the order *Bdellovibrionales,* the highest being *Bacteriovorax marinus* (82%). Higher sequence identities were observed between RKEM611^T and various taxa within the class *Deltaproteobacteria* such as, *Geobacter psychrophilus* (85%) and *Desulfovibrio senezii* (83%). Comparable sequence identities were also observed between taxa within the class *Alpharoteobacteria* such as, *Neptuniibacter caesariensis* (84%) and *Psychromonas macrocephali* (83%). Based on the evolutionary distance and phylogenetic relationship computed by the Jukes-Cantor distance model and NJ method RKEM611^T clustered within *Deltaproteobacteria* and demonstrated a closer association to members within the order *Bdellovibrionales* than any other order within *Deltaproteobacteria* (Figure 5.2). However no strong association to any family within the order was observed.

A previous study by Pineiro *et al.*[10] examined variations in the *rpoB* gene of BALO. They found that there was greater nucleotide variation when compared to the 16S rRNA gene, and that this variation allowed for a narrower delineation of phylogenetic groups within BALO. Similar to phylogenetic analysis employing the 16S rRNA gene, analysis of the RKEM611^T *rpoB* gene placed the strain within the *Bdellovibrionales* but did not reveal a strong association to any family (Figure 5.3).

Alignment and comparison of the secondary structure of BALO 16S rRNA reveals three key variations starting at nucleotide positions 180, 195, and 451 according to *E. coli* (J01695.2) numbering (Figure 5.4 and Figure 5.5). Members of the family *Bdellovibrionaceae* have

considerably shorter helices than all other BALO starting at nucleotide positions 195 and 451, as previously reported.[9] The secondary structure of the RKEM 611^T 16S rRNA exhibits similar length in helices at nucleotide positions 195 and 451 as members of the families *Bacteriovoracaceae* and *Peredibacteracea*. However, it differs from all other BALO by having a substantially shorter helix starting at nucleotide position 180. These variations in secondary structure further demonstrate that RKEM 611^T has no strong association with any particular family within the *Bdellovibrionales*.

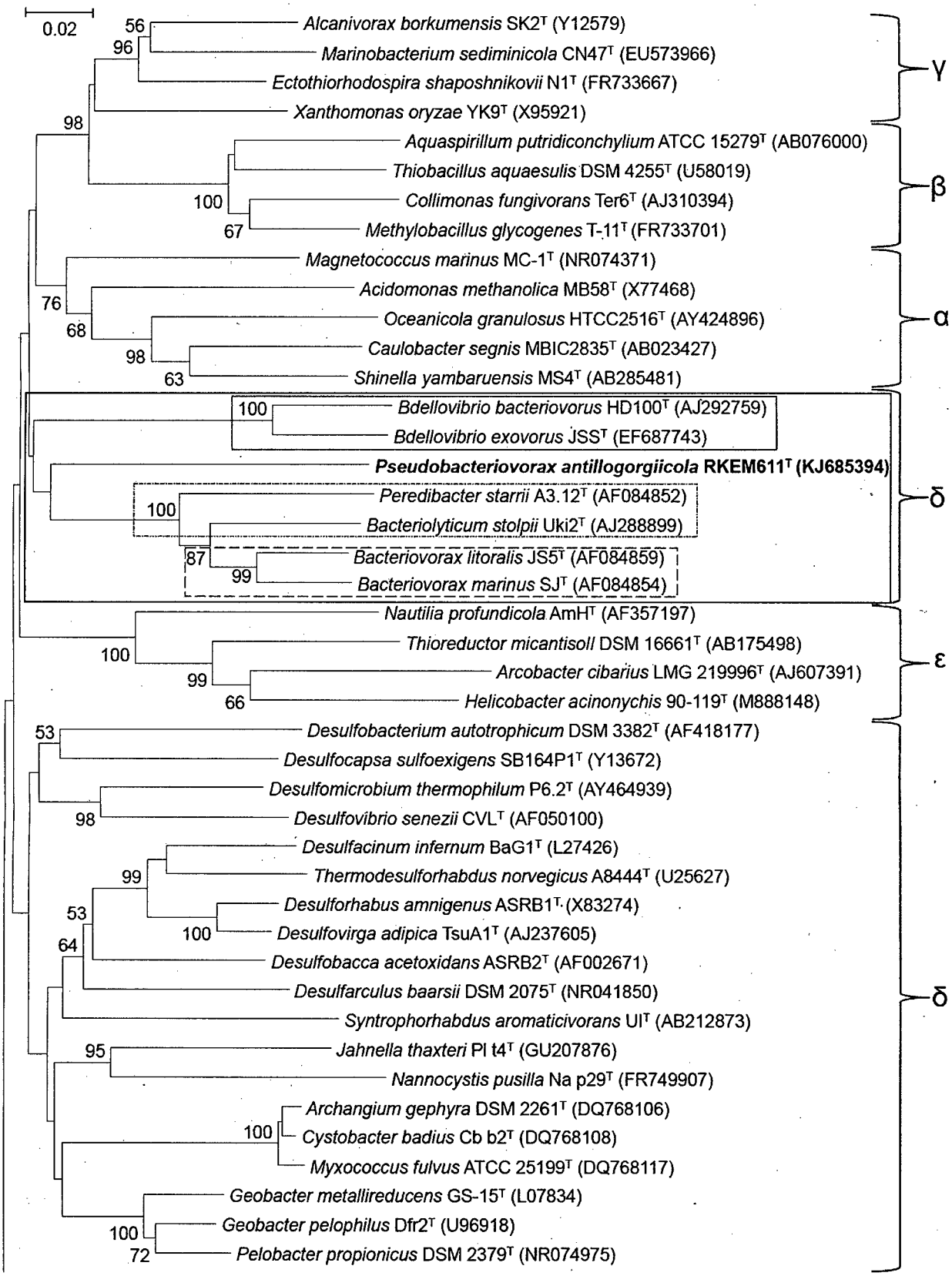
0.02
Alcanivorax borkumensis SK2T (Y12579)
Marinobacterium sediminicola CN47T (EU573966)
Ectothiorhodospira shaposhnikovii N1T (FR733667)
Xanthomonas oryzae YK9T (X95921)
Aquaspirillum putridiconchylium ATCC 15279T (AB076000)
Thiobacillus aquaesulis DSM 4255T (U58019)
Collimonas fungivorans Ter6T (AJ310394)
Methylobacillus glycogenes T-11T (FR733701)
Magnetococcus marinus MC-1T (NR074371)
Acidomonas methanolica MB58T (X77468)
Oceanicola granulosus HTCC2516T (AY424896)
Caulobacter segnis MBIC2835T (AB023427)
Shinella yambaruensis MS4T (AB285481)
Bdellovibrio bacteriovorus HD100T (AJ292759)
Bdellovibrio exovorus JSST (EF687743)
Pseudobacteriovorax antillogorgiicola RKEM611T (KJ685394)
Peredibacter starrii A3.12T (AF084852)
Bacteriolyticum stolpii Uki2T (AJ288899)
Bacteriovorax litoralis JS5T (AF084859)
Bacteriovorax marinus SJT (AF084854)
Nautilia profundicola AmHT (AF357197)
Thioreductor micantisoli DSM 16661T (AB175498)
Arcobacter cibarius LMG 219996T (AJ607391)
Helicobacter acinonychis 90-119T (M888148)
Desulfobacterium autotrophicum DSM 3382T (AF418177)
Desulfocapsa sulfoexigens SB164P1T (Y13672)
Desulfomicrobium thermophilum P6.2T (AY464939)
Desulfovibrio senezii CVLT (AF050100)
Desulfacinum infernum BaG1T (L27426)
Thermodesulforhabdus norvegicus A8444T (U25627)
Desulforhabus amnigenus ASRB1T (X83274)
Desulfovirga adipica TsuA1T (AJ237605)
Desulfobacca acetoxidans ASRB2T (AF002671)
Desulfarculus baarsii DSM 2075T (NR041850)
Syntrophorhabdus aromaticivorans UIT (AB212873)
Jahnella thaxteri PI t4T (GU207876)
Nannocystis pusilla Na p29T (FR749907)
Archangium gephyra DSM 2261T (DQ768106)
Cystobacter badius Cb b2T (DQ768108)
Myxococcus fulvus ATCC 25199T (DQ768117)
Geobacter metallireducens GS-15T (L07834)
Geobacter pelophilus Dfr2T (U96918)
Pelobacter propionicus DSM 2379T (NR074975)
γ
β
α
δ
ε
δ

Figure 5.2. Neighbor-joining phylogenetic tree based on 16S rRNA gene sequences from 46 type strains from within the phylum *Proteobacteria*. Evolutionary distances were computed using the Jukes-Cantor method, a total of 1150 nucleotide positions were used. Bootstrap values are expressed as a percentage of 1000 replicates, bootstrap values ≤ 50% are not shown. Bar, 2 substitutions per 100 base pairs. Brackets indicate the classes *Alpha(α)*-, *Beta(β)*-, *Gamma*(γ)-, *Delta*(δ), and *Epsilon*(ε)-*Proteobacteria*. The large black box indicates the order *Bdellovibrionales*. The small grey boxes indicate the families *Bacteriovoracaceae* (solid line), *Peredibacteraceae* (dotted line) and *Bdellovibrionaceae* (dashed line). The tree was generated using *Dinococcus radiodurans* MRP^T (Y11332), *Bacillus subtilis* DSM 10^T (AJ276351), and *Spirochaeta litoralis* DSM 2029^T (FR733665) as outgroups (not shown).

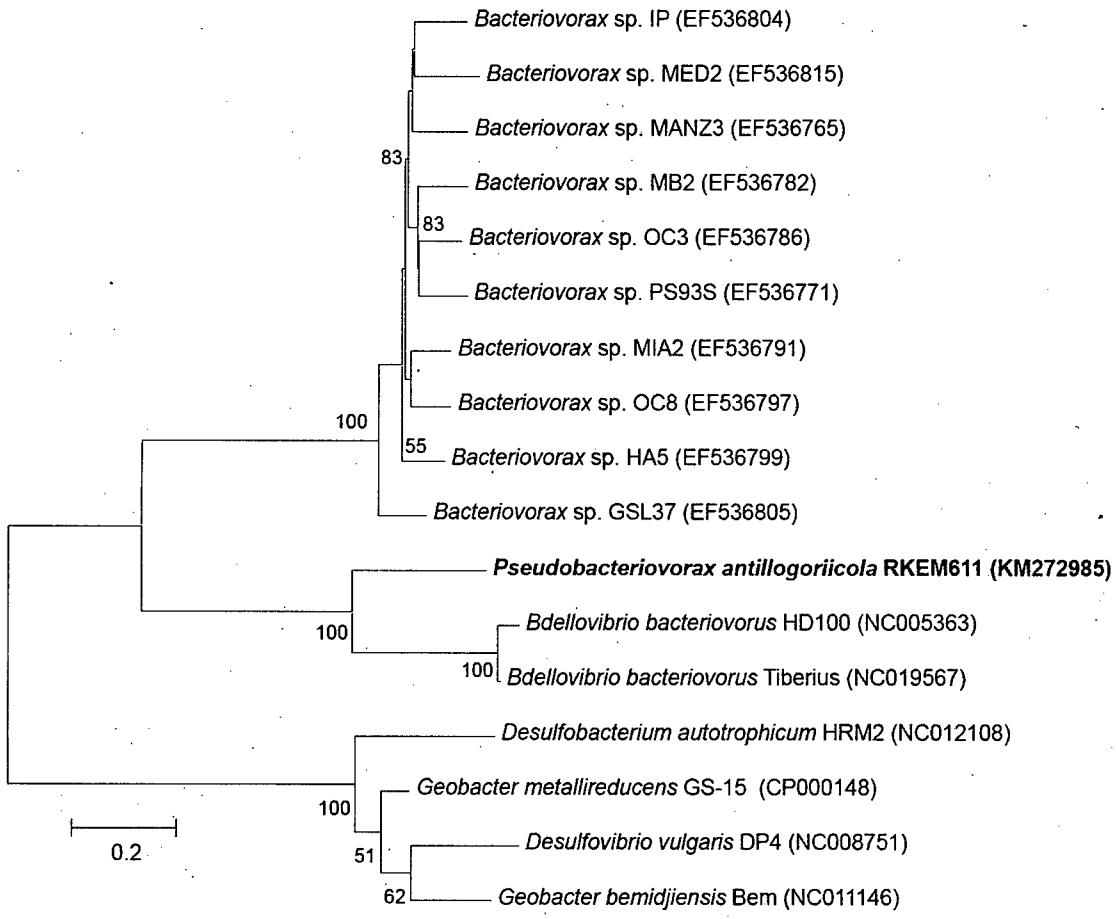

Figure 5.3. Neighbor-joining phylogenetic tree based on 17 *rpoβ* gene sequences from *Deltaproteobacteria*. Evolutionary distances were computed using the Jukes-Cantor method, a total of 973 nucleotide positions were used. Bootstrap values are expressed as a percentage of 1000 replicates, bootstrap values ≤ 50% are not shown. Bar, 20 substitutions per 100 base pairs.

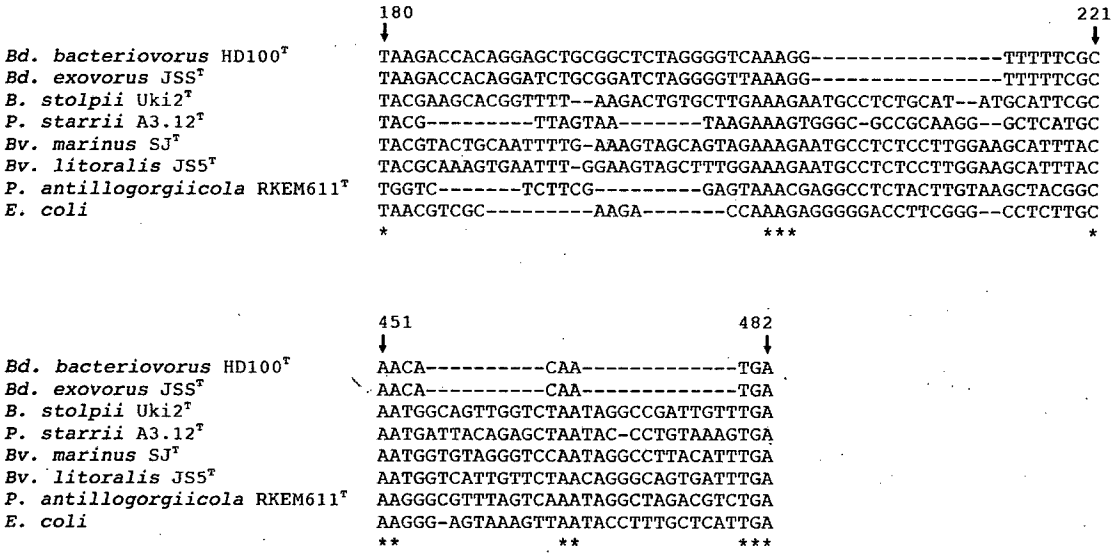

Figure 5.4. Clustal W multiple aligment of the 16S rRNA gene of type strains in the order *Bdellovibrionales*. Nucleotide positions are based on *E. coli* numbering (J01695.2)

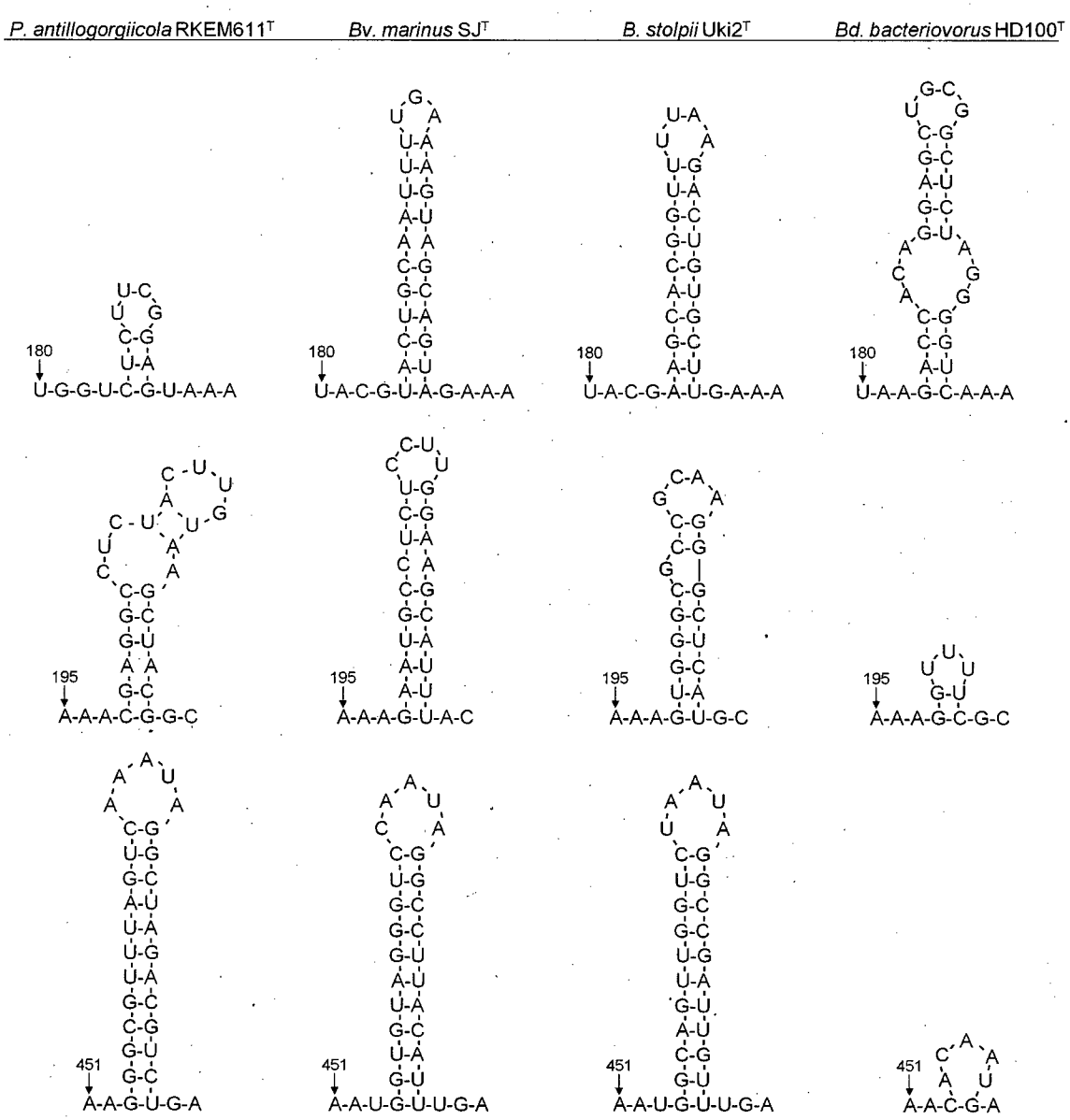

Figure 5.5. 16S rRNA secondary structure helicies for represenative bacterial type strains from each family within the order *Bdellovibrionales.* Nucleotide positions are based on *E. coli* numbering (J01695.2) between positions 180-221 and 451-482.

5.4 Formal Species Description

Based on the phenotypic similarities observed between RKEM611^T and other BALO it is clear that the strain is a member of the order *Bdellovibrionales*. However, based on the phylogenetic distance observed between the 16S rRNA genes and the variations in their secondary structure, the strain represents a member of a novel family. The name *Pseudobacteriovoraceae* fam. nov. and *Pseudobacteriovorax antillogorgiicola* gen. nov., sp. nov., are proposed for this taxon.

5.4.1 Description of *Pseudobacteriovorax* gen. nov.

Pseudobacteriovorax (Pseu. do. bac. te. ri. o. vo'rax. Gr. adj. *pseudês*, false; N. L. masc. n. *Bacteriovorax*, a bacterial genus name; N. L. masc. n. *Pseudobacteriovorax*, false *Bacteriovorax*) Consists of Gram-negative bacteria that are capable of predation on other Gram-negative bacteria. The description of this genus is the same as that of the type strain and only species in the genus, *Pseudobacteriovorax antillogorgiicola.*

5.4.2 Description of *Pseudobacteriovorax antillogorgiicola* sp. nov.

Pseudobacteriovorax antillogorgiicola (an.ti.lo.gor.gi.i'co.la N.L. n. *Antillogorgia* name of a zoological genus; L. masc. suff. -cola (from L.n. *incola*) inhabitant; N.L. n. antillogorgiicola, name of a zoological genus; N.L. n. *antillogorgiicola, Antillogorgia* inhabitant). Cells are Gram-negative, obligate aerobes, which exhibit a biphasic lifestyle. Cells were either vibriod with a single polar flagellum (0.4-0.6 μm in diameter and 1.0-2.0 μm in length), or filamentous with no flagellum (0.4-0.6 μm in diameter and 1.0-25.0 μm in length). Colonies are light yellow in colour after 48 h of growth on MA. Optimum growth occurs at 1-2 % (w/v) NaCl, 30-37°C, and pH 6.0-8.0. Enzymatic activities for catalase, oxidase, alkaline phosphate, esterase (C4), esterase lipase

(C8), leucine aminopeptidase, valine aminopeptidase, cystine aminopeptidase, acid phosphate, and phosphoamidase were detected. Carbon utilization of dextrin, glycogen, Tween 40, Tween 80, and D-galactose was detected. Resistant to the antibiotics methicillin, ampicillin, novobiocin, penicillin G, streptomycin, and vancomycin. Susceptible to kanamycin, neomycin, and gentamycin. The predominant cellular fatty acids were $C_{16:1}\omega 5c$, $C_{16:0}$, and the major respiratory quinone was menaquinone MK-6.

The type strain, RKEM611^T (=NCCB 100521^T, =LMG 28452^T) was isolated from the octocoral *Antillogorgia elisabethae* off the coast of San Salvador, The Bahamas. The DNA G+C content of the type strain is 46.3 mol%

5.4.3 Description of *Pseudobacteriovoraceae* fam. nov.

Pseudobacteriovoracaceae (Pseu.do.bac.te.ri.o.vo.ra.ce'ae., N.L. masc. n. Pseudobacteriovorax, a bacterial genus; suff. –aceae, ending to denite a family; N.L. fem. pl. n. *Pseudobacteriovoraceae,* the *Pseudobacteriovorax* family). The description of the family *Pseudobacteriovoracaceae* is based on the description of the genus *Pseudobacteriovorax* from the data of this study. The family consists of Gram-negative, obligate aerobes, which exhibit a biphasic lifestyle. Secondary structure of the 16S rRNA reveals longer helices starting at nucleotide positions 195 and 451 (according to *E. coli* numbering), as is observed in the family *Bacteriovoracaceae,* but a shorter helix at nucleotide position 180. The type genus is *Pseudobacteriovorax.*

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Chapter 6: Investigation of Secondary Metabolites Produced by Octocoral-Associated Bacteria

6.1 Introduction

6.1.1 Secondary Metabolites from Marine Bacteria

Marine bacteria have increasingly become a major focus in marine natural products (MNPs) research, as the rate at which secondary metabolites are being discovered is steadily increasing.[1-8] Marine prokaryotes have developed unique metabolic capabilities that enable them to live in various extreme ocean habitats. Over the course of the last few decades a variety of taxonomically diverse marine bacteria have been isolated that produce a broad range of chemically diverse compounds. While the taxonomic phylum *Actinobacteria* is by far the most prolific source of secondary metabolites,[9-12] novel compounds have also been isolated from phyla such as *Proteobacteria*, *Cyanobacteria*, and *Firmicutes,* as well as various unclassified bacteria.[13-21] The secondary metabolites produced by these bacteria are not only structurally diverse but also exhibit a wide variety of bioactivity, including but not limited to antimicrobial, anticancer, anti-inflammatory, antiparasitic, antiviral, and antioxidant activity.[22-29]

6.1.2 Chemical Dereplication

Even though the field of bacterial MNP research is rapidly growing and these metabolites continue to be an excellent source of bioactive compounds, finding novel secondary metabolites is becoming increasingly difficult. Many of these compounds are ubiquitous in the marine microbiome and isolation of previously discovered secondary metabolites is common. In order to address this issue, our lab developed a chemical dereplication process,[30] which allowed for the identification of unique metabolites in large batches of bacterial fermentations. This method uses a metabolomics approach and combined ultra-high performance liquid chromatography-high resolution mass spectroscopy (UHPLC-HRMS) data from bacterial fermentation extracts with

principal component analysis (PCA)[31] to identify unique metabolites. This is achieved by growing the bacterial isolates in small-scale liquid fermentations and analysing the extracts from these fermentations using UHPLC-HRMS. The data sets of metabolites are then bucketed based on the *m/z* ratio and retention time (RT). Metabolites are scored with a 0 or 1 depending on their presence or absence in a particular extract. This bucketed data is than subjected to PCA where bacterial isolates that produce the same metabolites group together, and isolates that produce unique metabolites group separately. The scores plot, which represents a view of the variance between data sets, allows for visualization of this separation. The greater the separation, the greater the difference of the metabolites that a particular strain produces compared to the others. The loadings plot, which is geometrically related to the scores plot describes the variance observed in the scores plot and is used to identify the buckets (*m/z* and RT) responsible for the observed separation. By using this method a large amount of bacterial extracts can be screened in an efficient and rigorous manner.

6.1.3 Overall Objective of Study

The overall objective of this study was to ferment and screen the secondary metabolites produced by the bacterial isolates in the culture dependent library (Chapter 4), as well as select isolates from an *Erythropodium caribaeorum* culture library. All fermentation extracts were subjected to the aforementioned chemical dereplication process in order to identify unique and possible novel secondary metabolites. In addition, as all the bacterial isolates used in this study were obtained from the octocorals *Antillogorgia elisabethae* and *E. caribaeorum* and there is a growing body of evidence to suggest that MNPs isolated from macroorganisms are actually biosynthesised by associated microorganisms.

All extracts were analysed for the presence of pseudopterosins and eleutherobins to determine if any of the isolates were capable of biosynthesising these pharmaceutically relevant MNPs under laboratory conditions.

6.2 Materials and Methods

6.2.1 Initial Small-Scale Screening

All bacterial isolates from the culture dependent library (Chapter 4), as well as *Pseudomonas* spp., *Pseudoalteromonas* spp., and *Ruegeria* spp. from a culture dependent library of bacteria isolated from *E. caribaeorum* (created by Brad Haltli - Research Manager, Nautilus Bioscience and Adjunct Faculty, University of Prince Edward Island) were subjected to small-scale (10mL) fermentations. Bacteria were grown from frozen glycerol stocks on Marine Agar (MA; 2216; BD Difco). Cultures from MA were inoculated into Marine Broth (MB 2216, BD Difco; 10mL) seed cultures (48 hours, 250 rpm, 30°C), 1% of seed cultures were inoculated into 10mL fermentations using four different media. A list of the media used in this study is summarized in Table 6.1. *Proteobacteria* were fermented in BFM1, BFM3, BFM5, and BFM11. *Firmicutes* and the one *Cytophagia* were fermented in BFM5, BFM6, BFM7, BFM11. *Actinobacteria* were fermented in BFM1, BFM3, BFM11, and ISP2. Media blank controls were included in each batch of fermentations.

Fermentations were grown for five days (250 rpm, 30°C), then extracted with EtOAc (10mL), dried under vacuum, and partitioned between equal volumes of 80% aqueous ACN and hexane. The ACN layers were dried down, re-suspended in MeOH to a final concentration of 0.5 mg/mL, and analysed using UHPLC-HRMS. UHPLC was performed using a Core-Shell 100 Å C_{18}

column (Kinetex, 1.7 μm 50 X 2.1 mm; Phenomenex, Torrance, CA, USA) with a linear solvent gradient over 5 min from 5% ACN in H_2O/0.1% formic acid to 100% ACN/0.1% formic acid followed by 3 min of 100% ACN/0.1% formic acid and an additional 2 min re-equilibration period of 5% ACN in H_2O/0.1% formic acid at a flow rate of 500μL/min. Eluent was detected on an Accela Thermo equiped with MS-ELSD-UV detection systems (Thermo Fisher Scientific, Mississauga, ON, Canada). ESI-MS monitoring (*m/z* 190-2000) in positive mode was achieved using an LTQ Orbitrap Velos Pro, ELSD on an LT-ELSD Sedex 80, and UV detection on an photodiode array (200-600 nm). The MS was operated under the following conditions: spray voltage, 3.0kV; capillary temperature, 320°C; S-Lens RF voltage, 66.0%; maximum injection time, 500ms; microscans, 3; injection volume, 10μL. The system was controlled by XCalibur software (Thermo Fisher Scientific).

Table 6.1. Fermentation media used in the screening of culture dependent bacterial isolates. All media were adjusted to a pH of 7.3 ± 0.2.

Media and Ingredients	Amount (g/L)
Bacterial Fermentation Medium 1 (BFM1)	
Dextrin	20
Soluble Starch	20
Beef extract	10
Peptone	5
$(NH_4)_2SO_4$	2
$CaCO_3$	2
Instant ocean	18
Bacterial Fermentation Medium 3 (BFM3)	
$MgSO_4*7H_2O$	0.5
KCl	0.5
K_2HPO_4	3
NaCl	5
Agar	0.4
Glycerol	12
Soy Peptone	5
Instant ocean	18
Bacterial Fermentation Medium 5 (BFM5)	
Pancreatic Digest of Casein	17
Enzymatic Digest of Soybean Meal	3
Sodium chloride	5
Dipotassium Phosphate	2.5
Dextrose	2.5
Instant ocean	18
Bacterial Fermentation Medium 6 (BFM6)	
Yeast Extract	5
Tryptone	5
$FeCl_2$-$4H_2O$	0.04
$MnSO_4$-H_2O	0.00034
$MgSO_4$-$7H_20$	5
NaCl	58.44
Instant ocean	18
Bacterial Fermentation Medium 7 (BFM7)	
KH_2PO_4	1
K_2HPO_4	1
$MgSO_4$-$7H_20$	0.2

$FeCl_3$	0.05
$CaCl_2$-$2H_2O$	0.02
NaCl	5
Glucose	2
Yeast Extract	2
Instant ocean	18
Olive Oil**	5
** Added individually to fermentation tubes/flasks to ensure even distribution	
Bacterial Fermentation Medium 11 (BFM11)	
Starch (potato)	10
Yeast Extract	4
Peptone	2
KBr Stock (20 g/L)	5 mL
FeSO4-7H2O (8 g/L pH 7)	5 mL
Instant ocean	18
International *Streptomyces* Project (ISP2)	
Yeast Extract	4
Malt Extract	10
Dextrose	4
Instant Ocean	18

6.2.2 Chemical Dereplication, Data Processing, and PCA

The data generated from the UHPLC-HRMS was processed using a modified version of a previously described chemical dereplication process.[30] Raw data files were converted to netCDF files through the use of a software module contained within XCalibur. The netCDF files were imported into mzMine 2.2 (VTT Technical Research Centre, Otaniemi, Espoo, Finland) and lists of masses with an intensity value greater than 1.0E5 for each file were generated. Exact masses were built into a chromatogram for continuous detection across scans. Ions were considered part of an isotopic pattern if they were within 0.005 *m/z* units and 0.3 min. The masses were bucketed based on retention time. Buckets were defined as 0.01 *m/z* units and 0.2 min. Buckets that were present in the media control files were removed from the bacterial fermentation data files. The remaining buckets were exported into a comma separated value file (.csv) (Microsoft® Excel® for Mac 2011). PCA was achieved using The Unscrambler software (CAMO Software, Oslo, Norway). Extracts that contained unique secondary metabolites grouped separately on the PC scores plot and the buckets responsible for the observed separation were identified on the PC loadings plot. The accurate mass for the $[M+H]^+$ adduct of each metabolite in the buckets was determined as some of the bucket *m/z* values corresponded to the $[M+Na]^+$, $[M+NH_3]^+$, or $[M+2H]^{2+}$ adducts. The accurate masses were screened through Antibase 2014 (Wiley-VCH, Hoboken, NJ, USA) and Scifinder® (Chemical Abstract Services, Washington, DC, USA) databases to identify those that belonged to previously reported secondary metabolites. Masses that were within ± 5ppm of the calculated masses,[32,33] and that had been previously isolated from close taxonomically related bacteria were considered a match. Standards of MNPs, pseudopterosins and eleutherobins, were analyzed on the UHPLC-HRMS and subjected to the same processing. Chemical barcodes were generated that contained the fermentation extract data

in addition to the MNP data. These barcodes were used to determine if any of the extracts contained those MNPs.

6.2.3 Scale-up Fermentations

For metabolites that could not be readily identified by their accurate mass through a database search, the fermentation of the bacterial isolate responsible was scaled up. The compounds of interest were purified and analysed by NMR and/or tandem MS. For each scale-up, the bacterial isolate was grown in six 1L fermentations (5 days, 250 rpm, 30°C), The fermentation broth was extracted using EtOAc and the extract was fractionated using reverse-phase (RP) flash chromatography on a CombiFlash® EX Prep (Teledyne, ISCO, Lincoln, NE, USA) equipped with a 43g C18 column (High Performance GOLD, RediSep Rf, Teledyne ISCO) and eluted at 30 mL/min with a linear solvent gradient from 10% MeOH in H_2O to 100% MeOH. The fraction containing the metabolites of interest was further purified using RP-HPLC with a Luna 110 Å phenyl hexyl column (5 μm, 250 mm x 10 mm, Phenomenex, Torrance, CA, USA) attached to a Thermo Surveyor containing an ELSD Sedex 55 (Thermo Fisher Scientific).

6.2.4 NMR Spectroscopy

NMR spectra were acquired on a Bruker Avance III 600 MHz NMR spectrometer (Bruker, Biospin Ltd., Milton, ON, Canada). Spectra were analysed using MestraNova Software v9.1 (Mestrelab Research, Galicia, Spain), chemical shifts (δ) were reported in parts per million (ppm) and are relative to the residual solvent signals for DMSO-d_6 (δ_H 2.50; δ_C 39.5). The signal multiplicities are reported with the abbreviations singlet (s), doublet (d), triplet (t), doublet of

triplets (dt), doublet of doublets (dd), doublet of doublets of doublets (ddd), quartet (q) and multiplets (m).

6.2.5 Marfey's Method

The absolute configuration of leucine in the new long chain *N*-acyl leucine produced by *Halomonas* sp. RKEM 883 was determined using Marfey's method.[34] The secondary metabolite in addition to L- and D-leucine standards (Sigma-Aldrich, Oakville, ON, Canada) were hydrolyzed using HCl, then derivatized with N-(2, 4-dinitro-5-fluorophenyl)-L-alaninamide (L-FDAA; Sigma-Aldrich). The reaction mixture was analyzed by UHPLC-HRMS using a Hypersil Gold 100 Å column (Thermo, 1.9 μm C18 50 mm X 2.1 mm). A flow rate of 400 μL/min and a linear gradient of 5% ACN in H_2O/0.1% formic acid to 40% ACN in H_2O/0.1% formic acid over 55 min followed by a rapid increase to 100% ACN/0.1% formic acid over 2 min then held for 3 min was used. The L-leucine standard eluted at 38.46 min and the D-leucine at 43.96 min.

6.2.6. Tandem Mass Spectrometry

Tandem MS spectra were obtained on a Thermo Orbitrap Velos mass spectrometer (Thermo Fisher Scientific) using a collision-induced dissociation energy of 35V. Spectra were collected between a *m/z* of 200 and 2000 using XCalibur software.

6.2.7. Bioactivity Screening

The new long chain N-acyl L-leucine was screened for bioactivity as previously described by Correa *et al.* 2011[35] against methicillin-resistant *Staphylococcus aureus* (MRSA; ATCC 33591), vancomycin-resistant E*nterococci faecium* (VRE, Ef 379, Wyeth), *Staphylococcus warneri*

(ATCC 17917), *Pseudomonas aeruginosa* (ATCC 14210), *Proteus vulgaris* (ATCC 12454), *Candida albicans* (ATCC 14035), and *Malassezia furfur* (ATCC 38593) at concentrations of 7.81 μg/ml, 15.6 μg/ml, 31.3 μg/ml, 62.5 μg/ml, 125 μg/ml, 250 μg/ml, and 500 μg/ml.

6.3 Results and Discussion

All bacteria present in the culture dependent library, as well as select bacteria from a library generated from *E. caribaeorum,* were fermented in four different media and metabolites were detected using UHPLC-HRMS. The data from each extract was grouped by taxonomic phyla (*Actinobacteria*, *Firmicutes*, and *Proteobacteria*) and analyzed using the aforementioned chemical dereplication process.[30] The bacterial extracts that grouped separately on the scores plot were identified as well as the buckets responsible for the observed separation. The accurate mass for the metabolite in each bucket was determined and screened against natural product databases.

6.3.1 Secondary Metabolites from *Actinobacteria*

All 49 *Actinobacteria* in the culture dependant library (Chapter 4) were fermented in ISP2, BFM3, BFM5 and BFM11, extracted with EtOAc, and analysed on the UHPLC-HRMS. After processing, a total of 1174 buckets were generated and subjected to PCA. Five of the fermentation extracts formed distinctly separate clusters when comparing the PCs (Table 6.2; Figures A.1.1-A.1.5). All the metabolites responsible for this clustering were putatively identified by their accurate mass as previously discovered secondary metabolites from *Actinobacteria* (Table 6.3; Figure A.2.1-A.2.5).

Streptomycetes sp. RKEM 1715 in BFM3 produced a family of compounds known as rhodopeptins, namely rhodopeptin C2 or C3 (**1a/1b**), rhodopeptin C4 (**2**), and rhodopeptin B5 (**3**).[36-38] These cyclic tetrapeptides were originally isolated from an *Actinobacteria* in the *Rhodococcus* genus and determined to have antifungal activity against *Candida albicans* and *Cryptococcus neoformans.*[36-38] *Arthrobacter* sp. RKEM 1637 produced coproporphyrin III (**4**) and zincphyrin (**5**) when grown in BFM3. While coporoporphyrin III is a ubiquitous metabolite[39,40] the zinc-containing form has only been detected in bacteria. Originally isolated from a *Streptomyces* sp., zincphyrin has been shown to have potent photosensitising activity in addition to acting as a histamine release inhibitor.[41,42] *Streptomyces* sp. RKEM 774 in ISP2 produced nocardamine (**6**),[43] a siderophore that has been isolated from bacteria in the *Nocardia*, *Pseudomonas*, *Streptomyces*, and *Citricoccus* genera.[43-46] In addition to iron chelation, norcaradamine has been reported to have various biological activity which include antitumor, antifungal, antioxidant, and inducing morphological changed in insect cells.[45,47-50] *Arthrobacter* sp. RKEM 1692 produced concanamycin C (**7**) and E (**8**) when grown in ISP2. The concanamycins were previously isolated from *Streptomyces* spp.[51,52] and have been shown to have antiarteriosclerotic activity, in addition to inhibiting the formation of cholesterol esters by inhibiting lysosome and endosome acidification.[47, 48] Lastly, *Kocuria* sp. RKEM 1608 in ISP2 produced kocurin (**9**),[53] a thiazolyl peptide that was originally isolated from a *Kocuria* sp. and reported to have antibiotic activity against MRSA, *B. subtilis*, and *E. faecium*.

Table 6.2 *Actinobacteria* fermentation extracts with unique secondary metabolites.

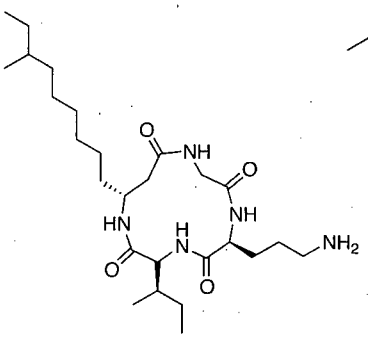
A total of 1174 buckets were analysed, and fermentation extracts that grouped separately in the PCA were identified on the scores plot and the buckets responsible were identified from the corresponding loadings plot.

Bacterial Isolate - Media	Buckets (m/z, RT)	Plot
Streptomycetes sp. RKEM 1715 - ISP2	496.3849, 4.2 510.4035, 3.3 524.4160, 2.8	PC-13/PC-7
Arthrobacter sp. RKEM 1637 - BFM3	655.2772, 3.1 717.1898, 3.3	PC-7/PC-1
Streptomyces sp. RKEM 774 - ISP2	601.3556, 2.0	PC-16/PC-11
Arthrobacter sp. RKEM 1692 - ISP2	845.4975, 3.7 860.4725, 4.0	PC-15/PC-1
Kocuria sp. RKEM 1608 - ISP2	758.1907, 3.6	PC-1/PC-5

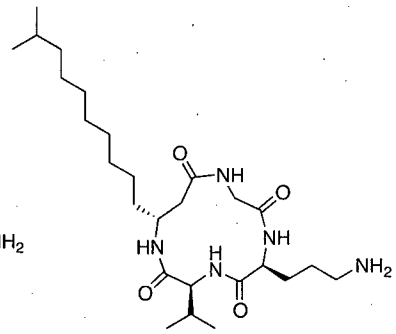
Table 6.3. Accurate mass of secondary metabolites produced by *Actinobacteria*.

Metabolites were identified by their accurate mass; a maximum mass difference of ± 5 ppm was tolerated.[32,33]

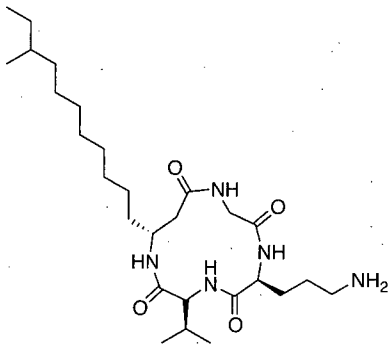
Bacterial Isolate - Media	Compounds	Molecular Formula	$[M+H]^+$ Exp.	[M+H]+ Calc.	Δ ppm	Reported Source Organism	Ref
Streptomycetes sp. RKEM 1715	Rhodopeptin C2/C3 (**1a/b**)	$C_{26}H_{50}N_5O_4$	496.3849	496.3857	1.61	*Rhodococcus* sp.	36-38
	Rhodopeptin C4 (**2**)	$C_{27}H_{52}N_5O_4$	510.4035	510.4014	-4.11		
	Rhodopeptin B5 (**3**)	$C_{28}H_{54}N_5O_4$	524.4160	524.4170	1.91		
Arthrobacter sp. RKEM 1637	Coproporphyrin III (**4**)	$C_{36}H_{39}N_4O_8$	655.2772	655.2762	-1.47	*Streptomyces* sp.	41
	Zincphyrin (**5**)	$C_{36}H_{37}N_4O_8Zn$	717.1898	717.1897	-0.17		
Streptomyces sp. RKEM 774	Nocardamine (**6**)	$C_{27}H_{49}N_6O_9$	601.3556	601.3561	0.85	*Streptomyces* sp.	54
Arthrobacter sp. RKEM 1692	Concanamycin C (**7**)	$C_{45}H_{75}O_{13}$	823.5178	823.5202	2.91	*Actinoalloteichus* sp.	51,52, 55
	Concanamycin E (**8**)	$C_{44}H_{72}NO_{14}$	838.4915	838.4947	3.82		
Kocuria sp. RKEM 1608	Kocurin (**9**)	$C_{69}H_{67}N_{18}O_{13}S_5$	1515.3738	1515.3733	-0.33	*Kocuria* sp.	53



(**1a**)



(**1b**)



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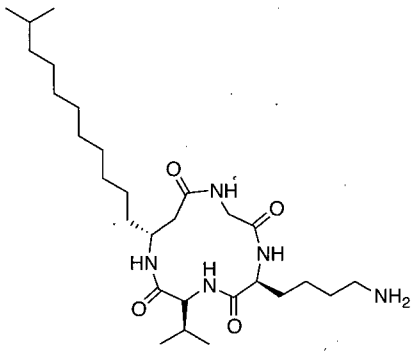

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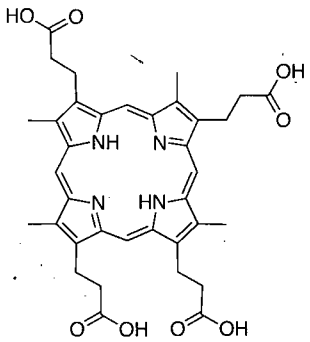

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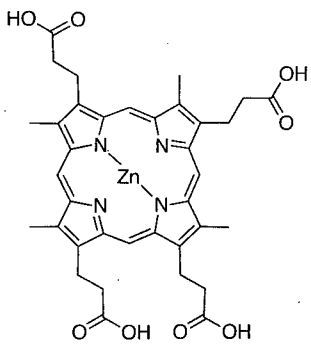

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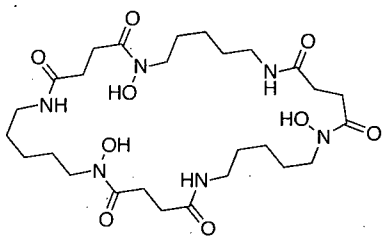

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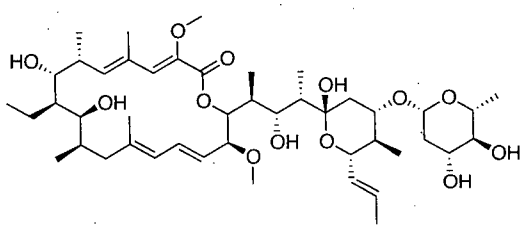
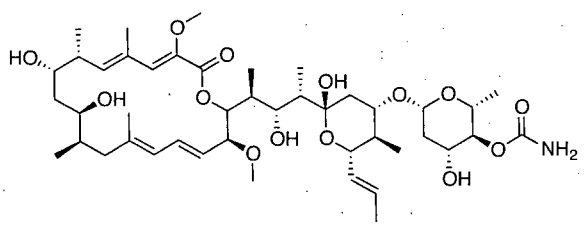
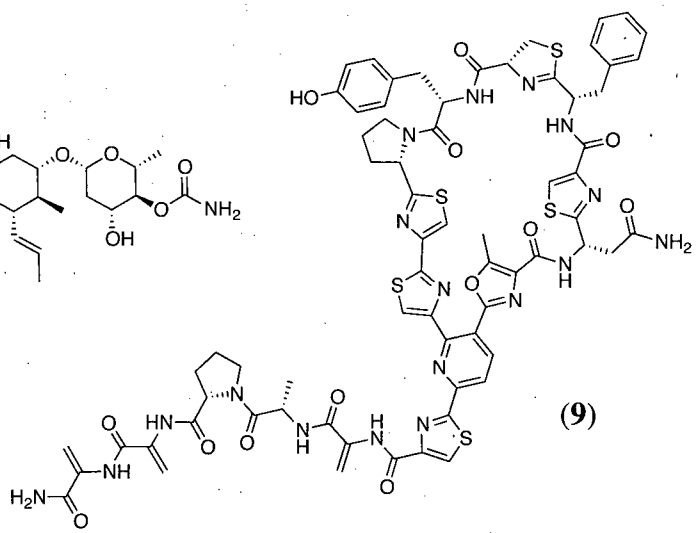






(**9**)

6.3.2 Secondary Metabolites from *Firmicutes*

All 22 bacterial isolates in the culture dependant library belonging to the phylum *Firmicute* were fermented in BFM3, BFM5, BFM6, BFM7, and BFM11, extracted with EtOAc and analysed on a UHPLC-HRMS. After processing, a total of 527 buckets were generated and subjected to PCA. Two of the fermentation extracts formed distinctly separate clusters when comparing the PCs (Table 6.4; Figures A.1.6 & A.1.7). All the metabolites responsible for this clustering were putatively identified by their accurate mass as previously discovered secondary metabolites from *Firmicutes* (Table 6.5; Figures A.2.6 & A.2.7)

The first, *Paenibacillus* sp. RKEM 768 in BFM5 produced baceridin (**10**), a cyclic peptide that had been previously isolated from a *Bacillus* sp. and was shown to have weak cytotoxic and antibacterial activity.[56] The second, *Bacillus* sp. RKEM 720 in BFM5 produced bacillamide B (**11**) and C (**12**).[57,58] The bacillamides are a family of alkaloids that were previously isolated from *Bacillus* spp. and were reported to have weak algicidal activity against *Cochlodinium polykrikoides*.[58-60,57,59]

Table 6.4. *Firmicutes* fermentation extracts with unique secondary metabolites.

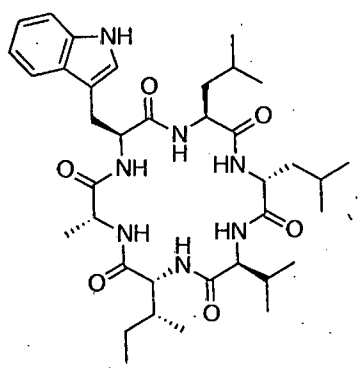
A total of 527 buckets were analysed, fermentation extracts that grouped separately in the PCA were identified on the scores plot and the buckets responsible were identified from the corresponding loadings plot.

Bacterial Isolate - Media	Buckets (m/z, RT)	PC plot
Paenibacillus sp. RKEM 768 - BFM5	718.4275, 3.7	PC-6/PC-4
Bacillus sp. RKEM 720 - BFM5	338.0931, 2.9 357.1378, 2.5	PC-3/PC-5

Table 6.5. Accurate mass of secondary metabolites produced by Firmicutes.

Metabolites were identified by their accurate mass; a maximum mass difference of ± 5 ppm was tolerated.[32,33]

Bacterial Isolate - Media	Compounds	Molecular Formula	[M+H]+ Exp.	[M+H]+ Calc.	Δ ppm	Reported Source Organism	Ref
Paenibacillus sp. RKEM 768	Baceridin (**10**)	$C_{37}H_{58}N_7O_6$	696.4457	696.4449	1.15	Bacillus sp.	56
Bacillus sp. RKEM 720	Bacillamide B (**11**)	$C_{16}H_{18}N_3O_2S$	316.1112	316.1114	-0.63	Bacillus sp.	57, 58
	Bacillamide C (**12**)	$C_{18}H_{21}N_4O_2S$	357.1378	357.138	-0.56		



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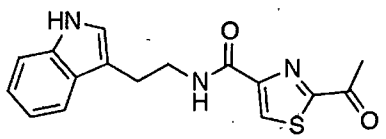

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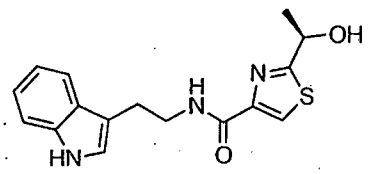


(12)

6.3.3 Secondary Metabolites from *Proteobacteria*

All 69 bacterial isolates present in the culture dependent library belonging to the phylum *Proteobacteria*, in addition to ten bacteria obtained from a culture dependent library built from *E. caribaeorum* were fermented in BFM1, BFM3, BFM5, and BFM11. The fermentations were extracted and analysed using UHPLC-HMRS, after processing a total of 2242 buckets were generated and analyzed using PCA. There were eight different fermentation extracts that grouped separately in the scores plots (Table 6.6; Figure A.1.8-A.1.13). The metabolites responsible for this observed separation could be identified by their accurate masses in four of the fermentation datasets (Table 6.7; Figures A.2.8-A.2.11). The first of which was *Pseudoalteromonas* sp. RKBH 282 in BFM5 which produced pseudoalteromone A (**13**) and B (**14**), cytotoxic metabolites previously isolated from other *Pseudoalteromonas* sp.[61,62] The second, *Vibrio* sp. RKEM 201 in BFM5 produced bahamamide (**15**) a cyclic *bis*-amide previously isolated from an unidentified marine bacterium.[63] The third, *Acinetobacter* sp. RKEM 817 in BFM5 produced andrimid (**16**), a non-ribosomal peptide-polyketide (NRP-PK) that has been isolated from various *Gammaproteobacteria* and has been reported to have mild antibiotic activity by inhibiting fatty acid biosynthesis.[64-67] The last, *Pseudoalteromonas* sp. RKBH 271 in BFM5 produced bromoalterochromide A (**17**). This chromopeptide previously reported from a *Pseudoalteromonas* sp. was shown to exhibit mild cytotoxic activity.[68] This bacterial isolate produced a different set of metabolites when grown in BFM3, these metabolites in addition to the ones produced by *Pseudoalteromonas* sp. RKEM 243 in BFM5, *Pseudomonas* sp. RKEM 545 in BFM 11, and *Halomonas* sp. RKEM 883 in BFM5 did not have hits when screened through the natural product databases. This was either due to the metabolite having a novel structure or that it was simply not in the database, as no database is entirely exhaustive. Regardless, all four of the

bacterial isolate fermentations were scaled up and the compounds were purified and identified either by NMR or tandem MS.

Table 6.6. *Proteobacteria* fermentation extracts with unique secondary metabolites.

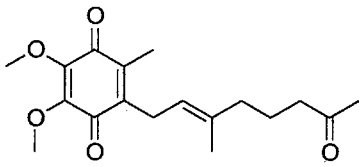
A total of 2242 buckets were analysed by PCA and fermentation extracts that grouped separately on the scores plot were putatively identified. The buckets responsible for that separation were identified from the corresponding loadings plot.

Bacterial Isolate - Media	Buckets (m/z, RT)	Plot
Halomonas sp. RKEM 883 - BFM11	314.2690, 4.4	PC-4/PC-6
Pseudoalteromonas sp. RKBH 271 - BFM3	456.2721, 4.6 442.2564, 4.4 428.2406, 4.1	PC-2/PC-10
Pseudoalteromonas sp. RKBH 271 - BFM5	846.2902, 3.1	PC-5/PC-7
Pseudoalteromonas sp. RKBH 282 - BFM11	343.1516, 3.6 277.1775, 3.0	PC-6/PC-7
Pseudoalteromonas sp. RKEM 243 - BFM5	380.0556, 3.6 265.0465, 3.5	PC-1/PC-3
Pseudomonas sp. RKEM 545 - BFM11	690.9231, 4.8	PC-1/PC-4
Vibrio sp. RKEM 205 - BFM5	357.2513, 4.2	PC-4/PC-5
Acinetobacter sp. RKEM 817 - BFM5	480.2493, 4.5	PC-3/PC-15

Table 6.7. Accurate masses of secondary metabolites produced by *Proteobacteria*.

Metabolites were identified by their accurate mass; a maximum mass difference of ± 5 ppm was tolerated.[32,33]

Bacterial Isolate - Media	Compounds	Molecular Formula	$[M+H]^+$ Exp.	$[M+H]^+$ Calc.	Δ ppm	Reported Source Organism (s)	Ref
Pseudoalteromonas sp. RKBH 282 - BFM11	Pseudo-alteromone A (**13**)	$C_{15}H_{27}O_3$	321.1697	321.1697	-0.06	*Pseudoalteromonas* sp.	61,62
	Pseudo-alteromone B (**14**)	$C_{18}H_{25}O_5$	255.1952	255.1955	-0.78		
Vibrio sp. RKEM 205 - BFM5	Bahamamide (**15**)	$C_{20}H_{35}N_2O_2$	335.2696	335.2701	-1.49	Unidentified marine bacteria	63
Acinetobacter sp. RKEM 817 - BFM5	Andrimid (**16**)	$C_{27}H_{34}N_3O_5$	480.2498	480.2493	-1.04	*Vibrio* sp. Pseudomonas sp. Enterobacter sp.	64-66
Pseudoalteromonas sp. RKBH 271 - BFM5	Bromo-alterochromide A (**17**)	$C_{38}H_{51}BrN_7O_{10}$	844.2916	844.28753	-4.82	*Pseudoalteromonas* sp.	68



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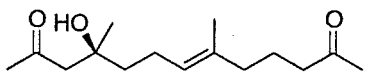

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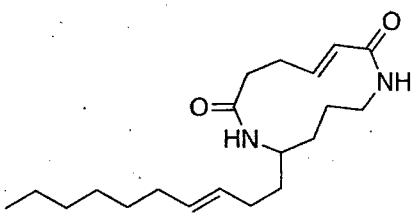

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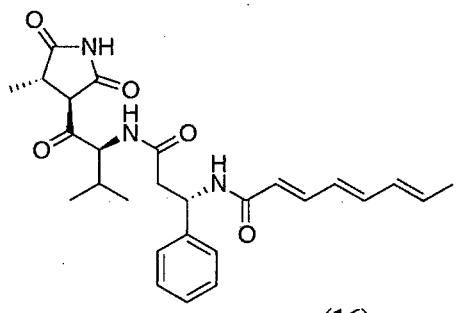

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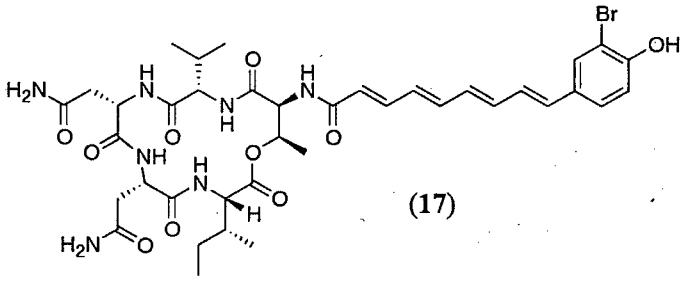


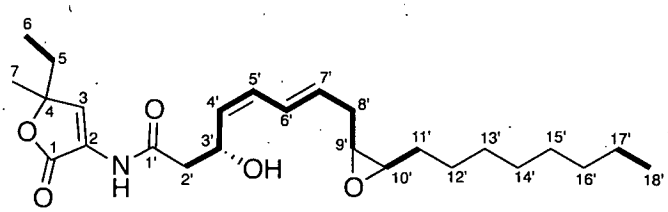
(17)

6.3.3.1 *Pseudoalteromonas* sp. RKBH 271 and the Korormicins

Fermentation of *Pseudomonas* sp. RKEM 271 in BFM3 was scaled up (6L) and the metabolites were purified using RP-flash chromatography and RP-HPLC. The metabolite with the $[M+H]^+$ of 434.2902 was obtained in the highest abundance (1.2mg) and therefore analysed using NMR. The ^{1}H and ^{13}C chemical shifts obtained from this compound matched those of korormicin A (**18**)[69-72] (Table 6.8; Figures A.3.1-A.3.3). Once the structure of korormicin A was determined the other metabolites were readily identified by accurate mass to be the analogs korormicin J (**19**) and K (**20**)[73] (Table 6.9). This family of NRP-PKs were initially isolated from *Pseudoaltetromonas* spp. and were shown to have moderate antibacterial activity against gram-negative bacteria.[69-72]

Table 6.8. Experimental and reported[69] ^{1}H (600 MHz) and ^{13}C (150 MHz) NMR data for korormicin A (18) in DMSO-d_6.

	Experimental			Reported	
Pos.	δ_C, type	δ_H (J, Hz)	COSY	δ_C, type	δ_H (J, Hz)
1				168.4, C	
2				125.1, C	
3	133.6, CH	7.39, s		133.9, CH	7.26, s
4				87.0, C	
5	30.9, CH_2	1.76, q (7.1)	H6	31.2, CH_2	1.74, q (7.3)
6	7.67, CH_3	0.76, t (7.1)	H5	8.0, CH_3	0.74, t (7.3)
7	23.8, CH_3	1.41, s		24.1, CH_3	1.37, s
NH		9.90, s			9.83, s
1'				170.1, C	
2'	43.6, CH_2	2.41, dd (5.2, 14.2)	H3'	44.0, CH	2.39, dd (5.4, 14.4)
		2.61, dd (8.2, 14.2)	H3'		2.59, dd (8.1, 14.4)
3'	63.5, CH	4.84, ddd (5.2, 8.2, 13.2)	H2', H4'	63.0, CH	4.83, ddd (5.4, 8.1, 9.0)
OH					5.09, d (4.4)
4'	132.6, CH	5.31, m	H3', H5'	132.7, CH	5.30, dd (9.0, 10.9)
5'	128.0, CH	5.94, t (11.0)	H4', H6'	128.0, CH	5.92, dd (10.9, 11.2)
6'	127.3, CH	6.48, dd (11.0, 15.1)	H5', H7'	127.6, CH	6.46, dd (11.2, 15.1)
7'	130.6, CH	5.71, dt (7.0, 15.0)	H6', H8'	130.9, CH	5.70, dt (6.8, 15.1)
8'	30.6, CH_2	2.28, dd (7.0, 13.5)	H7', H9'	30.9, CH_2	2.26, dd (5.9, 6.8)
9'	54.7, CH	2.92, dt (4.0, 6.2)	H8'	55.1, CH	2.90, dt, (4.2, 5.9)
10'	55.7, CH	2.88, dt (4.0, 5.9)	H11'	56.0, CH	2.87, dt, (4.2, 6.1)
11'	26.7, CH_2	1.48, m	H10'	27.2, CH_2	1.48, m
12'	25.8, CH_2	1.40, m		26.1, CH_2	1.38, m
13'	28.6, CH_2	1.20 ~ 1.35		28.92, CH_2	1.2 ~ 1.4, m
14'	28.6, CH_2	1.20 ~ 1.35		28.89, CH_2	1.2 ~ 1.4, m
15'	28.6, CH_2	1.20 ~ 1.35		28.6, CH_2	1.2 ~ 1.4, m
16'	31.0, CH_2	1.24, m		31.2, CH_2	1.22, m
17'	21.8, CH_2	1.26, m	H18'	22.1, CH_2	1.24, m
18'	13.62, CH_3	0.85, t (7.0)	H17'	13.9, CH_3	0.83, t (7.1)



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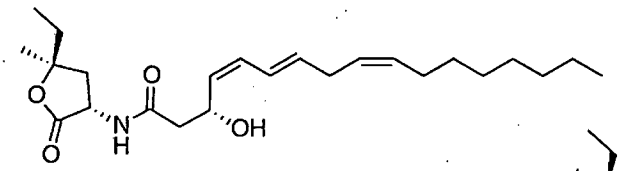

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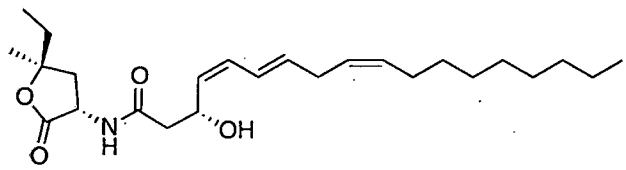


(20)

Table 6.9. Accurate masses of korormicins produced by *Pseudoalteromonas* sp. RKEM 271

Metabolites were identified by their accurate mass; a maximum mass difference of ± 5 ppm was tolerated.

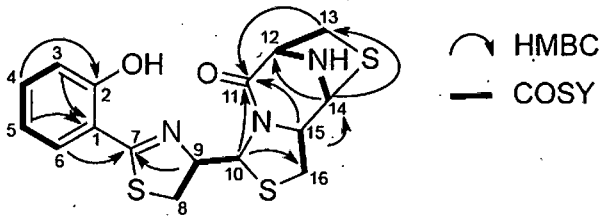
Compound	Molecular Formula	$[M+H]^+$ Experimental	$[M+H]^+$ Calculated	Δ ppm
Koromicin A (**18**)	$C_{25}H_{40}NO_5$	434.2902	434.2901	0.23
Koromicin J (**19**)	$C_{25}H_{42}NO_4$	406.2947	406.2952	-1.23
Koromicin K (**20**)	$C_{24}H_{40}NO_4$	420.3109	420.3107	0.48

6.3.3.2 *Pseudoalteromonas* sp. RKEM 243 and Ulbactins

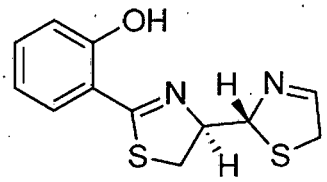
Fermentation of *Pseudoalteromonas* sp. RKBH 245 in BFM5 was scaled up (6L) and the metabolites were purified. The metabolite with the $[M+H]^+$ of 380.0556 was obtained in the highest abundance (3.4 mg) and analyzed by NMR. The structure of this compound was determined to be that of ulbactin E (**21**)[74] (Table 6.10; Figures A.3.4-A.3.7). Once the structure of ulbactin E was determined the other compound was identified by accurate mass to be the analog ulbactin A (**22**)[75] (Table 6.11). The ulbactins are a family of alkaloids that have been isolated from various bacterial genera including *Vibrio*, *Alteromonas*, *Pseudomonas*, and *Brevibacillus*, and have been shown to exhibit mild cytotoxic activity.[74-76]

Table 6.10. Experimental and reported[74] ^{1}H (600 MHz) and ^{13}C (150 MHz) NMR data for ulbactin E (21) in DMSO-d_6.

	Experimental				Reported	
Pos.	δ_C, type	δ_H (J, Hz)	COSY	HMBC	δ_C, type	δ_H (J, Hz)
1	115.90, C				116.4, C	
2	158.46, C				159.3, C	
3	116.89, CH	6.98, m	H4	C1, C2, C4, C6	117.4, CH	6.99, d (8.1)
4	133.68, CH	7.43, m	H3, H5	C1, C2, C3, C5 C6	133.4, CH	7.36, ddd (8.1, 7.6, 1.4)
5	119.28, CH	6.95, m	H5, H6	C1, C2, C4, C6	119.0, CH	6.88, dd (7.6, 7.6)
6	130.45, CH	7.43, m		C1, C6, C2, C7	130.9, CH	7.39, dd (7.6, 1.4)
7	173.42, C				173.9, C	
8	33.2, CH_2	3.24, dd (11.4, 6.6) 3.46, dd (11.4, 8.8)	H9	C7, C9, C10	33.9, CH_2	3.42, dd (9.5, 5.4) 3.45, dd (11.2, 8.5)
9	80.54, CH	4.94, td (8.6, 6.6)	H8, H10	C7, C8, C10	80.8, CH	4.87, ddd (8.5, 7.3, 6.0)
10	60.05, CH	5.42, d (8.4)	H9	C8, C9, C11, C15, C16	60.7, CH	5.76, d (6.0)
11	168.44, C				167.9, C	
12	64.61, CH	4.36, dd (6.4, 2.6)	H13, H17	C13, C14	71.3, CH	4.04, d (6.4)
13	39.12, CH_2	3.00, dd (10.4, 6.3) 3.29, d (10.3)	H12	C11, C12, C14	35.2, CH_2	3.32, d (10.8) 3.23, dd (10.8, 6.4)
14	65.34, CH	5.35, d (4.6)	H17	C12, C13, C15, C16	73.2, CH	4.62, d (4.5)
15	68.69, CH	3.91, dd (11.0, 5.0)	H16	C10, C11, C14, C16	69.5, CH	3.83, dd (10.9, 5.4)
16	33.4, CH_2	2.81, dd (11.0, 9.6) 3.08, dd (9.6, 5.0)	H15	C10, C13, C14, C15	3.47, CH_2	2.97, dd (9.5, 5.4) 3.01, dd (10.9, 9.5)
NH		3.86, t (4.2)	H12, H14			



(21)



(22)

Table 6.11. Accurate masses of ulbactins produced by *Pseudoalteromonas* sp. RKBH 245

Metabolites were identified by their accurate mass; a maximum mass difference of ± 5 ppm was tolerated.

Compound	Molecular Formula	$[M+H]^+$ Experimental	$[M+H]^+$ Calculated	Δ ppm
Ulbactin E (**21**)	$C_{16}H_{18}N_3O_2S_3$	380.0556	380.0556	0.00
Ulbactin A (**22**)	$C_{12}H_{13}N_2OS_2$	265.0465	265.0464	0.38

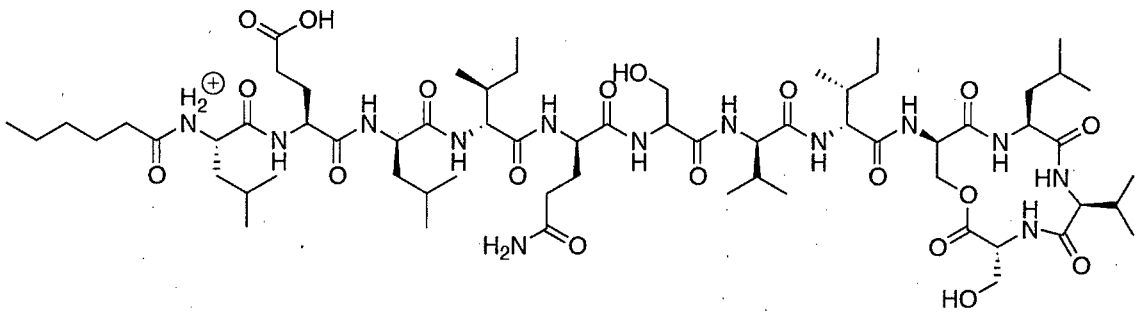
6.3.3.3 *Pseudomonas* sp. RKEM 545 and the putisolvins

Fermentation of *Pseudomonas* sp. RKBH 545 in BFM11 was scaled up (6L) and the secondary metabolites were purified using RP-flash chromatography and RP-HPLC. Although there was only one secondary metabolite responsible for the separation of this fermentation extract in the PCA plots, when scaled up there were two metabolites present. These had $[M+H]^+$ values of 1380.8376 and 1394.8545 (Figures A.2.14 & A.2.15). Using collision-induced dissociation tandem MS it was determined that these metabolites were putisolvin I and II (Table 6.12; Figure 6.1, 6.2).[77] These lipopeptides were previously isolated from a *Pseudomons* sp. and were reported to have biosurfactant activity.[77-79]

Table 6.12. Accurate masses of putisolvins produced by *Pseudomonas* sp. RKEM 545

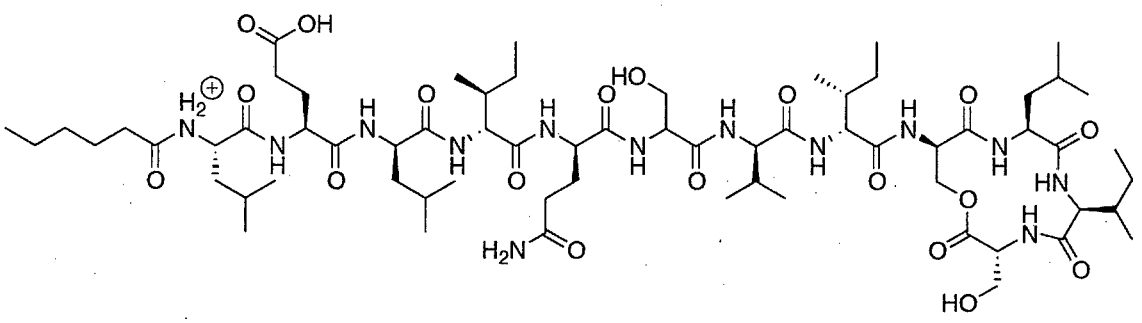
Metabolites were identified by their accurate mass; a maximum mass difference of ± 5 ppm was tolerated.

Compound	Molecular Formula	$[M+H]^+$ Experimental	$[M+H]^+$ Calculated	Δ ppm
Putisolvin I (**23**)	$C_{65}H_{114}N_{13}O_{19}$	1380.8376	1380.8348	2.03
Putisolvin II (**24**)	$C_{66}H_{116}N_{13}O_{19}$	1394.8545	1394.8505	2.87



Chemical Formula: $C_{65}H_{114}N_{13}O_{19}^{+}$
Exact Mass: 1380.8348

(23)



Chemical Formula: $C_{66}H_{116}N_{13}O_{19}^{+}$
Exact Mass: 1394.8505

(24)

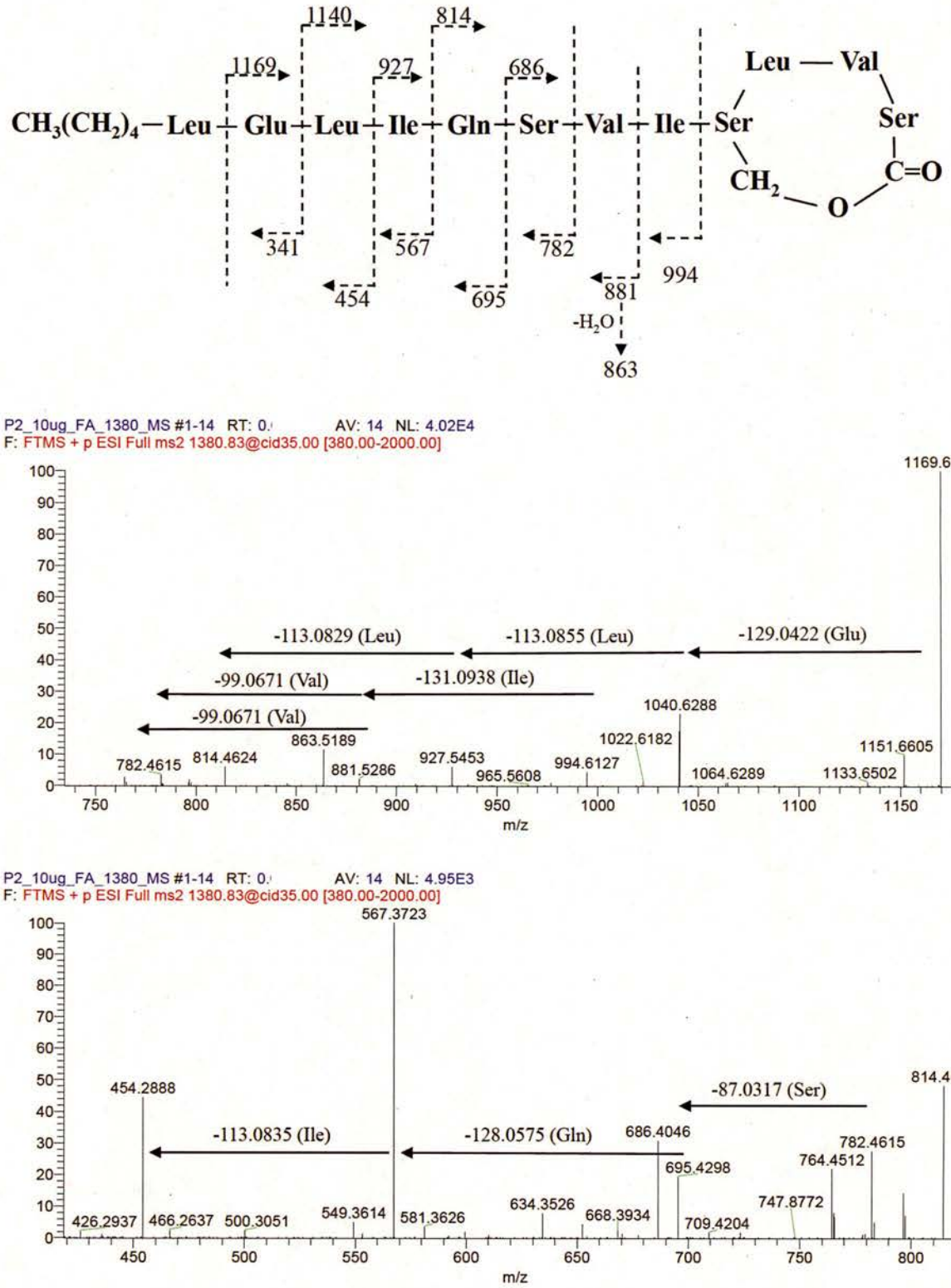


Figure 6.1. Tandem mass spectrum and fragment scheme of putisolvin I (23).

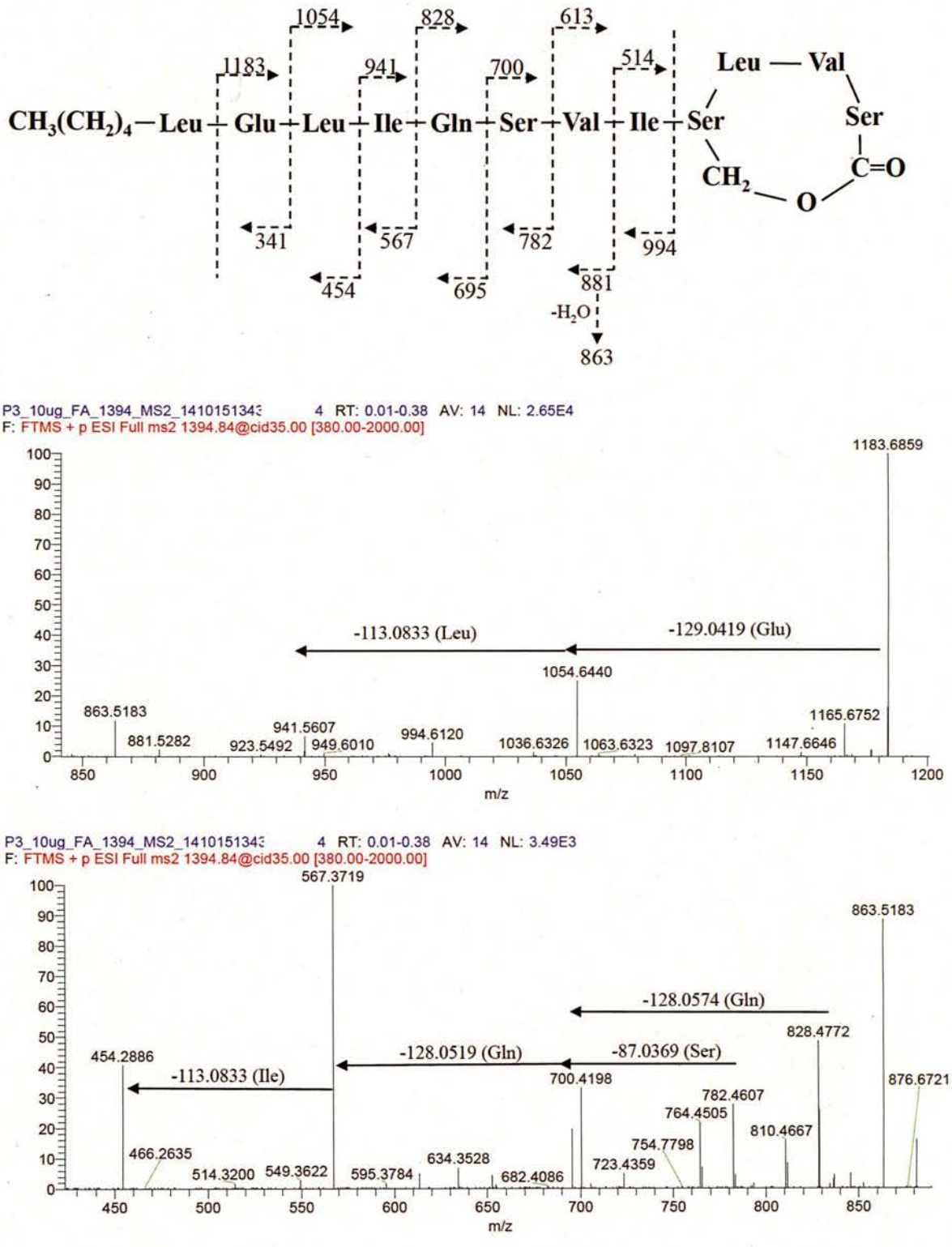
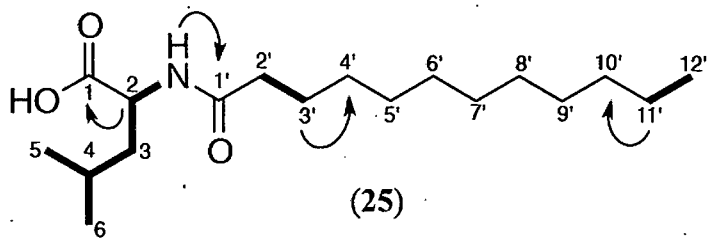


Figure 6.2. Tandem mass spectrum and fragmentation scheme of putisolvin II (24).

6.3.3.4 *Halomonas* sp. RKEM 883 and Identification of a New Long-Chain N-Acyl L-Leucine.

Fermentation of *Halomonas* sp. RKEM 883 in BFM5 was scaled up (6L) and 3.4 mg of metabolite **25** with a $[M+H]^+$ of 314.2690 was obtained. NMR data (Table 6.13) revealed the compound to be a long chain *N*-acyl L-leucine. Two distinct ^1H spin systems were identified by TOCSY spectra as a leucine and a fatty acid side chain. The leucine residue was assigned by COSY correlations between NH (δ_H 7.95) / H-2 (δ_H 4.31); H-2 (δ_H 4.31) / H-3 (δ_H 1.46); H-3 (δ_H 1.46) / H-4 (δ_H 1.61); H-4 (δ_H 1.61) / H-5 and H-6 (δ_H 0.81 and δ_H 0.87); and the HMBC correlations from H-2 to C-1 (δ_C 175.25). The fatty acid side chain was assigned by COSY correlations between H-2' (δ_H 2.07) / H-3' (δ_H 1.47); H-11' (δ_H 1.26) / H-12' (δ_H 0.85); HMBC correlations from NH (δ_H 7.95) to C-1' (δ_C 177.15); and the TOCSY spin system containing H-2' (δ_H 2.07) / H-3' (δ_H 1.47); H-11' (δ_H 1.26) / H-12' (δ_H 0.85)/six overlapping methylenes (δ_H 1.20-1.23). The absolute configuration of leucine was determined using Marfey's method[34] to be in the L-conformer (Figure 6.3).

This new secondary metabolite belongs to a family of compounds known as long chain N-acyl amino acids. These metabolites are commonly detected in *E-coli*-based soil eDNA libraries, to date N-acyl tyrosine, phenylalanine, tryptophan, and arginine have been reported.[80-82] This is the first report of a long chain N-acyl amino acid containing a leucine. These secondary metabolites have been reported to have mild antimicrobial activity.[82,83] Therefore, **25** was screened for antimicrobial activity and found to have very mild activity against MRSA, VRE, and *S. warneri* but only at a concentration of 500 μg/ml.



(25)

6.13. Experimental ^{1}H (600 MHz) and ^{13}C (150 MHz) NMR Data for a long chain *N*-acyl L-leucine in DMSO-d_6.

Pos.	C-Shift	H-Shift	COSY	HMBC
1	175.25, C			
2	51.02, CH	4.21, m	NH, H3	C1, C1'
3	40.89, CH_2	1.46, m	H2, H4	
4	24.05, CH	1.61, m	H3, H5, H6	
5	21.30, CH_3	0.82, d (6.5)	H4	C3, C4
6	22.94, CH_3	0.87, d (6.5)	H4	C3, C4
1'	177.15, C			
2'	35.08, CH_2	2.07, t (7.1)	H3'	C1', C3', C4'
3'	25.22, CH_2	1.47, m	H2'	C2', C4'
4' - 9'	28.36 - 29.01, CH_2	1.20 - 1.23, m		
10'	31.08, CH_2	1.23, m		
11'	21.91, CH_2	1.26, m	H12'	C10', C12'
12'	13.80, CH_3	0.85, t (7.0)	H11'	C10', C11'
NH		7.95, d (8.1)	H2	C1'

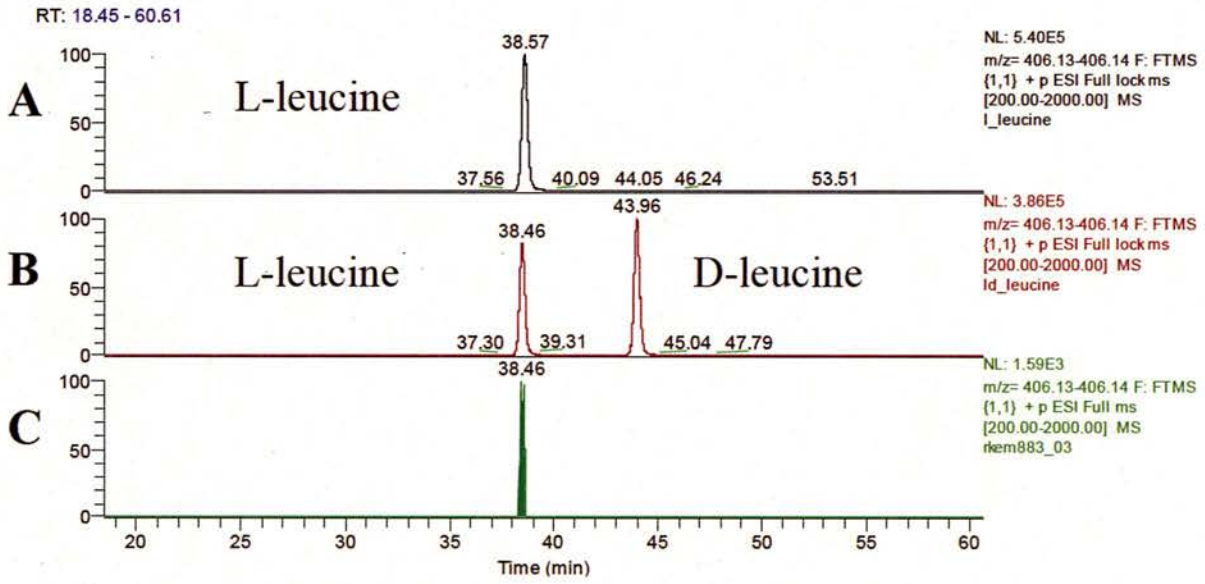


Figure 6.3. Chromatograms of Marfey's analysis for metabolite 25.

L-FDAA derivative L-leucine (A), L- and D-leucine (B), and metabolite **25** (C) UHPLC chromatograms.

6.3.4 Screening for the Presence of Pseudopterosins and Eleutherobins

It has long been suspected that secondary metabolites isolated from marine macroorganisms are in fact biosynthesised by associated microorganisms.[22,84] Therefore all the bacterial extracts were screened for the presence of pseudopterosins and eleutherobins, the MNPs associated with *A. elisabethae* and *E. caribaeorum*.[85-88] This was achieved by analyzing standards of pseudopterosins and eleutherobins using UHPLC-HRMS. The datasets were subjected to the same chemical dereplication process as the bacterial fermentation extracts. After which they were built into chemical barcodes to screen for metabolites with the same *m/z* and RT, an example of these barcodes is shown in Figure 6.4. Unfortunately, no pseudopterosins or eleutherobins were detected under the conditions used in this study in any of the bacterial isolates. However, the absence of these MNPs from these fermentations does not mean that they

are not of microbial origin, or that the bacteria screened in this research do not contain the biosynthetic machinery to produce these metabolites. It simply means that under the fermentation conditions used for this research no pseudopterosins or eleutherobins were detected, and that further research is required to determine the biosynthetic origin of these pharmaceutically relevant MNPs.

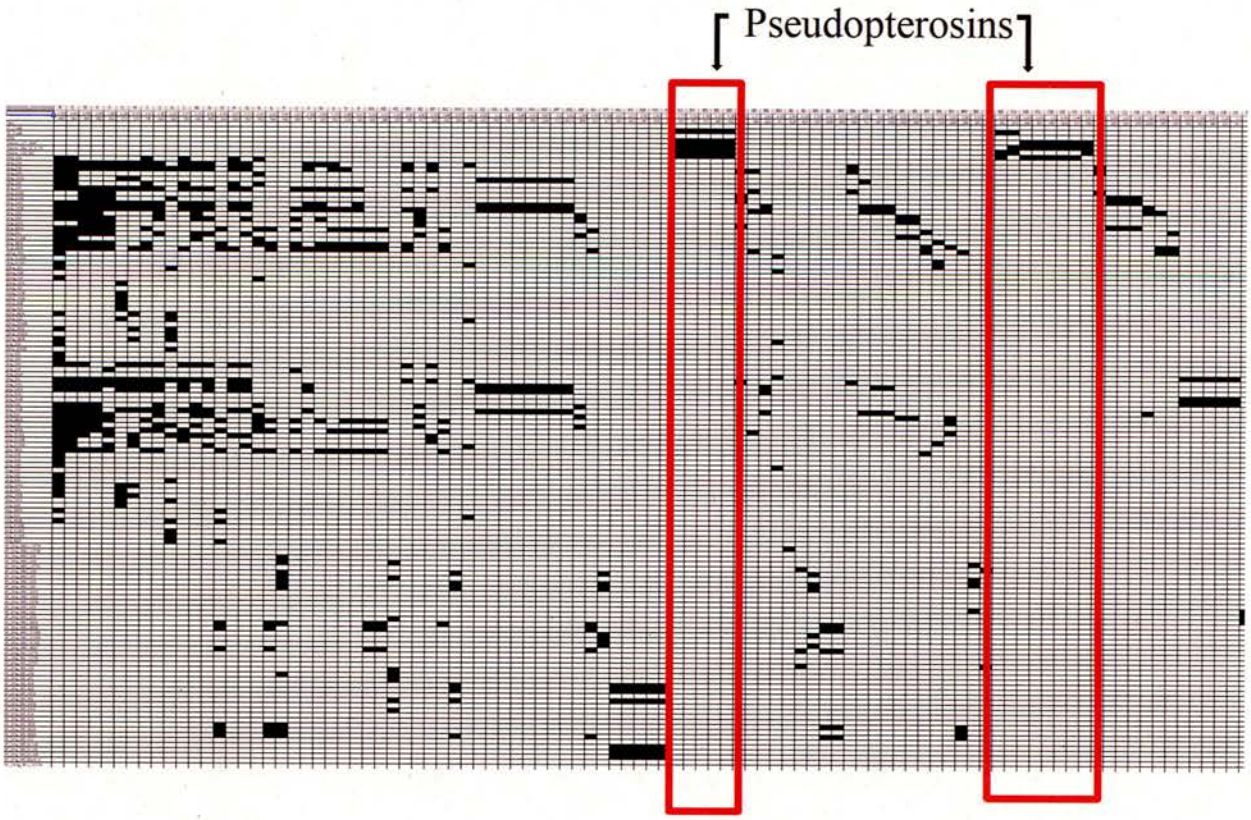


Figure 6.4. Example of chemical barcode containing pseudopterosin standards.

6.4 Conclusion

This study showed that bacterial isolates from octocorals have the potential to produce a broad array of chemically diverse structures with a wide range of biological importance. Using a chemical dereplication process to analyse all of the UHPLC-HRMS data generated from the

bacterial fermentations, 24 different known secondary metabolites were putatively identified. In addition, one new secondary metabolite, a long chain *N*-acyl L-leucine was discovered.

This study also highlighted the fact that culture conditions are central to secondary metabolite production. For example, *Pseudoalteromonas* RKEM 271 produced more bromoalterochromide A in BFM5 and more of the korormicins in BFM3. While both sets of metabolites were detectable in trace amounts in the other culture conditions, specific media components favoured the biosynthesis of these compounds. Therefore this study only scratches the surface of the secondary metabolites capable of being produced by this culture dependent library. Numerous other conditions can be explored in future studies to discover the untapped potential of these bacteria.

While no pseudopterosin or desmethyleleutherobin production was detected in this study, future studies should continue to explore the holobiont of these octocorals. With advancements in metagenomic sequencing and bioinformatic technologies, the true biosynthetic producer of these pharmaceutically important MNPs may be determined.

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Chapter 7: Overall Conclusions of Thesis Work and Future Directions of Octocoral Microbiome and Marine Natural Products Research

7.1 Summary of Thesis Research

7.1.1 Chapter 2: Spatial and Temporal Investigation of the Microbiome of the Caribbean Octocoral *Erythropodium caribaeorum*

This study characterized the microbiome of the octocoral *Erythropodium caribaeorum*.[1] Samples were collected from two locations at three different time points to investigate geographic and temporal variations in microbial communities, as well as to identify any putative core microbiome. This research found that there were no statistically significant variations in the microbial community structure between location and time of collection. Additionally, it was determined that *E. caribaeorum* possessed a high level of bacterial taxonomic diversity compared to other reported octocorals. However amid that high diversity the microbiomes had a dominant association with the class *Gammaproteobacteria*, in particular the genus *Endozoicomonas*. Three stable species-specific OTUs were present in all octocoral samples, confirming the hypothesis that there is a stable microbiome association with the octocoral *E. caribaeorum*. One of these, *Endozoicomonas* sp. OTU 001, was highly abundant and most likely plays an essential role in the biology of the *E. caribaeorum* holobiont. The data generated in this study serves as a valuable starting point for future investigations into the microbiome associated with *E. caribaeorum* and other octocorals in the Caribbean.

7.1.2 Chapter 3: Culture-Independent Investigation into the Microbiome of *Antillogorgia elisabethae* from San Salvador, The Bahamas, and the Microbial Communities Associated with the Holobiont, Algal Dinoflagellates, and Larvae

This study characterised the microbiome of *Antillogorgia elisabethae* collected from San Salvador, The Bahamas in addition to the microbial communities associated with the dinoflagellates and larvae of those samples. The holobiont samples examined in this study had statistically different bacterial community structure from the surrounding seawater and had a stable association with the genera *Endozoicomonas*, *Vibrio*, and *Pseudoalteromonas*. However, when compared to previously reported *A. elisabethae* holobiont microbiomes there was a high level of taxonomic heterogeneity and no stable taxonomic genera were detected.[2] Therefore rejecting the hypothesis that there is a stable microbiome association with the octocoral *A. elisabethae*. This suggests that this coral does not require a stable association with any specific bacterial species for survival.

The dinoflagellate and larval samples were determined to have a statistically different microbial community structure than the holobiont samples from which they were obtained. However they had similar microbial richness, diversity, and taxonomic composition at the genus level. This data has added to the growing body of research on *A. elisabethae* microbiomes and was the first to investigate the microbial communities associated with the dinoflagellates and larvae of this pharmaceutically important octocoral.

7.1.3 Chapter 4: Culture-Dependent Investigation of the Bacterial Communities Associated with *Antillogorgia elisabethae*

This study characterized the cultivatable bacteria from the octocoral *A. elisabethae* using two different techniques. The first investigated the taxonomic diversity that could be cultured from the holobiont, dinoflagellate, and larvae used in Chapter 2. From these samples a total of 89 unique bacterial species were obtained, the majority of which belonged to the class *Gammaproteobacteria*. When comparing the culture-dependent libraries to the culture-independent libraries for the holobiont, dinoflagellates, and larvae the recovery rates were 3.9%, 10.0%, and 7.5% respectively. In addition, 12 bacterial isolates were obtained from the dinoflagellate and larvae samples that were not obtained from the holobiont samples.

The second technique investigated the taxonomic diversity that could be obtained using microaerophilic and anaerobic culturing conditions. A total of 54 unique bacterial strains were obtained that had not been obtained in the previous aerobic culture-dependent library. Between the two culturing techniques used in this study, a total of 143 different species of bacteria were obtained. Of these, three were putatively novel species, two of putatively novel genera, and one bacterial isolate, *Pseudobacteriovorax antillogorgiicola* RKEM 611^{T}, confirming the hypothesis that *A. elisabethae* is a source of novel cultivatable bacteria.

7.1.4 Chapter 5: Species Description of *Pseudobacteriovorax antillogorgiicola* gen. nov., sp. nov., a bacterium isolated from the gorgonian octocoral *Antillogorgia elisabethae*, belonging to a novel bacterial family, *Pseudobacteriovoraceae* fam. nov., within the order *Bdellovibrionales*

This study taxonomically identified bacterial isolate RKEM 611 by comparing all available phenotypic, genotypic, and chemotaxonomic data of the closely related Bdellovibrio-and-like organisms.[3] Based on phylogenetic analysis of the 16S rRNA gene, in addition to phenotypic and chemotaxonomic characteristics, RKEM611 was determined to belong to a novel family within the order *Bdellovibrionales*. The names *Pseudobacteriovoracaceae* for the family and *Pseudobacteriovorax antillogorgiicola* for the genus and species were assigned. This research highlighted that octocorals can serve as reservoirs for taxonomically unique bacteria.

7.1.5 Chapter 6: Investigation of Secondary Metabolites Produced by Octocoral Associated Bacteria

This study investigated the secondary metabolites produced by bacteria from *A. elisabethae* and *E. caribaeorum* culture dependent libraries. A chemical dereplication process was employed to identify unique metabolites in the bacterial fermentation extracts belonging to the same taxonomic phylum. The previously reported rhodopeptins,[4-6] zincphyrin, [7] nocardamine,[8] concanamycins,[9-11] and kocurin[12] were detected in the *Actinobacteria* fermentation extracts. Baceridin[13] as well as bacillamide[14,15] analogs were detected in the *Firmicute* extracts. Pseudoalteromones,[16,17] bahamamide,[18] andrimid,[19-21] bromoalterochromide A,[22] korormicins,[23-26] ulbactins,[27-29] and putisolvins[30] were detected in the *Protoebacteria* extracts. Importantly, a new long-chain *N*-acyl L-leucine was isolated from *Halomonas* sp. RKEM 883 and determined to

have mild antibiotic activity against MRSA, VRE, and *S. warneri*. This research highlighted the diversity of secondary metabolites that can be obtained from octocoral-associated bacteria and confirmed the hypothesis that bacteria cultivated from *A. elisabethae* and *E. caribaeorum* can be a source of known and novel MNPs.

7.1.6 Overall Conclusions of Thesis Research

This thesis research provided valuable information about the microbiomes of *E. caribaeorum* and *A. elisabethae*. These Caribbean octocorals are keystone members of many reef communities,[31-33] and understanding the composition of their healthy microbiomes is important to understanding the health of the reef ecosystem in which they inhabit. Additionally *A. elisabethae* proved to be a unique reservoir of novel cultivatable bacteria as a wide range of taxonomic diversity was obtained. This was highlighted by a bacterial isolate that was determined to belong to an entirely new taxonomic family. Furthermore, the bacteria cultured from the octocorals in this thesis research proved to be an excellent source of secondary metabolites, producing many known compounds with various reported bioactivity, as well as the new long-chain *N*-acyl L-leucine. Although no pseudopterosin or eleutherobin production was detected through culturing of the octocoral associated bacteria, future metagenome sequencing studies of these octocorals should be employed to determine the true biosynthetic source of these important marine natural products.

7.2 Future Directions of Thesis Research

7.2.1 Octocoral Microbiomes

The overall field of microbial ecology, including octocoral microbiomes, has been greatly enhanced over the past few years by metagenomic sequencing. Advancements in high throughput next generation sequencing technologies and bioinformatics have enabled the sequencing of entire microbial genomes in complex environmental samples.[34-38] This has allowed for the identification of genes and a better understanding of their function in the environment.[39,40] Metagenomic research is particularly attractive in the field of natural products because it provides a means of exploring novel secondary metabolites from bacteria that are known to be present but are difficult to culture.[41,42] Furthermore, it allows for the identification of cryptic gene clusters that may not be expressed in cultivatable bacteria under laboratory settings.[43,44] Since the genetic information for the biosynthesis of these metabolites is generally clustered on the bacterial genome, it is therefore possible for the gene clusters to be cloned and expressed in a heterologous host to produce these metabolites of interest.[45-47] Therefore, the next step with regards to *A. elisabethae* and *E. caribaeorum* will be to sequence the metagenome of these octocorals and determine the true biosynthetic origins of pseudopterosins and eleutherobins, respectively.

7.2.2 Microbial Marine Natural Products

Advancements in high throughput screening along with automated purification and identification techniques has allowed the rate at which novel microbial secondary metabolites are being reported to steadily increase of over the last few years.[48-53] Furthermore, the rate at which these novel compounds are being discovered does not appear to be subsiding anytime soon,[54,55] as only

a fraction of the overall marine microbiome has been explored[56] and thus the potential for novel chemistry is largely untapped. However, even though the rate at which novel microbial metabolites being reported is increasing, the rate of bioactive metabolites is not. In fact the percentage of secondary metabolites with reported bioactivity has decreased over the last few years.[53] This highlights the need for new and innovative bioassays to be able to keep pace with the rate at which novel compounds are being discovered. The lack of reported bioactivity does not mean the compounds are inactive and bioactivity is often discovered in future studies.[57-59] Therefore further investigation into the bioactive potential of the long chain *N*-acyl leucine as well as other metabolites from the culture-dependent library is warranted.

The marine microbial community is a massive source of untapped novel chemistry; the bioactive potential of many known secondary metabolites is still waiting to be discovered. Natural products have been, and continue to be, an excellent source of drug leads as these metabolites have co-evolved with biological targets generating a wide range of pharmacophores with a high degree of selectivity. As Cragg and Newman stated in their 2013 natural products review, "Mother Nature has had three billion years to refine her chemistry and we are only now scratching the surface in exploring Nature's molecular diversity!"[60]

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Appendix A: Chapter 5 – Supplementary Information

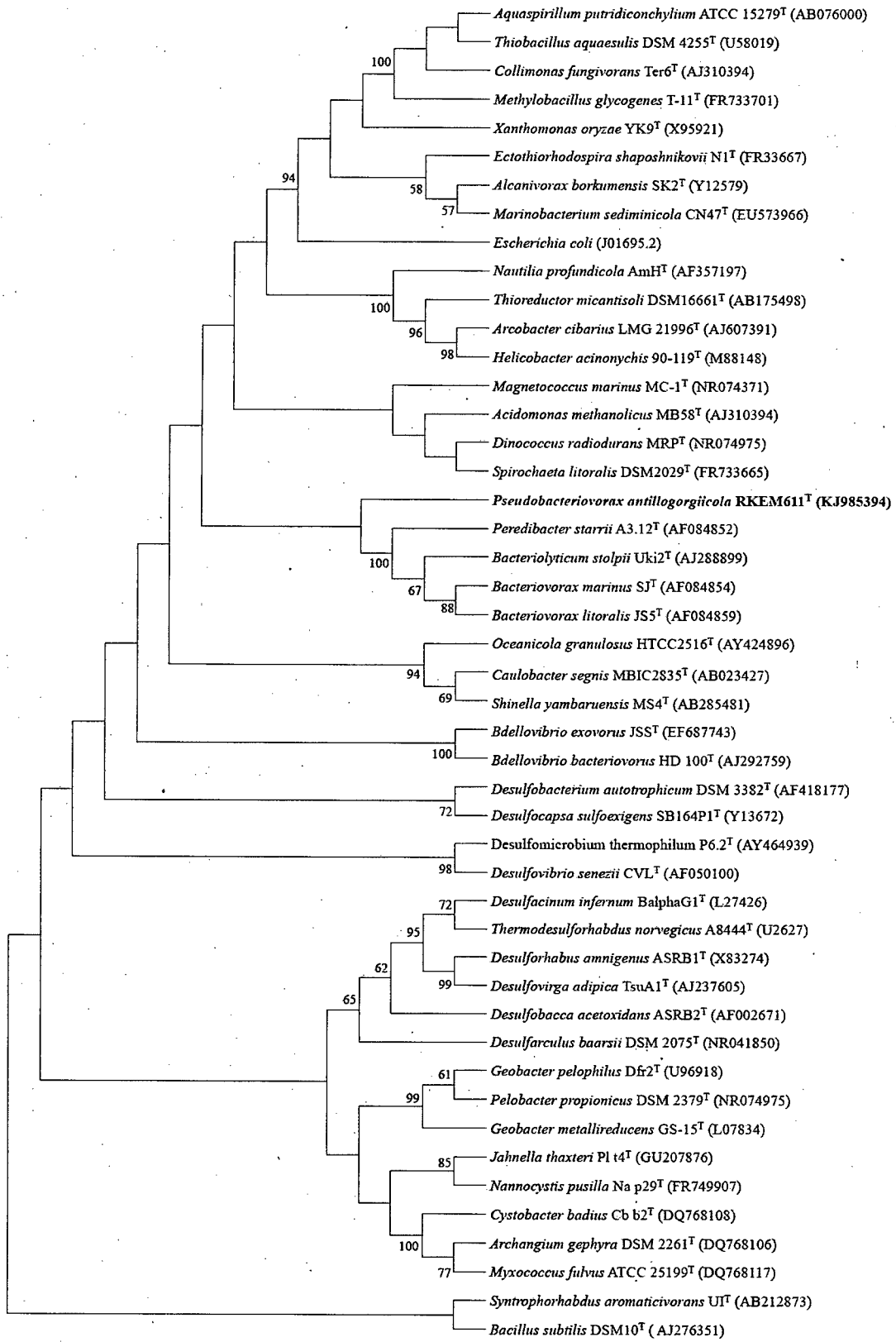

Figure A.1. Maximum-parsimony phylogenetic tree based on 16S rRNA gene sequences from type strains from with the phylum *Proteobacteria.*

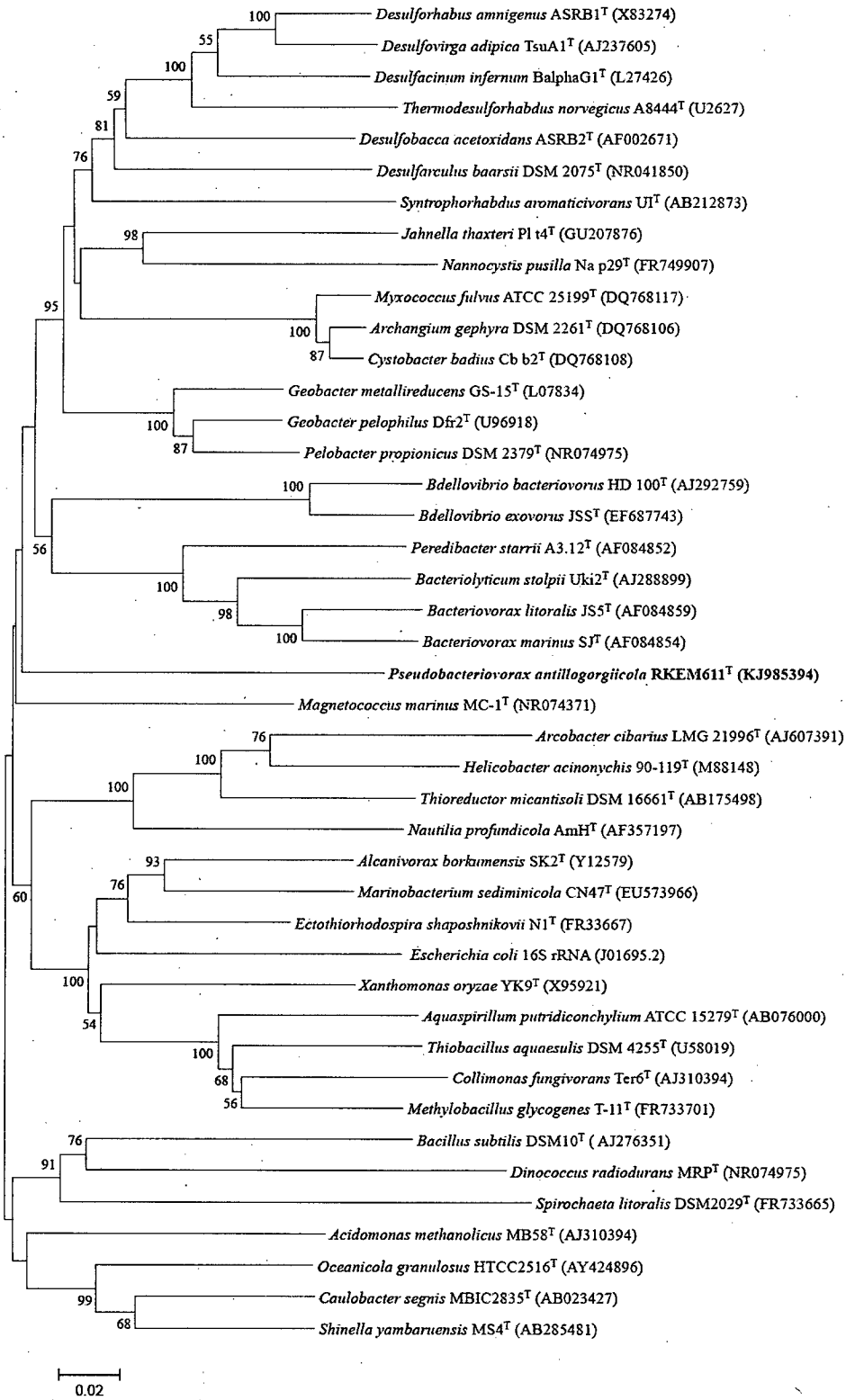

Figure A.2. Minimum-evolution phylogenetic tree based on 16S rRNA gene sequences from type strains from the phylum *Proteobacteria.*

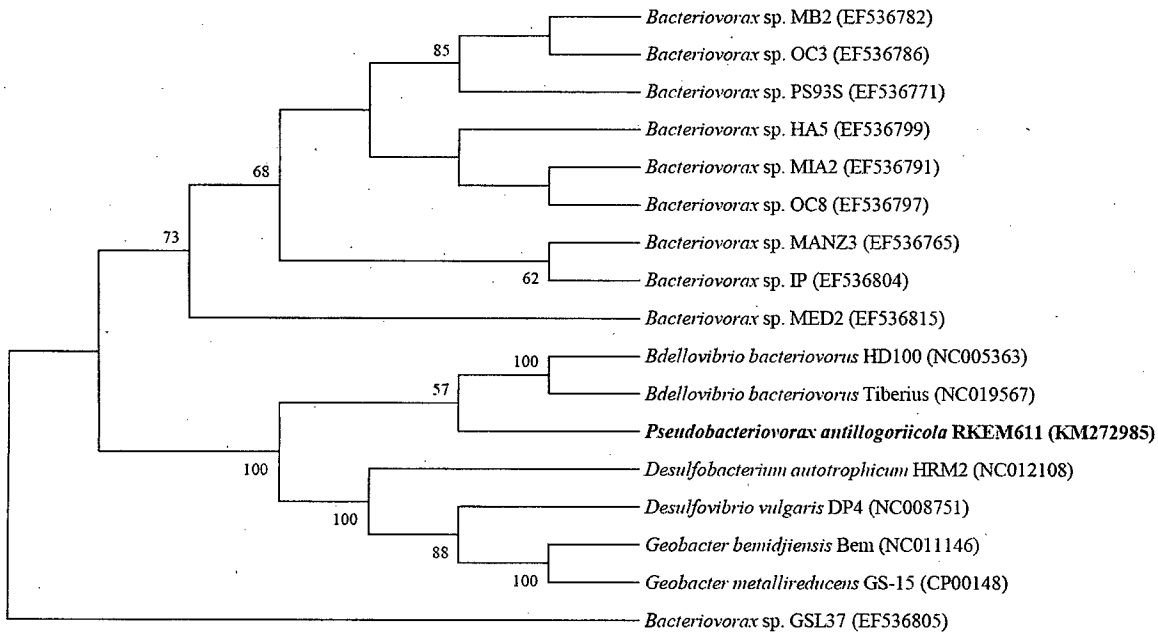

Figure A.3. Maximum-parsimony phylogenetic tree based on 17 *rpoβ* gene sequences from the class *Deltaproteobacteria*.

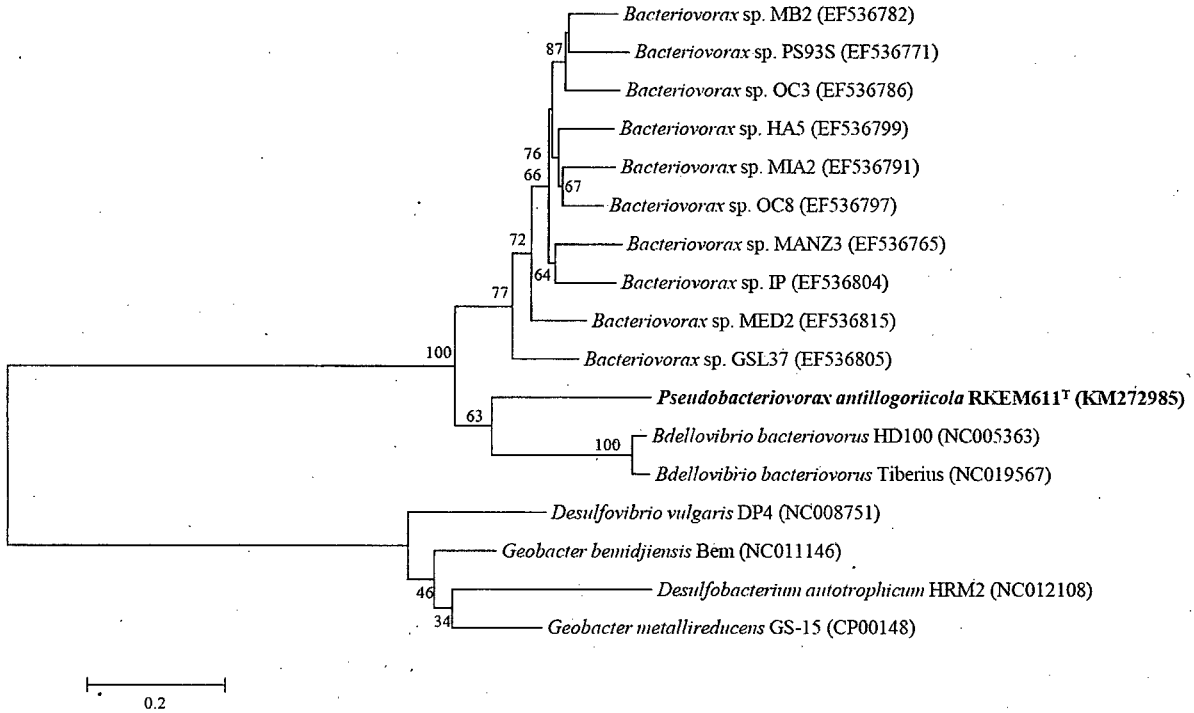

Figure A.4. Minimum-evolution phylogenetic tree based on 17 *rpoβ* gene sequences from the class *Deltaproteobacteria*.

Appendix B: Chapter 6 - Supplementary Information

B.1. Principal Component Analysis - Scores and Loadings Plots

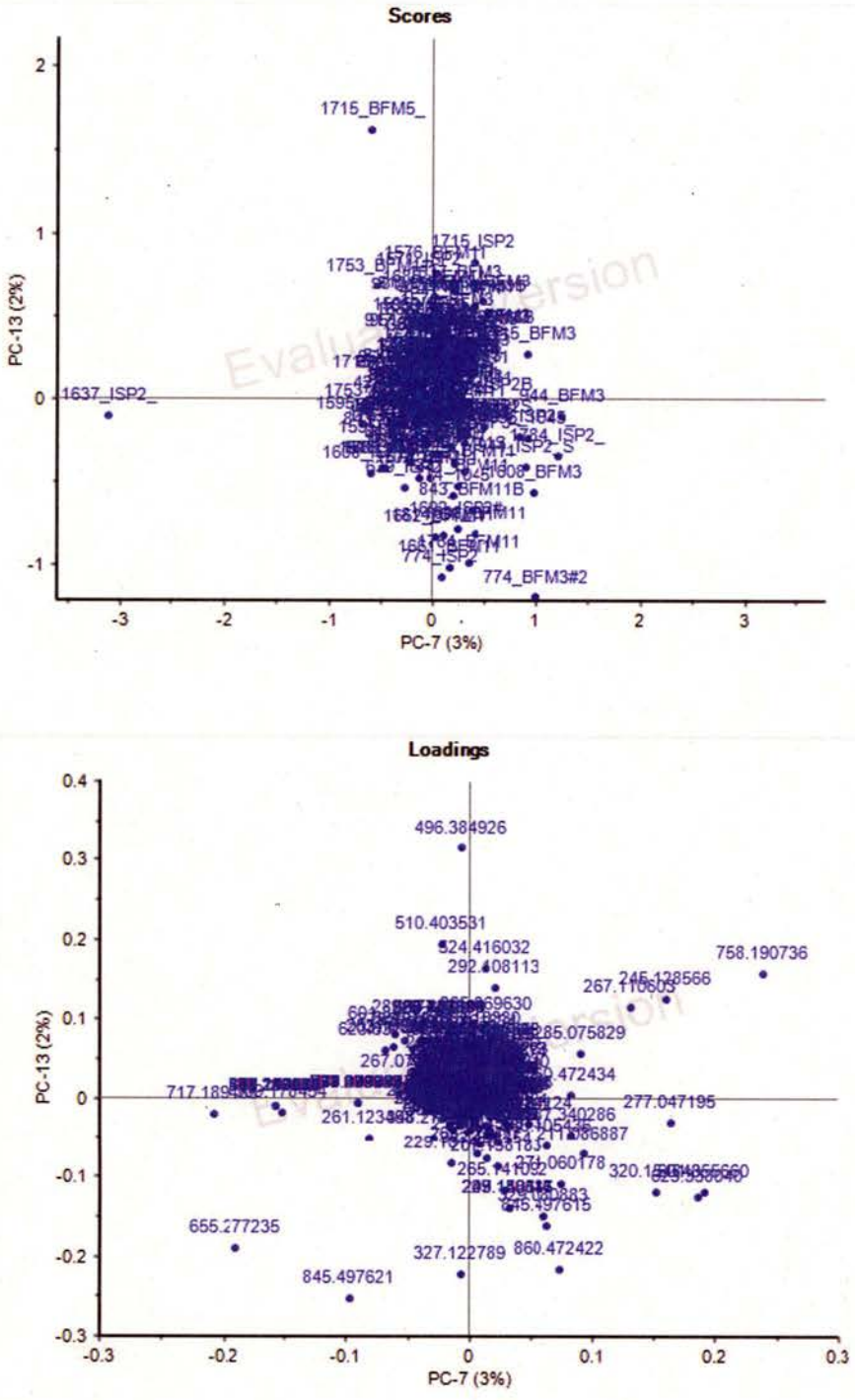

Figure B.1.1. Scores and loadings plots highlighting the production of the rhodopeptins.

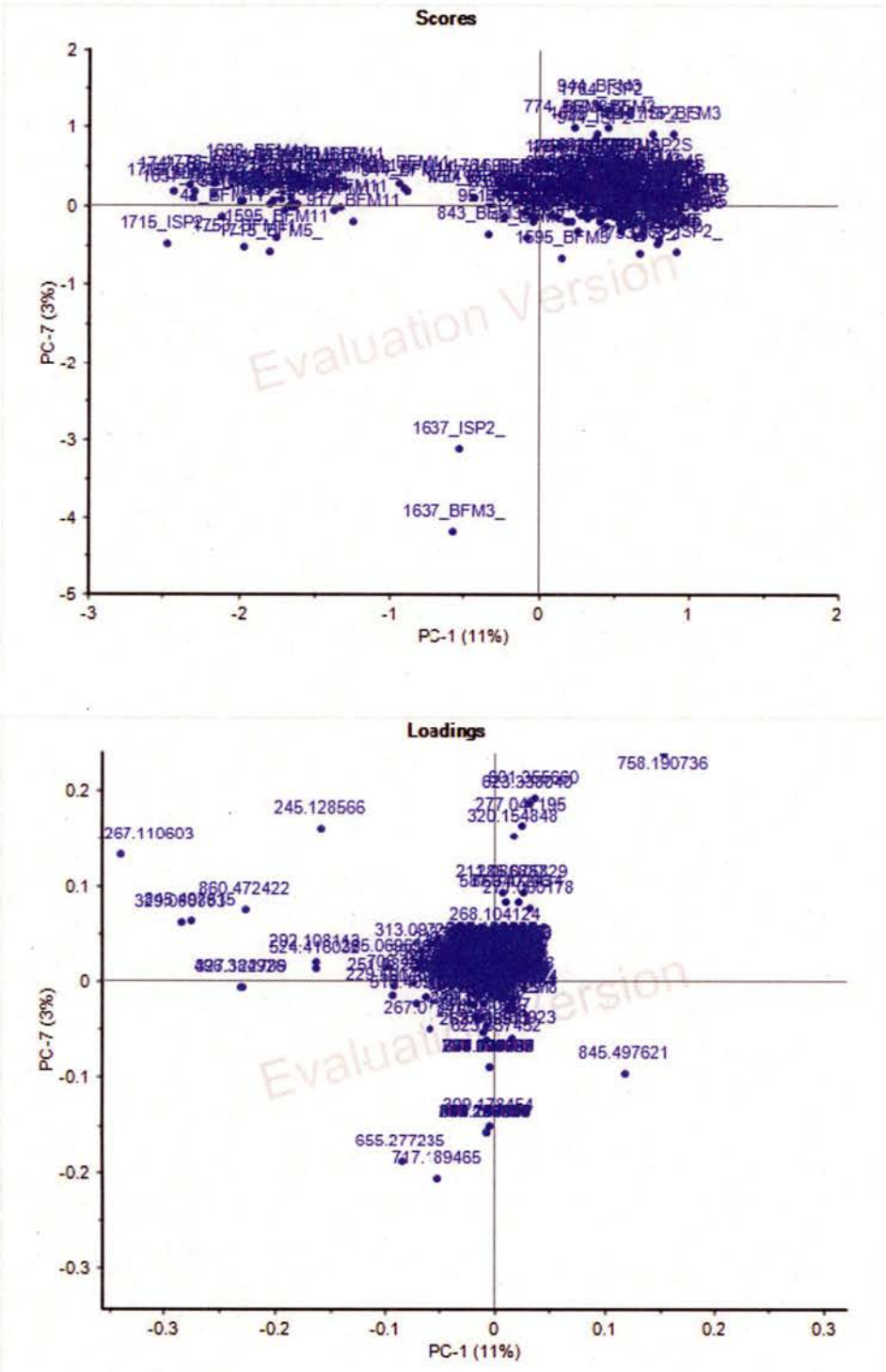

Figure B.1.2. Scores and loadings plots highlighting the production of coproporphyrin III and zincphyrin.

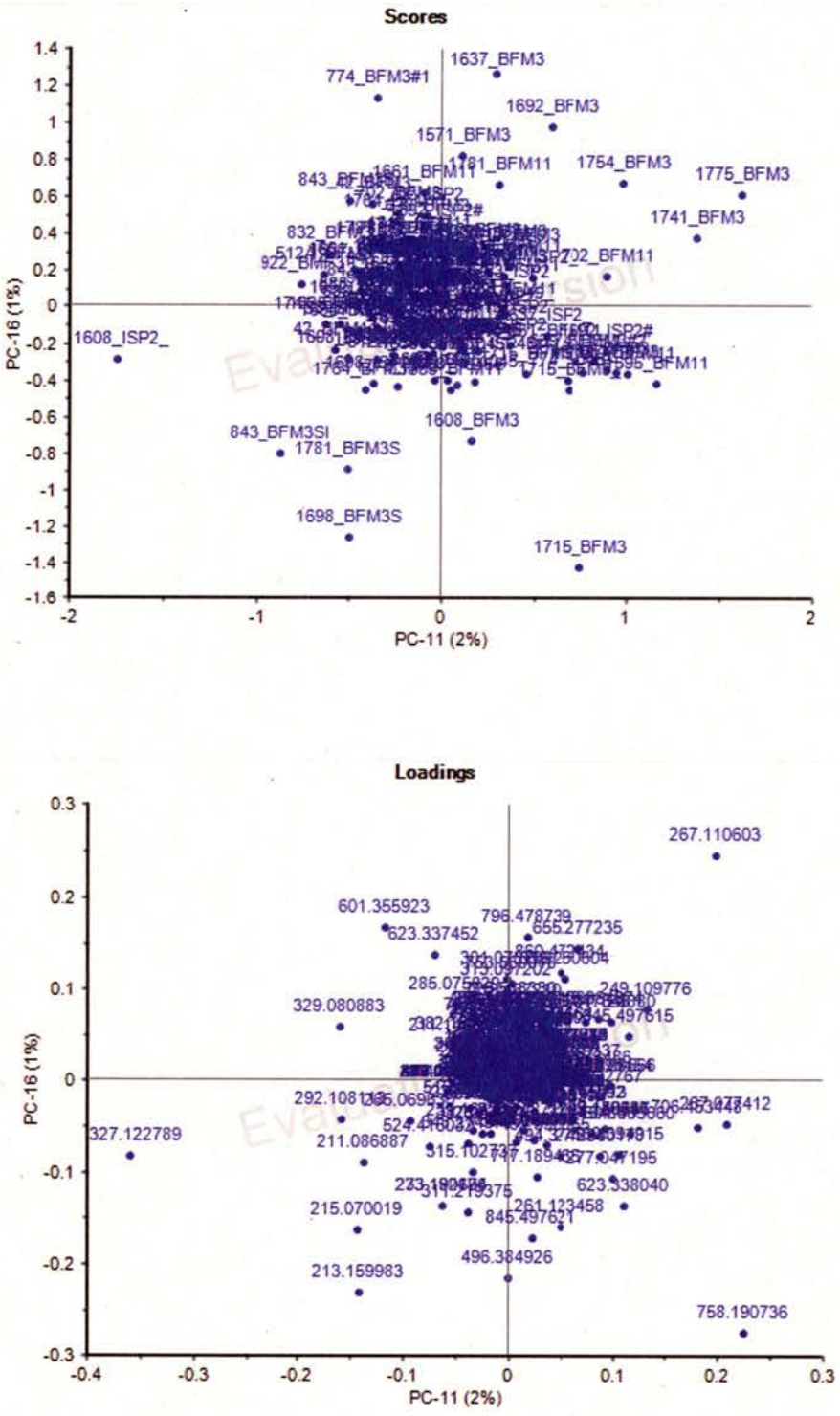

Figure B.1.3. Scores and loadings plots highlighting the production of nocardamine.

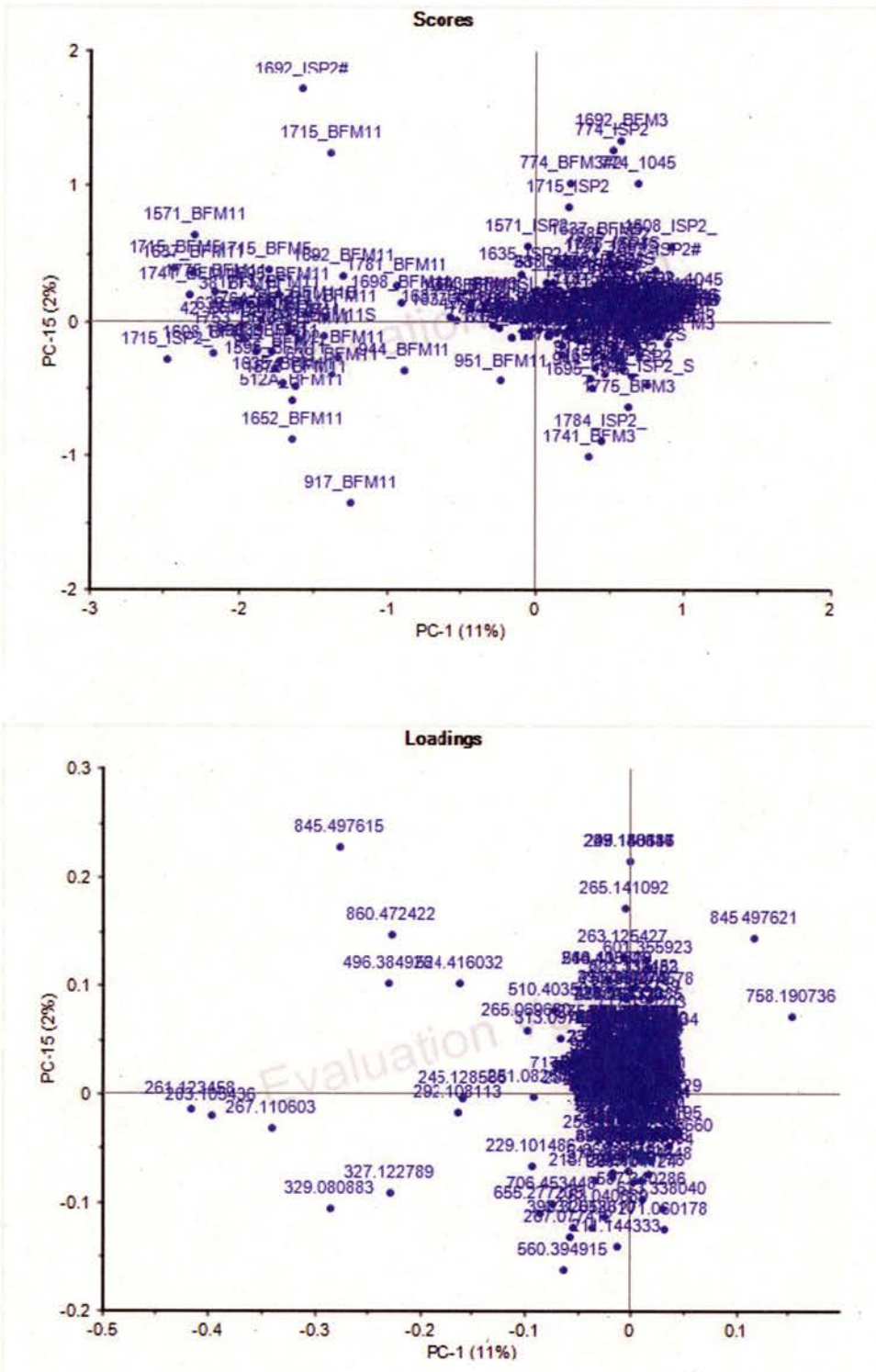

Figure B.1.4. Scores and loadings plots highlighting the production of concanamycin C and E.

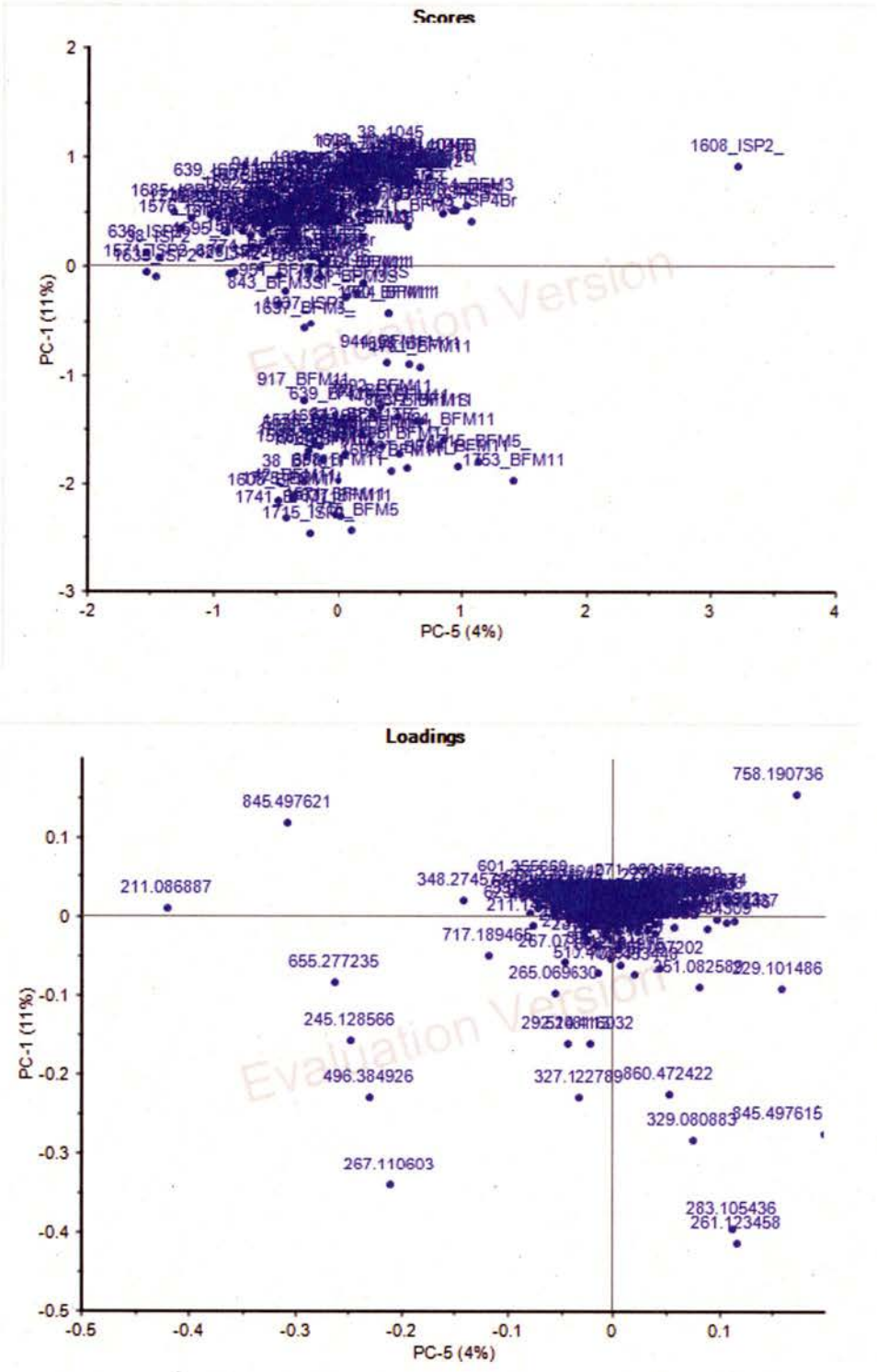

Figure B.1.5. Scores and loadings plots highlighting the production of kocurin

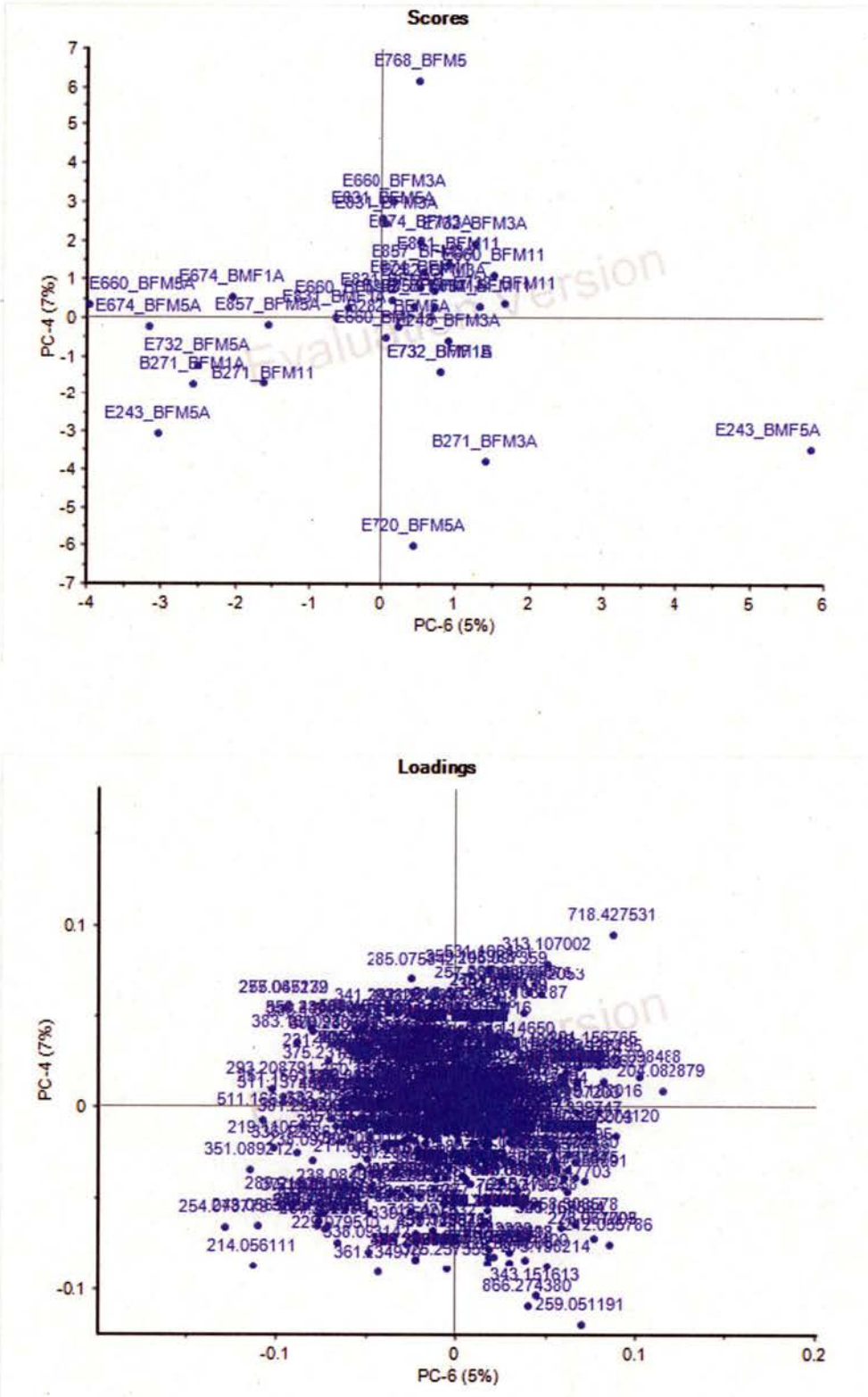

Figure B.1.6. Scores and loadings plots highlighting the production of baceridin.

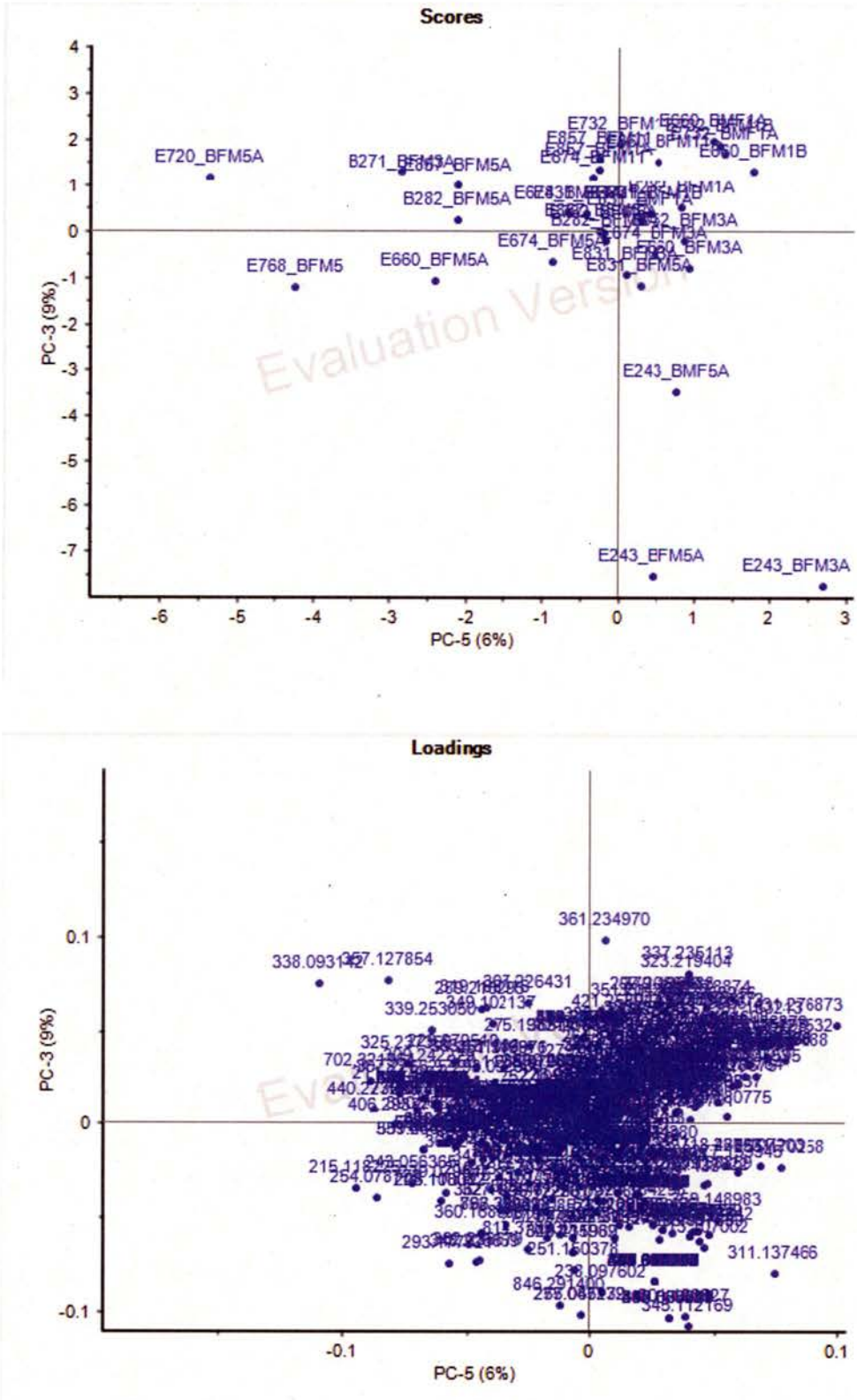

Figure B.1.7. Scores and loadings plots highlighting the production of bacillamide B and C.

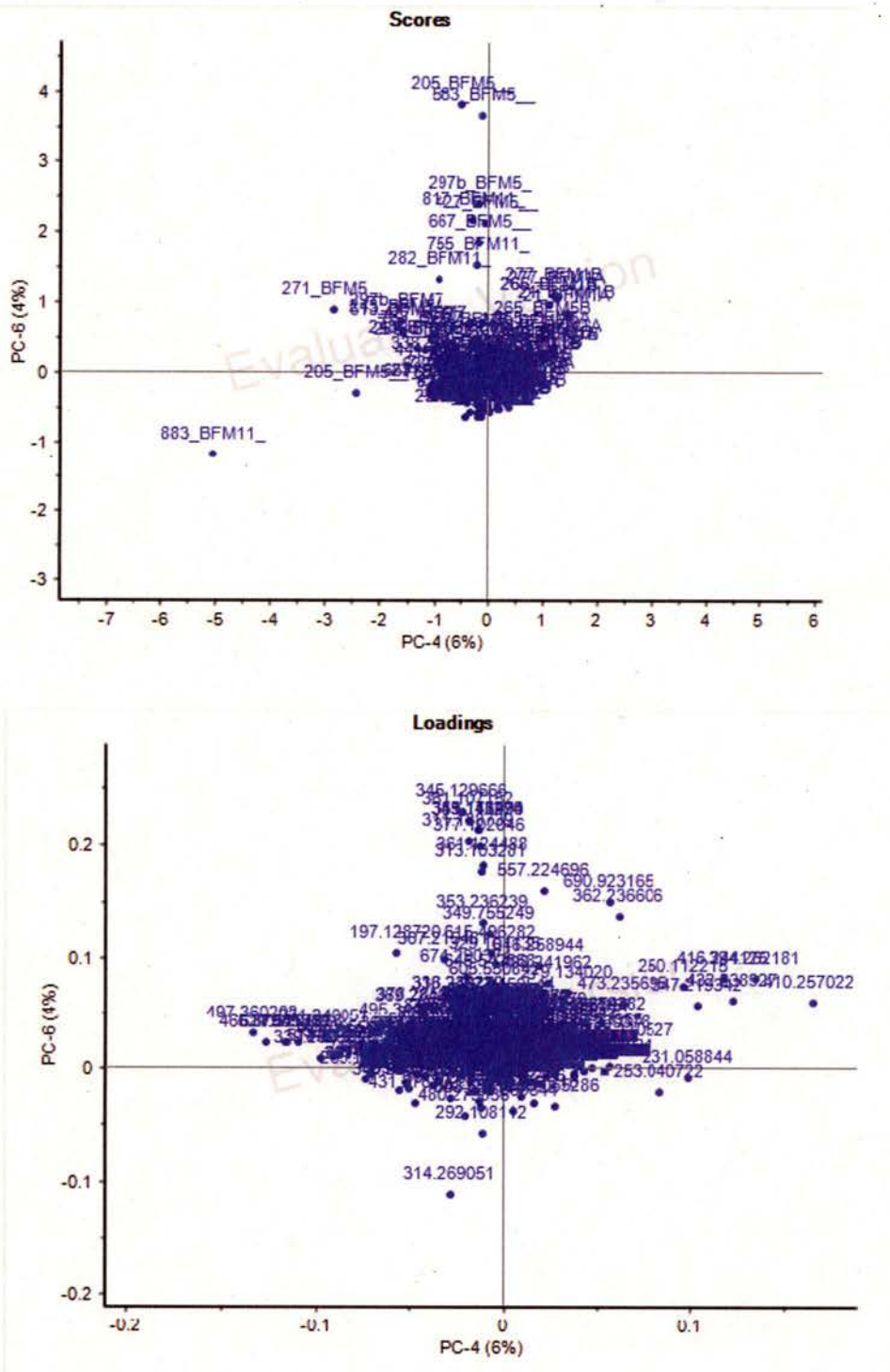

Figure B.1.8. Scores and loadings plots highlighting the production of long-chain N-acyl-L-leucine.

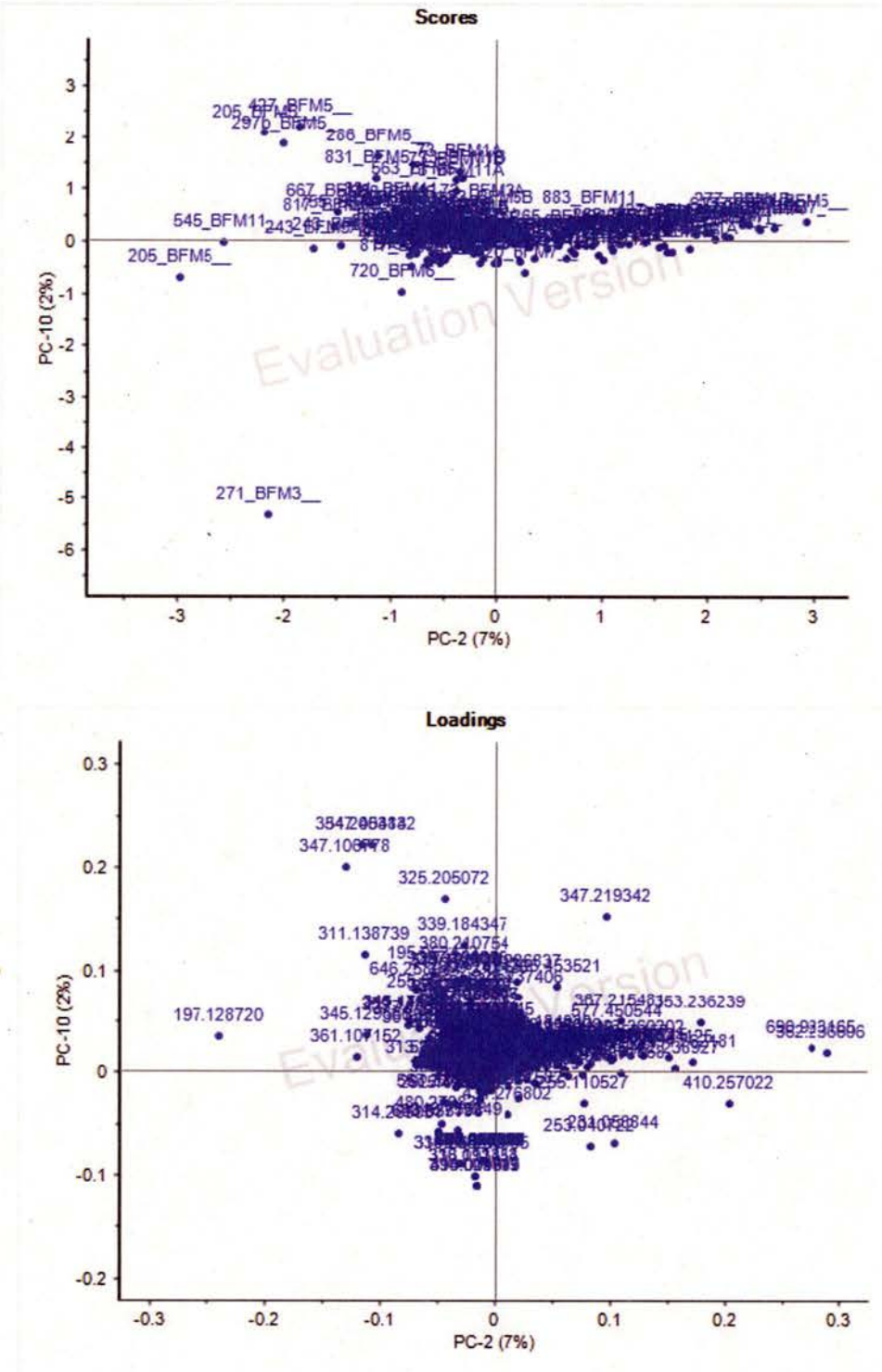

Figure B.1.9. Scores and loadings plots highlighting the production of korormicins.

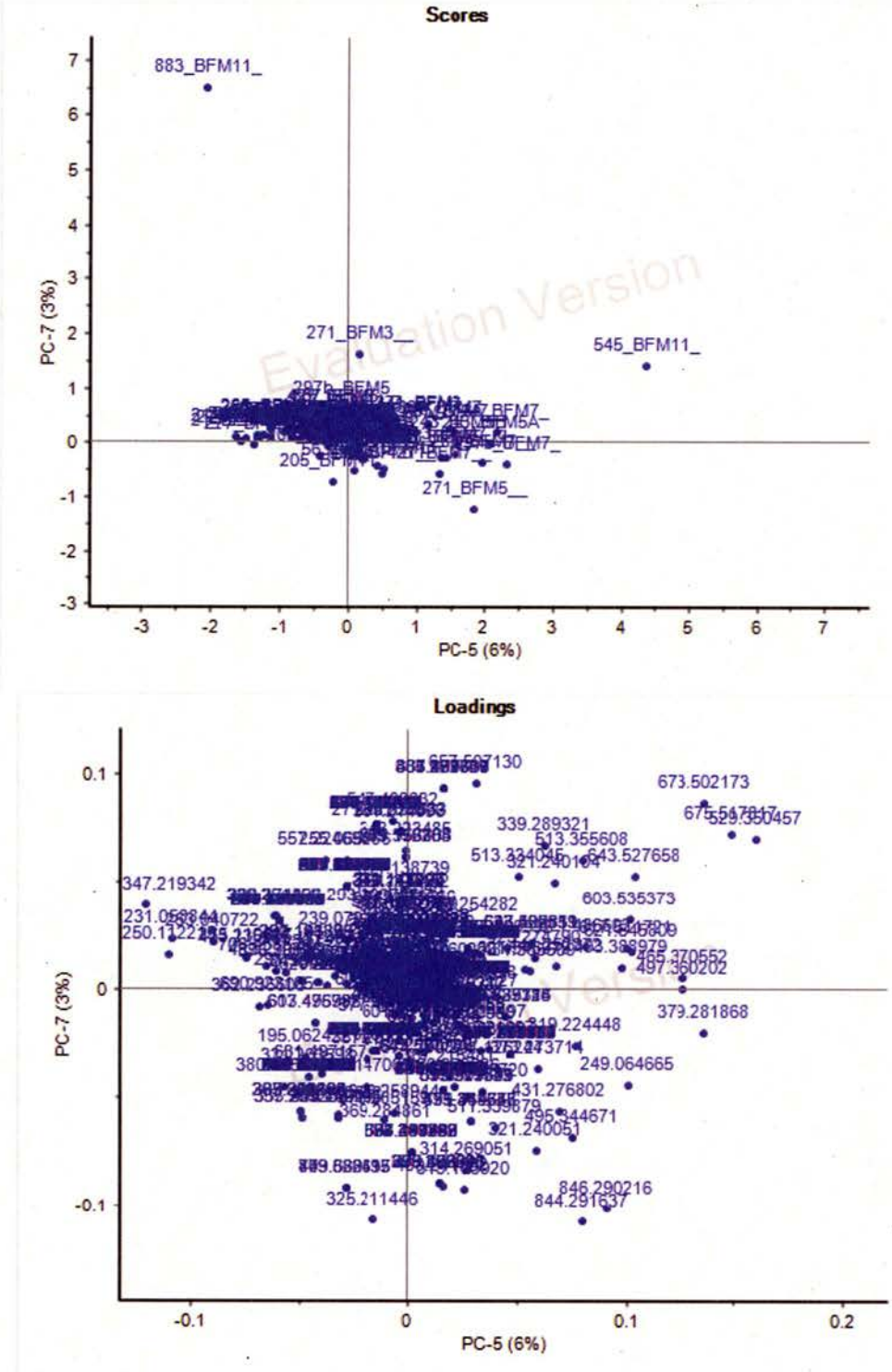

Figure B.1.10. Scores and loadings plots highlighting the production of bromoalterochromide A.

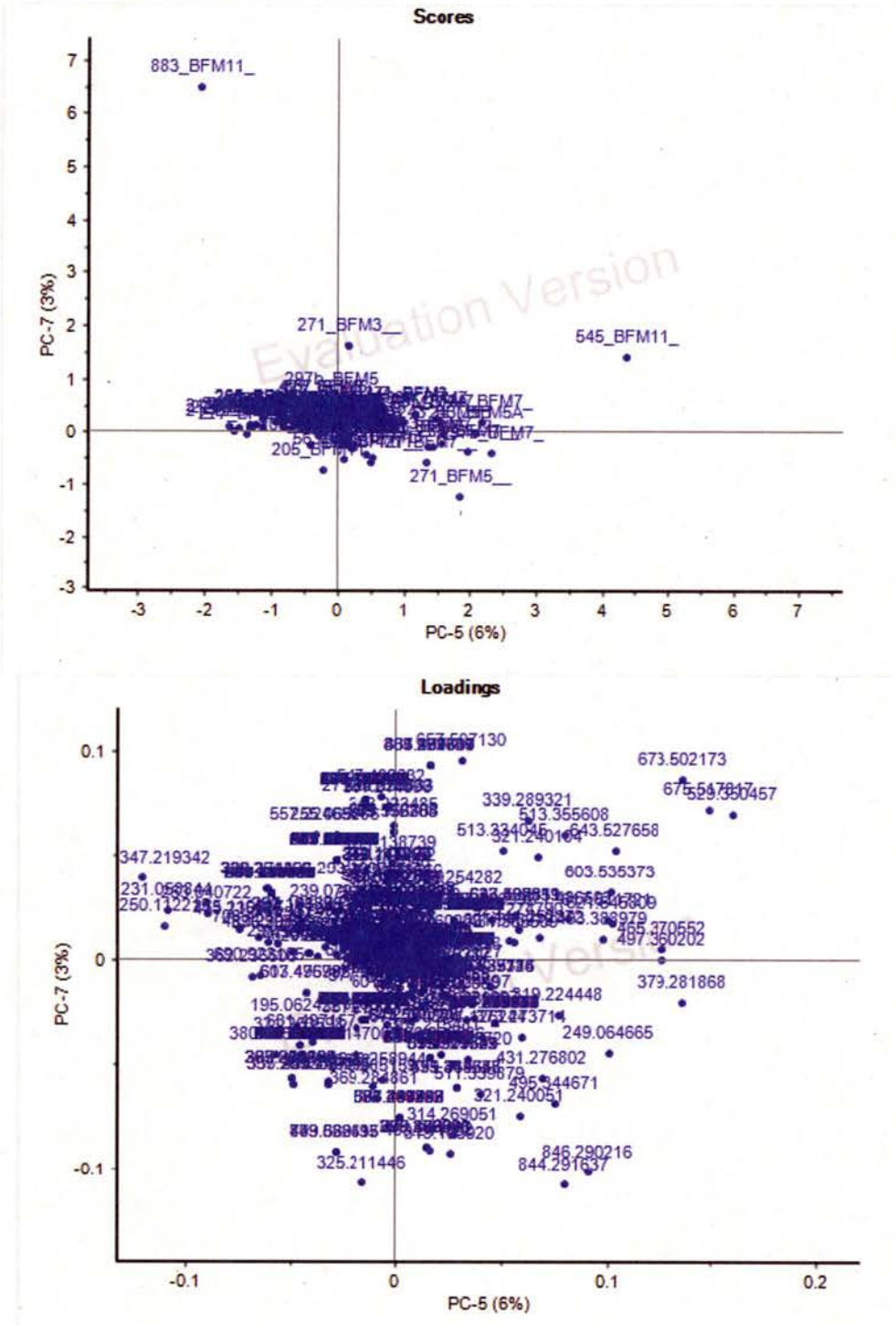

Figure B.1.11. Scores and loadings plots highlighting the production of pseudoalteromone A and B.

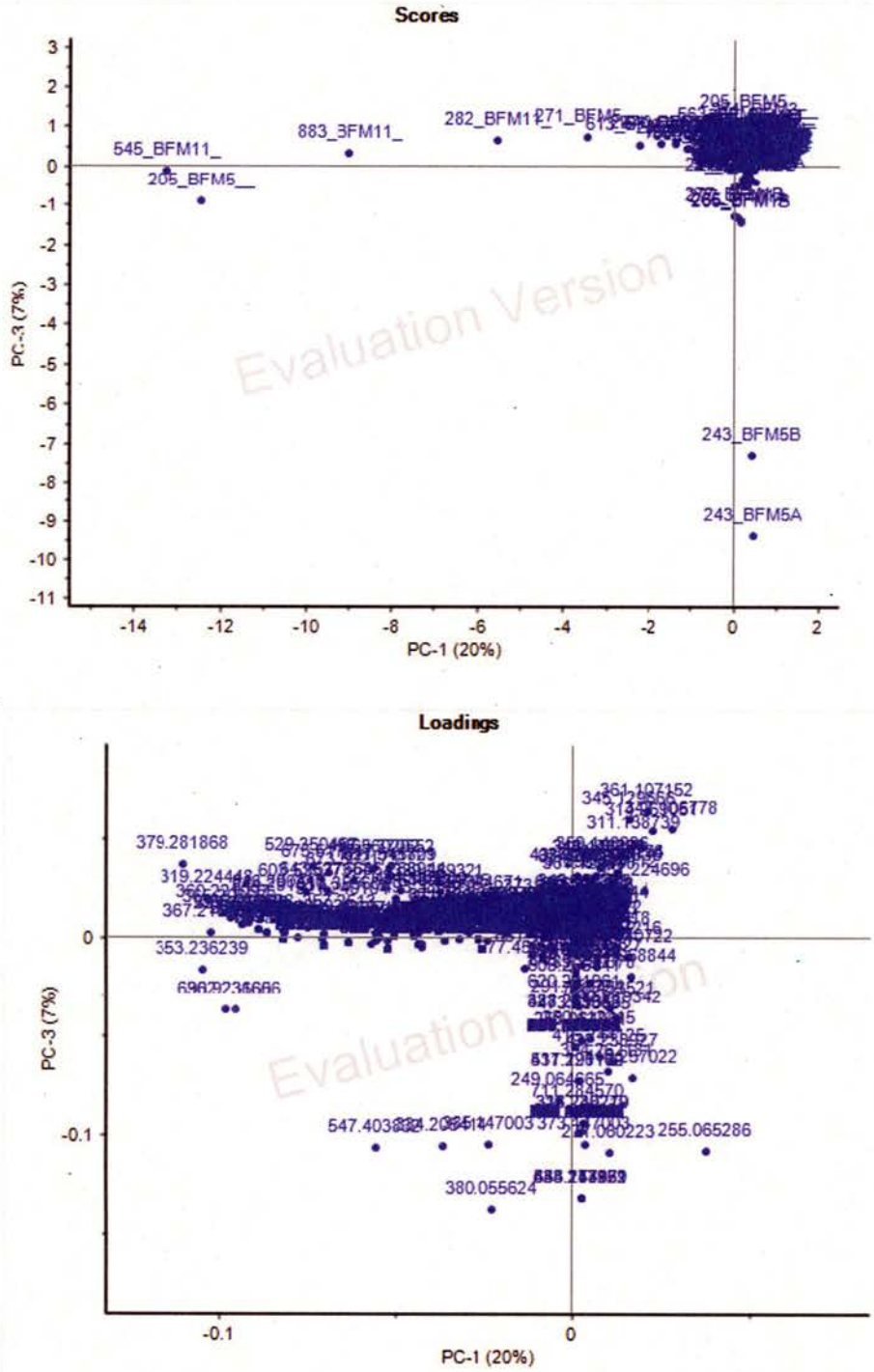

Figure B.1.12. Scores and loadings plots highlighting the production of ulbactin A and D.

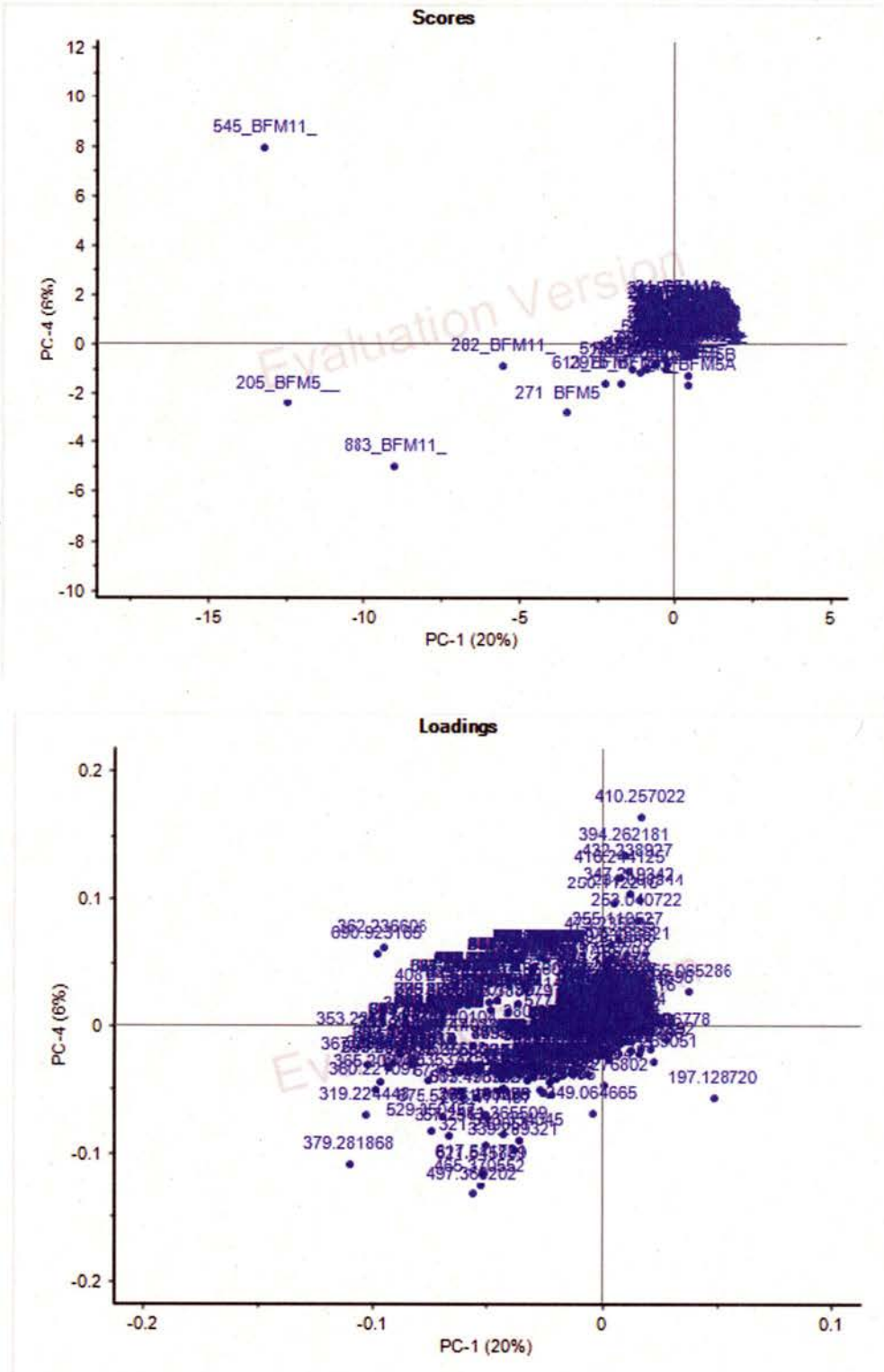

Figure B.1.11. Scores and loadings plots highlighting the production of putisolvin I.

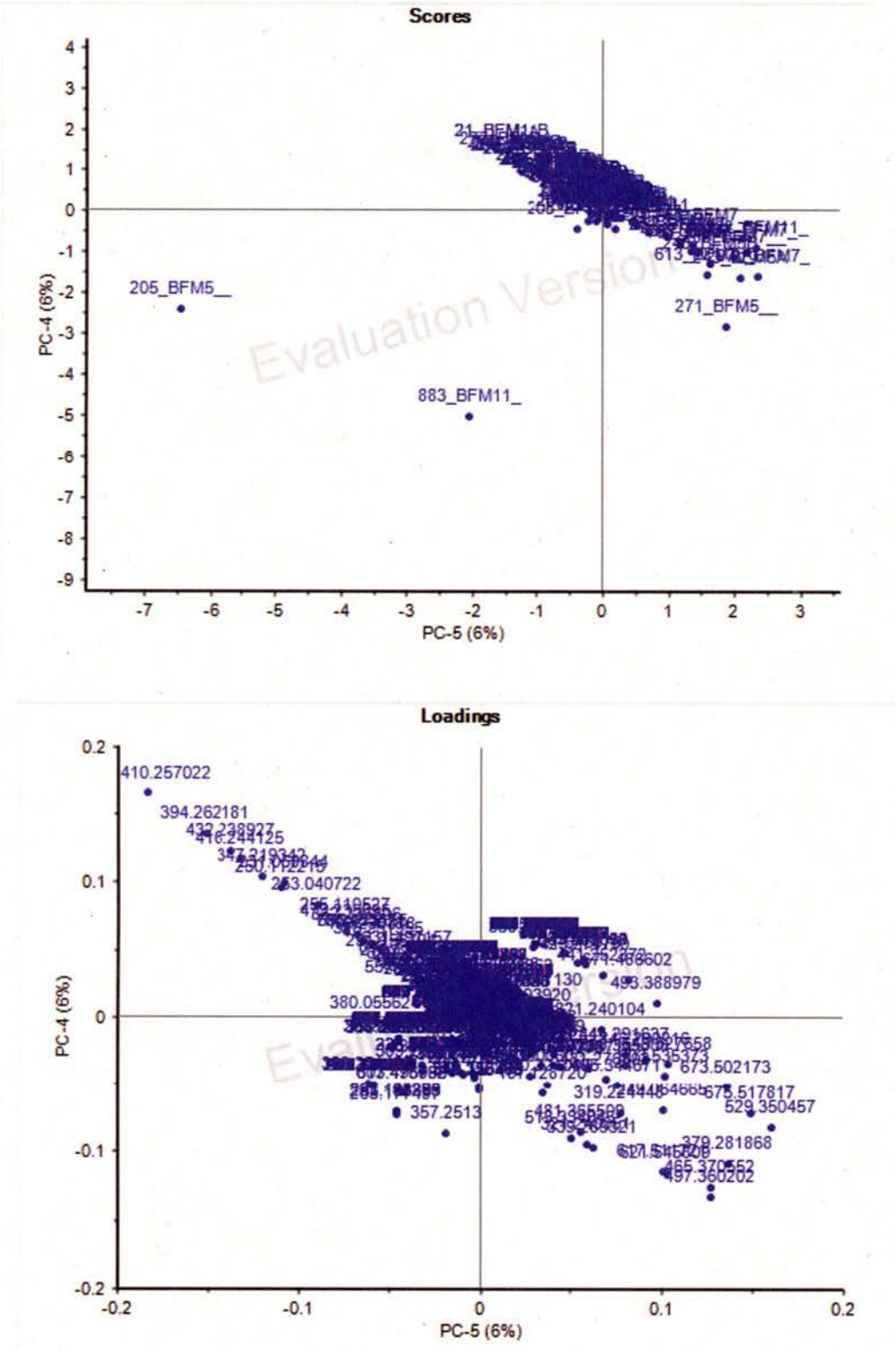

Figure B.1.12. Scores and loadings plots highlighting the production of bahamamide.

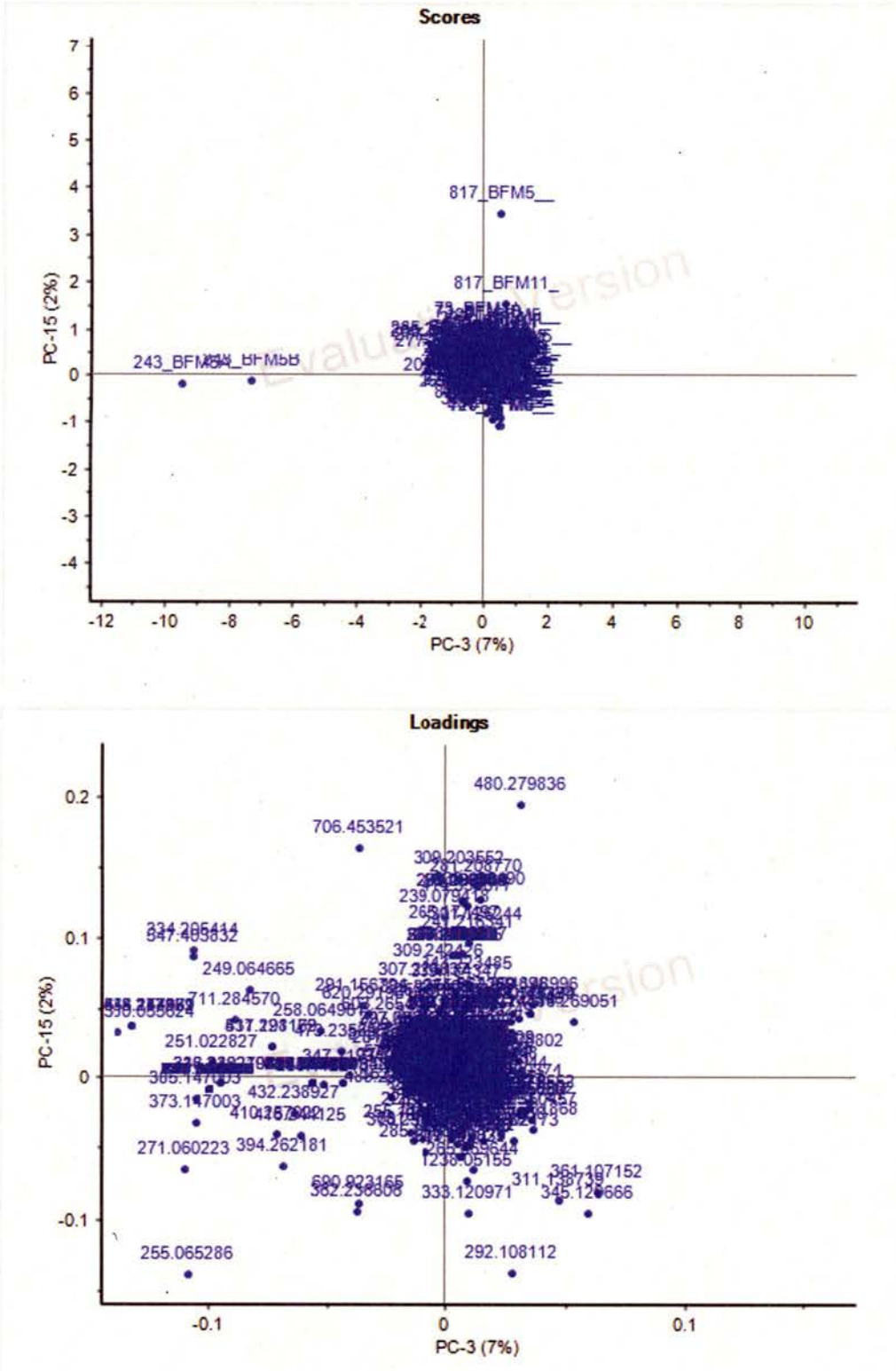

Figure B.1.13. Scores and loadings plots highlighting the production of andrimid.

B.2. Ultra High Performance Liquid Chromatography–High Resolution Mass Spectroscopy Data

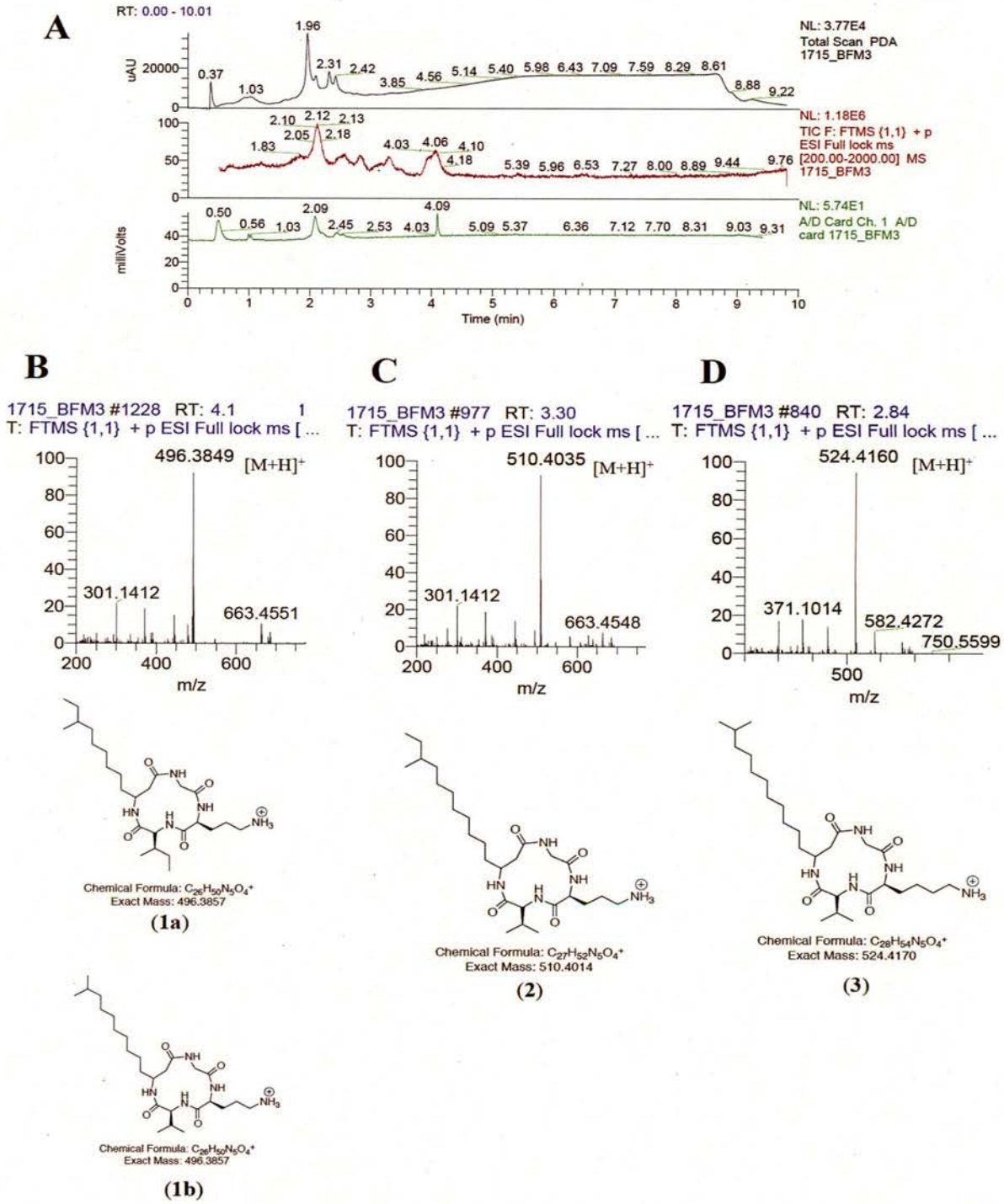

Figure B.2.1. (A) UHPLC-HRMS chromatogram of *Streptomycetes* sp. RKEM 1715 in ISP2 and mass spectra of (B) rhodopeptin C2/C3 (**1a/1b**), (C) rhodopeptin C4 (**2**), (D) rhodopeptin B5 (**3**).

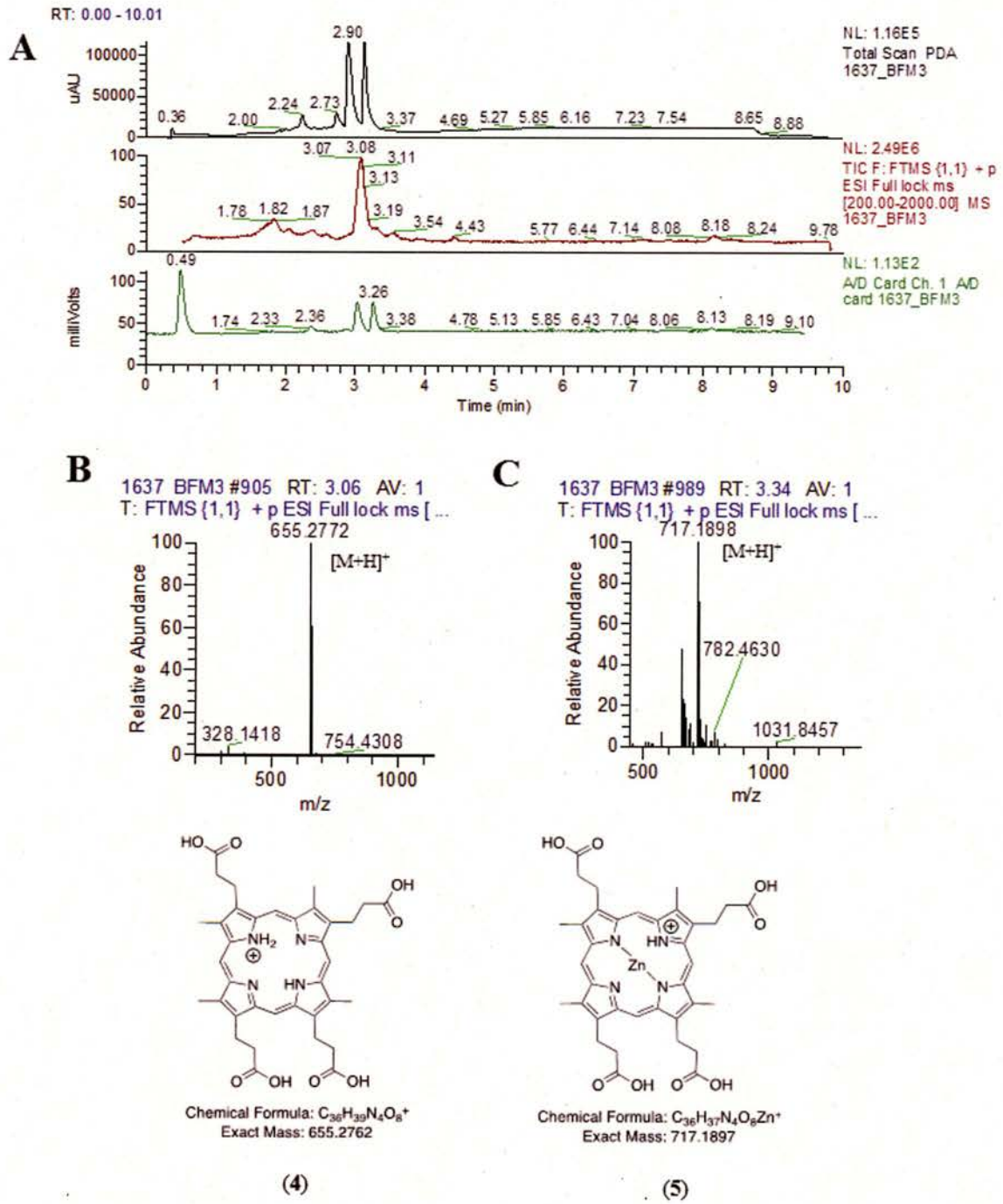

Figure B.2.2. (A) UHPLC-HRMS chromatogram of *Arthrobacter* sp. RKEM 1637 in BFM3 and mass spectra of (B) coproporphyrin III (**4**) and (C) zincphyrin (**5**).

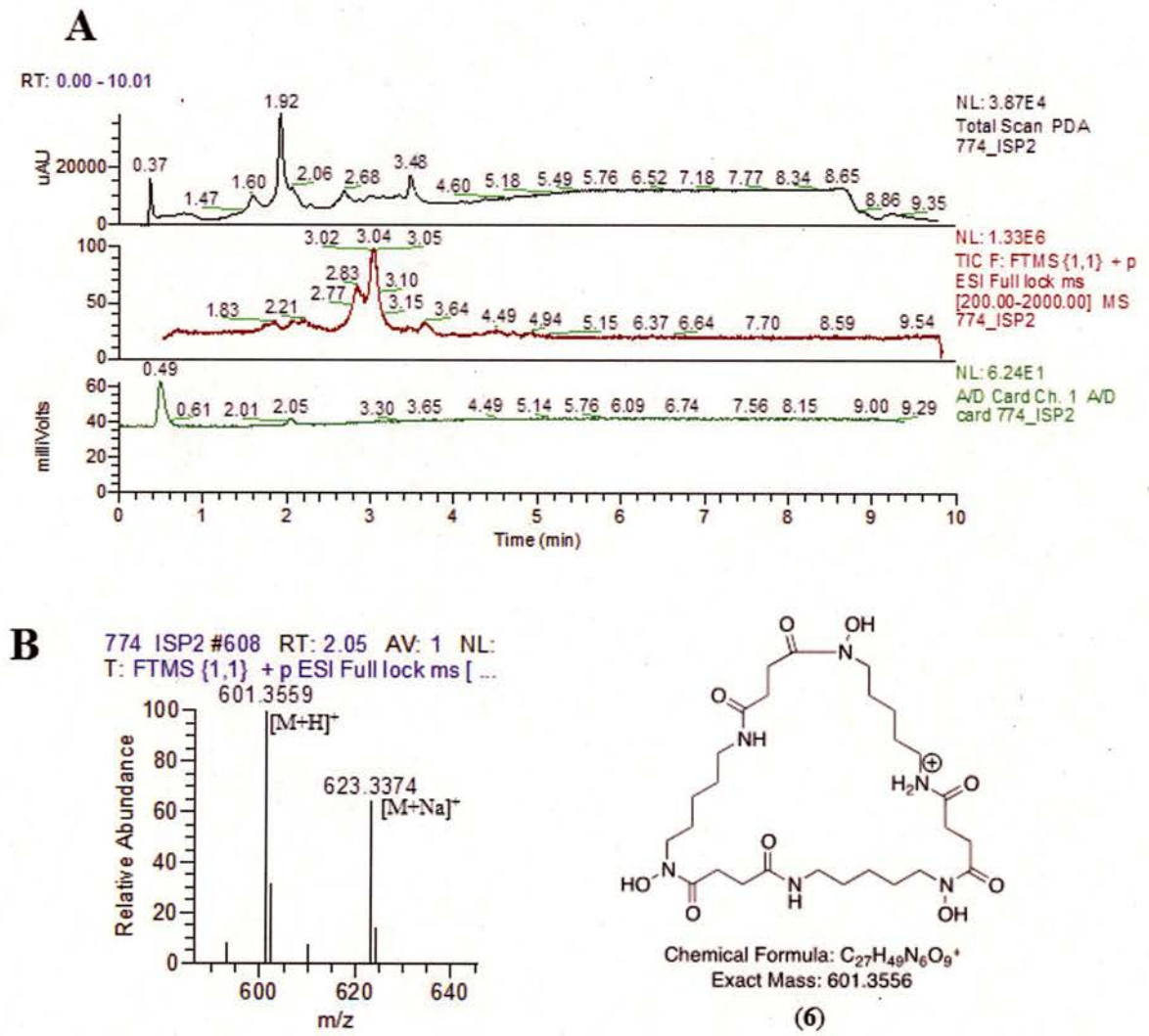

Figure B.2.3. (A) UHPLC-HRMS chromatogram of *Streptomyces* sp. RKEM 774 and ISP2 and mass spectra of (B) nocardamine (**6**).

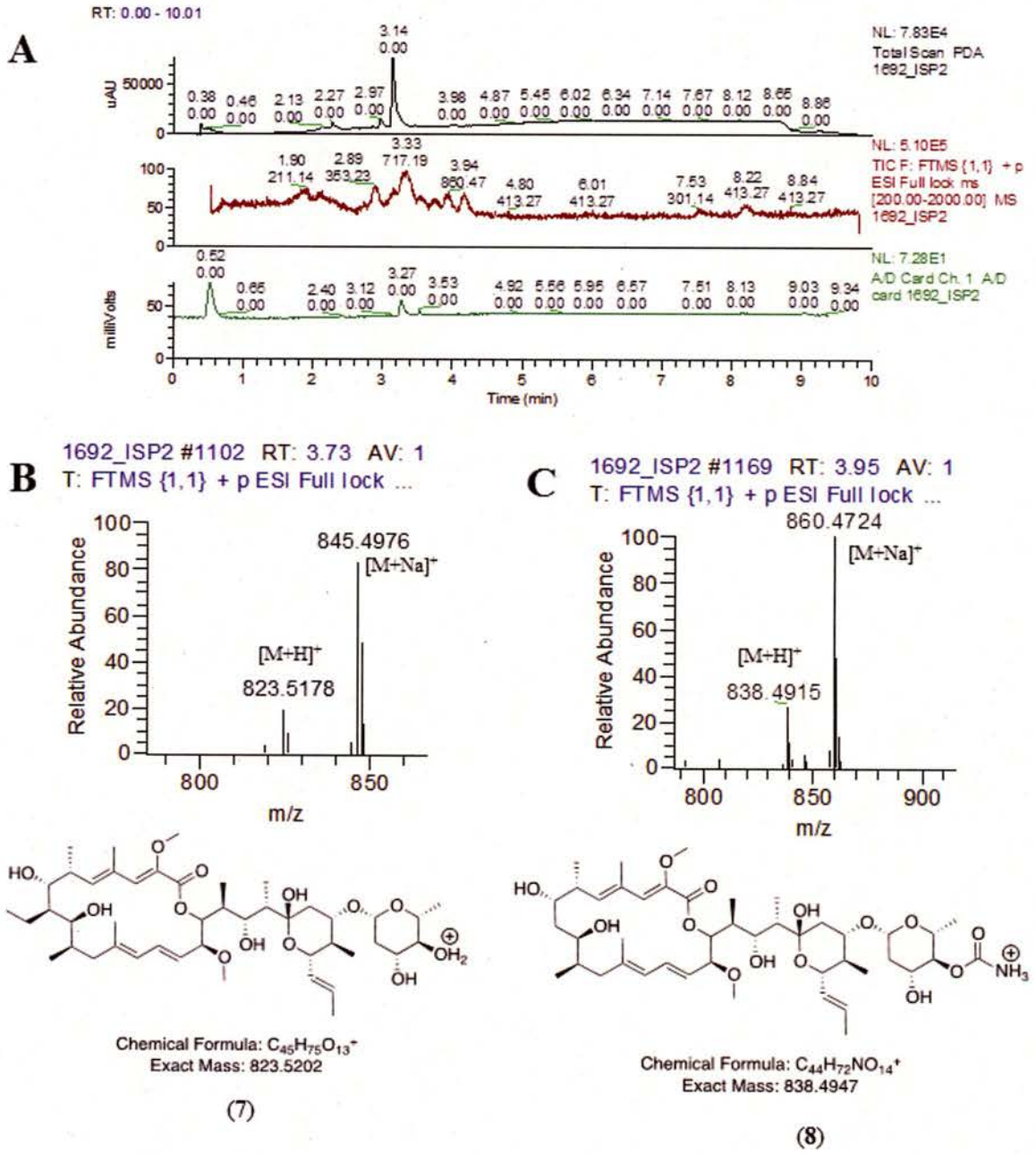

Figure B.2.4. (A) UHPLC-HRMS chromatogram of *Arthrobacter* sp. RKEM 1692 in ISP2 and mass spectra of (B) concanamycin C (**7**) and (C) concanamycin E (**8**).

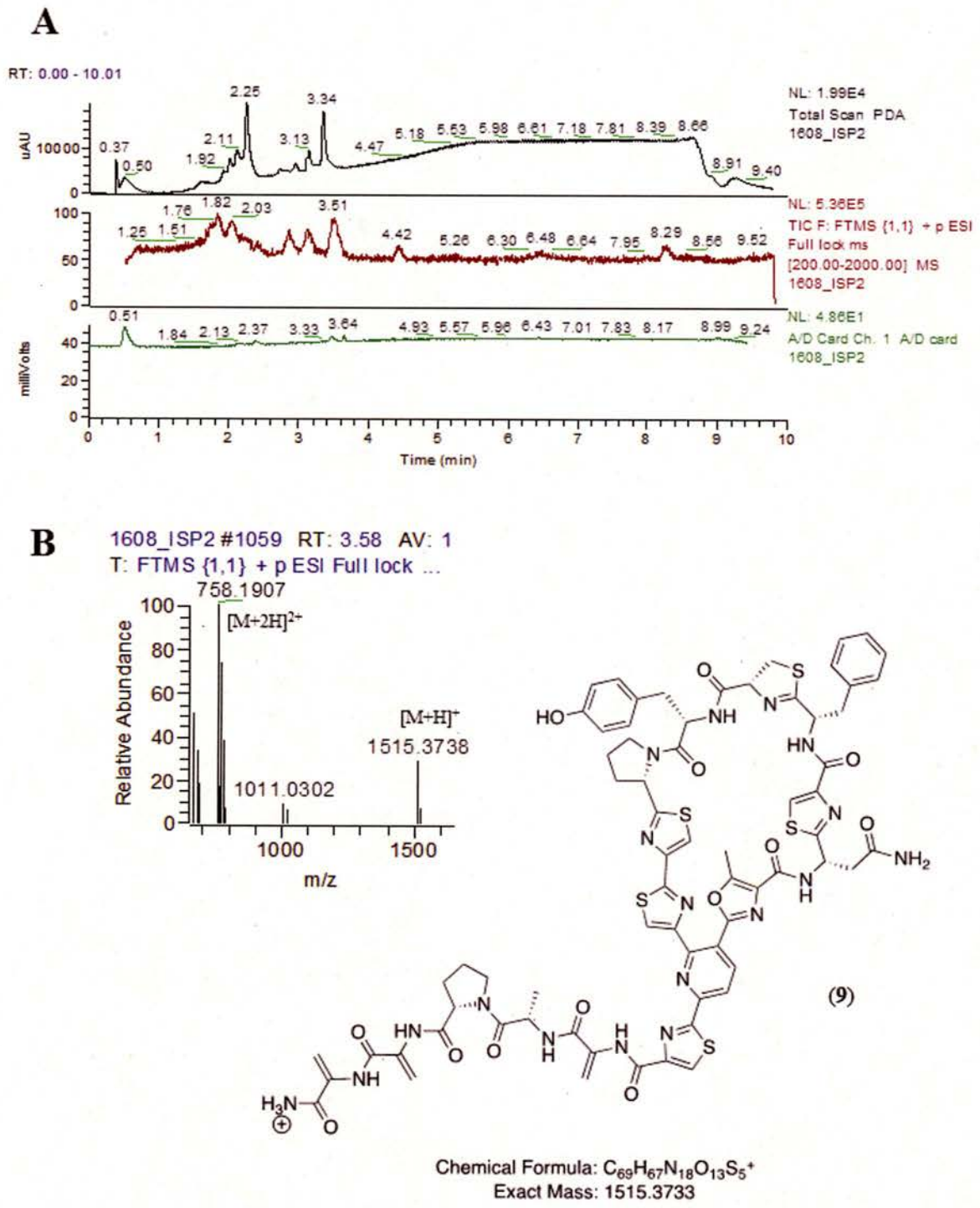

Figure B.2.5. (A) UHPLC-HRMS chromatogram of *Kocuria* sp. RKEM 1608 in IPS2 and mass spectra of (B) kocurin (**9**),

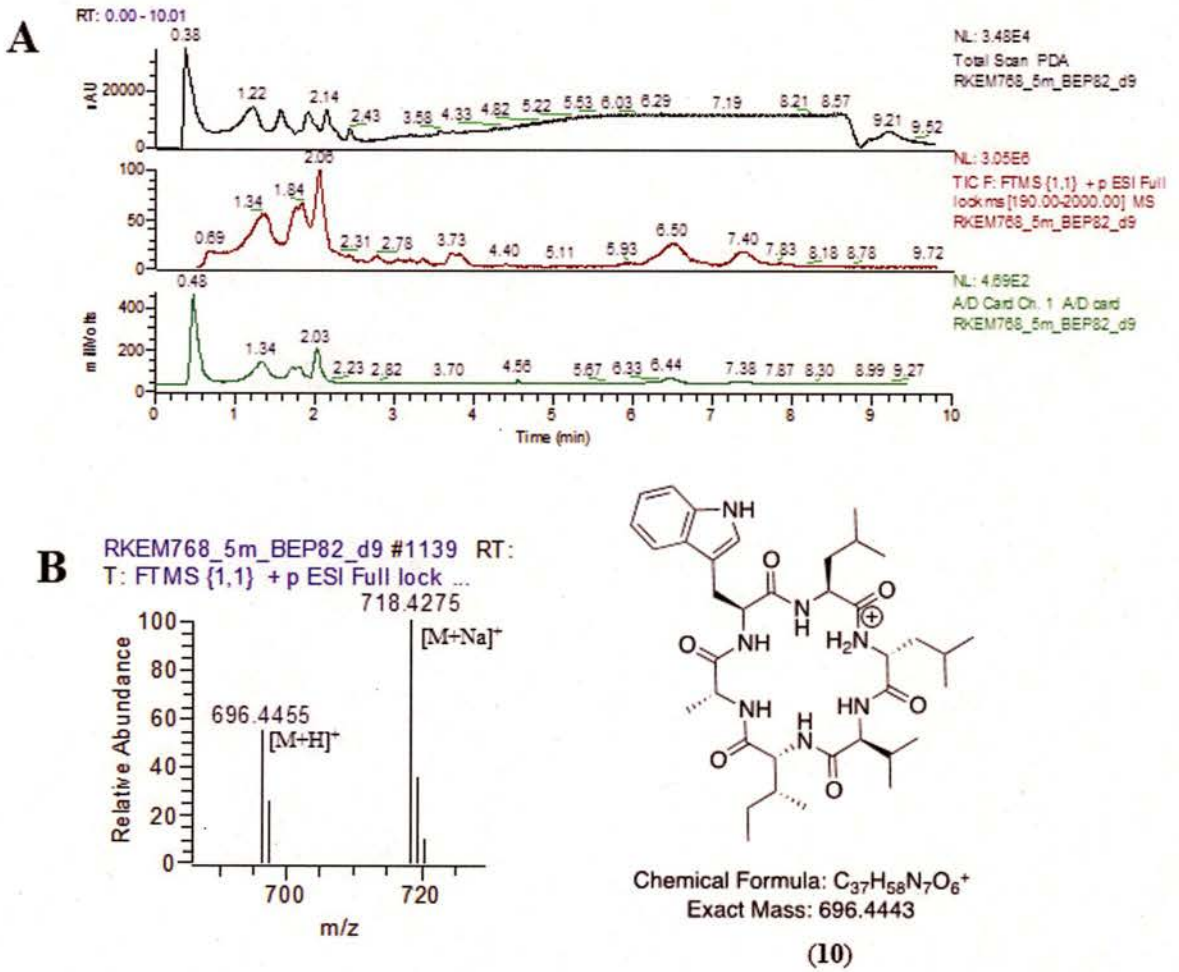

Figure B.2.6. (A) UHPLC-HRMS chromatogram of *Paenibacillus* sp. RKEM 768 in BFM5 and mass spectra of (B) baceridin (**10**).

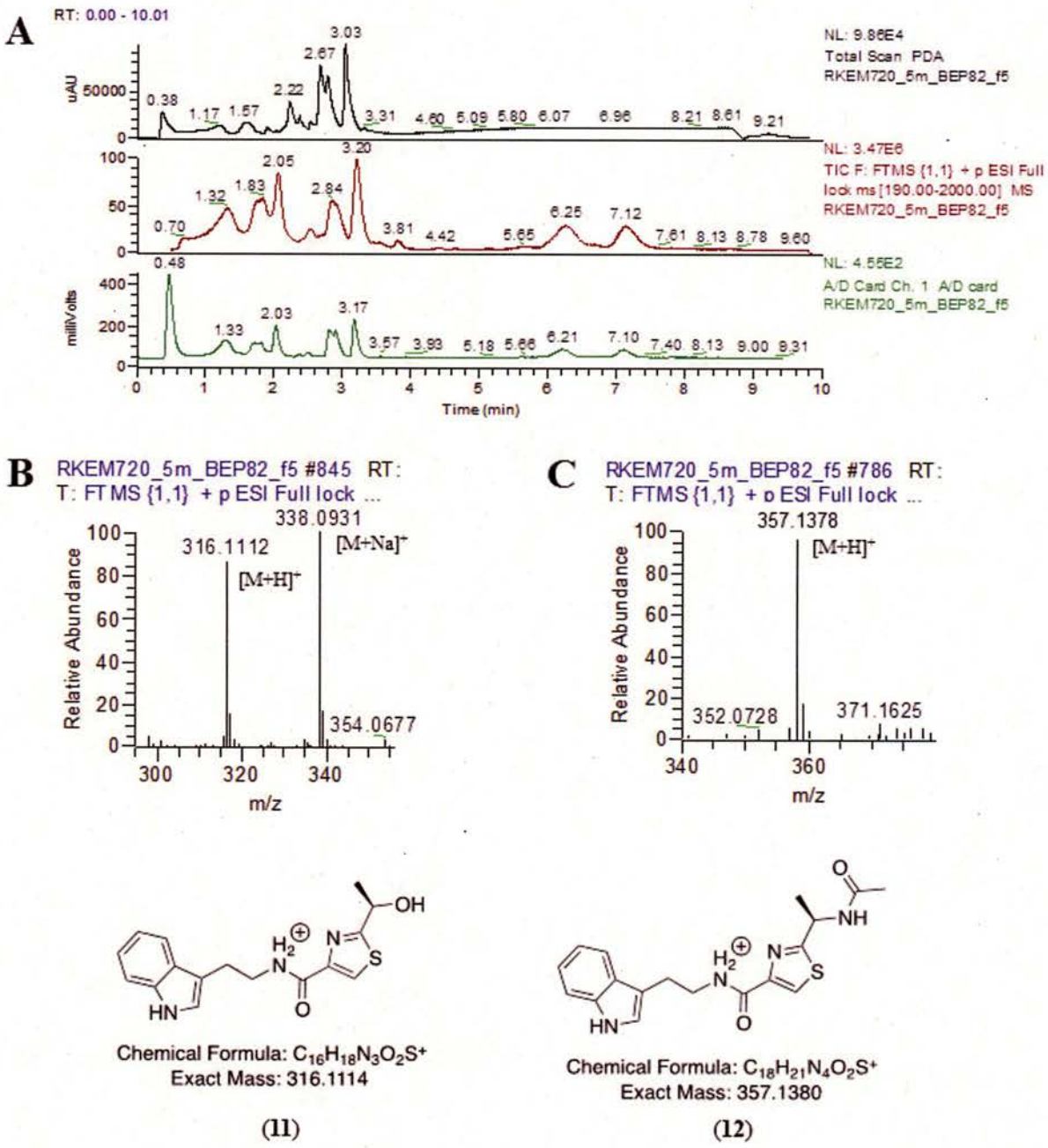

Figure B.2.7. (A) UHPLC-HRMS chromatogram of B*acillus* sp. RKEM 720 in BFM5 and mass spectra of (B) bacillaide B (**11**) and (C) bacillamide C (**12**).

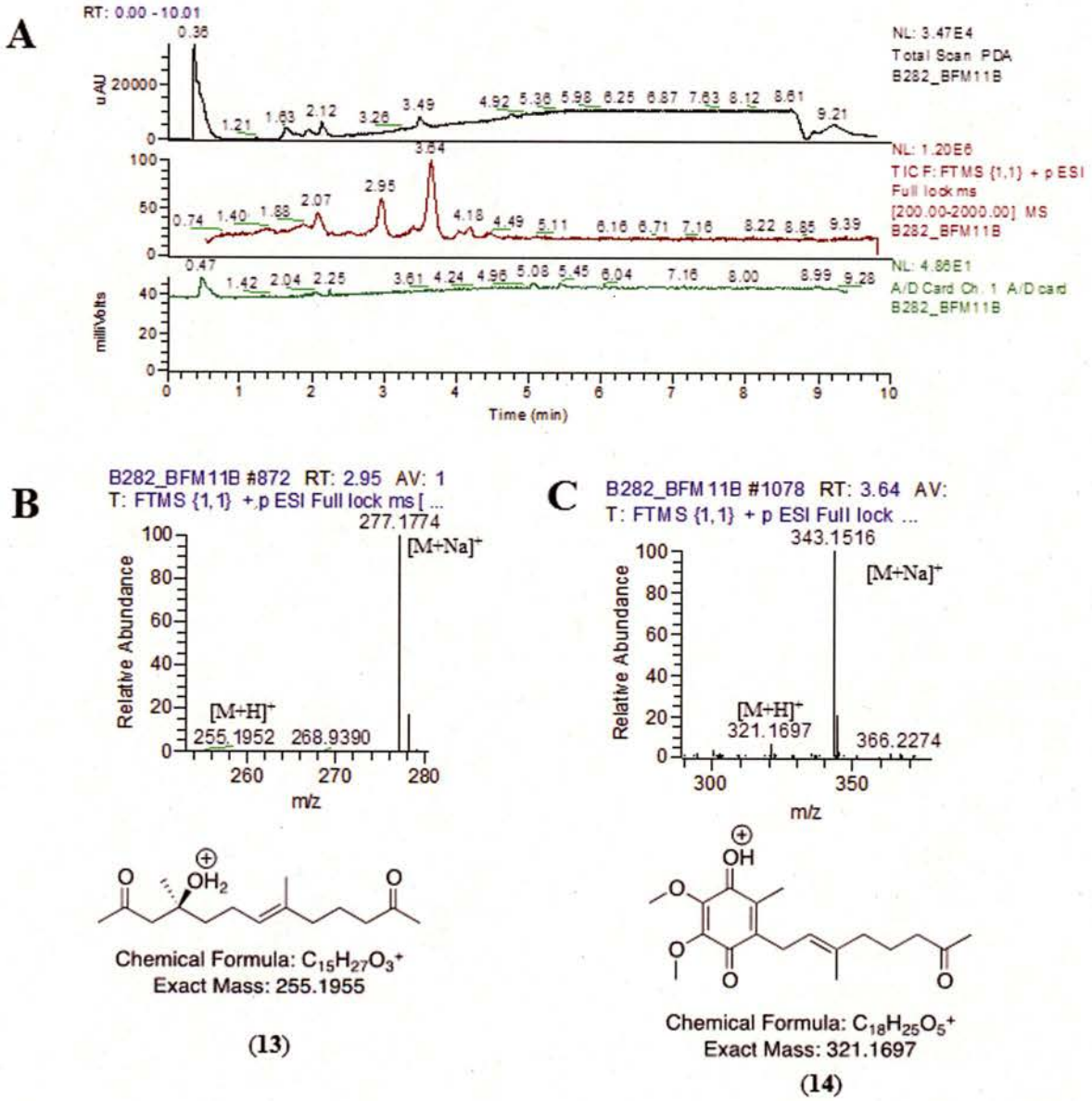

Figure B.2.8. (A) UHPLC-HRMS chromatogram of *Pseudoalteromonas* sp. RKBH 282 in BFM11 and mass spectra of (B) pseudoalteromone A (**13**) and (C) pseudoalteromone B (**14**).

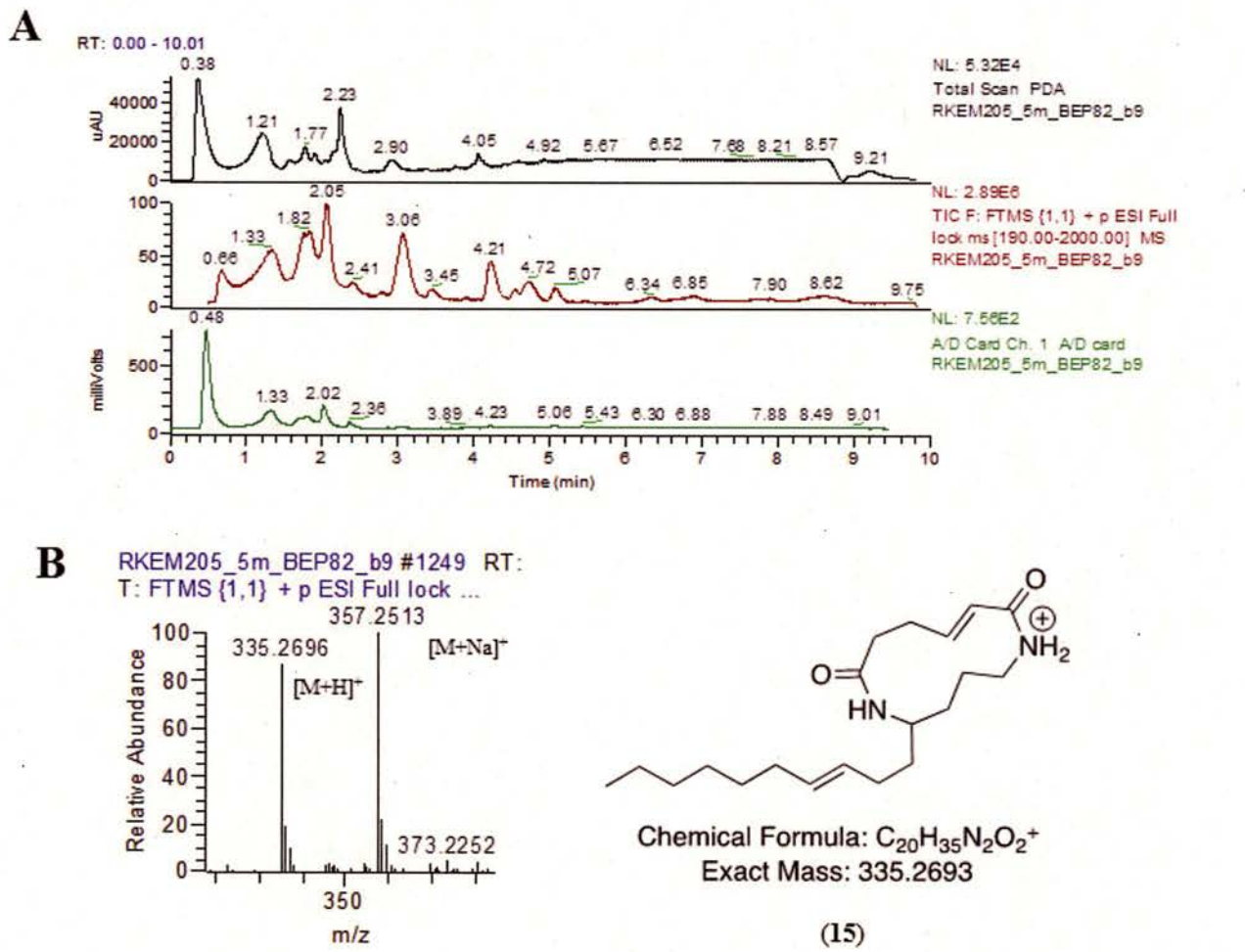

Figure B.2.9. (A) UHPLC-HRMS chromatogram of *Vibrio* sp. RKBH 205 in BFM5 and mass spectra of (B) bahamamide (**15**).

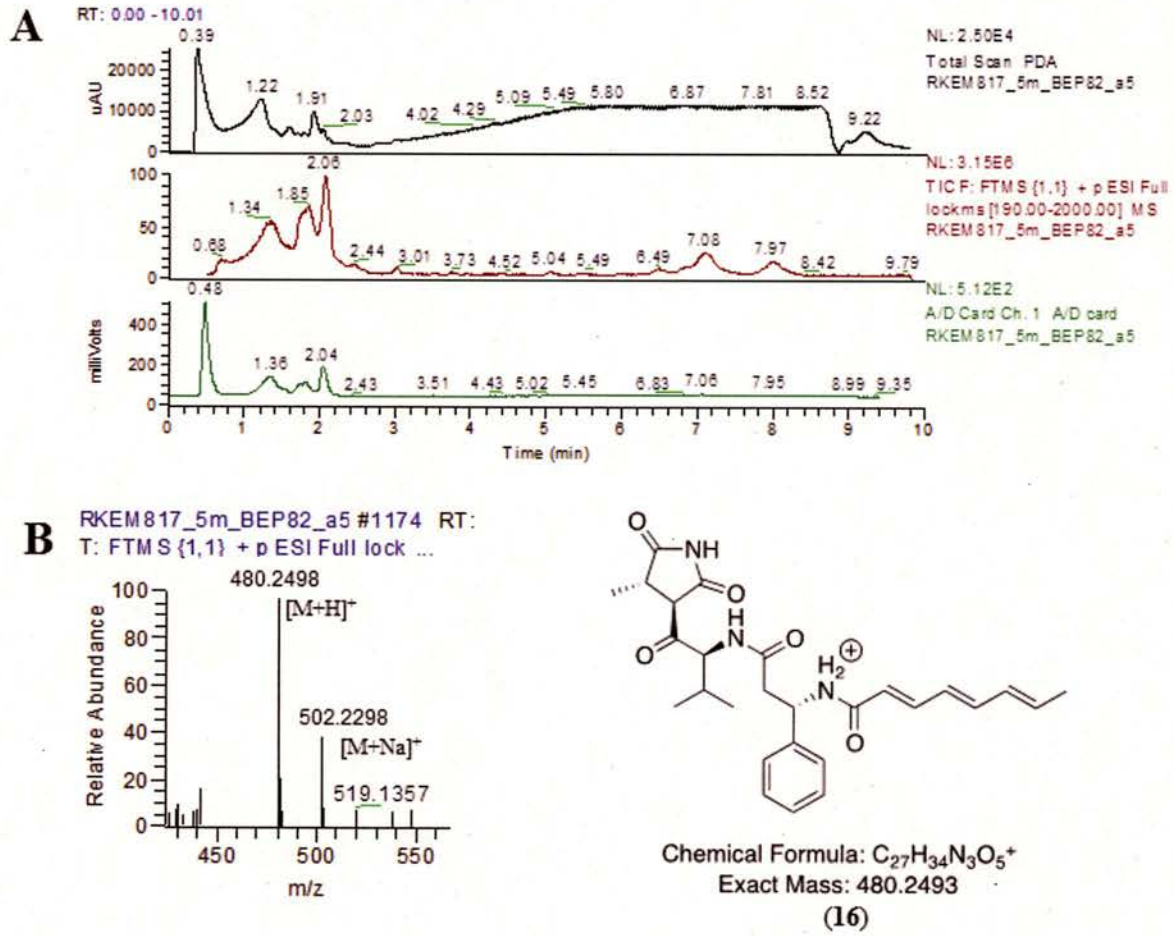

Figure B.2.10. (A) UHPLC-HRMS chromatogram of *Acinetobacter* sp. RKBH 817 in BFM5 and mass spectra of (B) andrimid (**16**).

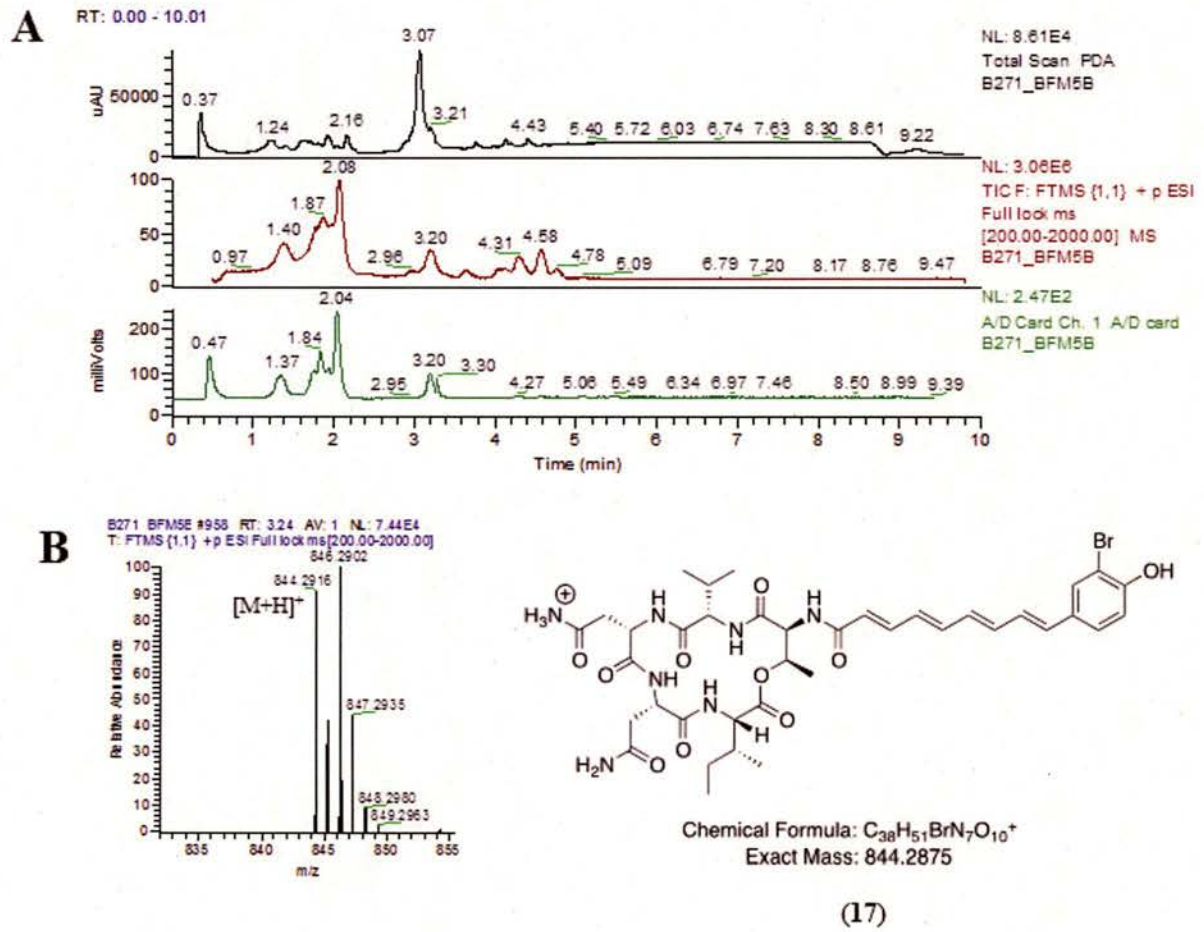

Figure B.2.11. (A) UHPLC-HRMS chromatogram of *Pseudoalteromonas* sp. RKBH 271 in BFM5 and mass spectra of (B) bromoalterochromide A (**17**).

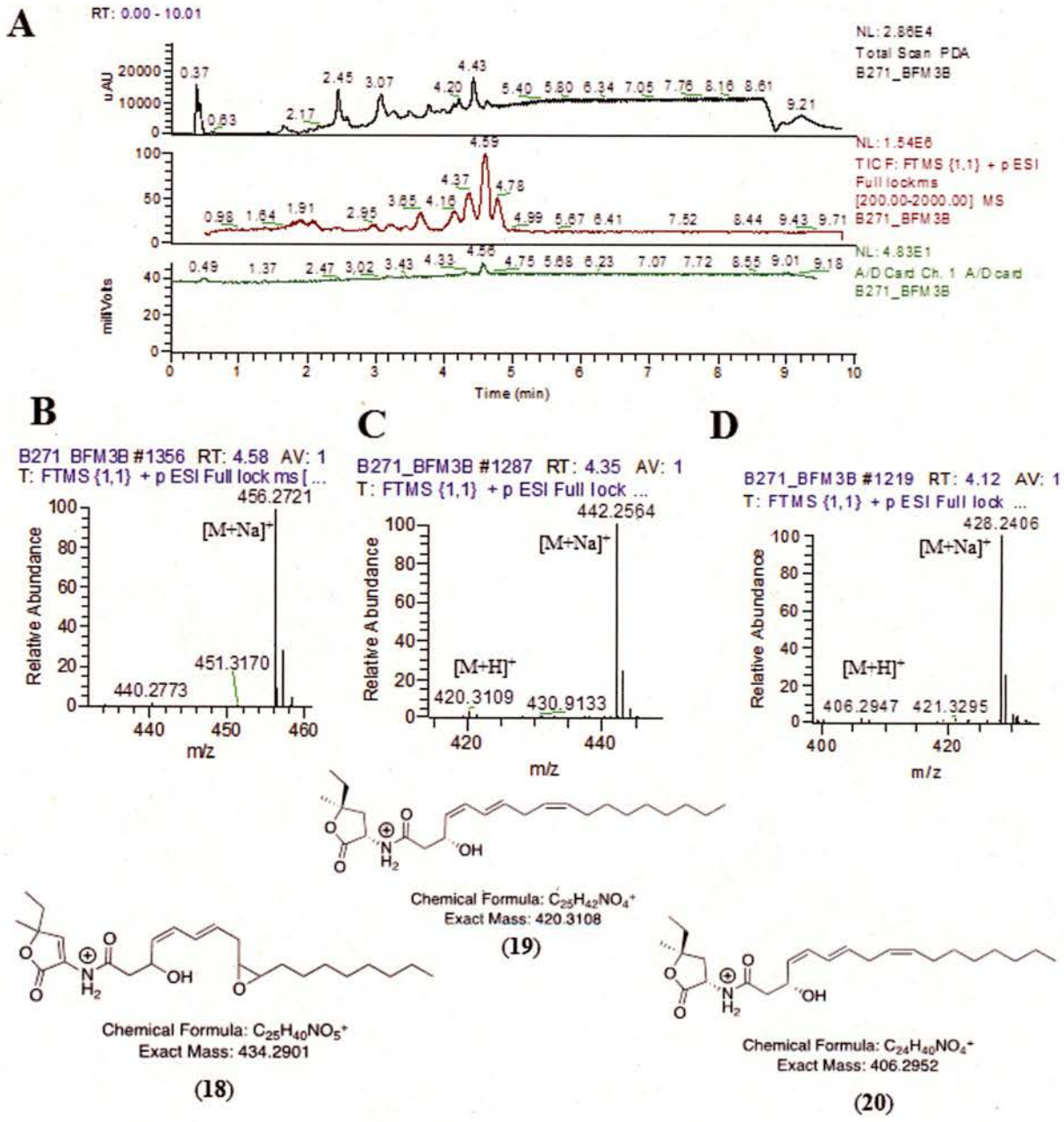

Figure B.2.12. (A) UHPLC-HRMS chromatogram of *Pseudoalteromonas* sp. RKBH 271 in BFM3 and mass spectra mass spectra of (B) korormicin A (**18**), (C) korormicin J (**19**), and (D) korormicin K (**20**).

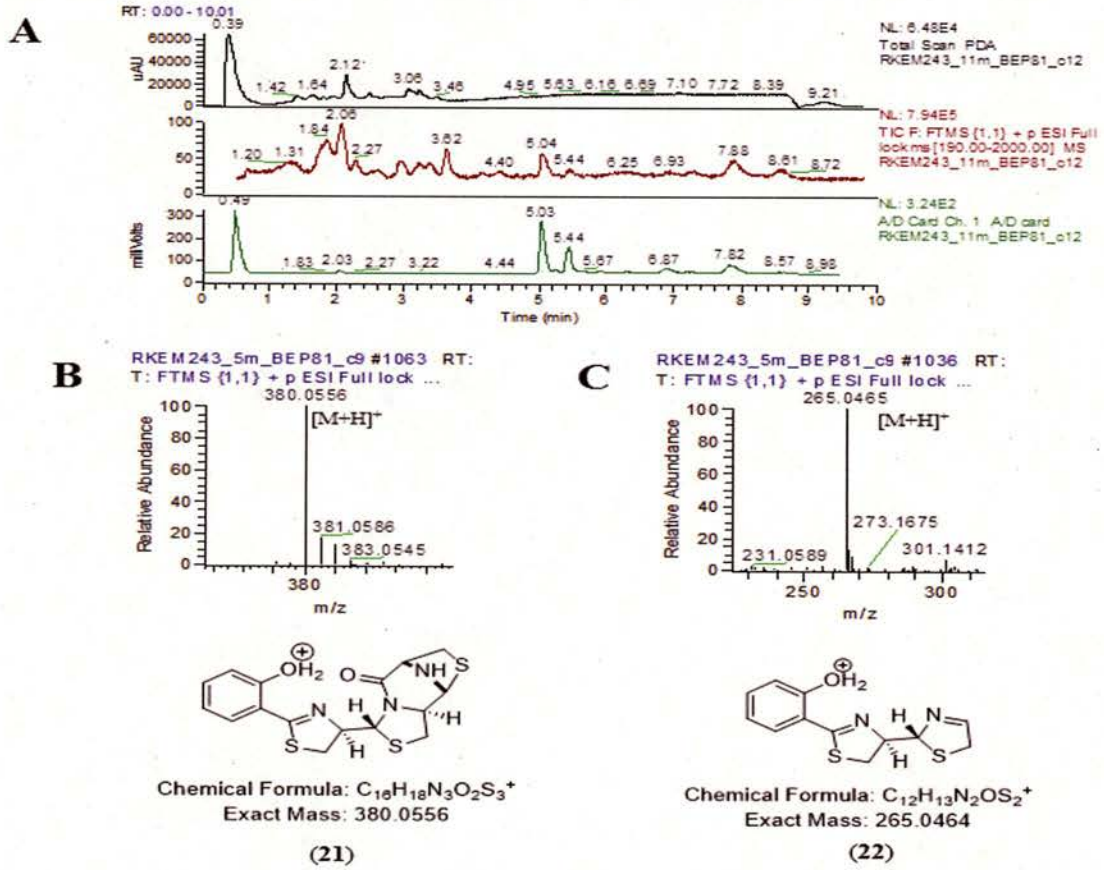

Figure B.2.13. UHPLC-HRMS chromatogram of *Pseudoalteromonas* sp. RKBH 243 in BFM5 and mass spectra of (B) ulbactin E (**21**) and (C) ulbactin A (**22**).

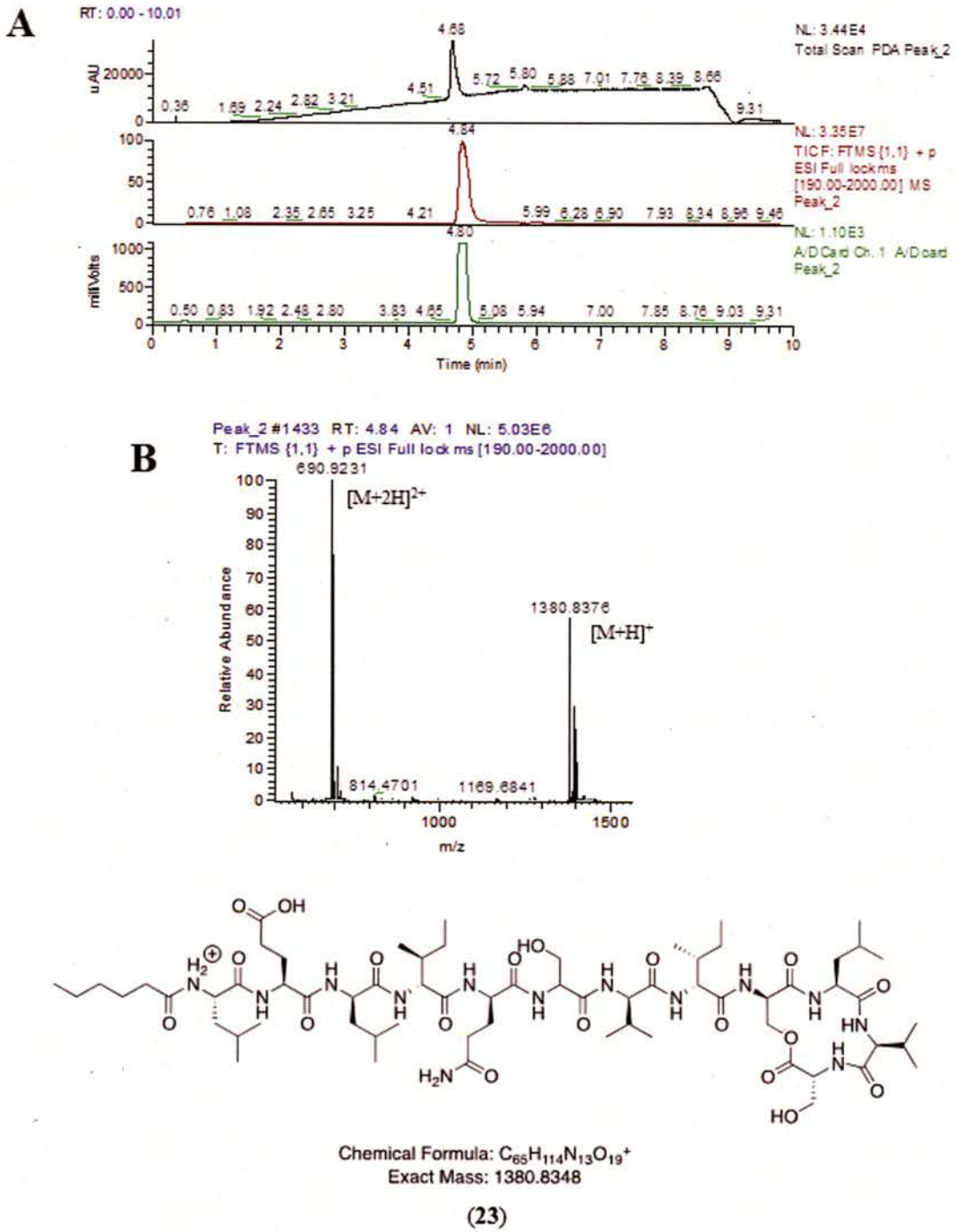

Figure B.2.14. UHPLC-HRMS and mass spectra of putisolvin I (**23**).

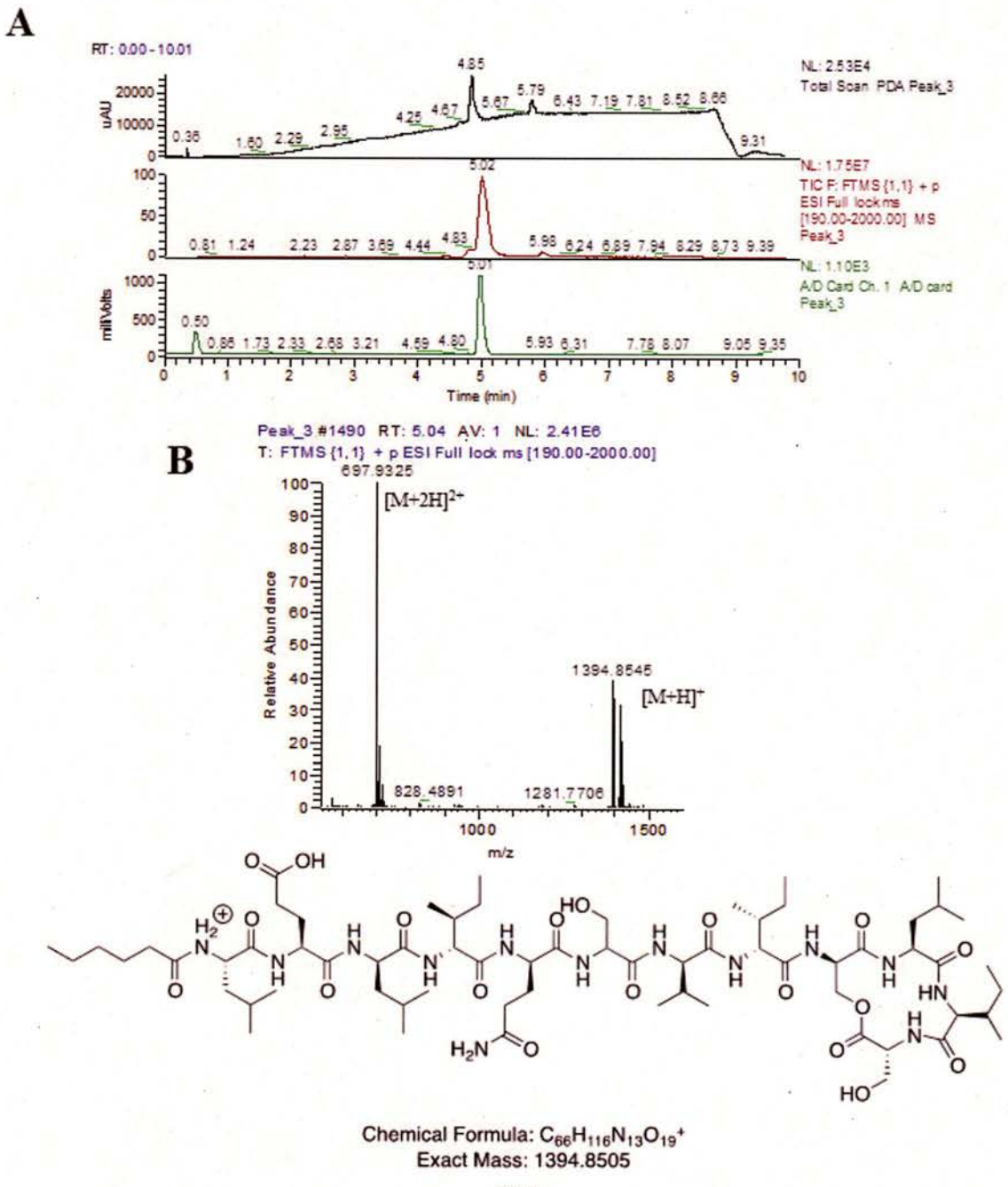

Figure B.2.15. UHPLC-HRMS and mass spectra of putisolvin II (**24**).

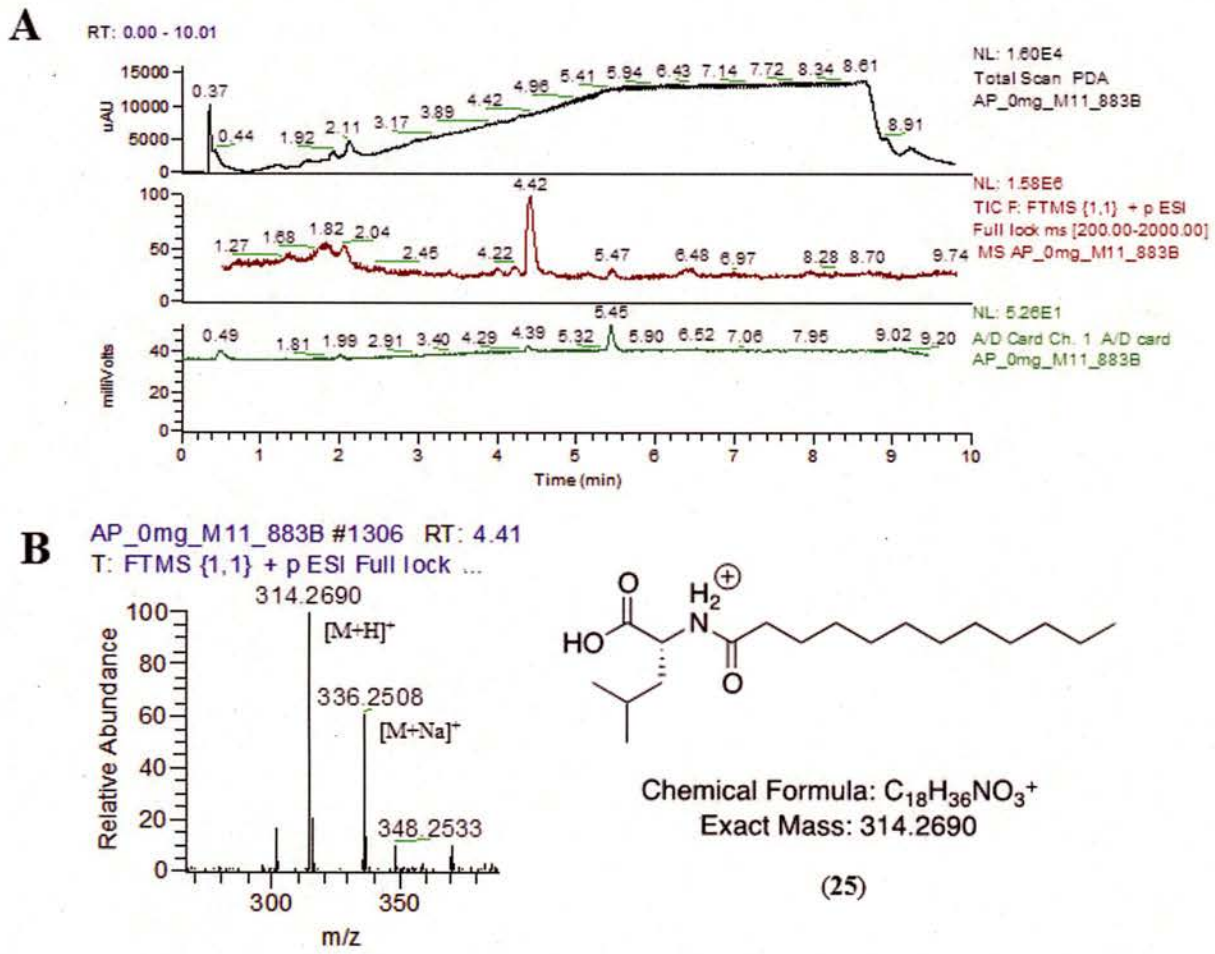

Figure B.2.16. UHPLC-HRMS chromatogram of *Halomonas* sp. RKBH 883 in BFM11 and mass spectra of compound (**25**).

A.3 Nuclear Magnetic Resonance Spectroscopy Data

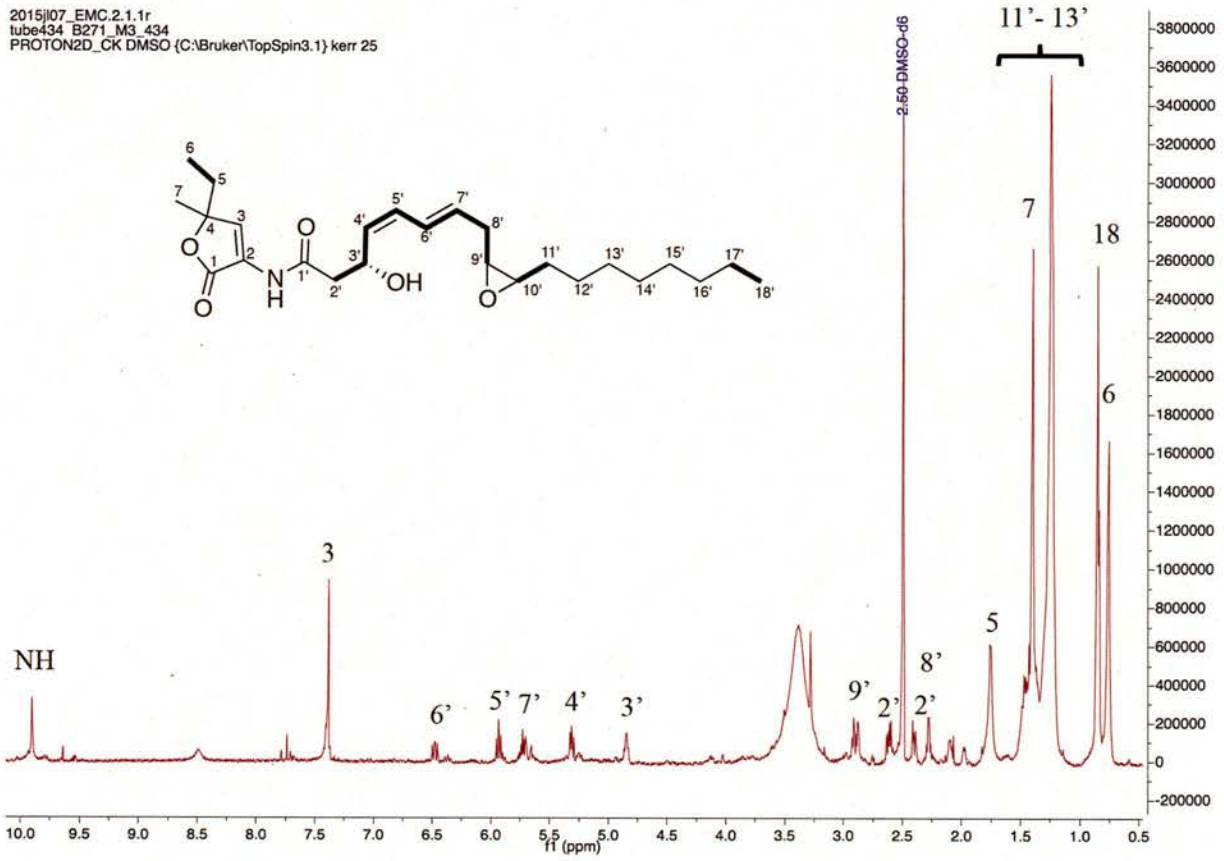

Figure B.3.1. ^{1}H NMR (600MHz, DMSO-d6) spectrum of korormicin A (**18**).

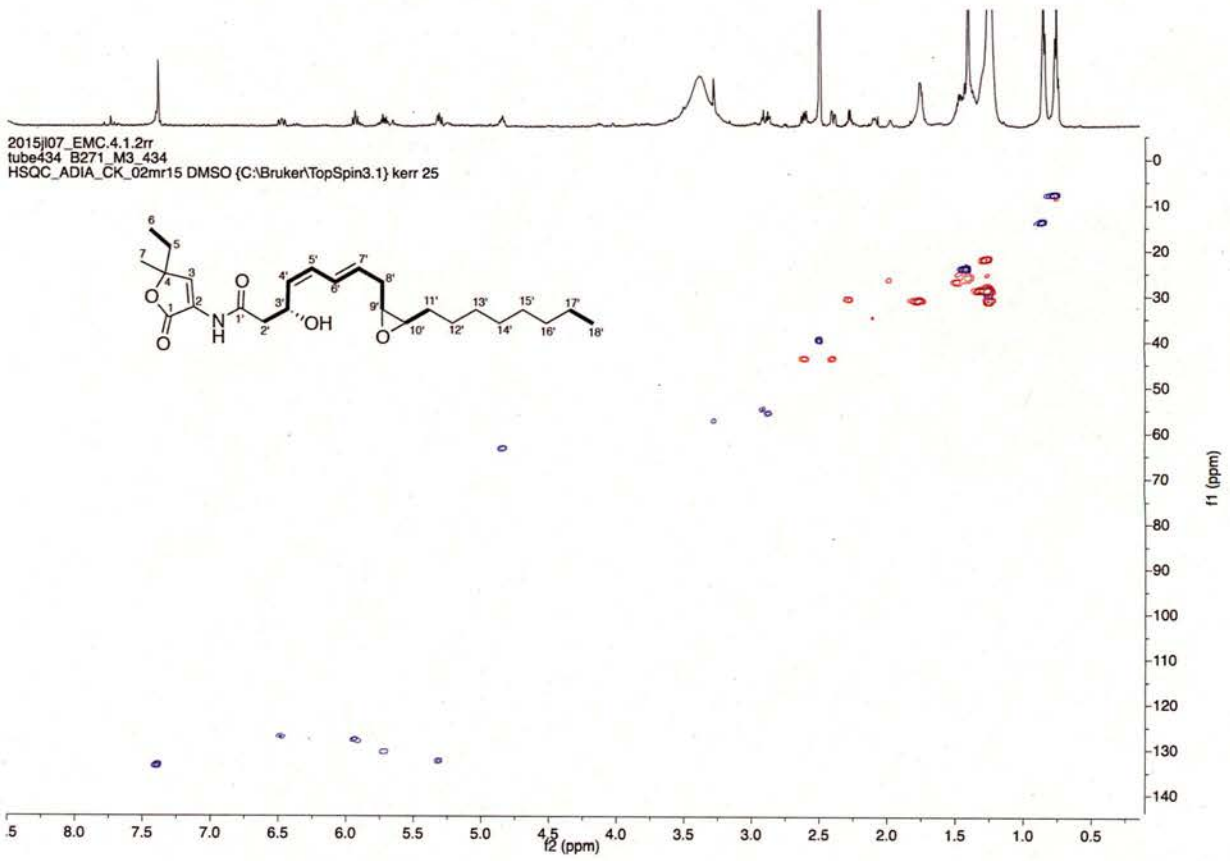

Figure B.3.2. HSQC spectrum of korormicin A (**18**).

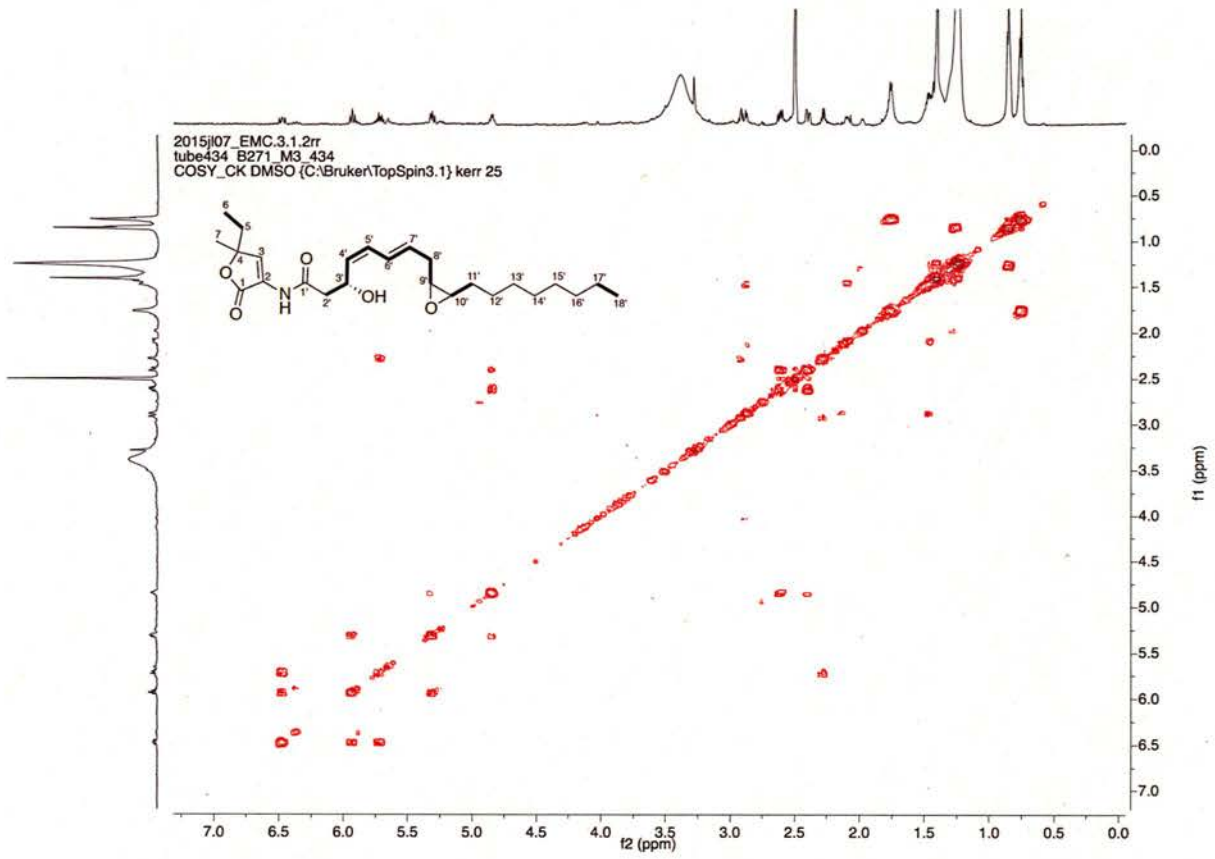

Figure B.3.3. COSY spectrum of korormicin A (**18**).

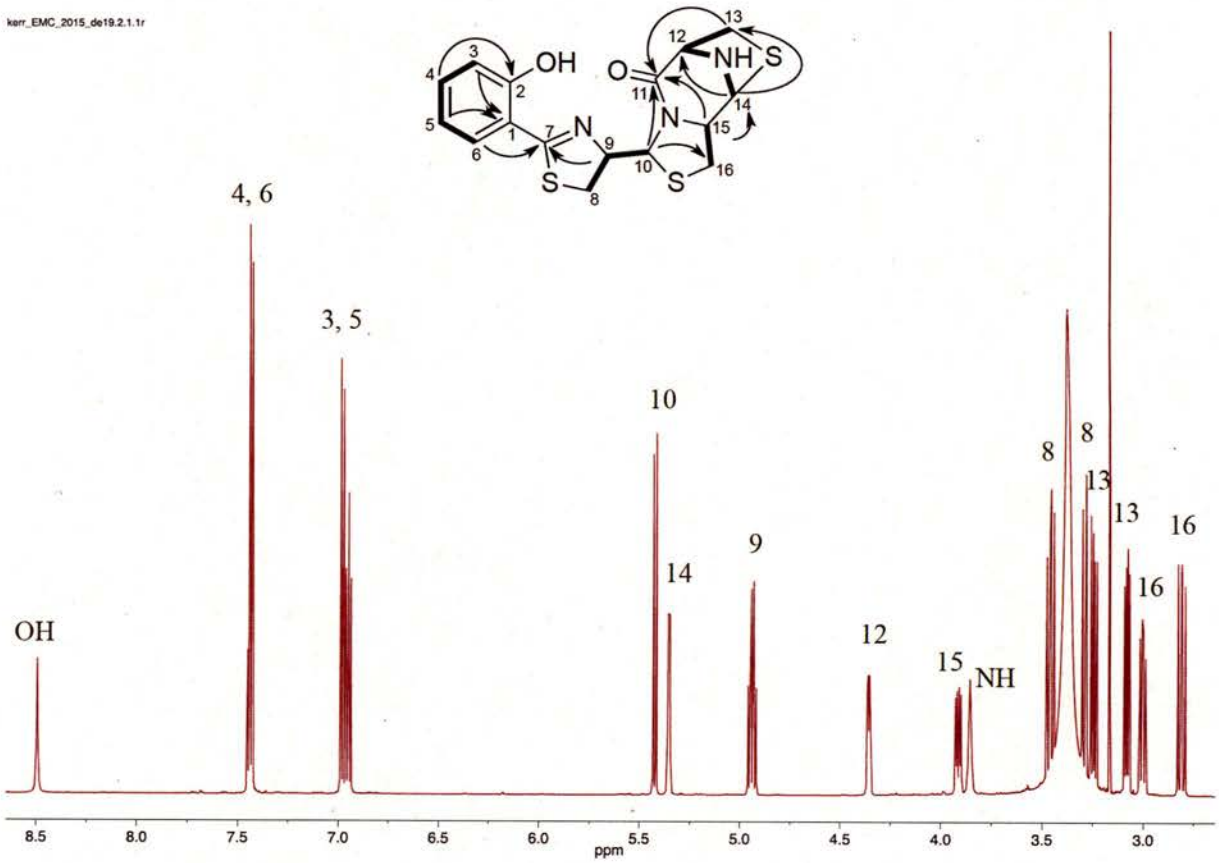

Figure B.3.4. ^{1}H NMR (600MHz, DMSO-d6) spectrum of ulbactin E (**21**).

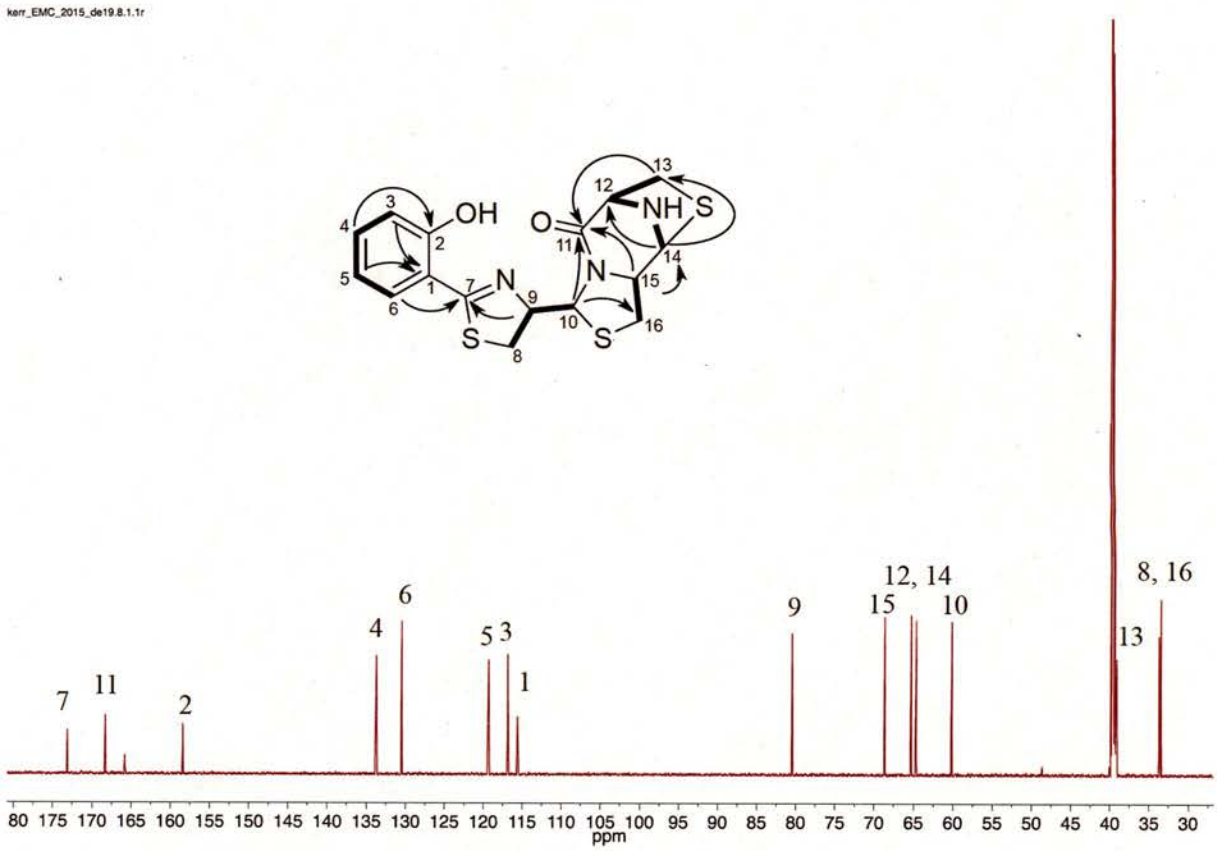

Figure B.3.5. ^{13}C NMR (150MHz) spectrum of ulbactin E (**21**).

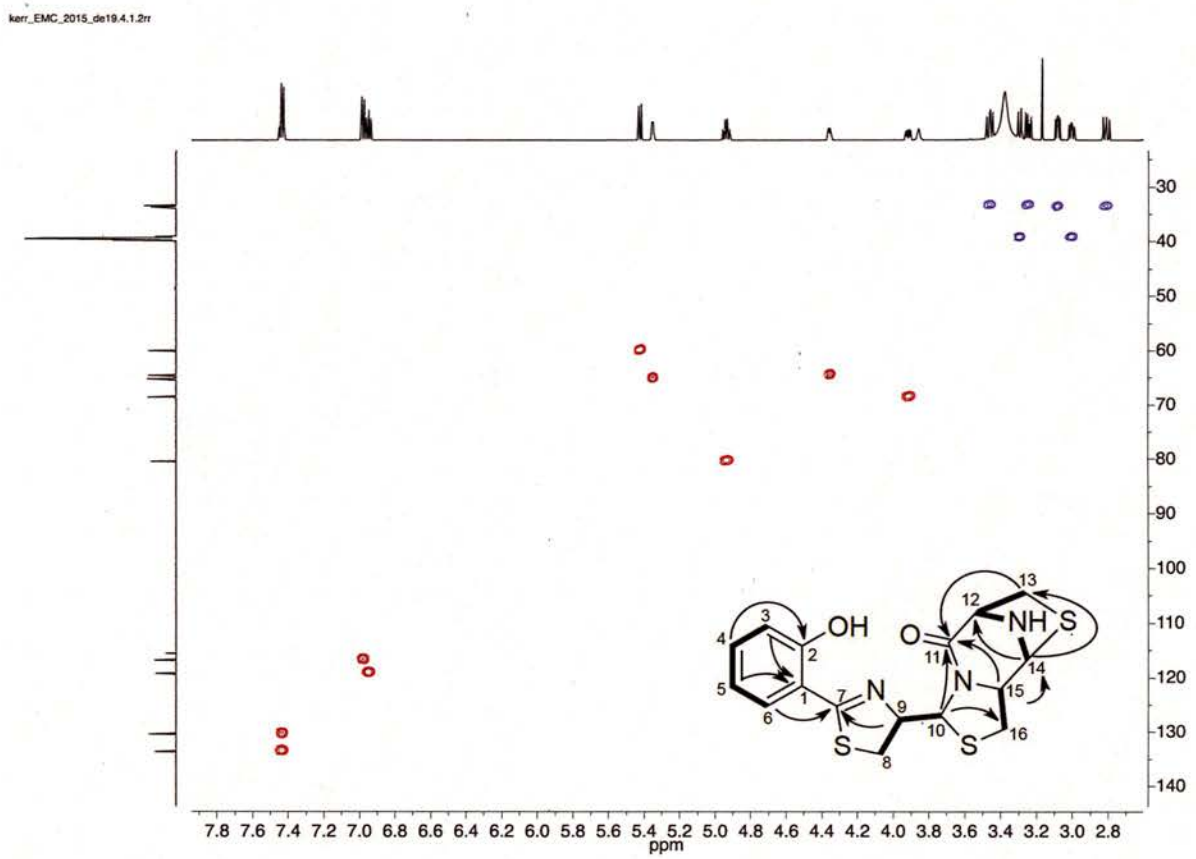

Figure B.3.6. HSQC spectrum of ulbactin E (**21**).

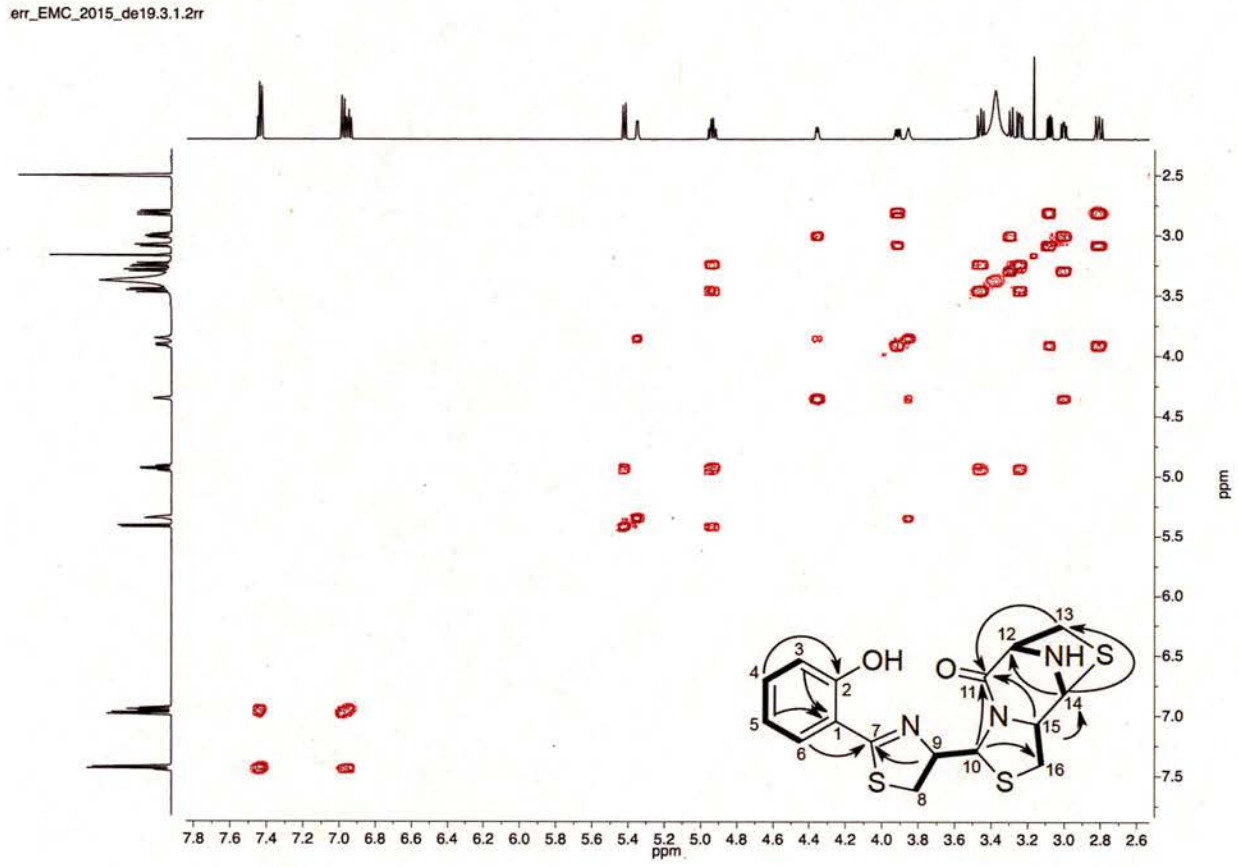

Figure B.3.7. COSY spectrum of ulbactin E (**21**).

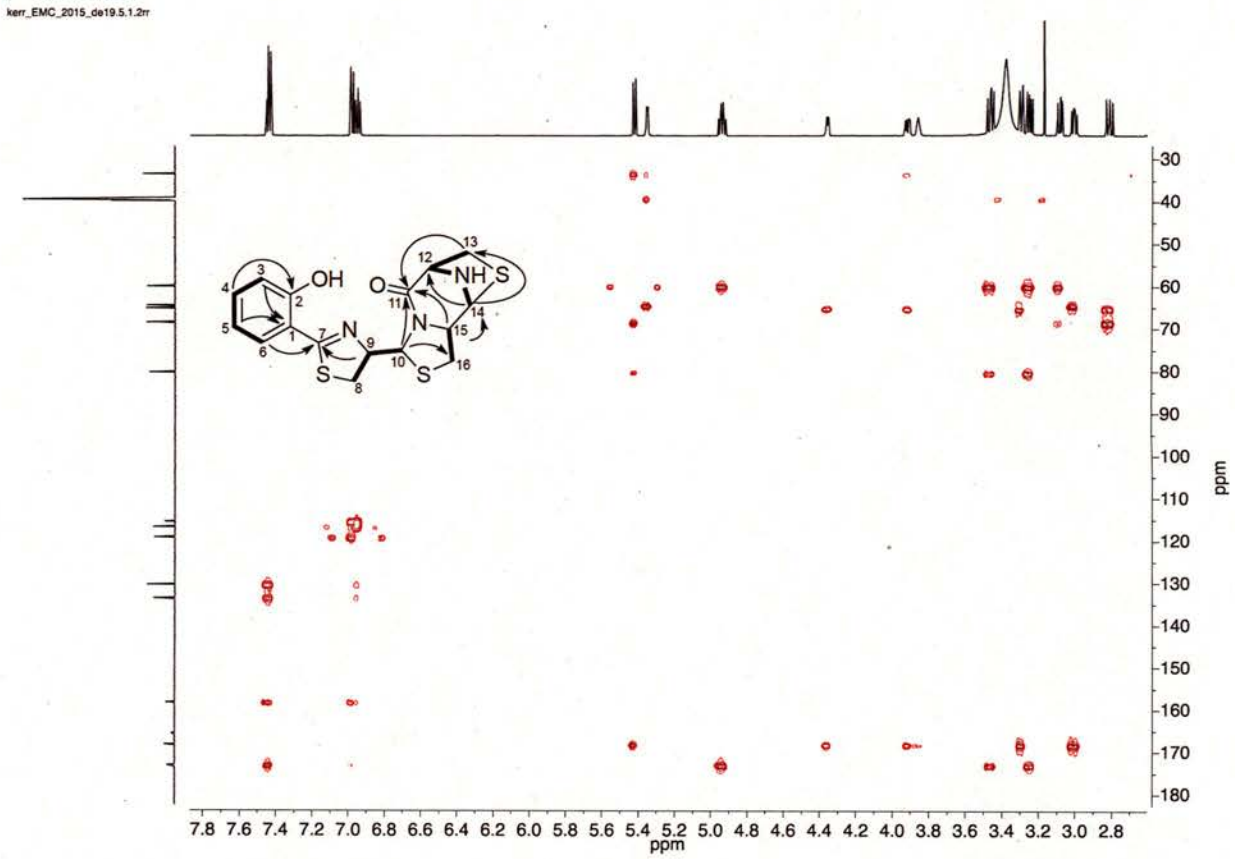

Figure B.3.8. HMBC spectrum of ulbactin E (**21**).

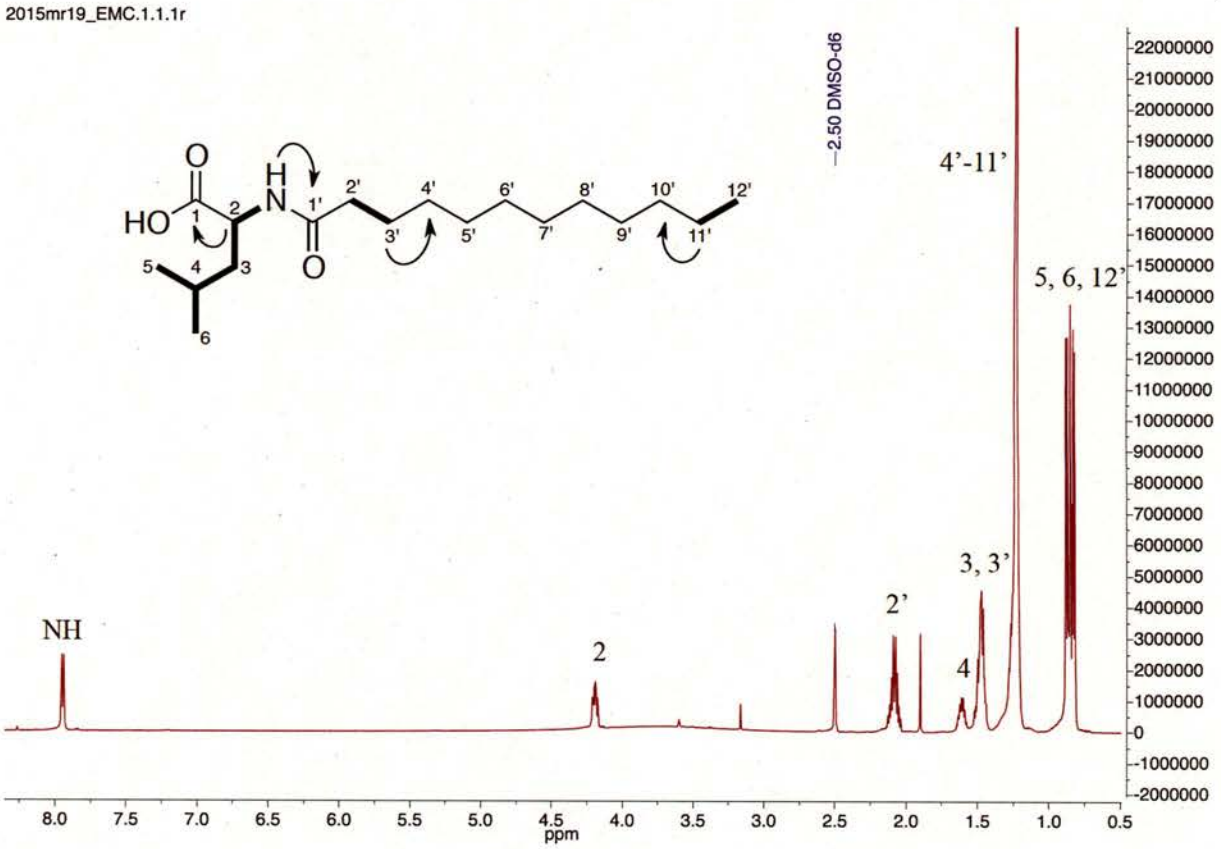

Figure B.3.9. ^{1}H NMR (600MHz, DMSO-d6) spectrum of long chain N-acyl L-leucine (**25**).

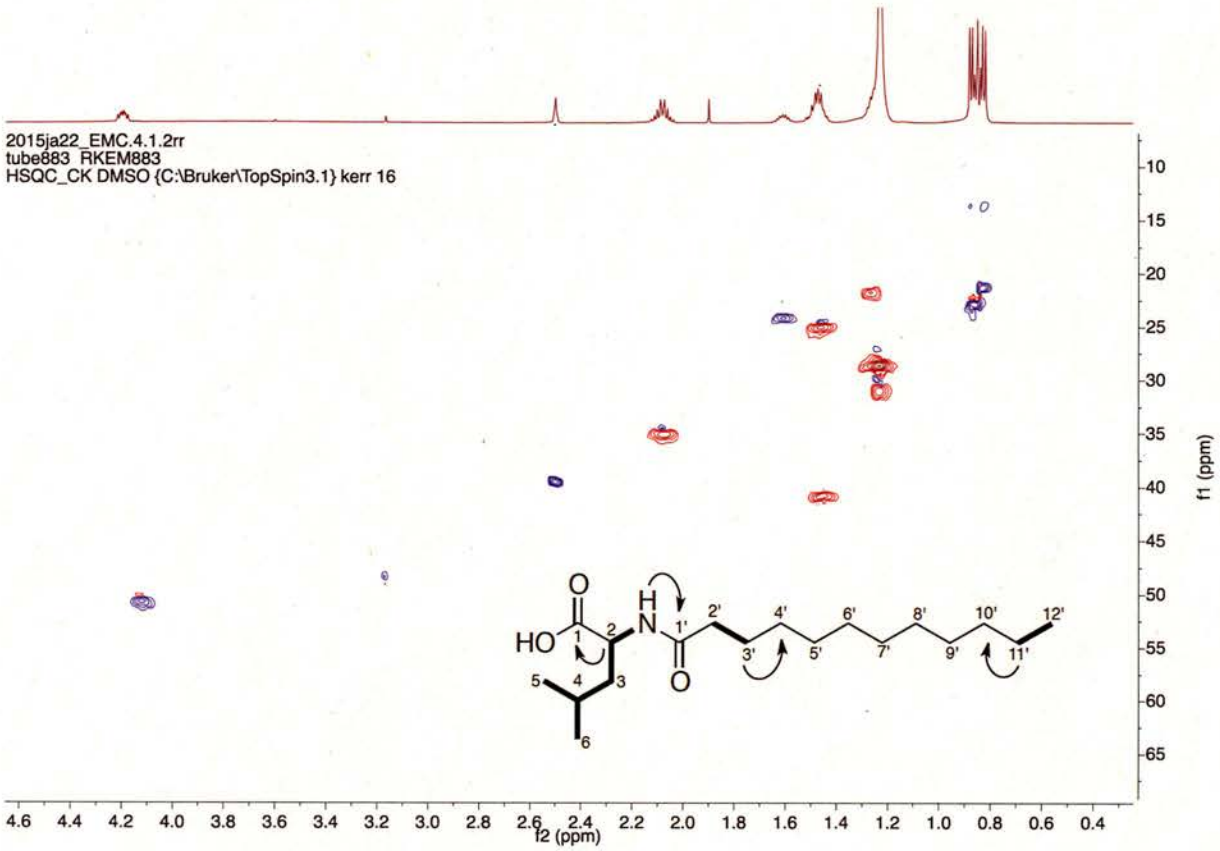

Figure B.3.10. HSQC spectrum of long chain N-acyl L-leucine (**25**).

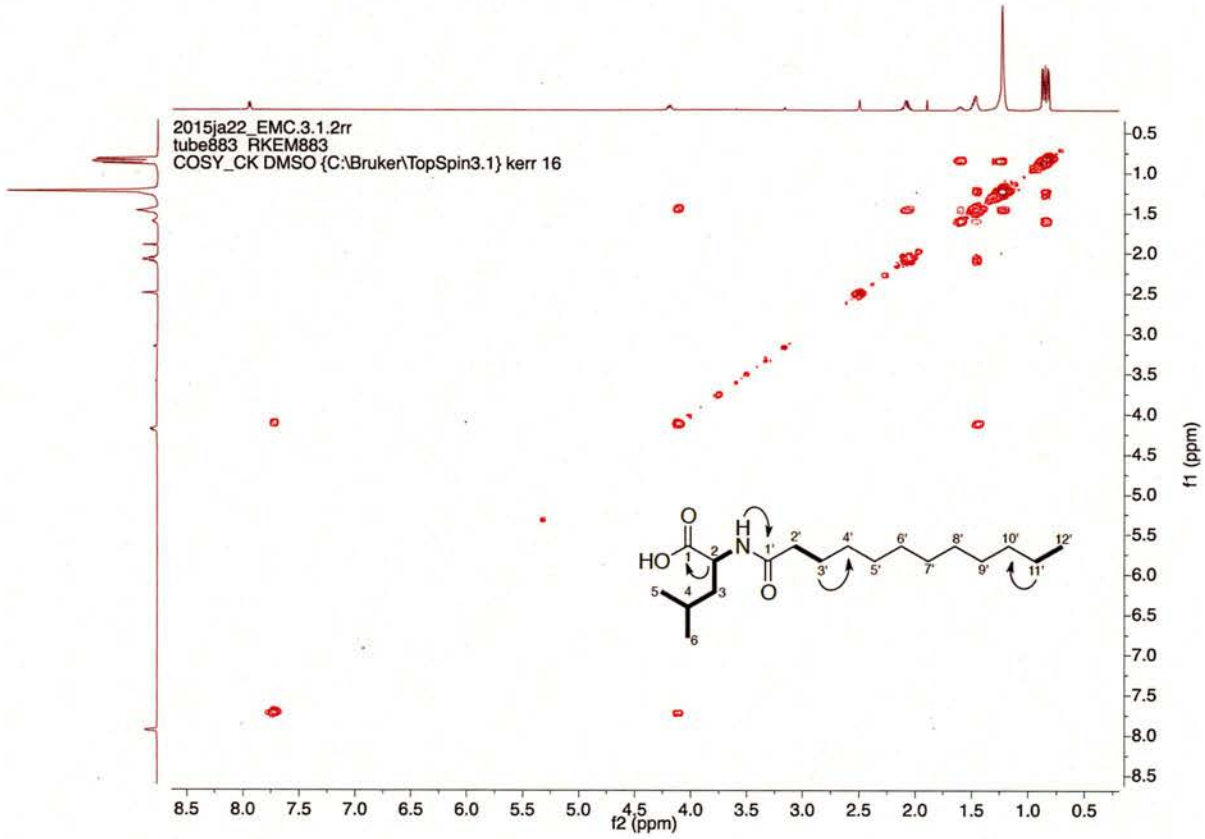

Figure B.3.11. HSQC spectrum of long chain N-acyl L-leucine (**25**).

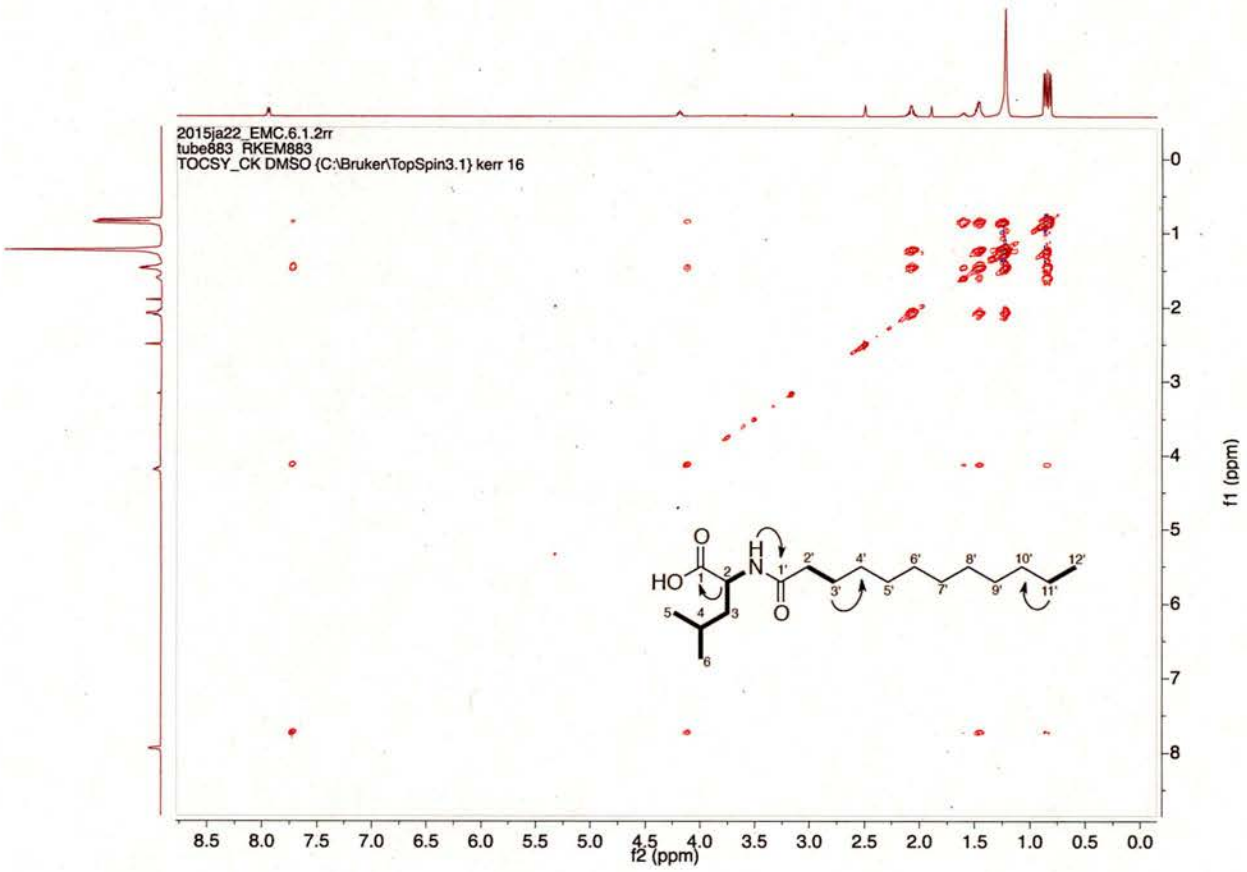

Figure B.3.12. TOCSY spectrum of long chain N-acyl L-leucine (**25**).

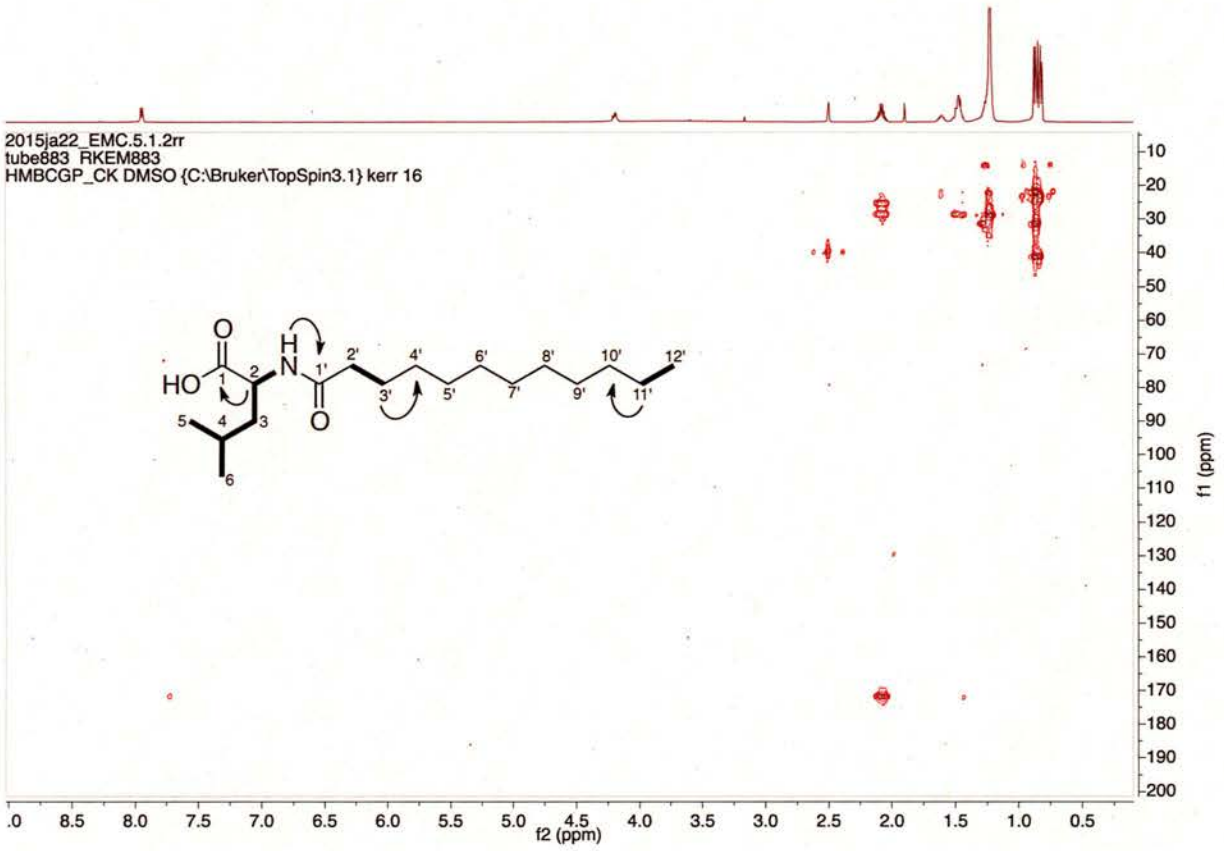

Figure B.3.13. HMBC spectrum of long chain N-acyl L-leucine (**25**).