Available online at www.sciencedirect.com
SCIENCE DIRECT®
Systematic and Applied Microbiology 28 (2005) 242–264
SYSTEMATIC AND APPLIED MICROBIOLOGY
www.elsevier.de/syapm

A molecular systematic survey of cultured microbial associates of deep-water marine invertebrates

Karen Sfanos[a,1], Dedra Harmody[a], Phat Dang[b], Angela Ledger[a], Shirley Pomponi[a], Peter McCarthy[a], Jose Lopez[a,*]

[a]*Division of Biomedical Marine Research, Harbor Branch Oceanographic Institution (HBOI), 5600 US Hwy. 1 Fort Pierce, FL 34946, USA*
[b]*US Horticultural Research Laboratory, Agricultural Research Service, USDA, Fort Pierce, FL 34945, USA*

Received 30 August 2004

Abstract

A taxonomic survey was conducted to determine the microbial diversity held within the Harbor Branch Oceanographic Marine Microbial Culture Collection (HBMMCC). The collection consists of approximately 17,000 microbial isolates, with 11,000 from a depth of greater than 150 ft seawater. A total of 2273 heterotrophic bacterial isolates were inventoried using the DNA fingerprinting technique amplified rDNA restriction analysis on approximately 750–800 base pairs (bp) encompassing hypervariable regions in the 5′ portion of the small subunit (SSU) 16S rRNA gene. Restriction fragment length polymorphism patterns obtained from restriction digests with *Rsa*I, *Hae*III, and *Hha*I were used to infer taxonomic similarity. SSU 16S rDNA fragments were sequenced from a total of 356 isolates for more definitive taxonomic analysis. Sequence results show that this subset of the HBMMCC contains 224 different phylotypes from six major bacterial clades (Proteobacteria (Alpha, Beta, Gamma), Cytophaga, Flavobacteria, and Bacteroides (CFB), Gram + high GC content, Gram + low GC content). The 2273 microorganisms surveyed encompass 834 α-Proteobacteria (representing 60 different phylotypes), 25 β-Proteobacteria (3 phylotypes), 767 γ-Proteobacteria (77 phylotypes), 122 CFB (17 phylotypes), 327 Gram + high GC content (43 phylotypes), and 198 Gram + low GC content isolates (24 phylotypes). Notably, 11 phylotypes were ⩽93% similar to the closest sequence match in the GenBank database even after sequencing a larger portion of the 16S rRNA gene (~1400 bp), indicating the likely discovery of novel microbial taxa. Furthermore, previously reported "uncultured" microbes, such as sponge-specific isolates, are part of the HBMMCC. The results of this research will be available online as a searchable taxonomic database (www.hboi.edu/dbmr/dbmr_hbmmd.html).

Keywords: Marine microorganisms; 16S rRNA; Culture collection; Sponge symbiosis

Introduction

Marine invertebrate filter feeders can harbor a great abundance of microbial diversity and biomass. For example, many marine sponges filter >20,000 l of water per day and appear to host microbial communities with

*Corresponding author. Tel.: +1 772 465 2400; fax: +1 772 461 2221.
E-mail address: Lopez@hboi.edu (J. Lopez).
[1]Current address: James Buchanan Brady Urological Institute, Johns Hopkins University School of Medicine, Baltimore, MD 21287, USA.

0723-2020/$ - see front matter © 2004 Elsevier GmbH. All rights reserved.
doi:10.1016/j.syapm.2004.12.002

a wide phylogenetic spectrum [29,30,36,65] that can comprise over 50% of the total sponge biomass [51,67]. Because marine invertebrates can accumulate microorganisms, samples collected from invertebrates provide a more diverse array of microbes than samples recovered from the water column [30,33,64,67]. In recent years, the deep sea has also proven to be a source of a surprisingly diverse abundance of microorganisms, including culturable, newly described species of γ-Proteobacteria [4], ε-Proteobacteria [7], and actinomycetes [9].

Small subunit (SSU) rRNA has emerged as a reliable tool for phylogenetics because it is present in all living organisms, functionally constant, and highly conserved [45,59,60]. It therefore serves as the "backbone" for the structuring of the second edition of Bergey's Manual of Systematic Bacteriology [22,37]. Restriction fragment length polymorphism (RFLP) analysis of the 16S SSU rRNA gene (also termed amplified rDNA restriction analysis (ARDRA)) has been used to rapidly distinguish microbial species in a variety of applications such as clinical laboratories [14,61,63], industrial wastewater [8,23], coral diseases [11], agricultural soils [44], lake sediments [12], saline mud volcanoes [69], and microbial communities in the marine environment [1,13,46,58].

The Harbor Branch Oceanographic Marine Microbial Culture Collection (HBMMCC) has been developed over the last two decades as a resource for drug discovery [5,47] and is one of the largest collections of marine-derived microorganisms. Prior to this survey, many of the isolates had not been characterized beyond microscopic, morphological, and Gram-stain identifications. The objectives of this study were to: (i) develop a rapid method to taxonomically inventory deep-water invertebrate-derived marine microorganisms in the HBMMCC, (ii) compare the relationships between the isolates described in this study to previously described marine bacteria, and (iii) assess the distribution of inventoried isolates across various host invertebrate species, depths, and geographic locales. The present study expands on previous work [42,49] by profiling approximately one-fifth of the deep-water (>110 ft seawater) bacterial isolates in the HBMMCC.

Materials and methods

The general scheme of the experimental design is depicted in Fig. 1. More detailed methodology is described below.

Microbe isolation and selection

The isolates used in this study were deep-water (>110 ft seawater) invertebrate- or sediment-associated bacteria maintained in the HBMMCC. Samples were collected from Aruba, the Bahamas, Barbados, Bonaire, Canary Islands, Cape Verde, Curacao, the Galapagos, the Gulf of Mexico, Honduras, Jamaica, Madeira, Puerto Rico, Turks & Caicos, the US Virgin Islands, and the USA using Harbor Branch Oceanographic Institution's underwater submersibles (Johnson-Sea-Link I and II). Bacterial isolation methods involved the sampling of invertebrate tissues using aseptic technique upon surfacing. Microbial isolates were sampled from a total of 38 invertebrate hosts plus sediment samples (Table 1). The taxonomy of most invertebrate hosts is resolved to the level of order or family, and ongoing taxonomic identifications will be continually updated in the online HBMMCC database (www.hboi.edu/dbmr/dbmr_hbmmd.html) [26]. The invertebrate tissue was ground in sterile seawater and the subsequent supernatant was diluted in sterile seawater before plating onto a series of media designed to recover a diverse range of heterotrophic microbes. Media ranged from extremely nutrient poor (60% seawater, 40% deionized water, trace metals, phosphate, agar), to nutrient rich (Difco Marine Agar 2216) and included a wide variety of carbon sources (e.g. chitin, simple and complex sugars, and mucin). Certain isolation media also included host tissue and other supplements designed to increase total microbial recovery [43]. In some cases, antibiotics were also employed for selective recovery of bacterial populations (e.g. nalidixic acid was used to reduce growth of Gram negative bacteria). The subset of the collection used in this survey was derived from 98 isolation media.

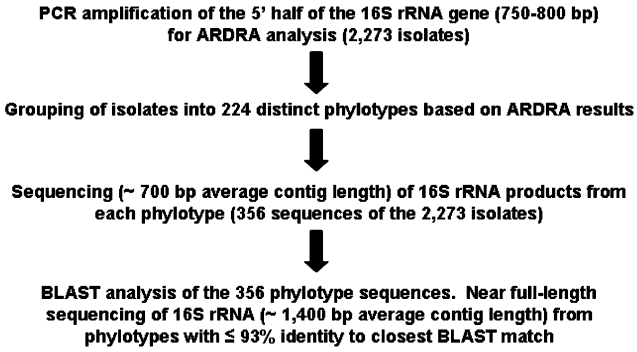

Fig. 1. Flow chart of experimental methods.

DNA extraction

Bacterial cells for DNA extraction were collected with a sterile 1 μl loop. The cells were added to 125 μl of Chelex-100 (Bio-Rad Inc.) made as a 5% solution in sterile distilled water. Total genomic DNA was then extracted using the standard protocol for Chelex-100 [15].

Table 1. Marine invertebrate sources of isolates used in this study

Phylum	Class	Order	Family	Identified isolates
Porifera	Demospongiae	Astrophorida	Ancorinidae (An)	107
			Calthropellidae (Ca)	12
			Geodiidae (Ge)	65
			Pachastrellidae (Pa)	119
		Dictyoceratida	Irciniidae (Ir)	17
			Thorectidae (Tr)	17
		Hadromerida	Placospongiidae (Pl)	41
			Polymastiidae (Pm)	12
			Suberitidae (Su)	14
		Halichondrida	Axinellidae (Ax)	220
			Desmoxyidae (Dx)	35
			Halichondriidae (Ha)	183
		Haplosclerida	Phloeodictyidae (Ph)	18
			Petrosiidae (Pe)	24
		Lithistida	Azoricidae (Az)	21
			Phymaraphinidae (Py)	8
			Scleritodermidae (Sc)	124
			Siphonidiidae (Si)	59
			Theonellidae (Tn)	138
			Vetulinidae (Vt)	9
		Poecilolsclerida	Acarnidae (Ac)	7
			Desmacellidae (Dc)	38
			Coelosphaeridae (Co)	244
			Mycalidae (My)	8
			Raspailiidae (Ra)	66
		Verongida	Pseudoceratinidae (Ps)	70
	Unidentified demospongiae (UD)			317
	Unidentified hexactinellida (UH)			65
Cnidaria	Anthozoa	Alcyonacea	Nephtheidae (Ne)	1
		Gorgonacea	Plexauridae (Px)	88
			Isididae (Is)	1
		Actinaria (sea anemone) (At)		6
Ectoproctoa (bryozoans)	Gymnolaemata	Ctenostomata	Vesicullariidae (Vs)	2
Mollusca	Gastropoda	Anaspidea (sea slug)	Pleurobranchidae (Pb)	4
	Gastropoda	Archeogastropoda (slit shell)	Pleurotomariidae (Pt)	18
Echinodermata	Holothuroidea (sea cucumber) (Ho)			35
	Echinoidea	Echinothurioidea (sea urchin)	Echinothuridae (Ec)	16
Annelida	Polychaeta (polychaete worm) (Po)			6
Sediments (Se)				38

Polymerase chain reaction (PCR)

Universal (consensus) 16S rRNA primers Ecoli9 5′-GAGTTTGATCCTGGCTCAG-3′ (equal to Lane [35] "27F" primer) and Loop27rc 5′-GACTAC-CAGGGTATCTAATC-3′ [36] amplified approximately 750–800 base pairs (bp) of the bacterial 16S rRNA gene (*E. coli* positions 9–804) as part of a rapid and cost-

effective method developed to screen thousands of isolates maintained in the HBMMCC. The fragment chosen for sequencing therefore encompassed four of the nine hypervariable (species-specific) regions (V1–V4) as defined by Neefs et al. [40]. Near full-length 16S rRNA gene products were generated only for phylotypes $\leqslant$93% similar to their closest GenBank match, using primers Ecoli9 and 1492R 5′-GGTTACCTTGTTACGACTT-3′ (*E. coli* position 1492) [53]. Standard PCR conditions were used as previously described [49]. A positive control (with previously amplifiable DNA) and a negative control (no template added) were run for every PCR performed. All PCR products were visualized by 1% agarose gel electrophoresis.

Amplified rDNA restriction analysis (ARDRA)

ARDRA (RFLP assay) was used as a primary screen for genetic variation in SSU PCR products [16]. Three tetrameric (4-base cutting) restriction endonucleases were used in order to increase the chances of detecting unique RFLP patterns: *Rsa*I, *Hae*III, and *Hha*I (Invitrogen). *Rsa*I and *Hae*III restriction patterns were obtained for all isolates. *Hha*I was used for samples that did not cut with either *Rsa*I or *Hae*III, or for instances where further distinction was necessary. The number of restriction enzymes used followed the results of Moyer et al. [39] who performed computer-simulated rRNA RFLP analysis and found that the use of three restriction enzymes can distinguish >99% of different bacterial taxa. RFLP results also verified the purity of the PCR products and/or cultured isolates by ensuring that digested fragments always added up to the expected length 16S rDNA fragment (~750–800 bp). Less than 10 isolates identified as contaminated were excluded from the study. Gel electrophoresis images were digitally captured on an Eagle Eye scanner (Stratagene, La Jolla, CA). The imager's accompanying software, RFLPscan (Scanalytics, Billerica, MA) was used to objectively calculate the molecular weight of each RFLP band.

Determination of phylotypes

The results of ARDRA assays were used to group isolates into "phylotypes" (sometimes abbreviated as "P1", "P2", etc). Each phylotype was defined as a group of isolates that had distinct *Rsa*I, *Hae*III, and/or *Hha*I restriction patterns.

DNA sequencing

Up to seven isolates from each phylotype were chosen for automated DNA sequencing to assure homogeneity of isolate identities within each phylotype. Sequences were obtained from both strands, edited into "contiguous" 16S rDNA fragments, and queried by Basic Local Alignment Search Tool (BLAST 2.0) against GenBank Release 2.2.9 (5/2004) [2]. The average contiguous sequence length for all runs was ~700 bp. Near full-length 16S rRNA contigs (~1400 bp average length) were obtained using overlapping primer pairs: Ecoli9 and Loop27rc for the first half of the gene, and SEQmidwayCG-F 5′-GTGTAGCGGTGAAATGCGTAG-3′ (50–60 bp upstream of Loop27rc) and 1492R for the remaining portion of the sequence. GenBank accession numbers for all new HBMMCC sequences are shown in Table 2.

Data analysis

RFLP band data were archived and queried using a Microsoft Access 97 database. Isolates with bands of similar molecular weight were grouped accordingly into phylotypes. Chimera formation was checked with the program CHIMERA_CHECK on the RDP [38].

16S SSU rDNA sequence alignments were made with CLUSTAL W [57] and are available from the authors upon request. Phylogenetic reconstructions employed either distance, likelihood or parsimony criteria using PAUP* version 4.0b10 [41,54]. However, due to the large genetic distances often involved in the SSU datasets, phenetic distances with the neighbor-joining algorithm were typically employed for phylogenetic reconstructions. Base composition was assessed with PAUP and MODELTEST [48] applied likelihood ratio tests to determine appropriate DNA substitution models for rRNA datasets. Gaps and SSU rRNA regions corresponding to loop 10, stem 11, and stem 18 in the *E. coli* secondary structure model [40] were typically difficult to align, and were therefore removed for most tree reconstructions.

Results

The use of ARDRA to identify phylotypes

Universal bacterial primers Ecoli9 and Loop27rc amplified 750–800 bp of 16S SSU rRNA from >99% of the isolates screened. A total of 2273 isolates were grouped into 224 different phylotypes based on the results of ARDRA assays (Table 2). For verification, 356 of the 2273 SSU rDNA amplicons were sequenced. Database queries indicated that the 224 identified phylotypes correspond to the following distinct taxa: 60 α-Proteobacteria (834 isolates), 3 β-Proteobacteria (25 isolates), 77 γ-Proteobacteria (767 isolates), 17 CFB (122 isolates), 43 Gram+ high GC content (327 isolates), and 24 different Gram+ low GC content bacteria (198 isolates) (Fig. 2, Table 2).

Table 2. Specific phylotypes identified in the HBMMCC

Phylotype #	HBOI ID[a]	No. (%) isolates[b]	No. seq[c]	Nearest taxonomic neighbor/Accession No.[d]	% sim.[e]	Accesssion No.[f]	Isolation source(s)[g]	Depth(s) (ft)	Geographic location(s)[h]
		834 (36.7)		**Alpha-Proteobacteria**					
1	J355	126 (5.5)	3	*Agrobacterium meteori*/D88527	99	AY362009	An, Ax, Co, Dc, Dx, Ec, Ge, Ha, Ho, My, Pa, Ph, Ps, Pt, Px, Sc, Se, Si, Su, Tn, Tr, UD, Vt	150–2429	A, Bh, Cu, GM, H, J, M, PR, TC, US, US-F
2	F813	3 (0.1)	1	*Agrobacterium tumefaciens* EBRI25/AY221181	99	AY362010	Dx, Ir, Ra	200, 1128	J, US-F
3	F921	2 (0.09)	1	*Ancylobacter* sp. DSM 1277/AY211515	99	AY362021	Ha, Po	1462, 2187	J
4	M914	1 (0.04)	1	*Azospirillum* sp. 5C/AF413109	98	AY371399	UD	2304	Bh
5[i]	S724	1 (0.04)	1	*Bartonella capreoli*/AF293389	91	AY371429	UD	1162	Cu
6	J586	376 (16.5)	4	Alpha proteobacter. MBIC3368/AB012864	99	AY364592	An, Ax, Az, Co, Dx, Ge, Ha, My, Pe, Pl, Ps, Ra, Se, Su, Tn, Tr, UD, Vt	150–2560	A, Bh, Bo, Cu, E, GM, H, J, PR, TC, VI, US, US-F
7	J345	5 (0.22)	1	*Blastomonas natatoria* strain 2.4/AJ299222	99	AY364594	Dx, Se, UD	200, 1394	Bh, US-F
8	F991	7 (0.3)	2	*Brevundimonas vesicularis*/AJ007801	99	AY364600	Co, Dc, Ho, UD	150–2236	GM, J, TC, US-F
9	S881	1 (0.04)	1	*Caulobacter crescentus* CB15/AE006011	99	AY371407	Se	1394	Bh
10	F996	9 (0.4)	2	*Caulobacter* sp. strain:MBIC1405/AB016847	99	AY367745	Ec, Ha, Ho, Px, UD	1039–2236	Cu, GM, J
11	K475	4 (0.18)	1	*Erythrobacter citreus*/AF118020	99	AY367755	Pe, Px	1162, 2013	Bo, Cu
12	F752	1 (0.04)	1	*Erythrobacter citreus* isolate HY-6/AJ294340	99	AY367756	Ir	1128	J
13	F761	62 (2.7)	3	*Erythrobacter flavus* strain SW-52/AF500005	99	AY371410	An, Ax, Az, Dc, Ec, Ge, Ha, Ho, Ir, Is, Pa, Pe, Ps, Px, Sc, Se, Si, UD, UH	250–3026	A, Ba, Bh, Bo, CV, Cu, Ga, GM, J, PR, TC, US, US-F
14	K384	4 (0.18)	1	*Erythrobacter* sp. MB-16/AF325446	99	AY367757	Ge, Pa, Px, UH	1000–1685	Bh, Cu, US
15	L259	1 (0.04)	1	*Erythrobacter* sp. AS-45/AJ391206	98	AY367758	Sc	1515	Bh
16	G265	1 (0.04)	1	*Erythrobacter* sp. MBIC4118/AB035545	98	AY371411	Pe	1110	J
17	K416	7 (0.3)	1	*Fulvimarina litoralis* HTCC2156/AY178863	96	AY368505	An, Ho, Ps, UD	692–2429	A, Bh, GM, M

18	L519	1 (0.04)	1	*Fulvimarina litoralis* HTCC2156/AY178863	94	AY371414	UD	1705	US
19	J356	3 (0.1)	2	Hydrothermal vent strain NF18/AF254107	99	AY368512	UD	1478	PR
20	J169	12 (0.5)	3	*Hyphomicrobium* sp. Ddeep-1/AB055793	98	AY368513	Ax, Ec, Ha, Ho, Pe, Ph, Se	259–2236	Bh, GM, J, PR, US-F
21	K488	4 (0.18)	4	*Mesorhizobium* sp. WG/AF156710	99	AY371420	Ca, Pa, Px	1162–2450	Cu, M
22	D701	5 (0.22)	1	*Mesorhizobium* sp. TUT1018/AB098586	100	AY368521	Ec, Pa, Px	1162–1904	Cu, J, M, US
23	R591	1 (0.04)	1	*Mesorhizobium* sp. GWS-SE-H229/AY332178	98	AY371423	Px	1162	Cu
24	P638	1 (0.04)	1	*Methylarcula* sp. BIO-24/AJ534207	97	AY368522	Sc	1513	Bh
25	E916	18 (0.8)	5	*Ochrobactrum anthropi* GH 1568/AJ276036	100	AY368533	An, Ax, Ca, Ge, Ha, Ir, Sc, UD, UH	1043–2429	Bh, Cu, J, M
26	J987	15 (0.66)	3	*Paracoccus marcusii*/AY159800	100	AY368534	Ax, Az, Ir, Pa, Px, Sc, Tn, UD	301–2815	Bh, Cu, E, H, J, TC, US-F
	M039					AY368535			
27	J364	3 (0.1)	1	*Paracoccus yeeii* strain G3060/AY014179	99	AY368536	Ha, UD	1030–1478	J, PR
28	R575	25 (1.1)	4	*Phyllobacteriaceae* bacterium NL21/AF534573	98	AY368540	An, Pm, Ps, Px	532–2322	A, Ba, Cu
29	S917	1 (0.04)	1	*Porphyrobacter* sp. KK351/AB033326	98	AY371424	Se	1394	Bh
30[i]	K018	1 (0.04)	1	*Rhizobium daejeonense*/AY341343	90	AY371436	UD	530	E
31	J211	7 (0.3)	1	*Candidatus Rhizobium massiliae*/AF531767	98	AY367744	Ax, Co, Dx, Px, Py, Su	150–1478	Cu, GM, PR, US-F
32	E913	7 (0.3)	2	*Rhizobium* sp. H-4/AF279889	98	AY368568	Po, UD	1238–2187	E, J
33	K376	1 (0.04)	1	*Roseivivax halotolerans*/D85831	96	AY368571	Sc	2128	Cu
34	J392	2 (0.09)	2	*Roseobacter gallaeciensis*/AY136134	99	AY368573	An	1525	PR
35	J486	15 (0.66)	3	*Roseobacter* sp. RED68/AY136132	96	AY368574	An, Pt, Px, Sc, Se, UD, UH	245–2980	Bh, Cu, PR, US
36	H264	6 (0.3)	1	*Roseobacter* sp. WHOI JT-08/AY349460	97	AY369978	Co, Px, Tr, UD	150–2025	Bh, Cu, GM, US-F
37	J504	4 (0.18)	2	*Roseobacter* sp. RED15/AY136124	99	AY369979	An, At	1525–2720	PR, TC
38	J483	9 (0.4)	2	*Roseobacter* sp. MED61/AY136107	100	AY369980	An, Co, Ho, Pa, Sc, UH	150–2980	Bh, GM, PR, US
39	H265	1 (0.04)	1	*Roseobacter* sp. RED1/AY136122	97	AY371428	Tr	217	US-F

Table 2. (*continued*)

Phylotype #	HBOI ID[a]	No. (%) isolates[b]	No. seq[c]	Nearest taxonomic neighbor/Accession No.[d]	% sim.[e]	Accesssion No.[f]	Isolation source(s)[g]	Depth(s) (ft)	Geographic location(s)[h]
40	J526	1 (0.04)	1	*Roseomonas genomospecies* 5/ AF533356	98	AY369981	Ha	735	PR
41	J484	8 (0.35)	1	*Ruegeria* sp. MB2/ AY005463	96	AY369983	An, Co, Px, Sc, UH	150–2815	Bh, GM, PR
42	N286	1 (0.04)	1	*Ruegeria* sp. AS-36/ AJ391197	97	AY369984	UD	245	US
43	N354	1 (0.04)	1	*Ruegeria* sp. AS-36/ AJ391197	96	AY371430	UD	245	US
44	E923	13 (0.57)	4	*Silicibacter lacuscaerulensis*/U77644	97	AY369990	Ax, Co, Ha, Sc, Su, Tn, UD, Vs	150–2187	GM, H, J, PR, US-F
45	L534	1 (0.04)	1	*Sphingomonas koreensis* JSS-26/AF131296	98	AY369992	Ax	1705	US
46	L538	3 (0.1)	1	*Sphingomonas* sp. SA-3/ AF327069	100	AY369991	Ax, Ha	1128, 1705	J, US
47	J560	2 (0.09)	1	*Sphingomonas* sp. P2/ AB091683	95	AY371451	Ha	747	PR
48	E986	5 (0.22)	3	*Stappia aggregata*/ D88520	99	AY369996	Dx, Pa	200–1685	J, US, US-F
49	F775	22 (0.97)	4	*Stappia aggregata*/ D88520	100	AY369997	An, Ax, Ca, Co, Ha, Ho, Ir, Po, Sc, Se, UD, UH	150–2905	Bh, Cu, GM, J, M, PR
50	L992	2 (0.09)	1	*Sulfitobacter pontiacus*/ AY159887	99	AF489286	Ge	1354	Cu
51	L553	6 (0.3)	1	Alpha proteobacter. MBIC3865/AB015896	100	AY362017	Ha, Pa, UD, Tr	217–1685	Bh, US, US-F
52	L801	1 (0.04)	1	Alpha proteobacter. MBIC1876/AB026194	98	AY362016	Si	1006	Cu
53	J487	1 (0.04)	1	Alpha proteobacter. NW4327/AF384141	99	AY369982	An	1525	PR
54	F820	1 (0.04)	1	Alpha proteobacter. PI_GH2.1.D7/AY162048	97	AY362018	Ha	1039	J
55	N268	1 (0.04)	1	Alpha proteobacter./ AF218241	99	AY370009	UD	245	US
56	H454	2 (0.09)	1	Marine bacterium Y4I/ AF388307	98	AY368572	Ha, Pa	259, 1685	US, US-F
57	L351	1 (0.04)	1	*Rhodobacteraceae* bacterium/AY442178	97	AY370003	Co	150	GM
58	L544	7 (0.3)	1	Marine bacterium HP29w/AY239008	98	AY370007	Co, Pa, Px, UH	150–2800	Bh, Cu, GM, US

59[i]	N272	1 (0.04)	1	Unidentified Alpha proteobacter. BD1-8/AB015520	93	AY371443	Pa	730	US
60	E172	1 (0.04)	1	*Parvibaculum lavamentivorans*/AY387398	98	AY370010	UD	2000	J
		25 (1.1)		**Beta-Proteobacteria**					
61	N317	18 (0.8)	3	*Alcaligenes faecalis* isolate 5659-H/AJ509012	100	AY362011	Ax, Co, Pa, Po, Px, Sc, UD, UH	150–2187	Cu, GM, J, US, US-F
62	L981	3 (0.1)	1	*Alcaligenes* sp. IS-18/AY346137	99	AY371437	Pe, Px, Tn	1162–2013	Bo, Cu, H
63	N123	4 (0.18)	2	*Bordetella petrii* strain DSM 12804/AJ249861	98	AY364595	Co, Ps	150–692	A, GM
		767 (33.7)		**Gamma-Proteobacteria**					
64	J332	2 (0.09)	1	*Acinetobacter calcoaceticus*/AF159045	99	AY362002	Pa, Px	1162, 1525	Cu, PR
65	E929	18 (0.8)	2	*Acinetobacter junii* DSM6964/X81664	99	AY362003	An, Ax, Az, Dc, Pa, Pl, UD	187–2590	Bh, J, TC, US-F
66	H742	25 (1.1)	2	*Acinetobacter venetianus*/AVE295007	99	AY362004	An, Ax, Dx, Pl, Sc, UD	187–2128	Bh, Cu, E, US-F
67	K649	1 (0.04)	1	*Aeromonas popoffii* LMG 17543/AJ223181	99	AY362008	UD	440	H
68	P663	3 (0.1)	2	*Alcanivorax* sp. Tak-1/AB053131	97	AY371398	Sc	1513	Bh
69	N331	14 (0.62)	4	*Alcanivorax* sp. PR-1/AB053132	99	AY362014	Co, Ha, Pa, Ps, Px, Py, Se, UH	150–2432	A, Cu, GM, M, PR, US
	K456					AY362013			
	K461					AY489287			
70	P653	2 (0.09)	1	*Alcanivorax* sp. Abu-1/AB053129	99	AY362012	Sc, UD	1394, 1513	Bh
71	D529	4 (0.18)	1	*Alcanivorax venusti* strain ISO4/AF328762	99	AY362015	Pa, UD	1394, 2450	Bh, M
72	J589	47 (2.1)	4	*Alteromonas macleodii* DSM 6062/Y18228	100	AY362020	An, At, Ax, Dc, Ge, Pa, Sc, Si, Tn, UD, UH	301–2905	Bh, Cu, CV, PR, TC, US, US-F
73	N352	2 (0.09)	2	*Alteromonas* sp. MED102/AY136118	99	AY371690	Pa, UH	730, 2980	Bh, US
74	N006	1 (0.04)	1	*Colwellia maris*/AB002630	94	AY367759	Co	150	GM
75	R675	4 (0.18)	2	*Halomonas boliviensis* strain LC2/AY245450	98	AY371415	An, Pm, Si	301–2322	Cu, US-F
76	K354	52 (2.3)	2	*Cobetia marina* KMM 734/AY628694	99	AY368511	An, Ax, Ca, Co, Ge, Pa, Ps, Ra, Sc, Si, UD	150–2450	A, Bo, CV, Cu, GM, M, PR, US, US-F
77	J436	4 (0.18)	2	*Halomonas meridiana* strain/AJ306891	99	AY368509	Az, Ha, Px, Si	1006–2000	Cu, J, PR, US

Table 2. (*continued*)

Phylotype #	HBOI ID[a]	No. (%) isolates[b]	No. seq[c]	Nearest taxonomic neighbor/Accession No.[d]	% sim.[e]	Accesssion No.[f]	Isolation source(s)[g]	Depth(s) (ft)	Geographic location(s)[h]
78	N280	2 (0.09)	1	*Halomonas* sp. MBIC2031/AB025599	99	AY368510	Pa	730	US
79	M394	1 (0.04)	1	*Halomonas ventosae*/ AY268080	97	AY371416	UD	2637	Bh
80	N362	2 (0.09)	2	*Idiomarina* sp. LA26/ AF513450	99	AY368514	Sc	2128	Cu
81	H453	1 (0.04)	1	Marine bacterium Tw-1/ AY028196	99	AY371418	Ha	259	US-F
82	F886	16 (0.7)	3	*Marinobacter lipolyticus*/ AY147906	98	AY368519	An, At, Ax, Ca, Ec, Ge, Ha, Ir, Pa, Sc, UD, UH	1128–2905	J, M, TC
83	R261	11 (0.48)	3	*Marinomonas vaga*/ X67025	97	AY368520	Ax, Ge, Sc, UD, Vt	120–2322	Bh, Cu, US-F
84	N276	21 (0.92)	3	*Microbulbifer cystodytense*/AJ620879	98	AY368556	Co, Ge, Ha, Se, Tn, UD	150–1490	Bh, GM, J, US
85[i]	N277	1 (0.04)	1	*Oceanospirillum maris hiroshimense*/AB006762	91	AY371442	Pa	730	US
86[i]	S018	1 (0.04)	1	*Oceanospirillum multiglobuliferum*/ AB006764	92	AY371422	Sc	2128	Cu
87	J246	5 (0.22)	1	*P.damselea* (wild isolate)/ X78106	99	AY368537	Ge, Ha	735–1043	Bh, PR
88	J551	8 (0.35)	1	*Photobacterium phosphoreum*/AY435156	95	AY368538	Ge, Ha, Ho, Ph, Sc	735–2264	Bh, Cu, GM, PR
89	J725	17 (0.75)	2	*Photobacterium* sp. HAR72/AB038032	96	AY368539	Ha, Ho, Pt, UD	693–1231	PR, VI
90	F925	3 (0.1)	1	*Pseudoalteromonas atlantica*/AB049728	100	AY368542	Ha, Sc, Tn	1011–2128	Cu, J, PR
91	B949	3 (0.1)	2	*A.luteoviolacea* NCIMB 1893 T/X82144	99	AY368543	UD, UH	696, 2980	Bh
92	M609	2 (0.09)	2	*Pseudoalteromonas piscicida* ATCC 15057/ X82215	100	AY371426	UD, UH	2905, 2980	Bh
93	J210	2 (0.09)	2	*Pseudoalteromonas* sp. EPR 2/AY394863	99	AY368544	Dx, Su	200	US-F
94	H720	71 (3.1)	7	*Pseudoalteromonas* sp. A28/AF227238	99	AY368545	An, Ax, Dx, Ha, My, Pa, Pt, Ra, Sc, Si, UD, UH	200–2980	Bh, CI, Cu, M, PR, US-F
95	F497	11 (0.48)	3	*Pseudoalteromonas* sp. PRLIST2/Y15323	99	AY368546	Ac, Ax, Ha, Sc, Se, UD	1016–2720	Bh, Cu, TC, US
96	G287	1 (0.04)	1	*Pseudoalteromonas* sp. KT0812A/AF239705	97	AY368547	Pe	1110	J

97	H756	1 (0.04)	1	*Pseudomonas aeruginosa* ATCC 27853/AY268175	99	AY368548	Ax	301	US-F
98	P664	1 (0.04)	1	*Pseudomonas balearica*/AF054936	99	AY368549	Sc	1513	Bh
99	J187	2 (0.09)	1	*Pseudomonas* cf. *monteilii*/AF181576	98	AY368550	Dx	200	US-F
100	J293	1 (0.04)	1	*Pseudomonas oleovorans*/D84018	99	AY368553	My	554	Bh
101	E762	8 (0.35)	1	*Pseudomonas pachastrellae*/AB125366	98	AY368557	Az, Ha, Ho, UD	259–2560	GM, J, TC, US-F
102	H786	17 (0.75)	4	*P.pseudoalcaligenes* (LMG 1225 T)/Z76666	99	AY368554	An, Ax, Ps, Ra, UD	200–692	A, Ba, US-F
	K458					AF489288			
	K433					AF489289			
103	H757	51 (2.2)	2	*Pseudomonas putida* KT2440/AE016782	100	AY368555	An, Ax, Dx, Ha, Pa, Px, Ra, Su, UD	200–2187	Ba, Bh, Cu, J, PR, US-F
104	H741	1 (0.04)	1	*Pseudomonas* sp. MBIC2027/AB030085	99	AY368558	Ax	301	US-F
105	J480	6 (0.3)	2	*Pseudomonas* sp. PB1/AF482708	98	AY368559	Co, Se, Tn, UD, Vs	150–1492	Bh, GM, H, PR, US-F
106	J451	8 (0.35)	1	*Pseudomonas* sp. CJ11064/AF500211	98	AY368552	Co, Ph, Se, Tn, UD	150–1525	Bh, GM, H, PR
107	J192	5 (0.22)	2	*Pseudomonas stutzeri* strain 28a42/AJ312165	99	AY368551	Ax, Ra	200, 254	US-F
108	E763	5 (0.22)	1	*Pseudomonas stutzeri* strain JJ/AF411219	99	AY368560	Ax, Co, UD	150–806	GM, US-F
109	M967	1 (0.04)	1	*Pseudoxanthomonas koreensis*/AY550263	99	AY368563	UD	2970	Bh
110	K512	9 (0.4)	4	*Psychrobacter pacificensis* NIBH/AB016058	99	AY368564	An, Az, Dc, Ra, Sc, UD	200–3026	Bh, Cu, H, J, PR, TC, US-F
111	K337	8 (0.35)	1	*Psychrobacter* sp. MJYP.15.12/AB094456	99	AY368565	Sc	2128	Cu
112	P672	11 (0.48)	1	*Psychrobacter submarinus* KMM 225/AJ309940	98	AY368566	Ax, Az, Ra, Sc, UD, UH	200–2815	Bh, J, PR, TC, US-F
113	R246	5 (0.22)	2	*Rheinheimera baltica* OSBAC5/AJ441082	97	AY368567	Sc, UD	112–1513	Bh, Cu
114	H411	5 (0.22)	1	*Shewanella fidelia* strain KMM3589/AF420313	99	AY369987	Ax, Ho, Ra, UD	250–2264	GM, US, US-F
115	H836	42 (1.8)	3	*Shewanella* sp. CL256/73/AF387346	99	AY369988	An, Ax, Ps, Ra, UD, UH	200–2980	A, Bh, E, PR, US-F
116[i]	H260	2 (0.09)	2	*Shewanella* sp. MR-4/AF005252	94	AY369986	Tr	217	GM
117[i]	N346	7 (0.3)	1	*Shewanella* sp. ANA-3/AF136392	92	AY371432	An, Ax, Ps, Sc, Si	200–1513	A, Bh. Cu, US, US-F
118[i]	H277	1 (0.04)	1	*Shewanella waksmanii*/AY170366	94	AY371431	Tr	217	US-F

Table 2. (*continued*)

Phylotype #	HBOI ID[a]	No. (%) isolates[b]	No. seq[c]	Nearest taxonomic neighbor/Accession No.[d]	% sim.[e]	Accesssion No.[f]	Isolation source(s)[g]	Depth(s) (ft)	Geographic location(s)[h]
119	J327	7 (0.3)	1	*Shewanella woodyi*/ AF003549	97	AY369989	An, Ax, Pa, Ph, Sc	259–2128	Cu, PR, US-F
120	F769	4 (0.18)	2	*Stenotrophomonas maltophilia* 10857/ AJ131117	100	AY369998	Ho, Ir, UD	1128–2590	Bh, GM, J, US
121	F802	11 (0.48)	3	*Stenotrophomonas maltophilia* 10989/ AJ131907	99	AY371433	Co, Ha, Ir, Po, Se, UD	150–2590	Bh, Cu, GM, J
122	P630	3 (0.1)	1	Marine bacterium Tw-3/ AY028198	96	AY362019	Ge, Sc	1043, 1513	Bh
123	H424	1 (0.04)	1	*Alteromonadaceae* bacterium BA-3/ AY643537	95	AY370004	Ax	259	US-F
124[i]	L193	1 (0.04)	1	Marine gamma proteobacterium/ AY386337	92	AY371439	Co	150	GM
125[i]	J505	1 (0.04)	1	Pseudomonas sp. YG-1/ AF441203	90	AY371435	An	1525	PR
126	H262	4 (0.18)	1	Uncultured gamma proteo. HOC27/ AB054161	96	AY370006	Pb, Tr	217, 254	US-F
127	H425	5 (0.22)	1	Uncultured gamma proteo. HOC2/AB054136	98	AY370008	Ax, Ha	259	US-F
128[i]	H433	2 (0.09)	2	Uncultured gamma proteo. HOC2/AB054136	93	AY371440	Pb	254	US-F
129	N066	1 (0.04)	1	Uncultured marine eubacterium HstpL43/ AF159674	94	AY371441	Co	150	GM
130	J462	1 (0.04)	1	*V.fisheri* (ATCC 7744 T)/ X74702	98	AY370011	Pa	1525	PR
131	J555	4 (0.18)	2	*V.mediterranei* (CIP 103203 T)/X74710	99	AY370012	Ha, Sc	259–2128	Cu, PR, US-F
132	J821	94 (4.1)	3	*Vibrio parahaemolyticus* Vp 27/AF388389	98	AY370013	An, Ax, Co, Dc, Dx, Ge, Ha, Ho, Pa, Pl, Ps, Pt, Sc, UD, UH, Vt	150–2980	A, Ba, Bh, Cu, GM, J, PR, US, US-F
133	D725	8 (0.35)	2	*Vibrio sp.* No.6/ AB089204	99	AY370015	Ax, Ha, Pa, Sc, UD	301–2128	Cu, M, PR, US-F
134	J608	20 (0.88)	3	*Vibrio* sp. NAP-4/ AF064637	99	AY371446	An, Co, Ge, Ha, Pt, Sc, Tn, UD, UH	150–2980	Bh, Cu, GM, M, PR
135	L536	1 (0.04)	1	*Vibrio* sp. 3d/AF388393	99	AY370017	Ax	1705	US
136	J684	1 (0.04)	1	*Vibrio* sp. R-14968/ AJ316168	99	AY370016	Tn	1011	PR

137	K883	1 (0.04)	1	*Vibrio* sp. LMG 20547/AJ316202	99	AY371447	Ge	1043	Bh
138	J312	1 (0.04)	1	*Vibrio* sp. OC25/AB038026	98	AY371448	Ph	1525	PR
139	J252	40 (1.76)	1	*Vibrio splendidus* biovar II/AB038030	99	AY370018	Ax, Ge, Ha, Ho, Pa, Pl, Pt, Ra, UD	187–2450	Bh, M, PR, US-F
140	H412	1 (0.04)	1	*Vibrio splendidus* strain 636/AY620972	99	AY370014	Ax	259	US-F
		122 (5.4)		**Cytophaga/Flavobacteria/Bacteroides (CFB)**					
141	K413	8 (0.35)	1	*Aequorivita ferruginea* SW49 T/AY027802	94	AY362005	Ps, Px	692, 1162	A, Cu
142	L979	55 (2.4)	2	*Bacteroidetes* bacterium GMDsbC3/AY162097	99	AF486815	An, At, Az, Ca, Dc, Ge, Pa, Pe, Se, UD, UH	1110–2970	Bh, Bo, J, M, TC
	M775					AY517542			
143[i]	A973	1 (0.04)	1	*Bacteroidetes* bacterium GMD16C10/AY162109	95	AY371406	An	532	Ba
144	H406	1 (0.04)	1	*Cytophaga* sp. I-377/AB073588	96	AY367750	Ax	259	US-F
145	K429	1 (0.04)	1	*Flavobacterium mizutaii* DSM 11724 T/AJ438175	99	AY367760	Ps	692	A
146[i]	R550	11 (0.48)	2	*Flavobacterium mizutaii* DSM 11724 T/AJ438175	90	AF489284	Ax, Pa, Ps, Px, Si, UD	692–2970	A, Bh, Cu, US
147	R564	1 (0.04)	1	*Flavobacterium* sp. V12.MO.200.17/AJ244699	100	AY367761	Si	1006	Cu
148	L303	1 (0.04)	1	*Flavobacterium* sp. 5N-3/AB017597	92	AY371412	Co	150	GM
149	F981	1 (0.04)	1	*Flexibacter aggregans* IFO 15974/AB078038	94	AY367762	Ec	1807	J
150[i]	S923	1 (0.04)	1	*Flexibacter aggregans* IFO 15974/AB078038	92	AY371438	Se	1394	Bh
151	E966	13 (0.57)	3	Marine bacterium MBIC1357/AB032514	99	AY368517	Ax, Ec, Ha, Sc, UD, Vt	1039–2187	Cu, J
152[i]	J873	1 (0.04)	1	Marine bacterium KMM 3937 (*Mesonia algae*)/AF536386	94	AY371419	Ho	1231	PR
153	K383	7 (0.3)	2	Marine CFB-group bacterium MBIC01599/AB086624	99	AF489285	Ha, Ps	692, 735	A, PR
	K439					AY367763			
154[i]	R634	7 (0.3)	2	Marine bacterium SCRIPPS_413/AF359548	94	AY371445	Px, Si	1006, 1162	Cu
155[i]	G847	7 (0.3)	2	*Flavobacteriaceae* bacterium/AY298788	94	AY368518	Ha, Ph, Se	886–2144	PR, TC

Table 2. (*continued*)

Phylotype #	HBOI ID[a]	No. (%) isolates[b]	No. seq[c]	Nearest taxonomic neighbor/Accession No.[d]	% sim.[e]	Accesssion No.[f]	Isolation source(s)[g]	Depth(s) (ft)	Geographic location(s)[h]
156	R966	1 (0.04)	1	*Flexibacteraceae* bact. KMM 6017/AY608410	99	AY371413	UD	110	Bh
157	J879	5 (0.22)	1	Uncultured CFB clone CD3D3/AY038388	99	AY370005	Ax, Co, Sc, Se, UD	150–1705	Bh, GM, PR, US
		327 (14.4)		**Gram + high GC content (Actinobacteria)**					
158	J012	8 (0.35)	1	*Aeromicrobium erythreum*/AF005021	97	AY362006	Ax, Co, Ec, Pa	150–1807	GM, J, US-F
159	J562	2 (0.09)	1	*Aeromicrobium erythreum*/AF005021	96	AY362007	Ax, Ha	301, 747	PR, US-F
160	K473	10 (0.44)	1	*Brachybacterium paraconglomeratum*/ AJ415377	99	AY364596	Ca, Co, Px, Tn, UD, UH	150–2800	Bh, Bo, Cu, GM, H, M
161	R604	2 (0.09)	1	*Brevibacterium avium* NCFB 3055/Y17962	94	AY364597	Px, UD	1162, 2970	Bh, Cu
162	J935	54 (2.4)	4	*Brevibacterium casei* (NCDO 2048)/X76564	97	AY364598	An, Ax, Co, Ha, Pa, Pe, Pm, Ps, Px, Sc, Si, Tn, UD, UH	150–2450	A, Bh, Bo, Cu, GM, H, M, PR, US
163	N311	4 (0.18)	1	*Brevibacterium casei* (NCDO 2048)/X76564	96	AY364599	Ax, Pa, UD	250–2102	Cu, US
164	R659	1 (0.04)	1	*Cellulomonas* sp. X7/ AF060791	96	AY367746	Px	1162	Cu
165	F781	7 (0.3)	2	*Cellulosimicrobium cellulans*/AB116667	99	AY367747	Ax, Ec, Ge, UD, UH	250–2905	Bh, J, M, US
166	R603	2 (0.09)	1	*Corynebacterium nigricans* 92-0360/ AF537608	99	AY367748	Si	1006	Cu
167	N138	2 (0.09)	2	*Corynebacterium* sp./ AF322369	99	AY367749	Co, Si	150, 1006	Cu, GM
168	L560	1 (0.04)	1	*M.nishinomyaensis*/ X87757	99	AY367751	Ha	1016	US
169	E241	9 (0.4)	2	*D.maris* (DSM 43102)/ X79291	99	AY367752	Ge, Pa, Tn, UD	1056–2956	Bh, E, H, M
170	J970	16 (0.7)	1	*Dietzia* sp. R32/Y08318	99	AY367753	Co, Ec, Ha, Ir, UD	150–1807	E, GM, J
171	F148	1 (0.04)	1	*Dietzia* sp. CIP104293/ Y08313	98	AY371409	Ne	2450	Bh
172	F867	1 (0.04)	1	*G.terrae* (DSM 43249)/ X79286	99	AY368506	UH	1807	J
173	J855	1 (0.04)	1	*Gordonia*-like sp. (strain J81)/X85244	100	AY368507	Ha	747	PR

174	B181	1 (0.04)	1	*Kocuria* sp. 2216.35.31/AB094467	98	AY371417	Ho	2236	GM
175	K372	15 (0.66)	3	*M.sedentarius*/X87755	99	AY368515	Co, Ge, Ir, Pa, Px, Si, UD, UH	150–2956	Bh, Cu, GM, J, M
176	D704	8 (0.35)	1	*Leucobacter komagatae*/AB007419	99	AY368516	An, Pa, Px	1162–2429	Cu, M
177	K454	22 (0.97)	2	*Microbacterium aerolatum*/AJ309929	98	AF489290	Ax, Ec, Ho, Pm, Ps, Px, Si, UD	250–2450	A, Cu, GM, J, M, US, US-F
178	L806	2 (0.09)	1	*Microbacterium foliorum* DSM 12966/AJ249780	97	AY368523	Pm, Px	1162, 2322	Cu
179	L262	1 (0.04)	1	*Microbacterium oleovorans*/AJ698725	99	AY368524	Sc	1515	Bh
180	E920	16 (0.7)	1	*Microbacterium oxydans*/Y17227	98	AY368525	Co, Ir, Pa, Po, Px, UD	150–2450	Cu, GM, J, M
181	F873	1 (0.04)	1	*Microbacterium paraoxydans* CF36/AJ491806	99	AY367754	UD	2187	J
182	K463	31 (1.36)	1	*Microbacterium* sp. VA22800_00/AF306835	96	AY368526	An, Dc, Ha, Pa, Pm Ps, Px, Sc, Si	532–2450	A, Ba, Bh, Cu, J, M
183	R535	1 (0.04)	1	*Xylanomicrobium cellulosilyticum*/AY062021	94	AY371450	Px	1162	Cu
184	K184	47 (2.1)	3	*Micrococcus luteus* SAFR-002/AY167858	99	AY371421	An, Ax, Az, Ca, Co, Dc, Ge, Ha, Pa, Pe, Sc, Tn, UD, UH	150–2815	Bh, Cu, E, GM, H, J, M, TC, US
185	J921	4 (0.18)	1	*Micrococcus luteus* HAMBI2408/AF501366	99	AY368527	Ge, Sc, UD, UH	624–1513	Bh, Cu, PR
186	H775	5 (0.22)	1	*M.halophytica* isolate DSM 43171/X92601	99	AY368528	Ax, Ho	277–2264	GM, US-F
187	L656	1 (0.04)	1	*Micromonospora* sp. N0093/AY221490	96	AY368529	Ha	1016	US
188	J313	3 (0.1)	1	*Mycobacterium manitobense*/AY082001	96	AY368530	Ph	1525	PR
189	J380	2 (0.09)	1	*N.alborubida*/X97882	99	AY368532	Py	1478	PR
190	R529	4 (0.18)	3	*Nocardiopsis metallicus* strain R2A/AJ420769	99	AY368531	Pt, Px, Si	1006–1231	Cu, PR
191	B951	7 (0.3)	2	*Pseudonocardia alni* IMSNU 20049 T/AJ252823	98	AY368561	Ax, Ha, Px, Ra, UD	254–1162	Bh, Cu, J, US-F
192	J561	3 (0.1)	1	*Pseudonocardia kongjuensis*/AJ252833	99	AY368562	Ec, Ha, Px	747–1807	Cu, J, PR
193	K004	2 (0.09)	1	*R.opacus*/X80630	96	AY368569	UD	747, 1238	E, PR
194	F786	1 (0.04)	1	*Rhodococcus ruber* M2/AY247275	100	AY368570	Ir	1128	J
195	L793	5 (0.22)	1	*Salinospora* sp. CNH646/AY040620	99	AY369985	Ax, Px, Si, UD	1006–2550	Bh, Cu, US

Table 2. (*continued*)

Phylotype #	HBOI ID[a]	No. (%) isolates[b]	No. seq[c]	Nearest taxonomic neighbor/Accession No.[d]	% sim.[e]	Accesssion No.[f]	Isolation source(s)[g]	Depth(s) (ft)	Geographic location(s)[h]
196	D721	1 (0.04)	1	*Streptomyces* sp. FXJ23/ AY314785	97	AY369999	Pa	1904	M
197	L732	13 (0.57)	1	*Streptomyces* sp. 40005/ AY295793	99	AY370000	Ax, Co, Ha, Pa, Py, UD	150–2800	Bh, Cu, GM, M, PR, US
198	J379	1 (0.04)	1	*Streptomyces* sp. YNUCC0233/AY552754	100	AY371434	Py	1478	PR
199	M618	1 (0.04)	1	*Terrabacter* sp. YK7/ AB070460	96	AY370001	UH	2800	Bh
200	K366	8 (0.35)	1	*Tsukamurella pulmonis*/ AF001011	99	AY370002	An, Pm, UH	1000–2429	CI, Cu, M
		198 (8.7)		**Gram+ low GC content (Firmicutes)**					
201	P313	46 (2)	1	*Bacillus benzoevorans*/ AY043085	99	AY364581	An, Ax, Ca, Ge, Ha, Pa, Se, Si, UD, UH	696–3002	Bh, Bo, Cu, J, M
202	D727	9 (0.4)	1	*Bacillus cereus* strain F 528/94/AJ577291	99	AY364589	Ax, Ha, Pa, UD, UH	301–2905	CI, J, M, PR, US-F
203	S942	1 (0.04)	1	*Bacillus decolorationis*/ AJ315075	97	AY371401	Se	1394	Bh
204	M608	2 (0.09)	1	*B.firmus*/X60616	99	AY364582	Se, UH	1394, 2800	Bh
205	H761	2 (0.09)	1	*Bacillus gibsonii*/ AB111933	100	AY364583	Ax	301	US-F
206	B126	1 (0.04)	1	*Bacillus macroides*/ AF157696	99	AY364585	Ho	2264	GM
207	L795	1 (0.04)	1	*B.methanolicus*/X64465 S42879	95	AY364586	Si	1006	Cu
208	E051	6 (0.3)	1	*Bacillus niacini*/ AB021194	99	AY364587	Co, Ha, Pa	150–1164	GM, J
209	H762	2 (0.09)	1	*Bacillus pumilus* strain KL-052/AY030327	99	AY364588	Ax	301	US-F
210	J383	10 (0.44)	2	*Bacillus* sp. MK03/ AB062678	98	AY371403	Pa, Ph, Sc, Se, Si	1006–2631	Cu, Ga, J, PR
211	H819	4 (0.18)	1	Bacterium str. 47083/ AF227837	99	AY364591	Ax, Ho, Pa	301–2264	GM, J, PR, US-F
212	J357	1 (0.0.4)	1	*Bacillus* sp. 98TH11316/ AY159884	97	AY371404	Pa	1525	PR
213	B940	49 (2.15)	3	*Bacillus* sp. N6/ AB043854	100	AY364590	Ac, An, Az, Co, Dc, Pa, Pe, Sc, Tn, UD	150–2956	Bh, Cu, GM, J, US, US-F
214	K396	2 (0.09)	2	*Bacillus* sp. AS-38/ AJ391199	98	AY371405	Sc	2128	Cu
215	F804	8 (0.35)	7	*Bacillus anthracis* Ames/ AE017025 AE016879	99	AY371400	Ec, Ha, Ho, Ir, UD, UH	1128–2905	GM, J

216	L794	1 (0.04)	1	*Bacillus* sp. KMM 3737/AY228462	99	AY364584	Si	1006	Cu
217[i]	K373	1 (0.04)	1	*Bacillus* sp. BH030062/AY553296	96	AY371402	UH	1000	Cu
218	D516	1 (0.04)	1	*Bacillus vietnamensis*/AB099708	99	AY371689	Ge	2427	M
219	H432	1 (0.04)	1	*Halobacillus* sp. MO56/AY553123	97	AY368508	Pb	254	US-F
220	H184	1 (0.04)	1	*Planococcus rifitiensis*/AJ493659	98	AY368541	Az	2560	TC
221	J318	33 (1.45)	1	*Staphylococcus haemolyticus*/X66100	99	AY369993	Ax, Co, Dx, Ge, Ha, Ir, Pa, Ps, Py, Sc, Si, Tn, UD, UH	150–2187	A, Bh, Cu, GM, H, J, PR, US, US-F
222	G779	5 (0.22)	3	*Staphylococcus pasteuri* ZA-b3/AF532917	99	AY369994	Dc, UH	2003, 3002	Bo, TC
223	J688	7 (0.3)	2	*Staphylococcus warneri* gene/Z26903	100	AY369995	Ha, Ho, Si, Su, UD	200–2236	Cu, CV, GM, J, PR, US-F
224	G304	4 (0.18)	3	Unidentified Hailaer soda lake bact. Z8/AF275715	98	AY364593	Pa, Pl, Tn	187–1490	J, US-F

[a]HBMMCC identification number of the isolate(s) sequence submitted to GenBank.
[b]Number (frequency) of isolates belonging to each phylotype.
[c]Number of isolates sequenced per phylotype.
[d]Closest GenBank taxonomic match.
[e]% similarity to closest GenBank match.
[f]GenBank Accession Number of the HBMMCC isolate.
[g]Abbreviations as defined in Table 1.
[h]Aruba (A), Barbados (Ba), Bahamas (Bh), Bonaire (Bo), Canary Islands (CI), Curacao (Cu), Cape Verde (CV), Ecuador (E), Galapagos (Ga), Gulf of Mexico (GM), Honduras (H), Jamaica (J), Madeira (M), Puerto Rico (PR), Turks and Caicos (TC), US Virgin Islands (VI), USA (US), and Florida (US-F).
[i]Isolate for which full-length sequence was obtained.

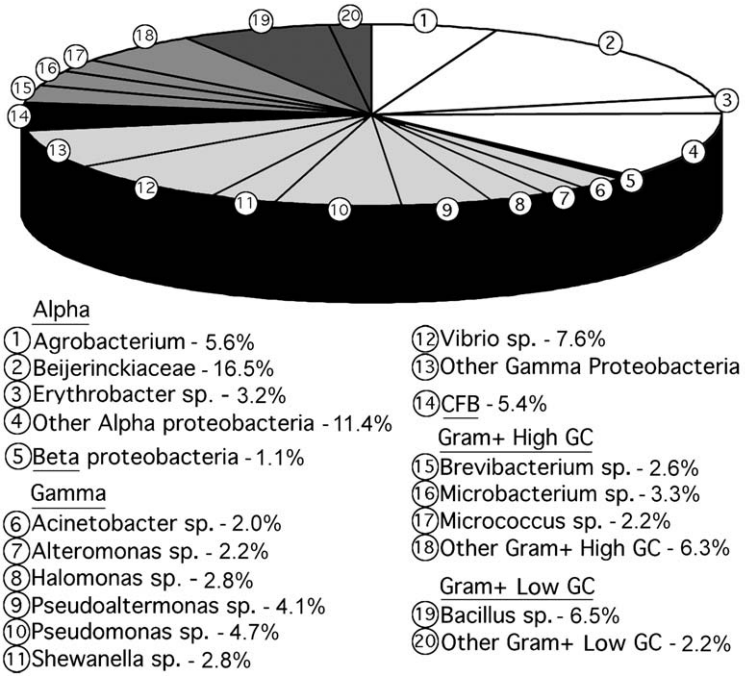

Fig. 2. Taxonomic distribution of the 2273 bacterial isolates inventoried by ARDRA in the present study. Shading reflects six major eubacterial subdivisions. For example, α-Proteobacteria (white) and γ-Proteobacteria (light gray) represent roughly 37% and 34% of the total survey, respectively. Common eubacterial groups in each pie slice are numbered as follows: α-Proteobacteria (1–4), β-Proteobacteria (5), γ-Proteobacteria (6–13), CFB (14), Gram + /high GC (15–18), Gram + /low GC (19–20). Percentages reflect the proportion of each respective group in this study.

Up to seven 16S rRNA gene products were sequenced from each phylotype to further verify that identical RFLP patterns also had the same closest rRNA sequence identity match (from database queries). We analyzed sequences from multiple isolates within five specific phylotypes by aligning and generating uncorrected pairwise distance matrices. Our preliminary results of *Alcanivorax* (phyotype 69, $n = 4$, avg. dist. = 0.61%), *Bacillus* (phylotype 215, $n = 6$, avg. dist. = 0.44%), *Ochrobacterium* (phylotype 25, $n = 4$, avg. dist. = 0.79%), *Pseudoaltermonas* (phylotype 94, $n = 5$, avg. dist. = 3.8%), and *Pseudomonas* (phylotype 102, $n = 4$, avg. dist. = 1.6%) sequences, showed that most pairwise distances were relatively low within a phylotype (<1.0%), with an average distance of 1.47% (range 0–5.3%) among all members of all groups. Many of the observed substitutions occurred near sequence termini, and can be attributed to poor alignments or base-calling near the primer sequences. However, *Pseudomonas* and *Pseudoalteromonas* sequences did appear to have a greater number of substitutions further downstream, thus generating the highest within-phylotype diversity among all groups examined. Both of these γ-Proteobactera genera encompass a large number of species, which might not be distinguishable using ARDRA and 16S rRNA sequence analysis alone. Overall, these data support the consistent phylotype grouping by ARDRA patterns.

Interesting trends among identified isolates

The 2273 microbial isolates were derived from at least eight different orders and 26 families of Porifera, plus sediment and non-poriferan samples [31] (Table 1). Since the taxonomy of some invertebrate specimens was subject to revisions after initial collections, distribution of microbes among host taxa was not always uniform. Overall, sponge orders Astrophorida (303 isolates), Halichondrida (438 isolates), Lithistida (359 isolates), and Poecilosclerida (363 isolates) yielded the largest numbers of isolates, while 215 microbes from non-Poriferan samples were included in this study.

Isolates most closely similar to the bacterial genus *Bacillus* (Table 2, phylotypes 201–218) and the phylotype most similar to an unidentified Alpha proteobacterium (phylotype 6) appeared to be readily culturable from most geographic locations as well as ⩾50% of the invertebrate hosts with more than two bacterial phylotypes. Phylotype 6 isolates (Table 2) comprised 376 (16.5%) of the surveyed isolates. The closest current sequence match of 99% in GenBank only provided taxonomic identification to the family level (*Beijerinckiaceae*).

In general, the composition of cultured isolates varied considerably between each source. For example, γ-Proteobacteria isolates dominated (approximately 88%) the culturable isolates from the sponge family

Raspailiidae, while α-Proteobacteria comprised about 80% of all surveyed eubacteria from the sponge family Theonellidae.

Eleven different HBMMCC phylotypes showed only ⩽93% similarity to the top GenBank database match after full-length sequencing (phylotypes 5, 30, 59, 85, 86, 117, 124, 125, 128, 146, and 150), while sequences from six different phylotypes most closely matched to previously "uncultured" bacterial taxa (phylotypes 59, 126–129, and 157). Although there are exceptions to the rule, in general, bacteria are considered different species if they share less than 97.5% 16S rRNA sequence similarity and members of different genera if they share lower than 93% sequence similarity [37,52]. Interestingly, the Cytophaga, Flavobacteria, and Bacteroides (CFB) clade contained a high proportion of interesting phylotypes. For example, 3 of the 17 total CFB phylotypes (including phylotypes 146, 148, and 150) had ⩽93% sequence similarity to the closest GenBank BLAST match. Although only partial 16S rDNA sequence data (772 bp) could be obtained for isolate L303 (phylotype 148), the sequence was only 92% similar to the closest GenBank match.

Phylogenetic analysis

Phylogenetic analysis was conducted primarily to identify the major eubacterial subdivisions (clades) in the HBMMCC, not necessarily to define specific relationships among all 224 phylotypes. The substitution model, Tamura and Nei, with a gamma distribution and invariable sites (TN + G + I), was chosen by MODELTEST for the SSU rRNA dataset: (a) all gaps omitted, or (b) only those gaps at selected hypervariable regions (see Methods). Genetic distances among taxa calculated with the Tamura Nei model [41] were fairly high and ranged from 0.02 to 0.9. Thus, the Tamura-Nei distance tree in Fig. 3 shows a representative subset of 54 phylotypes and the recapitulation of six major eubacterial subdivisions present in this survey. Similar topologies were generated with parsimony analyses on one dataset (all gaps omitted) and generally conformed to current eubacterial phylogenies [24,27], indicating robustness. Multiple low G + C Gram-positive isolates were used to root the phylogeny. β- and α-Proteobacteria separated into their own clades with 99% and 85% bootstrap support, respectively. Two representative "sponge symbionts" clustered together within the γ clade (phylotypes 126 and 127). The CFB sequences also form a distinct clade with 100% bootstrap support, containing a high number of unique and diverse isolates, some with long branch lengths such as R550 (phylotype 146) and J873 (phylotype 152). Some divergence may be a result of geographic separation (e.g. A973 and M775 from different Caribbean locations). The tree also includes several other unique isolates dispersed among multiple clades, such as previously uncultured bacteria H262 (Gamma), J879 (CFB) and N066 (Gamma). A more comprehensive phylogenetic analysis of these cultured phylotypes will be combined with various sponge-microbe, culture-independent derived sequences in a separate study.

Discussion

Previously described deep-sea marine microbes

The current profile of microbial SSU rRNA sequences from the HBMMCC gives a glimpse into the potential yield of the largest, and relatively unexplored habitat on earth (e.g. the ocean below 1000 m) [66]. Published accounts of the isolation and culture of deep-sea microorganisms stem mostly from marine sediments [9,55], hydrothermal vents [56], and seawater [4,17,32], but rarely from marine invertebrates [20,28,67]. To date, eubacteria isolated from deep-sea environments predominantly fall within the γ subclass of the *Proteobacteria* clade, and specifically within the genera *Shewanella*, *Mortiella*, *Colwellia*, *Photobacterium*, *Psychrobacter*, and *Pseudomonas* [5,17,34] as well as several species of Actinobacteria that have been selectively cultured for from marine sediments [9]. Taxonomic analyses of deep-sea microbial culture collections are rare [34,55]. Therefore, to date, this study represents one of the largest taxonomic inventories of culturable marine microbes ever conducted.

Efficacy of 16S rDNA sequencing in this study

A principal aim of this project was to develop a rapid screening protocol for the identification of the thousands of microbial isolates currently contained in the HBMMCC. Partial sequences were therefore used that encompass hypervariable regions of the eubacterial 16S rRNA gene that would both satisfy the requirement for a rapid screen (i.e. one sequencing run) as well as a sufficient taxonomic identity at least to the genus level. The isolates that were ⩽93% similar to the closest GenBank sequence match with the Ecoli9/Loop27rc partial sequence were also sequenced with the SEQmidwayCG-F/1492R primers to obtain nearly full-length contiguous sequences (18 isolates, Table 2). In general, most full-length rRNA sequence identities did not differ from data utilizing only the 5′ half of the rRNA gene, except that similarities increased by 1–2% similarity. However, this was expected since the region amplified by the SEQmidwayCG-F/1492R primers also contains several highly conserved regions [40]. Sequence matches did not deviate from the major clades (Alpha, Beta, Gamma, CFB, etc.) for any of the 18 isolates with full-length sequences.

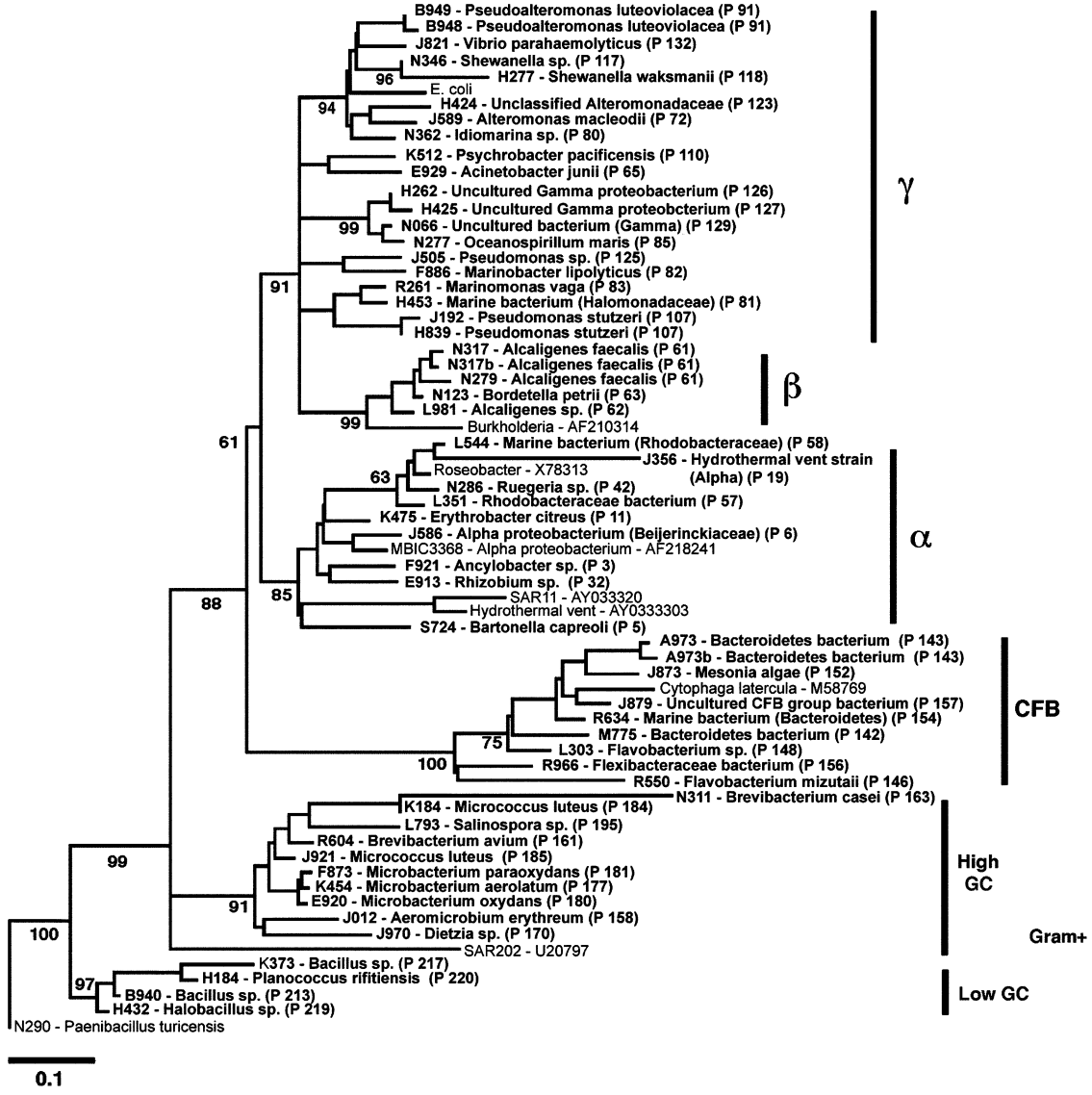

Fig. 3. Tamura-Nei phylogeny of 16S rRNA SSU sequences from 54 representative phylotypes of the HBMMCC. Reference sequences for each major clade are labeled with their respective GenBank accession number. The GenBank accession number of *E. coli* is V00348. Some sequences are replicated. A total of 676 nucleotides were used in the final reconstruction, with base frequencies of A (0.242), C (0.207), G (0.294), and T (0.255). The gamma-shaped parameter of 0.88, with 0.22 invariable sites, was used with the Tamura-Nei substitution model [41]. Bootstrap percentages $>50\%$ after 500 replications are shown at the nodes.

Efficacy of ARDRA in this study

The use of restriction enzymes is a proven method for rapidly screening stretches of nucleic acids for genetic variation [1,14,61]. In this study, RFLP of the 16S SSU rRNA gene successfully inferred 224 phylotypes from 2273 bacterial isolates, and was followed by DNA sequencing to confirm a distinct sequence match in subsequent GenBank database queries. Therefore, roughly one out of every 10 isolates surveyed from the HBMMCC had a unique combination of RFLP patterns for all three enzymes. Also, whenever multiple isolates from a single phylotype (designated by a specific set of RFLP patterns) were sequenced, the respective isolates were nearly identical. The analysis of within-phylotype 16S rRNA variation indicated relatively low 16S rRNA diversity as expected, supporting the capability of consistent grouping by ARDRA patterns. However, the results may also be taxa-specific, or be dependent on the geographic origin of each isolate in the group. For example, within the *Pseudomonas* phylotype 102, sequence variation followed the disparate geographic sources of each isolate—Florida (e.g. H673) or Aruba (K458). We acknowledge that 16S rRNA is not the best marker for determining within-phylotype diversity, and so more extensive pursuit of this question

was not performed here but rather should rely on other more variable loci or methods [3,5].

"Culturable" marine microbes

Since recombinant DNA technologies have made the isolation of individual 16S rRNA gene molecules from total environmental DNA possible [68], current research on marine microbes is highly biased towards "culture-independent" analyses of uncultured species. Although studies on unculturable microbes provide a more realistic estimate of microbial diversity in the natural environment [10], and our laboratory has an ongoing study of uncultured 16S rRNA sequences from various sponges, rRNA only provides genotypic information, which cannot reproduce the actual organism itself.

Previous studies to date have shown that culturable marine microbes from seawater fall predominantly within the γ subclass of the *Proteobacteria* clade [18,24]. This may be due to the finding that ZoBell's marine agar 2216 and other common bacteriological media selectively isolate Gram-negative chemoorganotrophs of the γ-Proteobacteria [24,42]. The results of this taxonomic survey differ from published research in that (i) the cultured HBMMCC microbes surveyed to date are dominated by members of the α subclass of *Proteobacteria* and (ii) the HBMMCC contains a high proportion of Gram+ microbial members (Fig. 2).

Although α-Proteobacteria have been reported as relatively uncommon in culture collections from seawater [24], recent studies have shown that some marine invertebrates can harbor, or be dominated by, members of this clade [6,64]. In fact, Webster and Hill [64] reported numerical dominance of an α-Proteobacterium designated strain NW001 (GenBank Accession # AF295099) in the sponge *Rhopaloeides odorabile*. This strain is almost identical to the 376 isolates designated as phylotype 6 in this study. At 16.5% of the 2273 isolates surveyed, this α-Proteobacteria-like phylotype was by far the most common bacterial isolate in the HBMMCC. Furthermore, α-Proteobacteria have been shown to be numerically dominant in the water column using culture-independent molecular techniques [21,25,62]. Likewise, members of the Gram+ taxa, and especially members of the Actinobacteria, can represent sizeable portions (17–30%) of the culturable (and unculturable) microbial associates of marine sponges [30,50,65]. The results of this study support these findings by showing that 23% of the isolates inventoried were Gram+ and roughly 14% were members of the Actinobacteria. Unique Actinobacteria, such as members of the genera *Rhodococcus*, *Dietzia*, *Gordonia*, *Corynebacterium*, and *Mycobacterium*, have been previously isolated from deep-sea environments [9]. The HBMMCC contains phylotypes with close sequence similarity to all of these genera (Table 2) as well as isolates similar to *Aeromicrobium*, *Brachybacterium*, *Brevibacterium*, *Cellulomonas*, *Dermacoccus*, *Kocuria*, *Kytococcus*, *Leucobacter*, *Microbacterium*, *Micrococcus*, *Micromonospora*, *Nocardiopsis*, *Pseudonocardia*, *Salinospora*, *Streptomyces*, *Terrabacter*, and *Tsusamurella.* Interestingly, although Actinobacteria comprised a major portion of isolates from several of the host invertebrates (such as cnidarians and members of the lithistid sponge family Siphonidiidae), by comparison no members of the Actinobacteria were found among the 38 isolates characterized from sediment samples (Table 2).

Patterns associated with cultured isolates

Although only a small fraction (roughly 13.4% or 2273 out of 17,000) of the isolates maintained in the HBMMCC have been taxonomically surveyed in this study, preliminary patterns appear with respect to the distribution of cultured microbes. For example, the major marine prokaryotic groups, such as the γ-Proteobacteria and, to a lesser extent, members of the CFB and α-Proteobacteria clades, are believed to have "cosmopolitan" distributions in the open ocean [19,24]. The most widely distributed phylotype genera in this study matches closest to *Beijerinckiaceae* (phylotye 6), *Erythrobacter* (phylotypes 11–16), *Bacillus* (phylotypes 201–218), and *Staphylococcus* (phylotypes 221–223). Members of the *Bacillus* and *Erythrobacter* genera are readily cultured from the marine environment [18,24]; however, none of the four taxa are necessarily known to be "widely" distributed throughout the oceans.

The fact that isolates closely related to *Bacillus* and *Staphylococcus* were found to be widely distributed among deep sea marine invertebrates undoubtedly raises the question of whether these isolates were derived from anthropogenic sources. Every effort was made to ensure that the specimens remained free of contamination prior to plating; however, it is possible that some of the marine specimens were contaminated with bacteria from sources such as the submersibles and divers involved in the collection process. We have kept these isolates in the analysis since we cannot determine, at this time, whether all or some are true members of the microbial flora of these invertebrates.

The question of host-specific symbiosis is beyond the scope of the current study, but other interesting associations will likely appear upon more extensive analyses that include a larger sampling of specific hosts, empirically varying culture conditions, and comparisons with culture-independent studies, which will enhance the value of present data in the future.

Interesting microbes of the HBMMCC

As Table 2 and the accompanying online database show, many interesting eubacterial taxa occur in this cross-sectional survey of the HBMMCC. These include previously uncultured, unidentified, and potentially "symbiotic" microbes. At least three different previously designated "sponge symbionts" within the γ-Proteobacterium clade (Table 2, phylotypes 126–128) now occur in the HBMMCC. Also, almost 1 out of every 10 HBMMCC isolates showed a different phylotype.

Although representing only a small proportion of the collection ($\sim$1%), the cultivation of several β-Proteobacteria similar to *Bordetella petrii* (phylotype 63) and *Alcaligenes faecalis* (phylotype 61) is interesting since β-Proteobacteria are generally not common in marine microbe collections [3]. Since some of these isolates were obtained from relatively shallow waters (150 fsw), a terrestrial origin is possible. Also, although *Alcaligenes* taxonomy can be problematic, all of the isolates identified as β-Proteobacteria formed a strong clade (Fig. 3).

In comparison, a considerable number of unique CFB-like members have been isolated in the HBMMCC (Table 2). These microbes are known for possible adaptations to cold oceans and deep seas [56].

Impact and future outlooks

Overall, full-length sequences from 11 different phylotypes were $\leqslant$93% similar to the closest GenBank database match and sequences from six different phylotypes were most closely matched to previously uncultured bacterial species. Furthermore, members of the genera *Ancylobacter*, *Blastomonas*, *Roseivivax*, *Roseomonas*, *Bordetella*, *Pseudoxanthomonas*, *Leucobacter*, *Pseudonocardia*, *Terrabacter*, and *Tsukamurella* are common terrestrial, freshwater, or pathogenic bacteria, which have rarely, if ever, been isolated from the marine environment. Since only a small percentage (0.1–1.0%) of microbial taxa can currently be cultured from the environment, virtually every niche of the oceans (e.g. shallow water, deep water, sediment, etc.) still serves as a potential source of novel marine microorganisms.

Biodiversity surveys of other microbial communities, such as cyanobacteria, Archaea, fungi, and most likely protozoans, known to be harbored by the $>$7000 marine sponge species [31], will likely continue to yield a rich catalogue of eubacteria, which were the subject of this study. Overall, this work has provided a substantial and important glimpse of the culturable microbial diversity found within marine invertebrates in the deep-sea environment.

Acknowledgements

This material is based upon work supported by the National Science Foundation under Grant No. DEB-0103668 to JVL and PJM. Any opinions, findings, and conclusions or recommendations expressed in this material are those of the authors and do not necessarily reflect the views of the National Science Foundation. This research was also supported by a Gertrude E. Skelly Charitable Foundation graduate fellowship to KSS. We thank Christine Politz, Katie Olds, Nicolas Joannin, Kathleen Janda, Dr. Amy Wright, and John Reed, and Harbor Branch Oceanographic Institution for their assistance and support. The manuscript was improved by comments on early drafts by Cheryl Peterson and Dr. Ute Hentschel, Dr. Wolfram Bruck and Dr. Robert Thacker. This manuscript is Harbor Branch Oceanographic Institution contribution HBOI #1569.

References

[1] S.G. Acinas, F. Rodriguez-Valera, C. Pedros-Alio, Spatial and temporal variation in marine bacterioplankton diversity as shown by RFLP fingerprinting of PCR amplified 16S rDNA, FEMS Microb. Ecol. 24 (1997) 27–40.

[2] S.F. Altschul, T.L. Madden, A.A. Schaffer, J. Zhang, Z. Zhang, W. Miller, D.J. Lipman, Gapped BLAST and PSI-BLAST: a new generation of protein database search programs, Nucleic Acids Res. 25 (1997) 3389–3402.

[3] O. Béjà, M.T. Suzuki, J.F. Heidelberg, W.C. Nelson, C.M. Preston, T. Hamada, J.A. Eisen, C.M. Fraser, E.F. DeLong, Unsuspected diversity among marine aerobic anoxygenic phototrophs, Nature 415 (2002) 630–633.

[4] I. Brettar, R. Christen, M.G. Hofle, *Rheinheimera baltica* gen. nov., sp. nov., a blue-coloured bacterium isolated from the central Baltic Sea, Int. J. Syst. Evol. Microbiol. 52 (2002) 1851–1857.

[5] A.T. Bull, A.C. Ward, M. Goodfellow, Search and discovery strategies for biotechnology: the paradigm shift, Microbiol. Mol. Biol. Rev. 64 (2000) 573–606.

[6] W.J. Burnett, J.D. McKenzie, Subcuticular bacteria from the brittle star *Ophiactis balli* (Echinodermata:Ophiuroidea) represents a new lineage of extracellular marine symbionts in the alpha subdivision of the class *Proteobacteria*, Appl. Environ. Microbiol. 63 (1997) 1721–1724.

[7] B.J. Campbell, J. Christian, J.E. Kostka, G.W. Luther, S.C. Cary, Growth and phylogenetic properties of novel bacteria belonging to the Epsilon subdivision of the *Proteobacteria* enriched from *Alvinella pompejana* and deep-sea hydrothermal vents, Appl. Environ. Microbiol. 67 (2001) 4566–4572.

[8] J. Cho, S. Kim, Increase in bacterial community diversity in subsurface aquifers receiving livestock wastewater input, Appl. Environ. Microbiol. 66 (2000) 956–965.

[9] J.A. Colquhoun, J. Mexson, M. Goodfellow, A.C. Ward, K. Horikoshi, A.T. Bull, Novel rhodococci and other

mycolate actinomycetes from the deep sea, Antonie Van Leeuwenhoek 74 (1998) 27–40.
[10] R.R. Colwell, U. Simidu, K. Ohwada, Microbial Diversity in Time and Space, Plenum Press, New York, 1996.
[11] R.P. Cooney, O. Pantos, M.D. Le Tissier, M.R. Barer, A.G. O'Donnell, J.C. Bythell, Characterization of the bacterial consortium associated with black band disease in coral using molecular microbiological techniques, Environ. Microbiol. 4 (2002) 401–413.
[12] A.M. Costello, M.E. Lidstrom, Molecular characterization of functional and phylogenetic genes from natural populations of methanotrophs in lake sediments, Appl. Environ. Microbiol. 65 (1999) 5066–5074.
[13] H. Dang, C.R. Lovell, Bacterial primary colonization and early succession on surfaces in marine waters as determined by amplified rRNA gene restriction analysis and sequence analysis of 16S rRNA genes, Appl. Environ. Microbiol. 66 (2000) 467–475.
[14] T. De Baere, R. de Mendonca, G. Claeys, G. Verschraegen, W. Mijs, R. Verhelst, S. Rottiers, L. Van Simaey, C. De Ganck, M. Vaneechoutte, Evaluation of amplified rDNA restriction analysis (ARDRA) for the identification of cultured mycobacteria in a diagnostic laboratory, BMC Microbiol. 2 (2002) 4.
[15] X. De Lamballerie, C. Zandotti, C. Vignoli, C. Bollet, P. De Micco, A one-step microbial DNA extraction method using "Chelex 100" suitable for gene amplification, Res. Microbiol. 143 (1992) 785–790.
[16] E.F. DeLong, Molecular phylogenetics: new perspective on the ecology, evolution, and biodiversity of marine organisms, In: K.E. Cooksey (Ed.), Molecular Approaches to the Study of the Ocean, Chapman & Hall, London, 1998, pp. 1–26.
[17] E.F. DeLong, D.G. Franks, A.A. Yayanos, Evolutionary relationships of cultivated psychrophilic and barophilic deep-sea bacteria, Appl. Environ. Microbiol. 63 (1997) 2105–2108.
[18] H. Eilers, J. Pernthaler, F.O. Glockner, R. Amann, Culturability and in situ abundance of pelagic bacteria from the North Sea, Appl. Environ. Microbiol. 66 (2000) 3044–3051.
[19] H. Eilers, J. Pernthaler, J. Peplies, F.O. Glockner, G. Gerdts, R. Amann, Isolation of novel pelagic bacteria from the German Bight and their seasonal contributions to surface picoplankton, Appl. Environ. Microbiol. 67 (2001) 5134–5142.
[20] L. Fieseler, M. Horn, M. Wagner, U. Hentschel, Discovery of the novel candidate phylum "Poribacteria" in marine sponges, Appl. Environ. Microbiol. 70 (2004) 3724–3732.
[21] J.A. Fuhrman, K. McCallum, A.A. Davis, Phylogenetic diversity of subsurface marine microbial communities from the Atlantic and Pacific Oceans, Appl. Environ. Microbiol. 59 (1993) 1294–1302.
[22] G.M. Garrity, J.G. Holt, The Road Map to the Manual, In: G.M. Garrity (Ed.), Bergey's Manual of Systematic Bacteriology, second ed, Springer, New York, 2001, pp. 119–166.
[23] F.B. Gich, E. Amer, J.B. Figueras, C.A. Abella, M.D. Balaguer, M. Poch, Assessment of microbial community structure changes by amplified ribosomal DNA restriction analysis (ARDRA), Int. Microbiol. 3 (2000) 103–106.
[24] S.J. Giovannoni, M.S. Rappe, Evolution, diversity, and molecular ecology of marine prokaryotes, In: D.L. Kirchman (Ed.), Microbial Ecology of the Oceans, Wiley-Liss, Inc., New York, 2000, pp. 47–84.
[25] J.M. Gonzalez, M.A. Moran, Numerical dominance of a group of marine bacteria in the α-subclass of the Class *Proteobacteria* in coastal seawater, Appl. Environ. Microbiol. 63 (1997) 4237–4242.
[26] A. Gunasekera, K.S. Sfanos, P.J. McCarthy, J.V. Lopez, HBMMD: an enhanced database of the microorganisms associated with deeper water marine invertebrates, Microbiol. Appl. Biotechnol. 66 (2005) 373–376.
[27] R.S. Gupta, The phylogeny of proteobacteria: relationships to other eubacterial phyla and eukaryotes, FEMS Microbiol. Rev. 24 (2000) 367–402.
[28] A. Haddad, F. Comacho, P. Durand, S.C. Cary, Phylogenetic characterization of the epibiotic bacteria associated with the hydrothermal vent polychaete *Alvinella pompejana*, Appl. Environ. Microbiol. 61 (1995) 1679–1687.
[29] M.G. Haygood, E.W. Schmidt, S.K. Davidson, D.J. Faulkner, Microbial symbionts of marine invertebrates: opportunities for microbial biotechnology, J. Mol. Microbiol. Biotechnol. 1 (1999) 33–43.
[30] U. Hentschel, J. Hopke, M. Horn, A.B. Friedrich, M. Wagner, J. Hacker, B.S. Moore, Molecular evidence for a uniform microbial community in sponges from different oceans, Appl. Environ. Microbiol. 68 (2002) 4431–4440.
[31] J.N.A. Hooper, R.W.M. Van Soest, Systema Porifera: A Guide to the Classification of Sponges, Kluwer Academic/Plenum Publishers, New York, 2002.
[32] E.P. Ivanova, L.A. Romanenko, J. Chun, M.H. Matte, G.R. Matte, V.V. Mikhailov, V.I. Svetashev, A. Huq, T. Maugel, R.R. Colwell, *Idiomarina gen*. nov., comprising novel indigenous deep-sea bacteria from the Pacific Ocean, including descriptions of two species, *Idiomarina abyssalis* sp. nov. and *Idiomarina zobellii* sp. Nov, Int. J. Syst. Evol. Microbiol. 50 (2000) 901–907.
[33] H.W. Jannasch, G.E. Jones, Bacterial populations in sea water as determined by different methods of enumeration, Limnol. Oceanogr. 4 (1959) 128–139.
[34] C. Kato, A. Inoue, K. Horikoshi, Isolating and characterizing deep-sea marine microorganisms, Trends Biotechnol. 14 (1996) 6–12.
[35] D.J. Lane, 16S/23S rRNA sequencing, In: E. Stackebrandt, M. Goodfellow (Eds.), Nucleic Acid Techniques in Bacterial Systematics, Wiley, New York, 1991, pp. 115–148.
[36] J.V. Lopez, P.J. McCarthy, K.E. Janda, R. Willoughby, S.A. Pomponi, Molecular techniques reveal wide phylogenetic diversity of heterotrophic microbes associated with *Discodermia* spp. (Porifera: Demospongiae), Mem. Queensland Museum 44 (1999) 329–341.
[37] W. Ludwig, H. Klenk, Overview: a phylogenetic backbone and taxonomic framework for procaryotic systematics, In: G.M. Garrity (Ed.), Bergey's Manual of Systematic Bacteriology, second ed, Springer, New York, 2001, pp. 49–65.

[38] B.L. Maidak, J.R. Cole, C.T. Parker, G.M. Garrity, N. Larsen, L. Bing, T.G. Lilburn, M.J. McCaughey, G.J. Olsen, R. Overbeek, S. Pramanik, T.M. Schmidt, J.M. Tiedje, C.R. Woese, A new version of the RDP (Ribosomal Database Project), Nucleic Acids Res. 27 (1999) 171–173.

[39] C.L. Moyer, J.M. Tiedje, F.C. Dobbs, D.M. Karl, A computer-simulated restriction fragment length polymorphism analysis of bacterial small-subunit rRNA genes: efficacy of selected tetrameric restriction enzymes for studies of microbial diversity in nature, Appl. Environ. Microbiol. 62 (1996) 2501–2507.

[40] J. Neefs, Y. Van de Peer, P. De Rijk, S. Chapelle, R. De Wachter, Compilation of small ribosomal subunit RNA structures, Nucleic Acids Res. 21 (1993) 3025–3049.

[41] M. Nei, S. Kumar, Molecular Evolution and Phylogenetics, Oxford University Press, Oxford, 2000.

[42] J.B. Olson, D.K. Harmody, P.J. McCarthy, Alphaproteobacteria cultivated from marine sponges display branching rod morphology, FEMS Microbiol. Lett. 211 (2002) 169–173.

[43] J.B. Olson, C.C. Lord, P.J. McCarthy, Improved recoverability of microbial colonies from marine sponge samples, Microb. Ecol. 40 (2000) 139–147.

[44] L. Ovreas, V. Torsvik, Microbial diversity and community structure in two different agricultural soil communities, Microb. Ecol. 36 (1998) 303–315.

[45] N.R. Pace, A molecular view of microbial diversity and the biosphere, Science 276 (1997) 734–740.

[46] M.F. Polz, C. Harbison, C.M. Cavanaugh, Diversity and heterogeneity of epibiotic communities on the marine nematode *Eubostrichus dianae*, Appl. Environ. Microbiol. 65 (1999) 4271–4275.

[47] S.A. Pomponi, The bioprocess—technological potential of the sea, J. Biotechnol. 70 (1999) 5–13.

[48] D. Posada, K.A. Crandall, MODELTEST: testing the model of DNA substitution, Bioinformatics 14 (1998) 817–818.

[49] K.A. Sandell (Sfanos), C.L. Peterson, D.K. Harmody, P.J. McCarthy, S.A. Pomponi, J.V. Lopez, Molecular systematic survey of sponge-derived marine microbes. Sixth International Sponge Conference Proceedings, Boll. Mus. Inst. Univ. Genova. 68 (2004) 579–585.

[50] D.L. Santavy, R.R. Colwell, Comparison of bacterial communities associated with the Caribbean sclerosponge *Ceratoporella nicholsoni* and ambient seawater, Mar. Ecol. Prog. Ser. 67 (1990) 73–82.

[51] D.L. Santavy, P. Willenz, R.R. Colwell, Phenotypic study of bacteria associated with the Caribbean sclerosponge, *Ceratoporella nicholsoni*, Appl. Environ. Microbiol. 56 (1990) 1750–1762.

[52] E. Stackebrandt, B.M. Goebel, Taxonomic note: a place for DNA-DNA reassociation and 16S rRNA sequence analysis in the present species definition in bacteriology, Int. J. Syst. Bacteriol. 44 (1994) 846–849.

[53] M.T. Suzuki, L.T. Taylor, E.F. DeLong, Quantitative analysis of small-subunit rRNA genes in mixed microbial populations via 5′-nuclease assays, Appl. Environ. Microbiol. 66 (2000) 4605–4614.

[54] D. Swofford, PAUP* Phylogenetic Analysis Using Parsimony (*And Other Methods). Version 4, Sinauer, Sunderland, MA, 2001.

[55] H. Takami, A. Inoue, F. Fuji, K. Horikoshi, Microbial flora in the deepest sea mud of the Mariana Trench, FEMS Microbiol. Lett. 152 (1997) 279–285.

[56] A. Teske, T. Brinkhoff, G. Muyzer, D.P. Moser, J. Rethmeier, H.W. Jannasch, Diversity of thiosulfate-oxidizing bacteria from marine sediments and hydrothermal vents, Appl. Environ. Microbiol. 66 (2000) 3125–3133.

[57] J.D. Thompson, D. Higgins, T.J. Gibson, CLUSTAL version W: a novel multiple sequence alignment program, Nucleic Acids Res. 22 (1994) 4673–4680.

[58] H. Urakawa, K. Kita-Tsukamoto, K. Ohwada, Microbial diversity in marine sediments from Sagami Bay and Tokyo Bay, Japan, as determined by 16S rRNA gene analysis, Microbiology 145 (1999) 3305–3315.

[59] Y. Van de Peer, J. Jansen, P. De Rijk, R. De Wachter, Database on the structure of small ribosomal subunit RNA, Nucleic Acids Res. 25 (1997) 111–116.

[60] P. Vandamme, B. Pot, M. Gillis, P. De Vos, K. Kersters, J. Swings, Polyphasic taxonomy, a consensus approach to bacterial systematics, Microbiol. Rev. 60 (1996) 407–438.

[61] M. Vaneechoutte, L. Vauterin, B. van Harsselaar, L. Dijkshoorn, P. De Vos, Considerations in evaluation of the applicability of DNA fingerprinting techniques for species differentiation, J. Clin. Microbiol. 37 (1999) 3428–3429.

[62] C.J. Venter, K. Remington, J.F. Heidelber, A.L. Halpern, D. Rusch, J.A. Eisen, D. Wu, I. Paulsen, K.E. Nelson, W. Nelson, D.E. Fouts, S. Levy, A.H. Knaop, M.W. Lomas, K. Nealson, O. White, J. Peterson, J. Hoffman, R. Parsons, H. Baden-Tillson, C. Pfannkoch, Y. Rogers, H.O. Smith, Environmental genome shotgun sequencing of the Sargasso Sea, Science 304 (2004) 66–74.

[63] M. Ventura, M. Elli, R. Reniero, R. Zink, Molecular microbial analysis of Bifidobacterium isolates from different environments by the species-specific amplified ribosomal DNA restriction analysis (ARDRA), FEMS Microbiol. Ecol. 36 (2001) 113–121.

[64] N.S. Webster, R.T. Hill, The culturable microbial community of the Great Barrier Reef sponge *Rhopaloeides odorabile* is dominated by an α-Proteobacterium, Mar. Biol. 138 (2001) 843–851.

[65] N.S. Webster, K.J. Wilson, L.L. Blackall, R.T. Hill, Phylogenetic diversity of bacteria associated with the marine sponge *Rhopaloeides odorabile*, Appl. Environ. Microbiol. 67 (2001) 434–444.

[66] W.B. Whitman, D.C. Coleman, W.J. Wiebe, Prokaryotes: the unseen majority, PNAS 95 (1998) 6578–6583.

[67] C.R. Wilkinson, Significance of microbial symbionts in sponge evolution and ecology, Symbiosis 4 (1987) 135–146.

[68] C. Woese, Bacterial evolution, Microbiol. Rev. 51 (1987) 221–271.

[69] M.M. Yakimov, L. Giuliano, E. Crisafi, T.N. Chernikova, K.N. Timmis, P.N. Golyshin, Microbial community of a saline mud volcano at San Biagio-Belpasso, Mt. Etna (Italy), Environ. Microbiol. 4 (2002) 249–256.