A MOLECULAR SYSTEMATIC SURVEY OF SPONGE-DERIVED MARINE MICROBES

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ABSTRACT

A systematic survey is being conducted to determine the microbial diversity held within the Harbor Branch Oceanographic Marine Microbial Culture Collection (HBMMCC). The collection consists of an estimated 16,500 marine microorganisms, with 11,000 from > 150 ft seawater. To date, around 1,000 microbes have been inventoried using the DNA fingerprinting technique RFLP (restriction fragment length polymorphism) on the small subunit (SSU) 16S (eubacterial) and 18S (fungal) rRNA gene. RFLP patterns obtained from restriction digests with the enzymes *Rsa*I and *Hae*III are used to "infer" taxonomic similarity of isolates. About 750 base pairs (bp) of the 5' portion of the 16S or 18S SSU rRNA gene was sequenced from representative isolates for more definitive phylogenetic analysis. Preliminary results show that the HBMMCC contains isolates from at least 6 major bacterial clades (Proteobacteria (Alpha, Beta, Gamma), CFB (Cytophaga, Flavobacteria, and Bacteroides), Gram+ High GC Content, Gram+ Low GC Content) with the possible discovery of novel microbial taxa.

KEY WORDS

Microbial systematics, sponge symbionts, RFLP.

INTRODUCTION

Stretches of nucleic acids can be highly specific to a particular strain or species of bacteria, as well as to related species or microorganisms with similar metabolic activities (LIESACK & STACKEBRANDT, 1992). Ribosomal RNA (rRNA) has emerged as one of the best candidates for phylogenetic studies because it is present in all organisms, functionally constant, and highly conserved (VANDAMME *et al.,* 1996). DNA fingerprinting techniques such as RFLP and REP-PCR have also been used as a means of rapidly screening and comparing prokaryotic environmental samples (VERSALOVIC *et al.,* 1994; URAKAWA *et al.,* 1999; DUNBAR *et al.,* 2001). The primary goals of this research project are: (1) to inventory 3000 deep-water invertebrate-derived microorganisms (90 % eubacteria, 10 % fungi) from the HBMMCC through RFLP screening and sequencing of universal 16S and 18S SSU PCR products, (2) to assess microbial diversity associated with various host invertebrate species across different habitats and geographic locales, (3) to compare the 16S rRNA diversity of cultured isolates with the "uncultured" microbial 16S rRNA diversity in the same invertebrate species and (4) to develop the HBOI Marine Microorganism Database

(HBMMD), a taxonomic database that will be available via the Internet (www.hboi.edu/dbmr/dbmr_hbmmd.html).

A total of 123 SSU PCR products have been sequenced to date. In this preliminary report, results show that the HBMMCC contains diverse bacterial isolates from at least 6 major bacterial clades (Proteobacteria (Alpha, Beta, Gamma), CFB, Gram+ High GC Content, Gram+ Low GC Content) as well as microbes that have a low percent similarity (<94 %) to known 16S rRNA gene sequences contained in the GenBank database (www.ncbi.nlm.nih.gov).

MATERIALS AND METHODS

Microbe Isolation

The isolates used in this research project were deep-water invertebrate-associated (primarily sponges) eubacteria and fungi maintained in the HBMMCC. The isolation methods used involved the sampling of the invertebrate via aseptic technique upon surfacing. 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 large variety of carbon sources (*e.g.* chitin, simple and complex sugars, and mucin). Media were designed to include both antibiotics and/or extracts of the host tissue.

The initial strategy to select samples was based on chronology, beginning with the most recent isolates (2001).

DNA Extraction

Bacterial cells for DNA extraction were collected with a sterile loop either directly from isolates maintained on agar slants or re-grown cultures of these isolates streaked on agar plates. 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.

Polymerase Chain Reaction (PCR)

PCR amplified the number of copies of the 16S and 18S small subunit (SSU) rRNA gene region. Universal eubacterial primers Ecoli9 5'-GAGTTTGATCCTGGCTCAG-3' and Loop27rc 5'-GACTACCAGGGTATCTAATC-3' and universal fungal (18S) primers UNIV-A 5'-CCTGGTTGATCCTGCCAG-3' and 579rc 5'-GCCCTCCAATGGATCCTCG-3' (LOPEZ *et al.,* 1999) amplified ~750 bp of the 5' end of the 16S and 18S SSU rRNA gene under standard PCR conditions (94° C for 2 min., 34 cycles of 94° C for 1 min. 53° C for 1 min. 72° C for 1 min., 72° C for 30 min., 4° C hold). A positive control (with previously 16S or 18S amplifiable DNA) and negative control (no template added) was run for every PCR performed. All PCR products were visualized by gel electrophoresis using ethidium bromide stained 1 % agarose gel (MetaPhor-FMC BioProducts) in 0.5X TBE buffer.

Restriction Fragment Length Polymorphism (RFLP)

RFLP was utilized as a rapid, primary screen for genetic variation in SSU PCR products. This method is a PCR-based fingerprinting technique in which an amplified DNA fragment is subject to digestion by a restriction endonuclease. Electrophoresis of the digestion products on an agarose gel (2 %) separated the sub-fragments according to size. This resulted in a species-specific banding pattern. The use of two 4-base cutting restriction endonucleases, *Rsa*I and *Hae*III (Gibco BRL) increases the chances of detecting unique RFLP patterns. Gel electrophoresis images are digitally captured on an Eagle Eye scanner (Stratagene, La Jolla,

CA). The molecular weight of each RFLP band is then calculated using the imager's accompanying software, RFLPscan (Scanalytics, Fairfax, VA).

Sequencing of 16S and 18S PCR Products

DNA templates were purified using a Qiagen QIAquick™ PCR Purification Kit and Sephadex (Amersham Biosciences) columns before cycle sequencing using an ABI Prism™ Big Dye® Terminator Cycle Sequencing Ready Reaction Kit. Automated sequencing using ABI DNA sequencers was conducted at the Interdisciplinary Center for Biotechnology Research (ICBR) at the University of Florida (Gainesville, FL). Once the sequence results were obtained and edited into contiguous 16S or 18S rDNA fragments, a query was performed using BLAST (Basic Local Alignment Search Tool) to find the closest taxonomic match in a non-redundant sequence database (ALTSCHUL *et al.,* 1997).

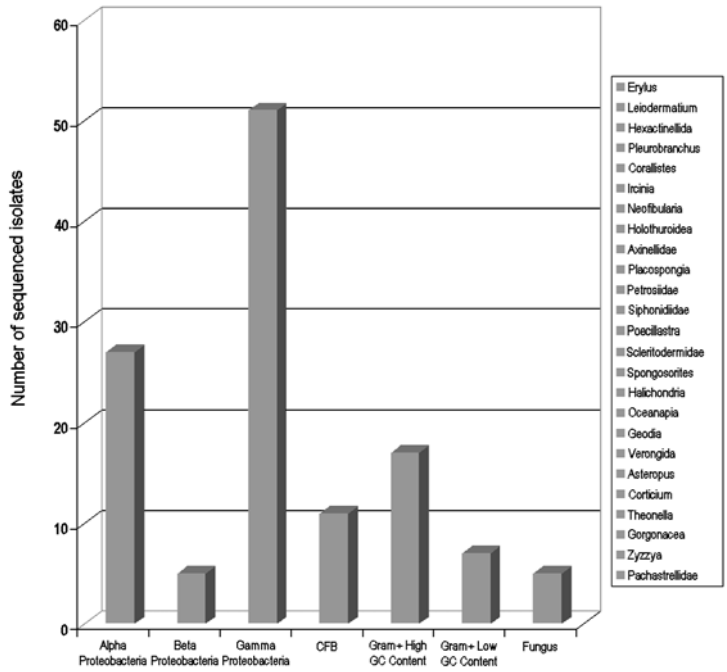

Fig. 1. The distribution of the sequenced isolates across each major bacterial clade (GARRITY & HOLT, 2001). The list on the right shows the invertebrate hosts from which the sequenced microbes were isolated.

RESULTS

RFLP band data for approximately 1000 isolates (stemming from at least 35 different species of invertebrate hosts) were stored and archived in a Microsoft Access 97 database where it could be sorted by bands of similar molecular weight. This analysis yielded 102 different "groups" of isolates with distinct *Rsa*I and *Hae*III RFLP patterns (*i.e.,* 102 different sets of combined RFLP patterns). Therefore,

roughly 10 % of the RFLPs performed featured a novel set of patterns. A group of isolates was considered "unique" if it contained less than 5 isolates with the same RFLP pattern, "intermediate" if between 5 - 10, or "common" if greater than 10 isolates exhibited the same patterns.

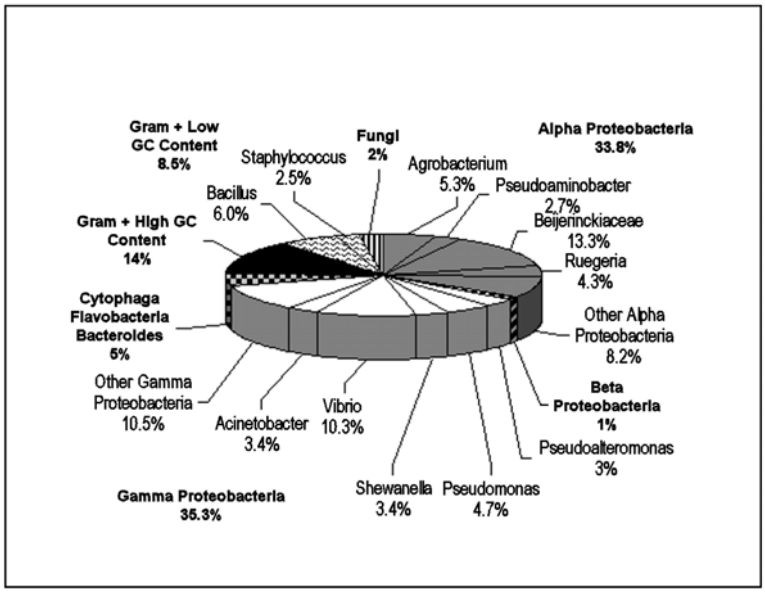

Fig. 2. The taxonomic distribution of the ~1000 isolates inventoried by RFLP to date as inferred by the *Rsa*I and *Hae*III RFLP patterns. Isolates were grouped based on these patterns, and representative 16S or 18S PCR products were sequenced from each group.

A portion of the 16S or 18S rRNA gene was sequenced from a total of 123 isolates representing each of the 102 different groups of isolates. In some cases, 2 isolates were sequenced from the unique and intermediate groups and up to 5 isolates were sequenced from each of the common groups to assure that isolates with the same RFLP patterns had the same taxonomic identity when subjected to rDNA sequence analyses. In all, 83 Proteobacteria, 11 CFB, 17 Gram+ High GC Content, 7 Gram + Low GC Content, and 5 fungal samples were sequenced (Fig. 1). BLAST searches indicated that at least 87 different species of microorganisms comprise the 1000 isolates inventoried to date. This includes 18 different species of α-Proteobacteria, 32 species of γ-Proteobacteria, 3 species of β-Proteobacteria, 10 CFB species, 16 Gram + High GC Content species, 4 Gram + Low GC Content species, and 4 different species of fungi (Fig. 2). Three CFB group species (*Flavobacterium*-like, *Cytophaga*-like, and *Sphingobacter*-like) and three γ-Proteobacteria species (an unclassified *Pseudomonas* and two *Oceanospirillum*-like species) had less than 94 % sequence similarity to the closest GenBank BLAST match (Fig. 3). Sequencing results also indicated that there are 50 "unique" groups, 9 "intermediate" groups, and 28 "common" groups of isolates. Approximately 12 % of the isolates inventoried

belong to unique groups, 7 % belong to intermediate groups, and 81 % belong to common groups.

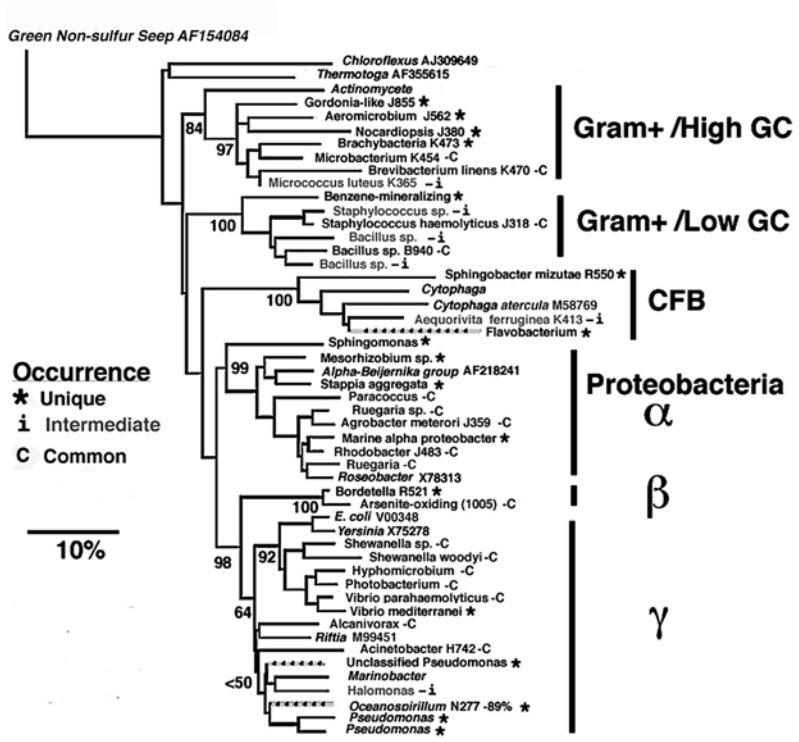

Fig. 3. Neighbor-joining phylogram based on 16S rRNA gene sequences of representative bacterial isolates. The tree was built with the PAUP 4* program (SWOFFORD, 1999) after deleting unalignable gapped regions, and bootstrap testing (with percentages shown at nodes). Isolates with sequences < 94 % similar to the closest GenBank BLAST match are shown with dotted lines.

DISCUSSION AND CONCLUSIONS

The preliminary results of this research indicate that the HBMMCC contains a diverse range of microbial isolates (at least 87 species so far as inferred by BLAST database queries of 16S and 18S rDNA sequences). The majority of this diversity lies within the Proteobacteria clade of bacteria. Approximately 70 % of the isolates inventoried to date by RFLP are thought to belong to the α or γ subclasses of *Proteobacteria* (Fig. 3). This predominance is most likely due to culture media and isolation conditions favoring the isolation of Gram-negative proteobacteria from the

marine environment (OLSON *et al.,* 2002). However, studies on "unculturable" marine microbes have also shown a numerical dominance by α-Proteobacteria (GONZALEZ & MORAN, 1997; WEBSTER & HILL, 2001).

To date, the amount of diversity is greatest within the γ-Proteobacteria (32 species) followed by the α-Proteobacteria and the Gram + High GC Content bacteria (18 species and 16 species respectively). Thus far, "unique" isolates of the HBMMCC (12 %) are comprised of 10 species of α-Proteobacteria, 2 species of β-Proteobacteria, 19 species of γ-Proteobacteria, 5 species of CFB group bacteria, 11 species of Gram + High GC Content species, and 3 species of fungi. There are less than 10 isolates of all of the CFB group bacteria. In contrast, all 4 of the Gram+ Low GC Content species are common (greater than 10 isolates) in the HBMCC.

Three of the sequenced γ-Proteobacteria species (an unclassified *Pseudomonas* and two *Oceanospirillum*-like species) showed respectively 90 %, 88 % and 89 % sequence similarity to the closest 16S rRNA gene sequence in the GenBank database. Similarly, a *Flavobacterium*-like (GenBank Accession AF486815), *Cytophaga*-like, and *Sphingobacter*-like (GenBank Accession AF489284) CFB group bacterium showed respectively 93 %, 89 %, and 89 % sequence similarity to their closest BLAST match. Since ribosomal RNA is highly conserved, prokaryotic species are generally differentiated by a 16S sequence similarity of less than 97 %. It is therefore very likely that the isolates with less than 94 % sequence identity in BLAST searches represent novel microbial taxa above the genus and family level. As this research continues, it is likely that the number of species identified will increase and that additional potentially novel microbial taxa will be discovered.

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REFERENCES

ALTSCHUL S.F., MADDEN T.L., SCHAFFER A.A., ZHANG J., ZHANG Z., MILLER W., LIPMAN D.J., 1997 - Gapped BLAST and PSI-BLAST: A new generation of protein database search programs. *Nucleic Acids Res.,* **25**: 3389-3402.

DUNBAR J., TICKNOR L.O., KUSKE C.R., 2001 - Phylogenetic specificity and reproducibility and new method for analysis of terminal restriction length fragment profiles of 16S rRNA genes from bacterial communities. *Appl. Environ. Microbiol.,* **67** (1): 190-197.

GARRITY G.M., HOLT J.G., 2001 - The road map to the manual. In G.M. Garrity (ed.), *Bergey's Manual of Systematic Bacteriology. 2nd ed.* Springer-Verlag, New York: 119-166.

GONZALEZ J.M., MORAN M.A., 1997 - Numerical dominance of a group of marine bacteria in the alpha-subclass of the class Proteobacteria in coastal seawater. *Appl. Environ. Microbiol.,* **63**: 4237-4242.

LIESACK W., STACKEBRANDT E., 1992 - Unculturable microbes detected by molecular sequences and probes. *Biodivers. Conserv.,* **1**: 250-262.

LOPEZ J.V., MCCARTHY P.J., JANDA K.E., WILLOUGHBY R., POMPONI S.A., 1999 - Molecular techniques reveal a wide phyletic diversity of heterotrophic microbes associated with the sponge genus Lithistid (Porifera : Demospongiae). *Mem. Queensl. Mus.,* **44**: 329-341.

OLSON J.B., HARMODY D.K., MCCARTHY P.J., 2002 - Alpha-proteobacteria cultivated from marine sponges display branching rod morphology. *FEMS Microbiol. Lett.,* **211** (2): 169-73.

SWOFFORD D., 1999 - *PAUP* Phylogenetic analysis using parsimony (*and other methods)*. Version 4. Sinauer, Sunderland, MA.

URAKAWA H., KITA-TSUKAMOTO K., OHWADA K., 1999 - Microbial diversity in marine sediments from Sagami Bay and Tokyo Bay, Japan, as determined by 16S rRNA gene analysis. *Environ. Microbiol.,* **145** (11): 3305-3315.

VANDAMME P., POT B., GILLS M., DE VOS P., KERSTERS K. SWINGS J., 1996 - Polyphasic taxonomy, a consensus approach to bacterial systematics. *Microbiol. Rev.,* **60** (2): 407-438.

VERSALOVIC J., SCHNEIDER M., DEBRUIJN F.J., LUPSKI J.R., 1994 - Genomic fingerprinting of bacteria using sequence-based polymerase chain reaction. *Methods Mol. Cell Biol.,* **5**: 25-40.

WEBSTER N.S., HILL R.T.**,** 2001 - The culturable microbial community of the Great Barrier Reef sponge *Rhopaloeides odorabile* is dominated by an alpha Proteobacterium. *Mar. Biol.,* **138** (4): 843-851.