CASE STUDY

High Throughput Cultivation for Isolation of Novel Marine Microorganisms

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MARINE NATURAL PRODUCTS

Natural products are organic molecules derived from plants, animals, or microorganisms, and represent the starting point for most of the anti-infective and anti-cancer drugs on the market today. Until recently, the majority of natural products has been isolated from terrestrial sources. During the last two decades, however, the rate of discovery of novel compounds has declined significantly, as exemplified by the fact that extracts from soil-derived actinomycetes have yielded unacceptably high numbers of previously described metabolites (Mincer et al., 2002). In addition to the redundancy and associated issue of de-replication, an innovation gap has been postulated as a cause for the dramatic reduction in small molecule novelty. Even today, most microbiologists are constrained by the use of traditional cultivation methods, which primarily target previously cultured microbes (“microbial weeds”). As a result, most pharmaceutical companies no longer place an emphasis on natural-product discovery as a source of lead compounds (Walsh, 2003).

In contrast, the marine environment is becoming increasingly appreciated as a rich and untapped reservoir of novel natural products (see Fenical, this issue). Bioactive compounds are frequently associated with marine invertebrates, including sponges, bryozoans, mollusks, and tunicates (Proksch et al., 2002). More recently, marine microorganisms have been recognized as a productive source of novel secondary metabolites. To date, the majority of natural products of marine bacterial origin have arisen from a small number of taxonomic groups that include *Streptomyces, Alteromonas, Pseudomonas, Vibrio, Agrobacterium*, and the cyanobacteria (Wagner-Döbler et al., 2002; Burja et al., 2001). Over the past decade, a consensus has developed among marine natural products chemists and chemical ecologists, who believe that most novel natural products found in extracts of marine invertebrates are synthesized, either in part or in their entirety, by the symbiotic microbes that are intimately associated with these marine metazoans.

THE EXAMPLE OF MARINE SPONGES

Marine sponges provide classic examples of microbial-macrofaunal partnerships that have been a productive source for the discovery of bioactive compounds (Faulkner, 2002). For example, Monks et al. (2002) found that extracts of eight out of ten different Brazilian sponge species exhibited anti-bacterial, anti-tumor, or anti-chemotactic activities. According to chemical ecologists, secondary metabolites are produced as part of a chemical arsenal designed to deter grazers by imparting toxicity or low palatability to the metazoan host. These marine invertebrate-microbial assemblages also produce toxic compounds to prevent colonization by other non-beneficial microbial species. Interestingly, some of the small molecules isolated from marine metazoans display a striking resemblance to prokaryotic-borne metabolites; it has thus been suggested that the source of many of these bioactives is in fact the symbiotic microorganisms (Piel et al., 2004).

One well-studied example is the synthesis of bryostatin-1 by the marine bryozoan, *Bugula neritina*, which has been directly linked to the presence of the uncultured bacterium *Candidatus Endobugula sertula* (Davidson et al., 2001). Similarly, *Bugula simplex* hosts the symbionts *Candidatus Endobugula glebosa*, which are linked to bryostatin production (Lim and Haygood, 2004). Such examples demonstrate the promise of marine symbiotic microorganisms as a viable source for the discovery of novel small molecules.

MICROBIAL CULTIVATION TECHNOLOGIES

Marine agar, a cultivation medium formulated by Claude ZoBell during the 1940s (ZoBell, 1946), has been extensively used for the isolation of marine bacteria. However, this medium contains organic carbon in concentrations much higher than those found in most natural environments; as such, strains isolated using marine agar are typically fast growing, and not always representative of cells that may play relevant roles *in situ*. Incubation times for the development of colonies from cells growing in rich media range from one day to one week.

Mincer et al. (2002) have addressed the high-carbon-content issue and the relatively short incubation times and have significantly improved the techniques for the isolation of novel marine actinomycetes strains from sediments. They employed several concentrations of organic carbon from diverse sources, longer incubation times, and the pretreatment of the samples with a heat shock to enrich for spore-forming microorganisms. As a result, novel lineages of marine actinomycetes have recently been isolated and cultured from diverse marine sediments. Extracts of some of these cultures have yielded promising molecules, some of which have been advanced to the pre-clinical trial phase by Nereus Pharmaceuticals (more information available at http://www.nereuspharm.com/overview.shtml). Similarly, Maldonado et al. (2005) selected carbon sources for the targeted isolation of marine actinomyces based on data generated by culture-independent studies on the *in situ* diversity as seen by ribosomal analysis of the actinomycetes present in the samples.

A number of new and innovative techniques have been developed in recent years to increase the efficiency of the isolation of novel microorganisms from the marine biosphere. These techniques include various modifications to growth media and end-point dilution methods using microtitre dish plate formats that allowed the cultivation of the ubiquitous marine bacterial clade SAR11 (Connon and Giovannoni, 2002; Rappé et al., 2002; Giovannoni et al., 2005). At Diversa Corporation (more information available at http://www.diversa.com/), a high-throughput cultivation (HTC) technology that employs agarose microcapsules to encapsulate single cells directly from environmental samples has been developed. The microcapsules are then transferred into mini fermentation columns for growth and microcolony development. The column is perfused with culture medium that contains nutrient concentrations similar to those seen in the natural environment from which the cells were collected (i.e., filtered sea water, diluted sponge extracts). One of the advantages of using this approach is the ability to enrich for slow-growing species. The use of fluorescence-activated cell sorting (FACS) enables the discrimination of slow-growing microbes retained in the microcapsules from fast-growing cells that overgrow and burst the microcapsule. This method has been suggested to be suitable for massively parallel cultivation of microorganisms for natural-product screening and drug discovery (Keller and Zengler, 2004).

UNIVERSITY OF HAWAII-DIVERSA COLLABORATION

The Hawaiian archipelago is a geographically isolated chain of oceanic islands with high biodiversity and endemism, and is considered to be an environmental "hot spot" in terms of its rich and often unique animal, plant, and microbial life forms. Consequently, Hawaiian marine invertebrates and their associated microbial biodiversity represent a promising resource for the discovery of novel bioactive molecules.

In 2004, Diversa Corporation signed a biodiversity access and collaboration agreement with the University of Hawaii; the goal was to access Hawaiian samples to cultivate novel microorganisms associated with marine metazoans, sea grasses, and ocean sediments. This discovery effort has been co-funded by the Oceans and Human Health Initiative of the National Oceanographic and Atmospheric Administration (NOAA) and the Centers for Oceans and Human Health of the National Science Foundation (NSF) and the National Institute of Environmental Health Sciences (NIEHS) of the National Institutes of Health. This collaborative research effort provides a unique opportunity to integrate Diversa Corporation's HTC technology with Hawaii's unique biodiversity and expertise in marine natural products chemistry. The strategy involves the employment of HTC to isolate novel strains of marine microbes, which will be screened for the presence of secondary metabolites exhibiting anti-tumor and anti-infective bioactivities. Qualified hits will then be dereplicated and undergo structure determination at the University of Hawaii (Manoa).

HIGH THROUGHPUT CULTIVATION OF MARINE SAMPLES FROM HAWAII

Samples of the sponge, *Mycale armata* (Figure 1A), were collected from Kaneohe Bay (Oahu, Hawaii), along with sediments sampled from various habitats (i.e., coral rubble, sea grass beds, a stream bed). The sponge and sediment samples were homogenized, and the associated microbial cells were separated using a Nycodenz cushion and differential centrifugation (Figure 1B). The crude cell pellets of living but uncultured microorganisms were then encapsulated in the agarose microcapsules (Figure 1C) as previously described (Zengler et al., 2002; Zengler et al., 2005). The capsules were transferred into mini fermentation columns equipped with a 0.22 μm filter at the inlet and an 8 μm filter at the outlet to allow free-living cells to be washed out of the system while retaining the microcapsules.

Culture medium was prepared by diluting (1/1000) sterile sponge homogenates or sediment extracts with filter-sterilized seawater. The samples were incubated over a period of five weeks and sorted (Figure 1D) with a MoFlo flow cytometry system (Cytomation) to array individual colony-containing microcapsules (Figure 1E) into 96-well plates containing marine broth. The growth of most strains is not inhibited by high marine broth carbon concentrations, and its inclusion allows the generation of the relatively large amounts of biomass required for anti-infective and anti-tumor screening.

The isolates were de-replicated via Fourier-transformed infrared (FT/IR) spectroscopy. Briefly, dense culture aliquots (3 mL) were spotted individually onto aluminum plates and allowed to air dry. The film formed on each plate produces a distinct FT/IR spectrum due to the composition of carbohydrate, lipid and protein. The FT/IR signature essentially produces a metabolic fingerprint of each microbial isolate and thus can be utilized to dereplicate strains by comparison of historical reference spectra from known strains. Unique cultures were then selected, and their 16S small subunit ribosomal gene sequenced in the case of bacteria or the internal transcribed spacer (ITS) sequenced in the case of fungi for higher-resolution taxonomic identification. Cultures of selected microbes were then scaled up to a volume of 1 L to generate sufficient biomass for screening.

As a parallel approach to HTC, environmental samples were plated onto marine agar (traditional isolation methodology) to com-

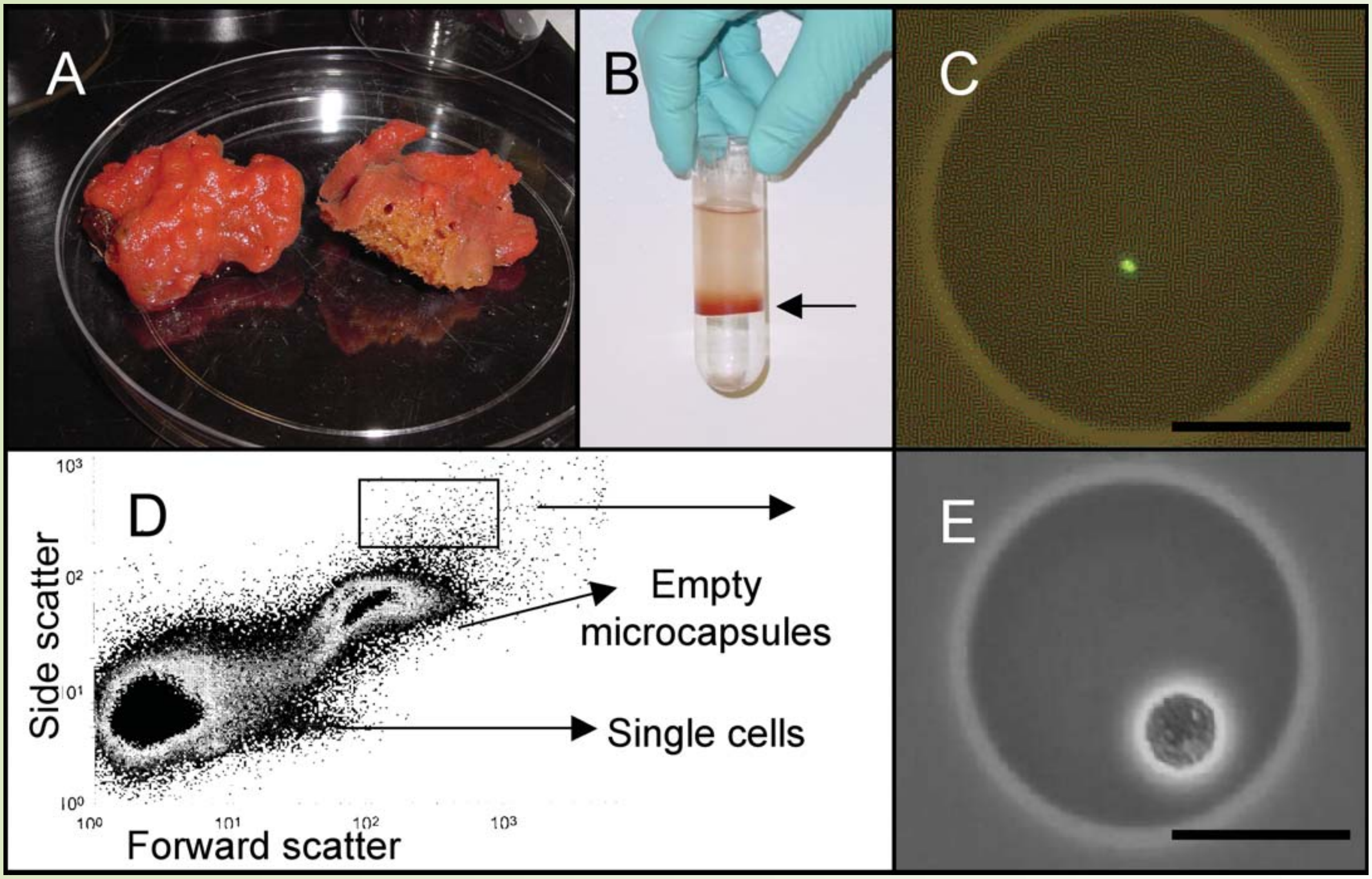

Figure 1. High-throughput cultivation of the sponge Mycale armata: (A) freshly collected specimen; (B) separation of bacterial cells by density gradient centrifugation (bacterial band indicated with an arrow); (C) encapsulation of single bacterial cells and visualization by fluorescence microscopy after SybrGreen™ staining (scale bar = 20 µm); (D) Flow cytogram of microcapsules after 5 weeks of incubation; and (E) bacterial colony growing within microcapsule (scale bar = 20 µm).

pare with the set of strains isolated using HTC technology. Similarly, for fungal strains, the samples were plated directly onto R2A medium with penicillin and streptomycin (50 µg mL^{-1}), respectively, to prevent bacterial growth and incubated for 30 days at 30°C.

BIOACTIVE MOLECULE DISCOVERY

To identify the selected strains, DNA was isolated from each culture followed by PCR (Polymerase Chain Reaction) amplification and sequencing of a 500 bp small subunit ribosomal RNA gene fragment (16S rDNA). Sequence data were used for phylogenetic tree construction using the neighbor-joining method (Figure 2). Phylogenetic analysis revealed that most strains belonged to the gamma and alpha *Proteobacteria* classes, one to the *Bacteroidetes*, and three to the *Bacilli* group. Novel strains were defined as those showing ≤ 98 percent nucleotide sequence identity to the closest relative in public databases. Strains with identical gene sequences were considered redundant, and only one was advanced for screening.

On the basis of this criterion, 42 percent of the HTC strains were novel as compared to 7 percent using the traditional agar plate isolation technique (Table 1). All of the strains were then grown in 1 L of marine broth at 30°C and 70 rpm of constant agitation for a period of 48 hours. The cultures were then pelleted by centrifugation and yielded ~ 3 g wet weight of cellular material for extract preparation and screening. Methanolic extracts were prepared and used for cytotoxity and anti-infective activity screening.

The cytotoxicity testing was performed with three cell lines that were selected by the National Cancer Institute (NCI) for pre-screening, prior to consideration of extracts for evaluation in the more comprehensive NCI panel of 60 cell lines. The pre-screen cell lines are MCF-7 (breast), SF268 (central nervous system) and H460 (small-cell lung). The assay was conducted at a standard screening concentration of 25 µg mL^{-1} crude extract, in triplicate in 96 well format using MTT (yellow tetrazolium salt) as an indicator of cell viability.

The anti-infective screen was carried out by assaying for the growth inhibition of three target species: *Escherichia coli*, *Staphylococcus*

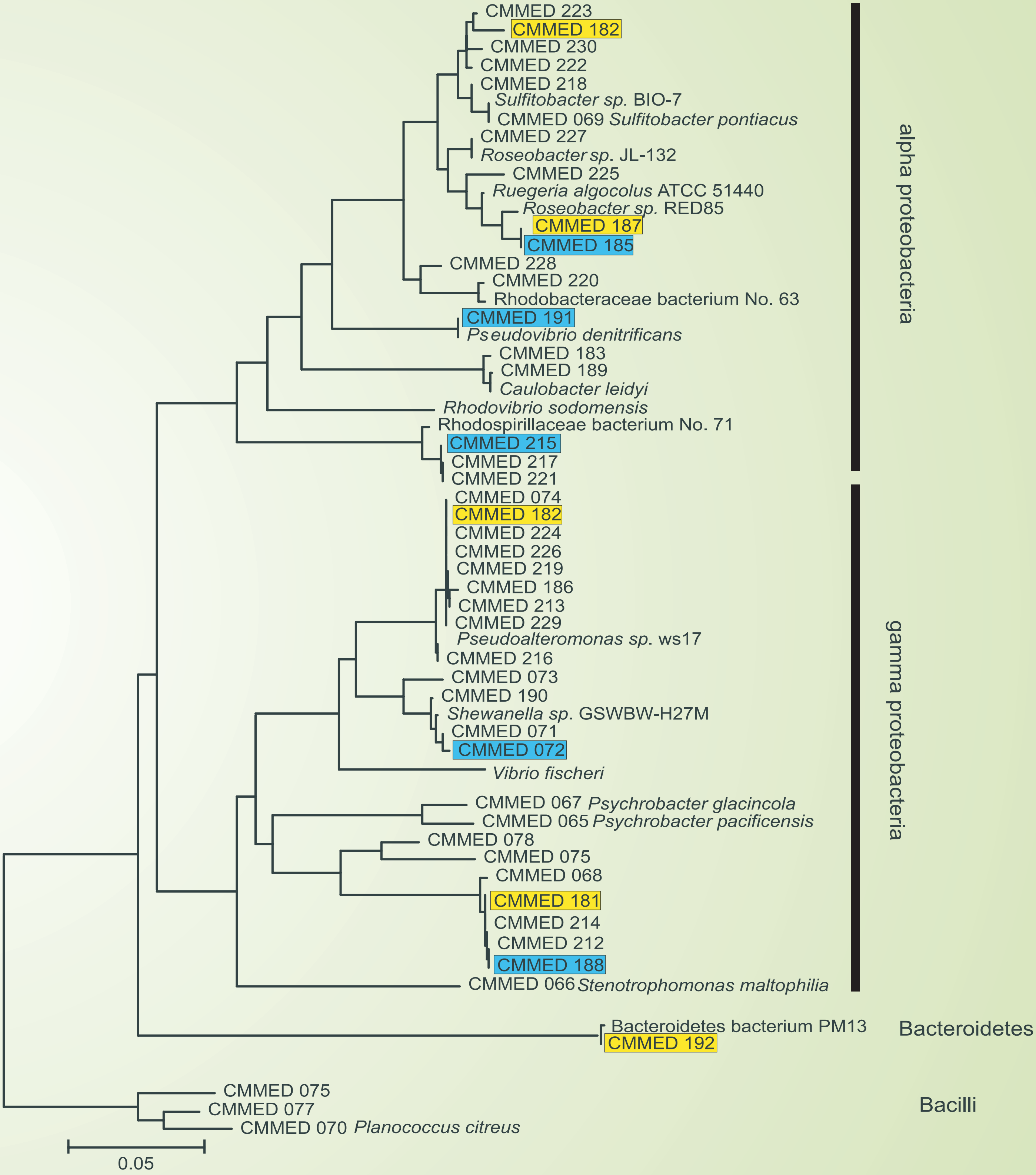

Figure 2. Neighbor-joining phylogenetic tree constructed from a 500 bp gene fragment derived from 16S sequencing of the bacterial isolates listed in Table 1. Some of the strains showed 100 percent sequence identity to the cultivated isolates and therefore share the same position in the phylogenetic tree (anti-infective hits are shown in yellow and anti-tumor hits are shown in blue).

Table 1. Summary of sequence identity and screening data for the bacterial and fungal isolates.

CMMED Culture	Closest Match	%ID NCBI
BAY SEDIMENT		
HTC Bacteria		
CMMED 224	*Pseudoalteromonas sp.* KM-Y92-001	99
CMMED 219	*Pseudoalteromonas sp.*	100
CMMED 220	Rhodobacteraceae bacterium No. 63	99
CMMED 225	*Ruegeria sp.* AS-36	98
CMMED 221	Rhodospirillaceae bacterium	99
CMMED 222	*Sulfitobacter pontiacus*	98
CMMED 223	*Sulfitobacter sp.* BIO-7	98
Agar Plate Bacteria		
CMMED 066	*Stenotrophomonas sp.*	100
CMMED 067	*Psychrobacter glacincola*	100
SEA GRASS SEDIMENT		
HTC Bacteria		
CMMED 212	*Marinomonas sp.* 6-2	99
CMMED 226	*Pseudoalteromonas sp.* KM-Y92-001	100
CMMED 213	*Pseudoalteromonas sp.*	100
CMMED 214	*Marinomonas sp.* 6-2	99
CMMED 227	*Roseobacter sp.* JL-132	99
CMMED 215	🟦 Rhodospirillaceae bacterium No. 71	98
CMMED 216	*Pseudoalteromonas sp.* A28	98
CMMED 217	Rhodospirillaceae bacterium	98
CMMED 218	*Sulfitobacter pontiacus*	98
Agar Plate Bacteria		
CMMED 072	🟦 *Shewanella frigidimarina*	99
CMMED 073	*Shewanella denitrificans*	97
CMMED 074	*Pseudoalteromonas sp.*	100
CMMED 070	*Planococcus citreus*	100
Agar Plate Fungi		
CMMED 231	*Trichoderma aureoviride*	97
CMMED 232	*Phialophora mustea*	98
CMMED 233	*Exophiala pisciphila*	98
CMMED 234	*Hypomyces aurantius*	93
CMMED 235	🟨 *Aspergillus elegans*	96
CMMED 193	🟦 *Mycospharaella macrospora*	99
CMMED 194	*Fusarium sp.*	98
CMMED 195	*Eupenicillum javanicum*	99
CMMED 196	🟦 *Fusarium sp.*	98
SPONGE *MYCALE ARMATA*		
HTC Bacteria		
CMMED 228	Rhodobacteraceae bacterium JC2049	98
CMMED 188	🟦 *Marinomonas protea*	98
CMMED 189	*Caulobacter leidyi*	99
CMMED 229	*Pseudoalteromonas sp.*	100
CMMED 190	*Shewanella sp.* GWS-BW-H27M	99
CMMED 191	🟦 Pseudovibrio denitrificans	100
CMMED 192	🟨 Bacteroidetes bacterium PM13	99
Agar Plate Bacteria		
CMMED 071	Alteromonadaceae bacterium P3	99
CMMED 075	*Agrobacterium agile*	99
CMMED 076	*Bacillus acetylicum*	99
CMMED 077	*Bacillus sp.*	99
CMMED 078	*Pseudomonas sp.*	99
CMMED 065	*Psychrobacter pacificensis*	100
CMMED 069	*Sulfitobacter pontiacus*	100
CORAL SEDIMENT		
HTC Bacteria		
CMMED 181	🟨 *Marinomonas sp.*	100
CMMED 182	🟨 *Sulfitobacter pontiacus*	98
CMMED 183	*Caulobacter leidyi*	99
CMMED 184	🟨 *Pseudoalteromonas sp.* KM-Y92-001	100
CMMED 185	🟦 *Roseobacter sp.* RED85	98
CMMED 186	*Pseudoalteromonas sp*	100
CMMED 187	🟨 *Roseovarius sp.* 2S5-2	98
CMMED 230	*Roseobacter sp.* LA7	98
Agar Plate Bacteria		
CMMED 068	*Marinomonas sp.*	100

🟨 Anti-infective
🟦 Anti-cancer

aureus, and *Candida albicans.* The screening results from bacterial strains indicated that nine hits were obtained from HTC strains and one from agar plate isolates. Among those ten, five were novel strains (Table 1), indicating the potential of discovering isolates that produce novel secondary metabolites. Two fungal strains exhibited anti-tumor and one anti-infective activity (Table 1).

CONCLUDING REMARKS

The use of improved cultivation approaches for the discovery of marine natural products from marine microbes is of paramount importance for the development of new pharmaceuticals (Bull et al., 2005). In this case study, we have shown that HTC technology can be used to isolate novel microbial strains of microorganisms from Hawaiian marine environments, some of which produce metabolites that possess anti-infective or cytotoxic activities. Dereplication analyses are currently underway to ensure that only activities generated by novel bioactive molecules are advanced for further investigation.

We are currently expanding the applicability of HTC to the exploration of marine ecosystems by enabling the use of diverse media at very high dilutions; mimicking the natural environment's concentrations and diversity of carbon sources may enhance the growth and development of heretofore uncultured species. In addition, we are applying HTC technology to other Hawaiian biotopes, including decaying algal material and a broader array of marine metazoans and other invertebrates, as well as cultivating anaerobic microbial flora associated with many diverse marine biotopes. The continued development of HTC technology shows promise for increasing the number of microbial cultures that can be probed for molecules with valuable biomedical applications.

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