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From Uncultured, Through Cultivation, Toward Environmental Proteomics: Cultivation of Uncultured Microorganisms in Diverse Marine Environments

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Uncultured microbial diversity

Epifluorescence microscopy and direct viable counting methods have shown that only 0.01-0.1% of all the microbial cells from marine environments form colonies on standard agar plates (13). Much of the discrepancy between direct counts and plate counts has been explained by measurements of microbial diversity that employed 16S rRNA gene sequencing without cultivation (1, 8, 11, 15, 23). The present consensus is that many of the most abundant marine microbial groups are not-yet-cultivated, that there is a need to cultivate these groups for genome-enabled studies of physiology, and that cultivation will probably require approaches other than standard plating methods. The widespread cloning of 16S rRNA genes from marine bacterioplankton has resulted in high numbers of sequences in public databases. Many of the most abundant phylogenetic groups of marine picoplankton, including the clades SAR86, SAR116, SAR202, SAR324, SAR406, marine *Actinobacteria*, and *Crenarchaeota* marine group I (14) still remain uncultivated, suggesting that further innovation will be needed for their successful cultivation.

Molecular ecological and phylogenetic analyses of 16S rRNAs, both with and without cultivation, have significantly expanded our view of microbial diversity. The number of major groups (lineages or phyla) within the domain *Bacteria* (24) increased from the 11 groups Carl Woese delineated in 1987 to 36 identifiable bacterial phyla in 1998 (16). Today, the domain *Bacteria* is composed of approximately 52 recognized phyla, including 26 candidate phyla which have exclusively 16S rRNA gene clone sequences obtained from environments without cultivation (21). While the number of candidate phyla increased from 14 in 1998 to 26 in 2003, only two phylum-level lineages, the phyla *Gemmatimonadetes* (26) and *Caldithrix* (19), have had representatives cultured during this period. We have recently isolated and identified two microorganisms which are the members of the novel phylum *Lentisphaerae* (9) - previously called VadinBE97 gene cluster, indicating many undiscovered bacterial phyla are to be investigated.

High throughput cultivation

Many attempts have recently been made to cultivate previously uncultured microorganisms by the

application of novel approaches. These include high throughput culturing (HTC) using dilution-to-extinction (10, 20), cultivation using a diffusion growth chamber (18), encapsulation of cells in gel microdroplets (25), and modified plating methods (12, 17). One striking success emerging from these efforts was the first cultivation of members of the SAR11 clade (20), but additionally many novel strains in the *Proteobacteria*, *Planctomycetes*, *Bacteroidetes*, *Acidobacteria*, and *Verrucomicrobia* were also cultivated. One reason for this success is thought to be the use of growth conditions which closely mimic the chemical composition of natural environments (10, 18, 25). Some of the strains obtained by HTC have already been taxonomically classified as novel genera in a novel phylum, order or family, and named *Lentisphaera*, *Parvularcula*, *Croceibacter*, *Fulvimarina*, *Robiginitalea*, and *Oceanicola* (2-7, 9).

The High Throughput Culturing (HTC) technique uses the concept of extinction culturing to isolate bacteria in microtiter dishes (3, 10). The system has been designed so that it has the capability of propagating and detecting microorganisms at growth substrate concentrations and cell numbers that are typical of natural waters. The HTC concept is based on the following hypotheses which might explain the difficulty of cultivating many microorganisms: 1) Some organisms are not easily cultivated because they grow slowly, or at best achieve low cell densities in culture, and therefore growth is not detected, 2) Some organisms are not easily cultivated because they can only grow in narrowly defined conditions that are not likely to be created by chance experimentation, 3) Some organisms are not easily cultivated because they require interactions with other organisms in consortia.

The procedure for isolating cells by extinction culturing is relatively simple. Water samples are diluted into prepared media by serial dilutions to a concentration of 1-5 cells per ml and distributed as 1 ml aliquots into 48-well plates. In these experiments marine bacteria were isolated and cultivated at *in situ* substrate concentrations, typically three orders of magnitude less than common laboratory media. To score the plates a technology developed for the HTC project is used to make cell arrays on polycarbonate membranes (10). Microtiter plates and a newly developed procedure for making cell arrays have been employed to raise the throughput rate and lower detection sensitivity, permitting enumeration of 200 μl aliquots from cultures at densities as low as 1×10^3 cells/ml. Robotic technology may be used to make the cell arrays. Positive wells are then transferred and consolidated on fresh plates, and re-isolated by the same procedure. The relatively high throughput rates achieved make it possible to screen physical and chemical variables to identify conditions that may promote novel forms of microbial life from diverse marine environments.

What microorganisms have been recently cultivated through HTC?

Previously uncultured Oligotrophic Marine *Gammaproteobacteria* (OMG) group

A total of 44 novel strains of *Gammaproteobacteria* were cultivated from coastal and pelagic regions of the Pacific Ocean using the HTC method. The organisms that we discuss here are *Gammaproteobacteria* that belong to previously detected environmental clusters (OM60, BD1-7, KI89A, OM182, and SAR92) retrieved from various marine environments, including different geographic areas and ecosystems. They are distantly related to the other major marine *Gammaproteobacteria* lineages, such as the SAR86 clade, *Vibrio*, *Alteromonas*, and *Oceanospirillum*. Eilers et al. (12) found that microorganisms related to KT71

comprised 8% of total microscopic counts. Phylogenetic analysis showed that the isolates fell into five ribosomal RNA clades, all of which contained rRNA gene sequences reported previously from seawater environmental gene clone libraries (SAR92, OM60, OM182, BD1-7, and KI89A) (**Fig. 1**). Bootstrap analyses of phylogenetic reliability did not support collapsing these five clades into a single clade, and they were therefore named the oligotrophic marine *Gammaproteobacteria* (OMG) group. Twelve cultures chosen to represent the five clades were successively purified in liquid culture and their growth characteristics were determined at different temperatures and dissolved organic carbon concentrations. The isolates in the OMG group were physiologically diverse heterotrophs, and their physiological properties generally followed their phylogenetic relationships. None of the isolates in the OMG group formed colonies on low- or high-nutrient agar upon their first isolation from seawater, while 7 of 12 isolates that were propagated for laboratory testing eventually produced colonies on 1/10 R2A agar. The isolates grew relatively slowly in natural seawater media (1.23-2.63 d^{-1}) and none of them grew in high nutrient media (> 351 mg C l^{-1}). The isolates were psychro-to-mesophilic, and obligately oligotrophic; many of them were of ultramicrobial size (<0.1 μm^3) (**Fig. 2**). This cultivation study revealed that sporadically detected *Gammaproteobacteria* gene clones from seawater are part of a phylogenetically diverse constellation of organisms mainly composed of oligotrophic and ultramicrobial lineages that are culturable under specific cultivation conditions.

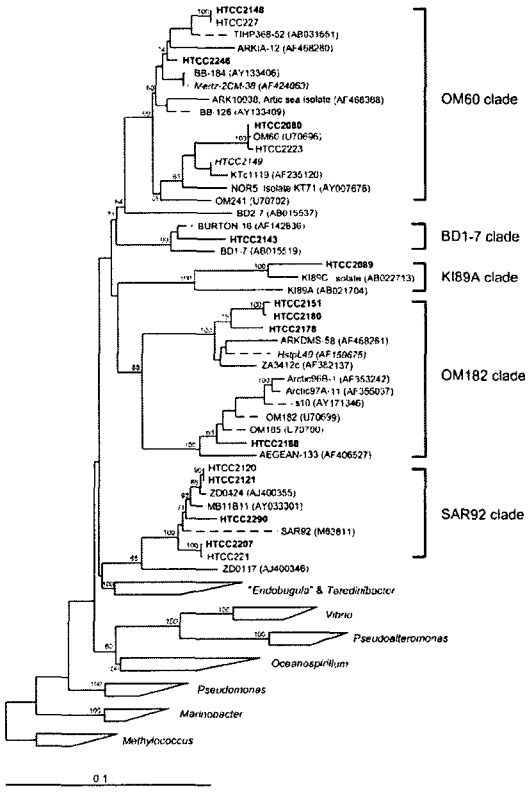

FIG 1. Neighbor-joining 16S rRNA phylogenetic tree showing relationships between the HTCC isolates and environmental sequences in the OMG group.

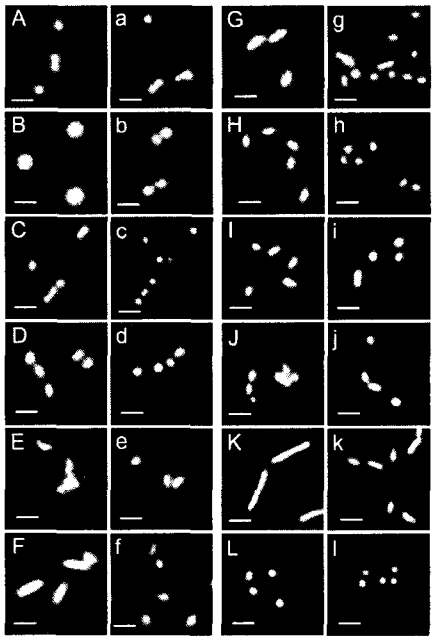

FIG 2. Epifluorescence micrographs of DAPI-stained exponential-phase (uppercase panel letters) and stationary-phase (lowercase panel letters) cells of HTCC isolates in the OMG group. Panels: (A, a) HTCC2148, (B, b) HTCC2246, (C, c) HTCC2080, (D, d) HTCC2143, (E, e) HTCC2089, (F, f) HTCC2151, (G, g) HTCC2180, (H, h) HTCC2178, (I, i) HTCC2188, (J, j) HTCC2121, (K, k) HTCC2290, (L, l) HTCC2207. Scale bars, 1 μm.

The 27th bacterial phylum *Lentisphaerae*

Two phylogenetically distinct marine strains producing transparent exopolymers (TEP) were cultivated from Oregon coast (9). When cultured in low-nutrient seawater media, these strains copiously produced Alcian Blue-stainable viscous TEP. Growing cells were attached to each other by the TEP in a three dimensional network **(Fig. 3)**. PCR employing 16S rDNA primers specific for the novel isolates indicated that they are indigenous to the water column of the Atlantic and Pacific oceans. The abundance of the isolates as determined by 16S rRNA dot blots, however, indicated that they are less than 1% of the total bacterial community. In phylogenetic analyses, the strains consistently formed a new phylum-level lineage within the domain *Bacteria*, together with members of the candidate phylum VadinBE97, which consists of *Victivallis,* the first cultured genus in the candidate phylum, and 16S rRNA gene clones from DNA extracted from marine or anaerobic terrestrial habitats **(Fig. 4)**. Five putative subgroups were delineated within this phylum-level lineage, including a marine group and an anaerobic group. The isolates are Gram negative, strictly aerobic, chemoheterotrophic, and facultatively oligotrophic sphere-shaped bacteria **(Fig. 5)**. The DNA G+C content of strain HTCC2155^T was 48.3 mol% and the genome size was 2.9 mb. It was proposed from these observations that the strains be placed into a new genus and a new species named *Lentisphaera araneosa* gen. nov., sp. nov., the cultured marine representative of the *Lentisphaerae* phyl. nov., and the phylum be divided into two novel orders named the *Lentisphaerales* ord. nov. and the *Victivallales* ord. nov.

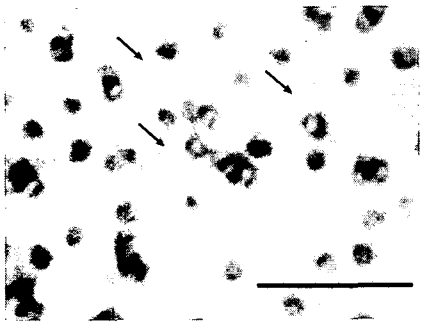

FIG 3. Alcian Blue-stained transparent exopolymer particles (TEP) produced by strain HTCC2155T. Arrows indicate 'slime' extracellular polysaccharides (EPS).

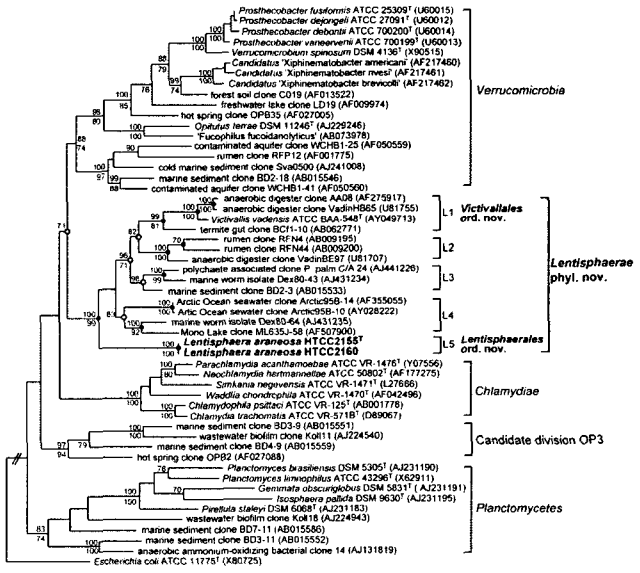

FIG 4. Maximum likelihood 16S rRNA phylogenetic tree showing the phylogenetic positions of the genus *Lentisphaera*, the orders *Lentisphaerales* and *Victivallales*, and the phylum *Lentisphaerae* within the other adjacent phyla. The closed circles and open circles at each node in the *Lentisphaerae* indicate recovered nodes in all three treeing methods and recovered nodes in two treeing methods, respectively.

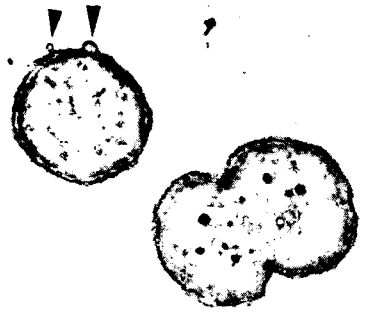

FIG 5. Ultra thin-sectioned transmission electron micrographs of cells of stain HTCC2155T. Triangles in the figure indicate buds or appendages around the cells. Scale bars; 1 μm.

Other HTCC isolates validly described and under description

We have applied the HTC technique mainly to seawater samples obtained from a coast of Oregon, the Pacific Ocean and the Western Sargasso Sea, the Atlantic Ocean. Approximately 5000 extinction cultures from 10 separate samplings of marine bacterioplankton were screened over the course of 3 years. Up to 12% of the cells collected from coastal seawater were cultured using this method, which is maximum three orders higher than obtained by traditional microbiological culturing techniques. Approximately 50 novel species, identified 16S rRNA gene sequences and phylogenetic analyses, were cultivated through the HTC approaches. Among the microorganisms cultured were several unique cell lineages that belong to previously uncultured or undescribed marine bacterioplankton clades known from environmental gene cloning studies. These cultures are related to the SAR11 clade, OM42-NAC11_7 gene clusters in the *Roseobacter* clade (*Alphaproteobacteia*), OM43 (*Betaproteobacteria*), OMG group and uncultured gamma proteobacteria clusters (*Gammaproteobacteria*), uncultured *Cyanobacteria*, uncultured *Actinobacteria*, uncultured *Bacteroidetes*, and novel bacterial phylum. These isolates generally did not form colonies on low- or high-nutrient agar upon their first isolation from seawater samples. In addition, relatively easily growing bacteria affiliated to the class *Alphaproteobacteria*, order *Rhodobacterales*, the families *Flavobacteriaceae* and *Nocardioidaceae* were isolated. Some of easily growing bacteria have been characterized by polyphasic approaches, resulting in the description of novel phylum-, order-, family-, genus level marine bacteria (2-7, 9). We have been now characterizing some marine bacteria, which grow only in seawater-based medium, using seawater medium-based polyphasic approaches. These approaches will give new insight of classification of marine oligotrophic microorganisms.

Proteomics of HTCC isolates and environmental samples

Several novel previously uncultured marine microorganisms in phototrophy, either aerobic anoxgenic phototroph in the *Gammaproteobacteria* or light-driven proton-pumping organism in the *Alphaproteobacteria*, were cultivated in the HTC method. A mass spectrometric protocol was developed to search for proteins related to phototrophy in these marine bacteria (22). ontaining whole proteomes. Several oligopeptides corresponding to photosynthetic reaction center genes were detected in the cell membranes of one isolate grown under the light by ESI/MS/MS. We have also found that the phototrophy-related proteins can easily be identified in whole bacterioplankton samples taken directly from the Pacific Ocean using mass spectrometric analysis. One peptide from this protein was identified with high confidence using the protocol developed for the cultured cells. One-dimensional gel electrophoresis was also used to separate proteins from the bacterioplankton samples prior to enzymatic digestion. The data shown above provide unequivocal evidence that phototrophy-related proteins are expressed in culture and support the conclusion that it is also expressed in coastal seawater. The data also demonstrate that LC/MS/MS can be applied to complex mixtures of proteins from environmental sources.

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