

New Cultivation Strategies Bring More Microbial Plankton Species into the Laboratory

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THE IMPORTANCE OF CULTURES IN THE AGE OF GENOMICS

It is difficult to describe the properties of a microorganism without first cultivating it. There has been a great deal of effort and considerable success in predicting microbial metabolic activities from genome sequences, (i.e., functional genes), assembling genomes from metagenomic data, testing the activities of cells in situ, and from studying complex microbial assemblages in the laboratory. But, the fact remains that the most efficient way to gather information about a microorganism is to study it in culture and relate that knowledge to field observations. Genomes are easily determined

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from pure cultures, and cultures often offer the best possibilities to test hypotheses that emerge from genome sequences or ecological studies. Figure 1 illustrates some of these relationships.

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The concept of “the assay” is an important part of microbial cultivation. It is a rare microbial cultivation experiment that does not succeed in growing something. The question is: what are you looking for? For decades, phytoplankton cultivation was a far more successful endeavor than the cultivation of marine bacteria simply because phytoplankton could be identified by their morphology or fluorescent properties. Bacteria are hard to tell apart by their appearances, so being handy with a microscope wasn't very helpful for determining whether a heterotrophic microbial isolate growing on a plate was a rare community member or an abundant species. By 1990, lots of heterotrophic marine bacteria

had been described, but there was virtually no certainty that any of them were abundant in the ecosystem. The problem was the plate count anomaly—microscopically counted cells in seawater out-

numbered colonies on plates by four orders of magnitude, leaving scientists in a quandary as to whether their isolates were important, but rarely formed colonies, or were rare specialists that could endure the conditions on a Petri dish.

The solution to this problem came from ribosomal RNA technology, which was applied to measure microbial diversity in most major ecosystems, including the ocean (Olsen et al., 1986). Ribosomal RNA sequences gave lists of organisms, a code for their identification, and their placement in phylogenetic trees. As it turned out, most of the organisms detected by this approach had no binomial name and had never been described, ostensibly because

they were “uncultivable” (Rappé and Giovannoni, 2003).

For marine bacteria, the one exception to the rule above was the oxygenic phototrophs—unicellular cyanobacteria and prochlorophytes—which were first described in 1979 and 1988 (Chisholm et al., 1988; Waterbury et al., 1979). These very abundant organisms were not easy to culture. But, they became the targets of focused cultivation efforts because their abundance could be confirmed by optical methods that detected their auto-fluorescence. This example illustrates the importance of cultivation approaches that are initiated with prior knowledge of the target organisms. Recent success in the cultivation of abundant heterotrophic bacterioplankton taxa could not have been successful without the large databases of 16S ribosomal RNA sequences that direct attention to important species.

CULTIVATED MARINE MICROORGANISMS BEFORE THE RIBOSOMAL RNA REVOLUTION

Since late in the nineteenth century, microbial cells in seawater have been studied, scrutinized, and enumerated based primarily on their ability to replicate and form colonies on solid growth media, as well as morphological characteristics of cells observed under a microscope. For as long as these cells have been domesticated in the laboratory, microbiologists have also sought to provide them names via the development of identification, description, and classification systems (i.e., a taxonomy) appropriate for marine microbes. Early systems relied upon such characteristics as cell morphology; motility; presence, number, and positioning of flagella; colony mor-

phology and color; ability to perform fermentative growth; and overall base composition, among others. In hindsight, it is now generally recognized that only a select few marine microorganisms present in any given seawater sample possess the ability to replicate and form colonies on solid media containing high levels of organic nutrients, which was the predominant medium and mechanism by which marine microbes were tradi-

tionally domesticated. This highly selective methodology resulted in the recovery of isolates with fairly uniform gross traits: the vast majority were Gram-negative chemoorganotrophs, motile via polar or peritrichous (uniformly distributed) flagella, straight or curved rods,

and frequently able to use nitrate as a terminal electron acceptor for facultative anaerobic growth.

The earliest reports to describe and name microorganisms isolated from seawater placed the more commonly recovered groups within the genera *Pseudomonas*, *Vibrio*, and *Spirillum* within the family Pseudomonadaceae; the genera *Achromobacter* and *Flavobacterium* within the family

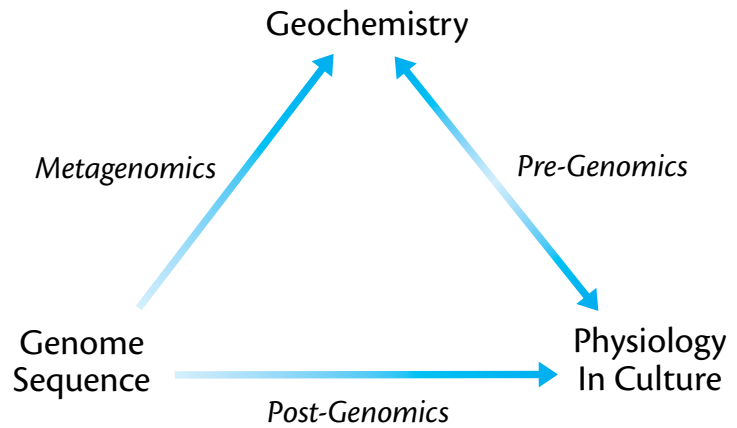


Figure 1. The role of microbial cultivation and genomics in oceanographic science. Genomics augments laboratory studies of microbial physiology that have the goal of predicting the geochemical activities of cells in nature. Metagenomics circumvents the need for cultures, but without cultures, hypotheses that arise from the scrutiny of genome sequences are more difficult to test.

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Achromobacteriaceae; and the genus *Bacillus* within the family Bacillaceae (see ZoBell, 1946, for a review). As additional morphological, physiological, and molecular (i.e., DNA base composition) traits were investigated over the ensuing 30 years, many genera and

species of marine origin were redistributed, renamed, or created de novo to accommodate newly discovered isolates and groupings. For example, in 1957, Campbell created the genus *Beneckea* to include marine microorganisms possessing a straight-rod cellular morphology with peritrichous flagellation, fac-

related clades (groups of organisms with a common ancestor, or a monophyletic group) of the gamma subclass of the Proteobacteria. One clade consists of the closely related genera *Vibrio* and *Photobacterium* (Baumann and Schubert, 1984), while the second consists of the original members of the

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ultatively anaerobic metabolism, and the ability to utilize chitin and grow fermentatively on glucose with the production of acid but no gas (Baumann et al., 1971; Campbell, 1957), though in 1980, it was subsequently abolished and its species included within the genus *Vibrio* (Baumann et al., 1980; Campbell, 1957). The end result of the era in marine microbiology preceding the widespread use of ribosomal RNA sequences in systematic studies was that most marine bacterial species were included within a fairly limited number of genera, including *Vibrio*, *Pseudomonas*, *Oceanospirillum*, *Aeromonas*, *Deleya*, *Flavobacterium*, *Alteromonas*, and *Marinomonas*.

The ribosomal RNA revolution brought with it a gradual refinement to the inter- and intra-generic relationships of cultivated marine microorganisms that continues to the present day. One noteworthy discovery was that a majority of the named species of culturable marine bacteria falls within two

genus *Alteromonas*, plus newly cultivated strains that have been subdivided into several different genera based in part on 16S rRNA gene sequence comparisons. Genera in this clade include the original *Alteromonas* plus *Pseudoalteromonas*, *Marinomonas*, *Shewanella*, *Glaciecola*, *Idiomarina*, *Colwellia*, *Thalassomonas*, *Oceanimonas*, *Ferrimonas*, and *Psychromonas*, among others (Ivanova et al., 2004). A second noteworthy discovery was that the characteristic helical cellular morphology considered to be the main delineating trait of members of the genus *Oceanospirillum* proved to be polyphyletic: species originally assigned to the genus *Oceanospirillum* have been shown to form several separate lineages in the gamma and alpha subclasses of the Proteobacteria based on 16S rRNA gene sequence comparisons, and have since been placed into a variety of new genera, including *Marinospirillum*, *Pseudospirillum*, *Oceanobacter*, and *Terasakiella* (Satomi et al., 2002; Satomi et al., 1998).

NEW APPROACHES PROVIDE NOVEL ISOLATES

New methods are leading to the isolation of many new microbial species, including strains that are important to oceanographers. These methods include simple modifications to traditional cultivation strategies, development of high-throughput culturing (HTC), combining cultivation with culture-independent methods for quick identification of isolates, design and use of novel growth chambers, and application of single-cell isolation procedures (Figure 2).

The simplest modifications to traditional media recipes, such as lowering the nutrient concentrations, amending nontraditional sources of nutrients, and increasing incubation times, led to the successful isolation of previously uncultured bacteria (Connon and Giovannoni, 2002; Eilers et al., 2001; Joseph et al., 2003; Stevenson et al., 2004). Ultimately, replicating the natural conditions (e.g., temperature, light, nutrients) as much as possible should lead to a higher probability of isolation. Furthermore, a good understanding of a target's metabolism is useful and a prerequisite for medium modifications; however, for the majority of uncultured representatives, only a 16S rRNA sequence is known.

The isolation of two novel pelagic gamma-Proteobacteria strains, KT71 and NOR5, from the North Sea was achieved by reducing the inorganic N and P sources in the growth media to the ambient levels in coastal waters (Eilers et al., 2001). In addition to reducing nutrient concentrations, prolonging the incubation period while continually removing newly formed colonies from the growth plates was instrumental in the isola-

tion of KT71 and NOR5. The removal of colonies allows the slower-growing targets to compete with faster-growing (“weed”) populations.

Amendments of nontraditional sources of nutrients, signaling molecules, or inhibitory compounds (for unwanted microorganisms) also show promise. For example, isolation of several heterotrophic bacterioplankton strains from the Central Baltic Sea was greatly enhanced by the addition of signal molecules (cyclic AMP and acyl homoserine lactone) (Bruns et al., 2002), and dormant *Micrococcus luteus* could be resuscitated by a cytokine-like growth-promoting factor from a spent medium of an actively growing culture of the same species (Mukamolova et al., 1998). In the isolation of the pathogenic bacterium, *Mycobacterium tuberculosis* (H37Ra), the addition of an aged culture supernatant increased the viability and initiated growth of *M. tuberculosis* in liquid culture (Sun and Zhang, 1999).

These examples clearly show that inter- and intraspecific interactions can influence the growth of microbial species. Microbial communities are viewed by many as complex and highly structured assemblies of interdependent populations connected to each other through a multitude of interactions that have evolved over billions of years of natural selection. The diverse metabolic capabilities of microbial species create essentially infinite possibilities for two (or more) microorganisms to perform reactions that neither partner alone is fit to do. Synergies based on commensalisms (symbiotic relationships where one organism benefits and the other is not harmed), cross-feeding, co-metabolism,

and other mutually beneficial interactions have been known for at least a century (Schink, 2002; Stams et al., 2006). The ability of many microorganisms to synchronize activities by intra- and interspecific signaling is a more recently discovered class of interactions (Bassler, 2002; Waters and Bassler, 2005).

The better-known examples of tight synergies between groups of different, often unrelated microorganisms are methanogenic (Chauhan et al., 2004; Conrad and Klose, 1999; Stams et al., 2005) and anaerobic methane-oxidizing consortia (Boetius et al., 2000). Concerted biodegradation of

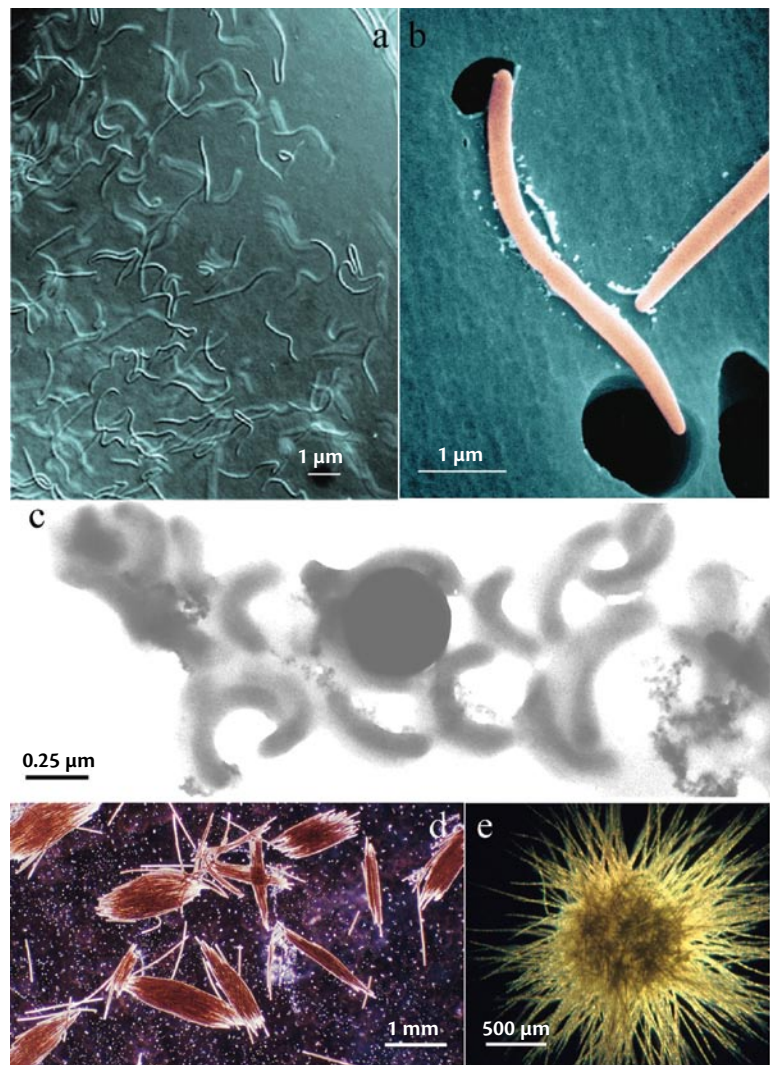


Figure 2. Examples of recent successful cultured isolates. a–b. DIC (a) and SEM (b) views of a marine isolate uncultivable on standard media but successfully isolated in diffusion chambers incubated in situ. The strain has 97% 16S rRNA gene similarity to *Arcobacter nitrofigilis*. Photos by S. Epstein and W. Fowle, Northeastern University c. SAR11 cells negatively stained alongside a 0.514 micron latex bead. Photo from Rappé et al., 2002 d–e. Tuft and puff morphologies of cultured diazotrophic cyanobacterium, *Trichodesmium erythraeum* (d) and *T. thiebautii* (e). Photos courtesy of Dr. H.W. Paerl, University of North Carolina at Chapel Hill, Institute of Marine Sciences

complex organic compounds by consortia of two and more members is also a well-established fact (Guevara and Zambrano, 2006; Pettigrew et al., 1990; Sanchez et al., 2005). Often, the interactions within consortia are based on one partner, or group of partners, stimulating the growth and activities of the other member(s) of the consortium by providing them with limiting electron donors, acceptors, or growth factors, or modifying the environment. However, this is unlikely to be a full list of possibilities as the nature of synergistic interactions in many other exciting consortia remains to be elucidated (e.g., apparently obligate phototrophic consortia) (Overmann and Schubert, 2002).

It follows that at least some microbial species may be incapable of growing in pure culture unless provided with metabolites of their natural partners. Because the natural milieu likely contains at least some such metabolites, growth media based on naturally occurring components show considerable

(1 to 10 cells of inoculum per culture) and examine the cultures for microbial growth by means of flow cytometry, which is effective for counting very dilute populations of cells. Two to sixty percent of cells from marine waters around Alaska and the Netherlands were reported to be culturable by this method when filtered, autoclaved seawater was used as the medium (Button et al., 1993; Schut et al., 1993). This work resulted in the description of two new oligotrophs, *Sphingomonas alaskensis* and *Cycloclasticus oligotrophus* (Button et al., 1998; Button et al., 1993; Schut et al., 1993).

S.A. Connon and coworkers reported a HTC method with dilution-to-extinction procedures in natural seawater media, and also applied a new array technology for rapidly screening isolates by epifluorescence microscopy (Connon and Giovannoni, 2002). This approach makes use of dilution to isolate the cells into microtiter dish wells. The new arraying procedures are a modifica-

phylogenetic surveys of ribosomal RNA genes cloned from environmental DNA. Among the microorganisms that were cultured, many were unique cell lineages and included cultures related to the clones SAR11 (alpha-Proteobacteria), OM43 (beta-Proteobacteria, related to the methylotroph *Methylophilus methylotrophus*), SAR92 (gamma-Proteobacteria), and OM60/OM241 (gamma-Proteobacteria).

Some newly described taxa that were isolated by high throughput dilution-to-extinction methods are: *Candidatus Pelagibacter ubique* (SAR11 clade), *Croceibacter atlanticus*, *Parvularcula bermudensis*, *Fulvimarina pelagi*, *Lentisphaera araneosa*, *Oceanicola granulosis*, *Robiginitalea biformata*, *Pelagibaca bermudensis*, *Maricola koreensis*, *Maricola bermudensis* and *Litoribacter rhodopsinicus* (SAR92 clade) (Button, 1998; Cho and Giovannoni, 2003a, 2003b, 2003c, 2004; Cho et al., 2004; Rappé et al., 2002; Schut et al., 1993; Vancanneyt et al., 2001; Wang et al., 1996).

Similar to a HTC dilution approach, Bruns et al. (2003) developed the MicroDrop® micro dispenser system, which significantly accelerates extinction culturing practices. This system inoculates 96-well microtiter plates with a highly reproducible droplet volume in less than a minute. When the system was compared with the conventional most probable number (MPN) approach, culturable efficiencies were systematically lower for the MicroDrop® technique; however, there is more replication in the MicroDrop® method, and therefore higher statistical significance (10 times lower standard deviation) (Bruns et al., 2003). It should be

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promise. The use of very small inocula into these media further enhances microbial recovery. D.K. Button pioneered the development of methods for isolating bacterial cultures in media made from natural seawater or lake water (Button et al., 1993). His approach was to dilute natural communities of microorganisms to extinction

tion of standard methods for counting cells on polycarbonate membranes, but because of the small dimensions of the filtration device, the procedure is effective with small volumes of very dilute cultures. Using the arrays and fluorescent in situ hybridization (FISH) enabled experimentalists to focus on high-priority organisms that emerged from

noted that both approaches (MPN and MicroDrop®) cultivated similar phylogenetic groups, including a numerically dominant, but not-yet-cultured beta-Proteobacterium strain.

Zengler et al. (2002) describe a novel strategy that separates individual microbes into microcapsules, then cultivates the capsules “together” in a chromatography column filled with amended growth media at a controlled flow rate. Growth is identified by microscopy, and microcapsules indicating growth are sorted by flow cytometry into microtiter dishes containing organic-rich media. This type of system allows interactions (i.e., cross-feeding of metabolites or signaling) to occur between the different microbes during the growth phase in the column while each remains separated in its “caged micro colony” and provides more than 10,000 bacterial isolates per environmental sample. Zengler et al. (2002) reported high cultivation efficiencies for bacterioplankton (and soil microbes), but no novel cultures were propagated.

The continuous search for cultivation methods that mimic the natural environment led to moving the cultivation effort into the environment itself and to developing devices to achieve this goal. The majority of these devices have been successfully applied to non-pelagic systems (i.e., sediments). Kaeberlein et al. (2002) designed a simple diffusion growth chamber with a stainless steel washer and two 0.03 µm-pore-size polycarbonate membranes. The growth chamber can be incubated in situ, where diffusion provides the microorganisms inside the chamber to interact with naturally occurring growth components.

A similar approach was adopted by Ferrari et al. (2005), where a sediment slurry was filtered onto a polycarbonate membrane filter and placed on top of a soil extract. In both systems, exchange of nutrients and other chemicals with the environment are allowed without the loss of cells; both led to successful

from the original environmental sample. This suggested the successful isolation of subpopulations; however, one caveat regarding cultivation by the FACS approach is the requirement for the use of a cell-surface molecule (lipid, protein, carbohydrate) to instill viability of the population after sorting.

Information, such as a microbe's nutritional requirements, enzyme expression in situ, and genetic potential, all can be used to predict optimal conditions for cultivation.

cultivation of a significant fraction of inoculated cells that did not grow on conventional media. Feasible alternatives for bacterioplankton would be to incubate in situ or to concentrate cells onto a membrane filter, then place the filter in a bottle with growth media. For example, planktonic iron-oxidizing bacteria were successfully isolated by floating a polycarbonate filter on modified media (De Bruyn et al., 1990).

The innovations described below are difficult to classify because they use “tricks” that involve neither natural media nor natural environment. Flow cytometry combined with fluorescent activated cell sorting (FACS) was used to quantify, fractionate, and isolate individual bacterial communities (Park et al., 2005). When this technique was applied to activated sludge and hydrothermal vent samples, subpopulations of cells obtained by FACS yielded reproducible denaturing gradient gel electrophoresis (DGGE) patterns that were different

Others have sorted populations of microbes for cultivation simply by picking individuals. For example, the cyanobacterial symbiont (*Calothrix* sp. SC01) of the diatom *Chaetoceros compressus* was identified by green and blue epifluorescence microscopy, then successfully isolated into culture using a micropipette (unpublished work of author Foster). Ziegler et al. (1990) gathered filamentous bacteria (*Gordona*, *Microthrix*) by micro-manipulation with special tools (Blackall et al., 1995; Tandoi et al., 1992), and others have used laser micro-dissection technology (Frohlich and Konig, 2000).

Populations that are easily identifiable and collected are not always easily maintained in culture. For example, the colonial diazotroph, *Trichodesmium* spp., evaded pure isolation for decades. Its eventual successful isolation required frequent monitoring, a specialized growth medium, chemical treatment (cycloheximide) during initial isolation, and subsequent special handling

and culture flasks for maintenance (Prufert-Bebout et al., 1993). Another interesting example is populations of the unicellular diazotrophs, *Crocospaera watsonii* (WH8501), formerly known as

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Synechocystis sp. (WH8501), which were first isolated offshore of the western tropical Atlantic Ocean in 1985 (Waterbury and Rippka, 1989); however, they were not recognized as important contributors in nitrogen fixation until a decade later (Zehr et al., 2001). Still other relevant examples are the many strains of *Synechococcus* and *Prochlorococcus* species that have been brought into pure culture. Currently, genomes for 13 *Synechococcus* (WH8102, CC9605, CC9902, PCC7942, PCC9311, WH5701, RS9916, RS9917, WH7805, BL107, MIT9220, M11.1, M16.17) isolates and 11 *Prochlorococcus* (CCMP1986, MED4, MIT9313, MIT9312, MIT9211, AS9601, MIT9303, MIT9515, NATL1A, MIT9301, MIT9202) strains have been sequenced (or in are in queue for sequencing), annotated, and made publicly available from the Joint Genome Project (<http://www.jgi.doe.gov/>), the TIGR Institute for Genomic Research (<http://www.tigr.org/db.shtml>), or the Venter Institute (<https://research.venterinstitutione.org/moore>).

In the last decade, culture-independent surveys have revealed an enormous amount of information on the uncultivated majority's diversity,

function, and distribution. Information, such as a microbe's nutritional requirements, enzyme expression in situ, and genetic potential, all can be used to predict optimal conditions for cultivation.

In comparison, attention to and focus on developing novel techniques in cultivation has been disproportionately low. New technologies specific for cultivation, including single-cell manipulation devices, high-throughput screening techniques, and simple modifications to traditional approaches, hold promise for targeting the isolation of microorganisms, but still require discrete attention to the study of new isolates.

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