

Incubation of Environmental Samples in a Diffusion Chamber Increases the Diversity of Recovered Isolates^{∇†}

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The majority of microorganisms from natural environments cannot be grown in the laboratory. The diffusion-chamber-based approach is an alternative method that allows microorganisms to grow in their natural environment. An inoculum is sandwiched between semipermeable (0.03- μm -pore-size) membranes of the chamber, which is then returned to the source environment. The chamber allows for a free exchange of chemicals with the external milieu by diffusion while restricting the movement of cells. We used freshwater pond sediment to inoculate diffusion chambers and petri dishes. The diffusion chambers were incubated on top of the sediment for 4 weeks. Both chamber and petri dish cultivation resulted in the isolation of numerous representatives of *Alpha*-, *Beta*-, and *Gammaproteobacteria*; *Actinobacteria*; *Firmicutes*; and *Bacteroidetes*. However, the diffusion-chamber-based approach also led to the isolation of species from rarely cultivated groups, such as *Deltaproteobacteria*, *Verrucomicrobia*, *Spirochaetes*, and *Acidobacteria*. Material from the chambers was also transferred to new chambers in order to learn whether this will increase the recovery of isolates. Several isolates could be obtained only from material transferred through multiple diffusion chambers. This suggests that continuous cultivation in diffusion chambers adapts some microorganisms for growth under otherwise prohibitive *in vitro* conditions.

The majority of microorganisms from environmental samples cannot be cultivated with the widely used laboratory methods (16, 22, 28). Large discrepancies between the number of CFU and direct cell counts were observed as early as the beginning of the last century (1, 10) and repeatedly confirmed in numerous publications (3, 8, 18, 36). Culture-independent molecular methods showed that uncultivated species represent the bulk of the microbial diversity on our planet (16, 28), with more than half of the discovered bacterial phyla having no or only a few cultured members (9, 22, 28). Gaining access to these microorganisms is of substantial basic and applied significance. Several cultivation approaches have been suggested and developed to address the “great plate count anomaly” (31). A high-throughput approach based on dilution to extinction with seawater as the substrate led to cultivation of representatives of the very abundant but previously uncultivated alphaproteobacterial clade SAR11 (11, 26). Another high-throughput approach successfully used single-cell encapsulation and cocultivation of environmental microorganisms to increase microbial recovery (35). The addition of cyclic AMP and homoserine lactones to the growth media also produced additional microbial isolates (5, 6, 7, 32). The manipulation of growth medium composition and increased length of incubation led to the isolation of *Acidobacteria* and *Verrucomicrobia* (12, 19, 20, 29, 32). However, the majority of environmental

microorganisms remain uncultivated, and this calls for the development of new isolation methods.

We developed a diffusion chamber approach (Fig. 1) that provided access to up to 40% of the cells present in a marine sediment environment (21). The approach is based on placing microorganisms in a diffusion chamber separated from the environment by 0.03- μm -pore-size membranes and incubating the chamber in the natural habitat of the target microorganisms. The membranes are permeable by molecules of various sizes as well as small viruses. Diffusion provides the cells with access to their naturally occurring growth components, including nutrients and possible signaling compounds, and removes the metabolic products. Preliminary observations (T. Kaeberlein and D. Nichols, unpublished) showed that some environmental microorganisms might acquire the ability to grow in a petri dish after repeated cultivation in a diffusion chamber. We confirm these observations by showing that one to several incubations in a diffusion chamber lead to an increase in the number and diversity of environmental isolates capable of growth *in vitro*.

MATERIALS AND METHODS

Sampling. Sediment and water samples were taken in the fall of 2004 from Turtle Pond, a small freshwater pond in Boston, MA. A freshly collected block of near-shore sediment was carefully placed in a 30- by 40-cm aquarium to form a 10-cm-thick layer. The sediment was kept in the lab under 5 cm of pond water with continuous aeration. Over the course of the growth experiment (16 weeks; see below), the sediment was replaced twice with freshly collected material.

Media. The media (all from Difco, Becton, Dickinson & Company, Sparks, MD) used in the isolation experiments were prepared with 1.5% Bacto agar and 10% sterile-filtered pond water. The following media were supplemented with the indicated carbon sources: medium A, no supplements; C, 0.01% Bacto Casamino Acids; E, 0.1% hot water extract from the source sediment (see below); CY, 0.01% Bacto Casamino Acids and 0.01% Bacto yeast extract; CYE, 0.01% Bacto Casamino Acids, 0.01% Bacto yeast extract, and 0.1% hot water extract from the source sediment; and Y, 0.01% Bacto yeast extract. Hot water extract was prepared by mixing sediment and deionized water in a 1:10 ratio. The

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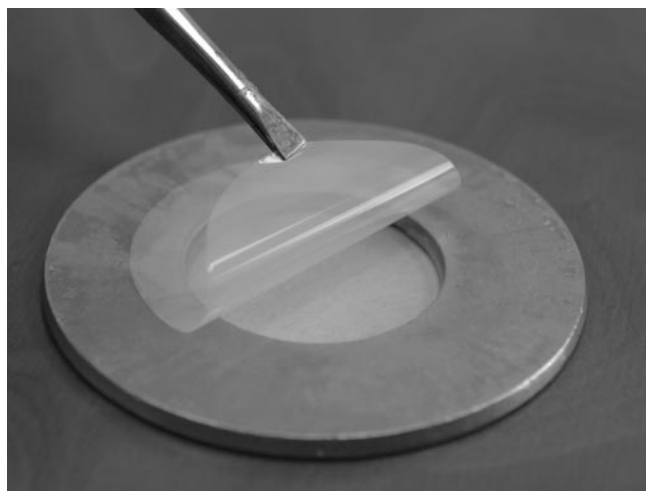


FIG. 1. General view of the diffusion chamber for in situ microbial cultivation. One polycarbonate membrane is attached to the bottom of the stainless steel O-ring, the other to its top surface. The inner space is filled with microbial cells mixed with agar.

mixture was autoclaved for 30 min, spun down (10 min, 11,000 rpm), and filter sterilized using 0.22- μ m-pore-size filters. In several experiments, a variation of the CYE medium in which the hot water extract was replaced by 0.01% humic acids (Sigma Aldrich, St. Louis, MO) was used. During the isolation process, the cultures were subcultured onto 0.1 \times LB (Sigma Aldrich, St. Louis, MO) according to the manufacturer's instructions and supplemented with 1.5% Bacto agar.

Cultivation and isolation. One-gram subsamples were diluted with sterile-filtered pond water and mixed with the media described above to a final dilution of 10^{-4} of the sampled material. This mixture was used to inoculate diffusion chambers with A, CY, and CYE media and to prepare pour plates (all media). The overall experimental design is outlined in Fig. 2.

The diffusion chambers (Fig. 1) were prepared as described previously (21). A 0.03- μ m-pore-size polycarbonate membrane (GE Osmonics Labstore, Minnetonka, MN) was glued to a stainless steel O-ring with silicone glue. A sample of environmental microorganisms was mixed with warm (45°C) agar, and 3 ml of the mix was placed on the membrane. After the agar solidified, the second membrane was glued to the other side of the O-ring, sealing the agar inside to form a diffusion chamber. Six first-generation diffusion chambers were prepared, two for each of the three media (A, CY, and CYE). The diffusion chambers were incubated on top of the sediment in the aquarium and flipped at 48-h intervals to prevent the buildup of anoxic conditions below the chambers. After 4 weeks of incubation, the chambers were opened. The material was homogenized by passaging it through a syringe equipped with a 25-gauge needle, diluted with sterile-filtered pond water, and mixed with the respective medium to a final dilution of 10^{-4} . The total volume of the mix

originating from a single chamber was 10 ml, of which 3 ml was used to inoculate one diffusion chamber of the next generation for an additional 4-week-long incubation in the aquarium. The rest of the mix was used to prepare one traditional pour plate. This procedure was repeated two more times, with the chamber-grown material inoculated into a single chamber of the next generation. The original sample of microorganisms was thus sequentially grown in four generations of diffusion chambers, subcultured at each step in petri dishes. Colonies growing in these petri dishes were further subcultured in CYE pour plates and/or streaked on 0.1 \times LB plates. The cell morphology of 15% of the isolates, which were randomly chosen, was examined for purity using a compound microscope equipped for differential interference contrast at $\times 400$ or $\times 1,000$ magnification.

Identification. Microbial identification was performed via sequencing of the isolate's 16S rRNA gene (24). The colony material was subjected to a freeze-thaw cycle (first -80°C , then 10 min at 65°C , 10 min at 96°C , 20 min at -20°C , and 10 min at 65°C) in 1 \times Tris-EDTA (10 mM Tris, 1 mM EDTA, pH 8; Sigma Aldrich, St. Louis, MO). This material was used as a template for PCR-enabled 16S rRNA gene amplification. Alternatively, the colony material was used directly as a template. If PCR failed to amplify the 16S rRNA gene, DNA was isolated as described in reference 17 or with a QIAGEN tissue kit (QIAGEN, Valencia, CA) according to the manufacturer's recommendations. The 16S rRNA gene was amplified using eubacterial primers 27F (AGA GTT TGA TCC TGG CTC AG) and 1492R (GGT TAC CTT GTT ACG ACT T) (24) with either a *Taq* polymerase (Promega, Madison, WI) or a Hot Start *Taq* system (QIAGEN, Valencia, CA). The PCR products were purified and sequenced commercially (Seqwright, Houston, TX) by fluorescent dye terminator sequencing using internal primer 357F (CCT ACG GGA GGC AGC AG) (27). The sequences were edited with 4Peaks (A. Griekspoor and Tom Groothuis, The Netherlands Cancer Institute) and grouped into strains and species if their 16S rRNA gene sequences were different by no more than 1 nucleotide and 3% of nucleotides, respectively. For each strain, the longest sequence was aligned to the ARB database (25) and added to the tree in the ARB database with the parsimony addition tool, and its position was used for strain identification.

Nucleotide sequence accession numbers. The sequences have been deposited in the GenBank database under the accession numbers EF636028 to EF636203.

RESULTS AND DISCUSSION

We cultivated environmental microorganisms from freshwater pond sediment by passaging them through four successive generations of diffusion chambers (Fig. 2). The sediment and the chamber-derived material were used to inoculate petri dishes for subsequent isolation of the bacteria. We then compared the richnesses of the isolates to evaluate the usefulness of the incubation in the diffusion chamber.

In total, we obtained 438 isolates representing 174 strains and 112 species from 10 bacterial phyla (Table 1; see the supplemental material). Seventy percent of the strains were obtained exclusively from the diffusion-chamber-derived ma-

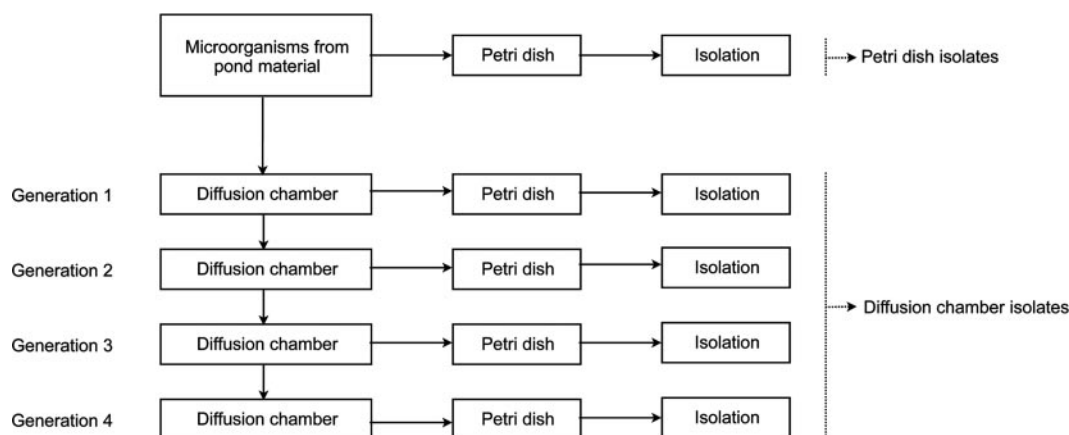


FIG. 2. Schematic overview of the experimental setup.

TABLE 1. Phylogenetic affiliation of the strains obtained by standard and diffusion-chamber-based approaches

Phylum	No. of petri dish-specific strains	No. of strains specific to diffusion chamber of:					No. of strains isolated by both methods	Total no. of strains isolated in this study
		Generation				All generations		
		1	2	3	4			
<i>Alphaproteobacteria</i>	20	8	13	6	9	36	6	62
<i>Betaproteobacteria</i>	3	25	22	8	8	63	3	69
<i>Gammaproteobacteria</i>	2			4		4	1	7
<i>Deltaproteobacteria</i>			1			1		1
<i>Bacteroidetes</i>	5	2	4			6	1	12
<i>Verrucomicrobia</i>			2	1	2	5		5
<i>Acidobacteria</i>			1	1		2		2
<i>Spirochaetes</i>				4		4		4
<i>Firmicutes</i>	5			1		1		6
<i>Actinobacteria</i>	5		1			1		6
Total	40	35	44	25	19	123	11	174

terial, 23% from the petri dish material, and 7% from the material cultivated using both approaches (Table 1). This indicates that the majority of the isolated strains were unique to their isolation approach and that in situ incubation substantially increased the overall number of isolated strains.

The diversity of the diffusion-chamber-derived strains exceeded that obtained from standard petri dishes. The strains from the diffusion chambers belonged to 10 different phyla: *Alpha*-, *Beta*-, *Gamma*-, and *Deltaproteobacteria*; *Bacteroidetes*; *Actinobacteria*; *Firmicutes*; *Spirochaetes*; and the rarely cultivated *Acidobacteria* and *Verrucomicrobia*, compared to only six phyla represented by the petri dish isolates (*Alpha*-, *Beta*-, and *Gammaproteobacteria*; *Bacteroidetes*; *Actinobacteria*; and *Firmicutes*) (Table 1). This result is in accordance with other studies using petri-dish-based methods and principally reporting isolates from the same four phyla (4, 14, 23, 33). We note that the diversity of the microorganisms actually growing in the diffusion chambers was likely even greater than that reported here because only the material grown in agar, not as biofilm on the membranes, was passaged to the chamber of the next generation.

The abundance distributions of the strains were different between the two cultivation techniques. *Betaproteobacteria* dominated the pool of the diffusion-chamber-derived strains (50%), and *Alphaproteobacteria* dominated the pool of petri

dish strains (49%). In general, *Betaproteobacteria* have been isolated in larger numbers when methods that attempted to mimic naturally occurring conditions, such as low-substrate concentrations, the addition of signaling compounds, and in situ incubation, were used (5, 6, 13, 15). All of these methods favor the growth of slower-growing oligotrophic species, often representing *k*-strategy and the ability to live in nutrient-poor environments (2, 13, 34). This might explain the isolation of *Verrucomicrobia* and *Acidobacteria* from material transferred through the diffusion chamber. A few members of both groups have been previously isolated by specific approaches, such as altering the medium composition (e.g., amending growth media with xylan), using a substrate concentration close to naturally occurring conditions, and prolonging incubation times (12, 15, 19). The historical data on microbial cultivation recorded in GenBank in the form of 16S rRNA gene sequences show that earlier studies recovered 109 isolates of *Verrucomicrobia* out of the total 110,322 isolates of *Bacteria* ("isolates" defined as a result of the search query "16S Verrucomicrobia/Bacteria not genome not clone not uncultured not unidentified"). Therefore, a probability of obtaining a single isolate from this phylum by a "standard" inventory of 174 strains is 0.174. Our survey of this size reports five new strains from the phylum. The probability of that occurring by chance alone is very small, 1.6×10^{-4} . Therefore, either the cultivation

TABLE 2. Genetic distance between the newly isolated and previously cultivated strains

Similarity (%) of the 16S rRNA gene with the closest cultured relative	No. of petri dish-specific strains	No. of strains specific to diffusion chamber of:					No. of strains isolated by both methods	Total no. of strains
		Generation				All generations		
		1	2	3	4			
100	3	1	3	1		5	6	14
99	8	15	9	4	6	34	2	44
98	7	11	9	7	4	31		38
97	5	5	13	3	5	26	1	32
96	5		3	3	2	8		13
95	3		2	2	2	6	1	10
94	5	1	1	4		6		11
93	1							1
92	1		2			2		3
91	1	2	1	1		4	1	6
90	1		1			1		2

method led to the isolation of bacteria underrepresented in other studies or the target habitat was uncharacteristically enriched with *Verrucomicrobia*. The latter is unlikely because standard approaches did not produce any verrucomicrobial isolates either in this study or in any other study conducted in a similar (freshwater) environment. Therefore, at least some microorganisms were likelier to be isolated in vitro by repetitive incubation in diffusion chambers than by employing other cultivation strategies. It follows that if the diffusion-chamber-based approach selected for certain microbial groups, its biases were different from that of traditional cultivation techniques.

We were interested in learning whether increasing the number of chamber-to-chamber transfers would result in the isolation of additional environmental species. We therefore followed the order in which the strains from the four successive generations of diffusion chambers appeared in the petri dishes. The majority of the chamber-derived strains were obtained from the first and second generations of the diffusion chambers (Table 1). *Alpha*- and *Betaproteobacteria* were detected in relatively high numbers at all steps of the experiments. However, representatives of *Verrucomicrobia* and *Acidobacteria* were isolated from the material grown exclusively in the second, third, and fourth generations of the chamber material. The isolation of these bacteria and the differences between the diffusion-chamber- and petri-dish-derived isolates suggest two different possibilities. First, representatives of these isolates were too rare at the beginning of the experiment and needed two rounds of enrichment in the diffusion chamber before appearing in the petri dishes in numbers sufficient for isolation. Second, their adaptation to growth in a petri dish required several growth events in the simulated natural environment of the chamber.

Both petri dish and diffusion chamber cultivation produced isolates of phylogenetic novelty (Table 2). A high number of all isolated strains (26%) likely belonged to new species (defined as strains sharing more than 97% 16S rRNA gene sequence identity) (30). The majority (59%) of these “new” strains were unique to the diffusion-chamber-based cultivation approach, 37% were unique to the standard petri dish technique, and 4% were isolated by both.

In conclusion, the cultivation strategy adopted here offers a reliable way to enlarge the access to the diversity of environmental organisms. In situ cultivation of environmental bacteria in the diffusion chamber either enriches species sufficiently for their subsequent isolation in petri dishes or adapts them for growth under prohibitive in vitro conditions. Irrespective, the in situ cultivation in diffusion chambers leads to the eventual in vitro cultivation of otherwise poorly cultivable species. We are currently investigating the molecular basis of this “domestication” process.

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