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Microcolony Cultivation on a Soil Substrate Membrane System Selects for Previously Uncultured Soil Bacteria

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Traditional microbiological methods of cultivation recover less than 1% of the total bacterial species, and the culturable portion of bacteria is not representative of the total phylogenetic diversity. Classical cultivation strategies are now known to supply excessive nutrients to a system and therefore select for fast-growing bacteria that are capable of colony or biofilm formation. New approaches to the cultivation of bacteria which rely on growth in dilute nutrient media or simulated environments are beginning to address this problem of selection. Here we describe a novel microcultivation method for soil bacteria that mimics natural conditions. Our soil slurry membrane system combines a polycarbonate membrane as a growth support and soil extract as the substrate. The result is abundant growth of uncharacterized bacteria as microcolonies. By combining microcultivation with fluorescent in situ hybridization, previously "unculturable" organisms belonging to cultivated and noncultivated divisions, including candidate division TM7, can be identified by fluorescence microscopy. Successful growth of soil bacteria as microcolonies confirmed that the missing culturable majority may have a growth strategy that is not observed when traditional cultivation indicators are used.

It is estimated that traditional methods of bacterial cultivation recover less than 1% of the bacterial species present (2). This culturable proportion is not representative of the total phylogenetic diversity, since members of 11 of 39 bacterial divisions have not been cultured yet (18). Analysis of 16S rRNA genes from environmental sources has resulted in identification of candidate divisions, such as candidate divisions TM6 and TM7, that have no cultured representatives, while for many other divisions only a few members have been cultivated (7, 12, 18). Considering the extent of functional diversity described for microbes and the numerous applications of their secondary metabolites, the biotechnological potential hidden among the 99% of the bacteria in soil that are nonculturable is immense. Hence, novel approaches to culture hitherto uncultured bacteria are necessary.

Traditional cultivation strategies are often selective, favoring faster-growing dominant species that are capable of colony formation or development of turbidity over slowly growing species. Additionally, culture media often supply excessive amounts of nutrients and in some cases may be toxic to target bacteria (7, 14, 20). Oligophilic "k-selected" bacteria have been described as bacteria that are adapted to growth in nutrient-poor environments, and they may represent a high proportion of the missing culturable diversity (29). Recently, the growth of microorganisms in a simulated natural environment or an environment with limited nutrients has enabled the growth of bacteria that have evaded cultivation for decades. This includes isolates belonging to bacterial divisions such as the ubiquitous marine bacterioplankton (SAR11) and marine methylotroph (OM43) clades (7, 14, 15, 22, 25). By simulating natural conditions, these strategies select for growth of bacteria that have been recalcitrant to cultivation using standard media.

In many of these studies traditional signs of bacterial growth, such as colony formation and turbidity, are poor indicators of cultivation as the majority of growth is microcolony CFU (mCFU) growth, which require microscopic identification (15, 27, 30, 32). For example, the majority of growth of marine bacteria (>99%) under environmental conditions was growth to only the microcolony stage (15), while up to 80% of soil bacteria grew to the microcolony stage only over a 63-day incubation period on dilute soil medium (30).

Here we describe a novel microcultivation method for soil bacteria that mimics natural conditions. Our approach relies on polycarbonate (PC) membranes as a growth support and a nonsterile soil slurry as the culture medium. The soil slurry is prepared in an inverted tissue culture insert (TCI) containing a fixed anopore membrane. This supplies nutrients for soil bacteria growing on the PC membrane while acting as a barrier to bacterial contamination. This soil slurry membrane system (SSMS) was used to cultivate methanotrophs from soil samples (28) and was modified in this study to facilitate growth of uncultured bacteria present in soil. The system provided evidence which supported the hypothesis that "k-selected" oligophilic bacteria were present and revealed specific soil bacteria which can be targeted for classical cultivation.

MATERIALS AND METHODS

Sample collection. A 500-g garden soil sample was collected from 15 cm below the surface at Macquarie University, Sydney, New South Wales, Australia, and was passed through a 2-mm-mesh sieve. The pH (24) and water content (expressed as a percentage of the dry weight) of the soil sample were determined on the day of collection to be pH 6.42 and 21%, respectively.

Microcultivation in a soil slurry membrane system. For microcultivation, a subsample of soil was diluted 1:200 in prefiltered (pore size, 0.2 μm) distilled H_2O and vortexed for 30 s. This was the bacterial inoculum used for the study. Sand particles were allowed to sediment for 1 min before 50 μl of the inoculum was placed into 10 ml distilled H_2O and filtered onto a 0.2-μm, white, isopore PC

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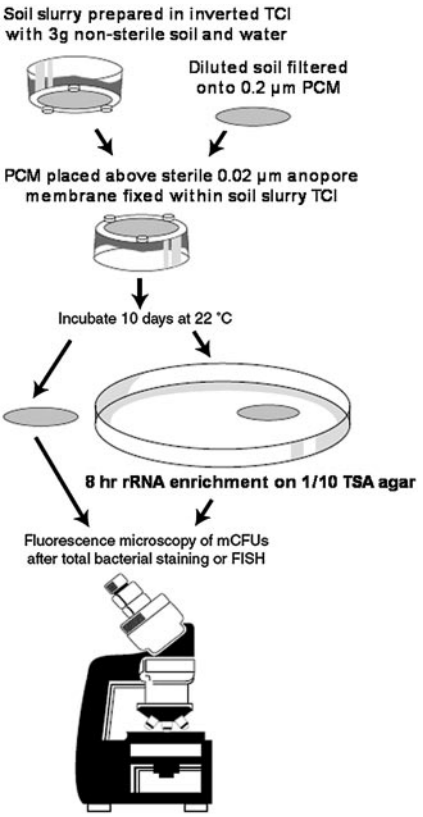

FIG. 1. Preparation of the microcultivation SSMS. A garden soil slurry is prepared in an inverted tissue culture insert containing a fixed anopore membrane, which supplies nutrients for soil bacteria growing on a PC membrane (PCM) while acting as a barrier to bacterial contamination. The PC membrane is inoculated with a soil filtrate and is placed on the inverted TCI, and growth is monitored.

membrane (Millipore, North Ryde, New South Wales, Australia) using a syringe filter holder (Sartorius, East Oakleigh, Victoria, Australia). Inoculated PC membranes were then placed on top of a sterile 0.02-μm anopore membrane, which was fixed within 25-mm TCIs (Nunc A/S, Roskilde, Denmark). Before addition of the PC membranes, the TCIs were inverted and filled with 3 g of the sieved garden soil (Fig. 1) that was wetted with 750 μl of prefiltered distilled H_2O. The soil was gently vortexed until the soil texture was broken down to a muddy soil slurry. A TCI was then placed upside down in a sterile six-well multidish (Nunc). The underside of the inoculated PC membrane was then placed on the TCI's fixed anopore membrane. The top side of the anopore membrane was also sterile and served as a barrier between the nonsterile soil slurry and the underside of the PC membrane, which was also sterile. The culture vessels were incubated at 22°C in the dark for 7 to 10 days. To confirm sterility, negative control preparations consisted of uninoculated PC membranes that were placed on top of the TCI growth support system containing nonsterile soil slurries.

DNA extraction from microcolonies and 16S rRNA gene amplification. PC membranes were removed from the TCIs after 10 days of incubation, and they were cut in half and placed into a sterile 1.5-ml microcentrifuge tube with sterile water (200 μl) that was prepared by autoclaving and filtration through a 0.22-μm sterile filter (Millipore, North Ryde, New South Wales, Australia). Each sample was vortexed vigorously to remove bacterial cells, and the membrane was then discarded. To ensure that the bacteria were removed, the PC membranes were counterstained with SYBR Green II as described below for total bacterial staining, and the presence of bacteria was determined. Cells were centrifuged for 5 min at 14,000 × *g*, washed once, and resuspended in 10 μl sterile water. Bacteria were lysed at 99°C for 5 min and centrifuged for 5 min at 14, 000 × *g*, and the entire supernatant was used for 16S rRNA gene amplification. Lysates (5 μl) were prepared for PCR by addition of 5 μl Gene Releaser (Bio Ventures, Inc., Murfreesboro, Tenn.) as described by the manufacturer. Negative controls containing only sterile water and only Gene Releaser were included in each set of reactions. The reaction tubes were heated on the high setting of a 650-W microwave oven for 7 min (4,550 W/min) in a microwave-transparent rack (Bioventures Inc.). An Erlenmeyer flask containing 100 ml of water was included as a microwave sink. DNA samples were heated to 80°C for 10 min prior to addition of a PCR master mixture. The PCR mixture consisted of 1.5 mM $MgCl_2$, each deoxynucleoside triphosphate at a concentration of 200 nM, 20 μg/ml RNase, 50 pmol of each primer (primers 27F and 1492R) (17), and 1 U Red Hot DNA polymerase prepared in the supplied PCR buffer (Advanced Biotechnologies, Surrey, United Kingdom). The PCR conditions were an initial denaturation step at 94°C for 3 min, followed by 35 cycles of 94°C for 30 s, 60°C for 30 s, and 72°C for 1.5 min and then a final elongation step of 5 min at 72°C (31). PCR products (ca. 1,450-bp fragment) were verified by electrophoresis on a 2% agarose gel.

Construction of a 16S rRNA gene clone library. PCR amplicons were ligated into the pGEM-T vector supplied with a T/A cloning kit as described by the manufacturer (Invitrogen, Carlsbad, CA). The ligation mixtures were transformed into INVαF competent cells supplied with the T/A cloning kit. Cells were then plated onto LB agar plates (NaCl, 10 g/liter; tryptone, 10 g/liter; yeast extract, 5 g/liter; agar, 20 g/liter; pH 7.5) containing X-Gal (5-bromo-4-chloro-3-indolyl-beta-D-galactopyranoside) for overnight incubation at 37°C. Forty white colonies were picked randomly from the transformation plates and dispensed into 100 μl LB broth (NaCl, 10 g/liter; tryptone, 10 g/liter; yeast extract, 5 g/liter; pH 7.5) containing kanamycin (50 μg/ml) and ampicillin (50 μg/ml) for overnight incubation. An aliquot of cells was placed into 100 μl sterile distilled water for screening by PCR. Clones were designated in the form PCF followed by the sample number (PCF1 to PCF40) according to the order in which they were picked.

Restriction fragment length polymorphism analysis and sequencing of 16S rRNA gene clones. PCR products were amplified from cloned rRNA gene fragments of interest using primers PCRr (CGCCAGTGTGATGGATATCT) and PCRf (AACGGCCGCCAGTGTGCTGG) (10). These primers targeted regions of the vector flanking the cloning site. Each reaction mixture contained 5 μl cell suspension, 50 pmol of each primer, 2 mM $MgCl_2$, and 1 U Red Hot DNA polymerase, and the PCR was carried out as described previously (10). Each selected PCR product (10 μl) was digested with Hinf1 and Rsa1 (Promega, Madison, WI) in a 20-μl mixture according to the manufacturer's instructions. Digests were electrophoresed on 2% agarose gels and stained with ethidium bromide. Two of each type of restriction fragment length polymorphism were selected for 16S rRNA gene sequencing.

Plasmids were purified from overnight cultures of selected clones using Wizard minipreps (Promega). The DNA sequence was determined using cycle sequencing, dye terminator chemistry, and primers 27F and r910 (5′CCCCGTCAATTC MTTTGAG) (10). Sequencing was carried out at the Macquarie University sequencing facility. The sequences were compared to sequences in the GenBank database using the BLAST program (www.ncbi.nlm.nih.gov/BLAST) and Biomanager (http://biomanager.angis.org.au). Sequence similarities and alignments were determined using appropriate programs in each database (Blastn and clustalX). The sequence analysis was last carried out in June 2005.

Total bacterial staining of PC membranes. For total bacterial staining, SYBR Green II RNA gel stain (1:1,000; Bioscientific, Gymea, Victoria, Australia) was used in Citifluor mounting medium (Leica Microsystems, North Ryde, New South Wales, Australia). An aliquot (15 μl) was placed on a section of a PC membrane surface and mounted directly on a microscope slide. An Olympus Fluoview FV 300 confocal microscope (Olympus, Mt. Waverley, Victoria, Australia) was used for visualization of microcolonies. Image analysis was carried out using the ImageJ free software (htpp://rsb.info.nih.gov/ij/).

FISH. A fluorescent in situ hybridization (FISH) protocol optimized for mCFUs on PC membranes was used, with slight modifications (3). Three oligonucleotide probes were used for FISH and were combined for multicolor identification. The eubacterial probe EUB338I (1), which targets most eubacteria, was labeled with fluorescein isothiocyanate, and two probes, TM7905 and TM7305, which were previously designed for candidate division TM7 (12), were labeled with Cy3. *Micrococcus luteus* ACM 975 and *Sphingomonas* sp. strain BF14 (= ACM 4962) were used as negative controls for hybridization specificity as they are reported to have only one mismatch with the TM7905 and TM7305 target sequences (12). To ensure that there was a FISH signal from the mCFUs on the membranes, growth of rRNA was enhanced by moving PC membranes to 0.1× tryptic soy agar (TSA) for 6 to 8 h of incubation before fixation with 4% paraformaldehyde. The enrichment step was vital for conventional FISH detection; however, the enrichment period could shift the microbial community present due to the presence of rich complex medium (8). Hybridization was performed at 46°C for 3 h at the recommended stringencies (12).

Secondary transfer for cultivation of pure microcolonies. A secondary transfer of single microcolonies was performed after identification of live microcolonies

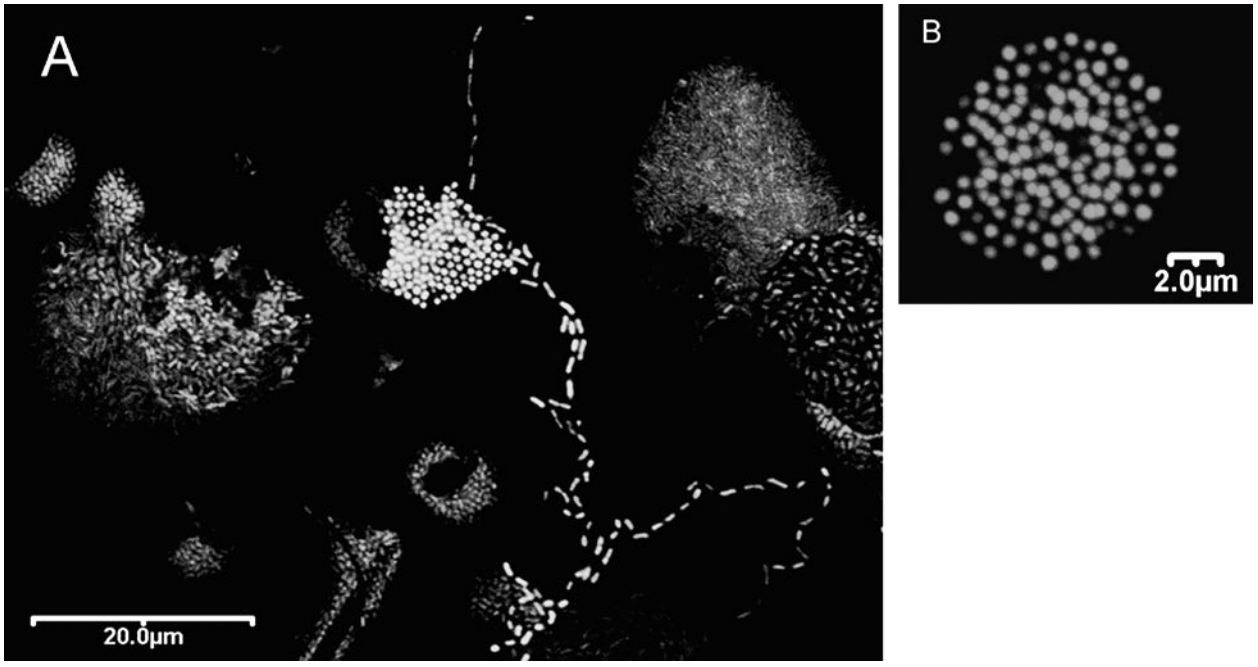

FIG. 2. Microcolony cultivation of bacteria grown on PC membranes using garden soil slurry as the culture medium. (A) Fluorescent staining (SYBR Green II) reveals several microcolony morphotypes growing in coculture after 10 days of incubation. (B) Presence of a common morphotype after 10 days of incubation.

by total bacterial staining. Fluorescence microscopy (magnification, ×200) was used to pinpoint and mark distinct microcolonies on a PC membrane. A sterile scalpel was then used to physically cut each marked microcolony and membrane out for secondary cultivation. Each isolated microcolony-membrane complex was placed into a sterile microcentrifuge tube with 200 μl of a sterile physiological salt solution (0.9% NaCl) and mixed. A portion of each diluted microcolony cell suspension (50 μl) was then transferred to a new TCI for 7 days of secondary SSMS cultivation. A second portion (100 μl) was also plated onto 0.1× TSA for 3 days of incubation at 22°C, and colony development was monitored.

Transfer of a pure secondary microculture onto dilute medium. A sterile 96-well microtiter plate (Nunc) was prepared with 200 μl of 0.01× Ravan medium (5 g/liter glucose, 5 g/liter peptone, 5 g/liter yeast extract, 5 g/liter sodium acetate, 2 g/liter pyruvic acid; pH 7 to 7.2.) (29). Following 7 days of secondary microcolony cultivation with the SSMS, growing cells were removed from the PC membranes by vortexing in a sterile physiological salt solution (500 μl). A portion (20 μl) of each cell suspension was plated into a microtiter well for incubation at 22°C for 7 days. The remaining cells were stored at −80°C in 10% glycerol. Growth was monitored by fluorescence microscopy by total bacterial staining of a portion (10 μl) of the cell suspension from each well. On day 7 a subsample (100 μl) of the successful microculture (isolate G2) was placed into a microcentrifuge tube for 16S rRNA gene PCR amplification and sequencing as described above for SSMS bacterial clones. The remainder of the microculture was stored at −80°C in 10% glycerol.

Nucleotide sequence accession numbers. Nucleotide sequences obtained in this study have been deposited in the GenBank database under accession numbers AY566234 (PCF1) and AY540760 to AY540773 (PCF2 to PCF39); the isolate G2 sequence has been deposited under accession number DQ113445.

RESULTS

Microcolony identification. Total bacterial staining using SYBR Green and confocal laser scanning microscopy revealed numerous microcolonies growing on the PC membranes. These microcolonies exhibited diverse morphologies, and variations in both cell numbers and sizes were observed in each field of view (Fig. 2A). On day 0, single cells and a small proportion of aggregated bacteria were observed (data not shown). On day 7 microcolonies, defined as three or more closely associated cells, were observed throughout each PC membrane. The majority of these microcolonies consisted of large numbers of cells that formed a single layer, in many cases with defined distances between individual cells, like a dispersal strategy for growth (Fig. 2B). Various morphotypes were observed; in some instances more than one cell type was present in the same microcolony, suggesting that cells were interacting (Fig. 2A). Following 10 days of microcultivation a dominant mCFU morphotype was observed that consisted of approximately 200 cells with a three-dimensional morphology (Fig. 2B). No macrocolonies were observed on the PC membranes. Bacterial development was to only the microcolony stage, which was invisible to the naked eye. No bacterial growth was observed on the negative control PC membranes after 10 days of incubation, and the top of the SSMS 0.02-μm anopore growth support remained sterile throughout the experiment.

Phylogenetic diversity of the bacterial domain. The diversity of the bacteria dominating the membrane after 10 days of SSMS incubation was assessed by 16S rRNA gene sequencing using only bacterial domain primers. A nucleotide sequence analysis of 40 clones revealed 15 different bacterial groups, each defined by >97% sequence homology. These groups were identified as members of the *Betaproteobacteria* (77.5% of the clones), *Gammaproteobacteria* (12.5%), *Actinobacteria* (5.0%), and candidate division TM7 (5.0%) (Table 1). The majority of the sequences exhibited the highest levels of similarity to 16S rRNA gene sequences recovered directly from environmental DNA rather than to 16S rRNA gene sequences from known, culturable species.

Several clones were closely related to *Aminomonas amino-*

TABLE 1. Phylogenetic diversity of 16S rRNA gene clones following soil substrate microcultivation of garden soil[a]

Isolate	Accession no.	Phylogenetic group (division, subdivision)	Closest match (accession no.)	Nucleotide identity (%)	Closest cultured organism (accession no., % identity)
PCF1 (8)[b]	AY566234	*Proteobacteria*, *Betaproteobacteria* (environmental samples)	Clone Crystalbog021G5 (AY792265)	93	*Aminomonas aminovorus* C2A1 (AY027801, 92%)
PCF2 (9)	AY540760	*Proteobacteria*, *Betaproteobacteria* (environmental samples)	Clone BIqi38 (AJ318162)	99	*Nitrosococcus mobilis* (M96403)
PCF4 (3)	AY540761	*Proteobacteria*, *Betaproteobacteria* (environmental samples)	Clone BIqi38 (AJ318162)	99	*Nitrosococcus mobilis* (M96403)
PCF10 (2)	AY540762	*Proteobacteria*, *Betaproteobacteria* (environmental samples)	Clone SM1G08 (AF445700)	98	*Pseudomonas saccharophila* (AB021407, 97%)
PCF12 (2)	AY540768	*Proteobacteria*, *Betaproteobacteria*	Clone HTCC349 (AY429717)	99	*Methylophilus methylotrophus* (AB13724, 95%)
PCF13 (1)	AY540769	*Bacteria* (environmental samples)	Clone 249ds10 (AY212697)	97	*Dechlorosoma suillum* (AF170348, 95%)
PCF14 (1)	AY540770	*Proteobacteria*, *Betaproteobacteria*	Clone HTCC349 (AY429717)	96	*Methylophilus methylotrophus* (AB193724, 95%)
PCF18 (1)	NA[d]	*Proteobacteria*, *Gammaproteobacteria* (*Pseudomonadales*)	P400Y-1 (AB076857)	100	*Pseudomonas citronellolis* (Z76659, 98%)
PCF24 (1)	AY540763	*Bacteria* (environmental samples)	Clone G12-12365 (AF525829)	99	*Schlegelella thermodepolymerans* (AY538707)
PCF25 (1)	AY540764	*Proteobacteria*, *Betaproteobacteria* (environmental samples)	Clone BIqi38 (AJ318162)	94	*Methylophilus methylotrophus* (AB193724, 92%)
PCF26 (3)	AY540771	*Proteobacteria*, *Gammaproteobacteria* (*Enterobacteriales*, *Enterobacteriaceae*)	*Enterobacter ludwigii* type strain EN-119T (AJ853891)	99	*Enterobacter ludwigii* type strain EN-119T (AJ853891)
PCF28 (2)	AY540765	*Bacteria*, *Actinobacteria* (environmental samples)	Clone AKYH854 (AY922162)	97	*Nocardioides jensenii* (Z78210, 96%)
PCF29 (2)	AY540766	*Bacteria* (environmental samples)	Clone pHAuB-34 (ABO72717)	98	*Aquabacterium* sp. strain Aquala (AF089856)
PCF30 (1)	AY540767	*Proteobacteria*, *Betaproteobacteria* (environmental samples)	Clone DSBR-C002 (AY238973)	89	*Aminomonas aminovorus* C2A1 (AY027801, 89%)
PCF33 (1)	AY540772	*Proteobacteria*, *Gammaproteobacteria* (*Pseudomonadales*, environmental samples)	Clone OSIL-17 (AB076873)	99	*Pseudomonas alcaligenes* isolate LB19 (AF390747, 98%)
PCF39 (2)	AY540773	Candidate division TM7 (environmental samples)	SBR1071 (AF268996)	95	NA
G2[c]	DQ113445	*Proteobacteria*, *Betaproteobacteria* (*Oxalobacteraceae*)	Type strain 76 (AY966000)	98	*Massiliaa timonae* isolate 97A4424 (AY157760, 97%)

[a] Partial 16S rRNA gene sequences were deposited in the GenBank database under accession numbers AY566234 (PCF1) and AY540760 to AY540773 (PCF2 to PCF39).
[b] The numbers in parentheses are the number of clones sequenced for each ribotype.
[c] The isolate G2 16S rRNA gene sequence was submitted to GenBank on 1 July 2005.
[d] NA, not applicable.

vorus, an unclassified β-proteobacterium that has been characterized only from sequence data and that falls in the *Methylobacillus* obligate methylotroph group (9). In the past, description of a new species from one sequence submission was possible, but a recent report recommends that more than one isolate be submitted before reliable classification of a novel species (13). In light of this report, *A. aminovorus* has not been validated yet. The lowest level of similarity was with PCF30 (89% sequence homology), suggesting that PCF30 is representative of a new family, while the dominant clone PCF1 exhibited 93% sequence homology to *A. aminovorus*, suggesting that it belongs to a new genus. Clone PCF28 exhibited 96% sequence similarity to *Nocardioides jensenii*, a previously cultivated member of the *Actinomycetales* isolated from Antarctica (26). Of the 40 clones analyzed, only 3 exhibited the highest levels of similarity (>96% nucleotide similarity) to cultured bacteria (PCF18, PCF26, and PCF33). Each clone was placed in the genus *Pseudomonas* or *Enterobacter* in the class *Gammaproteobacteria* on this basis.

A major finding was the presence of a microcultivated organism belonging to the candidate division TM7 lineage (95% similarity). Candidate division TM7 has previously been shown to be selectively enriched only in a laboratory bioreactor (12). Our TM7 clone, PCF39, exhibited 95% 16S rRNA gene sequence similarity to clone SBR1071, a member of subdivision 1 in the TM7 candidate division (4).

Identification of TM7 microcolonies. FISH was carried out with PC membranes enriched after 7 days of microcultivation, on which the density and size of mCFUs were found to be optimal for TM7 FISH analysis. The TM7 microcolonies on the PC membrane were identified using FISH with TM7-specific and eubacterial probes. Multicolor analysis revealed that several TM7 morphotypes were growing on the PC membranes (Fig. 3). Two TM7 morphotypes were dominant; one morphotype consisted of short rods with several hundred cells per colony (Fig. 3A), and the more common morphotype was large colonies consisting of long filamentous rods up to 15 μm long with less than 50 cells per colony (Fig. 3B). Image analysis of 20 fields of view for total cell counting resulted in analysis of approximately 12,000 cells. A comparison of total eubacterial fluorescein isothiocyanate-labeled, FISH-positive cell counts with TM7 Cy3-labeled, FISH-positive cell counts revealed that 6.7% of eubacterial cells were also TM7 positive on day 7. The initial genetic analysis revealed similar numbers, with 5% of the clones (2 of 40 clones) belonging to candidate division TM7 on day 10.

Cultivation of pure microcolonies. After 7 days of SSMS microcultivation, eight dominant microcolonies were isolated from a primary SSMS PC membrane using a manual manipulation technique. Secondary SSMS incubation was then employed for each microcolony isolated, which resulted in successful PC membrane subcultivation of only one isolate (isolate G2). Duplicate samples from each primary microcolony were also plated onto conventional nutrient-rich medium (0.1× TSA). No visible colony formation was observed for any isolate after 3 days of incubation on rich medium, suggesting that isolate G2 was a slowly growing, microcolony-forming bacterium which preferred limited substrates.

The SSMS-subcultured isolate (isolate G2) was next inoculated into a 96-well plate containing poor substrate medium (0.01× Ravan media), and growth was monitored by fluorescence microscopy. After 5 days of incubation motile rods were observed; therefore, the organism was capable of growth in minimal substrate media. Following 16S rRNA gene sequencing and comparison of a 1,421-bp fragment with appropriate databases, isolate G2 exhibited the highest level of similarity (98% similarity) (Table 1) to the novel bacterial species *Sinobacter plicatus* (19). This isolate, whose sequence has been submitted to GenBank, is yet to be validated as a new mesophilic soil bacterial species belonging to the family *Oxalobacteraeceae*. Interestingly, the closest recognized relative (97% similarity) is the recently emended species *Massilia timonae*, a gram-negative motile rod that has not been isolated from the environment yet (16, 21).

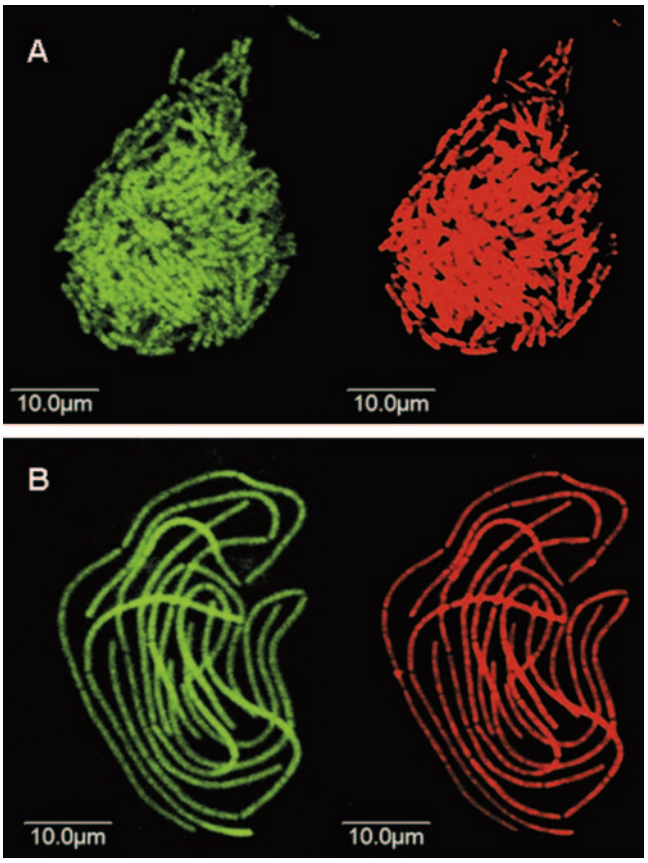

FIG. 3. Multicolor staining with FISH probes specific for TM7 bacteria (red) identifies two TM7 morphotypes that are stained by the eubacterial probe (green). (A) Faster-growing TM7 morphotype consists of small rods and several hundred cells per microcolony. (B) Larger TM7 microcolony consists of less than 50 long rods that are up to 15 μm long and is typical of filamentous TM7 lineage bacteria.

DISCUSSION

The soil slurry membrane system revealed that there was abundant growth of soil bacteria to the microcolony size, which was detectable only by microscopic visualization, confirming that organisms in dilute-substrate environments may have a growth strategy different from that revealed by classical measures of growth, such as turbidity and colony development

(6, 18, 27). This agrees with the previous findings that up to 99% of the colonies that grow on dilute media develop only to a microcolony size (29, 30) and that 300 times more microcolonies than macrocolonies have been observed on agar plates (15).

Oligophiles have been described as aquatic and terrestrial bacteria that have adapted to a "k-selected" life strategy, in which dispersal and slow growth are preferred over colony formation (27, 29). These bacteria exhibit higher growth yields in low-substrate media than in rich media and form small, microscopic colonies. Our method for microcultivation was adapted from a soil substrate membrane system that was developed for selective growth of methane-oxidizing bacteria (28). Soil is a complex substrate containing endemic organisms and organic and inorganic compounds. When the soil substrate system is used, a combination of these factors provides the limited carbon and energy sources and signaling molecules required for the growth of oligophilic microcolony-forming bacteria. Secondary SSMS microcultivation of isolate G2, combined with a lack of colony formation on rich medium, confirmed that this SSMS-microcultivated organism preferred a slow, "k-selected" growth strategy. Following the secondary transfer, growth of isolate G2 in a limited-substrate medium designed specifically for oligophilic bacteria was observed and resulted in genetic identification of the organism (29).

The SSMS-microcultivated bacteria ranged from bacteria belonging to recognized genera, including *Methylobacillus* and *Pseudomonas* (96 to 100% similarity), to bacteria with no cultivated representatives. Consequently, this method appears to recover organisms from the "unculturable" fraction of the microbiota, including members of candidate division TM7. TM7 was first discovered using environmental 16S rRNA gene sequences from a peat bog, and additional sequences were described from garden soil, batch reactor sludges, and a laboratory bioreactor (4, 5, 11, 12, 23). FISH with mCFU revealed several TM7 morphotypes growing in coculture on the PC membranes. The cell types visualized agreed with previous findings for TM7 bacteria found in a bioreactor (12). The diversity of microcolony-forming bacteria described here provides evidence that terrestrial oligophilic bacteria may contribute to the nonculturable diversity.

A multicolor approach with TM7 probes revealed that the cells hybridizing to TM7 probes, compared with the cells hybridizing to the eubacterial FISH probe, correlated well with the measures of phylogenetic diversity determined using 16S rRNA gene sequencing; therefore, the approach successfully cultivated bacteria that have evaded cultivation by traditional methodologies. This technique allows microcultivation of microcolonies in a controlled manner and in a shorter time than the time required for other recovery methods (25, 32). Identification of culturable groups of bacteria using this approach revealed bacteria that can now be targeted for classical cultivation strategies. Here, a combination of manual manipulation of SSMS bacteria and classical strategies resulted in the growth of one isolate belonging to *S. plicatus*, a novel organism recently submitted for validation. A poor success rate (one in eight) was observed following manual isolation of microcolonies, and this can be attributed to the fact that the method employed did not include microscopic dissection. Clearly, there is potential for direct recovery of higher numbers and greater success rates for obtaining pure microcolony cultures from PC membranes via micromanipulation using instruments like laser microdissection systems (Veritas, Mountain View, CA). This should allow more comprehensive biochemical and physiological characterization of undescribed bacterial isolates.

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