Cultivating previously uncultured soil bacteria using a soil substrate membrane system

Belinda C Ferrari[1], Tristrom Winsley[1], Michael Gillings[2] & Svend Binnerup[3]

[1]School of Biotechnology and Biomolecular Sciences, University of New South Wales, Sydney, New South Wales 2052, Australia. [2]Department of Biological Sciences, Macquarie University Sydney, Sydney, New South Wales 2109, Australia. [3]Department of Environmental Chemistry and Microbiology, National Environmental Research Institute, Frederiksborgvej 399, DK-4000 Roskilde, Denmark. Correspondence should be addressed to B.C.F. (b.ferrari@unsw.edu.au).

Published online 17 July 2008; doi:10.1038/nprot.2008.102

Most bacteria are recalcitrant to traditional cultivation in the laboratory. The soil substrate membrane system provides a simulated environment for the cultivation of previously undescribed soil bacteria as microcolonies. The system uses a polycarbonate membrane as a solid support for growth and soil extract as the substrate. Diverse microcolonies can be visualized using total bacterial staining combined with fluorescence *in situ* hybridization (FISH) after 7–10-d incubation. Molecular typing shows that the majority of microcolony-forming bacteria recovered using this protocol were resistant to growth using standard methods. The protocol takes <4 h of bench time over the 10-d period.

INTRODUCTION

Estimates suggest that <1% of total bacterial species can be recovered using standard laboratory methods of cultivation[1–3]. Culture-independent analyses reveal that readily culturable bacteria do not reflect the functional and phylogenetic diversity present within any environment[4,5]. There are now 54 bacterial divisions recognized through molecular phylogenetics, 13 of which have no pure culture representatives[1,6–8]. Some groups, such as the Acidobacteria and Verrucomicrobia, represent significant proportions of bacterial diversity in soil but are severely underrepresented, with only a handful of isolates obtained in pure culture[4,9–12].

In recent years, novel cultivation strategies have begun to address the problem of the unculturable fraction[1,7,9,13]. Cultivation on dilute nutrient media, using increased incubation times, or simulated natural environments has led to a resurgence in the field of microbial cultivation[7,9,12–14]. Initial studies focused on the marine environment, and in 2002, the use of sterile sea water as a substrate for growth enabled the successful cultivation of isolates from the highly ubiquitous marine bacterioplankton (SAR11), which was recalcitrant to standard cultivation attempts[7,15]. The combination of nutrient-poor marine environments with the use of 'diffusion chambers' has also facilitated the growth of previously 'uncultivated' slow-growing oligophilic marine bacteria[7,12,13].

Although several strategies exist for marine bacteria, the soil substrate membrane system (SSMS) was developed to mimic the natural terrestrial environment of soil bacteria[16]. The substrate used for cultivation consists of natural non-sterilized wet soil as the sole growth component. The soluble soil carbon components released after soil wetting is sufficient for microcolony-forming (mCFU) growth of oligotrophs whereas other protocols embed the sample in agar[13], use dilute artificial media[9] or sterilize the natural component used as substrate (e.g., sea or pond water)[12,11]. This not only facilitates the isolation and continued cultivation of the specimens of interest but also increases the selective nature of the medium against oligotrophic species. In the SSMS approach, microcolony formation occurs on a polycarbonate membrane (PCM) in immediate contact with the slurry of soil substrate. Having the bacterial sample on a membrane offers a number of advantages: (i) quantification of growing versus nongrowing bacteria is possible; (ii) different viability assays[17] or fluorescent molecular detection approaches can be applied[16,18] and (iii) the integrity of cells and microcolony morphologies remain intact and can be analyzed further by digital image analysis[19]. The SSMS simulates the terrestrial environment and selects for 'uncultivated' soil bacteria. Microcultivation occurs within 7–10 d, which is a shorter timeframe than dilution to extinction methods, and pure microcultures are obtained when the SSMS is combined with subculturing, which permits downstream molecular characterization of cultures. This protocol is also adaptable to different soil types, incubation temperatures and times.

The SSMS technique was adapted from a protocol developed for isolating methane-oxidizing bacteria[20] and selects for uncharacterized microcolony-forming bacteria from diverse divisions, including those with no or few pure cultured representatives such as the Candidate Division TM7[4,16,21]. A disadvantage of membrane microcultivation is that a fluorescence microscopy approach is needed to identify the cells[16,22]. This limits a more detailed molecular characterization of specific morphotypes or the isolation of individual microcolonies for generating pure cultures.

This protocol involves several stages (see **Figure 1** for a flowchart of the SSMS protocol). The first major stage of the protocol outlines the setup of the soil substrate and the inoculation of bacteria onto the growth membranes. Following the formation of mCFUs using the SSMS, additional stages include fluorescent confirmation of growth on PCMs by total bacterial staining and characterization of microbial groups present by fluorescence *in situ* hybridization (FISH). In addition, two direct DNA extraction procedures for bacteria present on growth membranes are included for studies aimed at molecular identification of communities present using this protocol. For those studies that aim to obtain pure cultures, subculturing from the SSMS into artificial media is also described.

This protocol is simple and cost-effective as it can be implemented into a laboratory without the need for specialized equipment such as fluorescence-activated cell sorters required by alternative methods[14]. When combined with subsequent cultivation in dilute media, a significant proportion of microcolonies have the ability to

switch from a slow-growing limited life strategy in a stable nutrient-poor environment, to one which can form a colony in a standard Petri dish[16,22]. This ability to switch or acquire the potential to grow on complex media following repeated microcultivation has been observed now in both marine and terrestrial environments[11,13,23,24]. A major advantage of the SSMS is that this ability to switch growth strategies occurs within a rapid timeframe compared to limiting dilution and diffusion chamber isolation methods[9,11–13].

The SSMS was originally carried out on a mesophilic environment[16]. The protocol can be adapted to improve the recovery of microbial species from different terrestrial environments by varying the soil type, incubation times and temperatures. The method has applications in isolation of novel species, recovery of isolates for bioremediation and bioprospecting for antibiotics. The SSMS offers the opportunity to specifically isolate novel soil bacteria that have been shown to be associated with pollutant degradation, by culture-independent approaches, but which have been resistant to cultivation using standard procedures[25,26]. For instance, the SSMS has been used recently on mercury contaminated soil to isolate difficult-to-culture mercury-resistant bacteria successfully[22]. The cultivation of novel isolates in this case led to the discovery of a new *merA* gene sequence which has applications in mercury degradation. In terms of detection methods for oligophilic bacteria, FISH has been adopted now for microcolonies on growth membranes for the rapid identification of antibiotic resistant bacteria in fresh water environments[27] and quantification of ammonium-oxidizing bacteria in soils[28].

Future improvements to automate recovery of individual microcolonies from growth membranes might include fluorescence-activated cell sorting or advanced micromanipulation of single microcolonies from the SSMS into artificial media. Combined, these single-cell strategies should increase the success rate of the isolation of pure cultures from the mixed microcolony-forming communities and should lead to a rapid increase in the numbers of previously unculturable organisms available for study.

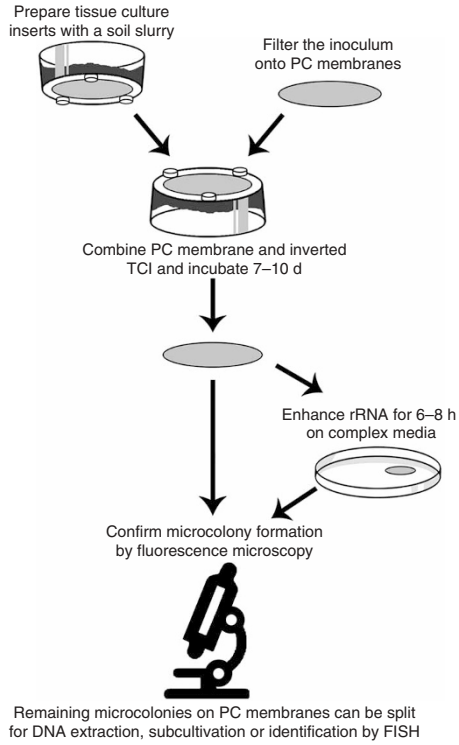

Figure 1 | Flow chart for microcultivation using the soil substrate membrane system. A soil slurry is prepared within an inverted tissue culture insert. This provides the substrate for growth while providing a barrier to contamination. A polycarbonate (PC) membrane provides the support for growth after being inoculated with a soil filtrate prepared from the same soil used to prepare the soil substrate. Growth is monitored after 7–10 d at 22–25 °C. The incubation period and temperature can be modified for specific soil types. FISH, fluorescence *in situ* hybridization. TCIs, tissue culture inserts.

Experimental design

Controls. Studies using the SSMS protocol must be designed to include appropriate controls. To ensure PCMs are not contaminated by the soil substrate within the tissue culture inserts (TCIs), a negative control growth membrane should be set up and monitored by total bacterial staining or FISH after the incubation period.

Sectioning of growth membranes. After incubation using the SSMS, growth membranes can be sectioned into quarters for analysis by either of the methods described below. Sectioning of membranes is dependant on the final outcome of the experiment. For instance, a study aimed at the molecular identification of microbial communities present after growth using the SSMS would first require confirmation of growth by total bacterial staining and fluorescence microscopy on a section of the PCM. If growth was successful, the remainder of the membrane can be used for direct DNA extraction and subsequent molecular identification.

FISH protocols (fixation conditions and probe design). FISH using rRNA targeted oligonucleotide probes is a important molecular tool used for the identification of bacterial groups in microbial ecology studies[3,29,30]. Detailed FISH protocols have been reported widely and include the use of different fixation conditions for gram-positive versus gram-negative bacteria[29,31,32]. Numerous reviews also discuss several aspects for FISH optimization including advanced probe design, hybridization, stringency and washing conditions[3,33]. Be sure to check current protocols for target microbial groups and adapt those methods to this protocol if required.

Although FISH has been carried out on membranes, slow-growing bacteria do not always contain the high number of ribosomes per cell required for obtaining high fluorescent signals[2,9,18]. Therefore, signal amplification methods may be required. Signal amplification through enhancement of the rRNA by incubation of growth membranes on nutrient rich agar plates is included in this protocol. However, the rRNA enhancement method described can alter the microbial population present on growth membranes[18]. Alternative amplification methods such as catalyzed reporter deposition-FISH could be adopted as these methods do not alter the microcolony-forming population present[18,29,34].

Methods for DNA extraction from PCMs. Two DNA extraction procedures have been successfully carried out on the microcolony-forming bacteria present following microcultivation on PCMs using the SSMS. The initial method removed cells from the growth membrane, followed by boiling of the cells to release the DNA[16].

Recently, a highly sensitive enzyme-based DNA extraction protocol proven to be efficient for isolating DNA from parasites[35] has been successfully adapted here for DNA extraction from PCMs. This method was superior as it enabled direct extraction from growth membranes without the requirement of a centrifugation step to separate the membrane from the cells of interest.

MATERIALS

REAGENTS

- Ultrapure Milli-Q water system (Millipore)
- 1 M Tris–HCl pH 7.4 (Bio-Rad, cat. no. T3253)
- 5 M NaCl (Ajax Finechem, cat. no. 465-500G)
- 10% SDS (Sigma-Aldrich, cat. no. L4390)
- 0.1% Low setting point agarose solution (Sigma-Aldrich)
- 96% Ethanol (Ajax Finechem, cat. no. 214-10L)
- Nail polish or slide sealing wax
- Formamide (Sigma-Aldrich, cat. no. 185906) ! CAUTION Poison and a mutagen. Avoid skin contact, eye contact, inhalation and ingestion. Work in a fume hood. Formamide waste should be collected and disposed of according to local rules and regulations.
- Tryptic Soy Broth (BD Biosciences, cat. no. 211825)
- Gelrite gellan gum (Sigma-Aldrich, cat. no. G1910)
- Glucose (Ajax Finechem, cat. no. 917-500G)
- Peptone (BD Biosciences, cat. no. 211677)
- Yeast extract (Oxoid, Thermo Fisher Scientific, cat. no. LP0021B)
- Sodium acetate (Sigma-Aldrich, cat. no. S2889)
- Tri-sodium citrate (Sigma-Aldrich, cat. no. S1804)
- Pyruvic acid (Sigma-Aldrich, cat. no. 107360)
- 4% Paraformaldehyde (Sigma-Aldrich, cat. no. 158127) ! CAUTION Irritant and toxic. Avoid skin contact, eye contact, inhalation and ingestion. Work in a fume hood. Paraformaldehyde waste should be collected and disposed of according to local rules and regulations.
- 1 M NaOH (Sigma-Aldrich, cat. no. S5881) ! CAUTION Highly caustic and is an irritant. Avoid skin contact, eye contact and ingestion.
- 100× SYBR Green II RNA Gel Stain (Invitrogen, cat. no. S-7564)
- Citifluor mounting medium (Leica Microsystems)
- *prepGEM* DNA extraction kit (ZyGEM Corporation Limited, cat. no. POR0200)
- 10× RAVAN medium stock (see REAGENT SETUP)
- FISH hybridization buffer (see REAGENT SETUP)
- FISH wash buffer (see REAGENT SETUP)

EQUIPMENT

- 2-mm Pore size sterile kitchen sieve
- 0.02-µm Pore size 25-mm anopore TCIs (Nunc) ▲ CRITICAL Various brands of TCI can be used in the protocol. TCIs with larger pore sizes (0.45 µm) such as those with PC or cellulose membranes (Millipore) can also be used for microcolony cultivation.
- 0.2-µm Pore size 25-mm isopore PCMs (Millipore, cat. no. GTTP02500)
- Benchtop vortex mixer (Bacto Laboratories)
- Sample filtration manifold (Carbon 14)
- Vacuum pump (Millipore)
- Whatman 25-mm glass fiber filter paper disks (Crown Scientific, cat. no. 1821025)
- Sterile 6-well multidish (Greiner Bio-One, cat. no. 657160)
- Zeiss Axioskop 2 epifluorescence microscope (Carl Zeiss)
- Microscope slides (Sail Brand)
- Small humidity chamber (e.g., contact lens case)
- 15- and 50-ml Falcon tubes (BD Biosciences, cat. no. 352097)
- Sterile 96-well microtiter plate (Greiner Bio-One, cat. no. 655185)
- Eppendorf compact thermomixer (Eppendorf)
- Eppendorf Mastercycler 5333 thermocycler (Eppendorf)
- Eppendorf benchtop centrifuge 5424 (Eppendorf)
- 0.2-µm Swinnex filter unit (Sartorius)
- Terumo 50-ml sterile syringe unit (Crown Scientific, cat. no. SS50ESA)

REAGENT SETUP

0.1% Low setting point agarose solution To make 100 ml of solution, add 0.1 g low setting point agarose to 100 ml Ultrapure Milli-Q water into a 250-ml Schott bottle and autoclave. The solution can be stored at room temperature (RT; 22 °C) for 1 month. The agarose solution must be boiled in the microwave before each use.

0.1× Tryptic Soy gellan gum plates To make approximately ten plates, begin with a 250-ml Schott bottle. When using Amyl media brand Tryptic Soy Broth, add 0.6 g to 200 ml Ultrapure Milli-Q water. Adjust the pH to 7, then add 6 g gellan gum powder, autoclave and pour plates. ▲ CRITICAL Gellan gum thickens the broth quickly and appears not to mix properly, mix as thoroughly as possible before autoclaving. The solution will homogenize further while being autoclaved. Plates can be stored at 4 °C wrapped in cling film for up to 2 months.

10× RAVAN medium stock To make 1 l of 10× stock medium, add glucose, 5 g l^{-1}; peptone, 5 g l^{-1}; yeast extract, 5 g l^{-1}; sodium acetate, 5 g l^{-1}; tri-sodium citrate, 5 g l^{-1}; pyruvic acid, 2 g l^{-1}; make up to 1 l in a Schott bottle with Ultrapure Milli-Q water, adjust the pH to 7. Autoclave and store the media in the refrigerator for several months. Use this at 0.1× concentration for all culturing. ▲ CRITICAL The media selected for subcultivation can be varied.

4% Paraformaldehyde To make 10 ml of 4% paraformaldehyde, add 400 µg solid paraformaldehyde powder to 10 ml Ultrapure Milli-Q water into a 15-ml tube. The powder will not immediately dissolve without the addition of 1 M NaOH. 1 M NaOH is then added dropwise until the solution is completely dissolved. This solution can be stored at −20 °C for 1 year. ! CAUTION Prepare this reagent in a fume hood with appropriate protective clothing.

FISH hybridization buffer To make 10 ml of the hybridization buffer, combine all reagents into a clean 15-ml tube: 1.8 ml of 5 M NaCl, 200 µl of 1 M Tris–HCl (pH 7.4), 7.9 ml of Ultrapure Milli-Q water and finally 10 µl 10% SDS. Mix well by gently shaking the tube. The hybridization solution can be stored at RT for several days. ▲ CRITICAL Care must be taken when adding and mixing SDS so as not to cause precipitation of salt in buffer. Do not vortex. Mix by gently inverting the tube. Formamide can be added to the buffer to increase the hybridization specificity of probes[3]. The selected FISH probes must be added to the hybridization buffer at a final concentration of 1 pmol μl^{-1} (refs. 3 and 16). ▲ CRITICAL Ensure that once probes are added, the solution is protected from light.

FISH wash buffer To make 10 ml of the wash buffer, combine in a sterile microfuge tube 1.8 ml of 5 M NaCl, 200 µl of 1 M Tris–HCl (pH 7.4), 7.9 ml of Ultrapure Milli-Q water and finally add 10 µl 10% SDS. The wash solution can be stored at RT for several days. ▲ CRITICAL Care must be taken when adding and mixing SDS so as not to cause precipitation of salt in buffer. Do not vortex. Mix by gently inverting the tube. Neither formamide nor probes are added to the wash buffer.

PROCEDURE

Preparation of TCIs and the soil slurry ● TIMING 1 h

1| Collect >20 g sample of soil. The pH and water content can be measured if needed downstream. This amount of soil taken will depend on the number of TCIs to be setup.

2| Pass the soil sample through a sterile 2-mm kitchen sieve to remove large particulate matter and repeat this process once more to homogenize the soil sample further. Using the cone and quarter method of sample homogenization, the entire 20 g is piled into a cone shape and divided into quarters. The opposite quarters are discarded and the two remaining quarters are mixed together to form a second cone. The process is repeated until the desired sample size of ~3 g of soil for each TCI is prepared. ▲ CRITICAL STEP Depending on the wet-weight of the soil used to prepare the TCI, more soil may be required to fill the inserts.

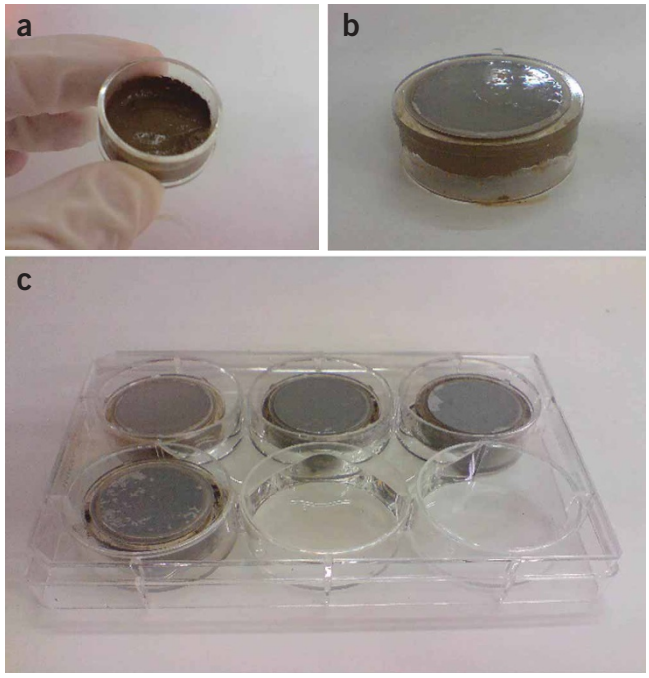

Figure 2 | Major steps involved in set up of the soil substrate membrane system. (**a**) The soil substrate is prepared within the tissue culture insert (TCI) by vortexing until an even slurry is formed. Polycarbonate (PC) membrane saturation will ensure optimal diffusion of nutrients through the membrane. (**b**) An inoculated PC membrane is placed on top of the inverted TCI, which contains the soil slurry. This allows diffusion of nutrients from the soil substrate to the bacteria present on the PC membrane. (**c**) Four soil substrate membrane system replicates are placed into a 6-well culture plate. To ensure humidity within the culture vessel, pipette sterile water underneath each TCI and into the spaces between wells. The vessel is then sealed with Parafilm and incubated for 7–10 d.

3| Take a sterile TCI and carefully place the weighed homogenized soil into it.
▲ CRITICAL STEP The membrane of the TCI is fragile and care must be taken as it will break easily. This insert must remain sterile.

4| Carefully pipette two to three drops of sterile physiological saline (0.9% NaCl) or water onto the soil within the TCI.

5| Turn on the benchtop vortex mixer to full speed and carefully touch the edge of the TCI to the edge of the vortex mixer plate so that the vibrations cause the soil and the water to change texture to become a soil slurry (**Fig. 2a**).
▲ CRITICAL STEP At this stage, it is necessary to take extra care because the TCI is fragile and the vortex mixer can cause the anopore membrane to break. It is important to note that the vibrations caused from the vortex can lead to soil falling out of the TCI. Care must be taken to prevent this as it could lead to sample contamination. This can be a long process; patience is required as the slurry will form gradually over 5–10 min. The time the slurry takes to form is dependant on the soil texture. To reduce the time required, preliminary slurries may be prepared in a 50-ml tube and sub-samples placed into the TCI. In this case, the soil slurry must still be vortexed within the TCI until all air bubbles are removed which means that the anopore membrane is completely saturated by the soil substrate (**Fig. 2a** and **b**).

6| Once the soil has formed an even slurry, pipette 500 µl Ultrapure Milli-Q water into the middle of a well within a sterile 6-well plate. Invert the TCI so that the membrane is facing upward and place it in the middle of the well over the sterile water (**Fig. 2c**). This water is used to prevent the TCI drying out during the incubation period.

7| Repeat this process for the number of TCIs required. Most cultivation attempts should be prepared in triplicate for each soil type studied.

Preparation and filtration of the inoculum onto PCMs ● TIMING 45 min

8| Weigh out a 3 g sub-sample of the same homogenized soil (from Step 2) to create the inoculum for the TCIs.

9| First, prepare a one in ten dilution of soil. To do this, place the soil into a sterile 50-ml tube with 30 ml of Ultrapure Milli-Q water and vortex the tube vigorously to mix the sample. Allow the large particulates to sediment by letting the sample stand for 30 s.

10| Aspirate 100 µl of the supernatant which contains the majority of bacteria and add it to 1 ml of Ultrapure Milli-Q water. Vortex the sample well before adding to the PCM. This 1 in 100 dilution of soil is the inoculum for the SSMS.

11| To filter the inoculum onto the PCM, set up a sample filtration manifold and attach the vacuum pump to its exhaust. Alternatively, a 25-mm Swinnex filter and a syringe can be used to filter the inoculum onto the PCM.

12| To support the PCM during filtration, place a 25-mm sterile glass fiber filter at each position required on the sample filtration manifold. Prewet the filter with 150 µl of Ultrapure Milli-Q water.
▲ CRITICAL STEP This will ensure PCM does not fold or break during the sample filtration process.

13| For every sample to be set up, place a sterile 0.2-µm PCM on top of the prewet glass fiber filter. Then, place a sterile stainless steel cylinder on top of each of the PCMs on the manifold. These cylinders hold a volume of 20 ml and are used to aid filtration of the inoculum onto the PCM.

14| Add 10 ml physiological saline (0.9% NaCl) or sterile water into each cylinder followed by 50 µl of the 1 in 100-fold soil inoculum. Mix by gentle pipetting to ensure an even distribution of cells on the PCM during filtration.

15| Turn on vacuum pump and open valve to draw the diluted inoculum through the PCMs. Close the valves as soon as the diluted inoculum has passed through the PCM.
▲ CRITICAL STEP Do not allow air to be drawn through PCM as this can cause bacteria to pass through or become embedded in the PCM.

Combining inoculated PCMs with preprepared TCIs ● TIMING 15 min
16| Carefully remove each PCM from the filtration manifold using sterile tweezers and place them gently on top of the inverted preprepared TCIs (**Fig. 2b** and **c**).
▲ CRITICAL STEP Make sure no bubbles become trapped between the PCM and the membrane of the TCI as this will prevent nutrient contact between the bacteria and the soil extract. If bubbles appear, carefully lift one edge of the PCM using tweezers and place back on the TCI.

17| Pipette 1 ml Ultrapure water in the spaces between the wells to prevent the SSMS from drying out during the incubation period (**Fig. 2c**).

18| Place the lid on the 6-well plate and seal the SSMS with Parafilm.

19| Incubate the system for ~7–10 d at 22 °C.
? TROUBLESHOOTING

Microscopic confirmation of microcolony formation on the PCM ● TIMING 40 min
20| After 7–10-d incubation, pipette 5 µl Ultrapure Milli-Q water into one-half of a Petri dish.

21| Remove one of the PCMs from a TCI and gently place it onto the drop of water. Using a sterile scalpel blade and forceps, cut out one quarter of the PCM carefully.

22| To keep microcolonies intact for fluorescence visualization, embed the PCM section in agarose by heating the 0.1% agarose solution in a microwave oven until it begins to boil.
? TROUBLESHOOTING

23| Pour a thin layer into the other half of the Petri dish and allow it to cool to ~30–40 °C. Using sterile tweezers, dip the quarter section of the PCM into the cooled agarose solution and then place it onto a piece of Parafilm. Allow the PCM section to dry in a 30 °C incubator.

24| To remove the PCM section from the Parafilm once it has dried, pipette 3 µl 96% ethanol onto the side of the PCM and let it absorb into the membrane so that it lifts away from the Parafilm.

25| For microscopic mounting of membranes, pipette 4 µl Citifluor onto a microscope slide. Gently place the PCM section onto the Citifluor. For total bacterial staining, pipette 4 µl Citifluor into the center of a microscope cover slip and combine with 1 µl of 100× SYBR Green II RNA Gel Stain. If required, pipette 1 µl Citifluor on each corner of the cover slip. Place the cover slip onto the PCM on the slide and seal the slide with nail polish or wax. Cells can be visualized immediately.

26| Confirm the presence of microcolony-forming bacteria using an epifluorescence microscope with the appropriate filter set for SYBR Green II emission (**Fig. 3**).
▲ CRITICAL STEP Abundant microcolony-forming bacteria are observed on a section of a PCM. The remaining PCM sections (from Step 21) can be analyzed further using FISH, molecular characterization after DNA extraction or subculturing (as described below).
? TROUBLESHOOTING

FISH on PCMs ● TIMING 15 h
27| Most FISH methods can be adapted to PCMs[16,29]. As microcolony-forming bacteria are slow growing, the rRNA content may need to be enhanced before fixation. To enhance rRNA, carefully place an entire PCM (from Step 19, or a PCM section) face up onto a 10% Tryptic Soy gellan gum plate and incubate for 6 h at 22 °C.

28| To keep microcolonies intact after rRNA enhancement, remove the PCM from the Tryptic Soy gellan plate and embed it in 0.1% agarose solution as described earlier (Steps 20–24).

29| For fixation, place a 25-mm glass fiber filter into a well of a sterile 6-well plate.

30| Pipette 350 µl 98% ethanol or 4% paraformaldehyde onto the filter paper.
▲ **CRITICAL STEP** Depending on the bacteria that the probes are designed for, the fixation step will vary. Generally, gram-positive bacteria require ethanol fixation and gram-negatives require paraformaldehyde[3,29]. This step should be modified to the target microbial population (see Experimental design).

31| Place the PCM section (from Step 28) gently on top of the filter paper. Place the lid on the 6-well plate, seal the vessel with Parafilm and place it at 4 °C for 4 h or overnight. The time for fixation depends on the FISH protocol being used and should be optimized for your microbial target group (see Experimental design).

32| To remove fixative from the PCM section after fixation, place 3 ml sterile water into the well of a sterile 6-well plate. Float each PCM section on the sterile water at RT for 30 min.

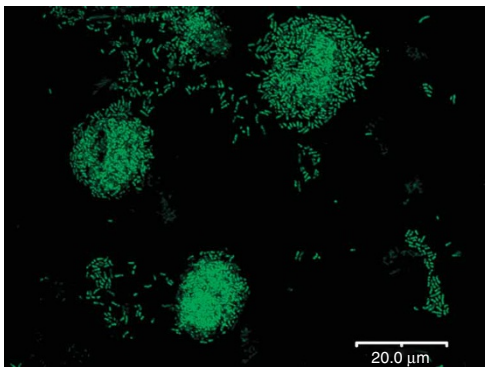

Figure 3 | Epifluorescence microscopy image of microcolony-forming soil bacteria present on growth membranes. Total bacterial staining with SYBR Green II reveals abundant bacterial growth as microcolonies only, invisible to the naked eye. When examined by epifluorescence microscopy, several morphotypes can be visualized per field of view after a 7–10-d incubation period.

33| Repeat this wash step by placing each PCM into a fresh well with 3 ml sterile water.
■ **PAUSE POINT** Fixed PCMs can be stored at 4 °C for several days.

34| Preheat a water bath or incubator to 46 °C.

35| According to the FISH protocol of choice, prepare 300 µl FISH hybridization buffer for every section of PCM to be analyzed. This includes the addition of FISH probes to a final concentration of 1 pmol μl^{-1}.
▲ **CRITICAL STEP** Be sure to check current protocols for specific microbial groups and adapt those protocols to this method if required (see Experimental design).

36| For FISH identification of microbial growth on PCMs, a universal bacterial probe can be used. EUB-338 (GCTGCCTCCCGTAGGAGT) has been used successfully for microcolony-forming bacteria[16,18]. The probes supplied need to be made up to a stock concentration of 100 pmol μl^{-1}. For example, if the stock probe is at 300 µM (300 pmol μl^{-1}) and each membrane section requires 3 µl probe at 100 µM (100 pmol μl^{-1}), dilute 1 µl stock probe with 2 µl hybridization buffer and add to the 300 µl hybridization buffer.

37| In a small humidity chamber, such as a contact lens case, place a 25-mm sterile glass fiber filter paper neatly in the chamber and pipette 300 µl FISH hybridization buffer onto it. This provides an interface that keeps the PCMs in contact with probes.

38| Carefully place the fixed PCM section onto the filter paper in the chamber with the bacteria facing upward. Seal the chamber tightly and incubate at 46 °C for 3 h. Ensure the samples are protected from light.

39| During the hybridization period, prepare 300 µl FISH wash buffer for each for every PCM section to be analyzed.

40| Following hybridization, prepare a fresh humidity chamber, place a 25-mm glass fiber filter paper and prewet the chamber with 300 µl of FISH wash buffer.

41| After hybridization, carefully transfer each PCM to a new humidity chamber containing the wash buffer. Protect samples from light. Seal the new chambers and incubate at 46 °C for 30 min in the water bath.

42| Repeat this wash process once more (Steps 39–41).

43| The PCM can be now counterstained with a total bacterial stain such as SYBR Green II total if required (Steps 25 and 26). For samples not counterstained, pipette 4 µl Citifluor onto a microscope slide and mount the PCM immediately.

44| Gently place the section onto the Citifluor. Pipette 4 µl Citifluor into the center of a microscope cover slip and 1 µl near each corner of the cover slip. Carefully place the cover slip on top of the PCM and seal with nail polish.

45| Confirm the presence of microcolonies using an epifluorescence microscope with appropriate filters for the excitation of fluorophores used for FISH.
? TROUBLESHOOTING

Preparation of PCMs for DNA extraction ● TIMING **10 min**

46| Pipette 5 µl Ultrapure Milli-Q water into a Petri dish. Remove one of the PCMs from the TCI (Step 20) or a 10% Tryptic Soy gellan gum plate (Step 27) and gently place it on the drop of the water.

47| Using a sterile scalpel blade, carefully separate a quarter section of the growth membrane.
? TROUBLESHOOTING

48| Carefully place the quarter section into the bottom of a sterile 0.5-ml microfuge tube using sterile tweezers. Ensure that the microbial population on the PCM is facing the middle of the tube.

DNA extraction from PCMs for subsequent molecular characterization

49| DNA extraction from PCM can be achieved by boiling (option A) or by *prepGEM* DNA extraction (option B).

(A) DNA extraction through boiling ● TIMING **Option A—15 min**

(i) Add 100 µl Ultrapure Milli-Q water to each tube from Step 48.

(ii) Use sterile tweezers to pull the membrane up to the top of the tube. Trap the edge of the membrane between the lip of the tube and the lid by closing the lid.

(iii) Vortex the tube vigorously for 2 min to remove cells from the membrane. Spin the sample at 12,000*g* for 10 min. Remove the PCM and discard.
? TROUBLESHOOTING

(iv) Resuspend the pellet in 20 µl Ultrapure Milli-Q water and boil at 99 °C for 5 min. Spin the sample at 14,000*g* for 3 min to pellet debris, which could interfere with the PCR.

(v) Use 2 µl of the supernatant to a 50 µl PCR. When targeting the 16S rRNA gene, a standard PCR used for microbial ecology studies can be used[16].

(B) *prepGEM* DNA extraction ● TIMING **Option B—50 min**

(i) Add 99 µl 1× *prepGEM* buffer 3 to each microfuge tube containing a PCM section from Step 48. Take care to ensure the membrane is completely covered by the buffer.

(ii) Add 1 µl *prepGEM* enzyme to each sample and mix the tube briefly by vortexing.

(iii) Place the tube in a thermocycler and program the following sequence: 37 °C for 15 min, 75 °C for 15 min and 95 °C for 15 min.

(iv) After completion of the extraction program, open each tube and use sterile tweezers to pull the membrane to the top of the tube. Trap the edge of the membrane between the lip of the tube and the lid by closing the lid.

(v) To remove membrane from the lysate, vortex the tube briefly and spin at 10,000*g* for 1 min. Remove the membrane and discard.

(vi) Finally, add 0.2 µl EDTA (0.5 M) to the lysate.
■ **PAUSE POINT** This lysate can be stored at 4 or −20 °C.

(vii) Use 2 µl of the lysate in a 50 µl PCR.

Removing live bacteria from PCMs for subcultivation ● TIMING **15 min**

50| To remove live cells from the PCM, repeat Steps 46–49A(iii). In this case, wash the cells off membrane into 500 µl of physiological saline (0.9% NaCl) as this should not affect the viability of the cells.
? TROUBLESHOOTING

51| Resuspend the pellet into 100 µl of sterile water or dilute media such as 0.01× RAVAN media designed for oligophilic bacteria[23]. For dilution to extinction culturing, obtain cell counts by microscopy or flow cytometry and dilute cells appropriately[9,36].
? TROUBLESHOOTING

52| A second round of subculturing can now be carried out by either a second round SSMS or limiting dilution using the resuspended cells. For limiting dilution to extinction, cells can be added into wells of a 96-well microtiter plate with 200 µl appropriate medium.

53| Incubate for 7–10 d at 22 °C and monitor for growth by microscopy after 3 d.

● TIMING

Steps 1–18, preparation of SSMS: 2 h
Step 19, incubation of the SSMS: 7–10 d
Steps 20–26, microscopic confirmation of microcolony formation: 40 min

FISH on PCM sections:
Step 27, rRNA enhancement: 6 h
Steps 28–33, fixation: >5 h
Steps 34–45, hybridization: 4 h
Steps 46–48, preparation of PCMs for DNA extraction: 10 min

DNA Extraction from PCMs:
Step 49A(i–v), 15 min; Step 49B(i)–(vii), 50 min
Removal of live cells for subculturing via SSMS or limiting dilution:
Steps 50–53, 15 min; Step 54, 7–10 d

TROUBLESHOOTING

Troubleshooting advice can be found in **Table 1**.

TABLE 1 | Troubleshooting Table.

Steps	Problem	Possible reason	Solution
19–26	Few microcolonies observed after the incubation period	Inoculum not high enough	In most cases, a 1 in 100 dilution results in even growth of microcolonies on the polycarbonate membrane (PCM) following 10 d of incubation. This can be modified according to the initial concentration of bacteria present in the soil (Steps 8–10). The dilution of the inoculum may be increased or decreased as required
		The soil slurry within the tissue culture insert (TCI) or the PCM dried out	Adding water to the 6-well plate should prevent the soil slurry from drying out over the course of the experiment (Steps 17 and 20). It is important to check the water levels and the humidity of the soil during the course of the experiment to ensure it does not dry out as this allows the nutrients to diffuse from the slurry to the surface of the PCM. If necessary, additional water can be added to the incubation chamber as required
		Lengthen the incubation time required for microcolony formation	The incubation time for the experiment can be increased to allow more time for microcolony formation, especially if lower temperatures are used during the incubation period
26	More single cells than intact microcolonies are present on the PCMs when examined by fluorescence microscopy	Microcolonies were disturbed during the staining process	0.1% low setting point agarose solution can be used to embed microcolonies on the PCM before fluorescence staining and microscopy (Steps 22–24). This will ensure that each microcolony is intact and that individual cells are not disturbed during the staining process
45	The fluorescent signal obtained after fluorescence *in situ* hybridization (FISH) is low	Slow-growing bacteria may have low levels of rRNA undetectable by standard FISH	An rRNA enhancement step may be necessary for visualization by FISH (Step 27). Care must be taken as this additional incubation step has been shown to alter the composition of the bacterial population present after soil substrate membrane system incubation[18]. Complex media may trigger the growth of fast growing species. Alternative FISH amplification methods can be used, such as catalyzed reporter deposition-FISH[18]
46–51	Not many cells are present for DNA extraction or subculturing	This wash step may not have removed the microbial cells that have adhered to the membrane	To monitor for effective removal of cells from membranes, the PCM can be screened. To do this, cut a small section of the PCM and observe by epifluorescence microscopy to ensure the majority of cells have been washed off the membrane (Steps 46–49A(ii)). If cells remain, repeat vortexing of the membranes to remove more cells. Alternatively, direct DNA extraction methods for PCMs can be used[35]

ANTICIPATED RESULTS

The SSMS results in abundant microbial growth observed as microcolonies only (**Fig. 3**). These microcolonies, defined as having three or more closely associated cells, are invisible to the naked eye, yet they vary in morphology and cell number. In most cases, several diverse morphotypes can be observed growing in coculture after 7–10 d of incubation. Molecular characterization has confirmed that this method selects for previously 'uncultured' bacterial species from several bacterial divisions including the Candidate Division TM7, which has no pure cultured representatives despite being ubiquitous in the environment[16,21]. When combined with micromanipulation and subculturing into dilute media, the SSMS provides a strategy for the recovery of pure microcultures for further characterization.

ACKNOWLEDGMENTS This work was supported by a Macquarie University Research Fellowship. We thank Debra Birch for her advice and support with confocal microscopy imaging of microcolonies.

Published online at http://www.natureprotocols.com/
Reprints and permissions information is available online at http://npg.nature.com/reprintsandpermissions/

1. Leadbetter, J.R. Cultivation of recalcitrant microbes: cells are alive, well and revealing their secrets in the 21st century laboratory. *Curr. Opin. Microbiol.* **6**, 274–281 (2003).
2. Green, B.D. & Keller, M. Capturing the uncultivated majority. *Curr. Opin. Biotechnol.* **17**, 236–240 (2006).
3. Amann, R.I., Ludwig, W. & Schleifer, K.H. Phylogenetic identification and *in-situ* detection of individual microbial cells without cultivation. *Microbiol. Rev.* **59**, 143–169 (1995).
4. Hugenholtz, P., Goebel, B.M. & Pace, N.R. Impact of culture-independent studies on the emerging phylogenetic view of bacterial diversity. *J. Bacteriol.* **180**, 4765–4774 (1998).
5. Handelsman, J. Metagenomics: application of genomics to uncultured microorganisms. *Microbiol. Mol. Biol. Rev.* **68**, 669–685 (2004).
6. Keller, M. & Zengler, K. Tapping into microbial diversity. *Nat. Rev. Microbiol.* **2**, 141–150 (2004).
7. Rappe, M.S., Connon, S.A., Vergin, K.L. & Giovannoni, S.L. Cultivation of the ubiquitous SAR11 marine bacterioplankton clade. *Nature* **418**, 630–633 (2002).
8. Sharma, R., Ranjan, R., Kapardar, R. & Grover, A. 'Unculturable' bacterial diversity: an untapped resource. *Curr. Sci.* **89**, 72–77 (2005).
9. Janssen, P.H., Yates, P.S., Grinton, B.E., Taylor, P.M. & Sait, M. Improved culturability of soil bacteria and isolation in pure culture of novel members of the divisions Acidobacteria, Actinobacteria, Proteobacteria, and Verrucomicrobia. *Appl. Environ. Microbiol.* **68**, 2391–2396 (2002).
10. Sait, M., Hugenholtz, P. & Janssen, P.H. Cultivation of globally distributed soil bacteria from phylogenetic lineages previously only detected in cultivation-independent surveys. *Environ. Microbiol.* **4**, 654–666 (2002).
11. Bollman, A., Lewis, K. & Epstein, S. Incubation of environmental samples in a diffusion chamber increases the diversity of recovered isolates. *Appl. Environ. Microbiol.* **73**, 6386–6390 (2007).
12. Connon, S.A. & Giovannoni, S.J. High-throughput methods for culturing microorganisms in very-low-nutrient media yield diverse new marine isolates. *Appl. Environ. Microbiol.* **68**, 3878–3885 (2002).
13. Kaeberlein, T., Lewis, K. & Epstein, S. Isolating 'uncultivable' microorganisms in pure culture in a simulated natural environment. *Science* **296**, 1127–1129 (2002).
14. Zengler, K. *et al.* Cultivating the uncultured. *Proc. Natl. Acad. Sci. USA* **99**, 15681–15686 (2002).
15. Morris, R.M. *et al.* SAR11 clade dominates ocean surface bacterioplankton communities. *Nature* **420**, 806–810 (2002).
16. Ferrari, B.C., Binnerup, S.J. & Gillings, M.R. Microcolony cultivation on a soil substrate membrane system recovers previously unculturable bacteria. *Appl. Environ. Microbiol.* **71**, 8714–8720 (2005).
17. Winding, A., Binnerup, S. & Sørensen, J. Viability of indigenous soil bacteria assayed by respiratory activity and growth. *Appl. Environ. Microbiol.* **60**, 2869–2875 (1994).
18. Ferrari, B.C., Tujula, N., Stoner, K. & Kjelleberg, S. Catalyzed reporter deposition-fluorescence *in situ* hybridization allows for enrichment-independent detection of microcolony-forming soil bacteria. *Appl. Environ. Microbiol.* **72**, 918–922 (2006).
19. Binnerup, S.J. *et al.* Ribosomal RNA content in microcolony forming soil bacteria measured by quantitative 16S rRNA hybridisation and image analysis. *FEMS Microbiol. Ecol.* **37**, 231–237 (2001).
20. Svenning, M.M., Wartiainen, I., Hestnes, A.G. & Binnerup, S.J. Isolation of methane oxidising bacteria from soil by use of a soil substrate membrane system. *FEMS Microbiol. Ecol.* **44**, 347–354 (2003).
21. Hugenholtz, P., Tyson, G.W., Webb, R.I., Wagner, A.M. & Blackall, L.L. Investigation of candidate division TM7, a recently recognised major lineage of the domain bacteria with no known pure-culture representatives. *Appl. Environ. Microbiol.* **67**, 411–419 (2001).
22. Rasmussen, L. *et al.* Cultivation of hard-to-culture subsurface mercury resistant bacteria and discovery of new *merA* gene sequences. *Appl. Environ. Microbiol.* April 25, e-pub ahead of print (2008).
23. Watve, M. *et al.* The 'K' selected oligophilic bacteria: a key to uncultured diversity? *Curr. Sci.* **78**, 1535–1542 (2000).
24. Simu, K. & Hagstrom, A. Oligotrophic bacterioplankton with a novel single-cell life strategy. *Appl. Environ. Microbiol.* **70**, 2445–2451 (2004).
25. Powell, S.M., Ferguson, S.H., Snape, I. & Siciliano, S.D. Fertilization stimulates anaerobic fuel degradation of antarctic soils by denitrifying microorganisms. *Environ. Sci. Technol.* **40**, 2011–2017 (2006).
26. Hamamura, N., Olson, S.H., Ward, D.M. & Inskeep, W.P. Microbial population dynamics associated with crude-oil biodegradation in diverse soils. *Appl. Environ. Microbiol.* **72**, 6316–6324 (2006).
27. Kenzaka, T., Yamagaguchi, N., Utrarachkij, F., Suthienkul, O. & Nasu, M. Rapid identification and enumeration of antibiotic resistant bacteria in urban canals by microcolony-fluorescence *in situ* hybridization. *J. Health Sci.* **52**, 703–710 (2006).
28. Hesselsoe, M. *et al.* Degradation of 4-nonylphenol in homogeneous and nonhomogeneous mixtures of soil and sewage sludge. *Environ. Sci. Technol.* **35**, 3695–3700 (2001).
29. Pernthaler, J., Glockner, F.O., Schonhuber, W. & Amann, R. Fluorescence *in situ* hybridization. In *Methods in Microbiology: Marine Microbiology* (ed. Paul, J.H.) 207–226 (Academic Press, London, 2001).
30. Amann, R.I., Krumholz, L. & Stahl, D.A. Fluorescent-oligonucleotide probing of whole cells for determinative, phylogenetic, and environmental studies in microbiology. *J. Bacteriol.* **172**, 762–770 (1990).
31. Stahl, D.A. & Amann, R. Development and application of nucleic acid probes. In *Nucleic Acid Techniques in Bacterial Systematics* (eds. Stackebrandt, E. & Goodfellow, M.) 205–248 (John Wiley and Sons, Chichester, UK, 1991).
32. Pernthaler, A. & Pernthaler, J. Fluorescence *in situ* hybridization for the identification of environmental microbes. *Methods Mol. Biol.* **353**, 153–164 (2007).
33. Amann, R. & Fuchs, B. Single-cell identification in microbial communities by improved fluorescence *in situ* hybridization techniques. *Nat. Rev. Microbiol.* **6**, 339–348 (2008).
34. Tujula, N.A. *et al.* A CARD-FISH protocol for the identification and enumeration of epiphytic bacteria on marine algae. *J. Microbiol. Methods* **65**, 604–607 (2006).
35. Ferrari, B.C., Power, M.L. & Bergquist, P.L. Closed-tube DNA extraction using a thermostable proteinase is highly sensitive, capable of single parasite detection. *Biotechnol. Lett.* **29**, 1831–1837 (2007).
36. Davey, H.M. & Kell, D.B. Flow cytometry and cell sorting of heterogeneous microbial populations: the importance of single-cell analyses. *Microbiol. Rev.* **60**, 641–696 (1996).