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Article

A new method for microbial cultivation and its application to bacterial community analysis in Buus Nuur, Mongolia*

Paper of the special Issue "Aquatic microbial ecology in the footsteps of Jürgen Overbeck"

Dawoon Jung[1, x], Eun-Young Seo[1, x], Slava S. Epstein[2], Yochan Joung[3], Joung Han Yim[4], Hongkum Lee[4] and Tae Seok Ahn[1, *]

With 3 figures and 4 tables

Abstract: We performed a comparative analysis of bacterial diversity in a saline soda lake using a novel cultivation method termed the filter plate microbial trap method (FPMT), and conventional techniques together with DGGE (denaturing gradient gel electrophoresis). The new method allowed for cultivation of representatives of seven bacterial taxonomic groups, including seven novel species. This compares favorably to the results from cultivation of representatives of four taxonomic groups and only one novel species using standard approaches. Neither culture collection matched the community composition revealed by DGGE but the FPMT method produced a more representative collection of strains. We conclude that FPMT is a powerful device for improving bacterial culturability and narrowing the gap between microbial diversity in nature and cultivable microorganisms.

Key words: bacterial diversity, DGGE, improved culturability, soda lake.

Introduction

In the past, bacterial community structure analysis relied mostly on conventional culture methods such as e.g. liquid enrichments, cultivation in Petri dish or membrane filters and for enumeration, the most probable number method. The success of these approaches is limited by "uncultivability" of the majority of microbial species, likely due to suboptimal media composition, allelopathy and other interactions between bacteria and other factors (Amann et al. 1995, Vartoukian et al. 2010).

Rapid development of culture-independent molecular tools such as the rRNA approach, FISH, DGGE (denaturing gradient gel electrophoresis), and others brought a new era in studies of microbial diversity (Christen 2008, Handelsman 2004). Their successes notwithstanding, cultivation and isolation of bacterial species remains essential for studying properties of individual microorganisms, obtaining biomass for whole genome sequencing, etc.

Recently, significant improvements have been achieved in microbial cultivation techniques, opening new opportunities for the study of microbial ecology and diversity. Modifying growth conditions, such as

Authors's addresses:

[1] Dept. of Environmental Science, Kangwon National University, Chunchon, 200–701, Republic of Korea

[2] Dept. of Biology, Northeastern University, Boston, MA, 02115, USA

[3] Dept. of Microbiology and Molecular Biology, Chungnam National University, Daejeon, 305–764, Republic of Korea

[4] Division of Life Sciences, Korea Polar Research Institute, KORDI, Incheon, 406–840, Republic of Korea

* Corresponding author; ahnts@kangwon.ac.kr

[x] D. J and E.-Y. S. contributed equally to the manuscript and are listed in alphabetical order

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addition of signaling molecules (Bruns et al. 2002, Bussmann et al. 2001), lowering nutrient concentrations (Janssen et al. 2002), extending incubation times (Davis et al. 2005) and using alternative gelling agents (Tamaki et al. 2005) led to isolation of many novel species. Other innovations aimed to better simulate the natural environment, such as using diffusion chambers (Kaeberlein et al. 2002), hollow-fiber membrane chambers (Aoi et al. 2009), ichips (Nichols et al. 2010) and minitraps (Gavrish et al. 2008, Sizova et al. 2012). These methods have significantly improved the recovery of 'unculturables' from the environment (Vartoukian et al. 2010).

In this study, we capitalize on the successes of the above approaches and design a new *in situ* culture method for isolating bacteria, termed the filter plate microbial trap (FPMT). The idea is to bring a gelling agent, such as agar, in contact with the environment (soil, marine sediment, etc.) but separate the two by a membrane (pore size 0.2–2 μm). The pores will allow microbes from the environment to penetrate the membrane and establish colonies in the medium. The expected advantage is that the membrane will allow for the exchange of growth factors between the natural habitat and agar. This should minimize the differences in the chemical environment between the two sides of the membrane, thus simulating the natural conditions in the growth medium. This should in principle allow for cultivation of species that are otherwise difficult or impossible to grow by conventional approaches.

FPMT has significant advantages compared to previous *in situ* cultivation methods. Firstly, since each chamber is independent from others, fast growing bacteria in a chamber are not able to spread to other chamber. Secondly, in other *in situ* cultivation devices, such as diffusion chambers, ichips, or the hollow fiber systems (Kaeberlein et al. 2002, Nichols et al. 2010, Aoi et al. 2009), samples including microorganisms must be added, whereas the FMPT is based on a different principle and is introduced into the environment cell-free and sterile. In this, our approach shares some similarity with the trap method (Gavrish et al. 2008), but with essential differences. Using our approach, the size of the opening through which microorganisms can enter the device can be manipulated, the filter membrane is aimed precisely at the microenvironment targeted for microbial isolation, and the technique is simpler in both design and field application.

To test the efficacy of the method, we used the FMPT method on samples from Lake Buus Nuur, Mongolia. Buus Nuur is a small, shallow, saline soda lake on dry steppes surrounded by saline soils. Because saline and soda lakes are unique ecosystems with high productivity and rates of biodegradation, we therefore considered Lake Buus Nuur a good model for bacterial community analysis (Ma et al. 2004). We used both conventional and FPMT-based cultivation to grow microorganisms from this lake, and compared the culture collections between themselves and also to a DGGE-based, culture-independent survey of this lake's microbial inhabitants.

Material and methods

Sample collection

Lake water, sediment and shoreline soil samples were collected on 11 September, 2011 from Buus Nuur (47° 46′ 40.72″ N, 107° 20′ 25.29″ E), Mongolia and then used as bacterial sources. The water temperature, salinity and pH at the sampling site were 12.1 °C, 10.9 g/L and 8.9, respectively. Chilled samples for cultivation were transported to the laboratory at Kangwon National University. The DNA extraction for molecular analysis was performed at the sampling site.

Media

At first, we used 4 alkaline media as follows; actinomycete isolation agar with 1 % NaCl, marine agar, mCMC agar and

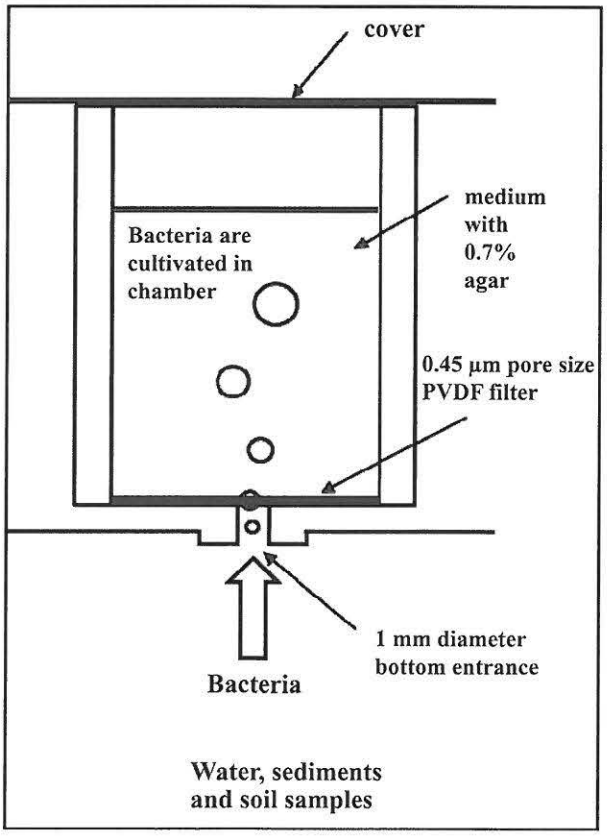

Fig. 1. Schematic diagram of the FPMT chamber.

R2A agar with 1% NaCl. Among these media, colonies on R2A medium were more diverse and abundant than those on other media. Therefore, we selected R2A medium for further experiments. The R2A medium was made with 1% NaCl, 15 agar, yeast extract 0.5, proteose peptone 0.5, casamino acids 0.5, dextrose 0.5, soluble starch 0.5, sodium pyruvate 0.3, dipotassium phosphate 0.3, magnesium sulfate 0.005 (g/L), and adjusted to pH 9.0 with 0.1 N NaOH.

FPMT device

The concept of FPMT is to cultivate microorganisms in a simulated natural habitat, following the ideas behind the diffusion chamber (Kaeberlein et al. 2002) and trap methods (Gavrish et al. 2008). However, the approach used in practice is quite different with the FPMT method. We used UNIFILTER filtration microplates (Whatman No 7700–1806, Germany) with 96 wells as the principle growth device. The bottom of each well is a hydrophilic polyvinylidenefluoride (PVDF) membrane (0.45-μm pore-size), which sits on a plastic support with a 1 mm diameter hole (Fig. 1). The latter provides a connection between the membrane and the outside environment. If filled with a gelling agent such as agar, and brought into contact with the target environment, each well becomes a small growth chamber for microorganisms penetrating the pores of the membrane. Note that nutrients, metabolites, and signal molecules will freely diffuse from the environment into the agar, mimicking the outside growth conditions. FPMT was prepared for growth experiments as follows. Prior to cultivation experiments, UNIFILTER filtration microplates were sterilized for 24 hrs under an ultraviolet lamp. The R2A media with 1% NaCl in 0.7% (wt/vol) agar was administered to each well and the upper part of the plate was sealed with a Breathe-Easy™ membrane (Diversified Biotech, USA) to prevent contamination from the air. Identical microplates with the bottom closed and sealed from the environment were used as a negative control.

Experimental design and cultivation of bacteria

A flow chart of growth experiments is shown in Fig. 2. For conventional cultivation, suspended sediment and soil samples (1 g/100 ml of 1× PBS) and water samples were serially diluted. The 100 μl aliquots from different dilutions were inoculated into R2A nutrient medium with 1% (wt/vol) NaCl in 1.5% (wt/vol) agar, placed into Petri dishes, and the plates were incubated at 12 °C. After 4 weeks, 37 colonies of different shape and color were selected for purification via subculture.

For FPMT-based cultivation, samples from the target environments were placed in 13 (width) × 19 (length) × 6 (depth) cm sterilized plastic boxes, and FPMTs were placed on top of the sediment or soil samples, or left floating on the water samples. The FPMTs were lifted at 48 hour intervals to supply air, and the soil/sediment samples were wetted with sterile water to prevent drying. After 4 weeks of incubation in the dark at

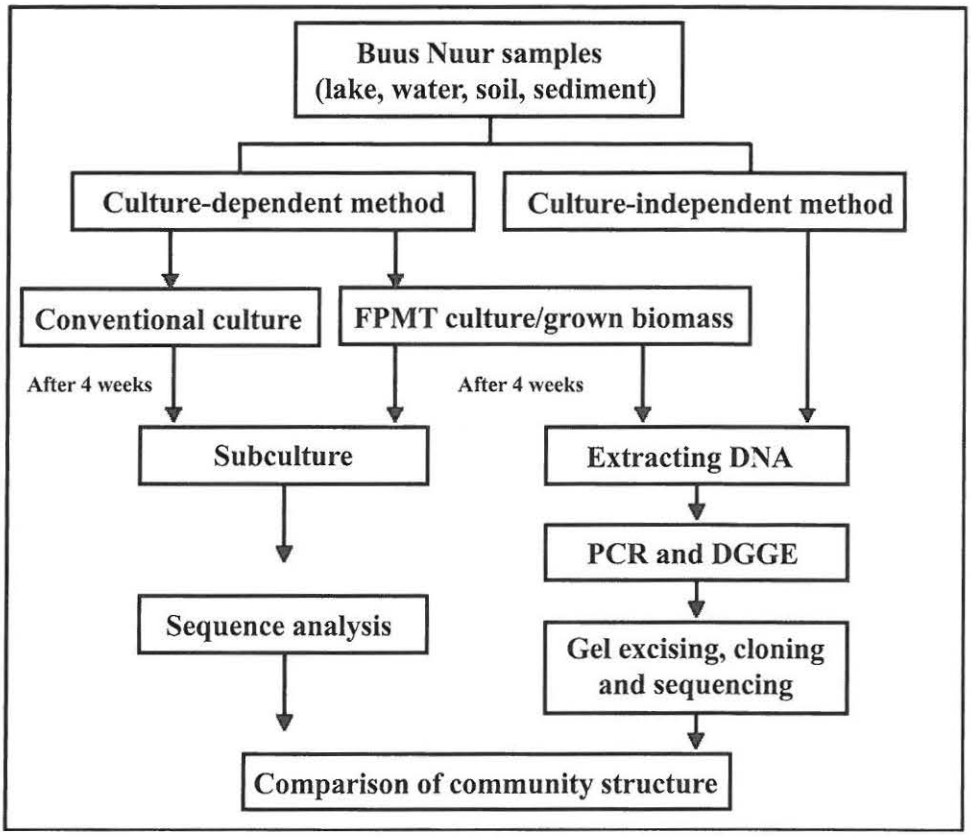

Fig. 2. Flowchart showing the experiment approach for bacterial community analyses with culture-dependent and molecular methods.

12 °C, FPMTs were carefully opened, and the agar with the grown microorganisms was removed from each well using a loop. The agar material was diluted in 3 ml of sterile water, homogenized by vortexing, and inoculated on R2A agar plates described above. For microbial purification and identification, 67 colonies were isolated.

Identification of isolates

Taxonomic identification was performed by sequencing 510 to 790 long fragments of the 16 S rRNA gene. The colony material was used directly as a template for PCR. The 16 S rRNA gene was amplified using the universal primers 27 F (5'-AGAGTTT GATCCTGGCTCAG-3') and 1492 R (5'-GGTTACCTTGT TACGACTT-3') with a Hot Start Taq system (QIAGEN, USA). The purified PCR products were sequenced commercially (Macrogen, Korea) by fluorescent dye terminator sequencing. The sequences were compared to databases in GenBank (http:// blast.ncbi.nih.gov/) by using the EzTaxon server 2.1 (www.eztaxon.org, Korea) to determine their closest relatives.

DGGE and sequencing

To compare bacterial diversity in the source environment and in FPMT-grown biomass, DNA was extracted from both the original samples and cultured biomass from the FPMTs. To obtain environmental DNA, three liters of lake water were filtered through a 0.2-μm pore-size cellulose nitrate membrane filter (diameter 47 mm; Whatman, Germany), with the follow up analyses performed as described by Massana et al. (1997). Twenty grams of composite soil and sediment samples were placed in sterile plastic bag and mixed vigorously, and 0.25-g subsamples were withdrawn for DNA extraction (Kowalchuk et al. 1998). In all cases, DNAs was extracted using MoBio Power Soil DNA extraction kit (MoBio Laboratories Inc., USA). PCR was done by the protocol of Edeborn & Sexstone (2007) and then DGGE was carried out using the method of Patil et al. (2001). The bands were excised and eluted for 24 hrs in sterilized distilled water at 4 °C. The eluted DNAs were transformed into *E. coli* DH5 α by the TOPO TA cloning protocol as suggested by the manufacturer. After confirmation of transformants, 3 transformed clones were randomly chosen, sequenced and 16 S rRNA gene sequence similarity were determined by using the MEGA-4.0 software (MEGA software, USA).

Results

Bacterial diversity obtained by culture-dependent methods

With conventional cultivation, we identified 37 isolates representing 18 species (Table 1). These species belong to 4 taxonomic groups, Actinobacteria (6 %), Cytophagia (17 %), Firmicutes (22 %) and Gammaproteobacteria (55 %). Several species appeared to be common as they were isolated from multiple sites.

Table 1. Phylogenetic affiliations of isolates with conventional culture on the basis of 16 S rRNA gene sequences.

Site	Taxonomic group	Closest species	% Similarity	No. of isolates
Water	Actinobacteria	*Cryobacterium psychrotolerans*	98	2
	Gammaproteobacteria	*Marinomonas arctica*	99	1
		Pseudoalteromonas citrea	98	1
		Pseudoalteromonas prydzensis	97	5
		Pseudoalteromonas tunicata	100	1
		Pseudomonas anguilliseptica	98	2
		Pseudomonas peli	98	1
		Rheinheimera perlucida	97	2
		Rheinheimera tangshanensis	98	1
Sediment	Cytophagia	*Algoriphagus alkaliphilus*	98	1
		Algoriphagus olei	98	1
		Rhodonellum psychrophilum	98	2
	Firmicutes	*Planococcus antarcticus*	99	1
		Planomicrobium psychrophilum	98	1
		Sporosarcina antarctica	100	1
	Gammaproteobacteria	*Halomonas andesensis*	100	1
		Rheinheimera perlucida	97–99	3
Soil	Actinobacteria	*Cryobacterium psychrotolerans*	98	1
	Cytophagia	*Rhodonellum psychrophilum*	98–99	2
	Firmicutes	*Carnobacterium pleistocenium*	100	2
		Planococcus antarcticus	99	1
		Planomicrobium psychrophilum	98	2
	Gammaproteobacteria	*Halomonas andesensis*	99	1
		Lysobacter concretionis	96	(1)

[a] The numbers in parentheses show the numbers of strains < 97 % 16 S rRNA similarity.

Table 2. Phylogenetic affiliations of isolates with FPMT culture on the basis of 16 S rRNA gene sequences.

Site	Taxonomic group	Closest species	% Similarity	No. of isolates[a]
Water	Actinobacteria	*Rhodococcus fascians*	100	1
		Salinibacterium xinjiangense	98	1
	Alphaproteobacteria	*Sphingomonas panni*	98–99	2
	Betaproteobacteria	*Hydrogenophaga palleronii*	98	1
		Massilia aurea	98	1
		Massilia consociata	97	1
	Cytophagia	*Hymenobacter rigui*	98	2
	Firmicutes	*Bacillus idriensis*	100	1
		Bacillus simplex	100	2
		Brevibacterium frigoritolerans	100	2
		Paenibacillus durus	96	(1)
		Planococcus rifietoensis	100	1
	Flavobacteria	*Flavobacterium haoranii*	96	(1)
	Gammaproteobacteria	*Pseudoalteromonas prydzensis*	98	1
		Pseudomonas cuatrocienegasensis	99	1
		Psychromonas arctica	91	(1)
Sediment	Actinobacteria	*Curtobacterium citreum*	99	1
		Flavobacterium oceanosedimentum	98	1
		Frigoribacterium faeni	98	1
		Microcella putealis	99	1
		Rhodococcus fascians	98–99	2
	Alphaproteobacteria	*Brevundimonas vesicularis*	100	1
		Novosphingobium soli	97	1
		Sphingomonas panni	99	1
	Cytophagia	*Algoriphagus alkaliphilus*	98	1
	Firmicutes	*Bacillus herbersteinensis*	99	1
		Bacillus niabensis	98	2
		Bacillus pseudofirmus	99	2
		Bacillus subterraneus	100	3
	Flavobacteria	*Flavobacterium haoranii*	95	(1)
	Gammaproteobacteria	*Alishewanella aestuarii*	96	(1)
		Ewingella americana	98	1
		Pseudomonas trivialis	100	1
		Pseudomonas xanthomarina	99	2
Soil	Actinobacteria	*Citricoccus nitrophenolicus*	99	1
		Jonesia quinghaiensis	100	3
		Zhihengliuella aestuarii	100	1
	Alphaproteobacteria	*Novosphingobium subterraneum*	100	1
		Rubellimicrobium mesophilum	98	1
		Sphingomonas hankookensis	98	1
	Betaproteobacteria	*Hydrogenophaga defluvii*	99–100	2
		Hydrogenophaga taeniospiralis	98	1
		Massilia brevitalea	98	1
	Cytophagia	*Algoriphagus boritolerans*	98	1
		Algoriphagus faecimaris	100	1
	Firmicutes	*Bacillus idriensis*	100	1
		Bacillus simplex	100	1
		Carnobacterium pleistocenium	100	1
		Planococcus antarcticus	99	1
	Flavobacteria	*Flavobacterium haoranii*	96	(1)
		Gillisia limnaea	98	2
	Gammaproteobacteria	*Pseudomonas cuatrocienegasensis*	99	1
		Reinekea aestuarii	95	(1)

[a] The numbers in parentheses show the numbers of strains < 97 % 16 S rRNA similarity.

These included *Cryobacterium psychrotolerans* isolated from lake water and soil, *Rheinheimera perlucida* isolated from lake water and sediments, and *Halomonas andesensis*, *Planococcus antarcticus*, *Planomicrobium psychrophilum* and *Rhodonellum psychrophilum* isolated from both soil and sediments.

With FPMT-based cultivation, we obtained 67 isolates representing 46 species from 7 taxonomic groups, Actinobacteria (20 %), Cytophagia (9 %), Firmicutes (24 %), Gammaproteobacteria (17 %) Alphaproteobacteria (13 %), Betaproteobacteria (13 %) and Flavobateria (4 %) (Table 2). No growth was detected in the negative control. *Flavobacterium haoranii* was isolated from all samples, *Bacillus idriensis*, *Bacillus simplex* and *Pseudomonas cuatrocienegasensis* were isolated from both lake water and soil, and *Rhodococcus fascians* and *Sphingomonas panni* were isolated from lake water and sediment. Multiple isolation of the same species from different samples was more common when we used the FPMT method compared to conventional methods. The rate of isolation of new species was different between the conventional and FPMT-based cultivation. Only 1 novel species, defined as a strain with < 97 % 16 S rRNA similarity to the closest known relative, was isolated by conventional cultivation, whereas seven novel species were obtained using FPMTs.

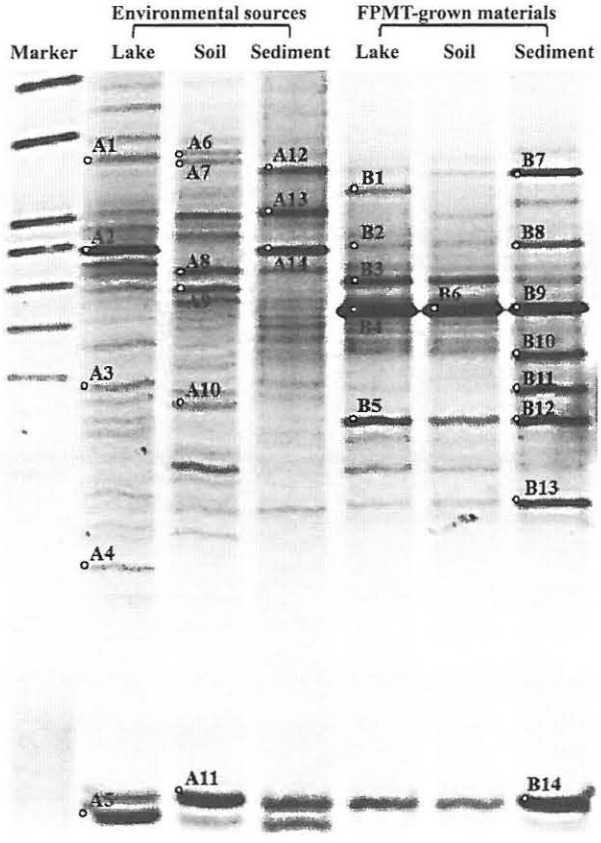

Fig. 3. PCR-DGGE profiles from environmental sources and FPMT-grown biomass.

PCR-DGGE-based bacterial community analyses and sequencing

The number of DGGE bands from the community DNA of environmental samples was higher than the number from the FPMT-grown biomass. The band profiles were also different from each other (Fig. 3). The environmental and FMPT DGGE profiles had 9 taxonomic groups in common (Alpha-, Beta-, Gamma- and Epsilonproteobacteria, Actinobacteria, Cyanobacteria, Cytophagia, Firmicutes and Flavobacteria). The most intense bands in environmental samples were band A2 (actinobacterium) and A5 (*Flavobacterium* sp.) in lake water, band A8 (Rhodobacteraceae) and A11 (actinobacterium) in soils, and band A14 (Unclassified group) in sediments (Table 3). Major bands that showed the highest intensity in the FPMT-grown biomass were bands B4, B6, and B9. All three were identified as cyanobacterium, and were notably absent from environmental sources. Other intense bands included band B7 (*Planococcus* sp.), B8 (Idiomarinaceae & *Sulfurospirillum* sp.), B10 (*Brevundimonas* sp. & Alteromonanadales), B11 (*Pseudomonas* sp. and Firmicutes), B12 (Firmicutes) and B14 (Alteromonanadales).

Several major bands were observed in the profiles of both environmental and FMPT DGGE. These included bands A12 and A13 which were identified as Desulfobulbacea and Rhodobacteraceae; B3 and B5 as *Trichococcus* sp. and *Aeromonas sobria*, respectively. Additionally, band A2 (actinobacterium) was observed in both the lake water and soil material and was positioned similarly to bands observed in the FPMT-grown biomass: A14 (Unclassified group), B2 (*Bacillus* sp., *Flavobacterium* sp.) and B8 (Idiomarinaceae). However, the intensity of the bands differed significantly between environmental samples and FPMT agar.

Some bands were unique to a single source of microorganisms. Band A1 (Cryomorphaceae, Firmicutes and *Flexibacter* sp.), A3 (*Tetrasphaera* sp.), A4 (actinobacterium and *Phenylobacterium* sp.), A6 (*Fluoribacter dumoffii* and *Legionella steigerwaltii*), A7 (actinobacterium), A9 (*Magnetococcus* sp. and *Ochrobactrum* sp.), A10 (actinobacterium, Coriobacteriaceae and Firmicutes), and A13 (Rhodobacteraceae and Ectothiorhodospiraceae) were observed only

Table 3. Sequence similarities to closest relatives and phylogenic affiliations of DNA recovered from DGGE gel.

Site	Band No.	Taxonomic group	Most closely related organism	Similarity	Acession No.
Environmental sources	A1	Flavobacteria	Uncultured Cryomorphaceae bacterium	99	JQ937370.1
		Firmicutes	Uncultured Firmicutes bacterium	100	HM215047.1
		Cytophagia	Uncultured *Flexibacter* sp.	100	FN860105.1
	A2	Actinobacteria	Uncultured actinobacterium	100	DQ662876.1
	A3	Actinobacteria	Uncultured *Tetrasphaera* sp.	100	JN867007.1
	A4	Actinobacteria	Uncultured actinobacterium	100	JN656922.1
		Unclassified	Uncultured Bacteroidetes bacterium	100	HM368331.1
		Alphaproteobacteria	*Phenylobacterium* sp.	96	AM411917.1
	A5	Flavobacteria	*Flavobacterium* sp.	100	DQ664234.1
			Uncultured *Flavobacterium* sp.	93	GU230410
	A6	Gammaproteobacteria	*Fluoribacter dumoffii* strain	100	HQ717439.1
			Legionella steigerwaltii strain	100	JF720405.1
	A7	Actinobacteria	Uncultured actinobacterium	97	HQ654258.1
		Unclassified	Uncultured soil bacterium	98	EF127899.1
	A8	Alphaproteobacteria	Rhodobacteraceae bacterium	99	AB550515.1
		Gammaproteobacteria	Uncultured *Legionella* sp.	97	EU700353.1
	A9	Alphaproteobacteria	Uncultured *Magnetococcus* sp.	93	GQ468519.1
			Uncultured *Ochrobactrum* sp.	100	HQ658042.1
		Unclassified	Uncultured bacterium	95	JN630828.1
	A10	Actinobacteria	Uncultured actinobacterium	98	FN669646.1
			Uncultured Coriobacteriaceae bacterium	98	JQ087160.1
		Firmicutes	Uncultured Firmicutes bacterium	99	EU281973.1
	A11	Deltaproteobacteria	*Desulfofustis glycolicus* strain	90	EF442939.1
		Actinobacteria	actinobacterium	100	HQ663351.1
	A12	Deltaproteobacteria	Desulfobulbaceae bacterium	100	HE600889.1
		Unclassified	Uncultured candidate division bacterium	99	FJ479934.1
	A13	Alphaproteobacteria	Rhodobacteraceae bacterium	100	HE818188.1
		Gammaproteobacteria	Uncultured Ectothiorhodospiraceae bacterium	96	AB286090.1
		Unclassified	Uncultured soil bacterium	97	EF127899.1
	A14	Unclassified	Uncultured Bacteroidetes bacterium	99	HM368331.1
FPMT-grown biomass	B1	Cyanobacteria	Uncultured cyanobacterium	99	FR648036.1
	B2	Firmicutes	*Bacillus* sp.	100	FR774584.1
		Flavobacteria	*Flavobacterium glaciei* strain	99	JQ692100.1
	B3	Firmicutes	Uncultured *Trichococcus* sp.	99	GU356323
	B4	Cyanobacteria	Uncultured cyanobacterium	100	FR648036.1
	B5	Cyanobacteria	Uncultured cyanobacterium	99	FR648036.1
		Gammaproteobacteria	*Aeromonas sobria* strain	100	JQ795757.1
	B6	Cyanobacteria	Uncultured cyanobacterium	100	FR648036.1
	B7	Firmicutes	*Planococcus* sp.	100	JQ907346
	B8	Gammaproteobacteria	Idiomarinaceae bacterium	100	JN652131.1
		Cyanobacteria	Uncultured cyanobacterium	100	FR648036.1
		Epsilonproteobacteria	*Sulfurospirillum cavolei* strain	100	NR041392
	B9	Cyanobacteria	Uncultured cyanobacterium	100	FR648036.1
	B10	Alphaproteobacteria	Uncultured *Brevundimonas* sp.	97	FR657545.1
		Gammaproteobacteria	Alteromonadales bacterium	99	EF554892.1
	B11	Firmicutes	Uncultured Firmicutes bacterium	99	JQ478525.1
		Gammaproteobacteria	*Pseudomonas* sp.	100	JQ229609.1
	B12	Firmicutes	Uncultured Firmicutes bacterium	99	JQ478525.1
	B13	Firmicutes	*Planococcus* sp.	100	JQ907346.1
	B14	Bacteroidetes	Rikenellaceae bacterium	97	AB362265.1
		Gammaproteobacteria	Alteromonadales bacterium	99	EF554892.1

in environmental samples. In contrast, band B1 (Cyanobacteria group) and B13 (*Planococcus* sp.) were detected only from the FPMT-grown biomass.

Discussion

FPMT-based cultivation allowed us to obtain a culture collection that was larger, richer, and more novel than that obtained by standard approaches. We ascribe this to the principle behind the use of *in situ* incubation devices in general, and FPMT in particular. First, during cultivation, chemical compounds that may be critical for microbial growth – but absent from standard media – freely exchanged between the environment and FPMTs. Second, allelopathy between colonies appears frequently in traditional laboratory cultivation but was seemingly minimized in FPMTs. In general, fast growing microbial strains repress the growth of slow growers (Watve et al. 2000). However in FMPT wells, growing bacteria are somewhat removed from the environment possibly reducing the effect of antimicrobials.

One interesting result is that FPMT approach led to isolation of Alphaproteobacteria, Betaproteobacteria and Flavobacteria whereas standard cultivation did not. Note that Betaproteobacteria were previously isolated in larger numbers using diffusion chambers that, analogously to FPMTs, mimicked natural conditions (Bollmann et al. 2007). In contrast, Gammaproteobacteria dominated the culture using traditional methods (54 % of isolates) but were a minor component of that obtained using FPMTs (15 %). Interestingly, Gammaproteobacteria including *Pseudomonas*, are thought to be a factor preventing cultivation of more diverse sets of bacteria in the laboratory because these fast-growing copiotrophic bacteria often have a negative impact on the slower growing oligotrophs (Hurst et al. 2007).

Furthermore, several microbial groups, such as Actinobacteria and Flavobacteria, were prominent in nature as indicated by the DGGE analyses and were also easily isolated via the FPMT approach – but not by standard cultivation. These are typical examples showing the limitations of conventional cultivation for understanding microbial diversity, and advantages of the new method described here. This indicates that FPMT culture may help close the gap between the large microbial diversity in nature and the much smaller diversity of currently existing culture collections.

On the other hand, a significant number of strains were detected in the environment using molecular approaches but were not cultivated by either of the cultivation approaches we used. It is possible that these microorganisms require specific growth conditions not present in our study. Isolating these strains in the future can be guided by what is known about biology of their close relatives. For example, the Cryomorphaceae group was recently proposed to have a marine and polar origin (Bowman et al. 2003, Lee et al. 2010). *Tetrasphaera* sp. is a known phosphate accumulating bacteria (Blackall et al. 2000). *Phenylobacterium* sp. is likely able to degrade recalcitrant material and was isolated using mineral salt media (Lingens et al. 1985). Properties of other species that are close relatives of those missed by our cultivation efforts may be informative as well. *Magnetococcus* sp. is known to mineralize magnetic iron minerals (Bazylinski & Frankel 2000). The Coriobacteriaceae group, while aero-tolerant, apparently grows only under anoxic conditions (Thomas et al. 2010). Representatives of Ectothiorhodospiraceae were co-isolated with a moderately halophilic and obligately methylotrophic bacterium (Tourvova et al. 2007). If environmental variables such as salinity, oxygen, and nutrients are altered to better match the requirements of the above species, it may be possible to isolate species absent from the culture collections we obtained here.

An interesting case is cyanobacterium species, grown via the FPMT approach but undetected in the environment by DGGE. It is possible that, during FPMT-based cultivation, short exposure to light was sufficient for these cyanobacteria to proliferate. Alternatively, they could have grown heterotrophically. Indeed, heterotrophic cyanobacterium species can be grown in prolonged darkness, interrupted by only 5 to 15 minutes of exposure to light per day (Anderson & McIntosh 1991). Our experiences in growing this unexpected bacterium suggest that if cultivation conditions such as light, nutrient medium, air supply vs anaerobiosis, pH, salinity, etc are adjusted differently in different trials, the probability of isolating novel species will increase.

The information available on microbial diversity in soda and salt lakes is limited. Because of well known limitations of standard cultivation approaches, only a minor component of soda lake communities has been cultivated. In a Chinese saline lake, a total of 18 bacterial isolates were obtained from lake water and sediment (Jiang et al. 2006). Among them, 14 isolates were related to genus *Halomonas* of the Gammaproteobacteria, two isolates were classified as Firmicutes, and another was classified as Actinobacteria. In East African soda lakes, most bacterial isolates belonged

to Gammaproteobacteria, including representatives of *Halomonas*, *Pseudomonas* and also *Bacillus* from Firmicutes group (Duckworth et al. 1996). Interestingly, when we applied conventional cultivation techniques, the results mirrored the published reports (Table 1). The interpretation is simple: for example, nutrient rich media are known to favor Gammaproteobacteria (Wagner et al. 1994), which can prohibit or mask growth of other bacteria, resulting in a significant under sampling of the natural microbial diversity. In this study, using the FPMT *in situ* cultivation method and oligotrophic medium, signifying a substantial departure from conventional methods, lead to a more diverse culture collection.

To overcome the limitations of culture-dependent methods, molecular techniques were adopted in several published studies. In an Indian soda lake, RFLP analysis and subsequent sequencing showed that 34 % of the clones were Firmicutes, followed in dominance by Proteobacteria and Actinobacteria (Wani et al. 2006). In three large alkaline and hypersaline lakes in Egypt, a high proportion of Firmicutes related clones in water and Alphaproteobacteria in sediment were observed (Mesbah et al. 2007). In this study, by DGGE analysis, Actinobacteria, Alphaproteobacteria and Flavobacteria were identified as dominant. The differences in bacterial diversity among saline and salt lakes uncovered by molecular techniques are likely due to several reasons. Firstly, geographic isolation and differences in salt composition and alkalinity might account for differences in microbial inhabitants. Secondly, sampling and methods of analyses, often specific to particular studies, might have specific biases leading to somewhat different pictures of detectable microbial diversity.

To summarize, we used one conventional and one novel (FPMT) cultivation approaches and cultivated representatives of 4 and 7 taxonomic groups, respectively. These corresponded to 27 and 47 % of the 15 taxonomic groups detected in the environment by DGGE (Table 4). This means that FPMT-based cultivation may help overcome some of the limitations of standard techniques. In addition, considering that most of the taxonomic groups unique to the list obtained by molecular methods comprise either photosynthetic or anaerobic bacteria, we expect that these groups could also be cultivated if the FPMTs incubation conditions are adjusted appropriately. In spite of this general enthusiasm we note that, as used here, the FPMT approach still appears to have biases in what species are selected for and against, and leads to cultivation of only a fraction of the entire microbial diversity. Further modifications of cultivation techniques are clearly needed to culture microbial populations detected so far by their molecular signatures. We suggest that the FPMT method we tested will be part of the evolution in methodological approaches. The newly proposed method is simple, straightforward, economical, and easy to use in both field and environmental settings.

Table 4. Bacterial taxonomic groups identified by different methods.

Culture-dependent		Culture-independent (DGGE)	
Conventional culture	**FPMT culture**	**From FPMT-grown biomass**	**From environment sources**
Actinobacteria	Actinobacteria	Actinobacteria	Actinobacteria
Cytophagia	Cytophagia	Cytophagia	Cytophagia
Firmicutes	Firmicutes	Firmicutes	Firmicutes
Gammaproteobacteria	Gammaproteobacteria	Gammaproteobacteria	Gammaproteobacteria
	Alphaproteobacteria	Alphaproteobacteria	Alphaproteobacteria
	Betaproteobacteria	Betaproteobacteria	Betaproteobacteria
	Flavobacteria	Flavobacteria	Flavobacteria
			Chlorobia
			Chloroflexi
		Cyanobacteria	Cyanobacteria
			Deltaproteobacteria
		Epsilonproteobacteria	Epsilonproteobacteria
			Sphingobacteria
			Spirochaetes
		Nitrospirae	Verrucomicrobia
4	7	10	15

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