Available online at www.sciencedirect.com
SCIENCE DIRECT®
Journal of Microbiological Methods 57 (2004) 379–390
Journal of Microbiological Methods
www.elsevier.com/locate/jmicmeth

The filtration–acclimatization method for isolation of an important fraction of the not readily cultivable bacteria

Martin W. Hahn[a,*], Peter Stadler[a], Qinglong L. Wu[a,b], Matthias Pöckl[a]

[a] *Institute for Limnology, Austrian Academy of Sciences, Mondseestrasse 9, Mondsee A-5310, Austria*
[b] *Nanjing Institute of Geography and Limnology, Chinese Academy of Sciences, 73 East Beijing Road, Nanjing 210008, PR China*

Received 3 November 2003; received in revised form 8 February 2004; accepted 10 February 2004

Available online 14 March 2004

Abstract

We developed a novel method, the filtration–acclimatization method (FAM), which enables the isolation and cultivation of an important fraction of the bacterial diversity, which is not cultivable by standard methods. The method consists of a filtration step, which removes most of the readily cultivable bacteria able to overgrow slowly growing bacteria, and an acclimatization procedure that provides a slow transition from the low environmental substrate concentrations to the high concentration of standard microbial media. So far, we isolated in total 65 strains from surface freshwater habitats by utilizing FAM. The isolates are affiliated with *Actinobacteria*, *Alpha-*, *Betaproteobacteria*, *Bacteroidetes*, and *Spirochaeta*. All isolates are pure cultures and form visible colonies on agar plates with high substrate concentrations. For further analysis, strains sharing more than a 97% 16S rRNA gene sequence similarity were grouped into one taxon. Based on sequence similarities, 88% of the obtained taxa can be considered to be undescribed species (< 97% similarity to closest species). The highest similarity value of the taxa to the respective closest related species ranged from 87.7% to 99.8%, and was on average 94.5%. For comparison we isolated, by direct plating of water samples on a rich agar medium, a similar number of taxa. Amongst these taxa the percentage of taxa, which can be considered to be undescribed species, was only half of the percentage found for the taxa isolated by FAM. More importantly, it was amongst the taxa obtained by the standard method no taxon that was closer related to an uncultured bacterium than to an isolate, while 56% of the taxa isolated by FAM were closely related to uncultured bacteria.

Keywords: Filtration–acclimatization method; Isolation of bacteria; 0.2 μm filtration; Freshwater bacterioplankton; Ultramicrobacteria

1. Introduction

Only a very small fraction of the existing microbial diversity has been cultivated till date (Hugenholtz et al., 1998). The majority of *Bacteria* and *Archaea* is resistant to cultivation by standard microbiological methods (Hugenholtz et al., 1998), and for most of those discovered members of this huge uncultured fraction, no more than the respective 16S rRNA gene sequences are known.

We developed a method which allows the isolation of important members of the thus far uncultivated bacterial diversity. The method consists of a filtration step and an acclimatization procedure. The filtration

* Corresponding author. Tel.: +43-6232-3125-29; fax: +43-6232-3578.
E-mail address: martin.hahn@oeaw.ac.at (M.W. Hahn).

0167-7012/$ - see front matter © 2004 Elsevier B.V. All rights reserved.
doi:10.1016/j.mimet.2004.02.004

through 0.2 μm is used for exclusion of opportunistic bacteria, which occur in situ with low numbers but rapidly overgrow in cultures the initially abundant bacteria. In contrast to standard isolation methods, our approach avoids the exposure of microorganisms to sudden increases in concentration of dissolved organic matter. Such quick and strong increases in substrate concentrations may lead to substrate shocks, which decrease the viability of bacteria adapted to low substrate concentrations. In our approach, bacteria were slowly acclimatized to higher substrate concentrations and finally cultured on substrate-rich agar plates. In contrast to other studies (Bussmann et al., 2001; Rappé et al., 2002; Connon and Giovannoni, 2002; Bruns et al., 2003), we neither avoided the use of high substrate concentration media, nor culturing of the isolates on solid media.

Our approach is not a quantitative approach aiming to increase the percentage of environmental bacteria growing in liquid or on solid media (i.e. not aiming to increase the culturability of bacteria in a particular environmental sample). The aim of our approach is simply to increase the diversity of cultured heterotrophic bacteria.

In this paper, we present the novel method in detail, and provide an overview of the taxa isolated thus far by FAM from diverse freshwater habitats. Furthermore, we compare the results of the performed FAM isolation experiments with an experiment performed by a standard isolation method (i.e. plating on agar plates).

2. Materials and methods

2.1. Isolation and cultivation of bacteria

2.1.1. Sampling of freshwater habitats

Water samples from 28 freshwater habitats including lakes, ponds, and running waters located in 11 countries were collected (Table 1). Some of the samples were filtered through 0.2 μm filters (see below) immediately after sampling, in other cases untreated samples were transported, and filtration was performed in the laboratory.

2.1.2. Media used for isolation of bacteria

For all FAM and standard plating isolation experiments NSY (nutrient broth, soytone, yeast extract) medium (Hahn et al., 2003) and inorganic basal medium (IBM) (Hahn et al., 2003) were exclusively used (Table 2). For FAM various mixtures of liquid NSY (3 g l^{-1}) and IBM media were used (see Fig. 1).

Table 1
Location and characteristics of sampled habitats

Sampled habitat(s)	Number of habitats	Number of sampling sites	Country	Abbreviation	Type of habitats	Climate
Lakes and ponds in the Salzkammergut lake district	9[a]	12	Austria	LSA	sub-montan lakes and ponds in pre-alpine region, oligo- to oligo-mesotrophic	temperate
Le Canal de Roanne a Digoin	1	1	France	RRD	small channel	temperate
Lake Victoria	1	3	Uganda, Tanzania	LVI	large mesotrophic lake	tropical
Lake Tanganyika	1	1	Zambia	LTA	large deep oligotrophic lake	tropical
Ponds, Dar es Salam	2	2	Tanzania	PDS	eutrophic ponds	tropical
Tana River near Sagana	1	1	Kenya	RTA	large river	tropical
Ponds, Uganda	2	2	Uganda	PUG	small eutrophic ponds	tropical
Lagos Managua	1	1	Nicaragua	LMA	eutrophic lake	tropical
Lake Taihu	1	3	P. R. of China	LTH	large shallow hypertrophic lake	subtropical
Yangtze River near Nanjing	1	1	P. R. of China	RYA	large polluted river	subtropical
Ponds, Suzhou	3	3	P. R. of China	PSU	small ponds in parks	subtropical
Pond, Beijing	1	1	P. R. of China	PBE	small pond in park	temperate
Pond, Sydney	1	1	Australia	PAU	small pond in park	temperate
Habitats in New Zealand	3	3	New Zealand	LNZ	lake, pond and river	temperate

Some of the investigated habitats were sampled repeatedly at distantly located sampling sites.
Some habitats were sampled repeatedly at the same site during different seasons.
[a] Some habitats were only sampled for the experiment with the standard isolation method.

Table 2
Media used for FAM and standard plating

Inorganic basal medium (IBM)	
Chemical	Concentration in medium [mg l^{-1}]
$MgSO_4 \cdot 7H_2O$	75.0
$Ca(NO_3)_2 \cdot 4H_2O$	43.0
$NaHCO_3$	16.0
KCl	5.0
$K_2HPO_4 \cdot 3H_2O$	3.7
Na_2EDTA	4.4
$FeCl_3 \cdot 6H_2O$	3.2
H_3BO_3	1.0
$MnCl_2 \cdot 4H_2O$	0.2
$NiCl_2 \cdot 6H_2O$	0.1
$ZnSO_4 \cdot 7H_2O$	0.02
$CuSO_4 \cdot 6H_2O$	0.02
$CoCl_2 \cdot 6H_2O$	0.01
$Na_2MoO_4 \cdot 2H_2O$	0.006
	pH 7.2
Liquid NSY medium (3 g l^{-1})	
add to the inorganic basal medium	Nutrient broth, 1 g l^{-1} Soytone peptone, 1 g l^{-1} Yeast extract, 1 g l^{-1} pH 7.2
Solid NSY medium (3 g l^{-1})	
add 1.5% agar	

For the final plating step of FAM, as well as for the standard method plating, NSY agar (1.5% w/v) plates with a substrate concentration of 3 g l^{-1} were used (Table 2).

2.1.3. Isolation of bacteria by the filtration–acclimatization method (FAM)

FAM consists of a filtration step and a subsequent acclimatization process. For the filtration step, either a sterile 0.2 μm syringe membrane filter (Minisart, Sartorius Göttingen, Germany), or a sterile 0.2 μm pore-size filter unit (Nalgene, Nalge Nunc International) were used. A few initial isolation experiments were performed by the incubation of 10 to 30 ml of the 0.2 μm filtrated water samples in 100 ml Erlenmeyer flasks. In the majority of performed experiments inoculum volumes of ≤ 1.0 ml of the 0.2 μm filtrates instead of the initially used ≥ 10 ml inoculum volume were used. In many experiments, we setup several (up to 120) parallel cultures which received different inoculum volumes (in the range of 0.01 to 0.1 ml) from the same 0.2 μm filtered water sample. For these experiments, sterile 24-well cell culture plates (Iwaki) were used instead of the Erlenmeyer flasks.

Bacteria contained in the inoculi were stepwise acclimatized to higher substrate concentrations by the stepwise addition of increasing doses of NSY medium (Hahn et al., 2003). During the acclimatization process, the cell culture plates were usually incubated without agitation at room temperature (22–26 °C) in the dark. In some of the first experiments, the cultures were incubated at 15 °C. Bacteria enriched in the cultures were isolated by plating on 3 g l^{-1} NSY agar (1.5% (w/v) agar) (Hahn et al., 2003). Isolates were purified by repeated culturing of single colonies in liquid NSY medium and plating of dilutions on agar plates. The purity of cultures was checked by microscopy. Pure cultures were subjected to deep-freezing preservation (10% (w/v) glycerol, −70 °C).

The entire culturing process was performed under aseptic conditions, and filter tips were used for all of the pipetting work.

Fig. 1 presents an optimized FAM protocol routinely used in our laboratory.

2.1.4. Direct plating of 0.2 μm-filtrated water samples on NSY agar plates

To test if bacteria contained in 0.2 μm-filtrated water samples are readily culturable, samples of 100 μl of filtrates were directly (i.e. without an acclimatization procedure) plated on 3 g l^{-1} NSY agar plates. This direct plating of 0.2 μm filtrates was performed in parallel to four FAM experiments. For both FAM and direct plating, the same volume was used for the inoculation of one cell culture plate well or one agar plate. In each of the four experiments, 24 wells and 24 agar plates were inoculated and incubated under the same conditions.

2.1.5. Isolation of bacteria by a standard isolation method (plating on NSY agar plates)

Samples of 100 μl of unfiltered water from eight lakes and ponds located in the Salzkammergut lake district (Table 1) were directly plated on 3 g l^{-1} NSY agar plates. After 10 days of incubation, 30 colonies were selected from the plates and streaked out separately on NSY agar plates. Subcultivation was repeated until pure cultures were obtained.

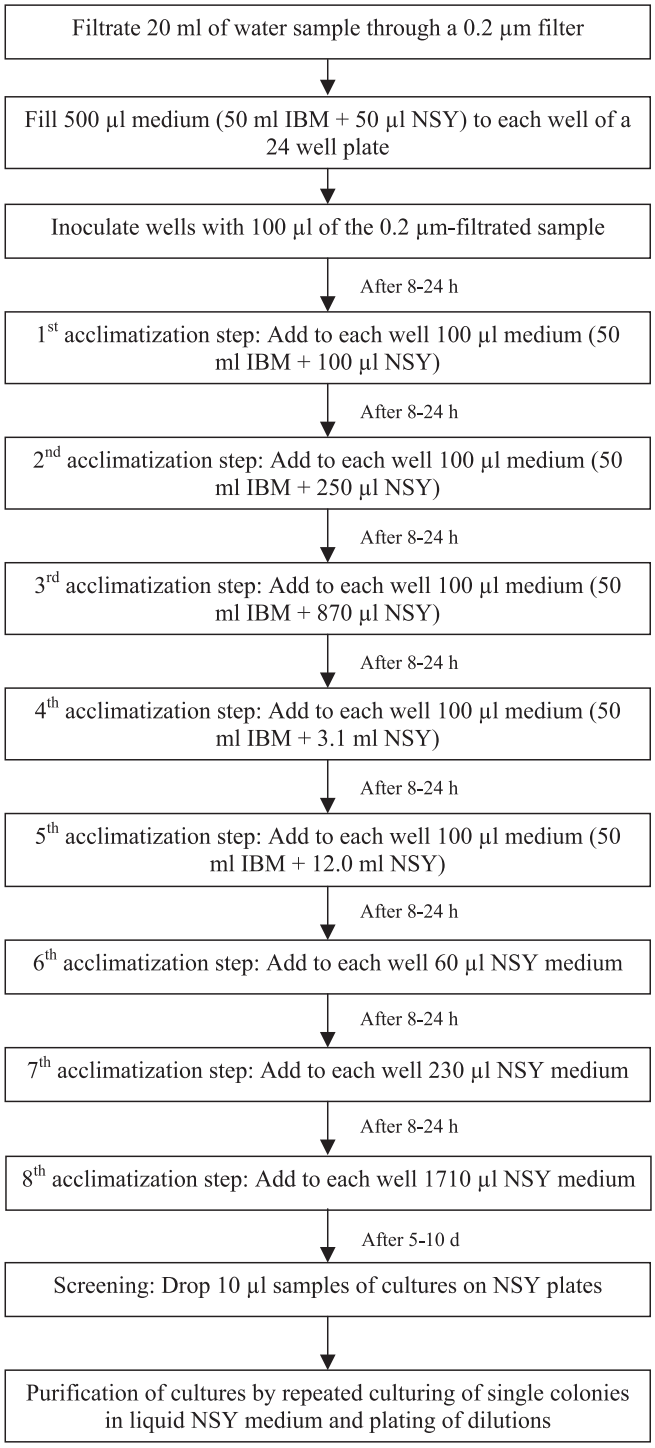

Fig. 1. Standard FAM protocol for freshwater samples. NSY medium with a concentration of 3 g l^{-1} is used. The composition of NSY and IBM media is shown in Table 2.

2.2. Epifluorescence microscopy

Water samples, enrichment cultures and cultures of isolates were investigated by epifluorescence microscopy. Samples were stained with 4′,6′-diamino-2-phenylindole (DAPI, Sigma) and filtered onto black 0.2 μm pore-size Nuclepore filters (Millipore) and viewed under UV excitation at a magnification of 1250 × (Zeiss Axioplan). Bacterial cell dimensions were measured by the image analysis system Lucia 4.51 (Laboratory Imaging, Prague, Czech Republic).

2.3. Sequencing of 16S rRNA genes and phylogenetic analysis

Amplification of the 16S rRNA genes of the isolates were performed as described previously (Hahn et al., 2003). PCR products were sequenced by MWG-Biotech (Ebersberg, Germany). In the case of almost all FAM isolates, the almost entire gene was sequenced, while in the case of the isolates obtained by the standard isolation method only ca. one third of the gene was sequenced. The program BLAST (http://www.ncbi.nlm.nih.gov/BLAST/ (Altschul et al., 1997)) was used to find all publicly available sequences closely related to the sequences obtained from the isolates. Complete and partial sequences of the isolates were submitted for comparison. Sequences of the isolates, as well as downloaded reference sequences were aligned by using the ARB software package (http://www.arb-home.de (Strunk et al., 1998)). Reconstruction and analysis of phylogenetic trees was mainly performed by ARB. Evolutionary distances were corrected for multiple substitutions by using the Jukes and Cantor algorithm. Filters were used to exclude sequence positions showing a variability of >50%. For construction of bootstrapped (1000 replicates) neighbor-joining trees the aligned sequences were exported (using the filters for exclusion of highly variable positions) to the software application Mega Version 2.1 (http://www.megasoftware.net (Kumar et al., 2001)). Sequence similarities were calculated by the program ARB without using the above mentioned filters.

2.4. Grouping into taxa

Isolates sharing sequence similarities of >97% and appearing in the phylogenetic analysis in the same cluster were grouped into one taxon. A taxon sharing >97% similarity and clustering with a validly described reference species was preliminarily considered to belong to the respective reference species. A taxon with similarities in the range of 94–97% appearing in the phylogenetic analysis in clusters containing a validly described species was preliminarily considered to belong to the respective genus. In the case of the isolates affiliated with the *Polynucleobacter necessarius* cluster (Zwart et al., 2002; Hahn, 2003), the above outlined rules were not applied due to the fundamental differences in lifestyle of the free-living isolates and the obligate endosymbiont *P. necessarius*.

2.5. Nucleotide sequence accession numbers

The GenBank accession numbers for sequences of strains isolated by FAM and standard plating can be found in Tables 3 and 4, respectively.

3. Results

3.1. Isolation of bacterial strains by FAM

We investigated more than 40 surface water samples from 23 freshwater habitats. In filtrates immediately analyzed after the filtration process only small C-shaped bacteria were detected. Initially we filtrated 10–30 ml of the sample and incubated the whole filtrate into flasks. Incubation of these samples at 15 °C throughout periods of a few days regularly resulted in the appearance of initially undetectable filamentous bacteria. These bacteria usually overgrew the C-shaped bacteria, even when no substrates were added. We isolated and characterized several strains of these filamentous bacteria. Data on morphology and cell sizes of some of these isolates were presented elsewhere (Wu et al., 2004). Most of the isolates demonstrated an unusual growth on agar plates. They initially formed small colonies on the agar surface and then penetrated into and spread further inside the agar. None of these filamentous strains showed pigmentation. Analysis of the 16S rRNA gene sequences of eight isolates revealed that seven are closely related (>99% sequence similarity) to each other and to *Hylemonella gracilis* (previously classified as *Aquaspirillum gracile*, (Spring et al., 2004)). The seven

Table 3
Taxa (>97% sequence similarity) which have been isolated by FAM from 0.2 μm filtered freshwater samples and their closest known relatives

Taxon	Num. of isolates	Acc. Number	Morphology	Origin	Group	Closest relative	Acc. number	Similarity [%]
MWH-NPG1	1	AJ565418	c	PDS (natural pond in Dar es Salam)	*Actinobacteria*	Isolate IFO15616	AB012590	95.6
						Leucobacter komagatae	AB007419	95.9
MWH-VicDar (Luna-1)	2	AJ565415, AJ565417	c	LVI (Muanza, Tansania), PDS (nat. pond)	*Actinobacteria*	*Clavibacter michiganensis*	U09763	92.5
MWH-Dar4 (Luna-1)	1	AJ565416	c	PDS (pond at university campus)	*Actinobacteria*	*Clavibacter michiganensis*	U09763	92.8
MWH-Ta3 (Luna-1)	1	AJ507468	c	LTH (Meiling Bay)	*Actinobacteria*	*Clavibacter michiganensis*	U09763	93.4
Luna-2 cluster isolates	19	AJ565412, AJ565413, AJ565414, AJ565435, AJ565436[a]	c	LSA, LTH, LMA, RTA, PUG, PSU, PAU	*Actinobacteria*	*Clavibacter michiganensis*	U09763	95–96
Nocardioides sp. MWH-CaK6	1	AJ565419	f	RRD (near Avrilly)	*Actinobacteria*	*Nocardioides* sp. SAFR-045	AY167844	99.1
						Nocardioides luteus	X53212	95.7
MWH-CaK2	1	AJ565420	c	RRD (near Avrilly)	*Alphaproteobacteria*	Uncultured Soil Clone 845-2	AF423296	93.7
						Sphingomonas echinoides	AJ012461	92.9
MWH-UniPo	2	AJ565421, AJ565422	c	PDS (pond at university campus)	*Betaproteobacteria*	Uncultured Clone UP9	AY080915	92.5
						Herbaspirillum seropedicae	Y10146	92.2
Hylemonella gracilis	7	AJ565423–AJ565429 AJ565429	f	LSA, LTH, LTA	*Betaproteobacteria*	*Hylemonella gracilis*	AF078753	>99
Hylemonella sp. WQH1	1	AJ565430	f	LSA (Lake Hallstadt)	*Betaproteobacteria*	*Hylemonella gracilis*	AF078753	96.3
P. necessarius subcluster C[b]	14	see reference[c]	c	LSA, PSU, RYA, LNZ	*Betaproteobacteria*	*Polynucleobacter necessarius*[d]	X93019	~ 99
						Ralstonia basilensis	AF312022	~ 93
P. necessarius subcluster D[b]	19	see reference[c]	c	LSA, RRD, PBE, RYA, LVI, LTH, LNZ	*Betaproteobacteria*	*Polynucleobacter necessarius*[d]	X93019	~ 96
						Ralstonia basilensis	AF312022	~ 93
MWH-CFBk5	1	AJ565431	f	LSA (Lake Mondsee)	*Bacteroidetes*	Uncultured Clone KD6-1	AY218749	88.4
						Sporocytophaga myxoco.	AJ310654	87.7
						Cytophaga hutchinsonii	M58768	86.9
Spirochaeta aurantia WQM4	1	AJ565432	f	LSA (Lake Mondsee)	*Spirochaeta*	*Spirochaeta aurantia*	M57740	99.5
Spirochaeta sp. MWH-HuW8	1	AJ565433	f	PSU (Pond Huqiu)	*Spirochaeta*	*Spirochaeta aurantia*	M57740	96.6
Spirochaeta sp. MWH-HuW24	1	AJ565434	f	PSU (Pond Huqiu)	*Spirochaeta*	*Spirochaeta aurantia*	M57740	95.8

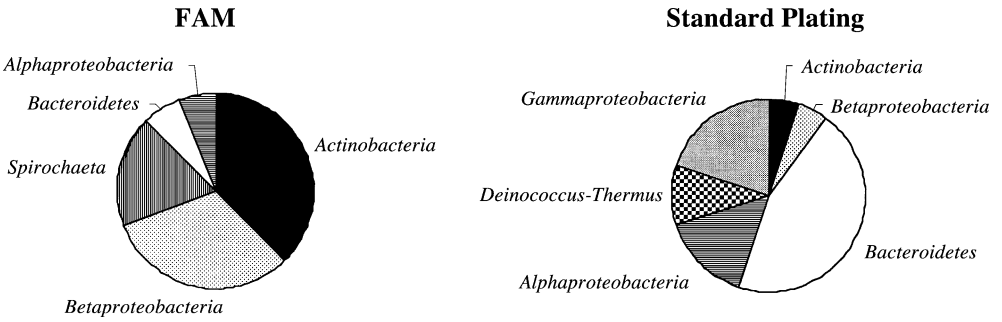

Fig. 2. Relative contribution (%) of phylogenetic groups to the total numbers of taxa obtained by FAM and standard plating of unfiltered water samples on NSY agar plates.

isolates were preliminarily considered to belong to this species (Table 3), which is thus far only represented by a single strain (Spring et al., 2004). The eighth strain is more distantly related to the other seven isolates and *H. gracilis* (ca. 96% sequence similarity), but clustered in phylogenetic analyses with those (data not shown). Therefore, this strain was preliminarily classified as *Hylemonella* sp. (Table 3). We assumed that most of the filamentous bacteria rapidly occurring in the filtrates belonged to these two taxa, and decided to avoid the investigation of more isolates morphologically resembling these eight isolated strains.

Consequently, the inoculum volume used in the isolation experiments was reduced. Instead of 10–30 ml inoculum volume 0.01–1.0 ml of the filtrated water sample was used for inoculation. This reduction resulted in an average decrease of the percentage of cultures showing growth of bacteria. While in initial experiments ($\geq$10 ml inoculum) all inoculated cultures showed growth, the percentage of positive cultures in the later experiments ($\leq$1 ml inoculum) ranged between 5% and 100%. Negative control cultures, which received no inoculum, always showed no growth, and in inoculated cultures typical airborne contaminants were not observed.

The decrease of inoculum volume resulted in a strongly decreased dominance of *Hylemonella* sp.-like filamentous bacteria in the enrichment cultures. This enabled the isolation of a large number of strains belonging to the C-shaped morphotype (Table 3). Most of the C-shaped strains possessed cell sizes smaller than 0.1 μm^3 (ultramicrobacteria), even when grown in media with a high substrate concentration. This trait was mainly found in *Actinobacteria* strains affiliated with the Luna-1 and -2 cluster (Hahn et al., 2003), and in *Betaproteobacteria* belonging to the *P. necessarius* cluster (Hahn, 2003). Furthermore, some filamentous bacteria, which showed no penetration of the agar, and some filamentous strains able to grow inside the agar, yet with pigmented colonies were isolated.

3.2. Diversity of isolated bacteria obtained by FAM

In total, 73 strains belonging to 16 different taxa (>97% sequence similarity) affiliated to the phyla *Actinobacteria*, *Bacteroidetes*, *Proteobacteria*, and *Spirochaeta* were isolated from the 0.2 μm filtrated freshwater samples (Tables 3 and 5). The majority of taxa was affiliated with the *Actinobacteria* (six taxa) and the *Proteobacteria* (six taxa) (Fig. 2). Five of the six *Proteobacteria* taxa belong to *Betaproteobacteria*, one to the *Alphaproteobacteria*.

Six taxa, including the three Luna-1 cluster (*Actinobacteria*) taxa (Table 3), the Luna-2 cluster (*Actino-*

Notes to Table 3:
Abbreviations for the locations of isolation can be found in Table 1. Num., number; c, C-shaped; f, filamentous; Acc., Accession number.
[a] Additional accession numbers can be found in Hahn et al. (2003).
[b] The subcluster classification of the *P. necessarius* cluster is presented in Hahn (2003).
[c] Accession numbers can be found in Hahn (2003).
[d] Obligate endosymbiont of a ciliate was never cultured without the host.

obacteria) taxon (Hahn et al., 2003), and the *P. necessarius* subcluster C and D taxa (Hahn, 2003) contain bacteria which were previously detected in diversity studies on freshwater bacterioplankton by culture-independent techniques (e.g. Zwart et al., 2002; Glöckner et al., 2000; Crump et al., 1999; Hiorns et al., 1997).

A comparison of the 16S rRNA genes of the isolates with reference species revealed a wide range of sequence similarity to the closest related validly described species. The highest values were 99.8% while the lowest value was 87.7%, and the average was of 95% sequence similarity (Table 3). Only 13%

Table 4
Taxa (>97% sequence similarity) which have been isolated by direct plating of unfiltered water samples on NSY agar plates

Taxon	Num. of isolates	Acc. number	Origin	Group	Closest species	Acc. number	Similarity [%]
Brevundimonas vesicularis	1	AJ585216	Pond T7	*Alphaproteobacteria*	*Brevundimonas vesicularis*	AJ007801	100
Sphingobium sp. PS-SwaK3	1	AJ585215	Lake Schwarzensee	*Alphaproteobacteria*	*Sphingobium herbicidovorans*	AB022428	95.9
Blastobacter sp. PS-HalK4	1	AJ585217	Lake Hallstatt	*Alphaproteobacteria*	*Blastobacter capsulatus*	X43072	98.6
Janthinobacterium lividum	2	AJ585218, AJ585219	Lake Wolfgangsee, Pond 1	*Betaproteobacteria*	*Janthinobacterium lividum*	AF174648	97.4, 99.7
Xanthomonas campestris	1	AJ585220	Lake Schwarzensee	*Gammaproteobacteria*	*Xanthomonas campestris*	AF501361	99.0
Aeromonas salmonicida	1	AJ585221	Lake Irrsee	*Gammaproteobacteria*	*Aeromonas salmonicida*	AE012540	98.6
Pseudomonas sp. PS-WolfK3	5	AJ585222–AJ585225	Lakes Wolfgangsee, Krottensee, Irrsee Hallstatt, and Pond 1	*Gammaproteobacteria*	*Pseudomonas* spp.[a]	-	99.8-100
Pseudomonas jessenii	2	J585226	Lake Wolfgangsee, Lake Schwarzensee	*Gammaproteobacteria*	*Pseudomonas jessenii*	AF501361	98.4, 100
Flavobacterium johnsoniae	1	AJ585227	Pond 1	*Bacteroidetes*	*Flavobacterium johnsoniae*	M59053	97.2
Flavobacterium sp. PS-WolfK1	2	AJ585228, AJ585229	Lake Wolfgangsee, Lake Krottensee	*Bacteroidetes*	*Flavobacterium johnsoniae*	M58781	95.2, 95.6
Flavobacterium sp. PS-IrrK3	1	AJ585230	Lake Irrsee	*Bacteroidetes*	*Flavobacterium johnsoniae*	AB078043	96.8
Pedobacter cryoconitis	1	AJ585231	Lake Hallstatt	*Bacteroidetes*	*Pedobacter cryoconitis*	AJ438170	97.9
Isolate PS-KroK1	1	AJ585232	Lake Krottensee	*Bacteroidetes*	*Pedobacter heparinus*	M11657	93.5
Chryseobacterium sp. PS-T7S1G	1	AJ585233	Pond T7	*Bacteroidetes*	*Chryseobacterium joostei*	AJ271010	96.2
Isolate PS-T12W	1	AJ585234	Pond T12	*Bacteroidetes*	*Flavobacterium gelidilacus*	AJ440996	92.2
Flavobacterium sp. PS-SwaK4	1	AJ585235	Lake Schwarzensee	*Bacteroidetes*	*Flavobacterium hibernum*	L39067	96.8
Flavobacterium limicola	1	AJ585236	Lake Hallstatt	*Bacteroidetes*	*Flavobacterium limicola*	AB075232	97.0
Deinococcus sp. PS-IrrK2	1	AJ585237	Lake Irrsee	*Deinococcus-Thermus*	*Deinococcus grandis*	Y11329	94.8
Deinococcus grandis	1	AJ585238	Pond T12	*Deinococcus-Thermus*	*Deinococcus grandis*	Y11329	98.3
Microbacterium sp. PS-PfuK3	1	AJ585239	Pond 1	*Actinobacteria*	*Microbacterium arborescens*	X77443	96.6

Num., number; Acc., Accession number.
[a] The five partial sequences show similarities >99% to two validly described *Pseudomonas* species.

(two taxa) of the obtained taxa could be preliminarily assigned to validly described species (Table 5). These two species, *H. gracilis* and *Spirochaeta aurantia* were previously reported to pass through filters with a small pore size (Canale-Parola et al., 1966; Breznak and Canale-Parola, 1975; Gerhardt et al., 1994).

Eighty-seven percent of isolated taxa (including the *P. nec*. subcluster C taxon) could not be assigned to a validly described species. Out of these 14 taxa, only 4 clustered with validly described species. The isolates belonging to these four taxa were preliminary assigned to the respective genera (Table 3). The remaining 63% of taxa could be considered as candidates for novel genera. Sixty-three percent of the isolated taxa are represented only by a single strain.

3.3. Direct plating of 0.2 μm-filtered samples on NSY agar plates

In total, four experiments were performed where the same number of 100 μl 0.2 μm-filtered samples were processed by FAM and direct plating in parallel. In each experiment, 24 agar plates and 24 cell culture plate wells were inoculated. Only in one of the four experiments resulted the direct plating in the formation of seven colonies, while FAM resulted in all four experiments in 8 to 15 positive cultures (33–63% of established cultures). The seven colonies were identified by analysis of partial 16S rRNA gene sequences as five strains related to *S. aurantia* and two strains distantly related to *Flexibacter litoralis* and *Flexibacter flexis* (*Bacteroidetes*).

3.4. Isolation of strains by direct plating of unfiltered water samples

In total, 27 strains were finally isolated and identified by analysis of partial 16S rRNA gene sequences (Table 4). The isolates grouped into 20 taxa (>97% sequence similarity). These taxa are affiliated with the *Bacteroidetes* (9 taxa), *Gammaproteobacteria* (4), *Alphaproteobacteria* (3), *Deinococcus-Thermus* (2), *Betaproteobacteria* (1) and *Actinobacteria* (1) (Fig. 2). None of the taxa showed a closer relationship to uncultured bacteria than to previously isolated strains (Table 5). Based on sequence similarities, 56% of the taxa could preliminarily be assigned to a described species. The average sequence similarity to the closest described species was 97.6%.

Table 5
Comparison of the results obtained by FAM and direct plating of unfiltered water samples on agar plates

	Plating (only from LSA)[a]	FAM	
		All isolates[b]	From LSA[c]
Number of strains	27	65	19
Number of taxa (>97% sim.)	20	16	7
Taxa represented by only one isolate (% of all)	80.0	62.5	42.9
Taxa closer to uncultured clone than to isolate (% of all)	0	56.3	57.1
Taxa with >97% sim. to closest species (% of all)	55.6	12.5	28.6
Taxa with 94–97% sim. to closest species (% of all)	34.4	37.5	28.5
Taxa with <94% sim. to closest species (% of all)	10.0	50.0	42.9
Similarity (%) with closest species (average of all taxa)	97.6	94.5	94.9

Direct plating was only performed for samples from LSA habitats. Therefore, results for all of the FAM isolates and results concerning the FAM isolates that were obtained from LSA habitats are shown. Sim., similarity.

[a] Direct plating of unfiltered water samples was only performed with samples from LSA habitats.

[b] All isolates obtained by FAM are considered (i.e. all habitats).

[c] Only isolates obtained by FAM from LSA habitats are considered.

4. Discussion

4.1. FAM enables the cultivation of not readily cultivable heterotrophic bacteria

The results of the experiments comparing the cultivation success of the direct plating of 0.2 μm-filtered samples with plating after the acclimatization procedure clearly demonstrate that FAM enables the cultivation of bacteria which cannot be cultured by standard methods.

Previous studies which also applied a filtration step for isolation of bacteria from freshwater habitats but lacked the acclimatization procedure, reported in total only the isolation of the two species *H. gracilis* and *S. aurantia* (Canale-Parola et al., 1966; Breznak and Canale-Parola, 1975; Gerhardt et al., 1994), which

were also isolated in our study. Therefore, it is highly likely that the isolation of the other 11 taxa (without the *Hylemonella* sp. and *Spirochaeta* sp. taxa) obtained in our study was only possible due to the application of the acclimatization procedure.

4.2. More than 50% of the taxa isolated by FAM represent previously uncultured bacteria

Fifty-six percent of the taxa obtained by FAM, but 0% of the taxa isolated by standard plating, are closer related to uncultured bacteria than to previously isolated bacteria (Table 5). Furthermore, 88% of the taxa obtained by FAM, but only 44% of the taxa isolated by standard plating can be considered to represent undescribed species. Both observations additionally demonstrate that FAM enables the isolation of heterotrophic bacteria, which cannot be cultured by standard techniques.

4.3. FAM enables the isolation of bacteria previously only known from investigations which used culture-independent methods

Six of the sixteen taxa isolated by FAM are closely related and cluster in phylogenetic trees with cloned sequences obtained in diversity studies focusing on freshwater habitats. Most of these clusters previously only contained environmental sequences cloned from several habitats (e.g. Hiorns et al., 1997; Crump et al., 1999; Glöckner et al., 2000; Zwart et al., 2002). Therefore, taxa affiliated with these clusters are assumed to represent abundant taxa. Despite of their cosmopolitan distribution and the assumed occurrence with high cell numbers in freshwater habitats, these organisms have not been isolated and cultivated before FAM was used (Hahn et al., 2003; Hahn, 2003).

4.4. FAM is selective

The FAM protocol applied in our study is selective for two reasons: the filtration step, and the growth conditions provided during the acclimatization procedure.

The 0.2 μm filtration excludes more than 90% of bacterial cells contained in the samples, and allows only thin C-shaped cells and thin filamentous cells with a flexible cell shape (e.g. spirochetes and *Hylemonella*-like morphotypes) to get into the filtrates. The latter morphotype is usually neither microscopically detectable in samples of freshwater bacterioplankton nor in the fresh filtrates. Bacterial cells resembling in size and morphology those C-shaped cells observed in fresh 0.2 μm filtrates and found amongst the isolates, comprise up to 40% of bacterioplankton in Lake Mondsee (unpublished data) but only a small fraction of this population of small C-shaped bacteria is passing through the 0.2 μm filters (Hahn et al., 2003).

The growth conditions provided in our experiments additionally excluded two groups of bacteria from being isolated: all bacteria, which cannot grow at higher substrate concentrations, and all bacteria, which cannot grow on the used NSY medium.

Besides this selectivity of the method, the results of our study are potentially influenced by our decision to avoid further isolation of *Hylemonella*-like bacteria. This selection against this morphotype might have resulted in the overlooking of novel taxa morphologically resembling the *Hylemonella* taxa.

The bacterioplankton of freshwater habitats is usually dominated by *Betaproteobacteria* and *Actinobacteria* (Glöckner et al., 2000). Interestingly, these two groups also dominated amongst the taxa isolated by FAM, while the set of taxa isolated by standard plating was dominated by *Bacteroidetes* and *Gammaproteobacteria* (Fig. 2). Positive selection of standard plating methods for *Gammaproteobacteria* is well documented (Wagner et al., 1993).

4.5. Adjustment of inoculum volume in order to avoid overgrowth of slowly growing strains

The FAM protocol as presented in Fig. 1 is an optimized protocol routinely used in our laboratory. This protocol yielded good results in isolation experiments with water samples from various freshwater habitats. In some cases, the usually used inoculum volume of 100 μl of 0.2 μm-filtered water sample per well resulted in the dominance of *Hylemonella*-like bacteria in almost all, or sometimes all wells. This overgrowth by *Hylemonella*-like strains hampered the isolation of other taxa. We observed that *Hylemonella* sp. and *Spirochaeta* sp. strains were sensitive to predation by the bacterivorous nanoflagellate *Ochro-*

monas sp. DS (Wu et al., 2004), while a small C-shaped *Actinobacterium* was protected against predation by this flagellate (Hahn et al., 2003). Therefore, we inoculated wells containing low numbers of C-shaped bacteria, but high numbers of *Hylemonella*-like strains with small inoculi from an axenic culture of the flagellate. In some cases, this resulted in a strong decrease of the filamentous bacteria, and finally in the isolation of C-shaped strains. In other cases, however, this strategy was not successful. Another strategy, which resulted more frequently in the isolation of strains suppressed in the presence of *Hylemonella*-like bacteria, is to reduce the inoculum volume per well (e.g. to 50 or 10 μl of filtrate) and to increase the number of inoculated wells (e.g. to 48–120 wells). This resulted in our experiments in a smaller percentage of positive wells, but also in a smaller proportion of cultures dominated by *Hylemonella*-like strains.

4.6. Transfer of bacteria grown in wells to solid media

Usually wells with growth can easily be detected by visual screening of the cell culture plate wells for turbidity. We used two different procedures for the transfer of the positive liquid cultures to agar plates. On the one hand, samples from positive wells were serially diluted and dilutions were spread with a Trigalski tool on agar plates. On the other hand, large numbers of positive cultures were screened for the presence of certain taxa by dropping 10 μl samples without them being spread on agar plates. Based on colony morphologies, coloration, and spreading behavior several taxa could be distinguished. Interestingly, the dropping procedure never yielded cultures of Luna-2 cluster bacteria (*Actinobacteria*), while only a few isolates affiliated with one of the *P. necessarius* subcluster taxa were found amongst the colonies grown after the spreading of samples (Hahn, 2003). Currently, it is not clear if this difference is due to the selectivity of the two procedures, or if other causes were responsible.

4.7. Future potential of FAM for isolation of novel taxa

The potential of FAM for the isolation of novel taxa is by far not exhausted. The FAM experiments performed thus far have exclusively focused on water samples from surface freshwater habitats. The method has yet to be applied to groundwater, sediment, marine or terrestrial samples. Furthermore, all of the experiments performed thus far have exclusively used the NSY medium. It is highly likely that the application of FAM to samples from other types of ecosystems and/or the use of other isolation media will result in the isolation of novel, till date uncultured taxa.

Another strategy for the isolation of novel taxa could be to apply filters with larger pore sizes in the filtration step. Alternatively, the acclimatization procedure could be combined with an alternative selection step, which removes those taxa able to overgrow strains of interest. One possibility could be to combine the acclimatization procedure with the dilution culture method (Button et al., 1993).

Even the further application of the standard FAM protocol to more samples from freshwater habitats can be expected to lead to the isolation of more novel freshwater taxa. This is clearly indicated by the high number of FAM isolated taxa, which are only represented by a single isolate. Isolation of novel taxa from freshwater habitats by way of FAM has by far not reached its full potential.

Acknowledgements

We are grateful to Grace Ssanyu Asiyo (Kampala, Uganda), Sihaba Ramadhan Mwaitega (Dar es Salaam, Tanzania), Haydée del Carmen Gross Guillen (Managua, Nicaragua), Bernerd Mulwa Fulanda (Sagana, Kenya), Godfrey Magezi (Jinja, Uganda), Wenchun Qu (Wollongong, Australia), Günther Stadler and Doris Hummer (Lenzing, Austria), Doris Slezak (Dauphin Island, USA), Robert Sinyinza (Mpulungu, Zambia), Christian Sturmbauer (Graz, Austria), and Rainer Kurmayer (Mondsee, Austria) for providing water samples. Jens Boenigk and two anonymous reviewers provided valuable comments on an earlier version of the manuscript.

The project was financed by the Austrian Science Fund (project P15655) and the Austrian Academy of Sciences. Q.L.W. was supported by a scholarship from the North–South Dialogue Program, Ministry of Foreign Affairs, Austria. Sampling of sites in the People's Republic of China was made possible by the

Chinese Academy of Sciences, the Nanjing Institute of Geography and Limnology, and the Austrian Academy of Sciences.

References

Altschul, S.F., Madden, T.L., Schaffer, A.A., Zhang, J., Zhang, Z., Miller, W., Lipman, D.J., 1997. Gapped BLAST and PSI-BLAST: a new generation of protein database search programs. Nucleic Acids Res. 25, 3389–3402.

Breznak, J.A., Canale-Parola, E., 1975. Morphology and physiology of *Spirochaeta aurantia* strains isolated from aquatic habitats. Arch. Microbiol. 105, 1–12.

Bruns, A., Nubel, U., Cypionka, H., Overmann, J., 2003. Effect of signal compounds and incubation conditions on the culturability of freshwater bacterioplankton. Appl. Environ. Microbiol. 69, 1980–1989.

Bussmann, I., Philipp, B., Schink, B., 2001. Factors influencing the cultivability of lake water bacteria. J. Microbiol. Methods 47, 41–50.

Button, D.K., Schut, F., Quang, P., Martin, R., Robertson, B., 1993. Viability and isolation of marine bacteria by dilution culture: theory, procedures, and initial results. Appl. Environ. Microbiol. 59, 881–891.

Canale-Parola, E., Rosenthal, S.L., Kupfer, D.G., 1966. Morphological and physiological characteristics of *Spirillum* gracile sp. n. Antonie van Leeuwenhoek. J. Microbiol. Serol. 32, 113–124.

Connon, S.A., Giovannoni, S.J., 2002. High-throughput methods for culturing microorganisms in very-low-nutrient media yield diverse new marine isolates. Appl. Environ. Microbiol. 68, 3878–3885.

Crump, B.C., Armbrust, E.V., Baross, J.A., 1999. Phylogenetic analysis of particle-attached and free-living bacterial communities in the Columbia River, its estuary, and the adjacent coastal ocean. Appl. Environ. Microbiol. 65, 3192–3204.

Gerhardt, H., Murray, R.G.E., Wood, W.A., Krieg, N.R., 1994. Methods for General and Molecular Bacteriology American Society for Microbiology, Washington, DC.

Glöckner, F.O., Zaichikov, E., Belkova, N., Denissova, L., Pernthaler, J., Pernthaler, A., Amann, R., 2000. Comparative 16S rRNA analysis of lake bacterioplankton reveals globally distributed phylogenetic clusters including an abundant group of *Actinobacteria*. Appl. Environ. Microbiol. 66, 5053–5065.

Hahn, M.W., 2003. Isolation of strains belonging to the cosmopolitan *Polynucleobacter necessarius* cluster from freshwater habitats located in three climatic zones. Appl. Environ. Microbiol. 69, 5248–5254.

Hahn, M.W., Lünsdorf, H., Wu, Q., Schauer, M., Höfle, M.G., Boenigk, J., Stadler, P., 2003. Isolation of novel ultramicrobacteria classified as *Actinobacteria* from five freshwater habitats in Europe and Asia. Appl. Environ. Microbiol. 69, 1442–1451.

Hiorns, W.D., Methé, E.A., Nierzwickibauer, S.A., Zehr, J.P., 1997. Bacterial diversity in Adirondack mountain lakes as revealed by 16S rRNA gene sequences. Appl. Environ. Microbiol. 63, 2957–2960.

Hugenholtz, P., Goeber, B.M., Pace, N.R., 1998. Impact of culture-independent studies on the emerging phylogenetic view of bacterial diversity. J. Bacteriol. 180, 4765–4774.

Kumar, S., Tamura, K., Jakobsen, I.B., Nei, M., 2001. MEGA2: molecular evolutionary genetics analysis software. Bioinformatics 17, 1244–1245.

Rappé, M.S., Connon, S.A., Vergin, K.L., Giovannoni, S.J., 2002. Cultivation of the ubiquitous SAR11 marine bacterioplankton clade. Nature 418, 630–633.

Spring, S., Jaeckel, U., Wagner, M., Kaempfer, P., 2004. *Ottowia thiooxydans* gen. nov., sp. nov., a novel facultatively anaerobic, N_2O-producing bacterium isolated from activated sludge, and transfer of *Aquaspirillum gracile* to *Hylemonella gracilis* gen. nov., comb. nov. Int. J. Syst. Evol. Microbiol. 54, 99–106.

Strunk, O., Gross, O., Reichel, B., May, M., Hermann, S., Stuckmann, N., Nonhoff, B., Lenke, M., Ginhart, A., Vilbig, A., Ludwig, T., Bode, A., Schleifer, K.-H., Ludwig, W., 1998. ARB: A Software Environment for Sequence Data. Department of Microbiology, Technische Universität München, Munich, Germany.

Wagner, M., Amann, R., Lemmer, H., Manz, H., Schleifer, K.H., 1993. Probing activated sludge with oligonucleotides specific for proteobacteria: inadequacy of culture-dependant methods for describing microbial community structure. Appl. Environ. Microbiol. 59, 1520–1525.

Wu, Q.L., Boenigk, J., Hahn, M.W., 2004. Successful predation of filamentous bacteria by a nanoflagellate challenges current models of flagellate bacterivory. Appl. Environ. Microbiol. 70, 332–339.

Zwart, G., Crump, B.C., Kamst-van Agterveld, M.P., Hagen, F., Han, S.K., 2002. Typical freshwater bacteria: an analysis of available 16S rRNA gene sequences from plankton of lakes and rivers. Aquat. Microb. Ecol. 28, 141–155.