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ORIGINAL ARTICLE

Viable ultramicrocells in drinking water

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Keywords
0·1–0·2 μm filtrate, biofilm, drinking water, tap water, ultramicrocells diversity.

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Abstract

Aims: To examine the diversity of cultivable 0·2 micron filtrate biofilm forming bacteria from drinking water systems.
Methods and Results: Potable chlorinated drinking water hosts phylogenetically diverse ultramicrocells (UMC) (0·2 and 0·1 μm filterable). UMC (starved or dwarf bacteria) were isolated by cultivation on minimal medium from a flow system wall model with polyvinyl chloride (PVC) pipes. All cultivated cells (25 different isolates) did not maintain their ultra-size after passages on rich media. Cultured UMC were identified by their 16S ribosomal DNA sequences. The results showed that they were closely related to uncultured and cultured members of the Proteobacteria, Actinobacteria and Firmicutes. The isolates of phylum Actinobacteria included representatives of a diverse set of Actinobacterial families: Micrococcaceae, Microbacteriaceae, Dermabacteraceae, Nocardiaceae and Nocardioidaceae.
Conclusions: This study is the first to show an abundance of cultivable UMC of various phyla in drinking water system, including a high frequency of bacteria known to be involved in opportunistic infections, such as *Stenotrophomonas maltophilia*, *Microbacterium* sp., *Pandoraea* sp. and *Afipia* strains.
Significance and Impact of the Study: Chlorinated tap water filtrate (0·2 and 0·1 μm) still harbours opportunistic micro-organisms that can pose some health threat.

Introduction

Ultramicrocells (UMC) are reduced forms of micro-organisms, usually associated with reductive cell division during starvation or stress, which can pass through a 0·2-μm filter (Torrella and Morita 1981; Bakken and Olsen 1987). Groundwater is known to contain unidentified oligotrophic, copiotrophic, ultrasmall bacteria and ultramicrobacteria (UMB) (Fischer and Velimirov 2000; Ross *et al.* 2001). Nutrient-depleted environments such as drinking water, appear to induce specific morphological changes to bacterial cells resulting in a reduction of bacterial size associated with starvation survival (Kjelleberg *et al.* 1982, 1983, 1993; Kurath and Morita 1983; Morita 1988; Heim *et al.* 2002). Mitchell (2002) determined that a 0·1-μm cell-size reduction can reduce energetic costs of chemotaxis 100 000-fold, greatly enhancing survival in nutrient-depleted environments.

Conventional cultivation methods fail to cultivate most environmental bacteria, although the majority of bacteria in nature are viable (Eilers *et al.* 2000; Zengler *et al.* 2002). Indeed, environmental microbial community studies are dominated by culture-independent analysis of the ribosomal gene sequences diversity. However, in order to understand the ecological roles of the organisms in their communities, a representative organism from each phylogenetic cluster should be cultivated and studied.

Recently, the presence of UMC has been reported in different oligotrophic environments such as marine and freshwater systems as well as in soil, subsurfaces terrestrial and in sample of kidney stones (Velimirov 2001). Several studies have detected freshwater bacterial UMC passing

through ultra-filters (Jones *et al.* 1999; Haller 2000; Hahn 2004). Miyoshi *et al.* (2005) determined 37 operational taxonomic units (mostly β-Proteobacteria), based on the amplification, cloning and sequencing of 16S ribosomal RNA gene sequences. Page *et al.* (2004) used high-throughput culturing (HTC) methods that relied on dilution to extinction in very-low-nutrient media (10% R2A medium) in order to obtain bacterial isolate representatives from Crater Lake, Oregon. However, they did not isolate representatives of Actinomycetes and the culturability rate was low. In the same year, Hahn *et al.* (2004) used the filtration–acclimatization method to isolate a total of 65 strains from surface freshwater habitats. The isolates were affiliated with Actinobacteria, α, β-Proteobacteria, 'Bacteroidetes' and *Spirochaeta*. Gich *et al.* (2005) used high-throughput cultivation combined with rapid and group-specific phylogenetic fingerprinting in order to recover bacterioplankton representatives of three different trophic status freshwater lakes, α-Proteobacteria, β-Proteobacteria, Actinobacteria, Firmicutes or Flavobacteria-Cytophaga lineage.

Safe water is essential for health, yet remains one of the most critical problems facing the world today. With the increase in water quality regulations and decrease in available fresh water supplies in the world, membrane filtrations and ultrafiltration technology are becoming an increasingly important role in drinking water treatment. The lack of simple methods for cultivating UMC hindered our knowledge about the ecology of UMC in drinking water. In this study the abundance, viability and diversity of UMC in potable drinking water had been investigated. Such studies will help us to understand the roles of those UMC in our drinking water and their potential role in the emerging of new pathogens. As well, *in situ* studies will shed light on the consequence of UMC surface attachment, biofilm formation and the ecology of the bacterial population of the water distribution systems, and so enable design of effective control strategies that will ensure safe, high-quality drinking water and sterile solution filtrates for medical usage.

Materials and methods

Location and water recourse

This study was performed on potable drinking water from the City of Shefa-Amr, Israel, whose water is supplied from the Sea of Galilee, by the National Water Carrier. The system supplies free chlorine at a concentration up to 0·5 mg l^{-1}, with chloride and nitrate concentrations below 600 and 70 mg l^{-1}, respectively.

Water from the Sea of Galilee is the source of the National Water Carrier. Some 60% of the water is supplied for household consumption. The water contains organic and inorganic materials that cause a high degree of turbidity and water pollution, which diminishes the disinfecting capability of the chlorine in the water carrier. As a result, a great amount of disinfectant is employed.

Selection of medium for bacterial growth and isolation from water

Tap water was filtered through 0·2 μm filter and the filtrates spread over different solid media such as Nutrient agar, various strengths of nutrient agar, R2A agar (Reasoner and Geldreich 1985) and Alpha agar (see below).

Modified minimal medium

Minimal medium (MM) had been prepared by adding 0·1% (v/v) of MMA and 0·01% of MMB stock solutions (Ihssen and Egli 2004). MMA (stock solution) consists of 6 μmol l^{-1} $MgSO_4$, 10 μmol l^{-1} $CaCl_2$, 20 μmol l^{-1} Na_2CO_3, 14 μmol l^{-1} $NaNO_3$, 10 μmol l^{-1} NH_4Cl, 1·75 μmol l^{-1} K_2HPO_4, 2·75 μmol l^{-1} EDTA (Na salt), 0·001% yeast extract and 0·001% peptone. MMB (stock solution) consists of 0·85 mmol l^{-1} $ZnSO_4{\cdot}7H_2O$, 7·1 mmol l^{-1} $MnCl_2{\cdot}4H_2O$, 0·086 mmol l^{-1} $Co(NO_3)_2{\cdot}6H_2O$, 1·6 mmol l^{-1} $NaMoO_4{\cdot}2H_2O$, 29·75 mmol l^{-1} citric acid·H_2O and 21·48 mmol l^{-1} ferric chloride $FeCl_3$.

Alpha agar

Alpha plates contain 0·1% (v/v) of MMA, 0·01% of MMB, 0·005% of Peptone from Enzymatic Digest of Soybean Meal (Difco) and 1·5% agar. The optimal amount of Peptone–Soybean Meal added to the MM was chosen after serial dilution of the Peptone percentage. Colony forming units were estimated from each medium with careful attention to the colonies morphological diversity. Comparison of bacterial diversity of the water filterable population by means of colony morphology and count showed advantage of Alpha medium over the various tested media.

Direct isolation of ultramicrocells from polyvinyl chloride pipe biofilm

Precipitation in water pipes and on surfaces in contact with water is a well-known phenomenon. To detect and cultivate UMC from the potable drinking water system precipitates, a 50-cm polyvinyl chloride (PVC) tube with a radius of 4 mm was cut from a water cooler that had been connected to the drinking water system for at least 6 years. The external surface of the pipe was sterilized with 70% ethanol solution and 1% potassium

permanganate. The inner side of the tube was rinsed with sterile water and the precipitation on the inner surface of the tube was removed using a sterile scalpel. The precipitates were suspended in 2 ml sterile water in order to release the attached bacteria and the suspension was filtered through a 0·2-μm filter to separate UMC from other precipitates and bacteria possibly present in the water. Filtered used in this study, 0·2 μm absolute rating cellulose acetate filter membrane and 0·1 μm absolute rating nylon filter membrane, Minisart high flow syringe filter (Sartorius, Goettingen, Germany).

The filtered solution (1·5 ml) was spread over the Alpha medium and incubated at room temperature for 3 weeks. Eight attached UMC with different colony morphology were isolated from the precipitate and were designated PVC1 to PVC8. The isolates were grown on rich medium and kept for DNA extraction.

Primary colonizers ultramicrocells isolation

In order to study the early or primary colonizer UMC, two separate flow systems were designed and used for isolation of UMC. The method consisted of two filtration steps which removed most of the readily cultivable bacteria that is able to overtake slowly growing bacteria and starved UMC. Each flow system was composed of a PVC tube measuring 20 cm in length and 0·4 cm in diameter, connected at the water entrance with two adjacent 0·2 μm filters and two 0·1 μm absolute rating filters. The first filter was placed in the middle of the system and the second filter at the end. The 0·2 μm filters were joined to amber latex tube which was connected directly to an alcohol disinfected faucet. Potable tap water was allowed to pass at a very slow rate (19 ml h^{-1}) through the filters and the PVC tubes (Flow System). In this manner, UMC in tap water were allowed to pass through the two 0·2 μm filters but were trapped on the 0·1 μm filter. The first 0·2 μm filter was replaced every 72 h because of clogging by water impurities. The flow rates values of 0·2 and 0·1 μm filters for water at pressure = 1 bar are 140 and 30 ml min^{-1}, respectively (Minisart high flow syringe filter, Sartorius, Goettingen, Germany).

One flow system experiment lasted for 72 h. Attached (biofilm) UMC that passed the 0·2 μm filters were isolated from the inner surface of the PVC pipe and were designated PVC(72h)1, 2, 4. Planktonic isolates from the trapped water were designated PVC(72h)5, 7, 8, 9, 11, 12, 15. The second system ran for 2 weeks, after which the 20 cm PVC tube linking the two filters 0·2 μm. The external pipe surface was disinfected with 70% ethanol solution and 1% potassium permanganate. The inner side of the tube was washed with 50 ml sterile water, and the attached UMC on the inner surface of the pipe were collected in sterile water and isolated on Alpha medium. The attached independent isolates that passed through the 0·2 μm and grew on Alpha agar were designated PVC(14d)1–13.

The four independent different colonies were isolated from the PVC pipe wall trapped between the two 0·1 μm filters and were assigned as PVC(0·1) A, A10, X and Z.

Ultramicrocell cultivation and isolation

Bacterial cells were isolated on fresh Alpha agar (MM containing 0·005% Peptone of Soybean Meal agar) plates. The plates were incubated at room temperature (22°C) in darkness for at least 3 weeks. UMC with different colonial morphology were isolated on Alpha and Nutrient agar for further study.

Microscopy

The inoculated Alpha agar plates were observed periodically with Axiovert 40 CFL, Carl Zeiss, Objectives LD 'Plan-Neofluar' 40×/0·60 Korr and Objectives LD 'Plan-Neofluar' 63×/0·75 Korr. The UMC colonies at the first isolation were very small microcolonies and could be detected by light microscope (Fig. 1a,b). However, with the passage of the isolates from Alpha agar to Nutrient agar they grew rapidly to form normal-sized colonies and normal size bacterial cells.

DNA extraction

Bacterial chromosomal DNA was extracted from each one of the UMC isolates by using QIAamp DNA Mini Kit (Qiagen GmbH, Hilden, Germany), following the manufacturer protocol.

PCR reactions

Amplification and sequencing of the 16S rDNA gene of the isolates were performed at a final volume of 50 μl. All PCR reactions contained the following reagents: A 50–100 ng of chromosomal DNA was added to PCR-MIX – 0·25 units of Vent DNA polymerase (New England BioLabs) × 10 buffer, 2·5 mmol l^{-1} of each dNTPs, 200 ng of each primer primer FP1 5′-AGAGTTTGATCCTGGCTCAG-3′ (27F; Tanner *et al.* 1998) corresponding to *Escherichia coli* positions 1–27, and RP1 5′-CCGGGTTACCTTGTTACGAC-3′, corresponding to *E. coli* nucleotide positions 1513 to 1491. DDW was added to a final volume of 50 μl. The PCR reaction was performed on an automated thermal cycler (Perkin-Elmer/Cetus). The two PCR primers used for the PCR reaction were 'universal' bacterial primers. Twenty-five cycles of PCR amplification were

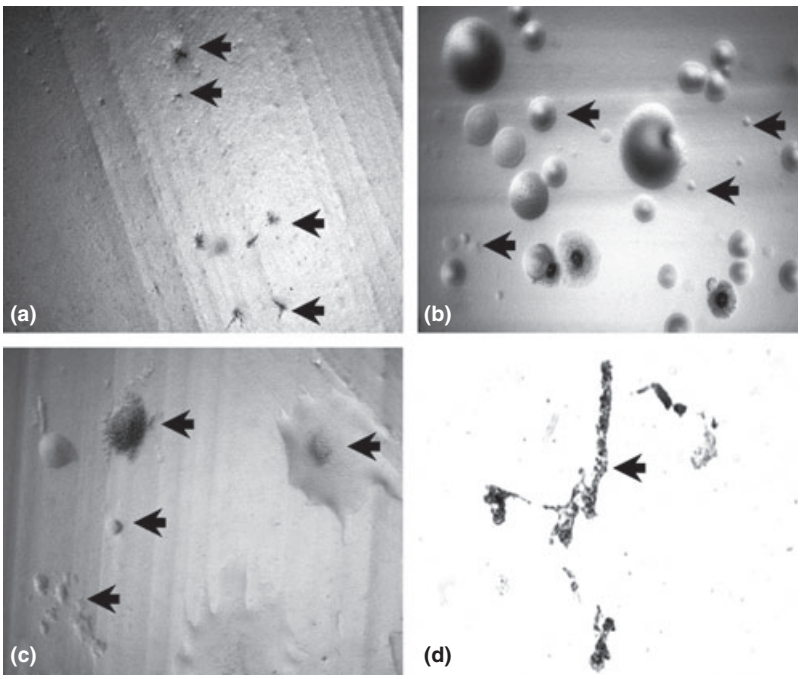

Figure 1 UMC microcolonies: (a) UMC isolated from 0·2 μm filterate of PVC pipe biofilm, and grown on Alpha agar for 7 days at room temperature. Arrows indicate the microcolonies development of the primary isolation (magnification ×400). (b) The same as above, UMC grown for 21 days. (c) 0·1 μm filterable UMC microcolonies. UMC colonies isolated from the inner surface of PVC pipe walls of the flow system and which filtered through 0·2 and 0·1 μm filters of the flow system. (d) Gram stain of fresh UMC smear from Alpha medium agar (×1000 magnification) (numerical aperture of 1·4), shows UMC aggregates beyond the limits of optical resolution.

conducted. Each cycle included an initial denaturing step at 94°C for 45 s followed by a 40-s annealing step at 47°C and a 1·5-min extension step at 72°C. The amplification cycles were preceded by a single denaturing step at 94°C for 3 min prior to the first cycle and included a final 72°C extension step for 8 min.

PCR product purification and sequencing

The PCR products were separated from free PCR primers using the QIAquick Spin Kit (Qiagen GmbH, Hilden, Germany) and sequenced directly using an ABI Prism Big Dye Terminator Cycle Sequencing Ready Reaction Kit (Applied Biosystems). The PCR products of the DNA templates were sequenced with ABI 377 DNA sequencer (Applied Biosystems) using the FP1 and RP1 primers.

Phylogenetic analysis

Sequence files chromatograms were edited and the final sequences were compared with GenBank sequences by using a basic local alignment search tool (Blast) for nucleotide homology search (NCBI). At the same time, a phylogenetic classification of all the clones was done online at Ribosomal Database Project II, release 9 (RDP II) (http://rdp.cme.msu.edu/index.jsp) into which each of the resultant 16S rDNA gene sequences was assigned to a set of hierarchical taxa and identified as member of the genera with the highest probability. All phylogenetic analysis of the 16S rDNA gene sequences of the isolated UMC were conducted by the interactive ClustalW (online multiple sequence global alignment program for DNA against all Database sequences) using a neighbour-joining method at EMBL-EBI and by the CLC Free Workbench for basic bioinformatics, products from CLC Bio for final editing and presentation.

Nucleotide sequences accession numbers

Sequences obtained in this study were deposited in EMBL Nucleotide Sequence Database under accession numbers AM421780–AM421805 (Table 1).

Results

Microcolonies isolation

Colony development in the first isolation step, from the PVC pipe on Alpha agar, was very slow and the bacterial populations behaved as a slow growing mycobacterial species. Because of minute size of the microcolonies (Fig. 1a,b), the first step of the bacterial colony isolation from Alpha medium had been carried out under the magnification of microscope with autoclaved Pasteur pipette with a heat-elongated tip. A Gram stain was performed and the bacterial cell size was examined under magnification × 1000 with emersion oil, but the bacterial size was very small and beyond the limits of optical resolution.

Table 1 Thirty-five independent cultivated UMC isolates from tap water. The phylogenetic affiliations and classification performed by RDP II and closest relative species 16S rRNA encoding gene was obtained by BLAST NCBI databank

Isolates	RDP II classification [classification reliability]	Closest relative species by sequence identity	16S rRNA sequence identities	No. of isolates	Accession no.
PVC1	*Rhodococcus* [100%]	*Rhodococcus* sp. A1XB1-5*	1027/1040 (98%)	1	AM421780
PVC2	*Microbacterium* [100%]	*Microbacterium* sp. Cr1†	942/942 (100%)	1	AM421781
PVC3	*Stenotrophomonas* [100%]	*Stenotrophomonas maltophilia* strain ZZ7‡	932/934 (99%)	1	AM421782
PVC5	*Aeromicrobium* [100%]	*Aeromicrobium tamlensis* SSW1-57§	920/931 (98%)	1	AM421783
PVC6	*Microbacterium* [100%]	*Microbacterium aurum*¶	865/872 (99%)	1	AM421784
PVC7	*Leucobacter* [100%]	*Microbacterium* sp. VKM Ac-2048**	860/871 (98%)	1	AM421785
PVC8	*Microbacterium*[100%]	*Microbacterium oxydans*††	878/880 (99%)	1	AM421786
PVC(72h)2	*Brevundimonas* [100%]	*Brevundimonas* sp.	763/775 (98%)	1	AM421787
PVC(72h)4	*Brachybacterium* [100%]	*Brachybacterium* sp.‡‡	894/900 (99%)	1	AM421788
PVC(72h)5	*Kocuria* [100%]	*Kocuria rosea* and marine sediment§§	777/779 (99%)	1	AM421789
PVC(72h)9, PVC(72h)1	*Silanimonas* [100%]	Uncultured *Xanthomonadaceae*¶¶	763/780 (97%)	2	AM421790
PVC(72h)11, PVC(72h)12	*Nocardioides* [100%]	*Nocardioides fulvus*	1332/1337 (99%)	2	AM421791
PVC(72h)15	*Dechloromonas* [88%]	Perchlorate-reducing bacterium RC1***	793/795 (99%)	1	AM421792
PVC(14d)1	*Caulobacter* [100%]	*Caulobacter* sp.	923/931 (99%)	3	AM421797
PVC(14d)2	*Sterolibacterium* [40%]	Uncultured bacterium DSSD13†††	923/928 (99%)	1	AM421798
PVC(14d)3	*Hyphomicrobium* [100%]	*Hyphomicrobium zavarzinii*	859/870 (98%)	1	AM421799
PVC(14d)4	*Kocuria* [100%]	Glacial ice bacterium‡‡‡ and *Micrococcus kristinae*	850/852 (99%)	1	AM421800
PVC(14d)5	*Brevundimonas* [99%]	Uncultured *alpha-Proteobacterium*	826/829 (99%)	2	AM421801
PVC(14d)6, PVC(72h)8	*Pandoraea* [47%]	Uncultured *beta-Proteobacterium*§§§	856/862 (99%)	2	AM421802
PVC(14d)8	*Brevundimonas* [100%]	*Brevundimonas* sp.	1232/1241 (99%)	3	AM421803
PVC(14d)9, PVC(72h)7	*Pandoraea* [35%]	Uncultured *beta-Proteobacterium*¶¶¶	790/799 (98%)	2	AM421804
PVC(0·1)A	*Staphylococcus* [100%]	*Staphylococcus epidermidis*****	650/656 (99%)	2	AM421793
PVC(0·1)10A	*Microbacterium* [99%]	*Microbacterium* sp. VKM Ac	718/720 (99%)	1	AM421794
PVC(0·1)X	*Afipia* [100%]	*Afipia* sp. TW-O10	927/937 (98%)	1	AM421795
PVC(0·1)Z	*Microbacterium* [100%]	*Microbacterium* sp. BBDP82 and oral strain C24KA††††	805/808 (99%)	1	AM421796

*Williams and Santo Domingo (2005).
†Wang (2006).
‡Zahir *et al.* (2006).
§Lee and Kim (2007).
¶Luna *et al.* (2002).
**Evtushenko *et al.* (2000).
††Giammanco *et al.* (2006).
‡‡Zhang (2006).
§§Milanesi *et al.* (2006).
¶¶Lee *et al.* (2005).
***Waller *et al.* (2004).
†††Williams *et al.* (2004).
‡‡‡Christner and Priscu (2006).
§§§Giammanco *et al.* (2006).
¶¶¶Connon *et al.* (2005).
****Miyoshi *et al.* (2005).
††††Paster *et al.* (2001).

Comparative analysis of 16S rRNA genes sequences

The results of the phylogenetic classification showed that isolates from PVC1-PVC8 were closely related to the class Actinobacteria, and the genus *Aeromicrobium*, *Leucobacter*, three *Microbacterium* isolates and *Rhodococcus*. Only one out of seven clones belongs to the class γ-Proteobacteria genus *Stenotrophomonas* (Table 1 and Fig. 2). The PVC4 isolated colony ceased to reproduce at the second passage and could not be re-cultivated. BLAST analysis of isolate PVC1-PVC8 16S rDNA gene sequences revealed many close matches to unidentified bacterial 16S rDNA gene clones in GenBank. The isolates were phylogenetically closest to class Actinobacteria: isolate PVC1 show 98% (1027/1040)

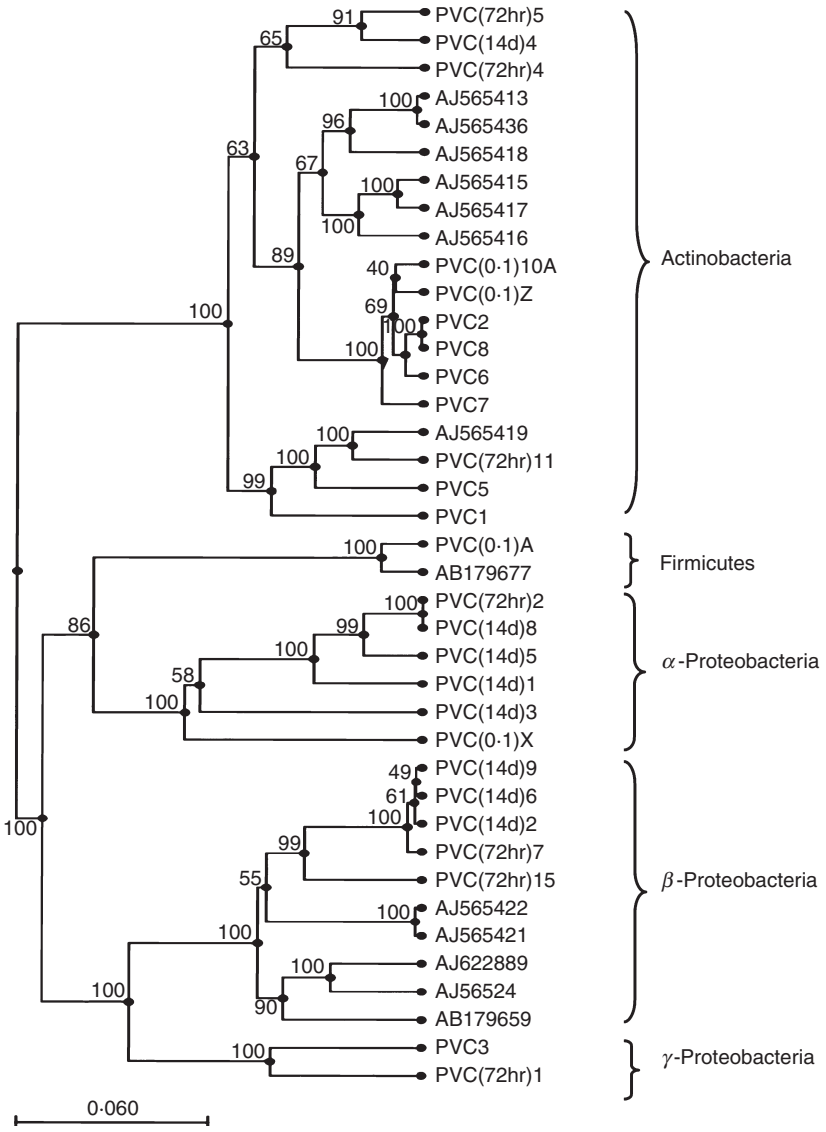

Figure 2 Phylogenetic relationship of 16S rRNA gene sequences of tap water and freshwater UMC. The tree represents diverse UMC isolated from tap water in regards to previously isolated by filtration-acclimatization method (cultured AJ clones, Hahn *et al.* 2004) and uncultured 16S rRNA gene clones, obtained from deep-groundwater micro-organisms that pass through 0·2-μm-pore-size filters (uncultured AB clones, Miyoshi *et al.* 2005). The scale bar corresponds to 6 base substitutions per 100 nucleotide positions. Bootstrap values at the main branching points are given.

identity with many *Rhodococcus* clones in the database, such as *Rhodococcus* sp. A1XB1-5 genotypes involved in bacterial aerobic BTEX degradation, and the same identity with uncultured bacterium clone B3NR56D2 isolated from model drinking water biofilm receiving chlorine or monochloramine residual (Williams and Santo Domingo 2005).

The PVC2 isolate was classified as a *Microbacterium* sp. and showed 100% (942/942) identity with an uncultured clone SC-35 sequenced from microbial communities inducing concrete corrosion and found in deep sea sediment (*Microbacterium* sp. Cr1, Wang 2006). The PVC5 isolate showed 98% (920/931) identity with *Aeromicrobium tamlensis* strain SSW1-57 (Lee and Kim 2007) isolated from dried seaweed and 98% (920/931) with *Aeromicrobium erythreum*. The PVC6 isolate showed 99% (865/872) identity with *Microbacterium aurum* and 99% (862/869) *Microbacterium esteraromaticum* isolated from activated sludge (Luna *et al.* 2002). PVC7 showed 98% (860/871) identical with plant-nematode Microbacteriaceae, *Microbacterium* sp. VKM Ac-2048 (Evtushenko *et al.* 2000). The PVC8 isolate showed 99% (878/880) identity with *Microbacterium oxydans* isolate OUCZ46 a polychlorinated biphenyl (PCB)-degrading bacterium, and *Microbacterium* sp. OVE isolate involved in interstitial pulmonary (Giammanco *et al.* 2006). The only isolate related to gamma-Proteobacteria class was a PVC3 isolate showed 99% (932/934) identity with *Stenotrophomonas maltophilia*. This species is an environmental bacterium increasingly involved in nosocomial infections and resistant to most antibiotics.

Early (72h) attached ultramicrocell tap water isolate

Ten independent microcolonies of UMC were cultivated. Three different isolates were attached to PVC wall of the flow system after 72 h from running the tap water, PVC(72h)1,2,4 *Silanimonas* (γ-Proteobacteria), *Brevundimonas* (α-Proteobacteria) and *Brachybacterium* (Actinobacteria), respectively. Isolates PVC(72h)5, 7, 8, 9, 11, 12 and 15 were isolated as planktons from the trapped water of the flow system. BLAST analysis of isolate 16S rRNA gene sequences revealed many close matches to bacterial 16S rRNA gene clones in GenBank (Table 1 and Fig. 2). The Actinobacterial isolates show high identity with many strains isolated from different environments such as marine sediment and fresco painting (Milanesi *et al.* 2006; Zhang 2006), and the Proteobacterial isolates show high identity with trichloroethene-contaminated groundwater (Connon *et al.* 2005), subsurface water of the Kalahari Shield, South Africa and slightly thermophilic and alkaliphilic gamma-Proteobacterium and with perchlorate-reducing bacterium (Waller *et al.* 2004; Lee *et al.* 2005).

Attached (14-day biofilm) ultramicrocell tap water isolates

Seventeen independent microcolonies of attached bacterial cells were cultivated after 14 days of running the flow system with tap water. The 16S rDNA sequences showed that 70% (12 isolates) of the isolates were phylogenetically related to Proteobacteria, 18% (three isolates) to Actinobacteria and 12% (two isolates) to Firmicutis. The 12 UMC isolates belong to phylum Proteobacteria divided into two classes: four isolates were identified as unclassified β-Proteobacteria, and another eight isolates belong to the class α-Proteobacteria, three isolates are related to the genus *Brevundimonas*, three to *Caulobacter*, one to the genus *Hyphomicrobium*, and one related to the genus *Afipia*. The three isolates related to Actinobacteria class belong to two different families: two isolates are related to Microbacteriaceae and to the genus *Microbacterium* and one isolate is related to the Micrococcaceae and the genus *Kocuria*. The Firmicutis related isolates were from the genus *Staphylococcus* (Fig. 2 and Table 1). BLAST search against the NCBI database sequences showed that UMC isolates are highly related to many uncultured bacteria from drinking water bacteria in a distribution system simulator (Williams *et al.* 2004; Christner and Priscu 2006), and trichloroethene-contaminated groundwater (Connon *et al.* 2005).

Four UMC isolates PVC(0·1)A, PVC(0·1)10A, PVC(0·1)X and PVC(0·1)Z were cultured from flow system walls downstream of the first 0·1 μm filter section (Fig. 1c). UMC isolate PVC(0·1)A 16S rRNA encoding gene sequence showed high identity 99% with uncultured *Staphylococci* sp. from different environmental isolates such as deep water, clean-room facilities, phenol contaminated soil, and in particular to *Staphylococcus epidermidis* strain S09 (Miyoshi *et al.* 2005). Isolate PVC(0·1)10A showed 99% identity with many *Microbacterium* sp. and, in particular, with *Microbacterium* sp. VKM Ac-1389 plant-nematode Microbacteriaceae. PVC(0·1)X showed high identity (98%, 927/937) with *Afipia* sp. TW-O10 isolated from Biofilm development and microbial communities of ozone-biological activated carbon filters for drinking water treatment and uncultured groundwater bacterium (98%, 933/945). PVC(0·1)Z showed identity of 99% (805/808) with *Microbacterium* sp. BBDP82 and *Microbacterium* sp. oral strain C24KA (Paster *et al.* 2001) (Table 1).

Ultramicrocell Actinobacteria cultivates of tap water

A total of 12 cultivates of UMC affiliated to Actinobacteria were isolated in this study. The isolates were mostly affiliated with the suborder Micrococcineae (six independent isolates belong to the family Microbacteriaceae and two isolates to the family Micrococcaceae), two isolates are affiliated with suborder Propionibacterineae and one isolate belongs to suborder Corynebacterineae. The phylogenetic affiliation of the Actinobacterial UMC to the known dominant freshwater Actinobacterial clusters (*acI*, *acII*, *acIV* and *acSTL*) (Warnecke *et al.* 2004, 2005; Allgaier and Grossart 2006) are shown in Fig. 3. The majority of all cultured Actinobacteria isolates are distinct from those of already-cultured and uncultured freshwater Actinobacteria based on their 16S rRNA encoding gene sequences. The tree shows that the UMC isolated from this study, belong to new clusters phylogenetically bordered between clusters *acII* and a*cSTL* clusters, and subclusters phylogenetically related to the *acII* and *acSTL* clusters (Fig. 3).

Discussion

Combination of ultrafiltration, surface attachment and Alpha agar plating greatly enhance the ability to isolate and cultivate diverse UMC from phyla and classes such as α-Proteobacteria, β-Proteobacteria, γ-Proteobacteria, Firmicutes and Actinobacteria. In particular, the diverse Actinobacteria family representatives have not been cultivated before from freshwater (Fig. 1 and Table 1). In addition, the cultivation of unclassified or poorly classified isolates PVC(14d)2, PVC(14d)6, PVC(72h)8, PVC(14d)9 and PVC(72h)7 related to uncultured beta-Proteobacteria belonging to *Pandoraea* sp. and a new

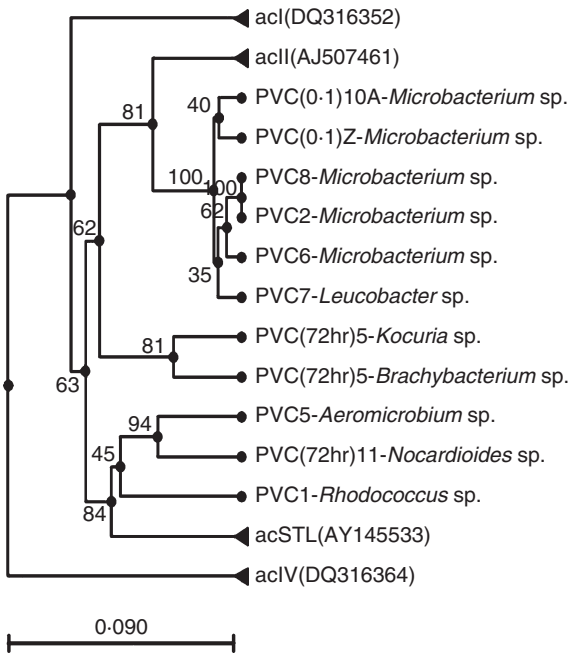

Figure 3 Phylogenetic relationship of 16S rRNA gene sequences of tap water UMC and freshwater Actinaobacterial planktons. Phylogenetic classification of UMC (dots) among known freshwater Actinobacterial clusters (triangles). The scale bar corresponds to 9 base substitutions per 100 nucleotide positions. Bootstrap values at the main branching points are given.

member of the *Sterolibacterium* lineage (Table 1), highlight the potential of this approach in the cultivation of uncultured starved freshwater bacteria or UMC.

The majority of bacterial cells in natural communities are either nonculturable by current cultivation methods or are present in a viable but nonculturable (VBNC) state (Oliver 2005). Most dwarfs are uncultured by current approaches and have not been identified phylogenetically. Studies of soil micro-organisms using electron microscopy have shown that the majority (72%) of soil microbes exist *in situ* as dwarf cells (Bae and Casida 1973). Uncultured dwarf bacterial (that can pass through a 0·45 μm filter) cells isolated from semiarid soil fell into four bacterial phyla, using 16S rDNA-based techniques: Proteobacteria, Firmicutes, Actinobacteria and the TM-7 group (Rutz and Kieft 2004). The Actinobacterial sequences were related to *Corynebacterium*, *Propionibacterium*, *Arthrobacter* and the *Blastoccocus-Geodermatophilus* groups. Tap water UMC or dwarf bacteria cultivation show that most of UMC cultures fell into similar phyla and classes as such as α-Proteobacteria, β-Proteobacteria and γ-Proteobacteria, Firmicutes, and Actinobacteria, which have not been cultivated before (Figs 2 and 3).

Nine out of twelve Actinobacterial ultramicocells cultures belonged to suborder Micrococcinaceae, which are aerobic, catalase-positive *cocci* and known to be pleomorphic, or exhibit a rod-coccus growth cycle. Only two UMC isolates belonged to suborder Propionibacterineae, which are a facultatively anaerobic (or aerotolerant), nonmotile rods, although cells may be coccoid (Table 1). It seems that the spherical or coccoid form of Gram positive bacteria could be an advantage for starvation survival in harsh environments, although, it is accepted that most cells with diameters equal to or below 0·2 μm are rods, while cocci are numerically of minor importance in natural aquatic systems (Siegele and Kolter 1992; Velimirov 2001). The presence of the Micrococcaceae PVC(72h)5, PVC(14d)4 and Staphylococci (PVC(0·1)A in both 0·2 and 0·1 μm filtrate tap water shows that viable ultra spherical cells are present in an aquatic environments and are able to reproduce on Alpha agar. The bacterial cell shape is a selectable feature that aids survival and nutrient access, attachment to surfaces and the need to escape predators (Young 2006). These are some of the forces that bacteria respond to by performing a type of calculus, integrating over a number of environmental and behavioural factors to produce a size and shape that are optimal for the circumstances in which they live.

This proposed UMC ultrafiltration and cultivation method largely relies on known phenomena that have been described previously but have not been adopted (Marshall 1985; Hermansson and Marshall 1985; Power and Marshall 1988; Bowden and Li 1997; Jones *et al.* 1999; Miteva and Brenchley 2005).

The continuous filtration of tap water in the flow system model that was used in this study might increase the chance of UMC encountering higher nutrient concentrations and surface interactions by passing through the filters ultra-pores, and hence cultivation of early plankton UMC isolates (Table 1). In addition PVC and latex tubes can be a significant source of nutrients leaching and are known to favour the viability of bacteria (Martínez-Martínez *et al.* 1990). However, cultivation of 0·2 μm filtrate of 10 ml deep groundwater (200 m) on Alpha agar reproduced 16 independent colonies of planktonic UMC. In addition the filtration of old PVC pipe biofilm isolates PVC1-8 in this work is more evidence that even without long filtration time, Alpha agar is capable of supporting growth.

It seems that microscopic observation of microcolony development on Alpha agar at early stages is a very important step for diverse UMC isolation and cultivation. All cultivated UMC isolates grew as microcolonies and were detected under microscopic magnification (up to ×400, Fig. 1). The microcolony development by starved bacteria and UMB was documented (Torrella and Morita 1981; Zengler *et al.* 2002) from seawater hetrotrophs, soil and clinical isolates from patients (Borderon and

Horodniceanu 1978; Trulzsch *et al.* 2003). The formation of microcolonies may be an effective mechanism that may allow bacterial biofilms to resist protozoan grazing and to persist in the environment (Matz *et al.* 2004; Sriramulu *et al.* 2005; Queck *et al.* 2006). Currently, researchers in fields of clinical microbiology studying bacterial biofilm development are using an artificial sputum medium (poor medium) to mimic growth of *Pseudomonas aeruginosa* in the cystic fibrosis lung habitat (Sriramulu *et al.* 2005).

Extensive attempts have been made to isolate typical freshwater bacterial representatives and in particular Actinobacteria, but without great success (Glockner *et al.* 2000; Pernthaler *et al.* 2001; Zwart *et al.* 2002). In Bruns *et al.* 2003; enriched two members of the Actinomycetales, which reached high numbers in the natural bacterioplankton assemblage by a complex cultivation approach. Hahn *et al.* (2004) were the first to describe the isolation of nine freshwater members of the class Actinobacteria and the family Microbacteriaceae. The nine isolate strains were UMB isolated from habitats representing a broad spectrum of ecosystems, ranging from deep oligotrophic lakes to shallow hypertrophic lakes.

The recent development of new oligonucleotide probes for different clusters of freshwater Actinobacteria (Warnecke *et al.* 2004, 2005; Allgaier and Grossart 2006) enables high-resolution analyses of freshwater Actinobacterial communities by fluorescence *in situ* hybridization (FISH) using specific probes. The abundance of lake-specific Actinobacterial lineages was determined by phylogenetic comparison of cloned 16S rDNA sequence fragments.

The growth state as well as the diversity and seasonal dynamics of Actinobacteria populations show that the majority of all obtained 16S rRNA encoding sequences belonged to the *acI* and *acIV* clusters. Several new clusters and subclusters were discovered (*acSTL*, *scB1-4* and *acIVA-D*), but with a total of a few existing isolates of freshwater Actinobacteria that belong to the *acII* cluster and to the newly proposed *acSTL* cluster (Allgaier and Grossart 2006). Those isolates were derived from totally different habitats, such as lakes, the German Wadden Sea, paddy soil, or a phenol-degrading bioreactor. However, as a result of the lack of cultivation methods, almost nothing is known about their physiology and hence their ecological role in the freshwater environment.

To my knowledge, this is the first article that shows culturable UMC and microcolony formation by phylogenetically diverse isolates such as Actinobacteria, α-Proteobacteria, β-Proteobacteria, γ-Proteobacteria and Firmicutes, from drinking and/or freshwater (Fig. 2 and Table 1), as well as the cultivation of different Actinobacteria family representatives, such as Nocardiaceae, Nocardioidaceae, Microbacteiaceae, Micrococcaceae and Dermabacteraceae (Fig. 3). The majority of cultured Actinobacteria isolated in this study are distinct from those of already-cultured and uncultured freshwater Actinobacteria based on their 16S rRNA encoding gene sequences; although, the phylogenetic tree (Fig. 3) shows that the UMC belong to new clusters and subclusters phylogenetically related and bordered between clusters *acII* and the newly proposed *acSTL* cluster (Allgaier and Grossart 2006).

This study describes an approach which consists of a size selective technique of ultrafiltration, bacterial surface attachment (to enable bacteria to change from a survival existence in the aqueous phase to an active growth mode at the solid surface). Microcolony cultivation and isolation make this approach an affordable simple technique for UMC cultivation, and shows that potable chlorinated drinking water hosts phylogenetically diverse UMC (0·2 and 0·1 μm filterable) which adhere to pipe walls and induce biofilm formation.

Although no UMC belonging to the enteric bacteria involved in waterborne infections have been isolated during this study, it is tempting to speculate that starvation survival and biofilm formation might be a strategy adopted by most bacteria inhabiting water. These include some opportunistic pathogens such as *S. maltophila*, *Microbacterium* sp., *Pandoraea* sp. and *Afipia* strains which have been isolated as UMC biofilm in this study and could be used as a good example.

Besides the environmental aspect of this study, it is known that tap water harbours potentially pathogenic micro-organisms that can pose a significant health threat to patients, especially those people with compromised immune systems and haemodialysis (Ledebo and Nystrand 1999; Gomila *et al.* 2005). For decades, hospital water sources have been known to be reservoirs of nosocomial pathogens, especially organisms of the *Pseudomonas* sp. yet guidelines for preventing such infections do not exist (Anaissie *et al.* 2003; Merlani and Francioli 2003). Nowadays, specialists in infectious diseases are considering point-of-use filters. They presume that the filters achieve a greater than 99% reduction in total heterotrophic plate count of bacteria in the immediate and postflush samples (Sheffer *et al.* 2005; Trautmann *et al.* 2005). They conclude that such filtration units would prevent exposure of high-risk patients to waterborne pathogens. However, the results of this study show that viable UMC (VBNC, starved and dwarf bacteria) from water systems could be cultured on Alpha agar, as early as 72 h post, 0·2 μm filtration. The presence of those UMC in water filtrates will remain undetected by the standard and total heterotrophic plate count.

From the results in this study, one might conclude that the filtration strategies may remove most microbes from water, but cannot prevent it. The ability of UMC to pass

through the 0·2 μm filters and even 0·1 μm filters might select for new emerging pathogens from diverse phyla such as *S. maltophilia* and *Pandoraea* sp. which are well recognized as pathogens among cystic fibrosis patients (Table 1; Vonberg and Gastmeier 2005). Using shower 0·2 μm filters for as short as 15 days (as recommended) can allow the passage for enough UMC and biofilm formation on the outer surface of the filters that might become a source of opportunistic bacteria. For these reasons, the proposed strategies of using 0·2 μm filters to reduce hospital-acquired infections should be re-examined.

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