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ORIGINAL ARTICLE

Isolation and characterization of low nucleic acid (LNA)-content bacteria

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Most planktonic bacteria are 'uncultivable' with conventional methods. Flow cytometry (FCM) is one approach that has been taken to study these bacteria. In natural aquatic environments, bacteria with high nucleic acid (HNA) and low nucleic acid (LNA) content are commonly observed with FCM after staining with fluorescent dyes. Although several studies have focused on the relative abundance and *in situ* activities of these two groups, knowledge on the growth of particularly LNA bacteria is largely limited. In this study, typical LNA bacteria were enriched from three different freshwater sources using extinction dilution (ED) and fluorescence-activated cell sorting (FACS). We have shown for the first time that LNA bacteria can be isolated and cultivated by using sterile freshwater as a growth medium. During growth, the typical LNA characteristics (that is, low-fluorescence intensity and sideward scatter (SSC)) remained distinct from those of typical HNA bacteria. Three LNA pure cultures that are closely affiliated to the *Polynucleobacter* cluster according to 16S rRNA sequencing results were isolated. Owing to their small size, cells of the isolates remained intact during cryo-transmission electronic microscopy examination and showed a Gram-negative cell-wall structure. The extremely small cell volume (0.05 μm^3) observed for all three isolates indicates that they are among the smallest free-living heterotrophic organisms known in culture. Their isolation and cultivation allow further detailed investigation of this group of organisms under defined laboratory conditions.
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Introduction

Planktonic bacteria are ubiquitous in aquatic environments and an average density of 10^6–10^7 cells ml^{-1} is typically observed in surface freshwater (Whitman *et al.*, 1998; Wang *et al.*, 2007). Not only are these organisms present in large numbers, but they also play a critical role in the turnover of organic matter and nutrient cycling in aquatic environments (Billen *et al.*, 1990). However, a large fraction of these planktonic cells are difficult to study outside of their natural habitats. Owing to the low-nutrient concentrations that prevail in freshwater environments, these cells are typically smaller, lower in cell number and less active than cells grown under laboratory conditions, and hence pose several difficulties to conventional cultivation and analysis methods. Microbiologists have used various different names and terminologies to classify aquatic bacteria. Terms like 'oligotrophs', 'viable-but-not-cultivable (VBNC) bacteria', 'ultramicrobacteria' and 'uncultivable bacteria' have all been used to describe various bacterial groups (for example, Oliver, 1993; Morita, 1997; Schut *et al.*, 1997; Kajander and Ciftcioglu, 1998). With the increasing use of flow cytometry (FCM) as a tool in aquatic microbiology research, two new terms have been assigned to planktonic bacteria visualized using this particular method. It started with the names of 'Group I cells' and 'Group II cells' proposed by Li and co-workers (1995), which were then renamed to 'low-DNA (LDNA) bacteria' and 'high-DNA (HDNA) bacteria', respectively, by Gasol *et al.* (1999), and again later modified to 'low-nucleic acid content (LNA) bacteria', and 'high-nucleic acid content (HNA) bacteria' by Lebaron *et al.* (2001). The terms 'LNA' and 'HNA' bacteria have since then been most widely used by researchers. This broad classification of the two groups is based on their distinctly

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different fluorescence intensity and sideward scatter (SSC) signals detected by FCM in combination with nucleic-acid stains (see Figure 1a; Gasol *et al.*, 1999; Lebaron *et al.*, 2001). Fluorescence intensity, in this respect, is used as an indicator of apparent cellular nucleic-acid content (Gasol *et al.*, 1999; Lebaron *et al.*, 2001) and SSC signals have been applied as an indication of cellular size (Lebaron *et al.*, 2001; Felip *et al.*, 2007). This classification is essentially method-specific; up to now, no other method has specifically recognized these two distinct groups. Nonetheless, numerous studies were published on the topic of LNA and HNA bacteria, focussing mainly on the relative abundance of LNA and HNA bacteria in aquatic environments, their *in situ* activities and phylogenetic identity (for example, Jellett *et al.*, 1996; Marie *et al.*, 1997; Zubkov *et al.*, 2001; Lebaron *et al.*, 2002; Jochem *et al.*, 2004; Longnecker *et al.*, 2005).

A review of these studies shows clear contradictions and controversies in many crucial points.

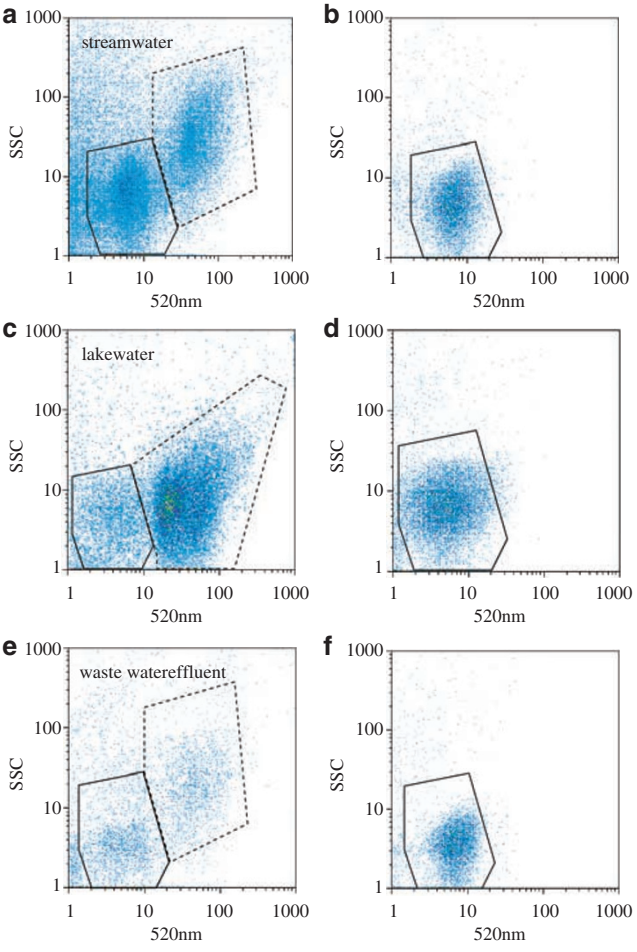

Figure 1 FCM dot-plots of the total bacterial community (**a**) and LNA bacterial enrichment culture (**b**) from stream water; the total bacterial community (**c**) and LNA bacterial enrichment culture (**d**) from lake water; the total bacterial community (**e**) and LNA bacterial enrichment culture (**f**) from wastewater effluent. The solid line indicates LNA bacteria and the dashed line HNA bacteria.

High-nucleic acid bacteria are usually regarded as the active part of the microbial metabolic group, whereas LNA bacteria are considered inactive (Lebaron *et al.*, 2001; Lebaron *et al.*, 2002; Servais *et al.*, 2003; Tadonleke *et al.*, 2005). However, there are contrasting reports showing that LNA bacteria are an active part of microbial communities in seawater (Zubkov *et al.*, 2001, 2005) and in freshwater (Nishimura *et al.*, 2005). This already poses the essential question of whether LNA bacteria represent a unique group of organisms, or whether they are merely a different physiological state of HNA cells, or even a combination of both (Bouvier *et al.*, 2007). In addition, all attempts to describe the phylogenetic differences between the two groups have so far resulted in contradictions. Some researchers reported that both groups are composed of the same dominant species (Servais *et al.*, 2003), whereas others have argued that they are phylogenetically different (Zubkov *et al.*, 2002). Furthermore, scenarios have been proposed in which bacterial cells may switch their phenotype from one group to the other (Bouvier *et al.*, 2007). These problems and contradictions stem at least partially from the fact that no LNA bacteria have been isolated until now. Having such isolates would facilitate a better understanding of the nature and importance of these bacteria.

In this study, we tested the hypothesis that LNA bacteria represent a unique and viable fraction of indigenous aquatic microbial communities. The specific objectives of this study were to (1) show the universal presence of LNA bacteria in freshwater; (2) show that LNA bacterial cells are able to grow under laboratory conditions without changing their unique LNA characteristics (that is, low-fluorescence intensity and low-SSC signal); and (3) isolate, cultivate and characterize bacterial communities/strains that maintain unique LNA characteristics throughout cultivation and that can be used for further studies. The experimental approach and results presented in this study may open a new page on the investigation and understanding of indigenous planktonic bacteria in aquatic environments.

Materials and methods

Source waters

Freshwater samples were collected in autoclaved Schott bottles (0.5 l) from six different water sources: Chriesbach stream (Dübendorf, Switzerland), Lake Greifensee (Uster, Switzerland), a small alpine stream (Luzern, Switzerland), non-chlorinated tap water (Dübendorf, Switzerland), groundwater (Vittel, France) and wastewater treatment plant effluent (Dübendorf, Switzerland). The temperature of the source waters was in the range of 4–12 °C at the time of sampling. All samples were kept below 8 °C during transportation and were processed within 24 h after sampling. Samples from all source

waters were analyzed using FCM to determine the total-cell concentration and specific-cell concentrations for LNA and HNA bacteria separately (as described below).

Flow cytometry

Flow cytometric measurements were carried out as described by Hammes *et al*. (2008). Bacterial cells were stained with 10 µl ml^{-1} SYBR Green I (1:100 dilution in dimethyl sulfoxide; Molecular Probes, Carlsbad, CA, USA), and incubated in the dark for 15 min at room temperature before measurement. Flow cytometry was carried out using a CyFlow Space instrument (Partec, Hamburg, Germany) equipped with a 200 mW laser, emitting at a fixed wavelength of 488 nm, and volumetric counting hardware. Green fluorescence was collected at 520 (± 20) nm, red fluorescence was collected above 630 nm and all data were analyzed with the Flowmax software (Partec). All samples were processed at a speed of 200 µl min^{-1}. Where necessary, samples were diluted before measurements in cell-free water, so that the concentration measured in the flow cytometer was always <500 events sec^{-1}. The specific instrumental gain settings for these measurements were as follows: green fluorescence = 450, red fluorescence = 550, SSC = 300. This setting was used for all experiments in this study. LNA and HNA cell populations were gated on the two-parameter dot-plot of green fluorescence (520 nm) against sideward scatter (SSC) and counted separately (see Figure 1). Geometrical mean (GMean) values for green fluorescence (520 nm) and SSC for both the LNA and HNA bacteria were recorded. All samples were measured in triplicate. The standard instrument error on FCM measurement was always below 3%. The detection limit of the instrument used in this study was about 200 cells ml^{-1} (Hammes *et al*., 2008).

Continuous cultivation of an indigenous LNA bacterial community

Indigenous LNA and HNA bacterial communities from stream water (Chriesbach stream) were separated by 0.45 µm filtration (see Supplementary information), and the resulting crude LNA fraction was used to inoculate 0.2 l bioreactors for continuous cultivation (Figure 2). Autoclaved and filtered (0.22 µm) stream water (from the same source) was supplied as growth medium (average dissolved organic carbon (DOC) = 3 mg l^{-1}; assimilable organic carbon (AOC) = 300 µg l^{-1}), and the reactors were operated at 12.0 ± 0.1 °C for 150 h at a dilution rate of 0.08 h^{-1}. The culture was continuously mixed at the speed of 100 r.p.m. The theoretical washout line was calculated as follows (Wick *et al*., 2002):

$$c_t = c_0 \times e^{-D \times t} \quad (1)$$

where c_t is cell concentration in the reactor at time t, c_0 is the initial cell concentration at time 0 and D is the dilution rate.

Samples from the reactor were taken at regular time intervals (every 10–20 h) and analyzed with FCM as described above. No substantial wall growth was observed in the reactor. The sterility of the medium was checked every 24 h with FCM.

Enrichment and isolation of LNA bacteria

LNA bacteria were enriched from three different source waters (Chriesbach stream, Lake Greifensee and wastewater effluent). Source waters were sampled with a 1-litre Duran flask (Schott, Germany), pasteurized (30 min, 60 °C) and filtered through 0.1-µm-pore-size sterile syringe filters (PVDF, Millipore, Billerica, MA, USA) to remove most particles. The filters were washed with at least 200 ml of carbon-free water before use to eliminate residual organic carbon (Vital *et al*., 2008). This sterile freshwater from each source was then used as the growth medium for the enrichment experiments. LNA bacterial communities from the three source waters were obtained by separation of LNA and HNA bacterial fractions with 0.45 µm filtration (see Supplementary Figure 1). The cells passing through this filter were used to inoculate further enrichment experiments using either the extinction-dilution (ED) technique or fluorescence-activated cell sorting (FACS) (Figure 2). Extinction-dilution experiments were carried out in carbon-free glass vials (as described in Supplementary information) containing 15 ml of sterile freshwater. The vials were inoculated with the filtrate (after determination of the cell concentration by FCM) to final cell concentrations of 100 cells ml^{-1}, 10 cells ml^{-1}, and 1 cell ml^{-1}, respectively. For each dilution, 24 vials were prepared (including three as blanks). Each inoculum was cultivated in the source water from where it originated. All the cultures were incubated at 20 °C for 14 days, which was sufficient to attain

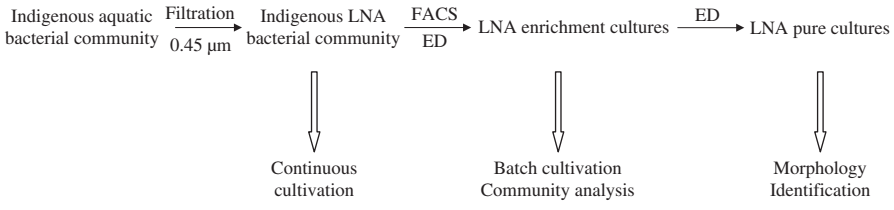

Figure 2 Experimental flow chart, indicating the terminology used and experiments carried out in the current study. FACS; fluorescence-activated cell sorting; ED; extinction dilution.

stationary phase, if growth occurred. After such a growth cycle, 1 ml samples were withdrawn from each vial of all dilution series and analyzed using FCM as described above. Cultures containing only LNA bacteria were selected for further ED and re-growth experiments. Such cycles were repeated until a stable LNA enrichment culture (that is, the culture that maintains the typical LNA characteristics) was obtained. In an alternative approach to obtain LNA enrichment cultures, cell sorting was employed using a BD FACS Aria instrument (Becton Dickinson, Franklin Lakes, NJ, USA). Samples were stained with $10\,\mu l\,l^{-1}$ SYBR Green I (Molecular Probes). LNA bacteria from three different sources (Chriesbach stream, Lake Greifensee and wastewater effluent) were sorted into 96-well plates (50 cells per well) containing 150 µl sterile source water (as described above) in each well. For each water source, three plates were used. After sorting, the plates were closed with their matching plastic cover and sealed with para-film to avoid evaporation. All the cultures were incubated at 20 °C for 14 days until stationary phase was reached. After incubation, a sample (50 µl) was withdrawn from each well and analyzed with FCM. Cultures containing only typical LNA bacteria were selected for further investigation. LNA enrichment cultures (obtained by either ED or FACS) were used for a first investigation of the growth properties of typical LNA bacteria and further isolation of LNA pure cultures (Figure 2). For the final step of achieving LNA pure cultures from the enrichment cultures, further ED experiments were carried out with initial concentrations of 1 cell per vial (as described above). The terms used to describe the cultures in each step and the experimental design are illustrated in Figure 2.

Batch growth characterization of LNA enrichment cultures

Three LNA enrichment cultures, obtained from three separate sources (Chriesbach stream, Lake Greifensee and wastewater effluent), were cultivated in sterile cell-free stream water (Chriesbach, Dübendorf) ($DOC = 2.6–3.3\,mg\,l^{-1}$; $AOC = 200–400\,\mu g\,l^{-1}$; pH = 7.7–7.9; conductivity = 402–502 µS). The water sample preparation procedure was described above. The initial cell concentration after inoculation was 1×10^4 cells ml^{-1} at all temperatures ranging from 12 to 37 °C in 20 ml carbon-free vials (with 15 ml medium; as described in Supplementary information). Aliquots (1 ml) were taken at regular time intervals ($t = 0$, 15, 24, 39, 68, 89, 95, 112, 119 and 138 h for cultivation at 12 °C and $t = 0$, 15, 23, 15, 39, 41, 47 and 68 h for cultivation at 20, 25, 30 and 37 °C) until stationary phase was reached. Samples were analyzed with FCM and for adenosine triphosphate (ATP) (as described in Supplementary information). All experiments were carried out in triplicate and without shaking. The specific growth rate (μ) based on cell number increase in each sample was determined as follows (Equation 2) (Vital *et al.*, 2007; Wang *et al.*, 2007):

$$\mu = (\mathrm{Ln}(n_t) - \mathrm{Ln}(n_0))/\Delta t \qquad (2)$$

where n_t and n_0 are the cell concentrations measured at two subsequent time points and Δt is the time interval between these points.

Comparison of batch culture growth

An enrichment of an indigenous bacterial community (Chriesbach stream, Dübendorf) that is normally used for the determination of AOC (Hammes and Egli, 2005; Vital *et al.*, 2007) was used as the reference HNA enrichment culture (see Supplementary Figure 2). A basic characterization of this HNA enrichment culture was documented earlier (Wang *et al.*, 2007). The HNA enrichment culture was used for a comparison with the growth characteristics of the LNA enrichment cultures. Both HNA and LNA enrichment cultures were cultivated in sterile cell-free stream water (Chriesbach, Dübendorf) (as described above). The initial cell concentration was 1×10^4 cells ml^{-1} and the initial medium volume was 15 ml. The comparison was carried out at 20 °C without shaking. Samples (1 ml) were taken every 2–10 h for FCM (as described above) and ATP determination (see Supplementary information). Average cell sizes were estimated from the GMean SSC values recorded using FCM (as described in Supplementary information).

Microbial community analysis

Catalyzed reporter deposition–fluorescence *in situ* hybridization (CARD–FISH) was carried out on LNA enrichment cultures according to the modified permeabilization protocol developed for freshwater bacterioplankton (Sekar *et al.*, 2003). The probes for bacteria, β-proteobacteria, α-proteobacteria, cytophaga–flavobacterium–bacteroides, and actinobacteria were used in the current study (Warnecke *et al.*, 2005). Details of the procedure are documented by Pernthaler and Pernthaler (2007). Counterstaining of CARD–FISH preparations with 4,6-diamidino-2-phenylindole (DAPI) and mounting were carried out as described earlier (Pernthaler *et al.*, 2002). Total bacterial abundances and the fractions of FISH-stained bacteria in at least 1000 DAPI-stained cells per sample were quantified at a 1000-fold magnification using an epi-fluorescence microscope (Leica, Wetzlar, Germany).

Total DNA was extracted from the three enrichment cultures and the corresponding source water samples using the UltraClean Water DNA Isolation Kit (Mo Bio, Carlsbad, CA, USA). Total bacterial community analysis was carried out by PCR-DGGE, using general bacterial primers P338F and P518r (Boon *et al.*, 2002). The processing of the DGGE gels was carried out with the Bionumerics software 2.0 (Applied Maths, Kortrijk, Belgium). The calculation of the dendrogram is based on the Pearson (product–

moment) correlation coefficient and the clustering algorithm of Ward (1963). Selected bands from the DGGE gel for the LNA enrichment cultures were cut out with a clean scalpel and added in 50 µl of PCR water. After 12 h of incubation at 4 °C, 1 µl of the PCR water was re-amplified with primer sets P338F and P518r (Boon *et al.*, 2002). Five µl of the PCR product was loaded on a DGGE gel (see above) and if the DGGE pattern only showed one band, it was sent out for sequencing. In case the DGGE band was not pure enough for sequencing, PCR fragments were cloned by using the TOPO TA cloning kit (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's instructions. DNA sequencing of the ca. 180 bp fragments was carried out by ITT Biotech-Bioservice (Bielefeld, Germany).

Rep-PCR genomic fingerprinting was carried out to discriminate the different isolates on a strain level, using the BOX-primers reported by Versalovic *et al.* (1994), and the BOX patterns were analyzed by TBionumerics software 2.0 (Applied Maths) as described above. Genomic DNA of LNA pure cultures was extracted (as described above), amplified and sequenced (see Supplementary information).

Scanning/transmission electron microscopy

LNA pure cultures were examined using both scanning electron microscopy (SEM) (see Supplementary information) and cryo-transmission electron microscopy (cryo-TEM). For cryo-TEM, a 4 µl aliquot of concentrated LNA pure culture was adsorbed onto an entirely carbon-coated grid (quantifoil, Jena, Germany), blotted with Whatman 4 filter paper and vitrified in liquid ethane at −178 °C. Frozen grids were transferred onto a Philips CM200-FEG electron microscope (Philips, FEI, Eindhoven, The Netherlands) using a Gatan 626 cryo-holder. Electron micrographs were recorded at an accelerating voltage of 200 kV and a magnification of ×50 000, using a low-dose system ($10e^{-}$ Å^{-2}) and keeping the sample at −175 °C. Defocus values were −3 µm. Micrographs were recorded on Kodak SO-163 films and then digitized with Heidelberg Primescan 7100 (Heidelberg Primescan, Heidelberg, Germany) at 4 Å per pixel resolution at the specimen level.

The cell volume of the individual bacterial cell was calculated based on the measurements from cryo-TEM pictures (Equation 3, Wang *et al.*, 2008) on the principle of a rod-shaped particle with spherical ends.

$$\text{Cell volume } (\mu m^3) = \frac{4}{3} \times \pi \times r^3 + \pi \times r^2 \times (l - 2r) \quad (3)$$

where r represents half of the smallest width and l represents the length of the bacterial cell.

Results

Presence of LNA bacterial communities in different aquatic environments

Typical LNA and HNA bacterial communities were observed in all aquatic samples tested, ranging from an extremely oligotrophic (alpine stream, DOC = 0.5 mg l^{-1}) to a relatively copiotrophic environment (wastewater effluent, DOC = 7.0 mg l^{-1}). The two communities are clearly distinguishable on FCM dot-plots of green fluorescence against sideward scatter (SSC) (Figure 1). LNA bacteria were numerically dominant (>50%) in the planktonic microbial communities from most sampled freshwaters, and there was no relation between their relative abundance and either the total bacterial abundance or the DOC content of the water (Table 1).

Continuous cultivation of an indigenous LNA bacterial community

A rough separation of HNA/LNA bacterial communities was achieved through 0.45 µm filtration, which retained about 90% of the HNA bacteria, while allowing most of the LNA bacteria to pass (Figure 2, Supplementary Figure 1). In a first experiment to test the viability of these separated LNA bacterial communities, we applied the above-mentioned filtration step and inoculated the filtrate into sterile river water. Growth of an indigenous LNA bacterial community was tested using continuous cultivation at a low dilution rate in sterile stream water at 12 °C, selected because of the low temperature (10–12 °C) recorded in the source water. The LNA bacterial community established in the continuous culture at a 'steady-state' level of 1.2×10^5 cells per ml over 150 h at a dilution rate of 0.08 h^{-1} (Figure 3). If all the cells in the LNA bacterial group were inactive or dead, they would have washed-out from the reactor under the continuous cultivation conditions used (Figure 3). During the entire period of continuous cultivation, the LNA bacterial community maintained its typical LNA characteristics, that is, low fluorescence intensity and low SSC signal (Figures 3b and c).

Enrichment and cultivation of LNA bacteria

Typical LNA bacteria were enriched and cultivated from three different freshwater samples with a batch growth assay using natural AOC from sterile fresh-

Table 1 Percentage of LNA bacteria in total bacterial communities from different aquatic environments

Source	*DOC (mg l^{-1})*	*Total cell concentration (10^6 cells ml^{-1})*	*Percentage of LNA*
Chriesbach stream	3.0 ± 0.3	3.21 ± 0.20	68% ± 2%
Alpine stream	0.5 ± 0.1	0.10 ± 0.00	74% ± 5%
Tap water	0.7 ± 0.1	0.15 ± 0.01	53% ± 1%
Groundwater	0.5 ± 0.2	0.31 ± 0.02	75% ± 3%
Lake Greifensee	3.2 ± 0.3	2.41 ± 0.23	23% ± 5%
Wastewater effluent	7.0 ± 0.5	10.6 ± 1.20	58% ± 2%

Results are shown in the format of average ± standard deviation calculated from at least three samples for each source location.

water (Hammes and Egli, 2005; Vital *et al.*, 2007; Wang *et al.*, 2007) in combination with FACS or the ED technique. The FACS/ED steps were essential, as we have observed that during a direct batch cultivation of indigenous bacterial communities, HNA bacteria always overgrew LNA bacteria (data not shown). Using FACS, 1 out of 24 samples from the Chriesbach stream showed positive growth of LNA bacteria after 14 days incubation (at least 10^5 cells ml^{-1}). However, no positive results were obtained for the samples from Lake Greifensee and the wastewater effluent. With the ED approach, all waters gave about three positive results out of 21 assays when inoculated with 10 cells ml^{-1}. However, all samples inoculated with 100 cells ml^{-1} showed either HNA bacterial growth or a mixture of LNA and HNA bacteria. The positive LNA samples were re-inoculated (10 cells ml^{-1}) into sterile natural freshwater and incubated for another 14 days. Such cycles were repeated until a stable LNA bacterial enrichment was achieved. Three enrichment cultures were obtained (Figure 1) from different aquatic environments. They are referred to as LNA enrichment culture A (enriched from the Chriesbach stream by FACS; Figure 1b), LNA enrichment culture B (enriched from Lake Greifensee by ED; Figure 1d) and LNA enrichment culture C (enriched from wastewater effluent by ED; Figure 1f). Their positions on FCM dot-plots correspond to the original LNA fraction in the water from which they were enriched (Figure 1 and Table 2).

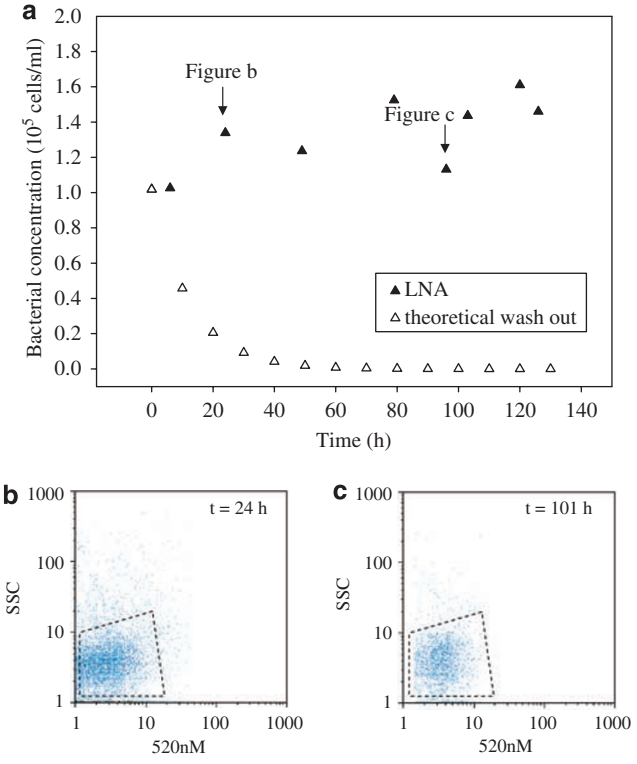

Figure 3 Continuous cultivation of a stream water LNA bacterial community with sterile stream water ($D = 0.08\,h^{-1}$) at 12 °C (**a**). The theoretical wash-out line was calculated based on the dilution rate and initial cell concentration. FCM dot-plots at $t = 24$ h (**b**) and 101 h (**c**) show that the LNA characteristics did not change.

After successful enrichment, the growth properties of the different LNA enrichment cultures were further characterized during batch cultivation. All three LNA enrichment cultures were capable of growing on natural AOC at different temperatures, while maintaining their typical LNA characteristics. Growth of LNA enrichment cultures was observed from 12 to 30 °C and a typical example for culture A is shown in Figure 4. Generally, a longer lag phase was observed for the growth at 12 °C compared with that at higher temperatures. The maximum specific growth rate (μ_{max}) increased with ascending incubation temperature up to 30 °C, whereas no growth was detected at 37 °C. The recorded μ_{max} values were as follows: 0.10 h^{-1} (12 °C); 0.23 h^{-1} (20 °C); 0.31 h^{-1} (25 °C); and 0.37 h^{-1} (30 °C). Irrespective of the cultivation temperature, all LNA enrichment cultures reached similar final cell concentrations when cultivated in the same water (Figure 4).

Comparison between batch growth of LNA and HNA enrichment cultures

During batch growth on sterile stream water, LNA and HNA enrichment cultures exhibited distinct characteristics. LNA and HNA enrichment cultures obtained from the same source water (Chriesbach stream) were compared for their growth properties with natural AOC at 20 °C. Similar lag phases were observed for both LNA and HNA enrichment

Table 2 Growth characteristics of three separately isolated LNA enrichment cultures and a HNA enrichment culture in sterile freshwater (see Materials and methods)

	μ_{max} (h^{-1})	*Green fluorescence (GMean)*	*SSC (GMean)*	*Estimated cell volume* (μm^3)	*Cellular ATP* ($\times 10^{-17}$ *gATP per cell*)	*ATP per volume* ($\times 10^{-15}$ *gATP per* μm^3)
LNA A	0.23 ± 0.06	7.4 ± 0.6	4.6 ± 0.3	0.01 ± 0.01	1.2 ± 0.3	1.2 ± 0.2
LNA B	0.19 ± 0.05	5.9 ± 0.6	5.1 ± 0.5	0.02 ± 0.02	3.2 ± 0.4	1.6 ± 0.3
LNA C	0.24 ± 0.06	8.2 ± 0.6	4.1 ± 0.4	0.01 ± 0	1.5 ± 0.3	1.5 ± 0.3
HNA	0.31 ± 0.08	40.7 ± 1.7	11.6 ± 0.9	0.13 ± 0.02	15.5 ± 2.6	1.2 ± 2.6

GMean values of green fluorescence and SSC are data of stationary phase cultures. Results are shown in the format of average ± standard deviation, calculated from triplicate samples ($n = 3$) from each culture. Cell volume was estimated from the SSC value of the enrichment cultures (Equation S1).

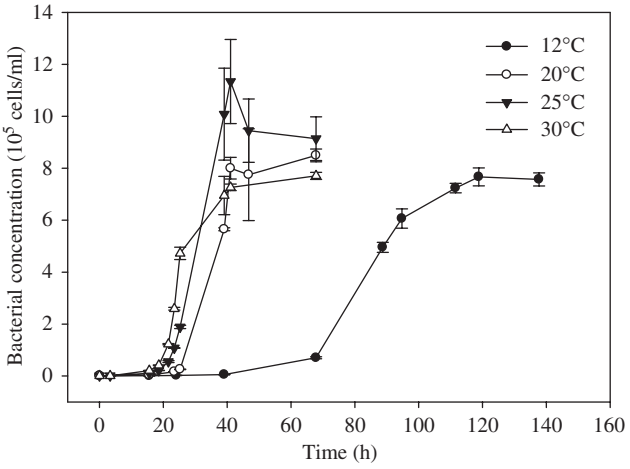

Figure 4 Batch growth of LNA enrichment culture A in sterile stream water at different temperatures. Error bars represent the s.d. for triplicate samples.

cultures (Figure 5a). The lag phase was followed by a short phase of exponential growth where the cells divided at a maximum rate for 2–3 generations, and then the rate gradually decreased until stationary phase was reached (Figure 5b). The maximum specific growth rate (μ_{max}) was higher for the HNA enrichment cultures ($0.31\,h^{-1}$) than that of the LNA enrichment cultures ($0.23\,h^{-1}$) (Figure 5b, Table 2). The HNA enrichment culture grew to a final concentration of 3×10^6 cells ml^{-1} and attained stationary phase after 60 h. The LNA enrichment culture grew only to half this density (1.6×10^6 cells ml^{-1}) and reached stationary phase already after 42 h (Figure 5a). The average cell size estimated from SSC values for the HNA enrichment culture showed an 18-fold change during the batch cultivation. In comparison, the change in cell size for the LNA enrichment cultures was much smaller (only 4-fold); however, a similar trend was observed (Figure 5a). During batch cultivation, the patterns in the specific growth rates were similar for LNA and HNA enrichment cultures (Figure 5b). The total ATP concentrations of both the LNA and HNA cultures increased concurrent to the total cell concentrations (Figure 5b). In general, the ATP concentration of LNA enrichment cultures was much lower than that of HNA enrichment cultures (0.04 vs 0.75 nM ATP at stationary phase), which can be directly attributed to the lower cell density and smaller apparent cell volume (Figure 5b and Table 2).

Phylogenetic diversity of LNA enrichment cultures

Using a dual approach of DGGE and CARD-FISH, we were able to pinpoint the identity of the three LNA enrichment cultures. The DGGE analysis showed the presence of multiple bands, with one or two dominant bands in all three LNA enrichment cultures (Figure 6a). However, the dominant bands

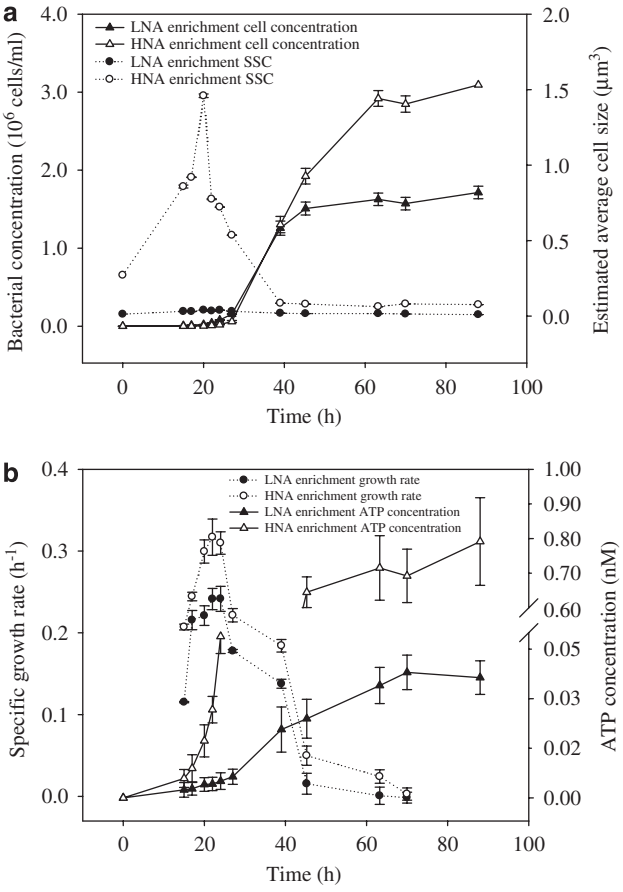

Figure 5 Growth comparison between LNA and HNA enrichment cultures. Cell concentration and size changes (**a**), and changes in specific growth rate and ATP concentration (**b**) during batch cultivation in sterile stream water at 20 °C. Error bars represent the s.d. for triplicate samples. Cell volume was estimated from the SSC value of the enrichment cultures (Equation S1).

in the LNA enrichment cultures (circled in Figure 6a) do not match the dominant ones in the total bacterial community, from where they originated (Figure 6a). Such results make sense in view of the highly selective procedures we carried out to enrich the LNA cultures. The dominant bands from each enrichment culture were further sequenced and compared with the sequences obtained from LNA isolates (see below). The CARD-FISH results corroborated the DGGE results. Most cells of the LNA enrichment cultures were affiliated to the β-proteobacteria cluster (80% for enrichment culture A, 72% for B and 88% for C). No α-proteobacteria were observed in all LNA enrichment cultures, but about 6% of enrichment culture B belonged to the Gram-positive domain Actinobacter and 3% of enrichment culture C hybridized with the Cytophaga–Flavobacterium probe. These results, especially the DGGE pattern, indicate that the enrichment cultures are dominated by one or two species. Hence, further purification steps were carried out to obtain LNA pure cultures.

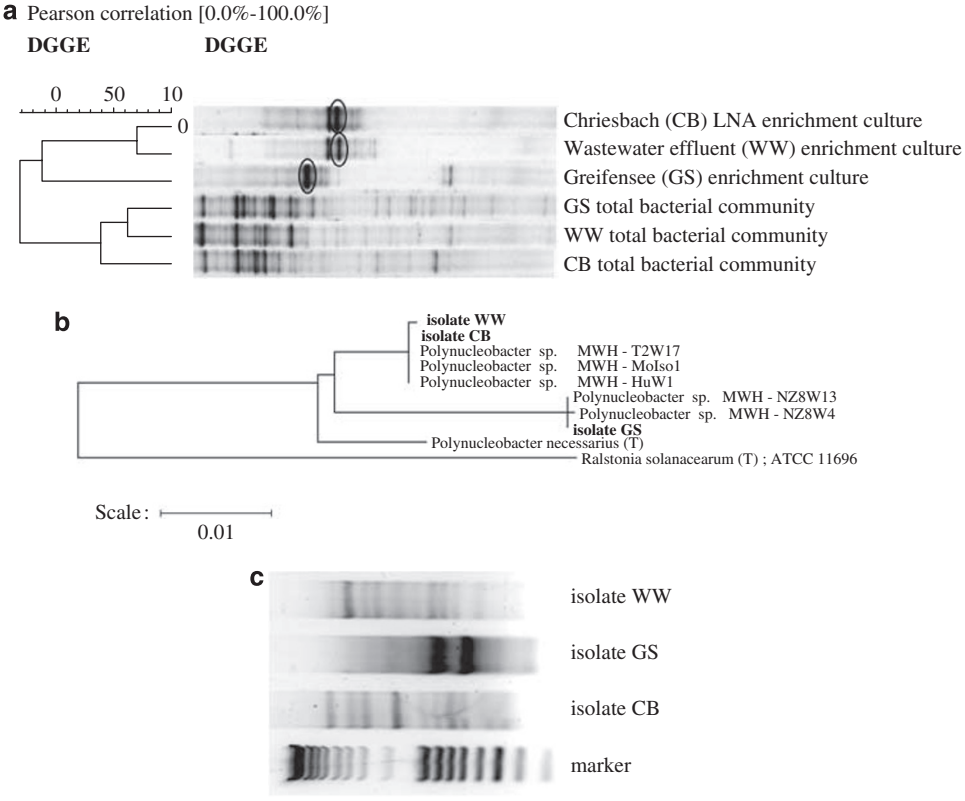

Figure 6 Phylogenetic analysis of LNA bacteria. (**a**) DGGE and clustering analysis of the water samples and the related LNA enrichment cultures. The distance matrix of all the possible gel tracks within the DGGE pattern was calculated by using the Pearson correlation. Bands that were further sequenced are encircled. (**b**) Phylogenetic tree of the three LNA isolates. (**c**) Rep-PCR of the LNA isolates from three different aquatic environments.

Isolation, cultivation, identification and morphological characterization of LNA pure cultures

Further ED (on average 1 cell per vial) was carried out using all three LNA enrichment cultures. Three isolates were obtained by this approach, that is, isolate CB (from enrichment A), isolate GS (from enrichment B) and isolate WW (from enrichment C). The 16S rRNA gene of each isolate was sequenced and the sequences were deposited in GenBank under the accession numbers EU780139, EU780140 and EU780141. The sequences matched 100% with those obtained from DGGE analysis of the dominant bands from the corresponding LNA enrichment cultures (Figure 6a), which confirms that the isolates were also the most dominant in the respective enrichment cultures. All three isolates were shown to be closely affiliated to the Polynucleobacter cluster (Figure 6b). Furthermore, repetitive extragenic palindromic-PCR (Rep-PCR) genomic fingerprinting of the three isolates indicated that they were different genotypes (Figure 6c).

The three pure LNA cultures were further examined with both SEM and cryo-TEM (Figure 7). Different cell shapes were observed for the different isolates. Isolate CB and WW appeared mainly as rod-shaped cells (Figures 7a, c, d and f), whereas those from isolate GS showed typically a vibriod morphology (Figures 7b and e). Cells of all isolates possess a cytoplasm full of ribosome particles and contain a diffuse nucleoid. The cell envelope was clearly seen around all cells. The excellent cell preservation of vitrified sample allowed visualizing the lipid bilayer of the plasma membrane (PM) and the outer membrane (OM) (inset in Figure 7f), showing that the cell envelope of these LNA isolates is structurally analogous to that of Gram-negative bacteria. The space between the PM and the OM (periplasmic space) is 20 nm wide and contains a layered structure similar to the peptidoglycan observed by cryo-sectioning of Gram-negative bacteria (Matias *et al.*, 2003). Furthermore, cells of isolate CB at different cellular cycle stages were imaged by cryo-TEM, documenting a clear cell division process (Figure 8).

During the sample preparation for SEM, the bacterial cells probably shrunk due to the dehydration. In contrast, the cells for imaging with cryo-TEM were frozen-hydrated in their native state. Owing to the well-kept shape and the volume preservation of the frozen-hydrated cells, the cell volume determination was carried out from the cryo-TEM micrographs. Cells of all isolates were in the range of 300–400 nm in width and 500–600 nm in length. The biovolume for the cells was on average below 0.05 μm^3. The periplasmic volume is relatively big for the cells. For isolate CB (Figure 7d)

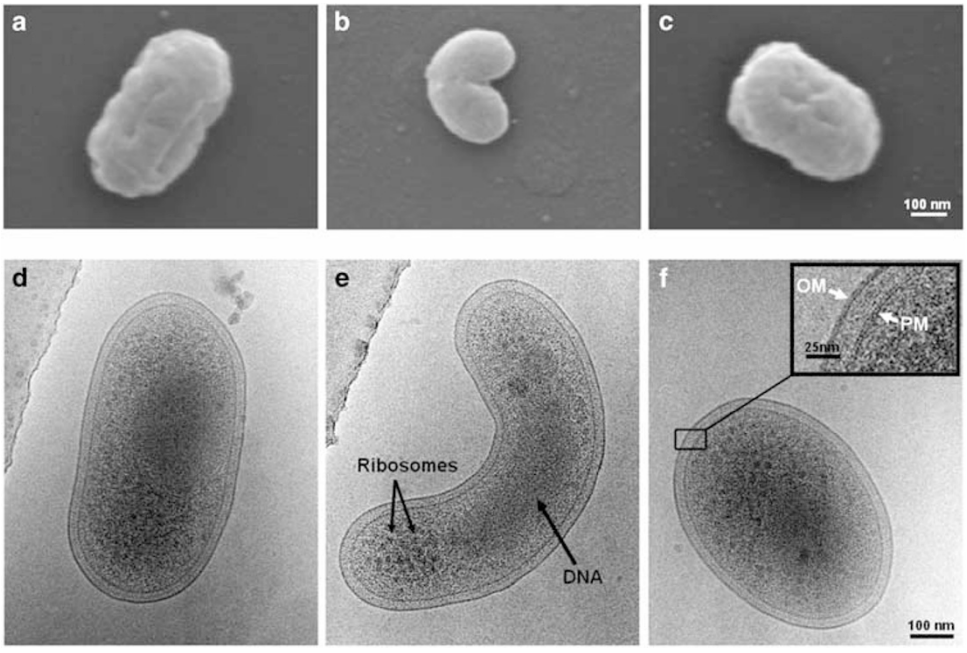

Figure 7 Scanning electron micrograph of LNA isolate CB (**a**), isolate GS (**b**) and isolate WW (**c**); and transmission electron micrograph of frozen-hydrated LNA isolate CB (**d**), isolate GS (**e**) and isolate WW (**f**). Inset in F is an enlarged micrograph to show the cell envelope structure of isolate WW.

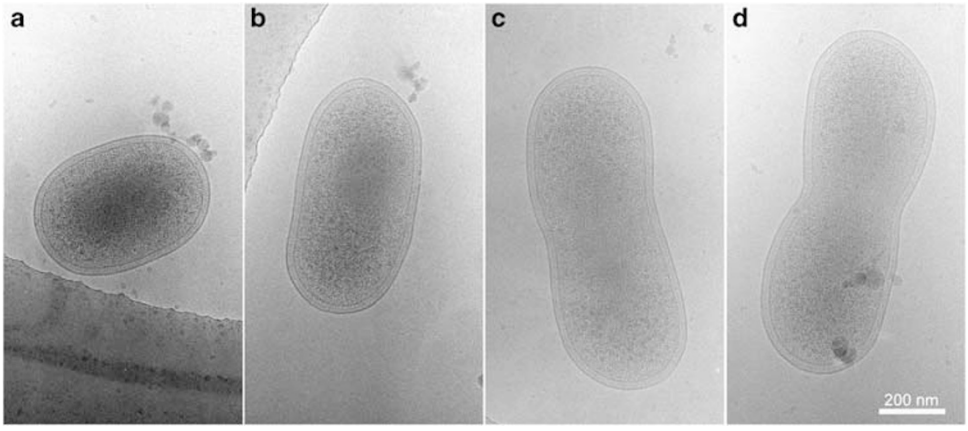

Figure 8 Transmission electron micrographs of four frozen-hydrated from LNA isolate CB at different stages of the division cycle (**a–d**).

it accounts for ca. 34% of the total cell volume, ca. 37% for isolate GS (Figure 7e) and ca. 23% for isolate WW (Figure 7f). The cell membranes are between 4–5 nm thick (inset in Figure 7f). The condensed dark region inside the cell is believed to be the chromosome DNA of the cell, which occupies >50% of the cytoplasmic area.

Discussion

Omnipresence of LNA bacteria in freshwater

The separation of natural aquatic microbiota into LNA and HNA bacterial groups is a typical FCM phenomenon based on cellular properties observed by FCM analysis after nucleic acid staining. This observation has been reported for virtually all aquatic environments, including sea water (that is, Li *et al.*, 1995; Gasol and Moran, 1999; Lebaron *et al.*, 2001; Jochem *et al.*, 2004), lakes and rivers (Nishimura *et al.*, 2005; Bouvier *et al.*, 2007), groundwater, drinking water and wastewater treatment plant effluents (Figure 1 and Table 1). Based on cell concentration, LNA bacteria and HNA bacteria are equally abundant in most aquatic microbial communities sampled in the current study (Table 1). Nonetheless, LNA bacteria are mostly reported to be the 'inactive' or even 'ghost' part of the microbial community (for example, Lebaron *et al.*, 2001; Servais *et al.*, 2003). In the present study, we

showed that LNA bacteria constitute a viable fraction of indigenous freshwater communities, and that bacteria that maintain unique LNA characteristics can be isolated and cultivated.

Cultivation of LNA bacteria

Despite the omnipresence of LNA bacteria in aquatic environments, there is little information on their growth and characteristics in the literature. To our knowledge, no LNA bacteria have been isolated or cultivated earlier. One of the main reasons for this is that LNA bacteria, like the majority of freshwater bacteria, cannot be cultivated in conventional growth media that employ high-nutrient concentrations. To circumvent this problem, we have used sterile river water as a growth medium. Sterile freshwater provides an undefined complex mixture of carbon compounds at low concentrations (in the range of $\mu gC\,l^{-1}$), similar to the natural habitat of many bacteria (Münster, 1993). Even though heat sterilization (either autoclaving or pasteurization) might alter the chemical properties of the water to a certain degree, sterile fresh/marine water has been proven to be a proper medium to cultivate 'uncultivable' bacteria from the natural aquatic environments (Rappé *et al*., 2002; Vital *et al*., 2007; Wang *et al*., 2007). We have shown that an indigenous LNA community can be maintained through continuous cultivation at low temperature in such sterile freshwater (Figure 3). The ability of the LNA bacteria to sustain a cell density of about 10^5 cells per ml over an extended period of time in continuous culture proved that a dominant fraction of the indigenous LNA bacterial community is actively multiplying without changing their LNA characteristics when provided with the correct cultivation conditions.

Enrichment of LNA bacteria through FACS or ED was a prerequisite for batch cultivation. All LNA enrichment cultures obtained in this manner showed their ability to grow in batch culture at various temperatures and maintain the typical LNA characteristics (Figures 4 and 5). Their growth rates were comparable to those of bacterial communities and other bacterial strains growing in carbon-limited freshwater (Hammes and Egli, 2005; Vital *et al*., 2007; Wang *et al*., 2007). It is interesting to notice that their optimal growth temperature was between 20 and 30 °C, which is much higher than the water temperature where they originated from (ca. 12 °C). This suggested that most of the planktonic bacteria in aquatic environments are not necessarily living at their optimum growth temperature (Morita, 1997; White *et al*., 1991; Rosenberg and Ben-Haim, 2002).

The successful cultivation of LNA bacteria indicates that the cultivation approach applied in the current study may also be used for cultivation of other so-called 'uncultivable' bacteria, which represent >99% of the natural microbial communities (Rappé and Giovannoni, 2003; Wang *et al*., 2007). Bouvier *et al*. (2007) proposed four scenarios for the relationship between LNA and HNA bacteria based on FCM parameters for each group, and suggested that each group consists of cells that are intrinsic to a group, and cells that may exchange between groups. Assuming the scenarios proposed by Bouvier and co-workers (2007) are right, we have found and cultivated strains that are intrinsic members of the LNA group bacteria and do not exchange with HNA bacteria.

Comparison between batch growth of LNA and HNA enrichment cultures

During the batch growth on sterile freshwater at the same temperature, the LNA enrichment cultures had lower-specific growth rates, smaller changes in average cell size and lower production of cell numbers in comparison with the HNA enrichment culture (Figure 5a and Table 2). The key question is then how these organisms can compete with HNA bacteria in natural environments. If the small cell size of LNA bacteria is taken into consideration, the biomass-specific production of LNA bacteria can be similar to that of HNA bacteria (Jochem *et al*., 2004). It has been reported that grazing pressure is heavier on middle-size class bacteria than on those with a small cell volume (Bernard *et al*., 2000; Grossart *et al*., 2008). Hence, the resistance of LNA bacteria to grazing may also partially explain why LNA bacteria, although having lower-specific growth rates, still persist in high numbers in most natural aquatic environments. Laboratory studies showed that *Cyclidium* species selectively graze on HNA communities (Tadonleke *et al*., 2005), and although small bacteria are also vulnerable to grazing, the grazing-related mortality was reported to be much lower (Boenigk *et al*., 2004).

During batch cultivation, LNA bacteria exhibit a less prominent size variation than HNA bacteria (Figure 5a). Still, distinct cell division and cell size change was observed by cryo-TEM (Figure 8), indicating a normal cell growth cycle. This phenomenon is again explainable by their extremely small cell size; changes within this size range are not easily detectable by FCM. In this study, flow cytometric SSC signals were used as an estimation of bacterial cell size (Felip *et al*., 2007; Vital *et al*., 2008). However, these estimations are used mainly for size comparison between LNA and HNA bacteria, and not for absolute cell size measurements.

The small cell volume of LNA bacteria is also closely linked to their low ATP content (Figure 5b and Table 2), which may explain why they are often regarded as an inactive or dead part of the microbial community (Berney *et al*., 2008). LNA bacteria have an apparently 10-fold lower ATP-per-cell content than HNA bacteria (Table 2). However, when the data are normalized based on cell volume, the ATP/biovolume for the LNA and HNA bacteria is quite similar ($1.2–1.6 \times 10^{-15}\,gATP\,\mu m^{-3}$). Our data suggest

that the ATP per microbial biovolume in active cells is a relatively constant value in the range of $1–2 \times 10^{-15}$ gATP μm^{-3} for both HNA and LNA bacteria when grown under similar conditions, which is similar to data reported recently (Eydal and Pedersen, 2007). Therefore, the failure to detect activity in LNA bacteria may be because of the limited sensitivity of the applied methods rather than their actual activity status.

Phylogenetic identity of LNA enrichment cultures

Observing that LNA enrichment cultures can grow actively with natural AOC and retain their LNA characteristics, the question 'what are they?' naturally follows. It has been documented earlier that the main bacterial population in freshwater environments consists of members of the β-proteobacterial group (Glockner *et al.*, 1999; Bruns *et al.*, 2003). Therefore, our finding that the cells of LNA enrichment cultures are affiliated mainly with β-proteobacteria is not surprising. The phylogenetic composition of LNA enrichment cultures, however, changes certainly with location (Figure 6a) and probably also with time, suggesting that also LNA bacterial communities in different aquatic environments may exhibit considerable diversity, which is consistent with earlier reports (Servais *et al.*, 2003; Longnecker *et al.*, 2005; Zubkov *et al.*, 2002).

Characterization of LNA pure cultures

One LNA pure culture was isolated from each of the LNA enrichment cultures in the current study, which corresponds to the dominant band shown by DGGE in the enrichment cultures (Figure 6a). The three isolates exhibit very similar growth properties as the enrichment cultures (Supplementary Figure 3). According to their 16S rRNA gene sequences and the REP-fingerprints, the three isolates are completely different strains, but are all closely related to the *Polynucleobacter* cluster (Figures 6b and c). Although the original *Polynucleobacter* type strain (*P. necessarius*) is a large and long obligate endosymbiotic bacterium having multiple nucleiods (Heckmann and Schmidt, 1987), several species of this genus were recently reported to be dominant in freshwater habitats (Page *et al.*, 2004; Hahn *et al.*, 2005). Recently, members of the *Polynucleobacter* linage were isolated from various sites by gradually enriching nutritional conditions during cultivation (Hahn, 2003). Although our isolates are phylogenetical by closely related to the *Polynucleobacter* spp. isolated by Hahn (2003), they have clearly different growth properties. Whereas the former grow (so far) at low-nutrient concentration only (Supplementary Figure 4), the latter form visible colonies on conventional high-nutrient agar. Further physiological and genetic data are required to confirm the exact phylogenetic position of these isolates and the relationship with their closest neighbors.

Cultivation and isolation of LNA bacteria not only facilitated investigation of their growth properties, but also gave us the chance to obtain their true portrait other than the cluster image on FCM dotplots. To date, no microscopic images have been published for pure LNA cultures. Here, for the first time, we report morphological characteristics of pure LNA cultures from freshwater (Figure 7). Owing to their extremely small cell size, there may be limited options of cell shape. Still, the cell shape of the three isolates covers quite diverse shapes, including a normal rod (Figure 7d), a vibrio shape (Figure 7e) and a short rod (Figure 7f). It is believed that surface-to-volume ratio is one of the reasons why most bacteria are rod-, filamentous- or vibrio-shaped, as non-spherical shape increases this ratio for the volume enclosed (Koch, 1996). Cell size estimated by electron microscopy indicates that the isolates obtained are among the smallest planktonic bacteria known in culture (Schut *et al.*, 1993; Hahn *et al.*, 2003). So far, a marine isolate affiliated with the SAR11 clade has been reported to have the smallest cell size (0.01 μm^3 estimated after glutaraldehyde fixation with TEM (Rappé *et al.*, 2002)). In comparison, our isolates from freshwater environments have a similar cell size (on average 0.05 μm,[3] with the smallest value of 0.01 μm^3 estimated with cryo-TEM). It is known that cells can shrink to a certain extent during the SEM sample preparation and therefore are smaller than the actual size. On the other hand, cells may slightly expand when trapped in very thin ice during cryo-TEM examination. Our results showed that the cell size estimated by SEM is up to five times smaller than that estimated by cryo-TEM (Figure 7). Hence, the accurate cell size probably lies between the estimation carried out by SEM and cryo-TEM. In this study, the cell sizes of LNA isolates were reported according to cryo-TEM, as it preserved a much better cell structure than SEM (Figure 7). Despite their small size, all LNA isolates have a proportionally larger periplasmic space (20–40% of the total cell volume) than that of *Escherichia. coli* (20% on average) (Graham *et al.*, 1991). Owing to their extremely small size, LNA bacteria only represent a minor part (5–10%) of the total biomass of the microbial community despite the fact that they have an equal share of cell abundance with HNA bacteria (Table 1).

Cryo-TEM is an optimal method for preserving biological structures (Dubochet *et al.*, 1988). Cryo-TEM of thin vitreous films has become a routine high-resolution technique for the study of isolated small particles such as viruses, liposomes, proteins and other macromolecular assemblies. This method is not suitable for larger objects ($>1\,\mu m$ in diameter) such as the normal-sized HNA bacteria. The most accurate cryo-technique for viewing prokaryotic ultrastructure involves the use of frozen-hydrated thin sections (Matias *et al.*, 2003; Al-Amoudi *et al.*, 2004). Owing to their extremely small cell size ($<500\,nm$ diameter), it was possible to obtain

cryo-TEM pictures of the frozen-hydrated whole LNA bacteria without thin sectioning, showing cell envelope structure and cytoplasmic content (Figure 7). To our knowledge, this is the first study showing the fine structure of frozen-hydrated whole bacteria. Our results show that cryo-TEM is a suitable tool to characterize the cell structure of bacteria with minute cell volumes.

LNA bacteria, 'oligotrophs' and 'ultramicrobacteria'
Until now, the concept of 'LNA bacteria' has remained confined to the field of FCM. However, the enrichment cultures and isolates obtained and characterized in the present study are not only representatives of the group of 'LNA bacteria', but also possess the properties of so-called 'obligate oligotrophs' (that is, they are unable to grow in rich media) (Supplementary Figure 4) and 'ultramicrobacteria/ nanobacteria' (that is, a cell size $<0.1\,\mu m^3$). Formerly attributed to the class of 'uncultivable bacteria' we have shown that they are perfectly cultivable in natural freshwater. All these names were created by researchers, based on isolated aspects of bacteria (for example, physiological properties, morphological properties) or different investigation techniques (for example, FCM, plating). Unfortunately, most of these names are often only vaguely defined and also used differently under various circumstances (Morita, 1997; Schut *et al.*, 1997). This may lead to confusion in understanding the true nature of the organisms studied and hamper the interpretation and exchange of knowledge among scientists.

Conclusions

In summary, we have documented the cultivation, isolation, morphological characterization and phylogenetic analysis of the LNA bacteria from different freshwater environments. We have shown that these organisms are alive, cultivable, extremely small and maintain unique LNA characteristics irrespective of growth phase. Future research will focus on the characterization of the growth properties of the LNA pure cultures isolated herein. The cultivation approach reported here is a valuable complementary method to the traditional assays applied in microbial ecological studies.

Acknowledgements

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