Diversion and phylogenetic relatedness of filterable bacteria from Norwegian tap and bottled waters

Short title: Filterable bacteria in Norwegian tap and bottled waters

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ABSTRACT

Numerous articles have documented the existence of filterable bacteria. Where filtration is the chosen method of sterilization for medicinal or media components, these bacteria will by definition render products non-sterile. They may further represent a health hazard to the end user. A wide-range of bacterial genera were found in bottled and tap water filtrates from 0.2 µm filters, including genera housing opportunistic pathogens (e.g. *Methylobacterium*) and endospore formers (*Paenibacillus*). Two municipal tap water isolates were only distantly related to named species. One of these grew on agar, and could potentially provide hitherto unharvested useful biological products. The other grew only in water, and failed to produce colonies on media targeting either heterotrophs or autotrophs. The present study is one of very few looking at filterable bacteria in bottled waters intended for human consumption and the first identifying the filterable portion. It extends the range of known habitats of filterable bacteria and provides data on two new or novel species.

Key words | bottled water, filterable bacteria, new species

INTRODUCTION

The knowledge that some bacteria present in aquatic environments can pass through filters with pore diameters of 0.45 and even 0.20 microns is not new. Reports of this phenomenon date back at least to the 1930s (Kendall 1931), and have since been recorded in many papers. Filters with the above mentioned specifications are commonly used for sterile filtration of growth media supplements (e.g. glucose), or medicinal components that do not survive the preferred heat sterilization. Filtration is not a guarantee of sterility and herein lies the importance of the field for the health sciences. As sterile products shall be sterile (i.e. free from viable microbes), the type of microorganisms passing through filters is in some respects not the issue, though of course some microbes represent a greater health risk than others and are potentially a greater problem in filtrates. A survey of the literature, while showing that most cases of filterable bacteria are typically environmental species, reveals that there are also cases of clinically important bacteria passing through filters (see Duda *et al.* 2012). Another interesting facet of the field is that some filterable bacteria have shown themselves to be new or novel species, indicating that fractionation by filtration may be useful in discovering new taxa and harvesting their products (Hahn *et al.* 2004; Nakai *et al*. 2013; Maejima *et al*. 2018). Perusing these studies, the inquisitive reader would also be struck by the sheer number of members of different genera which have been found in filtered samples. A common denominator seems to be the prevalence of different genera of Proteobacteria. This is perhaps unsurprising as these make up a major portion of the bacteria associated with aquatic sources. However, both within and outside the Proteobacteria, the variation in reported taxa is great (Lillis & Bisonette 2001; Miteva & Brenchley 2005; Fedotova *et al*. 2013). Clearly the ability to pass through filters intended for sterile filtration is a wide-spread phenomenon, suggesting that many genera have, or can achieve, a small enough size to do so. Some bacteria are constantly small regardless of their growth conditions (e.g. access to nutrients). These bacteria

are the true Ultramicrobacteria (UMB), a name first applied by Torella & Morita (1981) and subsequently expanded on and qualified particularly by Schut *et al*. (1997). Following on, Duda *et al*. (2012) have listed the following mandatory criteria for UMB: (1) ultra-small sizes (volume of less than 0.1 μm^3 for most cells in the populations); (2) maintenance of ultra-small cell size regardless of the growth conditions and culture development stage, and (3) small genome size (~3.2 to ~0.58 Mb). Other members of the domain Bacteria that pass ultrafilters but fail to meet one or more of the above criteria can then be referred to as "non-UMB filterable bacteria". Factors contributing to the production of the latter are many and multi-faceted, relating to issues such as culture-age and access to nutrients to name but two. For example, under low nutrient conditions, *Staphylococcus aureus* reduced its size by 40% (Watson *et al.* 1998; Chien *et al*. 2012). Given that filterable isolates represent a myriad of genera, size reduction as an adaptation to environmental stress or other factors is probably common in the bacterial world. The topic is well-surveyed in the review articles of Duda *et al.* (2012) and Ghuneim *et al.* (2018).

The great plate count anomaly (Staley & Konopka 1985) is restricting our access to potentially myriads of bioactive compounds produced during bacterial growth. One hurdle to growth on laboratory media is that fitter or faster growing strains may overgrow and suppress others. It is a reasonable assumption that this restriction may apply to UMB (e.g. because of smaller genomes, more limited metabolism and lower growth rates) and to filterable non-UMB cells (e.g. due to stress). It has, for example, been reported that filtration-cultivation techniques resulted in an enrichment for novel ultra-small-cell isolates originating from a glacial ice core (Miteva & Brenchley 2005). Filtration can reduce the competition from other strains, perhaps enabling growth of previously uncultured bacteria. The best known approach is the "filtration-acclimatization" method (Hahn *et al*. 2004). This consists of a filtration step, which removes most of the readily cultivable fraction able to overgrow slowly growing

bacteria, and an acclimatization period which provides a slow transition from the low environmental substrate conditions to the high concentration of standard microbial media.

The present study is concerned with bacteria originating in drinking water sources in Norway, with the focus on tap and bottled waters. In Norway, most municipal tap water is treated surface water. Under European Union and EEA law, bottled waters fall into one of three categories: "Bottled drinking water" (BDW) (European Community 1998); "Spring water" (SW) (European Community 1998, 2009) and "Natural Mineral Water" (NMW) (European Community 2009). NMW is characterized by a constant, rich level of minerals. Other differences between NMW and SW are of a more legislatorial nature. Pertinently, and unlike BDW, no treatment measures of NMW/SW are allowed to be taken to avoid the development of natural populations of bacteria arising from the source after bottling.

The aim of the work is to investigate the types of filterable bacteria present in Norwegian tap and bottled waters. Many of the latter originate from pristine low-temperature glacial regions which may be selective niches. Although there are several recent studies describing the bacterial content of European bottled waters and their sources (Casanovas-Massana & Blanch 2012; Hansen *et al*. 2013; França *et al*. 2015), we have only been able to find one other publication looking at filterable bacteria in bottled waters (Jones *et al*. 2002). To our knowledge, this study is the first of its kind looking at filterable bacteria in Norwegian brand bottled drinking waters and seemingly the first to identify filterable bottled water isolates.

METHODS

Water samples

One municipal tap water (treated surface water from Lake Maridal in Oslo, Norway) from the college, and four bottled waters from Norwegian sources bought from local retailers were included in the study. None of the waters had their origin in the same natural source. For the bottled waters the following were included: one BDW, two NMW and one SW.

Agar media

The following growth media were used in some or all of the experiments:

- R_2A agar (Thermo Fisher Scientific, Waltham, MA, USA – Oxoid): 10 and 25% full strength in municipal tap water. Noble agar (Merck-Sigma Aldrich, USA) was added to achieve a final agar concentration of 12 g/L prior to heat sterilization.
- CAS medium: 0.05 g Bacto™ casamino acids (Becton Dickinson, USA); 0.2 g glucose; 0.67 g yeast extract (Merck-Sigma Aldrich, USA) and 8.0 g phytagel (Merck-Sigma Aldrich, USA) as gelling agent in 1000 mL municipal tap water
- Water agar: 12 g/L Noble agar in municipal tap water.
- PE03 medium modified from Tamaki *et al*. (2005): the following analysis grade components were dissolved in 975 mL municipal tap water: 0.05 g sodium succinate, 0.05 g sodium acetate, 0.05 g yeast extract, 0.05 g Bacto™ casamino acids, 0.05 g sodium thiosulphate, 0.05 g ammonium sulphate and 1 mL trace element mix (Merck-Sigma Aldrich, USA, cat. no. 92949). After pH adjustment to 7.0 (5N NaOH), the solution was made up to 1000 mL and divided into two equal parts. To one part 5.0 g Noble agar was added and to the other 5.0 g Phytagel under stirring. After sterilization solutions were cooled to 50 ºC prior to pouring.

- Nitrosomonas broth and agar modified from Koops *et al*. (1991): Media were essentially made as previously described, but using trace element mix (Sigma-Aldrich, USA, cat. no. 92949), and phenol red (0.00125 g/L) in place of cresol red.
- ATCC medium 290 S6 medium for Thiobacilli: the medium was made exactly as described at the culture collection web site (ATCC, accessed October 2018) and made solid with 10 g/L Noble agar.

Filtration

All filtrations were performed in an ESCO class II type A2 biological safety cabinet (ESCO technologies Inc. Singapore) to avoid contamination from the surroundings. Filterable bacteria in water were isolated using two main approaches. In approach 1, tap water (1–5 L) was filtered through a Ø 90 mm 0.45 µm PVDF filter membrane (Durapore; Merck-Millipore, Germany) under vacuum (<10 kPa) using a Chemical Duty Pump (Merck-Millipore, Germany). The filtrate was re-filtered through a Ø 90 mm 0.22 µm polycarbonate membrane (Isopore; Merck-Millipore, Germany). Bacteria present in 100 mL of the second filtrate were captured on a Ø 47 mm 0.05 µm polycarbonate filter (Nucleopore; GE Healthcare-Whatman, UK) and the filter was transferred to CAS agar medium. A further 200 mL was filtered and the membrane was transferred to water agar. Plates were incubated for 3 weeks at 22±2 ºC, and a selection of colonies appearing to have different morphologies were identified.

In approach 2, tap water and bottled water samples (40 mL) were filtered through commercially available filter cartridges (0.2 µm) using low pressure applied with a 50 mL syringe. One filter per individual 40 mL sample was used. The sterile filtrate was incubated in 50 mL borosilicate tubes at 22±2 ºC, and tubes were sampled by spreading 0.1 mL portions onto agar media. In approach 2/experiment 1, 2–3 parallel portions of tap water and three bottled waters (NMW-1,2 and SW) were tested. Minisart® cellulose acetate filters were used

(SartoriusStedim, Germany). Filtered samples were gradually acclimatized from the tap water temperature ~5 to 22±2 ºC in a water bath, by increasing the temperature in increments of 2 ºC daily. Carbon in the form of suitably diluted "Nutrient broth number 3 Vegitone" (Merck-Sigma Aldrich, USA) was added every 2 to 3 days until 4 weeks. Each addition corresponded to 0.5–1.0 mg-C/L. Samples (0.1 mL) were plated out at 4 weeks onto 25% R_2A and incubated at 22±2 ºC for identification. In approach 2/experiment 2, triplicate samples of SW1, NMW2, BDW and TW were filtered. Fisherbrand Polyether sulfones filters were used (Thermo Fisher Scientific) for filtration. Tubes were gradually acclimatized to 22±2 ºC as described above, and samples (0.1 mL) were removed at 4, 8 and 10 weeks and spread on 10% R_2A.

Live/dead cell staining and fluorescence microscopy

For fluorescence microscopy, the FilmTracer™ LIVE/DEAD® Biofilm viability kit (Invitrogen, CA, USA) was used. In brief, 3 µL SYTO® 9 green fluorescent stain and the same volume of propidium iodide (red fluorescence) was added to 1 mL samples of water. Stained cells were captured on polycarbonate membranes and viewed in the microscope using standard techniques. Viable cells appear green and non-viable cells appear red.

Effect of nutrient starvation on filterability

A single colony was suspended in 10 ml autoclaved water of the type from which the isolate originated. From a 1 mL portion, two drops were gently pressed through a 0.2 µm filter onto a 10% R_2A plate. Two drops were also taken directly from the unfiltered suspension. The tubes were incubated at 22±2 ºC and testing was repeated at intervals over 12 weeks. For strains that grew in water but did not grow on agar, a 1 mL sample of the water culture was filtered

into new autoclaved water samples, and growth was monitored by epifluorescence microscopy.

PCR amplification and sequencing of 16S rDNA gene

The reaction mixture (50 µL) contained: 3 µL of 25 mM $MgCl_2$ (Promega, USA), 1.0 µL dNTPS 10 mM (Promega, USA), 10 µL HotStart DNA polymerase buffer, 0.2 µL HotStart DNA polymerase 5 U/µL (Promega, USA) 0.25 µL of each of primers 27f and 1492r (100 µM stock; standard a la carte sequencing primers from MWG Eurofins, Belgium) and PCR-grade water to 50 µL. To provide the template, a flame-sterilised steel pin was touched onto a bacterial colony and the pinpoint of material was transferred to the reaction mix. A different procedure was used for isolates which grew in water but not on agar. The template was 2 µL of concentrated cells made by pelleting bacteria from 1.2 mL of the water culture. After centrifugation, the supernatant was removed to leave about 50 µL. This was then vortexed briefly to re-suspend cells and 2 µL was used as template.

PCR conditions were: one cycle at 95 ºC (10 min) followed by 32 cycles 95 °C/L/min, 52 ºC/45 s and 72 ºC/1.5 min. This was followed by a final elongation of 72 ºC/12 min. Products were checked for purity by agarose electrophroresis and DNA concentrations were measured using Qubit™ dsDNA BR Assay Kit (Thermo Fisher Scientific). PCR products were sequenced on both strands at least twice. Sequencing was performed by a commercial laboratory (MWG, Eurofins, Belgium) using the PCR primers.

Sequences were aligned using Clustal Omega (Sievers *et al*. 2011) and the consensus region of overlap was used for purposes of identification. Sequences were analyzed and assigned to taxa using the RDP Naive Bayesian rRNA Classifier Version 2 with the default 80% confidence threshold (Wang *et al*. 2007). In addition, BLAST (Altschul *et al*. 1990) was used

to search the NCBI database. The “Reference RNA sequence” setting was used to assign the sequence to a named taxon.

PCR amplification and sequencing of an RpoB RNA polymerase (beta subunit) gene fragment

Consensus-degenerate primers targeting Proteobacteria were used to amplify a gene fragment of a strain which grew in water but not as colonies (see above). Partial amplication of the gene was achieved using the primers CGT GCA CCC CAC CCA YTA YGG NMG (forward) and CAC GGC CTG CCK YTG CAT RT (reverse) (Vos *et al*. 2012). The reaction mixture (50 µL) contained: 5 µL of 25 mM $MgCl_2$ (Promega, USA), 1.25 µL dNTPS 10 mM (Promega, USA), 10 µL HotStart DNA polymerase buffer, 0.4 µL HotStart DNA polymerase 5 U/µL (Promega, USA), and 0.375 µL of each of primers (100 µM stock), (MWG Eurofins), PCR-grade water to 48 and 2 µL template (made as described above). A touchdown PCR was performed with the following specifications: An initial denaturation 95 °C/10 min was followed by 34 cycles in which the annealing temperature was lowered from 64 ºC by 1 ºC in each round until it reached 55 ºC (34 cycles: 95 ºC/30 s; 64–55 ºC/45 s; 72 ºC/90 s). A final elongation step of 72 ºC/10 min completed the reaction. Quality control and measurements of DNA concentration were carried out as described for the 16S rRNA gene

Phylogenetic trees

Trees were generated using the Phyolgeny.fr (Dereeper *et al*. 2008, 2010) online tool in the A La Carte mode (www.phylogeny.fr/). Sequences were aligned using ClustalW (alignments with MUSCLE were also performed) in the program package and the alignment was curated with Gblocks using the most stringent setting. The tree was constructed using the maximum likelihood method (PhyML) with the approximate likelihood-ratio test (aLRT) setting for

branch support. The tree was visualized with the work-flow TreeDyn tool. For trees based on 16S rDNA genes, only curated, published sequences were included for comparison. These were chosen from the best hits obtained by searching against the RDP classifier database using the “Nomenclatural taxonomy, good quality” and “cultured/uncultured filters” (Wang *et al*. 2007). In addition, sequences were chosen from the NCBI database using the BLAST search motor (Altschul *et al*. 1990) set on “Reference RNA sequences”. For phylogenetic trees based on partial *rpoB* sequencing, sequences for comparison were obtained using the BLAST tool set on “highly similar sequences” (megablast). As this produced only four hits, other sequences were obtained using the “somewhat similar” setting (blastn) with the inclusion term “rpoB” to limit the search.

RESULTS

Filterable bacteria present in the capital's tap water were isolated initially using two approaches. In the first approach, pre-filtration through a 0.2 µm membrane was followed by cell capture on 0.05 µm filters (approach 1) using a filter manifold. The filters were then incubated on agar plates. In approach 2, samples were filtered through sealed, commercially available 0.2 µm cartridges followed by a filtrate acclimatization period, and subsequent spread-plating on agar media. Approach 1 (see above under "Methods") with tap water produced about 20 small colonies (100 mL sample; CAS-agar) and six minute colonies (200 mL sample; water agar) respectively. In total, six colonies with possibly different morphologies and taken from both plates were identified. All were Proteobacteria (Table 1). A *Hyphomicrobium* (identical sequence type) was found on both agars.

The use of open filters and a filter manifold may increase the likelihood of contamination from the surroundings or manifold itself. In subsequent tests (Approach 2), closed filtration cartridges and a period of acclimatization prior to plating were used. Approach 2/experiment 1 used also nutrient addition as a part of the acclimatization procedure.

In Approach 2/ experiment 1, the municipal tap water produced growth of a large number (OVG on low dilutions) of small colonies of probably two types (yellow and somewhat greyish). Several colonies of both types were sequenced. The greyish colony (betaproteobacteria) showed 97% similarity to a curated *Cupriavidus metallidurans* strain. The yellowish colonies were two closely related sequence-types designated filterable isolates 2a and 2b, due to their high degree of sequence similarity and common origin (Table 1). Alignment of these sequences using the multiple sequence alignment function of Clustal Omega showed 96% identity over the query coverage. Bottled water NMW1, 2 and SW were also tested in this round of experiment. One or a few colonies that spread quickly over the

agar surface were obtained from one or more parallel samples of each water type. Colonies with other morphologies were not obtained from the waters. Examination by microscope showed sporulating rods. Sequencing revealed identical (NMW1, SW1) or highly similar (NMW2) sequences aligning with *Paenibacillus glucanolyticus* (Table 1). In the second round of experiments (described below), similar colonies were obtained from some parallel tubes of all water types except NMW2.These were not identified further. Due to sequence similarity and the different sources of the *Paenbacillus* isolates, contamination of some tubes was suspected. In order to gain information on the matter, samples (about 10 mL in total) from the water bath used for acclimatization were spread on 10% R_2A. In addition 10 mL sterile water was forcefully pressed through each of six filters into 10% R_2A held molten at 45 ºC and into 50 mL Tryptone soya broth (Thermo Fisher Scientific, Oxoid). No growth was obtained on plates/broth from any filters. The water bath samples produced, as expected, plate cultures. However, none of the colonies were *Paenibacillus*-like.

In the second round of experimentation (Approach 2), no colonies were obtained from NMW2 and no cells were visible in concentrated samples viewed with the fluorescence microscope. One sample tube of municipal tap water contained dividing cells which were visible in microscope preparations, but which did not grow on agar (see below). Taken together, the SW samples produced three types of colonies on agar. These were flat, transluscent colonies with an uneven edge (*Commamonadaceae* possibly *Rhodoferax*), off-white colonies (*Afipia*) and pink colonies (*Methylobacterium*). BDW parallels produced various pink colonies showing some color variation (*Methylobacterium*; *Methylorubrum*), flat greyish colonies (*Curvibacter*) and small grey colonies in high numbers (*Burkholderiales*, possibly *Polaromonas*).

Potentially new species

Routine observations by fluorescence microscopy of samples which did not produce colonies (Approach 2/experiment 2) showed that one of three tap water parallels was populated with slender, dividing rods of similar morphology, here designated “filterable isolate 1”. Figure 1 shows the appearance of viable cells (staining green) at ×1000 magnification.

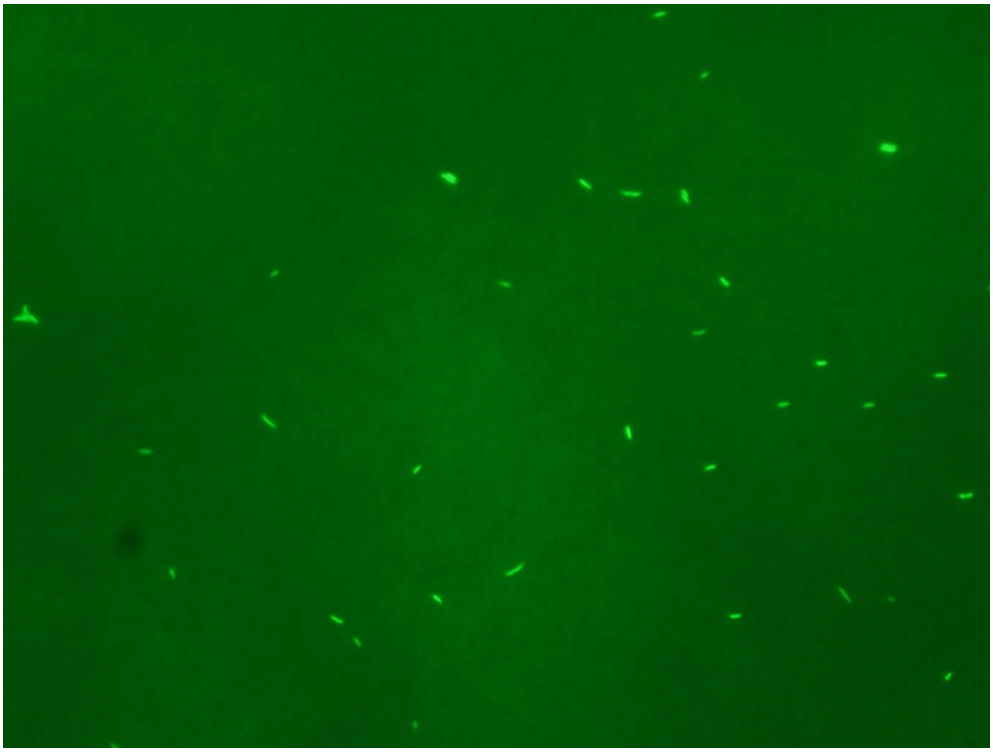

Figure 1 | Viable cells of “filterable isolate 1” originating from municipal tap water.

A sample concentrated for PCR produced only single products with primers for the 16S rDNA and RpoB genes, suggesting a pure culture. The PCR products were subsequently sequenced (see below). Whereas the culture regrew on multiple transfers to fresh tap water, all efforts to grow the bacteria on the laboratory media used for other isolates (R_2A, CAS, Water agar) failed even after incubations of up to four months. Furthermore, no growth was obtained on/in various classes of media designed to allow growth of autotrophs (see above under “Methods”). Plates were also incubated anaerobically, and under microaerophilic conditions using the Oxoid CampyGen system (Thermo Fisher Scientific). Neither approach

resulted in the growth of colonies on agar. Sequencing of the 16S rDNA gene showed that filterable isolate 1 belongs to the subclass betaproteobacteria (Table 1). Based on the almost complete 16S rDNA gene sequence the isolate was only 92% similar to another named species (*Denitratisoma oestradiolicum*). Its unique phylogenetic placement is shown in Figure 2.

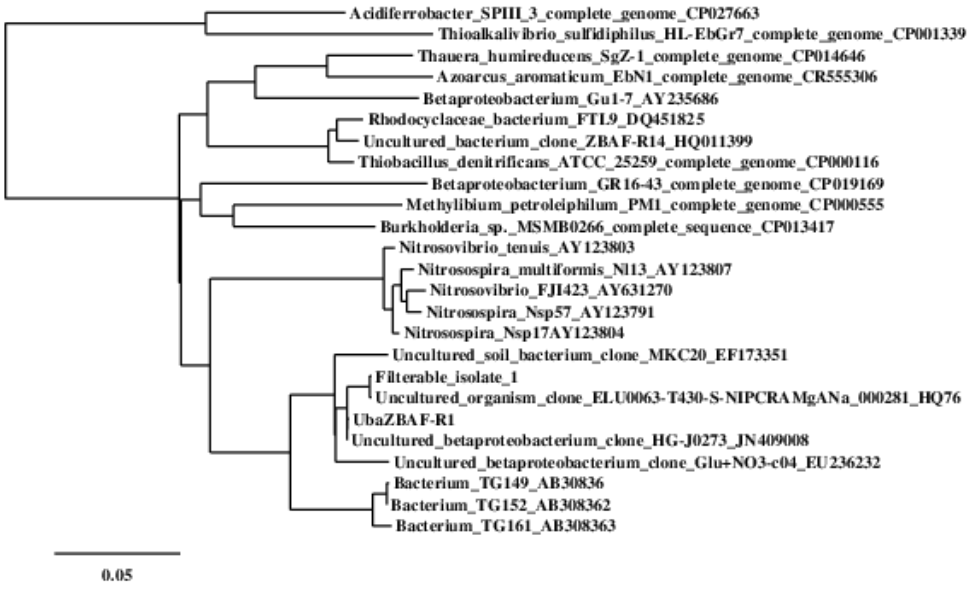

Figure 2 | Phylogenetic tree based on partial 16S rDNA gene sequencing showing the position of filterable isolate 1. Sequences were aligned using ClustalW (alignments with MUSCLE were also performed and gave essentially the same result). The tree was constructed using the maximum likelihood method (PhyML) with the approximate likelihood-ratio test (aLRT) setting for branch support.

A partial sequence of the isolate's RpoB gene showed most similarity with the acidophilic, autotroph *Acidoferrobacter* spIII (Table 1 and Figure 3). However, inclusion of the 16S rDNA gene from this species in Figure 2 suggests that *Acidoferrobacter* spIII is only distantly related to filterable isolate 1.

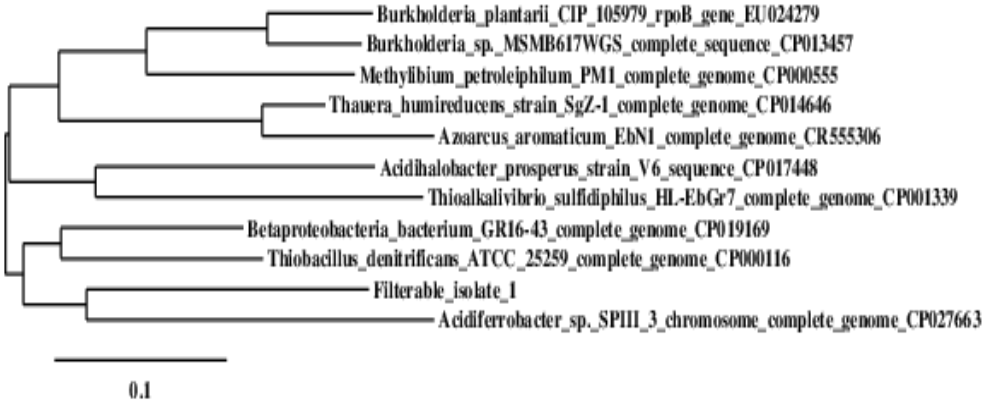

Figure 3 | Tree based on partial RpoB gene sequencing showing the position of filterable isolate 1. Sequences were aligned using ClustalW (alignments with MUSCLE were also performed and gave essentially the same result). The tree was constructed using the maximum likelihood method (PhyML) with the approximate likelihood-ratio test (aLRT) setting for branch support.

Filterable isolates 2a and 2b grew as yellowish, irregular colonies on R_2A. Multiple sequencing of several colonies showed two similar sequence types (96% identity of aligned sequences) with the same closest named affiliate (*Ohtaekwangia koreensis*). As indicated in Table 1, the partial 16S rDNA sequences were only 86 and 89% similar across the sequenced length to other named species. The phylogenetic placement of the strains is presented in Figure 4.

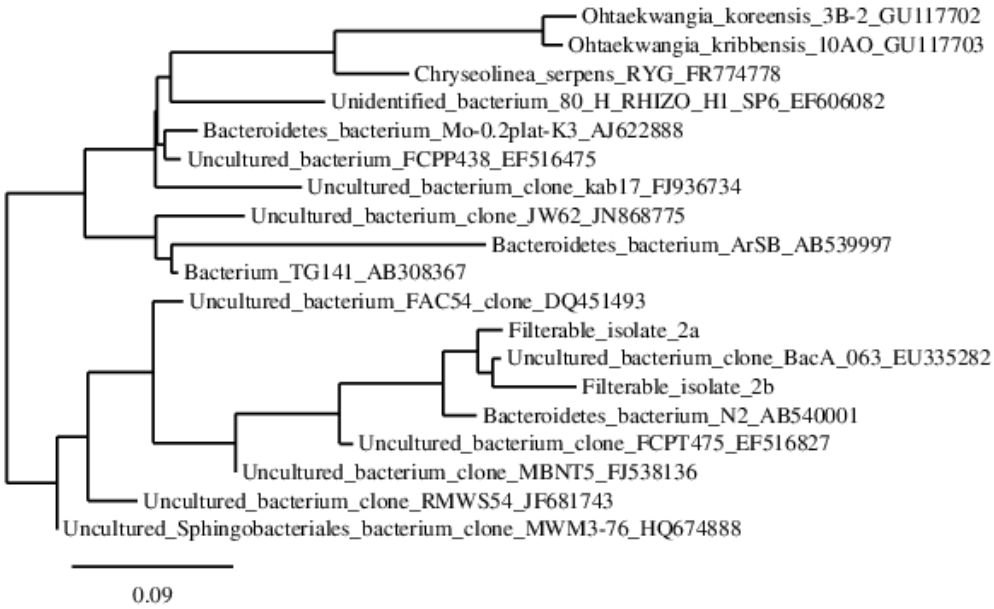

Figure 4 | Phylogenetic tree based on partial 16S rDNA gene sequencing showing the positions of filterable isolates 2a and 2b. Sequences were aligned using ClustalW (alignments with MUSCLE were also performed and gave essentially the same result). The tree was constructed using the maximum likelihood method (PhyML) with the approximate Likelihood-ratio test (aLRT) setting for branch support.

UMB or non-UMB filterable bacteria?

Filtration-starvation studies were performed with six isolates (see Table 1 for strains tested). None of the six chosen isolates were able to re-pass a 0.2 µm filter, suggesting that they are non-UMB types.

Salient points of the results are. Bacteria associated with the Proteobacteria represented the major part of the filterable population of a municipal tap water and four bottled waters. All but two Gram-negative isolates (filterable isolates 2a and 2b belonging to Bacteroidetes) were Proteobacteria, and all were in the sub-groups alpha- and betaproteobacteria (Table 1). The most commonly found bacteria in filtrates were members of the family *Methylobacteriaceae.* These were found in filtered SW, BDW, and TW (approaches 1 and 2), and probably two

genera (*Methylobacterium; Methylorubrum*) and several different species were present (Table 1). Filtrates of the municipal tap water seem to contain two new or novel species.

Accession numbers

Sequences for strains listed in Table 1 are deposited in GenBank under accession numbers: MK123807, MK12381-123822, MK123858-123860; MK123908, MK123946, MK124627-124634, M124972, MK130987, MK138352.

DISCUSSION

Non-UMB filterable bacteria are widespread in Norwegian waters. Our findings add to the publications documenting the presence of such bacteria in a wide-range of aquatic environments. The present study is the first of its kind in Norway, and seemingly the first that identifies bacteria in filtrates of bottled waters. We were only able to find one other study of filterable bacteria in bottled water (an NMW in the UK). Here, all isolates were Gram-negative, but none could be identified using the chosen method, the biochemical test API 20NE (Jones *et al*. 2002). Identifications in the present study are based on the now standard-approach of sequencing of the 16S rDNA gene. As in previous studies (see above under "Introduction"), bacteria associated with the Proteobacteria represented the major part of the filterable population. Probably several species of *Methylobacteriacea* (genera *Methylobacterium*; *Methylorubrum*) were represented. Other bacteria for which it was possible to provide an identification to the genus level included *Hyphomicrobium*, *Afipia*, *Curvibacter* and *Reyranella*. Also, these are among the many genera found in other studies of filtered water. However, as covered in the Introduction section, there is great variation from study to study with regards to the taxa isolated from filtrates. The study of Hahn *et al.* (2004) is close to the present work in terms of method and sample material. Table 1 in that publication is akin to Table 1 in this work, yet outside the large number of Proteobacteria detected in both studies, there is basically no similarity in genera reported. Every study on filterable bacteria seems to create unique results, indicating that the ability of bacteria to form filterable cells is a fundamental property of a great many taxa.

A salient finding was the relative preponderance of two genera belonging to the family *Methylobacteriaceae.* The genus *Methylorubrum* has only very recently (Green & Ardley 2018) been created to accommodate 11 species previously held in *Methylobacterium.* The

genus *Methylobacterium* has previously been detected along with many others in filtered groundwater (Lillis & Bissonnette 2001) and in the clone library obtained from filtered glacial ice (Miteva & Brenchley 2005). However, there is no indication in the literature that members of the *Methylobacteriaceae* are often small bacteria or true UMB. Indeed, even after prolonged maintenance under low nutrient conditions none of the six chosen isolates, including both a *Methylobacterium* and a *Methylorubrum* sp., passed again through a 0.2 µm filter. Other than their widespread presence in many water supplies (Rice *et al*. 2000), none of the publications on filterable bacteria single out *Methylobacteriaceae* as commonly found in filtrates. Thus, the present findings are novel, and it will be particularly interesting to see if future work on filtered bottled waters reveals the presence of *Methylobacteriaceae*. Given that genera of *Methylobacteriacea* were, with *Paenibacillus,* the most frequently found filterable bacteria from Norwegian waters, it is pertinent to ask if they may in low numbers represent a significant health risk, e.g. in medicinals filtered to achieve sterility. Kovaleva *et al*. (2014) have reviewed the medical significance of *Methylobacterium* (including species since transferred to *Methylorubrum*), with special focus on healthcare-associated infections. The authors inform that these genera cause healthcare-associated infections, including infections in immunocompromised hosts. Members of both genera seem to be widespread in the hospital environment, particularly in tap water and endoscope channels. They are aided in their persistence by the ability to form biofilms, a tolerance to disinfecting agents and an ability to withstand high temperatures and drying (Kovaleva *et al.* 2014). The ability to at least on occasion pass through filters intended for sterilization purposes may then be another factor contributing to their survival in the hospital environment. In the present study, we consider the finding of putative *Methylobacterium radiotolerans* (Table 1) particularly relevant. This species has been associated with several catheter-related bacteremias (Cazzavillan *et al*. 2009; Lai *et al*. 2011)

None of the other genera found in the filtrates described here are clearly and commonly associated with infections. La Scola *et al*. (2000) discuss some *Afipia* species as possible causes of nosocomial infections. Filterable *Curvibacter* species have been reported (Belova *et al.* 2012), and the genome of a UMB *Curvibacter* is now available (Ma *et al*. 2016). Strikingly, and particularly pertinent to the present work, is the report that *Hydrogenphaga pseudoflava* ATCC 700892 used as a control organism in microbial challenge tests of 0.2 µm membrane filters was in fact incorrectly named and is rather phylogenetically close to the *Curvibacter* (Kaesler *et al*. 2011). The same authors report that retention rates of this strain by different 0.2 µm filters vary, and only a 0.1 µm filter consistently achieved complete retention (Haake *et al*. 2012).

The occasional, but widespread (across the waters tested and rounds of testing) presence of *Paenibacillus* (*glucanolyticus*)-like sequences, could suggest a low level of extraneous contamination of some tubes. However, we were unable to find these bacteria in the most likely sources of contamination (filters and the water bath). The results thus suggest that the *Paenibacillus* isolates reported originated from the water samples. Future work could look at the relative abundance of *P. glucanolyticus* contra other *Paenibacillus* species in Norwegian and other waters. There are a few documented instances of *Paenibacillus* and other endospore-forming genera among filterable bacteria. A relatively large group of low-G+C Gram-positive bacteria spore-forming bacilli related to *Bacillus, Paenibacillus lautus*, and *Brevibacillus* were obtained from filtered glacial ice (Miteva & Brenchley 2005). Some of these were also related to previously reported Antarctic *Paenibacillus* isolates (Christner *et al*. 2003). Spore formation would be an obvious advantage for survival in extreme environments and several of the Norwegian brand waters also have an origin in cold sources. Bacteria belonging to the genus *Paenibacillus* have been isolated from a variety of sample materials, with many of the species being relevant to humans, animals, plants, and the environment.

They are occasionally present as opportunistic infections of immunocompromised individuals (Grady *et al*. 2016).

Two isolates from the municipal tap water appear to be new or novel species. This finding is in line with many other studies indicating that filtration, and especially filtration-acclimatization as in the present study (approach 2), is a means to isolate new and sometimes novel species. The nearest named relative to filterable isolates 2a and 2b is *O. koreensis.* The type-strain was isolated from a sand sample collected from the west coast of the Korean peninsula along with a *O. kribbensis* to which it shared 97.5% 16S rDNA gene sequence identity. A new genus was proposed to house these species (Yoon *et al*. 2011). Subsequently an isolate predicted to belong to the same genus was isolated from filtered lake water (Maejima *et al*. 2018). The members of the genus seen unremarkable with respect to their cellular properties (Yoon *et al*. 2011), and at present too little information is available to judge the importance of the current findings.

The closest named relative to filterable isolate 1 based on sequencing of the 16S rDNA gene was *D. oestradiolicum* (Table 1). However, the similarity was only 92% over 99% of the queried length. *D. oestradiolicum* is a Gram-negative denitrifying bacterium which was isolated from activated sludge of a municipal wastewater treatment plant (Fahrbach *et al*. 2006). It is also the sole member of the genus. However, unlike filterable isolate 1, *D. oestradiolicum* grew (slowly) on R_2A. As seen in the phylogenetic tree (Figure 2), the closest group of cultured heterotrophic bacteria were TG149, 152, 161, all reported by Tamaki *et al*. (2009). In the class *Betaproteobacteria,* these three strains, which also did not form colonies on agar plates, were grouped into a putative phylogenetic clone cluster at the order level that is not represented by any validly named species. However, the co-workers found that the strains did grow heterotrophicaly on ME03 medium (see above under “Methods”) solidified

with gellan gum (Phytagel) instead of agar. Filterable isolate 1 did not grow on ME03 made with Noble agar or phytagel, whereas control strains (*E. coli* and *Methylobacterium*) grew profusely on both. Attention was thus turned to the most closely related groups of bacteria capable of growth autotrophically (Figure 2). A cluster showing some degree of similarity to filterable isolate 1 included lithotrophic ammonia-oxidising genera (*Nitrosospira*, *Nitrosovibrio*, *Nitrososomonas*). A general purpose agar and broth medium for this group also failed to give growth the isolate, as did a standard medium for autotrophic, sulphur-oxidizing *Thiobacillus*. In an attempt to gain more information, the strain was also analyzed with respect to the RpoB gene (Table 1 and Figure 3). A partial sequence of the RpoB gen showed most, but low, sequence similarity with *Acidoferrobacter* spIII. The monospecies genus *Acidiferrobacter* was proposed in 2011 by Hallberg *et al*. (2011) with *Acidiferrobacter thiooxydans* m-1 (DSM 2392^{T}) as type strain. *A. thiooxydans*^{T} is able to grow chemolithoautotrophically by oxidation of ferrous iron or elemental sulfur, sulfide and tetrathionate, using either oxygen or ferric iron as terminal electron acceptors. However, the 16S rDNA gene sequence of this species suggested only a distant relationship to filterable isolate 1 (Figure 2). The RpoB gene, encoding the β-subunit of RNA polymerase, is a house-keeping gene which has shown itself to be a good candidate for phylogenetic analyses and identification of bacteria. This is especially so when studying closely related isolates (species and sub-species). The excellent review by Adékambi *et al.* (2009) provides a good introduction to the usefulness of RpoB gene-based classifications and identifications. The lack of accord between the 16S rDNA and RpoB-based identification in the present study may be explained by their different levels of resolution or, at least in part, by the relative paucity of RpoB-gene sequences and a dedicated RpoB database for comparison. However, although the sequencing data for the two gene classes are not mutually supportive with respect to the identity of filterable isolate 1, both reveal a similarity to autotrophic bacteria which would be

in keeping with our inability to grow the isolate heterotrophically. At the time of writing the identity of the strain remains unknown. We are currently trying to grow the bacteria in water in sufficient quantities with sufficiently low background DNA to attempt a complete genome sequencing. If filterable isolate 1 is autotrophic, this extends the reported range of physiological groups able to pass through 0.2 µm filters, and suggests that other filterable autotrophs will remain undetected by approaches using only organic carbon as electron and C-source.

CONCLUSIONS

In conclusion, the present study extends the knowledge on the types and origins of bacteria able to pass, at least intermittently, through membrane filters intended for use in sterile filtrations. Specifically it presents what appears to be the first identifications of filterable non-UMB bacteria originating in bottled waters. Key observations are that filterable bacteria were not present in all waters, and that in the samples where filterable bacteria were detected, the genera *Methylobacterium* (and the related *Methylorubrum*) and *Paenibacillus* were the most commonly found. Furthermore, we present two isolates that probably represent new species, providing further support for the idea that separation of indigenous bacterial populations based on size and acclimatization may be useful in the isolation of new and novel species. This is important with respect to species discovery and the potential harvesting of new bioactive compounds.

Table 1 | Phylogenetic affiliations of filterable bacteria

Source/approach	Sequence length (bp)	Phylogenetic affiliation[a]	Closest match (Accession number)	Similarity (%) (Accession number)	Closest species	Similarity (%)
BDW *App2/expt2*	610	Methylorubrum[c]	*M. rhodesianum* BE525[b] (LT601238)	100 (100)	*M. populi* BJ001[b] (CP001029)	100 (100)
BDW *App2/expt2*	509	Methylobacterium	*M. tardum* PH5 (LT617056)	100 (100)	*M. fujisawaense* (AB175634)	99 (100)
BDW *App2/expt2*	1394	Curvibacter	*C. fontanus* AQ12 (LT617056)	99 (100)	*C. fontanus* AQ9 (AB120963)	99 (100)
BDW *App2/expt2*	493	Burkholderiales	*Polaromonas europsychrophilia* 20BR6t3 (MH482339)	100 (100)	*P. europsychrophilia* B717-2 (KP013181)	100 (100)
NMW1 *App2/expt1*	856	Paenibacillus	*Paenibacillus* sp. Gh-134 (FJ233853)	99 (100)	*P. glucanolyticus* NBRC 15330 (AB680838)	99 (100)
NMW2 *App2/expt1*	989	Paenibacillus	*Paenibacillus* W10507 CP028366	100 (100)	*P. glucanolyticus* NBRC 15330 (AB680838)	99 (100)
SW *App2/expt2*	854	Paenibacillus[c]	*P. glucanolyticus* B2/38-2 (MG051306)	100 (100)	*P. glucanolyticus* NBRC15330 (AB680838)	99 (100)
SW *App2/expt2*	569	Methylobacterium[c]	*M. radiotolerans* EP27 (M6778733)	100 (100)	*M. radiotolerans* JCM 2831 (CP001001)	100 (100)
SW *App2/expt2*	559	Comamonadaceae	Rhodoferax K139 (AF288304)	100 (100)	*Limnohabitans parvus* 11-B4 (FM165536)	99 (100)
					R. saidenbachenensis (FJ755906)	99 (100)
SW1 *App2/expt2*	573	Afipia	*A. birgiae* 34632 (AF288304)	100 (100)	*A. birgia* 34632 (AF288304)	99 (100)

TW *App2/expt1*	942	Cytophagales ("Filterable isolate 2a")	Uncultured clone Kab17 (FJ936734)	96 (100)	Ohtaekwangia koreensis 3b-2 (GU117702)	87 (99)
TW *App2/expt1*	1386	Cytophagales ("Filterable isolate 2b")	Uncultured clone kab17 (FJ936734)	98 (100)	Ohtaekwangia koreensis 3b-2 (GU117702)	89 (100)
TW *App2/expt1*	385	Betaproteobacteria	*Cupriavidus metallidurans* 30 (JQ860104)	98 (100)	*C. metallidurans* CH34 (CP000352)	97 (100)
TW *App1*	1363	Bradyrhizobiaceae[c]	*Afipia massiliensis* SH41 (KY319044)	99 (100)	*Afipia birgiae* 34632 (AF288304)	99 (100)
TW *App1*	1362	Methylorubrum[c]	*M. rhodesianum* TMV13-6 (KY882056)	99 (99)	*M. rhodesianum* DSM5687 (AB175642)	99 (100)
TW *App1*	962	Hyphomicrobium	Hyphomicrobium P2 (AF14858)	100 (100)	Hyphomicrobium P2 (AF14858)	100 (100)
TW *App*	795	Reyranella	*Reynarella* sp. S49 (KY176929)	100 (100)	*R. massiliensis* 521 (EF394922)	99 (100)
TW *App2/expt2*	1407	Betaproteobacteria[c] ("Filterable isolate 1")	Uncultured clone ELU0063-T430-S- (AB680838)	99 (99)	Denitratisoma oestradiolicum (AcBE2-1)	92 (99)
TW *App2/expt2*	366	NA ("Filterable isolate 1")	Acidiferrobacter SpIII chromosome *(*CP027663.1)	79 (99)		

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AUTHOR CONTRIBUTIONS

Colin Charnock designed the study and performed most of the experiments. He is also responsible for most of the writing. The co-authors have contributed equally to the experimental work and its interpretation and in the writing of the study.