MICROBIAL CHARACTERIZATION OF DRINKING WATER SYSTEMS RECEIVING GROUNDWATER AND SURFACE WATER AS THE PRIMARY SOURCES OF WATER

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Abstract

Earlier descriptions of water distribution systems (WDS) microbial communities have relied on culturing techniques. These techniques are known to be highly selective in nature, but more importantly, they tend to grossly underestimate the microbial diversity of most environments. The goal of this ongoing study is to compare the microbial composition of a WDS receiving different sources of water. To circumvent some of the problems associated with culture-based techniques, sequence analysis of total community 16S rDNA clone libraries was performed. A total of 260 16S rDNA clones were analyzed in this study. Sequence comparisons with existing databases revealed that α-Proteobacteria and Mycobacterium sp. represented nearly 43% and 48% of the total clones examined, respectively. Other bacterial groups identified included members of the genera Legionella, Pseudomonas, and Agrobacterium. Seventy percent of the clones analyzed in this study showed at least 97% sequence identity with sequences available in current databases. While α-Proteobacteria is a numerically dominant group in chlorinated drinking water systems, the abundance of mycobacterial sequences in this study indicates that there are significant differences in microbial community structure between the WDS analyzed. These differences could be attributed to differences in water treatment or receiving water sources between the distribution systems. While some of the genera identified in this study have been associated with some public health risks, it should be noted that analysis of 16S rDNA clones does not confirm the presence of pathogenic strains of any organisms identified in this study, as current methods for detection and identification require several steps including selective enrichment, isolation, and final confirmation via in vitro studies. Future studies will focus on expanding sequencing databases to more accurately characterize these potential differences.

Keywords
Drinking water, 16S rDNA clones, phylogenetic analysis, chlorination

1. INTRODUCTION

The primary sources of drinking water are surface water and groundwater. Previous studies have shown that groundwater and surface water differ greatly with respect to types of microbes, organic content, and mineral quantities (Chapelle, 2000). Regardless of their microbial composition, source waters undergo a series of treatments before reaching the consumer as potable water. It is also known that different types of drinking water treatment processes have variable effectiveness with respect to removal of microorganisms (Norton and LeChevallier, 2000). Culture-based methods and biochemical techniques are commonly used

in attempt to characterize drinking water bacterial communities. Many of these phenotypic methods are time consuming and tend to grossly underestimate the microbial diversity within water samples. In order to avoid such problems, molecular techniques are now used to characterize and detect difficult to grow microorganisms and to monitor microbial pathogens such as *Cryptosporidium* sp*., Legionella sp.*, and *enteroviruses*. Many of these microorganisms have developed different mechanisms to resist disinfection treatment used by utilities providing potable water. For example, it is well documented that C*ryptosporidium* oocysts can be resistant to chlorine residual levels found in drinking water distribution systems. Additionally, bacteria may persist in the environment in a viable but non-culturable (VBNC) state. Due to the inability of VBNC bacteria to grow on artificial media, bacterial enumeration using culture technique can result in a gross underestimation of bacterial counts (Colwell et al., 1985).

Recently, a number of studies have relied on the use of 16S rRNA sequence analysis to characterize natural microbial communities. The 16S rRNA gene is vital to protein synthesis and therefore it has been maintained in all living organisms (Olsen et al., 1986). Moreover, the primary structure of this gene (that is, the DNA sequence) is highly conserved. As a consequence, microbial taxonomists have used sequence analysis of this gene to predict the phylogenetic affiliation of bacterial populations (Pace et al., 1986). Since it is possible to amplify this gene from whole community DNA extracts using universal primers, it is then possible to develop 16S rDNA clone libraries and study the composition of complex microbial communities without relying on culture-based techniques. Using this approach, scientists have been able to study the microbial network of a wide array of environments (Amman and Ludwig, 2000; Zoetendal et al., 2004; Leclerc and Moreau, 2002; Lawson, 2004; Dunbar et al., 1999; Bernhard and Field, 2000; Schmeisser et al., 2003; Eichler et al., 2006).

The goal of the study was to accurately characterize the microbial community structure within a drinking water distribution system using a 16S rDNA phylogenetic approach. Clone libraries were developed and sequenced to more accurately characterize microbial populations in drinking water samples. Water samples from the two existing drinking water distribution systems were used to extract whole microbial community DNA. The two distribution systems will be referred to as DSS1 (groundwater fed) and DSS2 (surface water fed).

2. MATERIALS AND METHODS

2.1 Sample Collection

The general experimental design used in this study is shown in Figure 1. Water samples were obtained directly from faucet heads located in two different sites within the drinking water distribution system of a metropolitan area. Site 1 is located in DSS1 while site 2 is within DSS2. The major differences between DSS1 and DSS2 are water source and treatment processes. The source for DSS1 is groundwater while the source of DSS2 is surface water. Faucets were run with the cold valve completely open for 5 minutes in order to flush out idle water from the pipes within the structure. Total chlorine concentrations within the distribution systems ranged from 0.76 to 0.94 ppm, as provided by publicly available information in the area of study. One liter polypropylene (Nalgene) bottles were used to collect water. A total of 5 liters were obtained from each sample location and transported to the laboratory in coolers. Samples were processed within three hours of collection.

2.2 Molecular Techniques

Prior to nucleic extractions, water samples were filtered through polycarbonate membranes (47 mm in diameter, 0.22 µm pore size; Osmonics Laboratory Products). Water samples from two locations within a municipal drinking water distribution system were used to extract whole microbial community DNA. Membranes were folded in half, placed in 2 ml tubes, and stored at -80C until used in extractions. DNA extractions were performed using UltraClean Soil DNA kit (MoBio Laboratories Inc., Solana Beach, CA). DNA extracted was stored at -20°C in a buffered solution provided in the DNA extraction kit. Bacterial community characterization was performed by 16S rDNA sequencing analysis. This gene, which is present in all bacteria, is considered a good phylogenetic marker due to the different levels of sequence conservancy present in the entire gene family. Microbial community DNA was used in Polymerase Chain Reaction (PCR) studies to develop 16S rDNA clone libraries. Primers 8F (5'-AGAGTTTGATCCTGGCTCAG-3') and 787R (5'-GGACTACCAGGGTATCTAAT-3') targeting universally conserved regions of the 16S rRNA gene were used to generate mixed community PCR products used to develop clone libraries. These primers generate a PCR product that cover more than half of the entire 16S rRNA gene (Lane, 1991) and is considered sufficient to provide an accurate phylogenetic affiliation for bacteria.

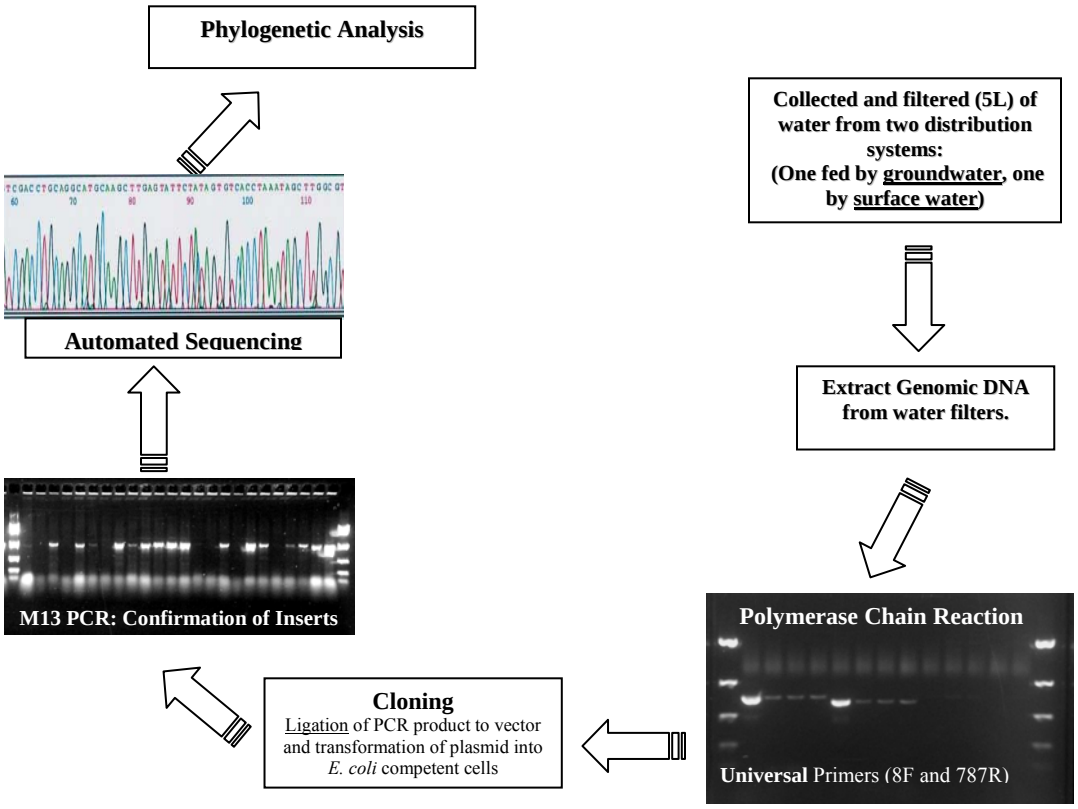

Figure 1. Experimental approach used in this study. The end result is the phylogenetic identification of bacterial populations directly from whole microbial community DNA extracts using 16S rDNA sequence analysis.

The PCR assays contained the following reagents per 50 µl: 5 U of *Ex Taq* DNA polymerase (TaKaRa Mirus Bio Corp., Madison, WI), 10X Buffer (5µl), dNTP mix (4µl), 8F and 787R primers (75 picomoles each), DNA template (2µl of community DNA extract), and Ultra Pure water (32.75 µl). The thermal cycler conditions used were as follows: an initial denaturation step of 3 min at 94°C, followed by 35 cycles of 1 min at 94°C, 1 min at 56°C, 1 min at 72°. An extension step of 7 min at 72°C was included at the end of the cycling run followed by a cooling step of 4°C. To confirm PCR product formation, products were screened using gel electrophoresis in 1% agarose (Fisher Scientific, Pittsburgh, PA) at 80 V for 100 min using GelStar™ as the DNA staining dye (BioWhittaker Molecular Applications, Rockland, ME).

2.3 TA Cloning

Prior to cloning experiments, mixed community PCR products were cleaned with a QIA-quick kit (Qiagen, Inc., Valencia, CA) and then cloned using a TOPO TA cloning kit (Invitrogen Corp., Carlsbad, CA). Transformed cells were grown on Luria-Bertani agar plates containing ampicillin (100 mg/ml) as selective agent. Colonies were selected from LB-ampicillin plates and screened for proper PCR size using M13 primers. Gel electrophoresis was used to confirm PCR products. Images were documented using a Kodak digital camera (model DC290 Zoom) and software package (Kodak 1D version 3.6.3). PCR products of expected size were cleaned as previously mentioned before use in sequencing reactions.

2.4 Sequence Analysis

Sequencing reactions were performed using cleaned PCR products and the ABI Big Dye Terminator kit following the manufacturer's instructions (Applied Biosystems, Foster City, CA). Sequence reactions were cleaned using DyeEx plates (Qiagen) and dried down using a lyophilization step. PCR products were then resuspended in 25 µL of Hi-Di™ Formamide (Applied Biosystems, Foster City, CA) and analyzed on an ABI 3730 xl DNA analyzer to obtain sequence data. Sequence data obtained through analysis was compared to sequences found in the NCBI database using BLASTn software (Altshul et al., 1997). A phylogenetic analysis was performed using a Neighbor Joining algorithm using the phylogenetic software ARB (http://www.arb.de.vu/). Sequencher 4.6 (Gene Codes, Ann Arbor, MI) was used to edit the contiguous sequences. Sequences were examined for chimeras with Chimera Check (Cole et al., 2003).

3. RESULTS AND DISCUSSION

DNA extractions yielded from 2 ng/µl to 13 ng/µl. The PCR assays generated products of the expected size further confirming the quality of the DNA extracted (Figure 2). These PCR products were used to develop 16S rDNA libraries using a TA cloning approach. A total of 260 16S rDNA clones generated from drinking water samples were obtained and analyzed in this study (Figure 3). Sequence comparisons with existing databases revealed that α-Proteobacteria and *Mycobacterium sp*. represented nearly 44% and 49% of the total clones examined, respectively (Figure 4). Other bacterial groups identified included members of the genera *Legionella*, *Pseudomonas*, and *Agrobacterium*. Approximately 30% of the sequences analyzed in this study showed less than 97 % identity with sequences in the currently available databases, suggesting the some drinking water bacteria represent novel bacterial species. In addition, nearly a third of sequences were closely related to uncultured bacteria suggesting that culture-based methods can underestimate the bacterial diversity of drinking water systems.

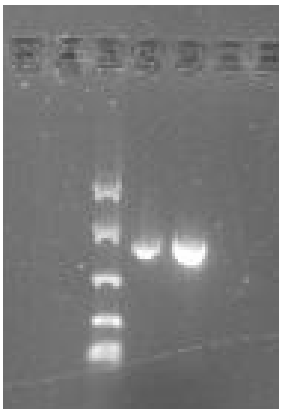

Figure 2. Gel agarose electrophoresis of PCR assay using 16S rDNA universal primers and drinking water microbial community DNA extracts. PCR products were approximately 800bp. The sizes of the DNA ladder are 2000 bp, 1000bp, 500 bp, 250 bp, and 100 bp (from top to bottom).

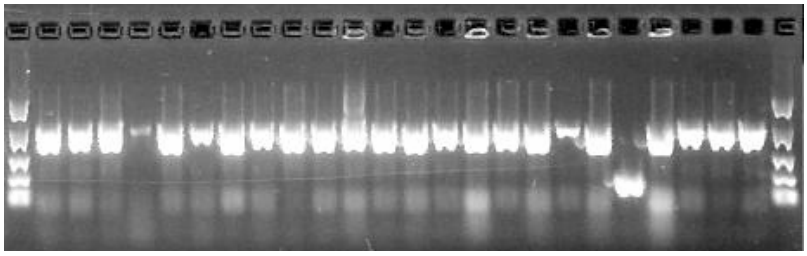

Figure 3. Gel agarose electrophoresis of clones analyzed in this study. Clones were transformed into *E. coli* competent cells and grown overnight. An aliquot (1 µl) of the suspended cells was used in to directly amplify inserts with M13 primers. Once insert was confirmed, PCR products were then cleaned and used in sequencing analysis.

Phylogenetic analysis of clones examined in this study, illustrate many similarities to previous studies pertaining to drinking water biofilms. For example, 16S rDNA sequences affiliated to mycobacteria, α-Proteobacteria, and *Legionella*-like organisms have been previously shown to be present in both drinking water biofilms and drinking water planktonic populations (Santo Domingo et al., 2003; Williams et al., 2004; Williams et al., 2005). Including this study, nearly identical sequences have been recovered from four independent studies from the same distribution system suggesting that these bacterial groups can be considered to be part of the normal microbiota of this drinking water system. Moreover, the isolation of mycobacteria and α-Proteobacteria (Covert et al., 1999; Falkinham et al., 2001) and the finding of rRNA-based clones affiliated to these bacterial groups (Keinänen-Toivola et al., 2006) suggest that some drinking water bacteria are capable of surviving the relatively harsh conditions found in drinking water.

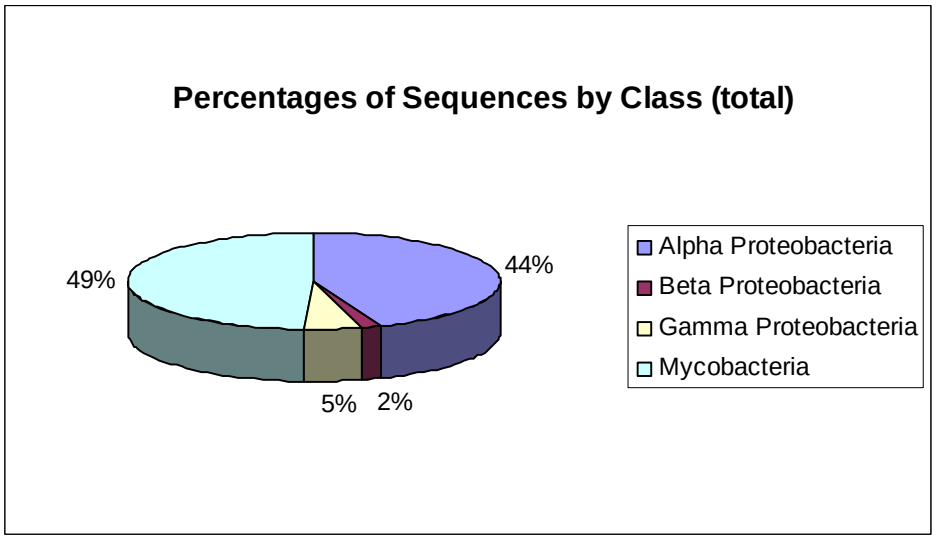

Figure 4. Summary of major bacterial groups present in drinking water samples as determined by phylogenetic analysis of drinking water clones obtained in this study.

Alignment with sequences available within the NCBI database (Genbank) showed that a significant number of sequences obtained in this study were related to the following mycobacterial species: *M. mucogenicum*, *M. massiliense*, *M. sacrum*, *M. gordonae*, and *M. gadium* (with sequence similarities of 98% and higher) (Figure 5). These clones represented 114 of the 260 clones of the clones examined. These data further supports several studies conducted on the occurrence of environmental non-tuberculosis mycobacterium (NTM) in drinking water using culture-based techniques (Covert et al., 1999). It should be noted that the percentage of mycobacteria related sequences obtained in this study is relatively high. Previous studies conducted on biofilms exposed to chlorinated drinking water have shown that mycobacterial species account for a smaller percentage of the species richness of the biofilm (Williams, et al. 2004). Moreover, drinking water samples originating from the distribution system receiving groundwater as the water source contained approximately three times the number of *M. sacrum* and *M. massiliense* like sequences than the samples obtained from the system receiving surface water (Figure 5). This data suggests that some of conditions in the distribution system might selectively favor the proliferation of some types of mycobacterial species. One possibility is the amount of assimilable organic carbon (AOC) as previous studies have shown that the density of mycobacteria increases with the increase of AOC (Falkinham et al., 2001). Other factors that might positively influence mycobacterial densities are heterotrophic bacterial counts, iron, water retention times and turbidity (Falkinham et al., 2001).

In general terms, the high numbers of mycobacteria and the different proportions of some members of this bacterial group in distribution systems are significant findings in light of the fact that several *Mycobacterium* species been associated with possible life threatening illnesses (Kline et al., 2004). Although not all environmental mycobacteria are pathogenic, some species identified in this study have been associated with diseases in humans. For example, *M. mucogenicum* was recently identified as the causative agent of neurological infections (Adekambi et al., 2006). This is an interesting finding as the latter species is considered a rapidly growing mycobacterial species while the mycobacterial strains of most public health concern are believed to be slow growing mycobacteria. While the molecular evidence presented in this study confirmed the presence of *Mycobacterium sp*. in this drinking water distribution system, there is no evidence that the identified strains are indeed pathogenic. This is one of the limitations of using 16S rDNA-based techniques for this bacterial group as they cannot differentiate between

pathogenic and non-pathogenic strains. The use of virulence genes is an alternative that warrants future consideration.

Similar to mycobacteria, the presence of *Legionella* does not immediately imply public health risk, as water has been the source of several *Legionella* species of little or no clinical relevance (Ohnishi et al., 2004). However, these results are interesting because they suggest that *Legionella* can survive the harsh environmental conditions within drinking water. One possible survival mechanism relates to the ability of this bacterial group to live as an intracellular symbiont of environmental protozoa (King et al., 1988). Indeed, chlorine disinfection studies have shown that bacterium-protozoan associations can provide chlorine-sensitive bacteria with increased resistance to free chlorine (King et al., 1988). Another mechanism relates to the ability of *Legionella* to grow within biofilms (Rogers et al., 1994). From a microbial water quality standpoint, these survival mechanisms are relevant to human health as they increase the persistence of potentially pathogenic bacteria in drinking water (Szewzyk et al., 2000; Parsek and Singh, 2003).

Phylogentic analysis was conducted using ARB software (Ludwig et al. 2004) (Figure 6). The sequences that closely match uncultured organisms are labeled on branches of the phylogenetic tree and are summarized in Table 1.

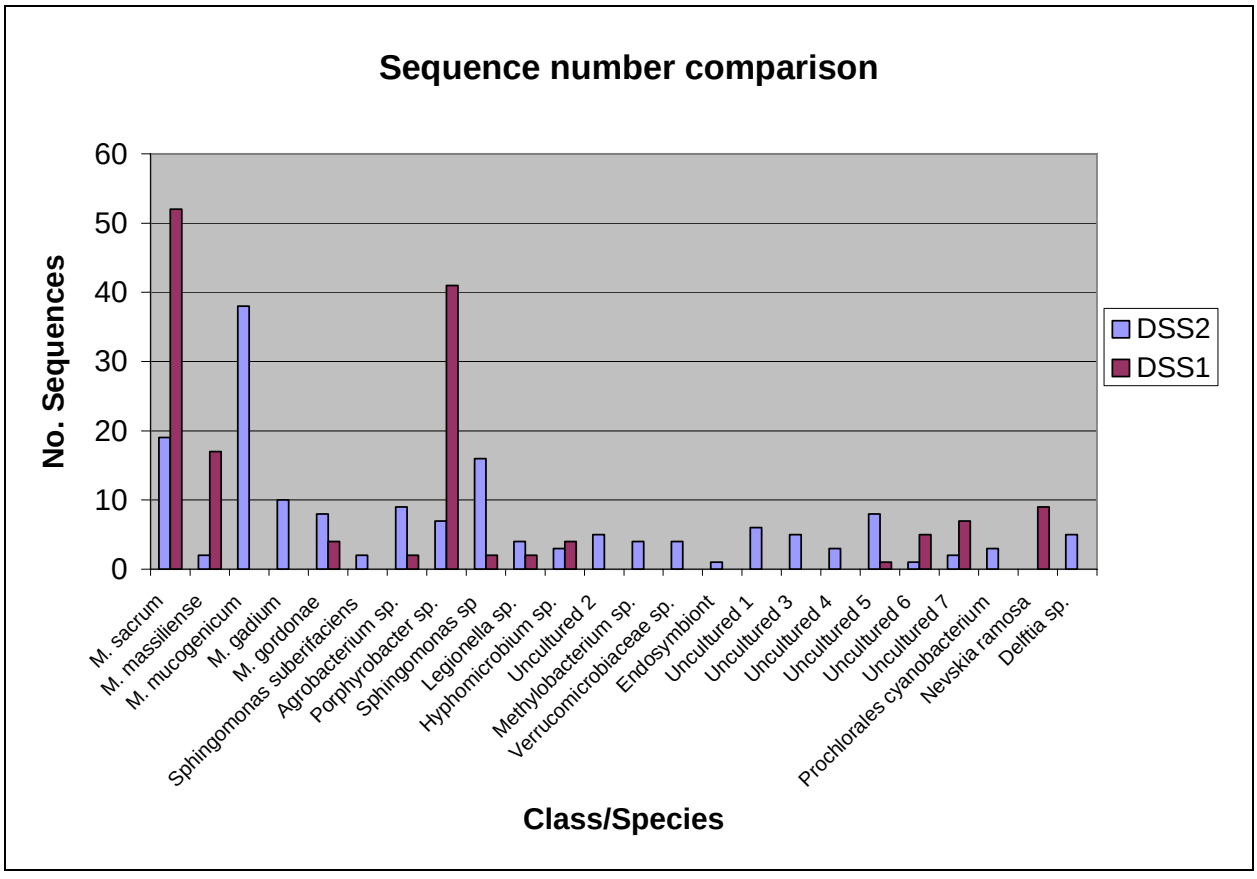

Figure 5. Presumed phylogenetic affiliation of sequences obtained in this study using Blastn.

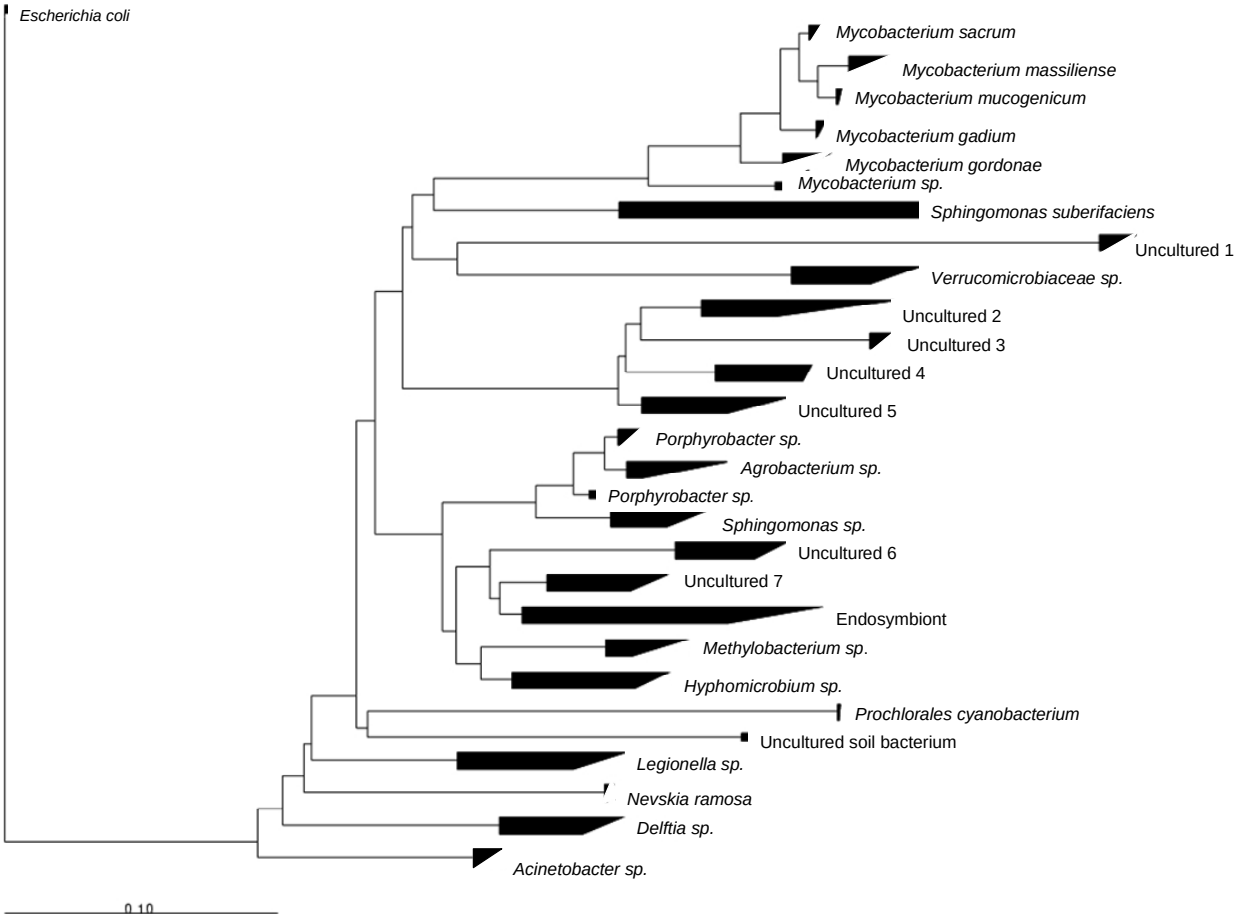

Figure 6. Neighbor joining phylogenetic tree constructed using ARB software (Ludwig et al., 2004). Solid blocks represent clusters of sequences recovered in this study and are labeled according to the closest relative.

Based on phylogenetic analyses, most of, the α-proteobacterial sequences were closely related to *Sphingomonas, Porphyrobacter, Hyphomicrobium,* and *Methylocystis* sp. In addition, some of the clones were closely related to sequences from yet to be cultured rhizosphere bacteria and to an endosymbiont of *Acanthamoeba*. *Sphingomonas* sp. have been found in abundance in chlorinated drinking water systems, not only in the United States but also in Europe (Norton and LeChevallier, 2000; Koskinen et al., 2000). In contrast, *Hyphomicrobium*-like bacterial sequences were found in chloraminated waters (Williams et al., 2004). These two microbial genera are known to survive and even thrive in oligotrophic conditions (i.e., low carbon concentrations), hence their presence in drinking water is not surprising. Interestingly, while *Sphingomonodaceae* sequences were obtained from both distribution systems, the distribution system using groundwater as the main water source contained 5 times more sequences than the system using surface water. *Methylocystis* and *Porphyrobacter* species are not normally associated with drinking water and perhaps are transitory members of this habitat. All together, these results provide further evidence on potential differences in community structure in different distribution systems.

Table 1. Summary of sequences obtained per uncultured group

Group	***Distribution System***	***Number of sequences***
Uncultured 1	DSS2	6
Uncultured 2	DSS2	5
Uncultured 3	DSS2	5
Uncultured 4	DSS2	3
Uncultured 5	DSS2 and DSS1	DSS2=8 and DSS1=1
Uncultured 6	DSS2 and DSS1	DSS2=1 and DSS1=5
Uncultured 7	DSS2	5

Sequences closely related to β-proteobacteria and γ-proteobacteria were also obtained in this study. Other studies have recorded the presence of both bacterial groups in drinking water systems (Eichler et al., 2006)**.** The β-Proteobacteria sequences obtained are closely related to *Delftia tsuruhatensis,* a bacteria isolated from activated sludge (100%) and *Aquaspirillum delicatum* (97%) (Shigematsu, et al. 2003). β-Proteobacteria have been mentioned as dominant members of drinking water systems. For example, using 16S rDNA fluorescently labeled oligonucleotide probes targeting different subclasses of β-Proteobacteria, Kalmbach et al. (1997) showed that three of the subclasses represented almost two thirds of the total bacteria in a drinking water sample obtained in Berlin. In contrast, in our study only two percent of all the recovered sequences were affiliated to this bacterial group. These differences could be attributed to the fact that bank filtration, and not chlorination, is the primary type of bacterial remediation of drinking water sources in Germany.

Microbial community structure and diversity of drinking water systems may be impacted by differences in source water, treatment unit processes, disinfection regimes, and effects of water residence time in a distribution network (Kerneis et al., 1995, Gibbs et al., 1993). Microbial growth in treated drinking water (and re-growth in water distribution systems) is commonly limited by the use of chlorine disinfection. However, high doses of chlorine can cause taste and odor problems and can react with organic matter causing the formation of disinfection byproducts. In addition to disinfection, microbial growth can also be reduced by removal of nutrients required for microbial growth (Volk and LeChevallier, 1999). In the United States, microbial growth is generally limited by assimilable organic carbon (AOC) (van der Kooij 1992). In countries like Finland and Japan, microbial growth in drinking water is limited by microbially available phosphorous (Lehtola, et al., 2002). Waterworks may have different water treatment unit processes depending on the quality of raw water being treated. These processes typically include chemical coagulation, slow or rapid sand filtration, ozonation and/or chlorination, and activated carbon filtration. The goal of future studies is to further understand the effect these processes have on the microbial composition and microbial activity in distribution systems, and how changes in community structure play a role (if any) on the occurrence and survival of microbial pathogens.

In conclusion, much of the sequence data obtained during the course of this study supports the notion that microbial community structure is dynamic and that it could be impacted by differences in the water biogeochemistry, disinfection treatment, and type of water source. The study also showed that certain bacterial groups tend to be relatively common members of WDS even though chlorine residual levels are maintained. It should be noted that the recovery of certain 16S rDNA sequences (e.g., mycobacteria and

Legionella spp.) from drinking water DNA extracts does not mean that these organisms can cause a serious threat to the consumer. Moreover, it should be noted that since the target molecule in this study was genomic DNA, it is not possible to determine if any of the populations identified are actively growing in situ. However, recent data using RNA (Keinänen-Toivola et al., 2006) and lipid biomarkers (Keinänen et al., 2003) as the target molecules as well as the identification of culturable isolates (Kalmbach et al., 1999; Williams et al. 2004) has provided evidence of the fact that some mycobacteria and proteobacterial populations are potentially active. In addition, these results suggest that there may be some differences of the drinking water treatment unit processes in the area of study, pertaining to the removal of these organisms. These findings may warrant a closer investigation of mycobacteria behavior pertaining to drinking water distribution systems.

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