J. Microbiol. Biotechnol. (2008), **18**(7), 1207–1215

JOURNAL OF MICROBIOLOGY AND BIOTECHNOLOGY

Occurrence and Molecular Differentiation of Environmental Mycobacteria in Surface Waters

Lee, Eun-Sook[1,2], Mok-Young Lee[1], Sun-Hee Han[1], and Jong-Ok Ka[2]*

[1]*Waterworks Research Institute, Seoul Metropolitan Government, 552-1 Cheonhodaero, Seoul 143-820, Korea*
[2]*Department of Agricultural Biotechnology, Seoul National University, Seoul 151-742, Korea*

Received: October 4, 2007 / Accepted: February 15, 2008

To investigate the occurrence and species diversity of mycobacteria in waters, surface water samples were collected monthly from the Han River and tap water samples at the terminal sites of the distribution system. Mycobacteria in each water sample were isolated by decontamination using cetylpyridinium chloride (CPC) and cultivation on Middlebrook 7H10 agar, and then identified by polymerase chain reaction-restriction fragment length polymorphism analysis (PRA) and sequencing of the 65-kDa heat-shock protein gene (*hsp65* gene). Mycobacteria were detected in 59% of the surface water samples and 26% of the tap water samples. Over half of the 158 isolates could not be identified by *hsp65* PRA and gene sequencing, and several identification discrepancies were observed between the two methods. The most frequently isolated species was *Mycobacterium gordonae* in surface water and *M. lentiflavum* in tap water. *M. avium* complex (MAC), the most important pathogen among environmental mycobacteria, was detected in the surface water samples but not found in the tap water samples. The result demonstrated that water is an important environmental source of mycobacteria and the combined application of *hsp65* PRA and sequencing was more reliable than *hsp65* PRA alone to accurately identify mycobacteria present in water.

Keywords: Environmental mycobacteria (EM), CPC, *hsp65* gene, molecular differentiation, MAC

Mycobacterium tuberculosis has long been recognized as the only clinically significant species among the member of genus *Mycobacterium* [6]. Recently, however, there have been increasing evidences that species of mycobacteria other than *M. tuberculosis*, which are also called environmental mycobacteria (EM) or nontuberculosis mycobacteria, are implicated in a variety of human diseases [4, 28, 29]. Environmental mycobacteria are common saprophytes in all ecosystems, including water, soil, food, dust, and aerosols [4, 7, 8, 12, 15]. In particular, EM species can multiply in the numerous water sources, including wastewater, surface water, recreational water, ground water, and tap water [7, 28, 29]. Piped water supplies are readily colonized by mycobacteria, and thus the biofilm in the water pipes may serve as a reservoir for these organisms [28]. Mycobacteria are resistant against common disinfectants and can tolerate wide ranges of pH and temperature, which allows them to persist in drinking water systems for long periods of time [7, 8, 15].

Several species of EM, such as *M. kansasii*, *M. marinum*, *M. xenopi*, and *M. avium* complex (MAC) consisted of *M. avium* and *M. intracellulare*, are opportunistic pathogens causing pulmonary and cutaneous disease, lymphadenitis, and disseminated infections in humans or animals [8, 15, 29]. These infections are more likely transmitted from environmental sources by ingestion, inhalation, and inoculation of mycobacteria [15, 29]. Specifically, MAC is a commonly found pathogen capable of causing pulmonary and other diseases in immunocompromised individuals [8, 15, 29, 30]. The United States Environmental Protection Agency (USEPA) listed MAC on the Contamination Candidate List (CCL) for drinking water as both health and treatment research priorities, particularly because of its resistance to chlorine, its ability to colonize pipes, and its likely occurrence in biofilms [30].

Investigation on mycobacteria, however, has been limited by the lack of appropriate methods, and thus few publications have reported the isolation and identification of mycobacteria in the drinking water systems in Korea. Because the majority of mycobacterial species grow slowly, a decontamination procedure is necessary to isolate them from water samples containing more rapidly growing microbes [18, 29]. Without a decontamination procedure, other microorganisms will rapidly overgrow on the media before mycobacterial growth is visible [8, 29]. In addition to the slow growth of mycobacteria,

*Corresponding author
Phone: 82-2-880-4673; Fax: 82-2-871-209;
E-mail: joka@snu.ac.kr

because of the complex species composition of the genus *Mycobacterium*, molecular techniques are needed for their identification.

In this study, both the cultivation and molecular methods were used to investigate the occurrence and species diversity of mycobacteria in surface and tap waters. The population density of mycobacteria in water was estimated by selective culture under decontamination control. The identification of mycobacteria was confirmed by comparison of patterns of the BstEII and HaeIII restriction fragments of the PCR amplification product of the 65-kDa heat-shock protein gene (*hsp65* gene) and by sequence analysis of the *hsp65* gene.

Materials and Methods

Sample Collection

We collected, monthly, six surface water samples at the Han River (Fig. 1) and six tap water samples at the terminal sites of the distribution systems in Seoul. All of the 84 samples of surface water and tap water were collected from November 2004 to December 2005. All samples were transported to the laboratory in an icebox, stored 4°C, and analyzed within 24 h.

Decontamination and Cultivation of Mycobacteria

One liter of tap water samples was filtered through a membrane filter (pore size 0.45 μm; Millipore, Bedford, U.S.A.). The membrane filter was put into a sterile tube containing 10 ml of tap water sample and was sonicated for 5 min, and then was decontaminated by 0.001% cetylpyridinium chloride (CPC; Sigma, St. Louis, U.S.A.). Ten ml of surface water was decontaminated by 0.02% CPC. After room temperature exposure for 5 minutes, samples were centrifuged at 3,000 ×*g* for 20 min, and then the supernatant was removed and the pellet was rinsed twice with sterilized phosphate buffer solution (PBS; pH 7.0). Samples were centrifuged again as described above. The pellet was resuspended in about 1 ml of remaining supernatant and plated on Middlebrook 7H10 agar (Difco, Sparks, U.S.A.) containing 10% (vol/vol) OADC enrichment (BBL, Sparks, U.S.A.) and 500 mg of cycloheximide (Sigma, St. Louis, U.S.A.). The plates were incubated at 37°C with 5% CO_2 for 1 month. Colonies on culture plates were preliminarily identified as mycobacteria based on Ziehl-Neelsen acid-fast staining. Colonies were enumerated and subcultured on Middlebrook 7H10 agar for further analysis.

DNA Extraction

DNA of isolates was extracted using an InstaGene matrix kit (Bio-Rad, Hercules, U.S.A.). A loopful of mycobacteria was suspended in 1 ml of sterilized water. The suspension was centrifuged for 1 min at 12,000 rpm and the supernatant was removed. Two hundred μl of InstaGene matrix was added to the pellet, and then it was incubated at 56°C for 30 min. The suspension was boiled at 100°C for 8 min and centrifuged at 12,000 rpm for 3 min. The supernatant was transferred to a new tube and stored at −20°C until use.

Polymerase Chain Reaction-Restriction Fragment Length Polymorphism Analysis (PRA)

Amplification of the *hsp65* gene was performed as described by Telenti *et al.* [26] with some modifications. The PCR mixture was prepared by combining 2 μl of DNA template and 1 μl each of primers tb11 (5'-ACCAACGATGGTGTGTCCAT-3') and tb12 (5'-CTTGTCGAACCGCATACCCT-3') in a One-Shot LA PCR mix tube (Takara Biomedical Co., Japan) to yield a final volume of 50 μl. The PCR condition consisted of one cycle at 95°C for 5 min, 35 cycles at 94°C for 1 min, 60°C for 1 min, and 72°C for 1 min, and a final extension step at 72°C for 5 min (Biometra, Germany). The 439-bp amplified product was detected on a 2% agarose gel. The amplified products were digested separately with restriction enzymes, BstEII and HaeIII (Bioneer, Daejon, Korea). The digestion reaction consisted of 5 μl of PCR products, 2 μl of 10× reaction buffer, and 5 U of restriction enzyme in a total volume of 20 μl. After incubation at 37°C for 1 h, restriction fragments were electrophoresed on a 3% agarose gel, using a 50-bp DNA ladder as a molecular size marker. Mycobacterial species were identified using published profiles [5, 9, 23] and/or the PRASITE [21].

Sequencing of the *hsp65* Gene

PCR products were purified with a SUPREC-02 kit (Takara) and were sequenced with an ABI Prism 3100 Genetic Analyzer

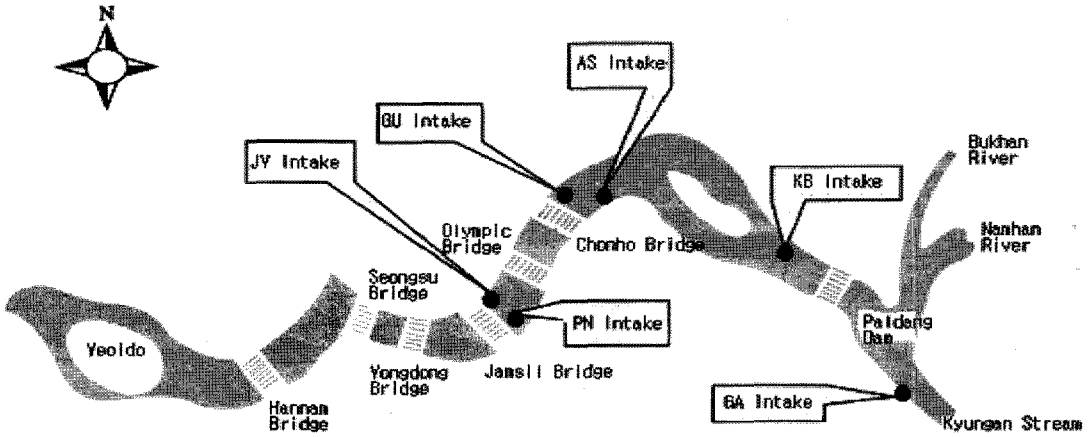

Fig. 1. Sampling sites collected from the Han River. Sampling sites (●): GA Intake, Gwangam (Paldang) Intake; KB Intake, Kangbuk Intake; AS Intake, Amsa Intake; GU Intake, Guui Intake; JY Intake, Jayang Intake; PN Intake, Pungnap Intake.

(Applied Biosystems, Foster City, U.S.A.) using BigDye terminator cycle sequencing ready kit (Applied Biosystems). Sequencing reactions consisted of 4 μl of BigDye ready reaction mix, 2 μl of 5× sequencing buffer, 1 μl of primer Tb11 or Tb12, 5 μl of template, and distilled DNase/RNase-free water to make a 20 μl reaction. The PCR conditions for sequencing were 25 cycles of 94°C for 10 s, 50°C for 5 s, and 60°C for 4 min. The sequencing products were cleaned according to the manufacturer's instructions (Applied Biosystems), suspended in 10 μl of HiDi formamide, heat denatured at 94°C for 2 min, placed on wet ice for 2 min, mixed, and analyzed with an ABI Prism 3100 Genetic Analyzer according to the manufacturer's protocol. Sequences were assembled and edited using SeqMan software (DNAStar). Determined sequences were analyzed using the Blast search program of the NCBI (http://www.ncbi.nlm.nih.gov) GenBank database [1, 3, 13, 14].

Physicochemical and Microbiological Characteristics of Water Samples

Total coliforms (TC), pH, temperature, conductivity, suspended solids (SS), biochemical oxygen demand (BOD), chemical oxygen demand (COD), dissolved oxygen (DO), total nitrogen (TN), total phosphorus (TP), NO_3-N, NH_3-N, and total organic carbon (TOC) were measured for the surface water samples according to *Standard Methods of Examination of Water and Wastewater* [2]. Heterotrophic plate counts (HPC), total coliforms (TC), *E. coli*, pH, turbidity, and free chlorine residuals were measured for the tap water samples

Statistical Analyses

Owing to the non-normal distribution of the variables, the results were analyzed by nonparametric tests (SAS for Windows version 8.0.1.). The relationships between microbial, chemical, and physical parameters and the concentration of mycobacteria in the water samples were studied by Spearman rank correlation analysis.

Nucleotide Sequence Accession Number

The *hsp65* sequences determined in the present study were deposited in GenBank under accession numbers EF591142 to EF591284.

RESULTS

Physiochemical and Microbiological Properties of Water Samples

Table 1 summarizes the physicochemical and microbiological properties of 168 water samples.

In the case of tap water samples, the pH was from 6.9 to 7.6, on average 7.3. HPC, TC, and *E. coli* were not detected and thus it appeared that microbes were controlled adequately by the water treatment. Free chlorine residual was from 0.24 mg/l to 0.97 mg/l, which satisfied the regulation limits (0.2 mg/l). Turbidity was from 0.10 NTU to 0.19 NTU, which also satisfied the regulation limits (0.5 NTU). For surface water samples, the average values of DO, BOD, COD, and SS were 11.6 mg/l, 1.6 mg/l, 2.6 mg/l, and 8.6 mg/l, respectively. The average values of TN, NO_3-N, NH_3-N, and TP were 2.415 mg/l, 2.070 mg/l, 0.089 mg/l, and 0.041 mg/l, respectively. The temperature was from 0.7°C to 24.8°C, on average 13.1°C. The pH value ranged from 7.0 to 9.2, on average 7.9, and TOC ranged from

Table 1. Physicochemical and microbiological properties of water samples.

Sample types	Parameter	Number of samples	Minimum value	Maximum value	Mean
Tap water	HPC (CFU/ml)	84	0	0	0
	TC (P/A[a])	84	A	-	-
	E. coli (P/A)	84	A	-	-
	Mycobacteria (CFU/l)	84	0	6.7×10^2	1[b]
	pH	84	6.9	7.6	7.3
	Free chlorine (mg/l)	84	0.24	0.97	0.64
	Turbidity (NTU[c])	84	0.10	0.19	0.13
Surface water	TC (CFU/100 ml)	84	8	4.2×10^4	4.2×10^{2b}
	Mycobacteria (CFU/10 ml)	76	0	6	1[b]
	Temp (°C)	84	0.7	24.8	13.1
	pH	84	7.0	9.2	7.9
	BOD	84	0.7	3.4	1.6
	COD	84	1.2	4.5	2.6
	DO	84	7.5	15.8	11.6
	TN	84	1.611	3.961	2.415
	NO_3-N (mg/l)	84	1.375	2.958	2.070
	NH_3-N (mg/l)	84	0.011	0.492	0.089
	TP (mg/l)	84	0.010	0.155	0.041
	TOC (mg/l)	84	1.34	3.11	2.11
	SS	84	1.2	27.6	8.6
	Conductivity	84	88	204	160

[a]P/A: Presence/Absence; [b]Geometric mean; [c]NTU: nephelometric turbidity units.

1.34 mg/l to 3.11 mg/l, on average 2.11 mg/l. TC was from 8 to 4.2×10^4 CFU/100 ml, on geometric mean 4.2×10^2 CFU/100 ml. The population density of TC was positively correlated with temperature ($r=0.34$, $p<0.005$).

Occurrence of Mycobacteria

A total of 168 water samples collected from surface and tap waters were analyzed. Mycobacteria were not detectable in 8 surface water samples owing to the overgrowth of background organisms. In all, 158 mycobacterial strains were isolated from the 160 water samples. Mycobacteria were isolated from 45 (59%) out of 76 surface water samples. The concentration of mycobacteria in the surface water samples ranged from 0 to 6 CFU/10 ml during the experiment. The concentration of mycobacteria in Guui and Jayang intake samples ranged from 0 to 5 CFU/10 ml. The mycobacterial concentration in Pungnap intake samples ranged from 0 to 3 CFU/10 ml. The mycobacterial concentration in Gwangam, Kangbuk, and Amsa intake samples ranged from 0 to 6 CFU/10 ml. The monthly average numbers of mycobacteria isolated among the six different sites were in the range of 1.0–1.4 CFU/10 ml, thus showing no significant differences among the six sampling sites. In the case of the tap water samples, 26% (22/84) of the samples were positive for mycobacteria. Ninety-one % of tap water samples showed low concentrations of mycobacteria, 0–6 CFU/l, and the peaks of 300 CFU/l and 670 CFU/l were found in only two samples.

Regarding the monthly changes of mycobacteria, relatively high levels of mycobacteria were observed in November (1–6 CFU/10 ml) and December (0–6 CFU/10 ml) of 2004 and in August (2–5 CFU/10 ml) of 2005 in the surface water. However, November and December of 2005 showed low levels (0–3 CFU/10 ml) of mycobacteria in the six sampling sites. In the case of the tap water samples, except for the two high peaks (300 and 670 CFU/l) of two samples in March, no significant differences were observed in the temporal distribution of mycobacteria. Thus, in spite of monthly fluctuations of mycobacterial populations, it did not appear that the occurrence of mycobacteria was intensified in any particular season in both surface water and tap water.

Relationships Between Mycobacteria and Water Quality Parameters

The population density of mycobacteria in the surface water samples was correlated negatively with their conductivity ($r=-0.27$, $p<0.02$). The concentrations of mycobacteria in all water samples also were not associated with the other physicochemical parameters of the water (data not shown). There were no correlations between mycobacteria and total coliforms in the surface water (data not shown).

Analysis of *hsp65* PRA

Ninety-six isolates obtained from 45 positive surface water samples were analyzed by *hsp65* PRA. Many of the isolates were identified as *M. gordonae* 4 (13%), *M. gordonae* 5 (4%), *M. gordonae* 3 (1%), *M. peregrinum* (4%), *M. flavescens* 3 (3%), *M. porcinum* (2%), *M. intracellulare* 4 (2%), *M. nonchromogenicum* 2 (2%), and *M. chelonae* 2 (2%) (Fig. 2). In addition, several isolates were identified as *M. hiberniae* (1%), *M. avium* (1%), *M. lentiflavum* 1/*simiae* 5 (1%), *M. simiae* 3/*intermedium* 1 (1%), *M. kansasii* 1 (1%), and *M. terrae* (1%). The other isolates (61%) could not be identified by the known PRA database for EM species (Fig. 2).

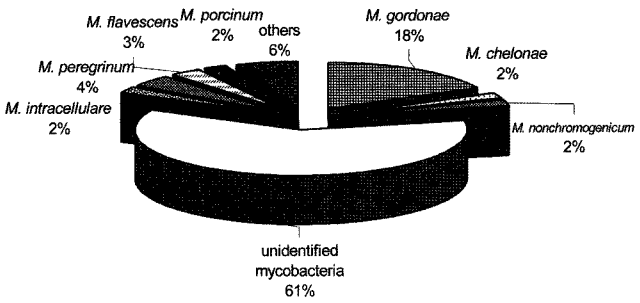

Fig. 2. Species distribution of mycobacterial isolates in surface water samples based on PRA patterns.

PRA identification of 62 isolates obtained from 22 positive tap water samples showed that 51% of the isolates were *M. lentiflavum* 1/*simiae* 5 and 47% of the isolates could not be identified by the known PRA database for EM species (Fig. 3). Seventy-nine % of the unidentified mycobacteria exhibited the same PRA patterns to each other (data not shown).

Sequence Analysis of the *hsp65* Gene

Among the 158 mycobacterial isolates, the *hsp65* gene sequences of 143 strains were useful for analysis of species identification. The *hsp65* gene sequence analysis revealed that 35 strains (42%) of the 83 isolates obtained from the surface water samples and 42 strains (70%) of the 60 isolates obtained from the tap water samples had high similarities of more than 97% with species type strains. The 35 isolates of the surface water samples were identified as *M. gordonae*, *M. terrae*, *M. kumamotonense*, *M. peregrinum*, *M. intracellulare*, *M. holsaticum*, *M. aichiense*, *M. phocaicum*, *M. arupense*, *M. brisbanense*, *M. chubuense*, *M. gilvum*, *M. lentiflavum*,

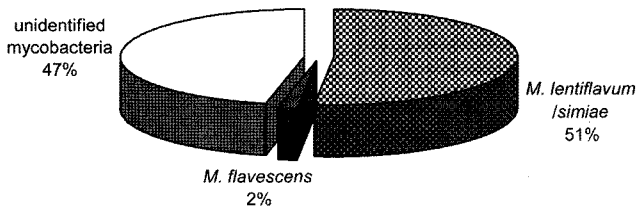

Fig. 3. Species distribution of mycobacterial isolates in tap water samples based on PRA patterns.

Table 2. Composition of mycobacteria identified by *hsp65* sequencing and PRA.

Sample types	*hsp65* sequencing		*hsp65* PRA	Identification consistency (n)
	Identification (n[a])	Similarity	Identification (n)	
Tap water	*M. lentiflavum* (13)	100%	*M. lentiflavum/simiae* (13)	Identity (13)
	M. lentiflavum (8)	99%	*M. lentiflavum/simiae* (7)	Identity (7)
			Unidentification (1)	
	M. lentiflavum (7)	98%	*M. lentiflavum/simiae* (4)	Identity (4)
			Unidentification (3)	
	M. lentiflavum (11)	97%	*M. lentiflavum/simiae* (4)	Identity (4)
			Unidentification (7)	
	M. triplex (3)	97%	Unidentification (3)	
Surface water	*M. aichiense* (2)	98%	Unidentification (2)	
	M. arupense (1)	99%	Unidentification (1)	
	M. brisbanense (1)	98%	*M. porcinum* (1)	
	M. chubuense (1)	98%	Unidentification (1)	
	M. gilvum (1)	98%	*M. flavescens* (1)	
	M. gordonae (1)	99%	Unidentification (1)	
	M. gordonae (5)	98%	*M. gordonae* (1)	Identity (1)
			Unidentification (4)	
	M. gordonae (2)	97%	*M. gordonae* (1)	Identity (1)
			Unidentification (1)	
	M. gordonae (2)	100%	Unidentification (2)	
	M. holsaticum (2)	99%	Unidentification (2)	
	M. intracellulare (1)	99%	*M. intracellulare* (1)	Identity (1)
	M. intracellulare (1)	99%	Unidentification (1)	
	M. kumamotonense (1)	98%	Unidentification (1)	
	M. kumamotonense (2)	97%	*M. chelonae* (1)	
			Unidentification (1)	
	M. lentiflavum (1)	100%	*M. lentiflavum/simiae* (1)	Identity (1)
	M. peregrinum (1)	100%	*M. peregrinum* (1)	Identity (1)
	M. peregrinum (1)	99%	*M. peregrinum* (1)	Identity (1)
	M. peregrinum (1)	98%	*M. peregrinum* (1)	Identity (1)
	M. phocaicum (2)	98%	Unidentification (2)	
	M. sp. (1)	98%	Unidentification (1)	
	M. gadium (1)	97%	Unidentification (1)	
	M. terrae (2)	97%	Unidentification (2)	
	M. terrae (1)	99%	*M. terrae* (1)	Identity (1)
	M. terrae (1)	100%	*M. nonchromogenicum* (1)	

[a]n represent the numbers of isolates identified as a particular species.

M. gadium, and *M.* sp. (Table 2). Twenty-two strains out of the 58 mycobacterial isolates unidentified by PRA in the surface water samples were identified at a species level by the *hsp65* gene sequencing. These were *M. gordonae* (8 strains), *M. aichiense* (2), *M. phocaicum* (2), *M. kumamotonense* (2), *M. gadium* (1), *M. terrae* (2), *M. holsaticum* (2), *M. arupense* (1), *M. chubuense* (1), and *M. intracellulare* (1) (Table 2).

The 42 isolates obtained from the tap water samples were identified as *M. lentiflavum* and *M. triplex* (Table 2). Sequence distance analysis between the type strains revealed that the type strain *M. lentiflavum* had 98.1% similarity with the type strain *M. triplex*. Considering that the type strains *M. gordonae* 3 and *M. gordonae* 1 showed 94.8% similarity, the above two type strains appeared to be closely related to each other.

When the identification results obtained by the *hsp65* PRA were compared with those obtained by the *hsp65* gene sequencing, identification consistencies were observed in 8 strains (23%) of the 35 isolates from the surface water samples and in 28 strains (67%) of the 42 isolates from the tap water samples (Table 2). Specifically, in the case of the tap water samples, 28 strains out of the 32 isolates identified as *M. lentiflavum* 1/*simiae* 5 by PRA were identified as *M. lentiflavum* by the *hsp65* gene sequencing. In contrast, *M. lentiflavum* 1 and *M. simiae* 5, which could not be distinguished by the PRA method because of their identical PRA patterns, were easily differentiated by the

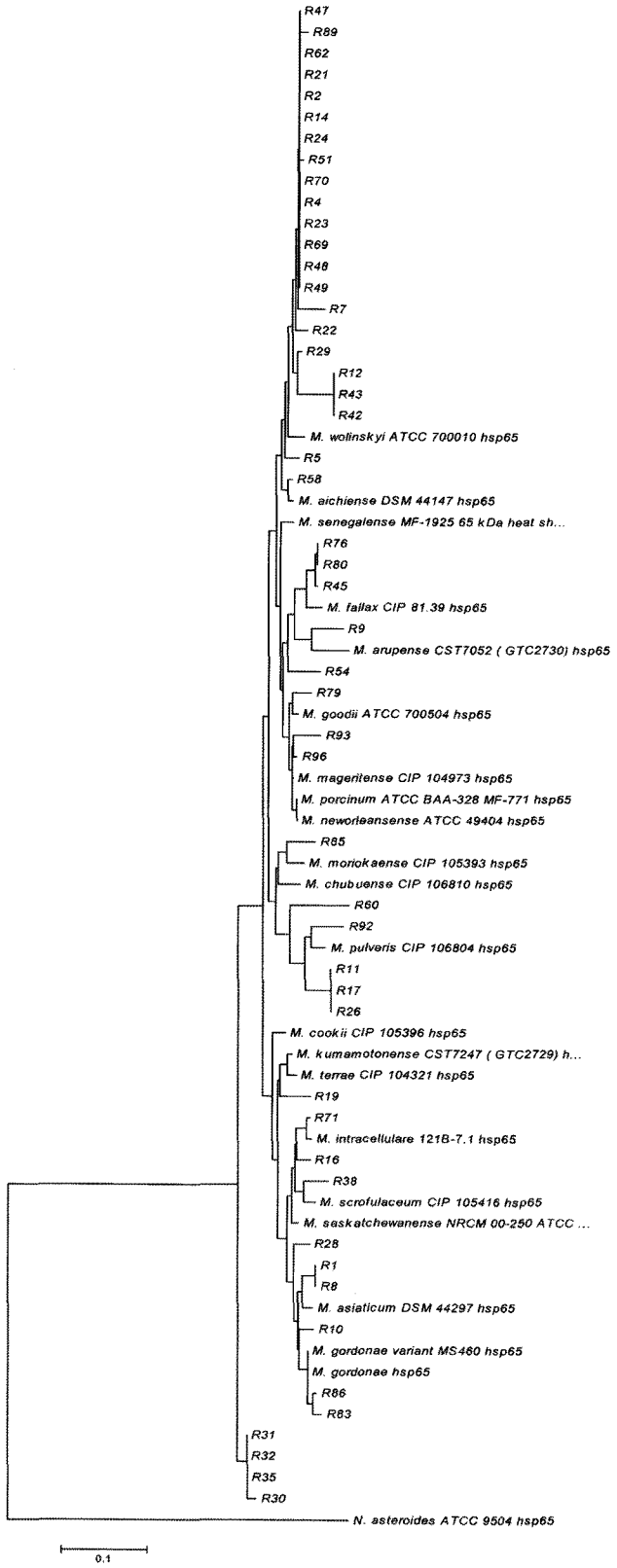

Fig. 4. Phylogenetic tree based upon *hsp65* sequences of surface water isolates and reference strains.
N. asteroides was used as an outgroup sequence. Scale bar=0.1% of difference. R, surface water isolates.

hsp65 gene sequencing. In addition, 14 strains of the 28 mycobacterial isolates unidentified by PRA in the tap water samples were identified at a species level, namely, *M. lentiflavum* (11 strains) and *M. triplex* (3), by the *hsp65* gene sequencing. Interestingly, the PRA patterns of the 11 isolates identified as *M. lentiflavum* by sequencing were different to the pattern of *M. lentiflavum* included in the PRA database. This indicated that the mycobacterial isolates identified as *M. lentiflavum* by sequencing could have diverse PRA patterns like *M. gordonae*, which are known to have at least nine *hsp65* restriction patterns [21]. The isolates showing low similarity (<97%) with type strains were assigned as *Mycobacterium* spp. and an unrooted phylogenetic tree was constructed for these isolates by the neighbor-joining method using MEGA 3.1. Forty-eight strains (58%) of the 83 isolates obtained from the surface water samples showed low similarity (<97%) with type strains. Among these isolates, 45 strains had similarity values between 96% and 90%, and only 3 strains had less than 89%. The surface water isolates having <97% sequence identity with type strains were related to variable species

Fig. 5. Phylogenetic tree based upon *hsp65* sequences of tap water isolates and reference strains.
N. asteroides was used as an outgroup sequence. Scale bar=0.1% of difference. T, tap water isolates.

of mycobacteria, such as *M. wolinskyi*, *M. asiaticum*, *M. gordonae*, *M. fallax*, and so on (Fig. 4). Many of these surface water isolates were most closely related to *M. wolinskyi*, whereas many of the isolates showing similarity of more than 97% were closely related to *M. gordonae*.

On the other hand, 18 strains (30%) of the 60 isolates obtained from the tap water samples showed low similarity (<97%) with type strains. Among these isolates, 14 strains had similarity values between 96% and 90%, and only 4 strains had less than 89%. The phylogenetic tree showed that most of the tap water isolates having <97% sequence identity with type strains were most closely related to *M. lentiflavum* and *M. triplex*, like the tap water isolates showing similarity of more than 97% (Fig. 5).

DISCUSSION

Environmental mycobacteria are common saprophytes in all ecosystems including diverse waters [4, 7, 8, 15]. Since these mycobacteria have recently been reported to be implicated in a variety of human diseases [4, 28, 29], their occurrence and species identification in waters have been a major concern for public health. However, little information is available for their occurrence in waters owing to their slow growth and lack of appropriate detection methods. In this study, culture-dependent and molecular methods were combined to accurately detect and identify environmental mycobacteria in surface and tap waters.

Decontamination procedure using cetylpyridinium chloride (CPC) is performed to remove or reduce the growth inhibition by other microorganisms present in the water samples. Several studies have been conducted to determine the optimum decontamination conditions [18, 20, 25]. Although the pre-treatment with CPC has been shown to promote the recovery of a wide range of mycobacteria with low rates of background contamination [7, 10, 25, 27], there is no clear consensus regarding the optimum conditions. In this study, mycobacteria were somewhat easily isolated from the water samples with the CPC treatment method used.

The *hsp65* gene is present in all mycobacterial species. It displays a higher degree of interspecies variability compared with the 16S rRNA gene, making the *hsp65* potentially useful for the identification of mycobacteria [11]. The *hsp65* PRA is an easy and rapid method and it can identify several mycobacterial species through one experiment using only two restriction enzymes [4, 9, 19, 23, 24, 26]. In addition, sequencing of the *hsp65* gene produces reproducible data in a relatively short time, and thus can successfully speciate *Mycobacterium* and identify new species [2]. In this study, when the species identifications obtained by PRA were compared with those obtained by the *hsp65* gene sequencing, a number of discrepancies were observed. Previous studies discussed several reasons associated with misidentification between the two methods [16, 22]. First, the reported sizes of restriction bands of the same species were different in the available PRA tables. For example, the HaeIII *hsp65* patterns for *M. terrae* are 190/140 in the studies of Telenti *et al.* [26] and Devallois *et al.* [9], whereas the patterns are 187/132/42/36 in the study of Brunello *et al.* [5]. The HaeIII patterns reported for *M. nonchromogenicum* 2 are 155 [9, 26], 145/91/50 [5], and 140/60/55 [21]. Likewise, the HaeIII patterns for *M. flavescens* 1 are reported as 140 [9, 26] and 140/55/50 [21]. The differences of restriction fragments obtained from the same species confused identification of mycobacteria and led to incorrect identification [5, 9, 11, 25]. Second, two or more mycobacterial species displayed similar PRA patterns, because fragments of similar sizes were not well discriminated in this method. For example, restriction patterns for *M. intracellulare* 2 are BstEII 240/210 and HaeIII 140/105/80 [21], which are very similar to those for *M. kansasii* 1 showing BstEII 240/210 and HaeIII 130/105/80 [9]. In addition, restriction patterns for *M. scrofulaceum* and *M. peregrinum* 2 are similar to each other [9, 26]. The size of the bands in these species differed by only ±10 bp, small differences that were difficult to discriminate for definite identification. Third, experience and skill of PRA handling affected interpretation of band sizes. The short running time of electrophoresis affected the resolution of bands of similar sizes and faint bands made interpretation of band sizes difficult. Highly polymorphic species also make it difficult to interpret PRA patterns. For example, *M. gordonae* exhibits nine different patterns by currently available literature [21]. Another difficulty with PRA is that a single base change may lead to the appearance or disappearance of a restriction site. Different restriction sites may therefore give different results [22]. PRA patterns of new species may not be added to the available database, which may cause additional identification discrepancies in results between *hsp65* sequencing and PRA.

Thus, although the *hsp65* PRA is a simple and a useful method for rapid identification of mycobacterial species, some species could not be correctly identified owing to deficient information of new species, difficulty in interpretation of band sizes, and so on. Therefore, for mycobacterial identification in environmental water, it is best to use the *hsp65* gene sequencing as well, rather than PRA alone. It is also important to standardize the PRA method and necessary to construct a new database by continuous combining of the restriction patterns and by uniting several PRA tables. In our results on the mycobacterial isolates, both *hsp65* PRA and sequencing showed low identification rates. This suggests that many species of environmental mycobacteria may be yet undiscovered, and PRA patterns and sequences of new species may not have been added to available databases.

In the previous study on occurrence of mycobacteria in environmental samples, Covert *et al.* [7] isolated 32

mycobacterial strains (36%) from 89 drinking water samples in distribution systems. The majority of samples (>80%) showed the colony counts of 1 to 20 CFU/500 ml and *M. mucogenicum* was the most frequently isolated organism. MAC organisms were included in 19% of the positive samples [7]. Chang *et al.* [6] investigated the tap water samples of a hospital in China. Ten of 49 tap water samples (20.4%) contained environmental mycobacteria. The isolates were identified as *M. szulgai*, *M. simiae*, *M. scrofulaceum*, *M. gastri/kansasii*, *M. gordonae* 2, *M. gordonae* 1, and *M. fortuitum*. Le Dantec *et al.* [15] reported that 104 (72%) of 144 water samples of water distribution systems in Paris were positive for mycobacteria [15]. Concentrations between 1 and 50 CFU/l were found in 78% of the samples. Fifty-five % of the positive samples could not be identified and *M. gordonae* was the most frequently identified species. Torvinen *et al.* [27] investigated some drinking water systems using the surface water and the ground water as the source water. The concentration of mycobacteria in water samples taken from the waterworks ranged from 10 to 30 CFU/l. The concentration of mycobacteria in water samples taken from the distal sites ranged from 10 to 3,500 CFU/l. *M. lentiflavum* and a previously unclassified group of mycobacteria were the most frequently identified species. In our study, as in the result of Torvinen *et al.* [27], *M. lentiflavum* was also the most frequently identified isolate from the tap water samples. This indicates that the organism may be ubiquitous in the drinking water.

M. avium complex (MAC), known as a human pathogen, was isolated at low levels from the surface waters and not isolated from the tap waters of this study. Moreover, other potentially pathogenic mycobacteria were not found in the tap water. Although all tap water samples of this study had sufficient free chlorine residuals (0.45–0.80 mg/l), some mycobacteria were recovered in the tap water. Our result showed that the species composition of mycobacteria in the surface water samples was different from that in the tap water samples, suggesting that one habitat of mycobacteria may be the biofilm of the distribution systems. Neither total coliforms nor heterotrophic plate counts were associated with the concentration of mycobacteria, showing that the typical microbial water quality parameters cannot be used as an indicator for the presence of mycobacteria in water.

In conclusion, our results suggest that water is an important environmental source of mycobacteria. Combined application of *hsp65* PRA and sequencing is more reliable than *hsp65* PRA alone to accurately identify environmental mycobacteria present in water. A number of strains still could not be identified by known PRA and sequence databases for environmental mycobacterial species, which necessitates to construct a new database by continuously combining *hsp65* restriction patterns and *hsp65* gene sequences. Since environmental mycobacteria are implicated in a variety of human diseases, it is necessary to conduct continuous mycobacterial monitoring not only on the drinking water system but also on the biofilm and to study the potential health risk of unidentified species.

Acknowledgment

This study was supported by the Waterworks Research Institute, Seoul Metropolitan Government and the BioGreen 21 Project.

References

1. Ahn, J. H., M. S. Kim, M. C. Kim, J. S. Lim, G. K. Lee, J. K Yun, T. S. Kim, T. S. Kim, and J. O. Ka. 2006. Analysis of bacteria diversity and community structure in forest soils contaminated with fuel hydrocarbon. *J. Microbiol. Biotechnol.* **16:** 704–715.
2. APHA. 1998. *Standard Methods for the Examination of Water and Wastewater*, 20th Ed. American Public Health Association, Washington, D.C.
3. Baek, K. H., B. D. Yoon, B. H. Kim, D. H. Cho, I. S. Lee, H. M. Oh, and H. S. Kim. 2007. Monitoring of microbial diversity and activity during bioremediation of crude oil-contaminated soil with different treatment. *J. Microbiol. Biotechnol.* **17:** 67–73.
4. Bland, C. S., J. M. Ireland, E. Lozano, M. E. Alvarez, and T. D. Primm. 2005. Mycobacterial ecology of the Rio Grande. *Appl. Environ. Microbiol.* **71:** 5719–5727.
5. Brunello, F., M. Ligozzi, E. Cristelli, S. Bonora, E. Tortoli, and R. Fontana. 2001. Identification of 54 mycobacteria species by PCR-restriction fragment length polymorphism analysis of the *hsp65* gene. *J. Clin. Microbiol.* **39:** 2799–2809.
6. Chang, C. T., L. Y. Wang, C. Y. Liao, and S. P. Huang. 2002. Identification of nontuberculous mycobacteria existing in tap water by PCR-restriction fragment length polymorphism. *Appl. Environ. Microbiol.* **68:** 3159–3161.
7. Covert, T. C., M. R. Rodgers, A. L. Reyes, and G. N. Stelma Jr. 1999. Occurrence of nontuberculous mycobacteria in environmental samples. *Appl. Environ. Microbiol.* **65:** 2492–2496.
8. Dailloux, M., C. Laurain, M. Weber, and P. H. Hartemann. 1999. Water and nontuberculous mycobacteria. *Wat. Res.* **33:** 2219–2228.
9. Devallois, A., K. S. Goh, and N. Rastogi. 1997. Rapid identification of mycobacteria to species level by PCR-restriction fragment length polymorphism analysis of the *hsp65* gene and proposition of an algorithm to differentiate 34 mycobacterial species. *J. Clin. Microbiol.* **35:** 2969–2973.
10. Falkinham, J. O., C. D. Norton, and M. W. Lechevallier. 2001. Factors influencing numbers of *Mycobacterium avium*, *Mycobacterium intracellulare*, and other mycobacteria on drinking water distribution systems. *Appl. Environ. Microbiol.* **67:** 1225–1231.
11. Häfner, B., H. Haag, H. K. Geiss, and O. Nolte. 2004. Different molecular methods for the identification of rarely isolated non-tuberculous mycobacteria and description of new *hsp65* restriction fragment length polymorphism patterns. *Mol. Cell. Probes* **18:** 59–65.

12. Izhar, U. H. K. and J. S. Yadav. 2004. Development of single-tube, cell lysis-based, genus-specific PCR method for rapid identification of mycobacteria: Optimization of cell lysis, PCR primers and condition and restriction pattern analysis. *J. Clin. Microbiol.* **42:** 453–457.
13. Ju, D. H., M. K. Choi, J. H Ahn, M. H. Kim, J. C. Cho, T. S. Kim, T. S. Kim, and J. O. Ka. 2007. Molecular and ecological analyses of biofilms of a full-scale aerated up-flow biobead process. *J. Microbiol. Biotechnol.* **17:** 253–261.
14. Kim, M. S., J. H. Ahn, M. K. Jung, J. H. Yu, D. H. Joo, M. C. Kim, *et al.* 2005. Molecular and cultivation-based characterization of bacterial community structure in rice field soil. *J. Microbiol. Biotechnol.* **15:** 1087–1093.
15. Le Dantec, C., J. P. Duguet, A. Montiel, N. Dumoutier, S. Dubrou, and V. Vincent. 2002. Occurrence of mycobacteria in water treatment lines and in water distribution system. *Appl. Environ. Microbiol.* **68:** 5318–5325.
16. Leão, S. C., A. Bernardelli, A. Cataldi, M. Zumarraga, J. Robledo, T. Realpe, *et al.* 2005. Multicenter evaluation of mycobacteria identification by PCR restriction enzyme analysis in laboratories from Latin America and the Caribbean. *J. Microbiol. Methods* **61:** 193–199.
17. Leclerc, M. C., N. Haddad, R. Moreau, and M. F. Thorel. 2000. Molecular characterization of environmental *Mycobacterium* strains by PCR-restriction fragment length polymorphism of *hsp65* and by sequencing of *hsp65*, and of 16S and ITS1 rDNA. *Res. Microbiol.* **151:** 629–638.
18. Livanainen, E., P. J. Martikainen, and M. L. Katila. 1997. Comparison of some decontamination methods and growth media for isolation of mycobacteria from northern brook waters. *Appl. Microbiol.* **82:** 121–127.
19. MaNabb, A., D. Eisler, K. Adie, M. Amos, M. Rodrigues, G. Stephens, W. A. Black, and J. Isaac-Renton. 2004. Assessment of partial sequencing of the 65-kilodalton heat shock protein gene (*hsp65*) for routine identification of *Mycobacterium* species isolated from clinical sources. *J. Clin. Microbiol.* **42:** 3000–3011.
20. Neumann, M., S. R. Roland, H. Claudia, and B. Katja. 1997. Comparison of methods for isolation of mycobacteria from water. *Appl. Environ. Microbiol.* **63:** 547–552.
21. PRASITE. http://app.chuv.ch/prasite/index.html.
22. Ringuet, H., C. Akoua-Koffi, S. Honore, A. Varnerot, V. Vincent, P. Berche, J. L. Gaillard, and C. Pierre-Audigier. 1999. *Hsp65* sequencing for identification of rapidly growing mycobacteria. *J. Clin. Microbiol.* **37:** 852–857.
23. Silva-Rocha, A., C. da Costa Leite, H. M. Torres, A. B. de Miranda, M. Q. P. Lopes, W. M. Degrave, and P. N. Suffys. 1999. Use of PCR-restriction fragment length polymorphism analysis of the *hsp65* gene for rapid identification of mycobacteria in Brazil. *J. Microbiol. Methods* **37:** 223–229.
24. Steingrube, V. A., J. L. Gibson, B. A. Brown, Y. Zhang, R. W. Wilson, M. Rajabopalan, and R. J. Wallace Jr. 1995. PCR amplification and restriction endonuclease analysis of 65-kilodalton heat shock protein gene sequence for taxonomic separation of rapidly growing mycobacteria. *J. Clin. Microbiol.* **33:** 149–153.
25. Stinear, T., T. Ford, and V. Vincent. 2004. Analytical methods for the detection of waterborne and environmental pathogenic mycobacteria, pp. 62. *In* S. Pedley, J. Bartram, G. Rees, A. Dufour, and J. A. Cotruvo (eds.), *Pathogenic Mycobacteria in Water.* World Health Organization (WHO), IWA Publishing, London, U.K.
26. Telenti, A., F. Marchesi, M. Balz, F. Bally, E. Botter, and T. Bodmer. 1993. Rapid identification of mycobacteria to the species level by polymerase chain reaction and restriction enzyme analysis. *J. Clin. Microbiol.* **31:** 175–178.
27. Torvinen, E., S. Suomalainen, M. J. Lehtola, I. T. Miettinen, O. Zacheus, L. Paulin, M. L. Katila, and P. J. Martikainen. 2004. Mycobacteria in water and loose deposits of drinking water distribution systems in Finland. *Appl. Environ. Microbiol.* **70:** 1973–1981.
28. USEPA. 2002. *Mycobacteria: Drinking Water Fact Sheet.* United States Environmental Protection Agency, Washington, D.C.
29. USEPA. 1999. *Mycobacteria: Health Advisory.* United States Environmental Protection Agency, Washington, D.C.
30. USEPA. 2000. *Technical Background Information for the UCMR*, pp. 6. United States Environmental Protection Agency, Washington, D.C.