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Original Article

Variation and Characterization of Bacterial Communities Contaminating Two Saunas Operated at 64℃ and 76℃

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This study was performed to analyze 6 day-term variations in bacterial communities contaminating the floor of two dry saunas that were operated at 64℃ (low temp) and 76℃ (high temp). Bacteria were sampled daily from the saunas for 6 days from Monday to Saturday. Genomic DNA was isolated directly from bacteria-collected cotton swabs. The diversity of the bacterial communities collected from the saunas was analyzed using thermal gradient gel electrophoresis (TGGE). The total numbers of DNA bands separated by TGGE for bacteria collected from the low temp and high temp sauna were 20 and 18, respectively, during the 6 days. Seven of 20 bacteria in the low temp sauna and eight of 18 bacteria in the high temp sauna were detected more than three times over the 6 experimental days. Twelve of the 26 bacterial genera contaminating the saunas were cross detected. Bacteria belonging to the genera *Moraxella* and *Acinetobacter* were selectively detected in the low temp sauna, whereas those belonging to *Aquaspirillum*, *Chromobacterium*, *Aquabacterium*, *Gulbenkiania*, *Pelomonas*, and *Aquitale*a were selectively detected in the high temp sauna. Three species of bacteria contaminating both the low and high temp saunas were thermophile or thermoduric. The results indicate that the sauna-contaminating bacteria may have been transferred from outside the saunas by user traffic but did not inhabit the saunas.

Key Words: Sauna-contaminant, Thermophile, Thermoduric, TGGE, Spore-forming bacteria

INTRODUCTION

We have previously characterized a specific bacterial community obtained by single-time and single point sampling from a sauna operated at 75~80℃ using thermal gradient gel electrophoresis (TGGE) (1). In that study, selectively isolated thermophilic bacteria grew maximally at 40℃ in both defined and complex medium but limited growth occurred at 50℃ in a defined medium and at 60℃ in a complex medium. Bacteria isolated from a 75~80℃ sauna were thermoduric but not thermophilic. However, the diversity and characteristics of the bacterial community were analyzed by single-time and single-point sampling may vary opportunistically according to the user number, residence time, and physiological condition.

Thermophilic bacteria are different from thermoduric bacteria by their ability to regenerate and grow under thermal conditions of 45~122℃ (2, 3). The survivability of thermoduric bacteria in a hot and dry sauna and under heat of pasteurization depends upon their ability to sporulate (4, 5). Spore-forming bacteria may be thermoduric or thermophilic. But, thermophilic bacteria can't grow at mammalian temperatures (30~40℃); however, thermoduric bacteria can grow at mammalian temperatures and generate spores at higher than mammalian temperatures (6~9). Dry

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saunas are generally operated at temperatures > 60℃, which may not be a proper temperature for thermophilic bacteria, but an environmental signal for thermoduric bacteria to generate spores. Dry saunas may not be nutritionally proper for bacterial growth but may be opportunistically contaminated with various bacteria and organic compounds by user traffic (10). User traffic may be the major cause of sauna contamination by bacteria, as human traffic supplies nutrients needed by specific thermoduric and thermophilic bacteria to opportunistically survive.

Theoretically, psychrophiles and mesophiles can't grow and inhabit the environmental conditions inside a sauna; however, spore-forming bacteria may survive for a particular period, considering that endospores are often highly resistant to chemical- and heat-treatment (11). Species belonging to the endospore-forming bacteria are found in both natural and artificial environments (12, 13). Endospore-forming bacteria are widely distributed in the natural environment such as soil and water. Most bacteria dwelling around humans may be transferred into a sauna through direct contact with the human body (14, 15).

In this study, the bacteria collected from two saunas on multiple occasions was analyzed using TGGE to compare the daily variations in the bacterial community during 6 days from Monday to Saturday and a conditional variation in the bacterial community based on the temperature difference between the two saunas.

MATERIALS AND MOTHODS

Bacterial sampling

A digital thermometer on the outside wall of the two saunas indicated that the internal temperatures were 64℃ and 76℃ in the low temp and high temp saunas, respectively. Bacteria were sampled from the floor of the dry sauna using sterilized cotton swabs. Samples were collected around 9 o'clock in the morning on December 24~29, 2012. An aluminum foil-wrapped cotton swab was opened immediately before sampling, and the floor was then deeply and broadly wiped with three cotton swabs per sample area. The sampling points were located at 30 cm from each wall in the central part because user traffic was mainly busy between the central part and each wall but not around the corners. The diameter of the sampling area was about 30 cm (1). In total, 15 swabs were taken and used for DNA extraction. The part of the cotton swab used for bacterial sampling was placed in a sterilized conical tube immediately after sampling, and the other end was removed and used in a previous study (1).

16S-rDNA amplification

Total DNA was directly extracted from the bacterial cells collected from the cotton swabs using a Genomic DNA extraction kit (Accuprep; Bioneer, Daejeon, Korea) according to the manufacturer's protocol. 16S ribosomal DNA was amplified via direct polymerase chain reaction (PCR) using the chromosomal DNA template and 16S-rDNA specific universal primers as follows: forward 5'-GAGTTGGATCCTGGCTCAG-3' and reverse 5'-AAGGAGGGGATCCAGCC-3'. The PCR reaction mixture (50 μl) consisted of 2.5 U Tag polymerase, 250 μM of each dNTP, 10 mM Tris-HCl (pH 9.0), 40 mM KCl, 100 ng template, 50 pM primer, and 1.5 mM $MgCl_2$. Amplification was conducted for 30 cycles of 1 min at 95℃, 1 min of annealing at 55℃, and 2 min of extension at 72℃ using a PCR machine (T Gradient model, Biometra, Göttingen, Germany).

TGGE

The 16S-rDNA amplified from chromosomal DNA was employed as the template for TGGE sample preparation. A variable region of 16S-rDNA was amplified using the forward primer 341f 5'-CCTACGGGAGGCAGCAG-3' and reverse primer 518r 5'-ATTACCGCGGCTGCTGG-3'. A GC clamp (5'-CGCCCGCCGGCGGGCGGGCGGGGCGGGGGCACGGGGGG-3') was attached to the 5'-end of the 341f primer (16). The PCR and DNA sequencing procedures were identical to the 16S-rDNA amplification conditions, with the exception of annealing temperature. The TGGE system (Bio-Rad, Dcode^{TM}, Hercules, CA, USA) was operated in accordance with the manufacturer's specifications. Aliquots (45 ml) of the PCR products were electrophoresed on gels containing 8% acrylamide, 8 M urea,

and 20% formamide in 1.5 ×TAE (Tris, acetate, and EDTA) buffer system at a constant voltage of 100 V for 12.5 h, followed by 40 V for 0.5 h, with a temperature gradient of 39~52℃. Prior to electrophoresis, the gel was equilibrated to the temperature gradient for 30~45 min.

Amplification and identification of the TGGE band

DNA was extracted from the TGGE band and purified with a DNA gel purification kit (Accuprep, Bioneer). The purified DNA was then amplified with the same primers and procedures used for TGGE sample preparation, except that the GC clamp was not attached to the forward primer. The amplified DNA was sequenced to identify the bacteria based on 16S-rDNA sequence homology using GenBank database.

RESULTS

TGGE patterns

The daily variations in the bacterial community collected from the floors of the low and high temp saunas were analyzed by the TGGE technique. The TGGE pattern for the bacteria contaminating the low temp sauna was not significantly different from that contaminating the high temp sauna (Fig. 1). A range of 5~10 species was observed daily in the low temp sauna, whereas 8~12 species contaminating the high temp sauna based on the DNA band number. Variations in DNA band number and DNA band position (migration distance on electrophoretic gel) were not patterned by specific days of the week. This result shows that the bacteria contaminating the saunas may be opportunistic and related to both user traffic and operating temperature of a sauna.

Identification of DNA separated by TGGE

Twenty and 18 bacterial species were separated by TGGE and identified by sequence homology in the low and the high temp saunas, respectively (Tables 1 and 2). Bacteria in *Moraxella* and *Acinetobacter* were selectively detected in the low temp sauna, whereas bacteria in *Aquaspirilum*, *Chromobacterium*, *Aquabacterium*, *Gulbenkiania*, *Pelomonas*, and *Aquitalea* were selectively detected in the high temp sauna during the 6-day sampling from Monday-Saturday. The bacterial diversity contaminating the saunas may be directly influenced by individual bacteria-contaminated users. An uncultured bacterium (JN883765) was detected daily in the low temp sauna, whereas *Neisseria flava* and uncultured bacterium (JN883765) were detected daily in the high temp sauna. The two bacteria that were detected daily in the low or high temp saunas commonly belonged to the normal microflora of specific organs of the human body (Table 3), suggesting that the bacteria detected in the saunas may be contaminants from user bodies but not residents in the saunas. The user's body may be the main source of bacteria which contaminate a sauna.

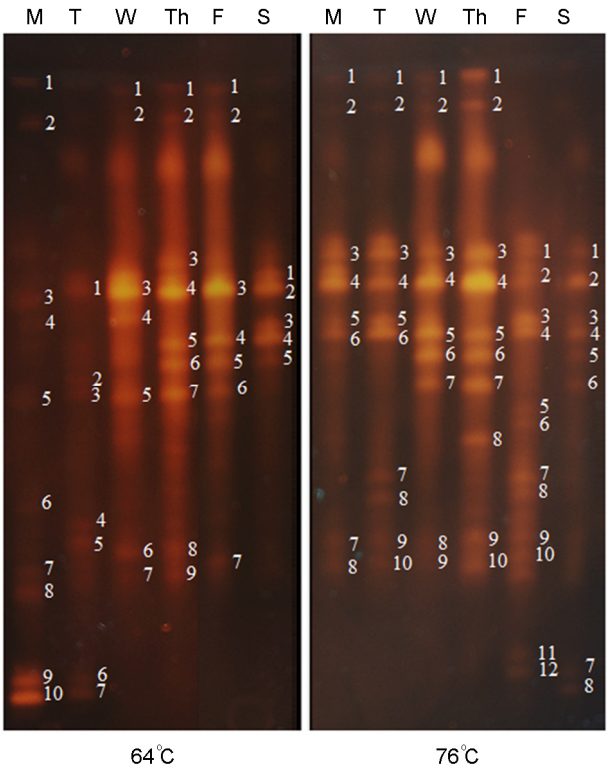

Figure 1. Thermal gradient gel electrophoresis (TGGE) profiles of 16S-rDNA isolated from bacteria that were collected from floors of two different saunas operated at 64℃ and 76℃. Each number on electrophoresis gel indicates DNA band originated from a specific bacterial species. Abbreviations: M, Monday; T, Tuesday; W, Wednesday; Th, Thursday; F, Friday; S, Saturday.

Table 1. Bacterial species identified based on sequence homology of 16S-rDNA extracted from electrophoresis gel (figure 1, 64℃). Thermal gradient gel electrophoresis was performed with the DNA extracted from the bacteria daily collected from low temp sauna from Monday to Saturday.

Bacteria (Genus or species)	Accession code	Homology (%)	Week: band number in TGGE gel					
			Mon	Tue	Wed	Thu	Fri	Sat
Bacterium ODP-193	AB084523	98	1					
Uncultured bacterium	**HE649228**	98			1	1	1	
Enhydrobacter aerosaccus	JX845725	98	2		2	2	2	
Neisseria flava	HF558370	98				3		
Moraxella cuniculi	**AJ247221**	99						1
***Moraxella* sp.**	**KC119125**	98						2
Uncultured bacterium	JN883765	98	3	1	3	4	3	3
Moraxella pluranimalium	**NR042666**	98	4		4			
Acinetobacter radioresistens	JF919868	97				5	4	4
Acinetobacter sp.	GU430989	98				6	5	5
Bacillus megaterium	JX311358	99		2			6	
Acinetobacter seohaensis	**EU420936**	98	5	3	5	7		
Uncultured bacterium	**JF237408**	99		4				
Acinetobacter beijerinckii	**JN644620**	98		5				
Uncultured *Acinetobacter*	**JN866218**	99			6	8		
Leptothrix sp.	JQ946028	97	6					
Uncultured bacterium	AB732642	98	7		7	9	7	
Uncultured bacterium	**JF661828**	97	8					
Deinococcus geothermalis	EU600161	97	9	6				
Uncultured bacterium	JN882031	99	10	7				

*Bold letters: Bacteria selectively detected in 64℃ sauna

General characteristics of the bacteria detected by TGGE

The general characteristics of the bacteria collected from the low and high temp saunas was tabulated based on genus or species information reported by other researchers or from a database. As shown in Table 3, most of the bacteria identified based on 16S-rDNA extracted from the TGGE gel and collected from both the low and the high temp saunas were not thermophiles except bacterium ODP-193-27 (17), *Bacillus megaterium* (18), and *Deinococcus geothermalis* (19). *Bacillus megaterium* is a spore-forming thermoduric bacterium but not a thermophile. Thermophilic and thermoduric bacteria were detected one to four times over the 6 experimental days, whereas mesophilic bacteria were detected less than three times during the entire sampling period. In contrast, *Neisseria flava* (20) was detected in the high temp sauna everyday over 6 days and an uncultured bacterium (JN883765) was detected in both saunas every day over the 6 days (21). Both bacterial species detected each day are known to contaminate specific organs of human body (20, 21); whereas most of the bacteria detected in both the high and the low temp sauna are known to inhabit natural habitats such as spring water, wastewater, soil, seawater, and lake water (22~31). From this result, it can be presumed that the human body may be the most suitable carrier for bacteria because the human body is a proper habitat for bacteria to temporarily survive or grow.

Table 2. Bacterial species identified based on sequence homology of 16S-rDNA extracted from electrophoresis gel (figure 1, 76℃). Thermal gradient gel electrophoresis was performed with the DNA extracted from the bacteria collected from high temp sauna from Monday to Saturday.

Bacteria (Genus or species)	Accession code	Homology (%)	Week: band number in TGGE gel					
			Mon	Tue	Wed	Thu	Fri	Sat
Bacterium ODP-193	AB084523	98	1	1	1	1		
Enhydrobacter aerosaccus	JX845725	98	2	2	2	2		
Neisseria flava	HF558370	98	3	3	3	3	2	1
Uncultured bacterium	JN883765	98	4	4	4	4	3	2
Aquaspirillum serpens	**AB680863**	100	5	5			4	3
Acinetobacter radioresistens	JF919868	97	6	6	5	5		4
Acinetobacter sp.	GU430989	98			6	6		5
Bacillus megaterium	JX311358	99			7	7		6
***Chromobacterium* sp.**	**HQ234407**	98					5	
Uncultured *Aquabacterium* sp.	**JQ288705**	98					6	
Gulbenkiania mobilis	**NR042548**	97				8		
***Pelomonas* sp.**	**AB730488**	97		7			7	
Leptothrix sp.	JQ946028	97		8			8	
Acinetobacter beijerinckii	JN644620	98	7	9	8	9	9	
Uncultured bacterium	AB732642	98	8	10	9	10	10	
***Aquitalea* sp.**	**JN208179**	97					11	
Deinococcus geothermalis	EU600161	97					12	7
Uncultured bacterium	JN882031	99						8

*Bold letters: Bacteria selectively detected in 76℃ sauna

DISCUSSION

Bacteria are ubiquitous organisms based on the diversity of their habitats and natural ecosystems, such as animals (intestine, skin, and genital organs), plants (leaves and roots) and man-made structures (32~36). The most popular man-made structures are buildings utilized as dwellings, businesses, and manufacturing plants, which may be habitats for heterotrophic bacteria due to the plentiful organic compounds and the proper environmental conditions (37). A sauna is a hot, dry room located inside a building, and the environmental conditions are not suitable for any organisms except thermophilic bacteria (1). Temperature is one of the major environmental factors influencing bacterial growth and has been artificially controlled to cultivate fermentation bacteria, suppress harmful bacteria, or destroy pathogenic bacteria (38~40). Generally, the temperature of a sauna is 60~80℃, which is sufficient to inhibit or stop growth of mesophilic bacteria except spore-formers (41).

The mesophilic bacteria that contaminated both the low and the high temp saunas may neither survive nor grow but the thermophilic bacteria (bacterium ODP-193-27, *B. megaterium*, *D. geothermalis*) can survive and grow in both saunas based on their characteristics (17~19). However, the growth of thermophilic bacteria contaminating both saunas must be experimentally verified because a sauna is an artificial location that satisfies only the temperature conditions for thermophilic bacteria (42). The ecological niche of bacteria is mineralization of organic compounds by parasitism, symbiosis, and saprophytism (43~45). The thermophilic bacteria that contaminated the saunas may

Table 3. General characters of bacterial genus or species identified based on sequence homology of DNA extracted from electrophoresis gel (Fig. 1). DNA used for thermal gradient gel electrophoresis was directly extracted from bacteria collected from 64℃ and 76℃ of dry saunas.

Bacteria (Accession code)	Detection days (LTS/HTS)	General characters or released information
Bacterium ODP-193-27	1/4	A thermophile capable of growing at 60~90℃, which were found in subsurface of hydrothermal vent (17)
Enhydrobacter aerosaccus	3/4	A heterotrophic, mesophilic, non-pathogenic, gas-vacuolated, and facultative anaerobic bacterium (22)
Neisseria flava	1/6	An non-pathogenic, mesophilic, and anaerobic bacterium that is often found in the upper respiratory tract surface in humans (20)
Moraxella sp.	1/0	A mesophilic and opportunistically infective bacterium, and some species belonging to this genus are commensal of mucosal surface (43)
Moraxella pluranimalium	2/0	Gram-negative, mesophilic, and heterotrophic bacterium that was isolated from sheep and pig (44)
Acinetobacter radioresistens	3/3	A mesophilic, non-spore-forming, aerobic, and soil-dwelled bacterium (23)
Chromobacterium sp.	0/1	A mesophile that inhabits in soil and natural water is sometime is found in foods (24)
Uncultured *Aquabacterium* sp.	0/1	It has been isolated and found from drinking water and freshwater spring (25)
Bacillus megaterium	2/3	This bacterium is able to survive in some extreme conditions such as desert environments due to the spores it forms. (18)
Acinetobacter seohaensis	4/0	A Gram-negative, non-motile, and mesophilic bacterium that was isolated from sea water of the Yellow Sea in Korea (26)
Deinococcus geothermalis	2/2	*This is* an extremely radiation resistant, moderately thermophilic bacterium (19)
Gulbenkiania mobilis	0/1	A mesophilic bacterium that was isolated from treated municipal wastewater (27)
Pelomonas sp.	0/1	A mesophilic, Gram-negative, non-spore-forming bacterium that was isolated from industrial wastewater and haemodialysis water (28)
Leptothrix sp.	1/2	A filamentous and mesophilic bacterium that resides in organic matter-plentiful aquatic environments (29)
Aquitalea sp.	0/1	A mesophilic, Gram-negative, and non-spore-forming bacterium that was isolated from humic-lake samples (30)
Uc. Bacterium (HE649228)	3/0	A bacterium that was detected in a uranium mine tailing sediment-water interface at Key Lake, Northern Saskatchewan
Uc. Bacterium (JN883765)	6/6	A bacterium belonging to normal gut microflora in human (21)
Uc. Bacterium (JF237408)	1/0	A bacterium belonging to human skin microflora related with atopic dermatitis (45)
Uncultured *Acinetobacter*	2/0	A Gram-negative, non-motile, and mesophilic bacterium that was isolated from Tibetan Plateau.
Uc. Bacterium (AB732642)	4/5	A bacterium detected in arsenic sediment
Uc. Bacterium (JF661828)	1/0	A bacterium belonging to microbial community in anaerobic digestion of carrot waste (37)
Uc. Bacterium (JN882031)	2/1	A bacterium that was found in crude oil (31)

*UC, Uncultured bacterium; LTS, Low temp sauna; HTS, High temp sauna

grow by saprophytism because most bacteria were randomly, occasionally, and discontinuously detected from both saunas over the 6 experimental days.

Individuals travel domestically or in foreign countries and

carry various bacteria between locations without realizing that they have contaminated their bodies, clothes, and baggage except in an emergency. Travelers are contaminated with various microorganisms during travel by contacting others, touching, visiting, and buying merchandise. The surface of the human body is a temporary habitat for bacterial growth due to the organic compounds excreted with sweat and sebum. Mesophilic bacteria detected from the saunas may have originated from natural or artificial environments; however, the thermophilic bacteria could have originated from a foreign country based on their general characteristics (Table 1).

In a previous study (1), bacterial samples were collected from a sauna operated at 75~80℃ on July 25, 2012 and most were thermoduric and spore-forming bacteria belonging to the *Bacillus* genus. The bacterial community collected from saunas during the summer is significantly different from that collected during the winter, which may be caused by differences in environmental conditions between summer and winter (46). Plentiful organic compounds, high temperature, and high humidity of a natural environment during the summer may be a more proper condition for *Bacillus* sp. than other bacteria. The seasonal differences in the bacterial communities that contaminated the saunas may not be a general phenomenon because sampling period, number, and area in each study were not equivalent.

In this study, the daily variation in bacteria contaminating the saunas may not be proportional to the number, frequency of use, or cleanliness condition of users but may be randomly and opportunistically influenced by the bacterial species contaminating the user's' bodies. The bacteria commonly and frequently detected from both saunas can survive on human body for a relatively longer time than those rarely detected in one of the saunas. General house conditions may be a more proper than natural environment for mesophilic bacteria to grow and for thermophilic bacteria to survive, considering the plentiful amount of organic compounds wasted from food.

Our results indicate that both the mesophilic and thermophilic bacteria detected from the saunas could have originated from the user's house and not their body because most people wash their bodies one or two times per day. No human pathogenic or harmful bacteria were in either sauna in this study. The bacteria detected from both saunas over the 6 days provided useful information about the origin of bacterial communities contaminating saunas because the saunas are not a proper and stable habitat for mesophilic bacteria to grow. Saunas may be a specific place to collect various bacteria from the human body but not permit growth of mesophilic bacteria. Most pathogens can grow at mammalian temperature but do not grow at the temperature of a sauna. Bacteria can convert from saprophytism to virulence only while growing continuously (47).

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