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Evaluation of 64°C and 76°C dry saunas as an environment for survival and growth of mesophilic and thermophilic bacteria

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ABSTRACT

In this study, the diversity and variations of bacteria that were sampled from the floor of dry saunas operated at 64°C and 76°C for 6 days (Monday–Saturday) wereanalyzed to determineif the bacteria contaminating the dry saunas can grow, survive, or die. Bacteria sampled from the 64°C sauna grew at 45°C and 60°C but those from the 76°C sauna didnot growat either45°C or60°C. Sixteen and eightspecies of bacteria grewat 45°C and 60°C,respectively. Eight of 16 species grown at 45°C werethermophiles and all bacteria grown at 60°C werethermophiles. DNA of non-growing bacteria sampled from a 76°C sauna wasdirectly extracted and analyzed by temperature gradient gel electrophoresis (TGGE). The non-growing bacteria included13 species based onthe band number of16S-rDNA separatedby TGGE. Thermophilescontaminating the64°C sauna were more frequently detected than mesophilesover the6 experimental days. Most of the mesophiles may have temporarily survivedand some of thermophilic bacteria survived for 4–5 days during sampling for the 6 days,whereas all bacteria contaminating the76°C sauna may lose their physiological function in a short time by drying out.

KEYWORDS

Dry sauna;
Thermophile;
Mesophile; TGGE;
Bacterial habitat.

INTRODUCTION

A sauna is designed to relax its users through sweating and has been used inhealthcare in many countries[1]. The operating temperature of dry saunas is generally 70–90°C, which is higher than mammalian temperature (35–40°C) and pasteurization temperature (63°C)[2,3]. Dry saunas may be oligotrophic because the source of organic compounds contaminating saunas is limited to sweat and usersecretions[4]. High temperature, dry air, and oligotrophic conditionsmay limit survival and growth of bacteria contaminatingdry saunas except spore-forming bacteria. Hot and dry air is used to heat the floor and walls, which may damage bacterial cells contaminating thewalls and floor[5].

Usersusuallymore frequently come in contact with the sauna floor than the walls and contaminate floor with sweat and skin secretions. Theoretically, non-spore-forming bacteria may lose their physiological function in 30 min at 63°C or15 sec at 72°C[6]. Accordingly, the sauna floor may not be the proper place for growth and survival of psychrophilic, mesophilic, and non-spore-forming bacteria that are transferred from outside thesauna through user traffic. However, some bacteria contaminating saunas may be maintainedby frequent contamination generated in proportion toentry and exit of users[7]. Thermophilic and thermoduric bacteria including spore-forming bacteria may also maintain aliving population during a certain period but may not consistently grow dueto insufficient water and the oligotrophic conditions[8].

A saunais anartificially designed thermal environment that is distinctly different from natural ecosystemsbut maybe similar to thermal environments in special places such asthermal vents, hot springs, and geothermal areas[9,10]. Bacteria inhabiting natural thermal-environments are thermophiles that not only can survive heat but also generally grow under the conditions from 45–122°C[11,12]. Thermophilic bacteria are distinguishedfrom thermoduric (spore-forming) bacteria based on a difference intemperature range for normal growth and survival[13,14]. Thermoduric bacteria can survivein thetemperature rangeof asauna and growordinarily in natural ecosystems, whereasthermophilic bacteria can grow at the temperature range of asauna and survive temporarily undernatural conditions[15,16].

Most bacteria inhabiting houses, parks, traffics, workplaces, and agricultural areascan contaminate the human body. Some bacteria that contaminate the human body may grow using secretions or simply survive without growth[17]. Any place where people reside orvisitis a potential source for cross contamination[18]. The cross contamination generated among people may increase bacterial diversity in facilitiessuch aspublic bath, public swimming pool, and public sauna[19]. Saunasmay be a temporary habitat for thermoduric and thermophilic bacteria transferredthrough user traffic dueto theconsistent thermalconditions. Diversity and variationsin natural bacterial communitiesmay be caused by seasonal fluctuationsinenvironmental factors such as temperature, water, nutrient, competitor, and symbiont,whereasdiversity in asauna may be caused by visiting frequency and the number of users. Users may be thesole source for bacteria and nutrients in saunas.

This study wasperformed to analyze diversity and daily variationsinbacteria contaminating two different saunas operated at 64°C and 76°C for 6 days from Monday–Saturday. Diversity may indicatethemicrobial sources for sauna contamination and the daily variation in saunas could helpidentify bacterial species that temporarily survive or periodically contaminate saunas. Difference in thebacterialcommunities contaminating the64°C and 76°C saunas may have been caused by differences insauna temperature.

EXPERIMENTAL

Bacterial sampling

Bacteria were sampled from floor of adry sauna using a sterilized cotton swab. Samples were collected at 10AMwhensome users were presenton February 18–23, 2013. An aluminum foil-wrapped cotton swab was opened immediately before sampling, and the floor was deeply and broadly wiped with twocotton swabs per sample area. The sampling points were located 30 cm from each wall in the central part whereusersfrequented but not around the corners, as shown in Figure 1A. The diameter of the sampling area was about 30 cm. One side end of the cotton swab used for bacterial sampling was placed in a sterilized conical tube immediately after sampling and the other end, which wasgripped bythe sampler,was removed,as shown in Figure 1B[1].

Cultureof the sampled bacteria

Ten cotton swabs were placedin 10 mL saline and thoroughly mixed using a vortex mixer for 5min. One ml of saline containing the bacteria sampled from the

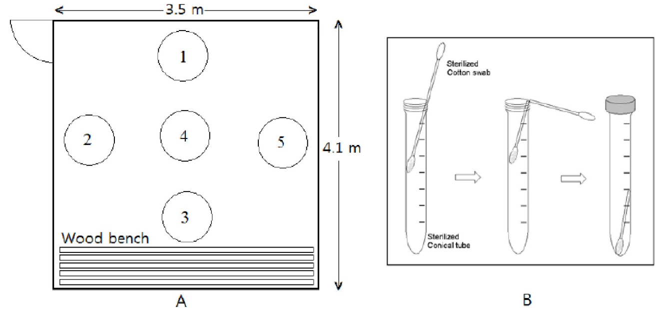

Figure 1: Location of sampling area in floor of dry sauna (A) and sampling method for protection of contamination from sampler's hands (B).

saunas was inoculated into 10 mL complex medium under aseptic condition and incubated at 45°C and 60°C for 24 h to selectively cultivate thermoduric mesophiles and thermophiles. The complex medium was composed of 2.5g/L yeast extract, 2.5g/L peptone, 9g/L glucose, 2g/L KH_2PO_4, and 9g/L NaCl. Medium pH was adjusted to 6.5 before autoclaving.

16S-rDNA amplification

Total DNA was extracted from the bacterial cells grown at 45°C and 60°C 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 52 -GAGTTGGATCCTGGCTCAG-32 and reverse 52 -AAGGAGGGGATCCAGCC-32 . The PCR reaction mixture (50 μl) consisted of 2.5 U Taqpolymerase, 250 ?M 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°C, 1 min of annealing at 55°C, and 2 min of extension at 72°C using a PCR machine (T Gradient model, Biometra, Göttingen, Germany).

Temperature gradient gel electrophoresis (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'-ATTACCGCGGCTGCT- GG-3'. A GC clamp (52 -CGCCCGCCGCGCGCGGCGGGCGGGGCGGGGGCACGGGGGG-3') was attached to the 52 -end of the 341f primer (20). 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™, 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 a 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°C. The gel was equilibrated to the temperature gradient for 30–45 minprior to electrophoresis.

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 identifythe bacteria based on 16S-rDNA sequence homology using GenBank database.

RESULTS

TGGE patterns

The diversity and daily variationsinbacteria that contaminatedthe 64°C sauna and selectively grew at 45°C and 60°C were analyzed by TGGE. The TGGE pattern forbacteria grown at 45°C (Figure 2A) was

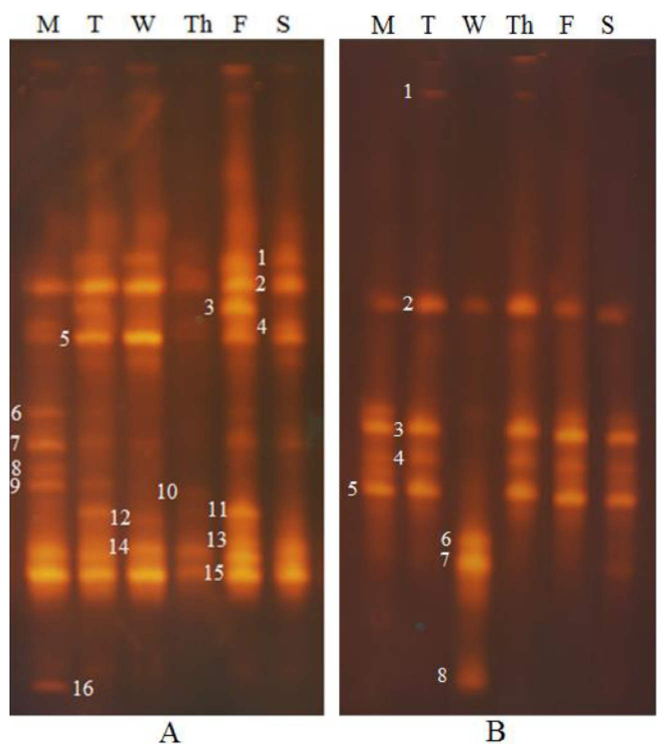

Figure 2 : TGGE profiles of 16S-rDNA obtained from bacteria that were cultivated at 45°C (A) and 60°C (B) for 24hr after collected from 64°C sauna. M, Monday; T, Tuesday; W, Wednesday; Th, Thursday; F, Friday; S, Saturday

significantly different from that at 60°C (Figure 2B). Sixteen species of bacteria grewat 45°C,andeightgrewat 60°C. The daily variationsin thebacterial species grown at 45°C werehigher than those at 60°C based on transition of the DNA band patternfrom Monday–Saturday. The bacterial samples collected from the 76°C sauna didnot grow at either45°C or60°C.

The diversity and daily variations of bacteria that contaminated the 76°C sauna were analyzed based on the TGGE pattern with DNA that was directly extracted from the bacterial samples. 16S-rDNA was obtained from 13 species of non-growing bacteria that contaminated the 76°C sauna based on the diversity of DNA bands shown in Figure 3. This result suggests that a dry sauna operated at 64°C may permit some bacterial species to grow but that 76°C may be a lethal environment for both mesophiles and thermophiles.

Characterization of bacteria contaminating the 64°C sauna

Sixteen and eight species of bacteria sampled from the 64°C sauna grew at 45°C and 60°C, respectively.

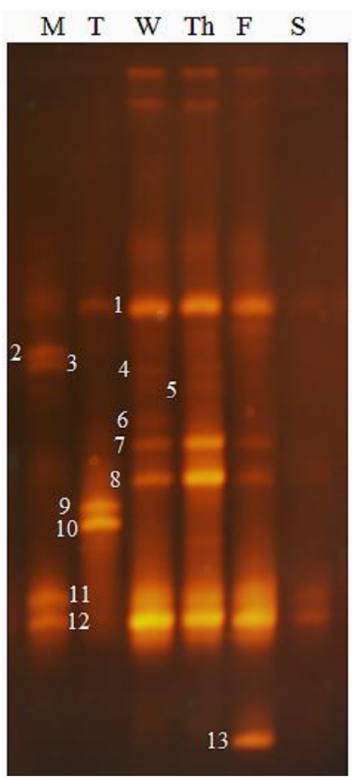

Figure 3 : TGGE profiles of 16S-rDNA obtained from bacteria that were collected from 76°C dry sauna without cultivation. M, Monday; T, Tuesday; W, Wednesday; Th, Thursday; F, Friday; S, Saturday

Seven of 16 bacterial species grown at 45°C were thermophiles and nine were mesophiles (TABLE 1). All mesophiles that were sampled from the 64°C sauna and that grew at 45°C existin natural ecosystems such as soil, water, and animal intestines. *Exiguobacterium*, *Gulbenkiania*, *Geobacillus*, *Anoxybacillus*, *Thermus*, and *Deinococcus*are typical thermophiles that can grow at pasteurization temperatures. All bacterial species grown at 60°C were thermophiles, as shown in TABLE 2 without exception. *Anoxybacillus*, *Geobacillus*, and *Thermus* grew at both 45°C and 60°C.

Characterization of bacteria contaminating the 76°C sauna

16S-rDNA form 13 species was separated from non-growing bacteria contaminating the 76°C sauna (TABLE 3). All bacteria identified based on DNA sequence homology were mesophiles. Most of these bacterial species exist in natural ecosystem and some originated from controlled environments for wastewater treatment, fermentation of foods, BTEX degradation, or phosphorus removal. Thus, mesophiles more frequently contaminated the 76°C sauna than that of thermophiles because the temperate zone is suitable for growth and propagation of mesophiles but not thermophiles. The 16S-rDNA of dominant bacteria can be competitively replicated when PCR is performed with genomic DNA extracted directly from the bacteria sampled from the sauna.

DISCUSSION

The temperate zone is not suitable for growth of thermophilic bacteria whose optimal temperature for normal growth is >45°C. In particular, outdoor temperaturesduringthe winter donot permit growth of either mesophiles orthermophiles. All bacteria including thermophiles donot grow, but rather may be dormant under lower temperaturesthan optimal during the winter[42]. Dormant bacteria may transferbetter thangrowing bacteria due to their survivability. Saunas may also be contaminated by bacteria transferredfrom thehuman body and clothes[43,44].

The bacteria sampled from the 64°C dry sauna were cultivated at 45°C or 60°C, which

TABLE 1 : Bacterial species identified based on sequence homology of 16S-rDNA extracted from TGGE gel (Figure 1). TGGE was performed with the DNA extracted from bacteria that are cultivated at 45°C for 24 hr after collected from 64°C dry sauna.

Band No.	Bacteria (GenBank Accession No)	Detection days	General characters
1	*Exiguobacterium Profundum*(JX535361)	WThFS	A facultatively anaerobic, halotolerant, moderately thermophilic and non-sporulating bacterium (21)
2	*Nitrosospira*sp. (GU189065)	M to S	A mesophilic and denitrifying bacterium that resides in ocean water, freshwater, aquarium water, deep-sea sediment. (22)
3	*Exiguobacterium* sp. (AY745850)	TF	Residing in Greenland glacial ice, hot springs at Yellowstone National Park, the rhizosphere of plants, and around food processing plants (23)
4	*Aquaspirillum serpens* (AB680863)	MThS	A mesophilic, non-motile, non-spore-forming, and aerobic bacterium that resides mostly in freshwater. (24)
5	*Paludiobacterium sp.* (HE981224)	M to S	Facultative anaerobic, Gram-negative, mesophilic, and motile bacterium that resides in natural environment (25)
6	*Gulbenkiania mobilis* (JN411342)	MTF	**A thermophilic bacterium that was isolated from treated municipal wastewater. (26)**
7	*Gulbenkiania* sp. (HF548444)	MTWFS	A Gram-positive and thermophilic bacterium that resides in natural environment (27)
8	*Bacillus vireti* (HQ897170)	M	A typical soil inhabiting and spore-forming mesophile (28)
9	*Chromobacterium* sp. (HQ234417)	MT	A free-living and mesophilic bacterium that resides in water and soil ecosystem. (29)
10	*Geobacillus caldoxylosilycus* (AY647283)	W	A Gram-positive and thermophilic bacterium that resides in natural environment (27)
11	Uncultured bacterium (HQ701533)	F	A mesophilic bacterium that colonizes as normal intestinal flora of humans and animal (30)
12	*Clostridium perfringens* (JX267121)	T	A spore-forming and mesophilic bacterium that resides in natural environments and intestinal tract of human. (31)
13	Bacterium NLAE-zi-P34 (JQ606884)	FS	A mesophilic bacterium isolated from enrichment culture of pig feces for degradation of cellulose and xylan (unpublished)
14	*Anoxybacillus* sp. (JF968626)	TWThF	**A thermophilic spore-forming bacterium that resides in geothermal soil. Optimum temperature and pH for growth are 61°C and 5.6, respectively. (32)**
15	*Thermus thermophiles* (HF558369)	M to S	**An extreme thermophile and Gram-negative bacterium that resides in hot spring and thermal vent (33)**
16	*Deinococcus geothermalis* (EU600161)	M	An extremely radiation resistant, moderately thermophilic bacterium. (34)

*** M, Monday; T, Tuesday; W, Wednesday; Th, Thursday; F, Friday; S, Saturday; * Bold letters: Thermophilic bacteria**

isthemaximumtemperaturefor mesophiles and the universal temperature for thermophiles, respectively[45]. Cultivatingsauna-originatingbacteria at 45°C and 60°Cis useful to select bacteria that can survive pasteurization. Eight of 16 bacterialspecies grown at 45°C were mesophiles and the others were thermophiles that may havecontaminated the sauna through user traffic. The bacteria grown in complex medium at 45°C probably temporarily survivedin the 64°C sauna because all bacteria cultivated in the complex medium at 60°C for 24 h were thermophiles. Some mesophiles that survivedin the 64°C sauna may have beenopportunistically isolated by swabbing during sampling before being completely destroyed by the high temperature.

Most of the bacteria were detected for 1–3 days but *Nitrospira* and *Paludiobacterium* were detected every day during the 6 days ofsampling. The number of users, frequency of traffic, time of use, sampling time, and sampling frequency may be opportunistic factorsfor specific bacteria to be sampled alive at a specified time (10 AM). The detection frequency of mesophiles and thermophiles sampled from the 64°C and 76°C saunas may be proportional to the contamination frequency and

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TABLE 2 : Bacterial species identified based on sequence homology of 16S-rDNA extracted from TGGE gel (Figure 2). TGGE was performed with the DNA extracted from bacteria that are cultivated at 60°C for 24 hr after collected from 64°C dry sauna.

Band No.	Bacteria (GenBank Accession No)	Detection Days	General characters
1	*Anoxybacillusbeppuensis* (KC310454)	TTh	A thermophilic spore-forming bacterium that was isolated from a hot spring reservoir secretes thermostable alpha-amylase. (31)
2	*Geobacillus* sp. (EU087702)	M to S	A thermophile and Gram-positive bacterium (35,36)
3	*Calorbacillus* sp. (DQ232876)	MTThFS	This bacterium is taxonomically similar to Anoxybacillus sp.
4	*Anoxybacillus* sp. (FJ744749)	MTThFS	A thermophilic spore-forming bacterium that reside in geothermal soil. Optimum temperature and pH for growth is 61°C and 5.6, respectively (31)
5	*Saccharococcus* sp. (JX457346)	MTThFS	A thermophilic, Gram-positive, and xylanolytic bacterium (37)
6	*Bacillussmithii* (JX157878)	W	A thermophilic, Gram-positive, alkaliphilic, and nitrile-hydrating bacterium that resides in hot spring. (38)
7	*Aeribacillus pallidus* (KC333048)	WS	This bacterium is taxonomicallyreclassified of *Geobacillus pallidus*. A thermophilic and Gram-positive bacterium. (39)
8	*Thermus thermophilus* (HF558369)	W	An extreme thermophile and Gram-negative bacterium that resides in hot spring and thermal vent (32)

***M, Monday; T, Tuesday; W, Wednesday; Th, Thursday; F, Friday; S, Saturday; * Bold letters: Bacterial community grown at both 45°C and 60°C**

TABLE 3 : Bacterial species identified based on sequence homology of 16S-rDNA extracted from TGGE gel (Figure 3). TGGE was performed with DNA that was directly extracted from theuncultured bacteria sampled from 76°C sauna.

Band No.	Bacteria (GenBank Accession No)	Detection Days	General characters
1	*Bacillus* sp. (GU429229)	M to S	A mesophilic bacterium belonging to human oral bacterial community (40)
2	*Aquaspirillum serpens* (AB680863)	M	A mesophilic, non-motile, non-spore-forming, and aerobic bacterium that resides mostly in freshwater. (24)
3	*Paludiobacterium* sp. (HE9812240	M	Facultative anaerobic, Gram-negative, mesophilic, and motile bacterium that resides in natural environment (25)
4	Uncultured *Nitrospira* sp. (GQ255611)	W	Ammonia-oxidizing bacteria inhabiting in surface water of constructed wetland (unpublished)
5	*Chromobacterium* sp. (DQ415656)	Th	A free-living and mesophilic bacterium that resides in water and soil ecosystem. (29)
6	*Aquitalea denitrificans* (AB682448)	W	A mesophilic wetland-inhabiting bacteria (unpublished)
7	*Gulbenkiania mobilis* (JN411342)	WThF	A mesophilic bacterium that was isolated from treated municipal wastewater. (26)
8	*Gulbenkiania* sp. (HF548444)	WThFS	A mesophilic bacterium that was isolated from treated municipal wastewater. (26)
9	*Bacillus vireti* (HQ897170)	T	A typical soil bacterium and mesophile (28)
10	*Staphylococcus* sp. (JX270830)	T	A halotolerant isolated from Korean traditional salt-fermented food (41)
11	Uncultured bacterium (GU933947)	MWThFS	A mesophilic bacterium used in study for biological phosphorus removal system (unpublished)
12	Uncultured bacterium (AY907870)	MWThFS	A mesophilic bacterium in study for removal of gaseous BTEX (unpublished)
13	*Bacillus* sp. (KC009573)	F	A mesophilic bacterium capable of growing in saline-alkaline soil (unpublished)

M, Monday; T, Tuesday; W, Wednesday; Th, Thursday; F, Friday; S, Saturday

contamination degree of specific bacteria. In natural temperate zone ecosystems, thermophiles may be less active and less diversethan mesophiles; however, they may not disappear from eithernatural orartificial environments. Accordingly, the possibility and frequency ofcontamination bythermophiles may be proportionally less than those of mesophiles. All bacteria that were sampled from the 76°C sauna and identified by TGGE technique were mesophiles.

The observation that bacteria sampled from the 76°C sauna didnot grow in complex medium at either45°C or60°C may be a critical clue to estimate optimal temperature for thermophiles contaminating64°C and 76°C saunas. The mesophiles and thermophiles contaminatingthe saunas through user traffic may temporarily and opportunistically survive at 64°C but may not be able to survive at 76°C. The optimal temperature for thermophiles isolated from a 64°C sauna is 50–60°C, according to reported information, which may be why all bacteria contaminating the76°C sauna did notsurvive and could notgrow when cultivated at 45°C and 60°C.

Conclusively, various mesophiles and thermophiles inhabiting natural ecosystems and the animal bodycan contaminate places that are nutritionally and environmentally suitable for bacterial growth. In particular, man-made environments may be more suitable than natural places for microorganisms transferredfrom thehuman body. The hot and dry sauna is a man-made environment, inwhich temperature is an essential factor to determineif the thermophilic bacteria inhabiting atemperate terrestrial ecosystem can surviveor die. The conditionsin a64°C saunaareoptimal for temperate thermophiles but thoseof a 76°C sauna areharsh. The 64°C sauna is a proper place to isolate and study thermophilic bacteria inhabiting temperate zonessuch asKorea without natural thermal conditions. Three species of thermophiles (*Anoxybacillus* sp. and *Geobacillus* sp.) originatingfrom the 64°C sauna were purely isolated and cultivated for industrial purposes. These bacteria growing at 60°C produce extracellular enzymes(glucoamylase and protease) that work at 60°C.

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