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MICROBIOLOGICAL INVESTIGATIONS ON THE WATER OF A THERMAL BATH AT BUDAPEST

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Thermal baths are unique aquatic environments combining a wide variety of natural and anthropogenic ecological factors, which also appear in their microbiological state. There is limited information on the microbiology of thermal baths in their complexity, tracking community shifts from the thermal wells to the pools. In the present study, the natural microbial community of well and pool waters in Gellért bath was studied in detail by cultivation-based techniques. To isolate bacteria, 10% R2A and minimal synthetic media (with "bath water") with agar–agar and gellan gum were used after prolonged incubation time; moreover, polyurethane blocks covered with media were also applied. Strains were identified by sequencing their 16S rRNA gene after grouping them by amplified rDNA restriction analysis. From each sample, the dominance of Alphaproteobacteria was characteristic though their diversity differed among samples. Members of Actinobacteria, Firmicutes, Beta- and Gammaproteobacteria, Deinococcus–Thermus, and Bacteroidetes were also identified. Representatives of Deinococcus–Thermus phylum appeared only in the pool water. The largest groups in the pool water belonged to the *Tistrella* and *Chelatococcus* genera. The most dominant member in the well water was a new taxon, its similarity to *Hartmannibacter diazotrophicus* as closest relative was 93.93%.

Keywords: cultivation, Gellért bath, well and pool waters, 16S rDNA, bacterial community structure

Introduction

Hot springs and thermal baths support diverse unique microbial communities. The pools of the thermal baths and spas are loaded with well or spring water. These baths are visited often for medical treatment or just for leisure purposes by many people. At the same time, except the control of the obligatory hygienic and the required chemical parameters, there are only a few investigations on these bath

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waters. Terrestrial hot springs located in Iceland, New Zealand, Japan, the United States (National Park of Yellowstone), Italy (area of Naples), and Russia (Peninsula of Kamchatka) are extensively studied [1].

In Hungary, there have been only a few research studies that studied the complete bacterial diversity of the well and pool waters of thermal baths yet [2–5]. The Gellért bath is located in Budapest (Hungary), where Europe's largest natural flowing water thermal system (Buda Thermal Karst System) is located [6]. The Rác, Gellért, and Rudas spas are supplied by the water of the spring group of Gellért Hill via drilled wells [4]. The well water is characterized by high Ca^{2+}, Mg^{2+}, HCO_3^-, and SO_4^{2-} [7]. The chemical parameters of the well water are controlled by the laboratory of Gellért bath (http://www.gellertfurdo.hu/viz-osszetetele)

The water of the well contains only limited organic carbon source, however, oligotrophic bacteria due to their metabolic flexibility are able to thrive under very low nutrient condition [8]. To cultivate oligotrophic microorganisms under laboratory conditions is still a challenge. It is estimated that less than 1% of bacteria can be cultivated in laboratory conditions [9]. There has been a focus in recent years on developing methods for the *in vitro* cultivation of those bacteria so far remained uncultured. In previous studies, increased incubation time [10], use of alternative gelling agent [11], *in situ* cultivation on media-supplemented polyurethane foam [12], or developed new media [13] were successfully used to cultivate uncultured microbes.

The aim of the present study was to reveal the cultivable bacterial communities of the well and pool waters of the Gellért bath using classical and special cultivation techniques.

Materials and Methods

Sampling

Sampling was carried out on 28.10.2013 from water that drains directly to the men's pool (originating from the well) and the men's pool water of Gellért bath, Budapest, Hungary. The temperature of the well water was 47 °C though at the sampling point water temperature was only 36 °C and the pH was 7.5. The well water flows without any treatment to the swimming pool. The pool water was 36 °C and the pH was 7.0. The thermal water is continuously flowing into the pool, while the excess water is being drained. The water sample (1–1 l) was aseptically taken from the well and pool waters into the previously sterilized screw capped flasks. The well water sample was taken from that tap, which continuously fills the

pool. The pool water sample was taken from the middle of the pool. During the sampling, 12 people were present in the pool water. Samples were taken to the laboratory in a cooler bag and processed within 1 h of sampling.

Determination of total cell count

To determine the total cell count, a DAPI staining procedure was applied, as described by Máthé et al. [14]. For the investigations, 1 ml of the pool water and 100 ml of the well water were filtered.

Cultivation

To cultivate and isolate bacteria, 10% R2A [15] and minimal synthetic media (prepared with own bath water) [13] with agar–agar or gellan gum were used. Also, 16 g agar–agar and 12 g gellan gum were used as a gelling reagent for 10% R2A and minimal synthetic media.

In case of 10% R2A with gellan gum, 0.3 g $CaCl_2$ and 0.6 g $MgSO_4$ were added to stabile gelling, but with agar–agar, no additional salts were needed. For minimal synthetic media with gellan gum, 0.5 g $CaCl_2$ and 0.75 g $MgSO_4$ or with agar–agar 0.32 g $CaCl_2$ were added for the same purpose.

During our studies, three different cultivation techniques were carried out. (1) The well and pool waters were directly spread to the 10% R2A and the minimal synthetic media after a serial dilution of samples. Media were prepared both with agar–agar and gellan gum. After incubation (9 days at 36 °C and 16 days at 25 °C), CFU values were estimated. (2) For the enrichment of slowly growing bacteria, 50–50 ml water sample was added to 250–250 ml liquid medium (10% R2A and minimal synthetic media). After 3 weeks of incubation (36 °C), serial dilution was done from the enrichment cultures and 100–100 µl of each were spread to 10% R2A and minimal media with agar–agar and gelrite. Plates were incubated at 36 °C for 7–14 days. (3) Polyurethane foam-based (PUF) traps were applied to enrich bacteria in 250–250 ml liquid medium (10% R2A and minimal synthetic media). PUF blocks were sterilized in autoclave at 121 °C for 20 min then soaked with hot agar–agar and gellan gum medium (10% R2A and minimal synthetic media), then these PUF blocks were put into the appropriate liquid media. After the incubation (3 weeks at 36 °C and 3 weeks at 25 °C), the PUF blocks were taken out from the enrichment media and pressed several times with sterile mortal. The liquid from the PUF blocks was spread to 10% R2A and minimal media after serial dilution. Plates were incubated at 36 °C for 4 days.

About 170 bacterial strains were randomly isolated from the plates, then purified and maintained on 10% R2A and minimal synthetic media with agar–agar or gelrite according to standard microbiological methods.

DNA extraction from the bacterial strains

DNA was extracted using glass beads for cell disruption. Cells were suspended in 100 µl diethylpyrocarbonate treated water in a 600 µl Eppendorf tube. Lysis of the cells was achieved by shaking for 2 min and 30 Hz by a MM301 mixer mill (Retsch, Haan, Germany) in the presence of 100 µl sterile glass beads. The raw lysate was denaturated for 5 min at 98 °C in a GeneAmp PCR System 2700 machine (Applied Biosystems). After centrifugation for 5 min at 10,000×*g*, the supernatant was used for the following PCR.

PCR amplification of the 16S rRNA genes of bacterial strains

The 16S rRNA gene fragments of bacterial DNA were amplified with universal primers 27F (5′-AGA GTT TGA TCM TGG CTC AG-3′) and 1401R (5′-CGG TGT GTA CAA GAC CC-3′). The temperature protocol of the PCR was performed as described by Anda et al. [5] except the elongation time, which took 45 s. The PCR mixture was the same, as previously described [5]. Amplification was carried out in a GeneAmp PCR System 2700 machine (Applied Biosystems). PCR products were checked by the same method, as previously described [5].

Amplified rDNA restriction analysis (ARDRA) to group the 170 bacterial strains

The amplified 16S rRNA gene fragments were digested using *Bsu*RI (GG CC) and *Msp*1 (C CGG) restriction enzymes (10 U μL^{-1}, Fermentas), as described by Massol-Deya et al. [16]. The fragments were separated by the same method, as previously described [5]. The samples with identical restriction fragment patterns (for both restriction enzymes) were grouped together. Group representatives and ungrouped samples were chosen for 16S rRNA gene sequence analysis to identify the selected bacterial strains.

16S rRNA gene sequence analysis

The partial 16S rRNA gene sequencing of the selected ARDRA representatives was performed using 27F (5′-AGA GTT TGA TCM TGG CTC AG-3′) and

1401R primers (5′-CGG TGT GTA CAA GAC CC-3′). Sequencing with Sanger method was carried out at LGC Ltd. (Berlin, Germany). The quality of chromatograms was checked manually with the help of the Chromas software (Technelysium Pty Ltd., Australia). Taxonomic relationships of the sequences were determined by EzTaxon database [17].

Results and Discussion

The temperature of the well originating water and the swimming pool water was the same (36 °C), the well water flows without any treatment to the swimming pool, and in the pool water, no disinfection process was done. Most probably, due to the anthropogenic effect, the quantity of cultivable heterotrophic bacteria was at least 10 fold higher in the pool (1.095×10^4 cells ml^{-1}) as compared to the well (6.34×10^2 cells ml^{-1}). The viable cell count was at least one to two orders of magnitude higher in the well (6.705×10^5 cells ml^{-1}) and 10 fold higher in the pool water (9.35×10^5 cells ml^{-1}), as compared to the plate counts.

Altogether, 170 bacterial strains (72 from the well and 98 from the pool water samples) were isolated and successfully maintained on different media. According to the 16S rRNA gene sequence analysis, the members of Alphaproteobacteria provided the main group among the identified bacterial strains in the well (40 strains) and also in the pool water (71 strains) (Figure 1), in main phylogenetic lineages, the community of the well and pool waters was similar. The similarities of the sequenced bacterial strains to their closest relatives are listed in Table I. Representatives of Actinobacteria, Firmicutes, Beta- and Gammaproteobacteria, Deinococcus–Thermus, and Bacteroidetes were identified. The ratio of the Firmicutes phylum was higher in the well water (14 strains), than in the pool water (4 strains). Representatives of Deinococcus–Thermus (*Deinococcus grandis*) appeared only in the pool water.

The isolated bacterial species were all heterotrophic, except the facultative phototrophic *Blastomonas natatoria* [18].

Altogether, 19 bacterial genera were isolated from the well water and 22 from the pool water. Based on the literature data, many of the detected taxa are able to fix nitrogen, e.g., *Pseudomonas azotiofingens* [19], *Rhizobium alkalisoli* [20], *Rhizobium straminoryzae* [21], and also *Hartmannibacter diazotrophicus* [22], which show only low similarity to our isolates. In oligotrophic environments, nitrogen fixation often has a great importance due to the limited nitrogen source in these oligotrophic habitats.

The largest bacterial groups in the pool water belonged to the genera *Tistrella* and *Chelatococcus*. As the pool water comes from the well, some

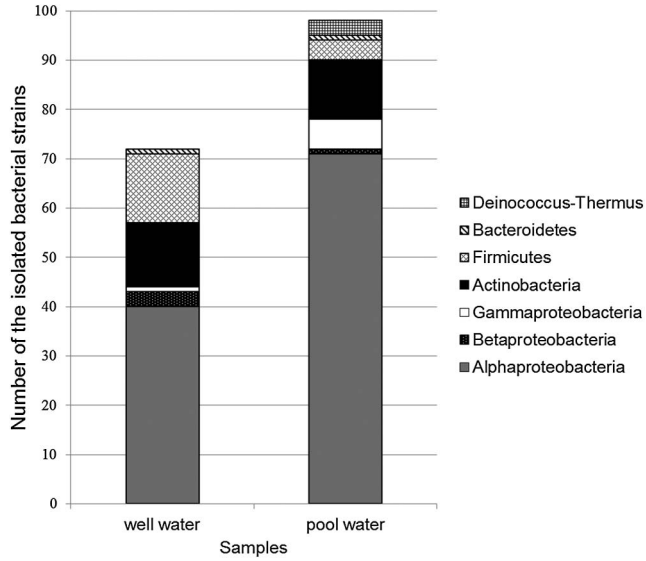

Figure 1. Phylogenetic distribution of cultivated bacterial strains from the well and pool waters of Gellért bath

bacterial genera (*Bacillus, Ferrovibrio, Nocardioides, Porphyrobacter, Pseudomonas,* and *Tistrella*) were detected at both sampling sites, but the overlap between the bacterial communities of the two sampling sites was little (Table I). Differences can originate from the fact that the original well water temperature is approximately 11 °C higher than that of the pool water but also it assumes an anthropogenic effect in the pool water.

Many of the detected taxa are able to utilize amino acids as sole carbon source, e.g., *Kinneretia asaccharophila* [23], *Acinetobacter baumanni* [24], *Fictibacillus nanhaiensis* [25], *Pannonibacter phragmitetus* [26], *Phenylobacterium falsum* [27], *Rhizobium alkalisoli* [20], *Porphyrobacter colymbi* [28], and *Pseudomonas knackmussii* [29]. These microbes may also utilize the degradation products of proteins of the human epithelial surface or human perspiration can occur in the pool water. Based on the literature data, *Dietzia cinnamea* isolated from the pool water can also utilize testosterone [30].

The anthropogenic effect seems to be also confirmed by other facts: some bacterial species (*Deinococcus grandis, Dietzia cinnamea,* and *Acinetobacter baumannii*) isolated only from the pool water (Table I), based on the literature data they were also detected only from human samples till now. Originally, *D. grandis* was isolated from human epithelial surface [31], *D. cinnamea* from a perianal swab of a patient with bone marrow transplant [30]. *A. baumannii* is an

Table I. Phylogenetic relatedness of ARDRA group representative and ungrouped bacterial strains. Strains with bold sign till now were described only from human sources. Notations in strain's signs: "B" originates from well water and "M" originates from the pool water. "Pu" means that the strain was isolated from polyurethane block. The small (diameter is less than 1 mm) bacterial colonies were isolated by the help of a sterile toothpick marked with "F". "R" or "M" means that the strain was isolated from 10% R2A or minimal synthetic medium. Strains isolated from a media solidified with agar–agar or gellan gum are marked with "A" or G". If a strain was isolated from the enrichment culture, it is marked with "D"

Selected representative strain (number of all isolates in well/pool)	Closest relative (EzTaxon)	Similarity %
Alphaproteobacteria		
PuMGM6 (0/1)	*Azospirillum rugosum* IMMIB AFH-6(T)	97.77
RAM1 (0/1)	*Blastomonas natatoria* DSM 3183(T)	100.00
FDRGB2/b (1/0)	*Brevundimonas vesicularis* LMG 2350(T)	97.24
FDRGM8 (0/1)	*Brevundimonas viscosa* F3(T)	99.88
FDMGM4 (0/1)	*Caenispirillum bisanense* K92(T)	99.61
DRAM1, FDRAM6, FDRGM16, FDRGM19, FDRGM6/a, FDRGM9, (0/22)	*Chelatococcus daeguensis* K106(T)	100.00
DRAB2, FDMAM7, FDRAB2, FDMGM9 (4/7)	*Ferrovibrio denitrificans* Sp-1(T)	100.00
DMAB1, DMAB2, DMAB3, DMGB13 (30/0)	*Hartmannibacter diazotrophicus* E19(T)	93.93
DMGB7 (1/0)	*Methylobacterium goesingense* iEII3(T)	99.39
RAM11 (0/1)	*Mycoplana dimorpha* IAM 13154(T)	96.83
DMGM8, FDMGM7 (0/3)	*Pannonibacter phragmitetus* DSM 14782(T)	99.66
MAB10 (1/0)	*Paracoccus siganidrum* M26(T)	98.19
DRAM15 (0/1)	*Phenylobacterium falsum* AC-49(T)	98.40
MGM5 (1/2)	*Porphyrobacter colymbi* TPW-24(T)	99.50
PuMAM10, PuMGM10 (0/4)	*Rhizobium alkalisoli* CCBAU 01393(T)	97.23
DMAM1 (0/1)	*Rhizobium straminoryzae* CC-LY845(T)	99.90
FDRAB3 (1/0)	*Sphingopyxis indica* DS15(T)	98.99
PuMGM1, FDMAM1, DMAM3, DMAM14, PuMAM2 (1/20)	*Tistrella mobilis* TISTR 1108(T)	99.64
Betaproteobacteria		
PuMGB3 (1/0)	*Caldimonas meghalayensis* AK31(T)	99.86
RAM12 (0/1)	*Kinneretia asaccharophilia* KIN192(T)	99.75
MGB6, MGB2 (2/0)	*Limnobacter thiooxidans* CS-K2(T)	99.87
Gammaproteobacteria		
RAM9 (0/1)	***Acinetobacter baumannii* ATCC 19606(T)**	99.88
MAM9, MAM2 (0/3)	*Pseudomonas alcaligenes* NBRC 14159(T)	99.76

Table I. (cont.)

Selected representative strain (number of all isolates in well/pool)	Closest relative (EzTaxon)	Similarity %
FDRAB19 (1/0)	*Pseudomonas azotifigens* 6H33b(T)	97.91
MAM12 (0/1)	*Pseudomonas balearica* SP1402(T)	99.64
RGM5 (0/1)	*Pseudomonas knackmussii* B13(T)	97.57
Firmicutes		
FDRAB14, FDRGB11, FDRGM2 (6/3)	*Bacillus licheniformis* ATCC 14580(T)	99.62
FDRGB12, FDRGB17, FDRAB12 (7/0)	*Brevibacillus choshinensis* DSM 8552(T)	97.93
RGM1 (0/1)	*Fictibacillus nanhaiensis* JSM 082006(T)	100
DRAB1 (1/0)	*Paenibacillus lautus* NRRL NRS-666(T)	99.00
Actinobacteria		
RAB11 (1/0)	*Brachybacterium paraconglomeratum* LMG	99.75
MGB4 (1/0)	*Corynebacterium humireducens* MFC 5(T)	99.00
RAM10 (0/1)	***Dietzia cinnamea* IMMIB RIV-399(T)**	99.62
DRAM3, MGM2, MGB5, FDMGM1, FDRAB7, FDRAM8/b (10/10)	*Micrococcus luteus* NCTC 2665(T)	100.00
RAM13 (0/1)	*Nocardioides daphniae* D287(T)	97.35
DMGB6 (1/0)	*Nocardioides furvisabuli* SBS-26(T)	98.50
Bacteroidetes		
PuMAB9, PuMAM9 (1/1)	*Sphingobacterium composti* 24M24(T)	99.47
Deinococcus–Thermus		
RAM20 (0/3)	***Deinococcus grandis* DSM 3963(T)**	98.49

opportunistic bacterial pathogen, primarily associated with hospital-acquired infections, e.g., pneumonia, wound infections, and urinary tract infections [32].

Bacillus licheniformis (which is a common bacterium in soils) detected both in the well and pool waters can also be opportunistic pathogen, can cause sepsis [33], aortic valve endocarditis [34], and food poisoning [35]. *Pseudomonas alcaligenes* detected only in the pool water was initially described as opportunistic pathogen, which causes endocarditis [36], although since then it has been detected from numerous environmental samples. One of the closest relative of strain FDRGB2b is the type strain of *Brevundimonas vesicularis* (97.24%), which is also a facultative pathogen that causes endocarditis [37], peritonitis [38], and septic arthritis [39]. In our case, FDRGB2b appeared only in the well water, and therefore its human origin is excluded. Moreover, it must also be stated that these taxa could be cultivated only after enrichment,

their real number is most probably very low in these waters. The most dominant member of the pool water was *Tistrella mobilis,* previously detected in deep sea of the Middle Atlantic Ridge [40] and in wastewater [41]. This bacterium produces an antimicrobial agent (didemnin) [42], which can be responsible for the dominant appearance of *Tistrella mobilis* also in the pool water of Gellért bath. The effect of this agent of the bacterial community structure needs further studies in our case; the antimicrobial effect of our strains was not yet tested.

Bacterial species previously detected in the swimming pool water (*Blastomonas natatoria* [43], *Pseudomonas alcaligenes* [44], and *Porphyrobacter colymbi* [45]) were also identified in the pool water of Gellért bath.

The dominant member of the well water belonged to a new taxon, according to the 16S rDNA sequences; its similarity to *Hartmannibacter diazotrophicus* as closest relative was only 93.93%. This bacterium occurred only in the well water.

The entire microbial community of the thermal water of pools and wells has been rarely studied. It has been mainly investigated by a hygienic view in the previous research studies. The whole bacterial community of an indoor warm-water (33 °C) therapy pool in an American Midwestern regional hospital has been studied with molecular methods [46]. During the February sampling, the dominant members of the pool water belonged to the Gammaproteobacteria and Bacteroidetes/Chlorobi and Firmicutes taxa. Our samples were investigated only by cultivation and it is hard to make comparisons with molecular results. Even so when we do it very carefully, the results show that in the pool of Gellért bath, the Alphaproteobacteria taxon was dominant (74.4%) and the members of Actinobacteria were also abundant (12.2%). These taxa were detected in the American therapy pool though in lower quantity as well. During the second sampling in the American hospital pool in August, the members of the *Sphingomonadaceae* family (Alphaproteobacteria) and the *Mycobacterium* genus (Actinobacteria) were dominant, Beta- and Gamma-proteobacteria, Actinobacteria, Bacillus, and Clostridia (Firmicutes) were also represented. In the pool water of the Gellért bath, these taxa were also isolated except the class Clostridia. Contrary to our sample, representatives of Deinococcus–Thermus phylum were not detected in the American therapy pool. Differences can also come from the different applied methods, though overlaps are obvious. Another swimming pool water of a college was studied with cultivation methods, where the dominance of the Firmicutes phylum was detected, and the members of Actinobacteria were identified as well [47].

Members of the Alphaproteobacteria class were also detected in Tunisian springs [1] in Yellowstone National Park [48] or in Diana/Hygieia spring (Hungary) [4], but less dominantly. Members of Beta- and Gamma-proteobacteria occurred frequently in thermal springs [1, 4], and in the well of Gellért bath, they were not

isolated in high number (4.2% and 1.4%). The second largest isolated group was related to Firmicutes (18.4%) phylum in the well water of Gellért bath.

From the well water, representatives of species *Caldimonas meghalayensis* and *Ferrovibrio denitrificans* were also identified. Both taxa were described from aquatic habitats, the former from a spring in India [49], while the latter one from a Russian spring [50]. In addition, members of the genera *Sphingobacterium* and *Sphingopyxis* were detected in a sulfurous well (called "Matty") in Harkány (Hungary) [2].

Conclusions

The thermal water of the well and pool waters have diverse bacterial communities. Our aim was not to reveal the hygienic state of the thermal bath; it is done regularly by the operators. At the same time, it must be stated that the anthropogenic effect has a great influence on the autochthon microbial community of the studied waters. On the other hand, even with enrichments, only a few facultative pathogenic microbes could be revealed, which underlines that the hygienic state of the studied water bodies is obviously good. Interestingly from the well water, novel taxa could be isolated, one is far from each known bacterial species. It underlines that oligotrophic environments have a hidden cultivable diversity that is worthy to study.

Conflict of Interest

There is no conflict of interest present connected to the work.

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