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PHYSIOLOGICAL AND MOLECULAR CHARACTERISTICS OF *Bacillus spp.* ISOLATED FROM WARM MINERAL WATERS IN VARNA, BULGARIA AS MODEL SYSTEM FOR ORIGIN OF LIFE

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AUTHORS' CONTRIBUTIONS

This work was carried out in collaboration between both authors. Author II designed the study, performed the statistical analysis, wrote the protocol and wrote the first draft of the manuscript. Authors II and NV managed the analyses of the study. Author NV managed the literature searches. Both authors read and approved the final manuscript.

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ABSTRACT

In Bulgaria, there are many mineral water springs with opportunities for research on different bacterial species. The present study aims to investigate the bacterial research of mineral waters in the region of Varna, Bulgaria. The results showed three species of genus *Bacillus* isolated from warm mineral waters, Varna, Bulgaria. The colonial and physiological characteristics of the isolated strains are defined. The isolated strains are identified by software processing with mass spectrometry MALDI-TOF MS *Versus* 16S rRNA Gene. The results obtained from the biochemical and molecular studies indicate that the isolated strains belong to different species of genus *Bacillus*.

In 2010 Ignatov researched hot mineral waters in Bulgaria and conducted comparative spectrum analysis with cactus juice. In the spectrum of cactus juice and the hot mineral water we observed five comparable local extremums (8.95; 9.67; 9.81; 10.47; 11.13 μ m), whereas there was only one (9.10 μ m) with the sea water. It is therefore justified to suggest that most probably life originated in hot mineral water. Ignatov and Mosin performed analyses with *Bacillus subtilis* with heavy water for analyses of the initial condition for origin of life in hot mineral water in primary atmosphere. According to their temperature they can be cold (up to 37° C), warm (from 37°C to 60°C) and hot (over 60°C). This is temperature standard in Bulgaria, European Union. In Varna there is place with stromatolites and cyanobacteria in warm mineral waters.

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This is the purpose the authors to make research of warm waters in Varna as model system for origin of life in warm and hot mineral waters.

Keywords: Mineral waters; selection; identification; sequencing; genus Bacillus.

1. INTRODUCTION

The studies contain the results from warm mineral water springs from Varna district on the Black Sea coast. Hydrocarbonate (HCO₃⁻) and calcium (Ca²⁺) ions in warm mineral waters are in the following quantities – 270 and 42 mg/ dm³. Stromatolites are also found near Varna – the first living organisms on Earth.

In 2010, Ignatov demonstrated that life may have originated in hot mineral water. He did this by spectral analysis of mineral water from the area at the base of an extinct volcano (Rupite) with large amounts of calcium and hydrocarbonate ions and a comparative analysis with a spectrum of cactus juice.

The authors research different species of the genus *Bacillus* in areas with a prehistory of the origin of life. The spectrum of microbial ecology got extended in the recent years, and now it includes extreme ambient such as thermal springs and sea depths. It allows analyses of the physiological conditions of naturally occurring microbial habitats. Abundance of bacteria and bacterial biomass are found in the soil, inland and sea waters. The determination of bacterial count in warm and thermal (hot) springs is scarce, and it is confined to determination of the total number of microorganisms.

Furthermore, most of the results refer to low-density bacterial populations. Nevertheless, the influence of microorganisms over the geochemical processes in thermal springs is well known.

Therefore the determination of active bacteria in the microbial communities of mineral waters is of particular importance.

In 2017, the Tara Djokic team demonstrated that the stromatolites in the Dresser Formation were located in an area with hot mineral water [1]. There were also shallow seas nearby. In 2019, the Baumgartner team explored the Dresser Formation rocks, which are 3.48 billion years old [2]. The Dresser Formation consists of layered structures of stromatolites. These stromatolites are found in hydrothermal-sedimentary layers that are not wellf ormed. The formation is indicative of biogenic origin.

Different methods are employed for the determination of the total number of cells. Most of the methods are based on color reactions. These mineral waters differ in physiochemical characteristics such as pH, oxidation reduction potential (ORP), water temperature, dissolved oxygen, electrical conductivity which defines the presence of different microbial species. The purpose of this work is isolation, selection and identification of bacteria strains from mineral springs in the region of Varna, Bulgaria.

2. MATERIALS AND METHODS

Representatives of the genus *Bacillus* produce substances with antimicrobial activity of different chemical nature [3,4]. The antimicrobial agents include peptides, amino acids, phospholipids, etc [5, 3].

2.1 Nutrient Media

The research is with LBG-agar. The composition in (g/dm³) is: tryptone-10, yeast extract-5,NaCl-10, glucose–10, agar-20.pHcorrected up to 7,5.Sterilization- 20 minutes at121°C.

Tryptone is formed by decomposition of casein by protease trypsin [6]. It is structured from peptides.

Triptone -10 g/dm^3

The triptone is used for the growth of microorganisms. It provides a source of amino acids in growing bacteria.

Yeast extract - 5 g/dm³

Yeast extracts consist of the cell contents of yeast without the cell walls. Yeasts are eukaryotic, single-celled microorganisms [6].

Sodium Chloride (NaCl) –10 g/dm³

Glucose -10 g/dm³

2.2 Methods of Analyses

Method for determination of total number of aerobic and facultative anaerobic bacteria – BDS EN ISO 6222: 2002

2.3 Device MALDI-ToF MS for Microbial Identification

Vitek[®] MS is an automated system for microbial identification of the company bioMerieux. The device

is based on innovative technology mass spectrometry MALDI-TOF MS Versus 16S rRNA Gene [7] is an abbreviation for Matrix-Assisted Laser Desorption/Ionization Time of Flight Mass Spectrometry. For the identification are needed microbial cultures. In the conducted analysis were used 48 h pure cultures cultured in Nutrient agar. [8, 9, 10] Each culture was mixed with a matrix over a special plate, positioned in a preparatory station with a bacteria protocol, inserted in the device and influenced by a laser. To control the process was used a standard strain of Escherichia coli ATCC 8739, which has well known characteristics. As a result were generated the so called MALDI-ToF spectra that were analyzed by the means of a software, and compared to existing profiles in the database.

2.4 Isolation of Total DNA

The isolation of DNA is conducted by the method of Delley, Moller, Hottingen, 1990 [11].

DNA hybridization techniques with specific DNA probes for the identification of bacterial and viral strains have beendeveloped. The main emphasis in constructing such DNA. Probes were to have quick and reliable tools to identify pathogenic material in clinical diagnosis. Thus, DNA probes have already been used forth identification of *Plasmodium falciparum, Yersiniaen-terocolitica, Salmonella typhi* and *Bacillus subtilis* [12]. *Haemophilus influenzae*, and other microorganisms and of DNA viruses and RNA viruses.

2.5 16SrDNAamplification

All PCR reactions are carried out using the PCR test – PCRVWR, volume of 25 μ l in Progene cycler (Techne, UK) according to the manufacturer's instructions, as in each reaction are used 50 ng of total DNA of the tested strain and 10pmol of primers. DNA of the assessed strain is amplified by using of universal primers for 16SrDNA- 27f (5'AGAGTTTGATCMTGGCTCAG3') and 1492r (5'ACCTTGTTACGACTT3').

The amplification program includes: denaturation - 95° C for 3min, 40 cycles- 93° C for 30 s, 55° C for 60 s, 72° C for2 min, extreme elongation – 72° C for 7min.

The PCR product obtained by amplification of 16Sr DNA is visualized by 2% agarose gel, colored by ethidium bromide solution (0.5 μ g/ml), of UVP Documentation System (U.K.).

From isolated total DNA is multiplied the gene for 16S rRNA by PCR – reaction by using of universal primers for multiplication of 16S rDNA. The obtained amplification products are purified and sequenced by "Macrogen Europe Laboratory", Holland. The comparative analysis of the sequences of the genes for 16S rRNA is conducted through the software CLC Sequence Viewer.

3. RESULTS AND DISCUSSION

3.1 Methods for Research of Stains of Bacillus

Via cultivating ina LBG-agar medium is determined the colonial characteristic of the selected strains (Table 1). Table 2 shows the growth of isolates from the mineral springs in Varna region in liquid growing medium for 24 – 48 h. Total DNA is isolated from 13 strains, the gene 16SpRNA is multiplied through PCR-reaction by using of universal primers for multiplication of 16S rDNA, and the obtained amplification products are purified and sequenced from "Macrogen Europe Laboratory", Holland.

The trial results reveal that 11 of the isolated strains are Gram-positive, spore-forming, and 2 are Gramnegative, non spore-forming. They develop at temperatures 3°- 45°C, as the best growth rate is reported at temperature of development 30°C. Total DNA is isolated from 3 strains, the gene 16SpRNA is multiplied through PCR-reaction by using of universal primers for multiplication of 16S rDNA, and the obtained amplification products are purified and sequenced from "Macrogen Europe Laboratory", Holland. The comparative analysis of sequences of genes for 16S rRNA is conducted through the software CLC Sequence Viewer. 13 strains differ by the sequence of the gene for 16S rRNA. The obtained sequences are processed by BLASTn software, and their species genus identification of strains is confirmed (Table 1, 2). Table 1 shows colonial characteristics of isolated strains from mineral warm springs from Varna region.

3.2 Behavior of *Bacillus subtilis* in Warm and Hot Mineral Waters

In 2010 Ignatov demonstrated the possibility of life being born in hot mineral water [13, 14]. In 2013 Ignatov and Mosin conducted research on the parameters of *Bacillus subtilis* as a model system for the origin of life in hot mineral water [15,16]. The mineral water is from Rupite, Bulgaria where there is

Name of water	Strain	Shape	Edges	Surface	Profile	Consistency	Color	Size	Visualization
1. Mineralspring (drilling No.R-83x KK "St. Constantine andElena") t=48°C, Varna region	N. Valcheva BAP 4-1	round to elliptical	serrated	smooth	convex	dry	whitish	3 – 4 mm average	
2. Mineral spring (R-1x "Aquarium") t=47°C, Varna region	N. Valcheva BS 5-1-1	round to elliptical	serrated	smooth	convex	dry	whitish	2 – 3 mm average	
3. Mineral Spring R- 106x "DomMladost" t=40°C, Varna region	N. Valcheva BV 5-1-2	round to elliptical	serrated	smooth	convex	dry	whitish	3 – 4 mm average	

Table 1. Colonial characteristic of isolated strains from mineral warm springs in Varna region

a variety of bacteria. Kamburova's team evidenced the presence of a new species of bacteria in the Rupite water. Czech scientists proved the presence of cyanobacteria in the Rupite water [17]. Cyanobacteria have also been proven in Varna [18]. The presence of calcium and hydrocarbonate ions, as well as cyanobacteria is at the foundation for the formation of the first living organisms – stromatolites [19,20,21].

The presence of divalent calcium and magnesium ions in warm and hot mineral water is involved in the activation of cortex-lytic enzymes during germination of *Bacillus subtilis* [22].

According to J. Szostak, the accumulation of organic compounds in open lakes is more possible compared to the ocean [23]. A hypothesis of Deamer, Damer and Kompanichenko emerges that hot springs associated with volcanic land masses have an ionic composition more conducive to self-assembly and polymerization than seawater [24].

In Bulgaria, there are stromatolites on the Black Sea coast, near Varna. This gives the authors reason to conduct research on mineral waters in this area. Research has been conducted by the authors on the water in Rupite. Interestingly, Kambourova's team isolated a new species of bacteria in Rupite water – *Anoxybacillusrupiensis sp.* Nov. [25, 26].

With the help of *Bacillus subtilis* in the presence of calcium carbonate, the creation of a biofilm that fills pores and cracks was studied. [27]. A similar process is observed in the formation of modern stromatolites [28]. The parameters of *Bacillus subtilis* as a model system for the origin of life with different amounts of deuterium are demonstrated by Ignatov and Mosin [16, 29, 30, 31].

In 2016 Bacillus sp. SG113 was isolated [32]. In 2020 the strains of geneses *Bacillus, Pseudomonas* and *Stenotrophomonas* were isolated in Rupite [33]. The stains of genus *Bacillus* are – *Bacillus subtilis, Bacillus vallismortis, Bacillus amyloliquefaciens, Bacillus megaterium* and *Bacillus simplex*. The strain *Pseudomonas fluorescens* was isolated. The strain *Stenotrophomonas maltophilia* was isolated.

Table 2. shows the growth of isolates from the mineral springs in Varna region in liquid growing medium for 24 - 48 h, at temperatures 3-50 °C.

Table 2. Growth of isolates from the mineral springs in Varna region in liquid growing medium for 24 –48 h, at temperatures 3 -50°C

Name of water source	Strain	Liner	Turbidity	Sediment	Spieces aerobes/anaerobes
1. Mineralspring (drilling	N. Valcheva	-	_	+	aerobes
No.R-83x KK "St.	BAP 4-1				
ConstantineandElena")					
t=48°C, Varna region					
2. Mineral spring (R-1x	N. Valcheva	+	_	+	aerobes
"Aquarium") t=47°C,	BS 5-1-1				
Varna region					
3. Mineral spring R-106x	N. Valcheva	+	+	+	facultative anaerobes
"DomMladost" t=40°C,	BV 5-1-2				
Varna region					

 Table 3. Species of genus identity of the strains isolated from mineral waters in Varna region, after processing the sequences of genes for 16SrRNA

Species of genus Subtilis in the examination of water from Varna, Bulgaria	Bacterial Profile	Result with MALDI-ToF MS identification (Percent (%) of reliable identification)	Result with MALDI-ToF MS identification (Percent (%) of reliable identification) (after 24 hours)	Result with MALDI-ToF MS identification (Percent (%) of reliable identification) (after 72 hours)
1.Bacillus altitudinis/pumilus	N. Valcheva BAP 4-1	99.9	99.9	99.9
2. Bacillus subtilis	N. Valcheva BS 5-1-1	99.9	99.9	99.9
3. Bacillus vallismortis	N. Valcheva 5-1-2	99.9	99.9	99.9

Table 3. is connected with Species of genus identity of the strains isolated from mineral waters in Varna region, after processing the sequences of genes for 16SrRNA

The following strains have been isolated during research – *Bacillus subtilis, Bacillus altitudinis/pumilus and Bacillus vallismortis* [34-39]. Microbiological. The presence of Bacteria Strains of *Bacillus* is described in hot mineral water [40]. It also has its practical applications [41, 42].

4. CONCLUSION

The experimental research determined the species of the strains isolated from warm mineral springs in Varna region. The isolated strains belong to spore forming bacteria of the genus *Bacillus*.

The research shows the following strains of genus Bacillus – Bacillus subtilis, Bacillus altitudinis/pumilus and Bacillus vallismortis.

In 2012 *Bacillus subtilis* was used by Ignatov and Mosin as a model system for studying the possible effects for origin of life with different concentration of deuterium in the water.

The main conclusion from physicochemical parameters is the large amount of calcium and hydrocarbonate ions in the water of Varna. They are an integral part of the first living organisms – stromatolites.

In Bulgaria stromatolites have been found in the PobititeKamani location near Beloslav, Varna district [43, 44]. The place is near the Black Sea coast.

There is a great variety of bacteria in the water of Varna, which is consistent with the idea of warm spring water as a medium for origination of life and living matter.

COMPETING INTERESTS

Authors have declared that no competing interests exist.

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