AUTECOLOGY OF MICROORGANISMS OF COASTAL ECOSYSTEMS OF THE DEAD SEA

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Abstract.. From coastal ecosystems of the Dead Sea (vertical steep gorge around of the Dead Sea, clay-salt plain and a black highly mineralized muds) *Bacillus licheniformis, B.subtilis* subsp. *subtilis, Bacillus* sp., *Staphylococcus hominis* strains have been isolated. All strains grew at 0-10 % of NaCl in medium, in a range of 20-50°C. Resistance to UV has been revealed in all the investigated bacteria. Lethal doses of UV (LD_{90} and $LD_{99,99}$) for spore-forming strains were 100-150 and 1100-1500 J/m^2, respectively; for strain 6t1 (does not form spores) - 50 and 370 J/m^2. Some strains of genus *Bacillus* had strong antagonistic effect on conditionally pathogenic test cultures *Staphylococcus aureus* and *Candida albicans*. Among the isolates discovered strains which showed lipase or amylase or elastase activity. These properties of the bacteria may be used in various biotechnology.

Keywords: extremophilic bacteria, Dead Sea, halo- and thermo-tolerance, resistance to UV, antagonism.

INTRODUCTION

The Dead Sea is a terminal lake located on the border between Jordan, the Palestinian Authority and Israel. Since the beginning of the 20th century the water budget of the Dead Sea has been negative, leading to a continuous decrease in the water level. Due to the continuous evaporation of the Dead Sea, Na^+ precipitates out as halite while Mg^{2+},whose salts are more soluble, is further concentrated and has become the dominant cation [1]. Recently, the complex system of underwater springs in the Dead Sea has been discovered. A more detailed exploration revealed that these springs harbor microbial communities with much higher diversity and cell density than reported to date for the Dead Sea, including dense biofilms covering sediments and rocks around the springs [2]. In water of the Dead Sea various groups of halophilic microorganisms have been revealed: aerobic and anaerobic chemoorganotrophic bacteria, cyanobacterium [3]. The majority aerobic bacteria from the Dead Sea belong to a *Halobacteriaceae* family (domain *Archaea*) [4], and also to Gram-negative and Gram-positive halophilic bacteria species (domain *Bacteria*) [5, 6]. At least 70 species (in 26 genera) of Oomycota (Chromista), Mucoromycotina, Ascomycota, and Basidiomycota (Fungi) were isolated from near-shore localities and offshore stations, including from deep waters. Aspergillus and Eurotium were most often recovered. *Aspergillus terreus, A. sydowii, A. versicolor, Eurotium herbariorum, Penicillium westlingii, Cladosporium cladosporioides, C. sphaerospermum, C. ramnotellum,* and *C. halotolerans* probably form the stable core of the community [7]. However microorganisms of coastal ecosystems of the Dead Sea are a little studied.

Aims of investigations – isolate microorganisms from the Dead Sea coastal ecosystems and to determine their resistance to UV radiation, high temperature and salinity, and to explore their antagonism to conditionally pathogenic bacteria and yeast.

MATERIALS AND METHODS

Objects of research are the bacteria isolated from ecosystems of the Dead Sea costal (Israel). The bacteria were recovered from the samples of vertical steep gorge around of the Dead Sea, clay-salt plain and a black highly mineralized muds. For revealing aerobic chemoorganotrophic bacteria medium NA (Nutrient Agar, HiMedia Laboratories Pvt. Ltd) was used. On medium NA was added nystatin (50 mg/l) for mushrooms growth repressing. The plates were incubated at 42ºC (till 10 days). Only those morphotypes of bacteria which dominated in the natural samples, have been isolated. The pure cultures were obtained by two-four successive transfers on NA.

Morphology of cells studied by microscopy of the alive and Gram stained preparations, spores were revealed with the methods given in the work [8]. As the basic criterion for definition of various morphotypes of colonies was the set of following signs served: pigmentation, allocation of the water-soluble pigment, formation of extracellular slime, a consistence, the dimension, air and substrate mycelium presence and other characteristic signs.

Physiological and biochemical properties of bacteria were studied by using test system for identification of bacteria API Coryne and API 20A of bioMérieux company SA (France), according to the manufacturer's instructions.

Sequences of 16S rRNA genes defined on automatic sequenator ABI310A (ABI PRISM 310

Genetic Analyzer) in Centre "Genomics" of the Siberian Branch of the Russian Academy of Sciences in the Novosibirsk city. The received sequences of 16S rRNA genes of Dead Sea isolates compared to those of the microorganisms deposited in database GenBank, using a package of program BLAST. Phylogenetic position defined at construction dendrograms which show position of a studied strain among closely related species (program BLASTN 2.2.28+, subprogram Tree view, algorithm neighbour joining).

Growth temperature range and NaCl tolerance of bacterial isolates. Growth of the isolates in a temperature range of 10-70°C was studied in liquid NB medium. The strains were cultivated at 10, 15, 20, 30, 50, 55, 60 and 70°C for 2 to 5 days. The tolerance for NaCl (g/l medium: 0, 20, 50, 100, 150, 200, 250) was tested in NA medium. After inoculation, the tubes were incubated at 42°C for 2 to 10 days.

Resistance to an ultraviolet radiation (UV-C). Tenfold delutings (from 10^{-2} to 10^{-7}) of bacterial suspensions have been put (0.05 ml) evenly on plates (NA medium). Open plates have been placed on distance of 1 m from an irradiation source (UV lamp, λ=254 nanometer). Duration of UV irradiation compounded from 1 till 60 minutes (40-2400 J/m^2). After an irradiation strains were incubated at 42°C. UV irradiation and the further incubation of the irradiated bacteria have been made in the dark to avoid a photo repair. The number of bacteria surviving after UV irradiation were counted after 1-2 days. In initial suspension (before UV irradiation) the number of cells has been defined by seeding of bacterial suspension from the same tenfold delutings of bacterial suspensions on NA medium. Bacterial survival after UV irradiation were evaluated by changing the percentage of surviving cells from their original amount.

Hydrolytic enzyme activities. The α-amylase activity of isolates was determined after the growth on 1% (w v^{-1}) of soluble starch, and detected as clear zone that surrounds colonies after adding iodine reagent [9]. Lipolytic activity was tested by hydrolysis of 1% (v v^{-1}) Tween 60 in a basal agar medium and was indicated by the appearance of a visible precipitate from the deposition of crystals of the calcium salt formed by the fatty acid liberated by the enzyme [10]. A lipase formation indicates the presence of opaque zone of calcium salts of fatty acids released from Tween 60 that are around the colony or stroke. Elastase activity was defined calorimetricly by the intensity of colouring of solution at enzymic hydrolysis of elastin [11]. One unit of activity defined as the amount of enzyme which catalyzes the hydrolysis of 1 mg of substrate per minute.

Activity of antibiotic biosynthesis has been defined by a method of the delayed antagonism [12]. As test cultures conditionally pathogenic bacteriums have been used: *Escherichia coli* ATCC 25592, *Staphylococcus aureus* 209p, *Pseudomonas aeruginasa* 4141, and also yeast *Candida albicans* UCM У-690, which were incubated in a thermostat (37°C, 1 days). Zones of growth retardation test cultures was measured after 24 hours incubation. Lack of growth of the test culture for stroke (at 24 hours) indicating an antagonistic effect on the test bacteria.

RESULTS

The characteristic strains. It is shown that the total number of aerobic heterotrophic bacteria in the coastal ecosystems of the Dead Sea is low and amounts to $1.4 \cdot 10^3$-$2.6 \cdot 10^5$/g of sample. For ecophysiological studies were selected seven strains that dominate in the samples. All strains are aerobic chemoorganotrophic bacteria. Colonies were large, light-brown (sometimes not pigmented), pasty, dim, rippled, round, the correct or irregular form, deckle-edged (an exception - a strain 6t1). Cells – Gram-positive, spores have been found in six strains (tab. 1).

Table 1. The list of the investigated strains isolated from coastal ecosystems of the Dead Sea.

No of strain	Presence of spores	The characteristic of samples of the Dead Sea coastal ecosystems
1t2	+	Vertical steep gorge around of the Dead Sea which consist of the stones bridged by clay. The organic matter maintenance was 5-10 g/kg of the sample
1t3	+	
1t5	+	
6t1	-	The salty soil near the brook which flows through clay-salt plain to the Dead Sea
6t2	+	
7t1	+	Black mud with the high maintenance of minerals which has been culled in the same place. It is used as a medical mud.
7tk3	+	

To identify isolated strains, besides studying of their morphological and physiological properties, comparative analysis of nucleotide sequences of the 16S rRNA genes of various strains has been carried out. For this purpose we used program BLASTN (version 2.2.28+) and the GenBank database (tab. 2). The received result was specified at the construction of phylogenetic tree, showing position of the investigated strain among closely related species (Neighbor Joining algorithm, subprogram Tree view, program BLASTN 2.2.28+). As a result, the strain 6t1 is related to the species *Staphylococcus hominis*, 7t1 - to *Bacillus licheniformis*, 7tk3 - to *Bacillus subtilis subsp. subtilis*. These results are consistent with the morphological and physiological characteristics of the strains. Strains 1t2, 1t3, 1t5 and 6t2 on the basis of morphological and physiological properties can be ranked to the genus Bacillus.

Table 2. The relatively closest species for investigated isolates by results of sequence of the 16S rRNA genes

No of isolate	The relatively closest species	
	The species, No of strains, (GenBank accession No)	Relatedness, %
6t1	*Staphylococcus hominis* subsp. *hominis* (NR036956.1)	98.6
6t2	*Bacillus licheniformis* DSM 13 (NR_074923.1)	97.7
7t1	*Bacillus licheniformis* DSM 13 (NR_074923.1)	99.8
7tk3	*Bacillus subtilis* subsp. *subtilis* DSM 10 (NR_027552.1)	99.8

Autecology. We studied the resistance to extreme factors in isolated strains (UV radiation, salt tolerance, thermo tolerance).

Resistance to UV was displayed by all isolated bacteria (Fig. 1). For comparison on a figure is citied the conforming data for sensitive to UV collection strain *E. coli* Bs-1. In a legend the numbers of isolated strains and collection strain *E. coli* are designated.

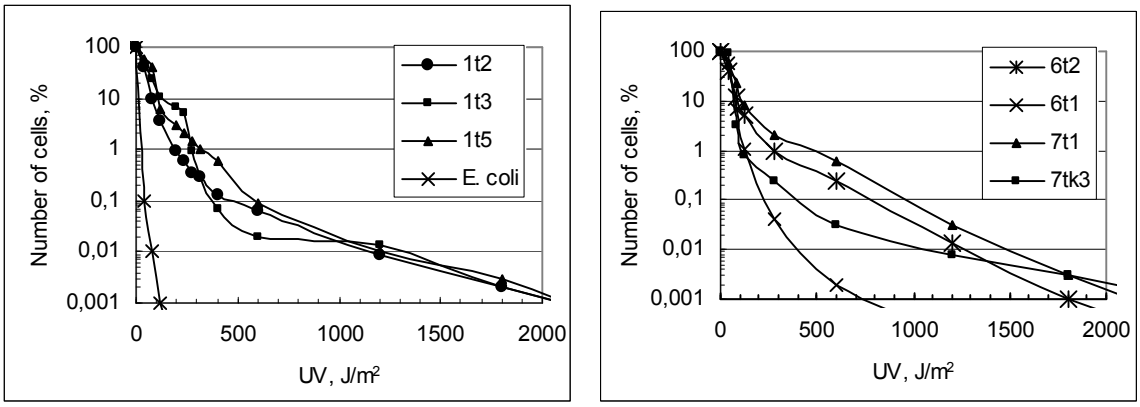

Fig. 1. Survival of the isolated strains after different doses of UV irradiation

To compare the sensitivity to UV different strains on the curves representing the variation of the amount of surviving cells from doses UV, calculated LD_{90} and $LD_{99,99}$, i.e. UV dose at which the killed 90 and 99.99% of cells, respectively (tab. 3). Lethal doses of UV (LD_{90} and $LD_{99,99}$) for spore-forming strains were in a range of 100-150 and 1100-1500 J/m^2, respectively; for strain 6t1 (does not form spores) – 50 and 370 J/m^2, for control collection strain *E. coli* Bs-1 – 10 and 80 J/m^2.

As follows from the data presented (tab. 3), the investigated strains grew at 0-10% NaCl in medium, i.e., the strains isolated from coastal ecosystems of the Dead Sea, were moderately halophilic bacteria. These typically rank microorganisms which grow in medium containing 3% to 15% NaCl [13]. All investigated strains grew in a range 20-50°C, at 55°C some have grown (tab. 3), i.e. they are thermo tolerant. Growth was absent at 15°C and lower.

The synthesis of biologically active substances. We have investigated the antagonism of spore-forming bacteria to the test cultures conditionally pathogenic microorganisms, representatives Gram-negative and Gram-positive bacteria and yeast

Candida. It is established that the isolated bacteria did not display antagonism in relation to Gram-negative bacteria (*Escherichia coli* and *Pseudomonas aeruginosa*). However, *Bacillus* sp. 1t2, 1t3 and 1t5 showed antagonism to *Staphylococcus aureus* and *Candida albicans* (Tab. 3). Zones of growth inhibition of test cultures were 30 and 28 mm, respectively.

Hydrolytic enzymes production by the bacterial isolates was determined qualitatively by the agar diffusion method. All *Bacillus* strains were capable to hydrolyze starch (α-amylase activity) and some showed lipase activity. Elastase was found in strains of Bacillus licheniformis 6t2 and 7t1, and the enzyme activity was very high and amounted to 23 and 35 U/ml, respectively.

Table 3. Ecophysiological properties of the investigated bacteria

No of strain	Presence of spores	Lethal doses of UV, J/m^2		Grow in a range		The antago-nist	Enzyme activity	
		LD_{90}	$LD_{99,99}$	NaCl, %	T,°C		*α-amylase	lipase
1t2	+	100	1200	0–10	20–50	+	+	+
1t3	+	120	1300	0–10	20–55	+	+	-
1t5	+	150	1200	0–10	20–50	+	+	+
6t1	-	50	370	0–15	20–50	nd	-	-
6t2	+	100	1250	0–10	20–55	-	++	-
7t1	+	150	1500	0–10	20–50	-	+	-
7tk3	+	100	1100	0–10	20–50	nd	+++	-

*α-amylase: size of light zone surrounding the colony after the addition of iodine reagent, mm 3-4 (+), 5-7 (+ +) 8-12 (+ + +)

DISCUSSION

It is shown that the total number of bacteria in samples of Dead Sea coastal ecosystems is much less than in similar ecosystems in other regions (such as the Black Sea coast, the coast of Antarctica, and the like), which is caused, probably, by the action of extreme factors in the investigated region. Most strains isolated from coastal ecosystems Dead Sea belong to the genus *Bacillus*, which was confirmed by phylogenetic analysis. Taxonomic status of these strains: *Bacteria; Firmicutes; Bacilli; Bacillales; Bacillaceae; Bacillus.*

Next, we examined the autecology isolated strains. Autecology studies the interaction of individual strains (or species) and biotic and abiotic factors of the environment. It is, first of all, the high content of salt, elevated levels of solar radiation, temperature factor, resulting in clay-salt plains, steep vertical cliffs around the Dead Sea and the air is heated to 40-60°C and above. Thus we assumed that since the Dead Sea coastal ecosystems are almost always open to the sun, the bacteria that live in these ecosystems must be resistant to UV. In fact, all isolated bacteria were resistant to high doses of UV. Lethal doses of UV (LD_{90} and $LD_{99,99}$) for spore-forming strains were in the range 100-150 and 1100-1500 J/m^2. The strains isolated from the Dead Sea coastal ecosystems were also thermo tolerant and moderately halophilic bacteria. We also have studied ability of isolated strains to synthesise some biologically active substances, such as antibiotics and hydrolases. Strains of *Bacillus* sp. showed antagonism to *Staphylococcus aureus* and *Candida albicans.* All *Bacillus* strains showed α-amylase activity and some showed lipase activity. Elastase activity of two strains of *Bacillus* was approximately the same as the activity of elastases known bacterial producers. This is very interesting, because elastase can dissociate elastin fibers of connective tissue and may therefore be potential to be used in medicine.

Thus, the study autecology strains of *Bacillus*, isolated from the coastal ecosystems of the Dead Sea, has shown that they are moderate halophiles, thermo tolerant and resistant to high doses of UV radiation. These properties seem to have formed as a response to extreme factors in the region. Among the isolates discovered strains that were antagonists conditionally pathogenic Gram positive bacteria and yeast *Candida*; as well as strains which showed lipase or amylase or elastase activity. These properties of the bacteria may be used in various biotechnology.

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АУТЕКОЛОГИЯ НА МИКРООРГАНИЗМИ ОТ КРАЙБРЕЖНИТЕ ЕКОСИСТЕМИ НА МЪРТВО МОРЕ

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Резюме. От крайбрежните екосистеми на Мъртво море (вертикални стръмни проломи, глинесто-солена равнина и черна силно минерализирана кал) бяха изолирани щамове *Bacillus licheniformis, B. subtilis* subsp. *subtilis, Bacillus* sp., *Staphylococcus hominis*. Беше установен растеж при всички щамове в среда, съдържаща 0-10% NaCl, при температури 20-50°C. Всички изследвани бактерии бяха резистентни при УВ-облъчване. Леталните УВ-дози за спорообразуващите щамове бяха съответно 100-150 и 1100-1500 J/m^2; за неспорообразуващ щам 6t1 - 50 и 370 J/m^2. Някои щамове от род *Bacillus* показаха силен антагонистичен ефект спрямо условно-патогенните тест-култури *Staphylococcus aureus* и *Candida albicans*. Може да се предположи, че устойчивостта към екстремни фактори на микроорганизмите от крайбрежните екосистеми на Мъртво море се е сформирала под влияние на абиотичните (физични и химични) фактори, типични за този регион.

Ключови думи: екстремофилни бактерии, Мъртво море, хало- и термотолерантност, устойчивост към УВ, антагонизъм

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