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PHYLOGENETIC ANALYSIS AND AUTECOLOGY OF SPORE-FORMING BACTERIA FROM HYPERSALINE ENVIRONMENTS

Multi-resistant to extreme factors spore-forming bacteria of Bacillus genus are isolated from hypersaline environments of the Crimea (Ukraine) and the Dead Sea (Israel). Phylogenetic analysis showed distinction of dominating extremophilic culturable species in studied regions. In Crimean environments they are B. mojavensis and B. simplex, in the Dead Sea ecosystem - B. subtilis subsp. spizizenii, B. subtilis subsp. subtilis, B. licheniformis and B. simplex. Isolates are simultaneously halotolerant and resistant to UV radiation. Strains isolated from the Dead Sea and the Crimea environments were resistant to UV: LD_{90} and $LD_{99.99}$ made 100-170 J/m^2 and 750-1500 J/m^2 respectively. Spores showed higher UV-resistance ($LD_{99.99}$ - 2500 J/m^2) than the vegetative cells. However, the number of spores made 0.02-0.007% of the whole cell population, and should not significantly affect the UV $LD_{99.99}$ value. Isolates of both environments were halotolerant in the range of 0.1-10% NaCl and thermotolerant in the range of 20-50 °C, and didn't grow at 15 °C. Survival strategy of spore-forming bacteria from hypersaline environments under high UV radiation level can be performed by spore formation which minimize cell damage as well as efficient DNA-repair systems that remove damages.

K e y w o r d s. Hypersaline environments, Bacillus, phylogenetic analysis, UV-resistance, halotolerance.

Under natural conditions, microorganisms from diverse environments exist under the influence of numerous extreme physical, chemical and climatic factors. Previously, we studied the effect of extreme factors such as UV radiation, high temperature and salinity on psychrotolerant bacteria of polar region (Antarctica) and identified resistant and sensitive strains [2, 3]. In the current work microbiological studies are expanded to steppe and desert regions possessing extreme natural conditions - hypersaline environments of Crimea (Ukraine) and the Dead Sea (Israel). The aim the study is to determine the taxonomic position of spore-forming bacteria isolated from hypersaline environments of Crimea and the Dead Sea, describe and compare their resistance to extreme factors. We hypothesized that aboriginal microbial habitats could perform environments-specific resistance to abiotic factors and form an adaptive response to their action - halotolerance, thermotolerance, resistance to UV radiation.

Material and methos. *Research objects* were the microbial strains of hypersaline environments of Crimea (mineral therapy mud of estuary lakes near Saki and Pribrezhnoe settlements) and the Dead Sea (mineral therapy mud and clay-salty cliffs), isolated at 42ºC. For isolation and cultivation of bacteria was used Nutrient Agar medium (NA), HiMedia Laboratories Pvt. Ltd. adding 50 mg/L of pharmaceutical nystatin to suppress the growth of filamentous fungi.

Isolation of dominant bacteria. To identify dominant bacteria serial tenfold dilutions (10^{-2}–10^{-8}) of natural sample inoculum were seeded on solid nutrient medium. The dominant strains were isolated from the last sample dilutions (10^{-5}–10^{-7}) performing 5-10 separate colonies per plate.

Isolation of pure cultures was carried out by conventional methods [1] inoculating Petri dish with serial tenfold dilutions of microbial suspensions (0.1 ml), growing on solid medium, subsequent selection of individual colonies and their reisolation. The culture purity was checked by microscopy.

Morphological and cultural properties. Cell morphology (shape, size, mobility, and spores) was studied by standard microscopy methods of living and Gram-stained preparations [1]. Spores were detected by negative staining Peshkov method [1]. Strain cultural properties (pigmentation, water-soluble pigment and extracellular mucus production, texture and size of the colonies, the presence of air and substrate mycelium etc.) were determined by culturing (42ºC) on agar nutrient medium (NA).

Phylogenetic analysis. Sequencing 16S rRNA genes of isolates from hypersaline environments of the Crimea and the Dead Sea was held by staff of the Limnological Institute of Russian Academy of Sciences. Obtained 16S rRNA gene sequences were used to determine closely related species and phylogenetic analysis of bacterial isolates. Sequences were compared with GenBank database, using the BLAST package software. Related species were determined by calculating the pairwise similarity (%) based on ratio of matching / analyzed nucleotide of 16S rRNA genes of each isolate with the reference bacteria. Phylogenetic position was determined by constructing a tree showing the taxonomic status of the studied strains among closely related and typical species (program ClustalX 2.1, Mega v. 6.00). Phylogenetic analyses of gene sequence data were conducted using the neighbor-joining (NJ) method. The reliability of internal branches was assessed from 1000 bootstrap pseudoreplicates.

The temperature range of extremophilic strain growth was studied using liquid NB medium in 10 ml tubes. The strains were cultured at 10–70°C for 2-5 days in stationary conditions. Visual changes, lag phase duration, rate of biomass production and growth patterns were recorded and assessed with 1-3-day intervals.

Halotolerance was studied on NaCl concentration range (0.1–250 g/L) by plating strain onto agar media containing listed NaCl concentrations. After inoculation, the plates were incubated at an optimal growth temperature. Results were assessed after 2-10 days of growth.

UV-resistance (UV-C) was determined as described previously [2]. Spore UV-resistance was studied on heated bacterial suspensions [1]. For this purpose, diurnal (24- hour) and 4-day bacterial suspensions in sterile vials were heated in a water bath (20 min, 100°C) [1]. Next active non-pasteurized (active cells and spores) and heated pasteurized (sole spores) suspensions were seeded onto plates with nutrient agar and exposed to UV-radiation. Survival rate was estimated both for active bacterial population and spores by method described in [2].

Results and discussions. Strains of spore-forming bacteria are isolated from hypersaline environments of the Crimea and the Dead Sea regions. 6 dominating isolates (Table. 1) were selected for identification of taxonomic position and study their resistance to extreme factors typical to specified ecosystems

Table 1.

List of dominating strains and sampling sites

Isolate No	Sampling site (region, environment)
Crimea (Ukraine)	
2s1	Black mineral therapy mud, saline lake (Saki settlements)
3s2	Black mineral therapy mud, lake Sasyk (Pribrezhnoe settlements)
the Dead Sea (Israel)	
7t1, 7t2	Black mineral therapy mud
1t3, 1t4	Saline clay formations from the cliff

All isolates were gram-positive, spore-forming, aerobic chemoorganotrophic bacteria. Oval-shaped endospores did not inflate cell and possessed central or terminal location. Halo- and thermotolerant strains form on the surface of liquid medium dense, folded film.

Strains 3s2 and 1t4 are similar in cell morphology and colony shape. Strain 3s2 - oval hyphae-forming cells up to 15 microns, breaking down into short filaments and individual cells 0.8 × 1 microns. Colonies are brown, shiny and pasty, convex, 2-5 mm diameter. Strain 1t4 - straight or curved rods, form a segmented hyphae, 1.5-2.0 × 10-30 microns. Colony characteristics are identical to strain 3s2.

Strain 2s1. Cells – cocci, size 3 microns. Colonies light brown, pasty, wrinkled, irregular edges, up to 20 mm.

Strains 1t3, 7t1, 7t2 are similar in cell and colony morphology. Cell are rods 1.5-2.0 × 0.7-1.0 mm, motile. Colonies are large, light-brown (sometimes non-pigmented), pasty, wrinkled, round, regular or irregular, with jagged edges. Listed properties of isolated strains testify to their close relation with Bacillus genus. To clarify their taxonomic position was conducted phylogenetic analysis based on 16S rRNA gene sequencing.

Phylogenic analysis. To detemine the taxonomic position of the isolates we performed the comparative and phylogenetical analysis of the nucleotide sequences of 16S rRNA genes of our isolates and the 16S rRNA genes of the reference bacteria from database GenBank. For the comparative analysis the BLASTN (2.2.28+) software application was used. Related species for own isolates were detected by calculating pairwise similarity (%) of 16S rRNA gene sequences of each isolate to the reference bacteria from GenBank. Based on the data obtained, closely related strains (species) for the studied bacteria were detected (Table 2).

Table 2.

Comparative analysis of the pairwise similarity of 16S rRNA genes of the extremophilic bacteria from hypersaline environments with the ones of the bacteria in the GenBank database.

Strain№	Bacterial species, the most related to the studied strains, according to software application BLASTN 2.2.28+	
	Species, strain № (GenBank accession No)	similarity, %
2s1	**Bacillus mojavensis* IFO15718 (NR_24693)	99.5
	Bacillus subtilis subsp. *inaquosorum* BGSC 3A28^T (NR104873)	99.4
	[Brevibacterium] halotolerans DSM 8802^T (NR_042638)	99.4
3s2	*[Brevibacterium] frigoritolerans* DSM 8801^T (NR042639)	99.8
	**Bacillus simplex* DSM 1321^T (NR_042136)	99.7
	Bacillus muralis LMG 20238^T (NR_042083)	99.6
1t3	**Bacillus subtilis* subsp. *spizizenii* NRRL B-23049^T (NR024931)	99.9
	[Brevibacterium] halotolerans DSM 8802^T (NR_042638)	99.8
	Bacillus mojavensis IFO15718^T (NR_024693)	99.7
1t4	**Bacillus simplex* DSM 1321^T (NR_042136)	99.4
	[Brevibacterium] frigoritolerans DSM 8801^T (NR_042639)	99.3
	Bacillus muralis LMG 20238^T (NR_042083)	98.9
7t1	**Bacillus licheniformis* DSM 13^T (NR_074923)	99.8
	Bacillus aerius 24K^T (NR_042338)	99.3
	Bacillus atrophaeus 1942^T (NR_075016)	98.1
7t3	**Bacillus subtilis* subsp. *subtilis* DSM 10^T (NR_027552)	99.8
	[Brevibacterium] halotolerans DSM 8802^T (NR_04263)	99.6
	Bacillus vallismortis DSM11031^T (NR_024696)	99.6

In the comparative analysis (Table 2) for each of the isolates three most close related strains are offered. Most isolates had approximately equal level of pairwise similarity with several related strains (species). Therefore in Table 2 related species that comply with the most close species, obtained in the result of the following phylogenetical analysis (Fig. 1) are pointed out (*).

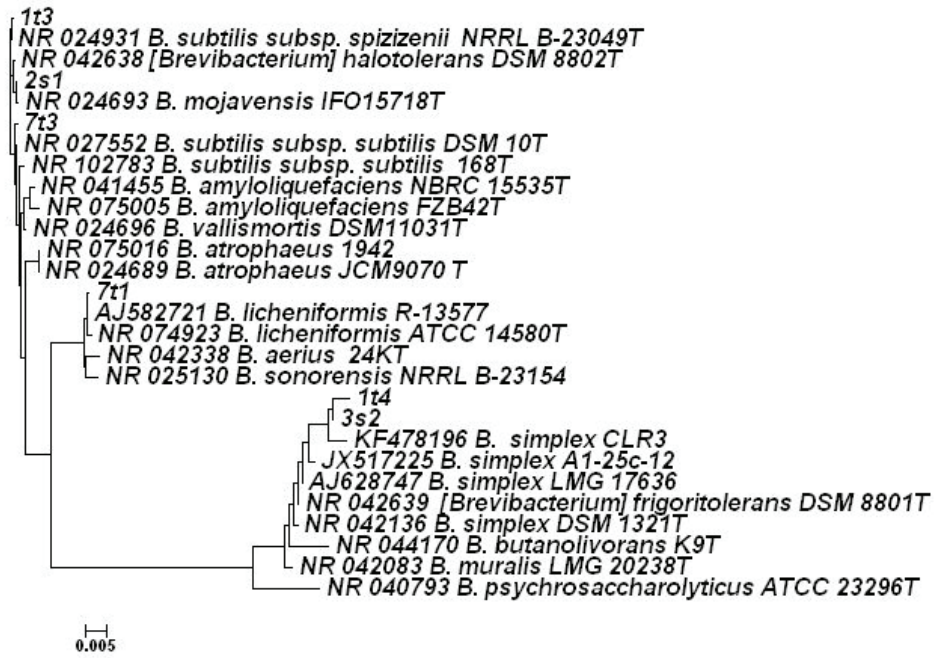

Fig. 1. Phylogenetic tree developed by NJ method (ClustalX 2.1, Mega v. 6.00 software) basing on 16S rRNA sequences of strains *Bacillus* isolated from hypersaline environments of Crimea (strains 2s1 and 3s2) and the Dead Sea (strains 1t3, 1t4, 7t1, 7t3).

Note. The scale corresponds to 5 substitutions per 1000 of nucleotide pairs

During the comparative analysis of the pairwise similarity of 16S rRNA genes of isolated bacteria, with the ones of the bacteria from GenBank database we also formed the extended list of closely related and typical strains of the corresponding species for each isolate. The 16S rRNA gene sequences of the bacteria from the list were used for constructing of the phylogenetic trees (Neighbor Joining algorythm, bootstrap NJ tree), software applications ClustalX 2.1, Tree view, Mega v. 6.00), which allowed detection of the taxonomic position of isolates. The results of the phylogenetic analysis (Fig. 1) confirmed that studied strains are the species of the genus *Bacillus*, their taxonomic position is Bacteria; Firmicutes; Bacilli; Bacillales; Bacillaceae, *Bacillus.*

The spore-forming bacteria from the hypersaline lakes of Crimea could be assigned to the following species: 2s1 – to *B. mojavensis,* 3s2 – to *B. simplex* (Fig. 1, Table. 2). The spore-forming thermotolerant bacteria from the Dead Sea ecosystems could be assigned to the species, as follows: 1t3 – to *B. subtilis* subsp. *spizizenii*, 1t4 – to *B. simplex,* 7t1 – to *B. licheniformis* and 7t3 – to *B. subtilis* subsp. *subtilis* (Fig. 1, Table. 2). Thus in the studied hypersaline lakes the *Bacillus* species are dominating.

Resistance to extreme factors. The resistance to UV radiation of the bacteria, dominating in the hypersaline ecosystems of Crimea and the Dead Sea, has been studied. All studied spore-forming bacteria showed resistance to UV radiation (Fig. 2).

To compare the sensibility to UV of different monocultures from the hypersaline lakes of Crimea and the Dead Sea, on the dose curves representing the dependance of

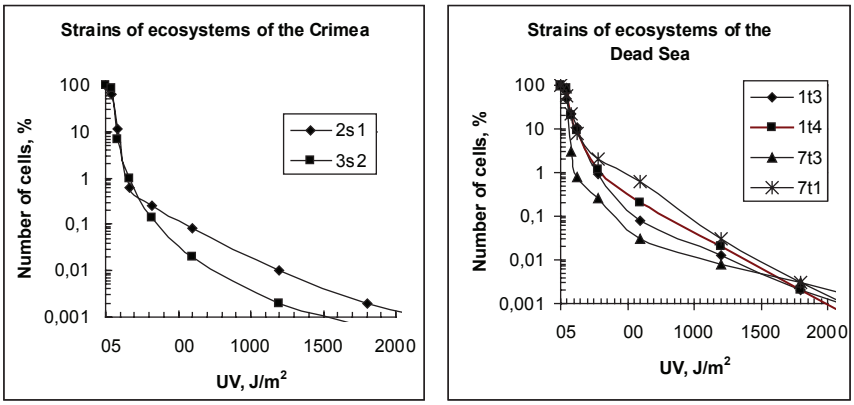

Fig. 2. Survival of strains isolated from hypersaline environments of Crimea and the Dead Sea, after action of UV-radiation.
Note. The legend numbers marked strains

the quantity of survived cells from the UV doses, we calculated LD_{90} and $LD_{99.99}$ – the UV doses that cause death of 90% and 99.99% of the cells accordingly (Table 3). The lethal UV doses (LD_{90} and $LD_{99.99}$) for the spore-forming strains of the Bacillus genus made respectively 100–170 and 750–1500 J/m^2.

As follows from the data presented in Table 3 the studied strains were growing in the range of NaCl concentration 0.1-10%, strain 3s2 was the exclusion (it was growing at presence of 0.1-5% NaCl). Thus, strains, isolated from the hypersaline Crimea and the Dead Sea ecosystems belong to moderate halophylic (halotolerant) bacteria. This group typically includes microorganisms growing at presence of 3%- 15% NaCl [6]. They are widely occurred in various environments, such as hypersaline lakes, saline soils, saltwork reservoirs, salt mines etc.

Table 3.

Ecophysiological properties of bacteria from the Dead Sea hypersaline environments

Strain №	Species	Lethal doses UV, J/m^2		Growth range	
		LD_{90}	$LD_{99.99}$	NaCl, g/l	t,°C
2s1	*Bacillus mojavensis*	100	1200	0.1-100	20-50
3s2	*Bacillus simplex*	100	750	0.1-50	20-50
1t3	*Bacillus subtilis* subsp. *spizizenii*	170	1300	0.1-100	20-50
1t4	*Bacillus simplex*	150	1400	0.1-100	20-50
7t1	*Bacillus licheniformis*	150	1500	0.1-100	20-55
7t3	*Bacillus subtilis* subsp. *subtilis*	100	1200	0.1-100	20-50

Temperature range of growth for isolated bacteria was studied (Table 3). The strains were incubated on the liquid medium in the test-tubes under stationary conditions. The growth was observed in the form of dense, rugous film, or in the form of thin crumbly smooth film. All the studied strains were growing in the range of 20-50°C and can be considered as thermotolerant. Upper and lower temperature limits are 55°C and 15°C. At 55°C the single strain possessed ability to grow; at 15°C growth was not observed.

During analysis of the UV impact on the survival of spore-forming bacteria from hypersaline ecosystems of Crimea and the Dead Sea we were taking into account, that the damage minimization under the UV radiation could be provided by spore formation. For instance *Bacillus subtilus* spores are protected by a thin protein layer from the damaging effect of UV-B and UV-C and solar UV. Layer-free mutants were very sensitive to these factors [4]. *Bacillus subtilus* spores include DNA-bound protein

protecting the spore DNA from the brakes induced by UV or desiccation [5]. Therefore appeared a question whether the survival of studied spore-forming bacteria under the UV radiation could be supported exclusively by high resistance of spores?

Thereby we studied separately UV spores and active cell population. Spore selection was carried out by heating active strain population at 100°C. The results showed (Fig. 3) that number of spores is 4-5 orders lower than the general number of the active cells; spores make 0.02–0.007 % of the total cell population (Table 4).

Table 4.

Lethal doses of UV for: (v) total cell population before heating and (s) heated cell population (contains only spores) of *Bacillus mojavensis* 2s1

Experiment variants	Culture age, days	UV, J/m^2		CFU/ml		
		LD_{90}	$LD_{99.99}$	Total number of cell	Spores	
					Number	%
v	1	100	1200	600 000 000		
s	1	100	2400		44 000	0.007
v	4	70	600	1 200 000 000		
s	4	180	1400		270 000	0.02

To determinate the lethal doses of UV we built graphs exhibiting, in per cent, the survival under different doses of UV radiation of all the cell population (before heating) and spores (after heating) (Fig. 4). The 4-day culture was more sensitive to UV than a 1-day one (Fig. 4, Table 4). This may be connected to lower physiological activity of «old cultures» (4-days) cells. It was shown that for the spores the lethal dose of UV ($LD_{99.99}$) was practically twice higher comparing vital populations independently on their age (Table 4), i.e. spores are more resistant to UV. At the same time, the presence of 0.02–0.007 % spore cells of the number of all microbial cell population (Table 4) could not make a significant impact on the UV lethal dose ($LD_{99.99}$) for the whole cell population where vegetative cells make 99.980–99.993%. The similar results were obtained other isolated *Bacillus* strains.

Apparently, the survival strategy of the studied spore-forming bacteria under the effect of UV radiation (DNA-damaging factor) is provided by the damage minimizing mechanism (spore presence) and the effective mechanism of DNA damages reparation. Basing on main autecology principles (study impact of external factors on the viability of the whole cell population), at the evaluating action of certain extreme factor, the main indicator is survival rate of the whole population independently on variety of

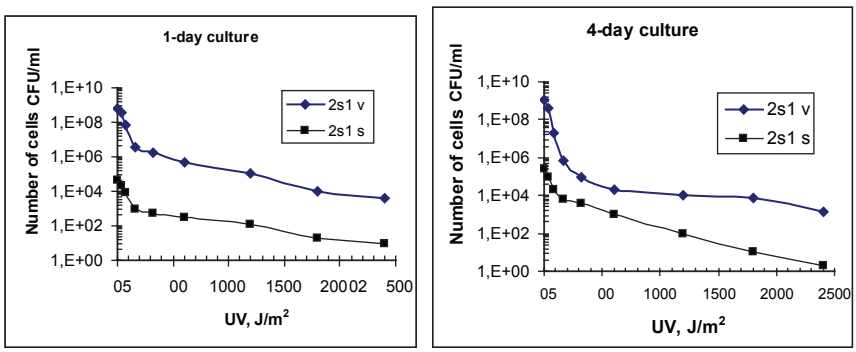

Fig 3. Total number of survived cells of *Bacillus mojavensis* 2s1 population under various UV doses

Note. v –cell population before heating, s – heated cell population (contains only spores).

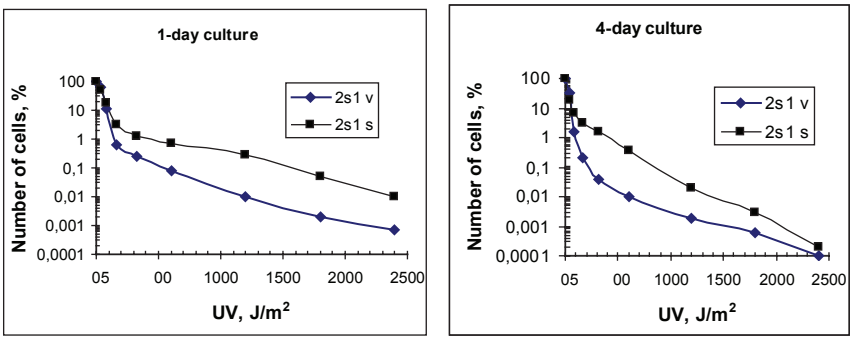

Fig. 4. Number (%) of survived cells of *Bacillus mojavensis* **2s1 at various UV doses**
Note. See Fig 3.

survival mechanisms. Therefore from the ecological point of view it does not matter what cells (vegetative or spores) provide population survival. The presence of spores could be crucial for the population survival under the UV radiation in the case when vegetative cells do not possess effective mechanisms of DNA damage reparation.

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ФИЛОГЕНЕТИЧЕСКИЙ АНАЛИЗ И АУТЭКОЛОГИЯ СПОРООБРАЗУЮЩИХ БАКТЕРИЙ ИЗ ГИПЕРСОЛЁНЫХ ЭКОСИСТЕМ

Р е з ю м е

Из гиперсолёных экосистем Крыма и Мёртвого моря изолированы спорообразующие бактерии преимущественно рода *Bacillus.* В результате филогенетического анализа показано, что изоляты из Крыма можно отнести к видам *B. mojavensis* и *B. simplex,* из экосистем Мёртвого моря – к *B. subtilis* subsp. *spizizenii, B. subtilis* subsp. *subtilis, B. licheniformis* и *B. simplex.* Изоляты из гиперсолёных экосистем Крыма и Мёртвого моря резистентны к УФ радиации. Летальные дозы УФ ($ЛД_{90}$ и $ЛД_{99.99}$) для них составляли, соответственно, 100–170 и 750–1500 Дж/м^2. Споры более устойчивы к УФ ($ЛД_{99.99}$ до 2500 Дж/м^2), чем вегетативные клетки. Вместе с тем, количество спор составляет 0.02–0.007 % от числа клеток всей микробной популяции, и не должно существенно повлиять на значение $ЛД_{99.99}$ УФ для всей популяции. Изоляты из гиперсолёных экосистем Крыма и Мёртвого моря были галотолерантными (росли в диапазоне 0.1-10% NaCl в среде) и термотолерантными (росли в диапазоне 20-50°C, при 15°C рост отсутствовал). Стратегия выживания при высоком уровне УФ радиации спорообразующих бактерий из гиперсолёных экосистем может осуществляться как за счёт спор, которые минимизируют повреждения, так и эффективных систем репарации, которые устраняют повреждения ДНК.

К л ю ч е в ы е с л о в а. Гиперсолёные экосистемы, бактерии, филогенетический анализ, УФ-резистентность, галотолерантность.

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ФІЛОГЕНЕТИЧНИЙ АНАЛІЗ І АУТЕКОЛОГІЯ СПОРОУТВОРЮЮЧИХ БАКТЕРІЙ З ГІПЕРСОЛОНИХ ЕКОСИСТЕМ

Р е з ю м е

З гіперсолоних екосистем Криму і Мертвого моря ізольовані спороутворюючі бактерії переважно роду *Bacillus.* Внаслідок філогенетичного аналізу показано, що ізоляти з Криму можна віднести до видів *B. mojavensis* і *B. simplex,* з екосистем Мертвого моря – до *B. subtilis* subsp. *spizizenii, B. subtilis* subsp. *subtilis, B. licheniformis* і *B. simplex*. Ізоляти з гіперсолоних екосистем Криму і Мертвого моря резистентні до УФ радіації. Летальні дози УФ ($ЛД_{90}$ і $ЛД_{99.99}$) для них становили, відповідно, 100–170 і 750–1500 Дж/м^2. Спори більш стійкі до УФ ($ЛД_{99.99}$ до 2500 Дж/м^2), ніж вегетативні клітини. Разом із тим, кількість спор становить 0.02–0.007 % від числа клітин всієї мікробної популяції, і не повинно суттєво вплинути на значення $ЛД_{99.99}$ УФ для всієї популяції. Ізоляти з гіперсолоних екосистем Криму і Мертвого моря були галотолерантними (росли в діапазоні 0.1-10 % NaCl в середовищі) і термотолерантними (росли в діапазоні 20-50°С, за 15°С ріст був відсутній). Стратегія виживання за високого рівня УФ радіації спороутворюючих бактерій з гіперсолоних екосистем може здійснюватися як за рахунок спор, які мінімізують ушкодження, так і ефективних систем репарації, які усувають пошкодження ДНК.

К л ю ч о в і с л о в а: Гіперсолоні екосистеми, бактерії, філогенетичний аналіз, УФ-резистентність, галотолерантність.

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