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Moderate thermophilic chemoorganoheterotrophic bacterium in surface layer of anthropogenic grounds of industrial estate area of Al-Mafraq, Jordan

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Surface of oil-contaminated soil from Industrial Estate of Al-Mafraq city, Jordan, was investigated for the presence of aerobic oil-degrading moderately thermophilic bacteria. A pure culture of spore – forming aerobic chemoorganogeterotrophic rod shaped bacterial isolate, designated as strain j3n, was obtained. Phylogenetic analysis of the 16S rRNA gene sequence revealed that strain j3n is closely related to gram-positive bacteria of *kaustophilus – thermoleovorans* cluster of *Geobacillus* genus. Strain j3n grew aerobically with oil, hexadecane, benzoate and acetate. Growth data indicated that utilization of hexadecane but not of oil and benzoate might be under catabolite repression control. Possibility of a regulation of alkane degradation by acetate in aerobic thermophilic gram-positive bacteria of *Geobacillus* spp. was shown for the first time.

Keywords: aerobic; thermophilic; chemoorganoheterotroph; *Geobacillus*; oil; alkanes; ground surface; carbon catabolite repression.

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Умеренно термофильная хемоорганогетеротрофная бактерия из поверхностного слоя антропогенного грунта промышленной зоны г. Аль-Мафрак, Иордания

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Поверхность нефтезагрязненной почвы промышленной зоны г. Аль-Мафрак (Иордания) была исследована на предмет выявления присутствия аэробных нефтеокисляющих умеренно термофильных бактерий. Чистая культура спорообразующих аэробных хемоорганогетеротрофных бактерий, штамм j3n, была выделена из одной пробы. Филогенетический анализ последовательности нуклеотидов гена 16S рРНК показал, что штамм j3n принадлежит к грамположительным бактериям группы *kaustophilus – thermoleovorans* рода *Geobacillus*. Особенности роста штамма в средах с разными субстратами показали, что использование гексадекана, но не нефти и бензоата, в присутствии ацетата могло контролироваться механизмом катаболитной репрессии. Возможность регулирования разложения алканов ацетатом с участием этого механизма аэробными термофильными грамположительными бактериями *Geobacillus* spp. показана в первый раз.

Ключевые слова: аэробные; термофильные; хемоорганогетеротроф; *Geobacillus*; нефть; алканы; поверхность почвы; катаболитная репрессия.

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BACKGROUND

Two sources of energy create environments with elevated temperatures suitable for thermophilic microorganisms. The heat derived from the inner Earth creates habitats for thermophiles (hot springs and deep-sea hydrothermal vents) that are non-uniformly located in the geothermal and volcanic areas of tectonically active zones of the Earth [1]. Energy from outside of the planet mostly coming from the Sun heats the surface of terrestrial environments, thus, creating new ecological niches for thermophiles [2]. These habitats promote spatial distribution of moderate thermophiles far away from their primary sources [3–5]. Though data about occurrence of moderate thermophiles in solar-heated environments is accumulating [6–9], our understanding of the occurrence and function of various thermophiles in solar-heated surface environments is far from clearness.

Jordan possesses many geothermal springs and wells with water temperature varying from 20°C to 68°C [10, 11]. Microbial population of these water sources was investigated with culture-dependent and independent techniques and some of thermotolerant and moderate thermophiles of genera *Geobacillus*, *Anoxybacillus* and *Thermomonas* were shown to be present [12–22].

Arid regions comprise the largest ecosystems in Jordan. The Jordanian high total annual sun derived irradiance [23] can promote heating of surface arid area sufficient for the survival of moderate thermophiles that may distributed around geothermal area [4, 24, 25]. Therefore, it is possible to expect the presence of moderate thermophiles in the Jordan area distant to geothermal springs and wells.

The aim of the study was to check upper layer of arid type of ground of Industrial Estate of Al-Mafraq (Jordan) for the presence of moderate aerobic chemoorganoheterotrophic thermophiles by cultivation-dependent approach and to characterize phylogeny and physiological properties of isolated bacteria.

MATERIALS AND METHODS

Three surface ground samples polluted with oil were collected in sterile plastic Falcon 50 ml tubes on the 2nd of April of 2017. The sampling sites with coordinates 32.321634 N and 36.227748 E, 32.322284 N and 36.229237 E, 32.321780 N and 36.225798 E designated as J1, J2 and J3, correspondingly, were located in the Industrial Estate of Al-Mafraq, Jordan. The conditions of the sampling sites were semi-arid and dry. The samples were stored at 4°C until investigation.

Mineral medium of Voroshilova and Dianova [26] was used for enrichment and cultivation of bacteria with following modification: ammonium chloride (1 g/L) replaced ammonium nitrate and trace elements solutions [27] were added (1 ml/l). Organic compounds supplied to the mineral medium as electron donors and carbon sources for growth of bacteria were: oil (2 vol%), hexadecane (2 vol%), benzoate (5 mM) and acetate (10 mM). Cultivation procedures were done at 60°C in the dark.

Pure cultures of bacteria were isolated by streaking cultures on the surface of solid organic rich medium Nutrient Agar (HiMedia Laboratories, Mumbai, India). Purity of the culture was checked by observing growth of colonies of bacteria during cultivation of the strain on this medium and by microscoping of cells under light microscope Axiostar Plus (Zeiss, Jena, Germany). Phase-contrast equipment of the microscope allowed to observe cellular morphology and the presence of spores without fixation and staining procedures.

Growth of bacteria was followed by measurement of optical density of culture at wavelength of 578 nm in cuvette of 1 ml with 1 cm of light path on Spectrophotometer PE-3000 UV (Ekroschem, Saint Petersburg, Russia). Growth experiments were done in duplicate and time point measurements were done twice.

Determination of the sequence of 16S rRNA gene was performed at Evrogen Company (Moscow, Russia) using standard protocols for DNA extraction, gene sequence amplification with universal bacterial primers 27F and 1492R and PCR products sequencing. Phylogenetic analysis was done via BLAST option of NCBI database [28] and phylogenetic tree was constructed using Mega 7 software [29]. The 16S rRNA gene sequence was deposited in GeneBank® NCBI under accession number MW913412.

RESULTS

Enrichment of aerobic heterotrophic thermophilic microorganisms was performed by the cultivation at 60°C of 0.1 g of each sample in tubes with 4.5 ml of medium supplied with organic growth substrates: acetate, hexadecane and oil. After 3 days of incubation, bacteria of sample j1 grew with all growth substrates and j3 – only with oil. Sample j2 (with all growth substrates) and j3 (with acetate and hexadecane) did not show growth after 7 days of incubation. Addition of ~1 g of soil into these variants did not help to initiate growth of bacteria. Bacteria from sample j1 stopped to grow in the second transfer of cultures into the new portions of medium. Stable enrichment culture growing in liquid medium with oil with bacteria from sample j3 developed after several successive transfers followed by dilution of culture in the same medium to extinction. The last positive tube showing growth on oil was used for isolation of pure culture of oil – degrading microorganisms by plating the culture on solid organic rich medium. All grown colonies were of the same type: white, round, lens shaped with smooth surface. Bacteria from one separately grown colony showed growth in the liquid medium with oil. Microscoping of the culture revealed the presence of rod shaped cells. Some of cells showed formation of terminal endospores. The culture did not grow at 40°C (7 days of incubation). Thus, obtained data indicated that the pure culture of moderately thermophilic organoheterotrophic oil-utilizing bacterium, designated as j3n, was isolated.

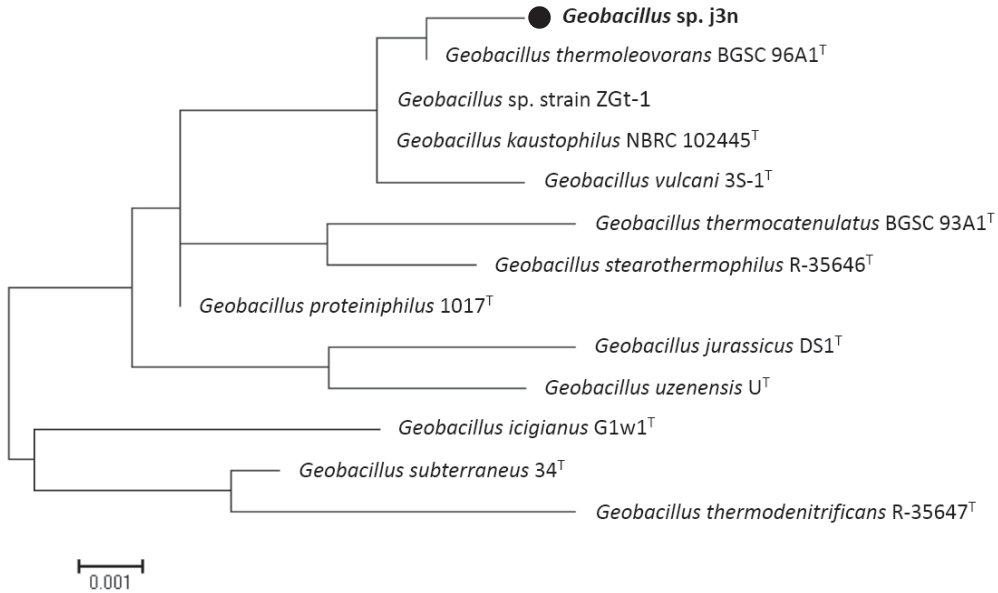

Fig. 1. Phylogenetic position of strain j3n based on the 16S rRNA gene sequences. Clustering was inferred by using the Maximum Likelihood method based on the Tamura–Nei model [30]. The tree with the highest log likelihood is shown. Bar – 0.001 substitutions per nucleotide position

The nucleotide sequence obtained from the 16S rRNA gene of strain j3n was 1396 nucleotides long and after removal of bad quality sequence at the beginning final length of the sequence was 1369 nucleotides. Blast search of database of NCBI revealed affiliation of strain j3n within the family *Bacillaceae* of the class *Bacilli* of the *Firmicutes*. The closest cultivated relatives of strain j3n were spore – forming bacteria of the cluster of *Geobacillus kaustophilus – thermoleovorans* (Fig. 1).

Substrates utilization tests with the culture pre-grown on acetate confirmed the ability of the strain j3n to grow by benzoate, oil and hexadecane utilization (Fig. 2). Inoculation of control medium without organic substrate showed little growth of bacteria at the beginning of cultivation that used acetate introduced into the control medium with inoculum. Growth on acetate started without lag-phase while growth on oil, benzoate and hexadecane started after lag-phase that was quite short in the case of oil and benzoate and growth on hexadecane started only after depletion of acetate added into the medium with inoculum.

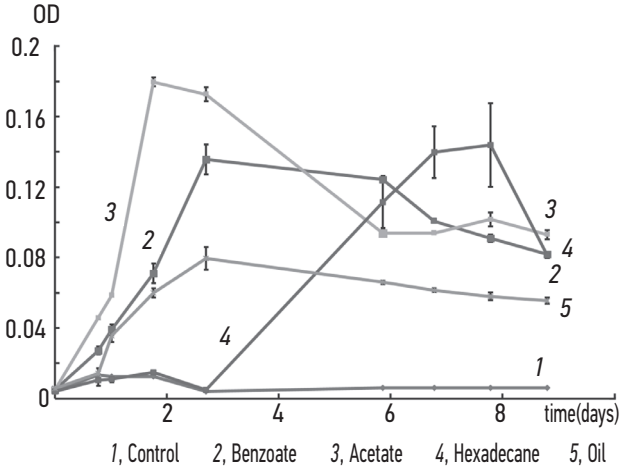

Fig. 2. Growth of strain j3n (shown as change of optical density) by acetate, hexadecane, benzoate and oil utilization. Medium without organic substrates inoculated by bacteria served as control. Axis: Y – OD (optical density of culture at 578 nm), X – time of cultivation (days)

DISCUSSION

Thermophilic microorganisms including *Geobacillus* spp. Inhabited worldwide distributed "hot spots" [2, 31]. Isolated by us *Geobacillus* sp. strain j3n indicated occurrence of thermophiles in semi-arid area distant to hot springs. Thus, evidences are accumulating that *Geobacillus* spp. occur quite often in non-geothermally heated environments [6, 8, 9, 31]. These bacteria could be found all over the planet starting from geothermally heated areas in the North and ending in the South of the planet. Distribution of bacteria as dust of "hot spots" via atmosphere could be one of possibilities to inoculate new habitats [3, 31–33]. After arrival on the surface of the ground thermophiles might survive long at moderate temperatures or even sporadically grew if temperature arose from sun heating [3, 5]. The origin of strain j3n is unclear. Though, isolated from Zara hot spring *Geobacillus* sp. strain ZGt-1 [18] is not a very close relative of strain j3n (Fig. 1) it is possible to assume that strain j3n might arrive from hot springs areas that are located to the North and to the South from Al-Mafraq [10].

Additional studies are required and to clarify phylogeny of the strain j3n on the basis of other conserved genes sequences or genomes comparison [34–37].

Strain j3n was able to grow by hexadecane, benzoate and oil degradation and, therefore, has a potential to participate in

remediation of hydrocarbon-contaminated soils in arid areas. Growth data (Fig. 2) showed that acetate is more preferred growth substrate than hexadecane and suggested that degradation of this alkane might be regulated by catabolite repression mechanism [38]. This is first report about observation of the possibility of repression of alkane utilization by acetate in the species of the genus *Geobacillus*. Repression of alkane degradation by acetate was earlier described for mesophilic gram-negative bacteria, as *Pseudomonas aeruginosa* and *Burkholderia cepacia* [39, 40].

The major global transcriptional regulators of carbon catabolite repression systems in gram-positive bacteria are LacI family proteins that are often associated with sugar metabolism [41, 42]. Proteins from families ArsR, GntR, LysR and TetR could also specifically regulate alkane degradation in gram-negative and gram-positive mesophilic bacteria [43–46]. Though global analysis of metabolism of some *Geobacillus* spp has been performed [47, 48], fine mechanisms of regulation of carbon metabolism in these bacteria are not elucidated, yet. It is possible to envisage that carbon catabolite regulation in thermophilic *Geobacillus* spp. proceeds via already known mechanisms because the genes of regulatory proteins of families LacI and GntR, were detected in the genome of the closest relative of strain j3n *G. kaustophilus*s train HTA426 [49, 50]. The presence of LacI regulator in *Geobacillus* spp. may explain earlier observation of inhibition of glucose utilizing moderately thermophilic bacteria that are capable to degrade homo- and heterocyclic aromatic compounds [51]. We searched deposited in NCBI database genome of *Geobacillus* sp. strain ZGt-1 isolated from hot spring not far from the area of our investigation [18] and found genes of proteins of LacI, TetR and LysR families. Similar genes might be present in the genome of strain j3n and further investigation is required to elucidate the mechanism of acetate dependent catabolite repression of alkane degradation.

CONCLUSIONS

Present study provides evidences for the presence of moderate thermophilic species of microorganisms in the surface layer of industrial type of grounds of the regions with semi-arid conditions. They may survive in such environments under unfavorable conditions (dryness or low temperature) as dormant cells or as spores. It is possible to envisage that sporadically wetting and heating from solar energy events may cause these microorganisms to function. Further studies are required to provide clarify a question about role of these microorganisms in semi-arid ecosystems.

Results of our investigation showed for the first time that alkane degradation by the strain j3n could be regulated in the presence of acetate possibly via mechanism of carbon catabolite repression. Growth on oil and an aromatic compound as benzoate seems not to be repressed in the presence of acetate.

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