Journal of the Serbian Chemical Society
JSCS@tmf.bg.ac.rs • www.shd.org.rs/JSCS
J. Serb. Chem. Soc. *89* (3) 321–334 (2024)
JSCS–5723
Original scientific paper
Published 24 March 2024

PCR-based detection of alkane monooxygenase genes in the hydrocarbon and crude oil-degrading *Acinetobacter* strains from petroleum-contaminated soils

AYŞE EREN, FATMA MATPAN BEKLER and KEMAL GÜVEN*

Department of Molecular Biology and Genetics, Faculty of Science, Dicle University, 21280 Diyarbakır, Turkey

(Received 7 July, revised 8 August, accepted 18 August 2023)

Abstract: Bacterial strains D11, E1 and E2 isolated from petroleum-contaminated soils were found to be members of *Acinetobacter* genus revealed by 16S rRNA gene sequence analysis and phenotypic characteristics. After incubation for 5 days, about 43, 9 and 12 % of total petroleum hydrocarbons of crude oil were degraded by strains D11, E1 and E2, respectively, and determined by GC–MS analysis. Moreover, about 70 and 76 % of single hydrocarbon hexadecane was degraded by the strains D11 and E1 after 3 days of short incubation time, respectively, while the strain E2 degraded about 48 % of single hydrocarbon pentadecane. By using PCR-based method, gene sequences of the strains D11 and E2 showed similarity to alkane 1-monooxygenases from *Acinetobacter* sp. BUU8 *alkM* with 93.06 and 92.72 %, respectively, while the sequence similarity of strain E1 was 95.84 % to *Acinetobacter* sp. 826659. The present study of hydrocarbon biodegradation by *Acinetobacter* strains may provide a good advantage in bioremediation process.

Keywords: petroleum-degradation; bacteria; GC–MS analysis; *alkM* gene.

INTRODUCTION

There is a continuous great concern about petroleum hydrocarbons causing pollution in the environment, produced from various anthropogenic activities, such as accidental petroleum spills during transport and refining of oil products, industrial discharge and leakage from underground tanks.[1,2] Toxic oil products are known to pose a serious threat to marine habitats, fishery and human health, leading to an imbalance in the ecosystem and thus harming the wildlife which takes years or even decades to recover.[3,4] Aliphatic *n*-alkanes, formed by the reduction of organic material during the geochemical formation of petroleum, are hydrocarbons found in crude and refined oils.[5]

* Corresponding author. E-mail: kemalg@dicle.edu.tr
https://doi.org/10.2298/JSC230707053E

A greater amount of aliphatic alkanes is released into the environment and thus the bioavailability and toxicity of *n*-alkanes vary according to the chain length.[6] Since crude oil and its products are biodegradable, bioremediation for cleaning up oil-contaminated sites is considered as an efficient and cost-effective technique compared to chemical and physical treatments. Recently, bioremediation of oil-contaminated sites has been an important task for scientists, carrying out many studies using pure culture or mixed bacterial consortia which degrade petroleum hydrocarbons.[4,7,8] In the environmental biotechnology using biodegradation and bioremediation processes, various kinds of microorganisms including bacteria and fungi have been employed.[9] The bacteria using hydrocarbons as a carbon source and energy for their growth have been studied in detail, such as *Alcaligenes*, *Bacillus*, *Corynebacterium*, *Pseudomonas*, *Methylomonas*, *Micrococcus*, *Methylobacterium*, *Nocardia*, *Rhodococcus*, *Achromobacter*, *Acinetobacter*, *Arthrobacter*, *Flavobacterium*, *etc*. Members of these groups have been evaluated for usage in bioremediation processes.[10–13] Among these, biodegradation studies have been also focused on *Acinetobacter* species such as *Acinetobacter baylyi* ADP1,[14,15] *Acinetobacter* sp. MUB1,[16] *Acinetobacter oleivorans*,[17] *Acinetobacter* sp. LS-1,[18] *A. baumannii*[19] and *Acinetobacter johnsoni.*[20] As identified in *Acinetobacter*, alkane monooxygenases encoded by the *alkM* gene and composed of alkane hydroxylase (*alkM*), rubredoxin (*RubA*) and rubredoxin reductase (*RubB*) are key enzymes that catalyze the terminal oxidation of *n*-alkanes to alcohols.[14–16,21–23]

In this study, we aimed to study and characterise *Acinetobacter* strains isolated from petroleum-contaminated sites in Batman province of Turkey and to evaluate the hydrocarbon biodegradation potential of the isolated bacterial strains.

EXPERIMENTAL

Collection of samples

The samples were collected from petroleum-contaminated soils around the petroleum wells (Southern Raman1. and 237. petroleum stations). The crude oil used in the experiments was obtained from Batman petroleum refinery. The Basal medium (BM) contained per L: phosphate buffer (5.0 mL), magnesium sulphate (3.0 mL), calcium chloride (1.0 mL), ferric chloride (1.0 mL), as well as 1.0 mL mineral elements solution containing $ZnSO_4 \cdot H_2O$, $MnSO_4$ and $(NH_4)_6MoO_{24} \cdot 4H_2O$. Filtered crude oil (1 %) was transferred to the BM by using a 0.2 mm pore size filter as the energy source. The petroleum-contaminated soils (1 g) around the petroleum wells were transferred into BM in the presence of crude oil (1 %) and then incubated in a shaking water bath at 120 rpm at 30 °C for 5 days. After incubation, aliquots (1 mL) from each sample were transferred to freshly prepared BM plus crude oil (1 %) for preparing subculture. Following two subculture cycles, the microorganisms were let to grow on solid medium (nutrient broth agar) for 5 days of incubation. The morphological appearance of the colonies growing on the plates was examined and different colonies were selected and retransferred to BM in the presence or absence of crude oil for 5 days. The isolated strains

were characterized as colonies with different morphologies for petroleum hydrocarbon degradation and utilising hydrocarbons for the carbon and energy source.

Morphological, biochemical and physiological characteristics

Gram staining was carried out by the method of Dussault. [24] The temperature range of 10–45 °C was used for growth in liquid medium, whereas the pH range tested was 4.0–12.0. The initial pH of media was adjusted with acidic and basic solutions. Urease activity testing was carried out as described by Lányi.[25] Procedures described within Bergey *et al*.[26] and Claus and Berkeley,[27] were followed to determine different biochemical properties such as indole, oxidase, citritase, catalase and urease activity as well as motility of the studied isolates.

Preparation of genomic DNA extraction and 16S rRNA analyses

Bacterial cells (D11, E1 and E2) were cultured overnight in NB medium under optimum conditions. After incubation bacterial culture was centrifuged at 14,000 rpm for 10 min and bacterial pellet was resuspended in Tris-EDTA (TE) buffer. Genomic DNA was isolated from tested bacteria using protocol supplied by the manufacturer of the Bacterial Genomic DNA Extraction Kit. DNA concentrations were determined using a UV/Vis spectrophotometer (S60 Double Beam, Libra Biochrom). All DNA extracts were stored at –20 °C. 16S rRNA encoding genes were amplified by PCR from isolated chromosomal DNA (1 μL) using universal primers (27F and 1492R, Sentebiolab). PCR amplification was performed in PCR reaction mixture (25 μL) containing; primers (20 pmol for F and R), 0.3 mM dNTP's mixture and Taq DNA polymerase (2.5 U, Sigma) in the supplied buffer (10X, Sigma). The PCR temperature program used was: initial denaturation at 95 °C 5 min, 40 cycles of denaturing at 94 °C for 1 min, annealing at 55 °C for 1 min, and extension at 72 °C for 2 min and the final extension at 72 °C for 7 min, using thermal cycler (T100, BIO-RAD). Isolated chromosomal DNA samples and PCR products were subjected to analysis with agarose gel electrophoresis using 1 % of agarose gels containing red safe gel stain and visualized using a transilluminator (Wuv-M20, Daihan Scientific). PCR products were extracted from the gel using QIAquick gel extraction kit (QIAGEN).

Phylogenetic analysis

Sequence analysis of 16S rRNA gene sequences of hydrocarbon-degrading isolates was performed by BM Laboratory system (Ankara, Türkiye). Sanger Sequencing was performed in the Macrogen Netherlands laboratory using the ABI 3730XL Sanger sequencing instrument (Applied Biosystems, Foster City, CA) and the BigDye Terminator v3.1 cycle sequencing kit. Reads obtained with primers 27F and 1492R were contiguous using the CAP contig assembly algorithm in BioEdit software to generate a consensus sequence. The sequences of the 16S rRNA gene for all strains were determined (D11: 1407 bp, E1: 1383 bp; E2: 1393 bp). The Blast search tool on National Centre of Biotechnology (NCBI, http://www.ncbi.nlm.nih.gov) was used to compare and search for homology of these sequences in the GenBank database. The 16S rRNA gene similarities were retrieved from the database, determining most closely related strains. The Kimura-2 parameter algorithm Kimura[28] was used to calculate distance matrices. The phylogenetic tree was built using the neighbor-joining method according to Saitou and Nei.[29] The bootstrap consensus tree was set at 100 replicates to represent the evolutionary history of taxa. Evolutionary analyses were performed in MEGA11.[30] All strains were deposited in GenBank database.

PCR amplification of alkM gene

CLUSTALW, available from NCBI, was used to align all complete sequences of the *alkM*.[31] Consensus regions were used to design degenerate primers by identifying conserved regions in alkM nucleotide sequences. The purified DNA was screened by PCR to detect alkane degradation genes (*alkM*). Therefore, the*t alkM* gene was amplified using the primers *alkM*-F (5'-CCTGTCTCATTTGGCGCTCGTTCCTACAGG-3') and *alkM*-R (5'--GTGATGATCTGAATGTCGTTGTAACTGG-3'). PCR reactions mix (25 µL) were prepared containing 10X PCR Buffer with MgCl2 (Sigma), 0.5 µM primer *alkM*-F and *alkM*-R, dNTP's (0.2 mM of each, Sigma), Taq polymerase (0.05 U mL^{-1}, Sigma) and H_2O for PCR (Sigma). The amplification program consisted of a denaturation at 94 °C for 4 min, denaturing at 94 °C for 30 s (35 cycles), annealing at 55–65 °C for 1 min (using gradient), followed by extension at 72 °C for 7 min using a thermal cycler. PCR products were confirmed by running on 1.0 % agarose gel containing red safe staining and visualized using a transilluminator. For sequencing, PCR products were purified using gel extraction kit.

Detection of alkane monooxygenase gene alkM

PCR products of *alkM* gene from the strains were sequenced on both strands by the commercial services of Sentebio lab (Ankara, Türkiye). Analysis of PCR products was done by an automated genetic analyser and sequences were aligned and compared with other *alkM* sequences of *Acinetobacter* available in the GenBank database using the Clustal X program and all sequences were compared using a Blast search tool database[32] on NCBI. Phylogenetic trees of aligned sequences were constructed with MEGA11 software.[30] Nucleotide sequences were translated into amino acid sequences using the Transeq tool (on the website of the European Bioinformatics Institute) and compared directly with the Protein Database. Conserved areas within coding nucleotide sequences were analysed by CD-Search[33] while using the BLASTP tool on NCBI to check if sequenced *alkM* genes have conserved motifs. The *alkM* protein sequence was modelled by the SWISS-Model workspace (https://swissmodel.expasy.org). The Ramachandran plot was evaluated with Verify 3D and Procheck.[34]

Growth of bacterial strains in the presence of hydrocarbons and crude oil

A fresh culture of all strains grown in BM liquid medium for 24 h was obtained, followed by centrifugation and washing with BM. This process was repeated 3 times. Bacteria were then incubated in BM (25 mL) in the presence of crude oil (1 %) for 5 days and in single hydrocarbons (1 % hexane, heptane, octane, decane, pentadecane, hexadecane, toluene and squalene from Sigma) for 3 days under optimum growth conditions. Following incubation, the bacterial growth was determined by spectrophotometer at 600 nm. Each data point represents the mean of at least three experiments.

GC Analyses of degradation of crude oil and hydrocarbons by the strains

The determination of both crude oil and single n-alkanes degradation such as hexadecane and pentadecane by bacterial strains were performed by using gas chromatography–mass spectrometry (GC–MS). Bacterial cells precultured overnight in NB medium were transferred to a 100 mL Erlenmeyer flask containing 25 mL of BM in the presence of 1 % crude oil or *n*-alkanes and incubated under optimum conditions at 30 °C. Following incubation, the bacterial cells were removed, and petroleum hydrocarbons remaining in the BM were used for calculating the degradation ratio of single hydrocarbons and those within crude oil. Petroleum hydrocarbon fractions remaining in the culture medium after incubation time were analyzed by GC–MS with a flame ionization detector (FID, HP 6850, Hewlett Packard). Authentic stan-

dards were used to determine individual hydrocarbon fraction components by matching the retention times.

RESULTS AND DISCUSSION

Morphological, physiological and biochemical characteristics

Two bacterial strains designated as strains D11 and E2 were isolated from the petroleum station Southern Raman 237, while the strain E1 was isolated from Southern Raman1 in Batman province of Turkey, and all strains were found to degrade and use hydrocarbons within crude oil to grow (Fig. 1).

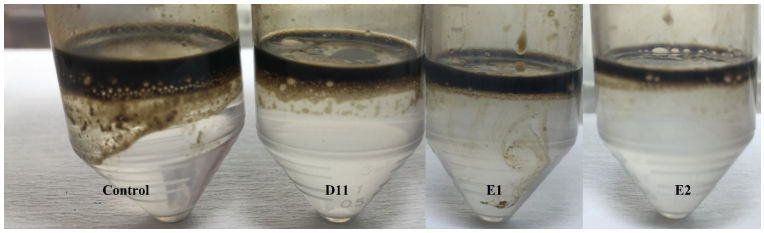

Fig. 1. Demonstration of growth and biodegradation by the strains D11, E1 and E2, compared to abiotic control at 1 % crude oil as carbon source.

The strain D11, E1 was Gram-negative, motile, coccobacilli, while E2 strain was Gram-negative, non-motile and coccobacilli (see Table S-I of the Supplementary material to this paper.). The comparison of phenotypic properties of the strain D11, E1 and E2 with some *Acinetobacter* strains in Table S-I reveal the similarities of both strains to each other and to other *Acinetobacter* species, phenotypically and biochemically, based on the results obtained. As Henrichsen[35] stated, the name "*Acinetobacter*" means "motile rod" and it has been noted in previous studies that a nonmotile phenotype is a common feature in this genus, but it was also noted that the motility of a few *A. calcoaceticus* strains was very conditional and observed. As D11 and E1 was found to be motile, there are several studies on *A. baylyi* and *A. gerneri*,[36] *Acinetobacter* sp. BT1A,[37] describing motile phenotypes of *Acinetobacter* members. As shown in Table I, all strains were gram-negative and aerobic. Moreover, the characteristics of oil--degrading strains D11, E1 and E2 were interestingly very similar of which the catalase, citrate and starch test results were clearly positive, whereas, oxidase, urea and indole test results were negative. The difference was that gelatine hydrolysis test was positive for E1, but negative for D11 and E2. The biochemical test results such as starch hydrolysis, catalase and citritase activities of D11, E1 and E2 strains were similarly positive as in most *Acinetobacter* species: positive starch hydrolysis also in *A. junii* strain VA2[38] and *Acinetobacter* sp. strain BT1A,[37] positive catalase and citritase activities also in *A. soli*, sp. Nov,[39] *Acinetobacter* strain USTB-X.[40] The growth values of strain D11 and E1 for the

temperature, pH and NaCI tolerance were found to be very close and observed to be 20–40 °C (optimum 35 °C), pH 5.0 and 10.0 (optimum pH 8.0–10.0 for D11, optimum pH 9.0–10.0 for E1) and tolerated up to 3 % NaCl, respectively. Moreover, the temperature and pH values for the strain E2 were also found to be 20––40 °C (optimum 35 °C), but pH growth range was 4.0–10.0 (a wide optimum range of 4.0–10.0), and tolerated up to 5 % NaCl. Compared with previous studies on other *Acinetobacter* species, the optimum pH values obtained were 8.0 for *A. soli*, sp. Nov,[39] and 8.5 for *Acinetobacter* sp. strain S2.[41] Similar temperature values as in the present study were reported as 20–40 °C for *Acinetobacter* strain USTB-X[40] and *A. junii* strain VA2.[38]

TABLE I. *alkM* Gene sequences and conserved domains within coding nucleotide sequences

Isolate	Related bacteria strains gene	Base pairs long	Nucleotides similarity, %	Description	Interval	*E*-value
D11	*Acinetobacter* sp. BUU8 (UniprotKB (A6N7F9)	651	93.06	The membrane fatty acid desaturase (Membrane_FADS)-like CD includes membrane FADSs, alkane	66–281 744–938	1.21×10^{-16} 2.15×10^{-19}
E1	*Acinetobacter* sp. 826659 (A0A013TK13)	471	95.84	The membrane fatty acid desaturase (Membrane_FADS)-like CD includes membrane FADSs, alkane	33–293 799–1542	2.00×10^{-20} 1.87×10^{-57}
E2	*Acinetobacter* sp. BUU8 (UniprotKB (A6N7F9)	448	92.72	The membrane fatty acid desaturase (Membrane_FADS)-like CD includes membrane FADSs, alkane	36–170	9.51×10^{-8}

Phylogenetic analysis

The amount of Genomic DNA isolated from D11, E1 and E2 strains was 25––71 μg/mL and each determined to be >10 kb in size on 1 % agarose gel. Reads obtained with primers 27F and 1492R were contiguous to form a consensus sequence. The contig of forward and reverse sequences, contig assembly algorithm (CAP) was used in BioEdit software to perform this process. The analysis of 16 S rRNA gene sequence revealed that the most similarity of the strain D11 (1407 bp) was to *A. pittii* (99.93 %) and E1 (1383 bp) to *A. pittii* (100 %), while the strain E2 (1393 bp) was similar to *A. calcoaceticus* (100 %), Fig. 2.

GenBank accession numbers of the sequences of the strains D11, E1, E2 are MT374264, MT374266 and MT374267, respectively. The tree was generated

using Blast program for sequence alignment and by the neighbor joining method using MEGA11 software. *Exiguobacterium* sp. (KM077136) was used as the outgroup.

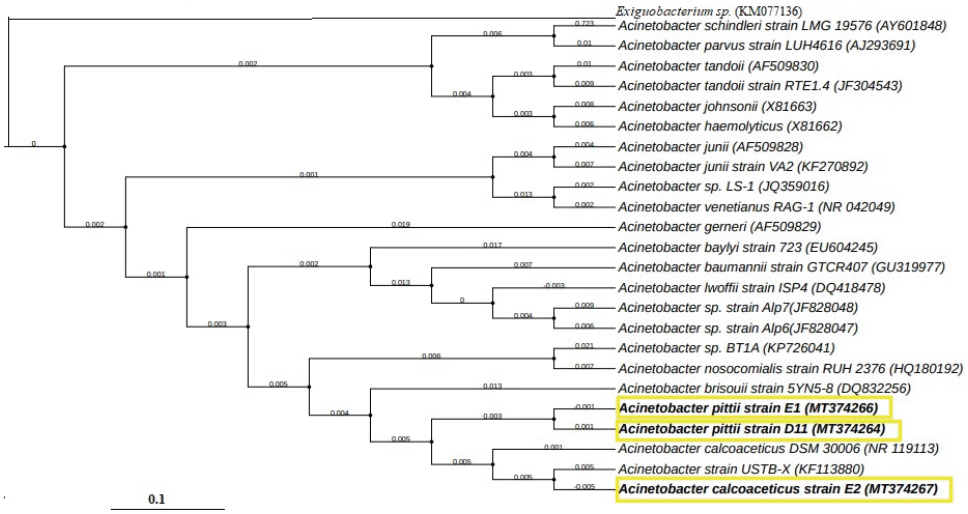

Fig. 2. The 16S gene sequence analysis of the strains D11, E1 and E2.

Detection of alkane monooxygenase gene alkM

The nucleotide and the predicted amino acid sequences for the *alkM* genes were compared using several databases as described in the previous section. The results of base pairs long and nucleotides similarity with related bacterial strains are presented in Table I.

Analysis of the derived amino acid sequences of alkane 1-monooxygenase from tested strains showed a high degree of similarity to that of other *Acinetobacter* strains. Conserved domains within coding nucleotide sequences were analysed with CD-Search using tool on NCBI. All sequences showed similarity the membrane fatty acid desaturase (accession no. cl00615) and the regions of the conserved domains of the tested strains are also given in the Table I. It was previously reported that members of *Acinetobacter* species possess many metabolic capabilities including degradation pathways.[42] For example, the biodegradation studies carried out on *Acinetobacter* species include: *A. baylyi* ADP1,[14,15] *Acinetobacter* sp. MUB1[16] and *A. oleivorans.*[17] *Acinetobacter* species isolated from petroleum contaminated sites have been reported to possess *n*-alkane degrading genes including alkane monooxgenase, alkane hydoxylase and dioxygenase genes which are the key enzymes of metabolic process in the remediation oil pollution,[18,19,22,23,43] Taking into account the amino acid sequences of the *alkM* protein from strains D11, E1 and E2, three-dimensional (3D) model was analysed

with SWISS-Model workspace through a comparative analysis of the Ramachandran Prochek software for each *alkM* protein model generated. Alignment of region linked to substrate specificity for the *alkM* from tested bacteria (D11, E1 and E2) and other related species (*A. baylyi* (O31250), *Acinetobacter* sp. SJ-2 (K4HWF9), *Acinetobacter* sp. BUU8 (A6N7F9), *Acinetobacter* sp. (Q9XBM0), *Acinetobacter* sp. 826659 (A0A013TK13), *A. calcoaceticus* (F0KMZ3), *Alkanindiges hydrocarboniclasticus* (A0A1S8CT34) was performed by using the UniprotKB server. Phylogenetic analysis of strains and related species was performed by the Neighbor joining method based on *alkM* gene sequences.

In Fig. S-1a–e of the Supplementary material, strain D11 (651 bp) sequences showed similarity to alkane 1-monooxygenase. Strain D11 sequences showed similarity to alkane 1-monooxygenase from *Acinetobacter sp.* BUU8 (UniprotKB (A6N7F9) with 93.06 %. The three-dimension (3D) model of alkane monooxygenase (*alkM*) of the *A. baylyi* (UniprotKB O31250) was used as reference.

The data shown by the Ramachandran graph (94.55 %) reveal the identity/similarity of primary, secondary and tertiary structures between the strain D11 and *A. baylyi* (UniprotKB O31250). Alkane monooxygenase gene sequences of strain E1 (471 bp) showed similarity to alkane 1-monooxygenase of *Acinetobacter* sp. 826659 (A0A013TK13) with 95.84 % and Ramachandran graph was found to be 83.33 % (Fig. S-2a–e of the Supplementary material).

In Fig. S-3a–e of the Supplementary material, strain E2 gene sequences (448 bp) showed similarity to alkane 1-monooxygenase from *Acinetobacter sp.* BUU8 (UniprotKB A6N7F9) with 92.72 % while the Ramachandran graph was 100 %. As van Beilen *et al.*[44] stated, many alkane hydroxylase homologues have been characterized in addition to *alkM* genes in studies conducted in *A. calcoaceticus* strains EB104, 69-VA and NCIMB 8250 and also *Acinetobacter* sp. 2769A related to *alkM* genes. Phylogenetic analysis of *alkM* amino acid sequences, in these studies, showed high sequence variation between them and clearly differentiated *alkM* genes from *alkB* genes. Therefore, most of these genes not found in *Acinetobacter* genus have been named *alkB* gene.

Growth of bacterial strains in the presence of crude oil and various hydrocarbons

The strains were tested by incubation in the presence of various short and long chain hydrocarbons and crude oil, namely hexane, heptane, octane, decane, pentadecane, hexadecane and squalene, as well as toluene cultivated in BM medium at 1 % concentrations under optimum conditions. As it can be seen in Fig. 3, the strains D11, E1 and E2 clearly use certain hydrocarbons as carbon and energy sources for growth on both 1 % crude oil and single hydrocarbons tested.

Fig. 3 also shows that the strains D11 and E1, significantly degrade hexadecane, among the aliphatic hydrocarbons tested. The strains also grow at low

rates on decane and pentadecane, but not in short chain hydrocarbons such as hexane, heptane or octane. However, the growth for strain E2 was remarkable in the presence of long chain hydrocarbon pentadecane, while the growth was relatively higher in hexadecane and squalene, compared to control. Sun *et al.*[45] reported that the aerobic degradation of *n*-alkanes by bacteria needs alkane monooxygenase encoded by *alkM* gene which catalyzes the terminal oxidation of *n*-alkanes to alcohols, suggesting that the transcription of *alkM* in bacterial strains directly influences the degradation of *n*-hexadecane.

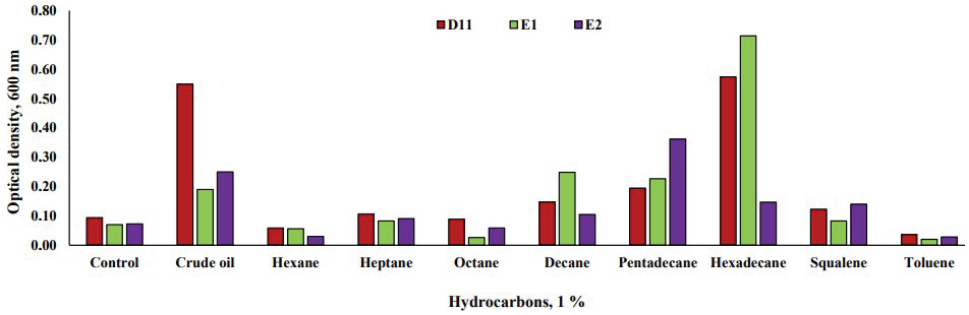

Fig. 3. Growth of strains D11 (red), E1 (green) and E2 (purple) in BM supplemented with crude oil (incubated for 5 days) and various hydrocarbons (incubated for 3 days) at 1% concentrations as carbon sources. Each data represents the mean of at least 3 different experiments

Degradation of crude oil and hydrocarbons by the strains determined by GC–MS analyses

Members of *Acinetobacter* sp. are capable of utilizing a broad range of *n*-alkanes with varying chain lengths (C10–C40) as a sole carbon source.[46] Furthermore, *Acinetobacter* species are dominant bacteria in most petroleum contaminated soils,[47,48] as well as in various environments including sea waters and sediments,[20,49] in waste waters[50] and in streams.[51]

Different degradation rates (%) of crude oil and alkane hydrocarbons by various *Acinetobacter* species are summarised in Table II, as well as the degradation results obtained in the present study. The remaining amount of hydrocarbons in the culture medium, analysed by GC–MS, were used for calculating degradation rates of crude oil and alkane hydrocarbons (1 %). The comparisons with the undegraded control showed that the strains D11, E1 and E2 degraded 43, 9 and 12 % of total petroleum hydrocarbons, respectively, within crude oil after short incubation time of only 5 days. The growth of the strains D11, E1 and E2 in the basal media with hexadecane or pentadecane was remarkable as shown in Fig. 3, and the gas chromatographic analysis of two single hydrocarbons were also carried out for each strain Fig. S-4a–e of the Supplementary material. Fig. S--4b shows that as much as 70 % of single hydrocarbon hexadecane (1 %) was

degraded by the strain D11, compared to abiotic control (Fig. S-4a), while 76 % of hexadecane was degraded by the strain E1 (Fig. S-4c). Moreover, as can be seen in Fig. S-4e, the strain E2 degraded about 48 % of single pentadecane (1 %) after 3 days of short incubation time, compared to abiotic control (Fig. S-4d).

TABLE II. The degradation of petroleum hydrocarbons by members of *Acinetobacter*

Isolate	Isolation of bacteria from	Substrate tested	Degradation rate %	Incubation time, days	Literature
Acinetobacter sp. strain Y9	Sea mud	C9–C22 *n*-alkanes from diesel oil (4 %)	53.28	7	Min *et al*.[23]
Acinetobacter sp. strain S2	Wastewaters	Diesel oil	50.62	7	Sawadogo *et al*.[41]
Acinetobacter sp. Strain BT1A	Crude-oil contaminated soil	Crude oil (1 %)	83	7	Acer *et al*.[37]
Acinetobacter baumannii MKS2	Crude-oil contaminated soil	Crude oil (1 %)	43	13	Muthukamalam et.al.[19]
Acinetobacter sp.	Crude-oil contaminated soil	Crude oil	75 (of C20, C21 and C22) 50 (of shorter chains)	7	Zheng *et al*.[52]
A. pittii H9-3	Crude-oil contaminated soil	Crude oil (1 %)	36.8	21	Wang *et al*.[43]
A. pittii strain D11	Crude-oil contaminated soil	Crude oil (1 %) Hexzadecane (1 %)	43 (c. oil) 70 (hexadecane)	5 3	Present study
A. pittii strain E1	Crude-oil contaminated soil	Crude oil (1 %) Hexzadecane (1 %)	9 (c. oil) 76 (hexadecane)	5 3	Present study
A. calcoaceticus strain E2	Crude-oil contaminated soil	Crude oil (1%) Pentadecane (1 %)	12 (c. oil) 48 (pentadecane)	5 3	Present study

There have been many studies reported in the literature related to biodegradation rates of hydrocarbons by various bacterial species. For example, Tapilatu *et al*.[53] reported that *Alcanivorax venustensis* strains and some other Gram-positive bacteria preferably degraded *n*-hexadecane (40–63 %), but other genera tested seemed to prefer *n*-heptadecane (8–44 %), after long time incubation of 30 days. Many other bacterial species isolated from contaminated sites were found to degrade oil hydrocarbons at varying degradation rates as: *Pseudomonas* sp. degraded 67.57 % of the oil (1 %) and *Micrococcus* sp. with 52.95 % after 25 days of incubation period.[54] *Pseudomonas* sp. had shown 49.93 % of diesel oil degradation in 20 days against 0.5 % of diesel.[55] Six isolated bacterial strains identified

as *P. alcaligenes* (HDB-6), *Bacillus thuringiensis*, *Pseudomonas alcaligenes* (HDB-4), *Pseudomonas mendocina*, *Bacillus flexus* and *Lysini bacillus* sp. were found to degrade between 37.88 and 54.01 % of crude oil after 14 days.[56] There have also been several studies on hydrocarbon biodegradation rates using *Acinetobacter* species. Throne-Holst *et al*.[46] determined that *Acinetobacter* strain can use carbon sources in the range of decane and tetracontane long-chain *n*-alkanes. Min *et al*.[23] reported that *Acinetobacter* strain Y9 degrades *n*-alkanes in the range of C9–C22 as sole carbon sources degrading 53.28 % in 7 days of incubation. *Acinetobacter* sp. BT1A was also found to degrade 83 % of 1 % crude oil in 7 days.[37] In more recent studies, various *A. baumannii* strains were found to degrade an average of 43 and 61% of oil alkanes, respectively.[19,57]

CONCLUSION

A wide variety of microorganisms including bacteria that can degrade hydrocarbons within petroleum were isolated from oil-contaminated soils. Bacteria are the most dominant microorganisms determined in microbial ecology degrading petroleum hydrocarbons. Among bacteria, *Acinetobacter* is recently found to be most common genera that is able to degrade hydrocarbons. The members of *Acinetobacter* species studied were found to biodegrade crude oil and sole hydrocarbons. Gene sequences of the strains D1, E1 and E2 showed similarity to alkane 1-monooxygenases from *Acinetobacter* sp. *alkM*. The present study seems to be effective in degrading *n*-alkanes in shorter incubation time and further microbial consortium studies, with the related strains, may provide a good advantage in bioremediation of oil-contaminated soil.

Acknowledgement. This study, a part of Ayse EREN's PhD project, was supported by Dicle University Scientific Research Projects Coordination Unit (Project no.: FEN.19.014).

SUPPLEMENTARY MATERIAL

Additional data and information are available electronically at the pages of journal website: https://www.shd-pub.org.rs/index.php/JSCS/article/view/12469, or from the corresponding author on request.

ИЗВОД

ДЕТЕКЦИЈА ГЕНА ЗА АЛКАН-МОНООКСИГЕНАЗУ У *ACINETOBACTER* СОЈУ КОЈИ РАЗЛАЖЕ УГЉОВОДОНИКЕ И СИРОВУ НАФТУ ПРИМЕНОМ PCR МЕТОДЕ НА ЗЕМЉИШТА КОНТАМИНИРАНА НАФТОМ

AYŞE EREN, FATMA MATPAN BEKLER и KEMAL GÜVEN

Department of Molecular Biology and Genetics, Faculty of Science, Dicle University, 21280 Diyarbakır, Turkey

Бактеријски сојеви D11, E1 и E2 изоловани из земљишта контаминираних нафтом су припадници *Acinetobacter* рода, што је утврђено секвенцирањем гена за 16S rRNA и фенотипском карактеризацијом. Након инкубације 5 дана, GC–MS анализом је утврђено да је око 43, 9, односно 12 % укупних угљоводоника из сирове нафте разложено соје-

вима D11, E1, односно E2, редом. Такође, око 70 и 76 % хексадекана је разложено сојевима D11 и E1 након 3 дана инкубације, док је сој E2 разложио око 48 % пентадекана. PCR методом генског секвенцирања утврђена је 93,06 %, односно 92,72 %, сличност гена за алкан-1-монооксигеназу сојева D11 и E2 са геном из *Acinetobacter* sp. BUU8 *alkM*, док је сличност секвенце соја E1 95,84 % са геном *Acinetobacter* sp.826659. Резултати ове студије биодеградације угљоводоника сојевима *Acinetobacter* могу наћи примену у поступку биоремедијације.

(Примљено 7. јула, ревидирано 8. августа, прихваћено 18. августа 2023)

REFERENCES

1. R. Bajagain, Y. Park, S. W Jeong, *Sci. Total Environ*. (2018) 1236 (https://doi.org/10.1016/j.scitotenv.2018.01.212)
2. S. J. Varjani, *Bioresour. Technol*. **223** (2017) 277 (http://dx.doi.org/10.1016/j.biortech.2016.10.037)
3. Z .Zhang, Z. Hou, C. Yang, C. Ma, F. Tao & P. Xu, *Bioresour. Technol*. **102** (2011) 4111 (https://dx.doi.org/10.1016/j.biortech.2010.12.064)
4. M. B. Yakubu, *Afr. J. Biotechnol*. **6** (2007) 2735 (https://doi.org/10.5897/AJB2007.000-2437)
5. K. Kloos, J. C. Munch, M. Schloter, *J.Microbiol. Methods* **66** (2006)486 (https://doi.org/10.1016/j.mimet.2006.01.014)
6. C. O. Gill,C. Ratledge, *J. Gen. Microbiol*. **72** (1972) 165 (https://doi.org/10.1099/00221287-72-1-165)
7. D. M. Al-Mailem, N. A. Sorkhoh, H. Al-Awadhi, M. Eliyas, S. S. Radwan, *Extremophiles* **14** (2010) 321 (https://doi.org/10.1007/s00792-010-0312-9)
8. F. Chaillan, A. Le Flèche, E. Bury,Y. Phantavong, P. Grimont, A. Saliot, J. Oudot, *Res. Microbiol*. **155** (2004) 587 (https://doi.org/10.1016/j.resmic.2004.04.006)
9. S. J. Varjani, D. P. Rana, A. K. Jain, S. Bateja, V. N. Upasani, *Int. Biodeterior. Biodegrad*. **103** (2015)116 (http://dx.doi.org/10.1016/j.ibiod.2015.03.030)
10. R. K. Jain, M. Kapur, S. Labana, B. Lal, P. Sarma, D. Mhattacharya, I. S.Thakur, *Curr. Sci*. **89** (2005) 101 (https://www.jstor.org/stable/24110436)
11. B. Thapa, A Kumar KC, A. Ghimire, *Kathmandu Univ. J. Sci. Eng. Technol*. **8** (2012) 164 (https://doi.org/10.3126/kuset.v8i1.6056)
12. W. Xia, H. Dong, C. Zheng, Q. Cui, P. He, Y. Tang, *RSC Adv.* **5** (2015) 102367 (https://doi.org/10.1039/C5RA17137G)
13. K. Sadighbayan, M. M. Assadi, A. Farazmand, A. R. Monadi, N. Aliasgharzad, H. Mobaiyen, Ramin Zadghaffari, *Adv. Biores*. **7** (2016) 57 (https://soeagra.com/abr/march_2016/11f.pdf)
14. A. Ratajczak, W. Geissdoerfer, W. Hillen, *Appl. Environ. Microbiol*. **64** (1998) 1175 (https://doi.org/10.1128/AEM.64.4.1175-1179.1998)
15. A. Ratajczak, W. Geissdoerfer, W. Hillen, *J. Bacteriol.* **180** (1998) 5822 (https://doi.org/10.1128/JB.180.22.5822-5827.1998)
16. S. Phrommanich, S. Suanjit, S. Upatham, S. V. Grams, M. Kruatrachue, P. Pokethitiyook, G. Korge, A. Hofmann, *Microbiol. Res*. **164** (2009) 486 (https://doi.org/10.1016/j.micres.2007.03.002)
17. J. Jung, E. L. Madsen, C. O. Jeon, W Park, *Appl. Environ. Microbiol*. **77** (2011) 7418 (https://doi.org/10.1128/AEM.05231-11)

18. H. Liu, J. Yao, Z. Yuan, Z. M. Yuan, Y. F. Shang, H. L. Chen, F. Wang, K. Masakorala, C. Yu, M. Cai, R. E. Blake, M. M. F. Choi, *Int. Biodeterior. Biodegrad.* **87** (2014) 52 (https://doi.org/10.1016/j.ibiod.2013.11.005)
19. S. Muthukamalam, S. Sivagangavathi, D. Dhrishya, S. S. Rani, *Braz. J. Microbiol.* **48** (2017) 637 (https://doi.org/10.1016/j.bjm.2017.02.007)
20. F. Bendadeche, M. B. B. Hamed, S. Ayad, *J. Environ. Eng. Sci.* (2019) 131 (https://doi.org/10.17265/2162-5298/2019.04.001)
21. W. Geissdoerfer, R. G. Kok, A. Ratajczak, K. J. Hellingwerf, W. Hillen, *J. Bacteriol.* **181** (1999) 4292 (https://doi.org/10.1128/jb.181.14.4292-4298.1999)
22. Y. Liu, A. Ding, Y. Sun, X. Xia, D. Zhang, *Front. Environ. Sci. Eng.* **12** (2018) 3 (https://doi.org/10.1007/s11783-018-1064-5)
23. S. Min, L. Qun, S. Xian-rong, H. Deng-yong, H. Ying, S. Zhan, W. Qing-Rong, *Afr. J. Microbiol. Res.* **6** (2012) 3936 (https://doi.org/10.5897/AJMR12.278)
24. H. P. Dussault, *J. Bacteriol.* **70** (1955) 484 (https://doi.org/10.1128/jb.70.4.484-485.1955)
25. B. Lányi, *Methods Microbiol.* **19** (1988) 1 (https://doi.org/10.1016/S0580-9517(08)70407-0)
26. D. H. Bergey, N. R. Krieg, J. G. Holt, *Bergey's manual of systematic bacteriology*, Williams and Wilkins Co, Baltimore, 1989
27. D. Claus, and C. W. Berkeley, in: *Bergey's manual of systematic bacteriology*, 1986, p. 1105
28. M. Kimura, *J. Mol. Evol.* **16** (1980) 111 (https://doi.org/10.1007/BF01731581)
29. N. Saitou, M. Nei, *Mol. Biol. Evol.* **4** (1987) 406 (https://doi.org/10.1093/oxfordjournals.molbev.a040454)
30. K. Tamura, G. Stecher, S. Kumar, *Mol. Biol. Evol.* **38** (2021) 3022 (https://doi.org/10.1093/molbev/msab120)
31. J. D. Thompson, D. G. Higgins,T. J. Gibson, *Nucleic Acids Res.* **22** (1994) 4673 (https://doi.org/10.1093/nar/22.22.4673)
32. S. F. Altschul, T. L. Madden, A. A. Schäffer, J. Zhang, Z. Zhang, W. Miller, D. J. Lipman, *Nucleic Acids Res.* **25** (1997) 3389 (https://doi.org/10.1093/nar/25.17.3389)
33. A. Marchler-Bauer, M. K. Derbyshire, N. R. Gonzales, S. Lu, F. Chitsaz, L. Y. Geer, S. H. Bryant, *Nucleic Acids Res.* **43** (2015) 222 (https://doi.org/10.1093/nar/gku1221)
34. R. A. Laskowski, M. W. MacArthur, D. S. Moss, J. M. Thornton, *Appl. Crystallogr.* **26** (1993) 283 (https://doi.org/10.1107/S0021889892009944)
35. J. Henrichsen, *Acta Pathol. Microbiol. Scand., B* **83** (1975) 179 (https://doi.org/10.1111/j.1699-0463.1975.tb00090.x)
36. Y. S. Kang, W. Park, *Environ. Microbiol.* **12** (2010) 1304 (https://doi.org/10.1111/j.1462-2920.2010.02175.x)
37. Ö. Acer, K. Güven, F. Matpan Bekler, R. Gül-Güven, *Bioremediat. J.* **20** (2016) 1 (https://doi.org/10.1080/10889868.2015.1096898)
38. Q. Zhang, D. Wang, M. Li, W. N. Xiang,V. Achal, *Front. Earth Sci.* **8** (2014) 58 (https://doi.org/10.1007/s11707-013-0415-6)
39. D. Kim, K. S. Baik, M. S. Kim, S. C. Park, S. S. Kim, M. S. Rhee, Y. S. Kwak, C. N. Seong, *J. Microbiol.* **46** (2008) 46 (https://doi.org/10.1007/s12275-008-0118-y)
40. H. Yuan, J. Yao, K. Masakorala, F. Wang, M. Cai, C. Yu, *Environ. Sci. Pollut.* **21** (2014) 2724 (https://doi.org/10.1007/s11356-013-2221-9)
41. A. Sawadogo, O. C. Harmonie, J. B. Sawadogo, A. Kaboré, A. S. Traoré, D. Dianou, *J. Environ. Prot.* **5** (2014)1183 (https://doi.org/10.4236/jep.2014.512115)

42. S. Yoshida, K. Tazaki, T. Minamikawa, *Phytochemistry* **14** (1975) 195 (https://doi.org/10.1016/0031-9422(75)85036-9)
43. Y. Wang, Q. Wang, L. Liu, *Int. J. Environ. Res. Public Health*. **16** (2019) 188 (https://doi.org/10.3390/ijerph16020188)
44. J. B van Beilen, Z. Li, W. A. Duetz, T. H. M. Smits, B. Witholt, *Oil Gas Sci. Technol.* **58** (2003) 427 (https://doi.org/10.2516/ogst:2003026)
45. J.-Q. Sun, L. Xu, Y.-Q.Tang, F.-M. Chen, X.-L. Wu, *Bioresour. Technol*. **123** (2012) 664 (https://doi.org/10.1016/j.biortech.2012.06.072)
46. M. Throne-Holst, A. Wentzel, T. E. Ellingsen, H. K. Kotlar, S. B. Zotchev, *Appl. Environ. Microbiol.* **73** (2007) 3327 (https://doi.org/10.1128/AEM.00064-07)
47. P. Anbu, M. J. Nouh, D. H. Kim, J. S. Seo, B. K. Hur, K. H. Min, *Afr. J. Biotechnol*. **10** (2011) 4147 (https://academicjournals.org/journal/AJB/article-full-text-pdf/467B98830582)
48. A. M. Tanase, R. Ionescu, I. Chiciudean, T. Vassu, I.S toica, *Int. Biodeterior. Biodegradation* **84** (2012) 150 (https://doi.org/10.1016/j.ibiod.2012.05.022)
49. D. Cerqueda-Garcíaa, J. Q. García-Maldonadob, L. Aguirre-Macedoc, U. García-Cruz, *Mar. Pollut. Bull*. **150** (2020) 110775 (https://doi.org/10.1016/j.marpolbul.2019.110775)
50. H. Li, X. L. Wang, B. Z. Mub, J. D. Gu, Y. D. Liu, K. F. Lin, S. G. Lu, Q. Lu, B. Z. Li, Y. Y. Li, X. M. Du, *Int. Biodeterior. Biodegradation* **76** (2013) 49 (https://doi.org/10.1016/j.ibiod.2012.06.007)
51. S. A. Adebusoye, M. O. Ilori, O. O. Amund, O. D. Teniola, S. O. Olatope, *World J. Microbiol. Biotechnol*. **23** (2007) 1149 (https://doi.org/10.1007/s11274-007-9345-3)
52. J. Zheng, J.*Q. Feng, L. Zhou, S. M. Mbadinga, J.-D. Gu, B.-Z. Mu, *World J. Microbiol. Biotechnol*. **34** (2018) 1 (https://doi.org/10.1007/s11274-018-2417-8)
53. Y. Tapilatu, M. Acquaviva, C. Guigue, G. Miralles, J. C. Bertrand, P. Cuny, *Lett. Appl. Microbiol.* **50** (2009) 234 (https://doi.org/10.1111/j.1472-765X.2009.02766.x)
54. T. Nikhil, V. Deepa, G.Rohan, B. Satish, *Int. J. Environ. Res*. **2** (2013) 48 (http://www.isca.me/IJENS/Archive/v2/i2/8.ISCA-IRJEvS-2012-092.pdf)
55. S. K. Panda, R. N. Kar, C. R. Panda, *Int. J. Environ. Sci*. **3** (2013) 1314 (https://doi.org/10.6088/ijes.2013030500001)
56. X. Tian, X. Wang, S. Peng, Z. Wang, R. Zhou, H. Tian, *Water Sci. Technol*. **78** (2019) 2626 (https://doi.org/10.2166/wst.2019.025)
57. I. Jerin, M. Rahi, T. Sultan, M. S. Islam, S. A. Sajib, K. M.H oque, M. A. Reza, *Arch. Microbiol*. **203** (2021) 5075 (https://doi.org/10.1007/s00203-021-02469-2).