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Genomic and Metabolic Adaptations of Hydrocarbon-Degrading Bacteria in Iraq's Surface Waters

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Abstract: Oil exploration and extraction operations have contributed significantly to hydrocarbon contamination in surface water in Iraq. Molecules which acquire an adaptive properties through the genomic and metabolic changes of indigenous bacteria able to live in hydrocarbon polluted areas of the world are investigated. Water samples were gathered from twelve distinct polluted locations. GC-MS was used to determine the amount of hydrocarbons in the water. Chemical oxygen demand (COD) was measured to determine the level of pollution. *Citrobacter freundii* and *Bacillus cereus* were isolated and identified using 16S rRNA sequencing and VITEK 2. Bioinformatics analysis and whole-genome sequencing (WGS) were used to identify genetic mutations in hydrocarbon-degrading pathways. Metabolomic and proteomic analyses assessed functional adaptations. Phylogenetic analysis showed that these strains are genetically related to industrial and coastal bacteria from China and India. Statistical analyses of variance (ANOVA) and Pearson correlation were performed to verify that the results were robust. This study provides valuable insights into microbial adaptation in hydrocarbon-impacted habitats, and assesses the suitability of such strains in bioremediation technologies.

Keywords: Hydrocarbon pollution, genetic adaptations, *Bacillus cereus*, *Citrobacter freundii*, 16S rRNA,

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Introduction

Globally, hydrocarbon pollution of aquatic ecosystems is a major environmental and ecological threat. Surface water affected by oil extraction operations in Iraq is highly sensitive and exposed to hydrocarbon pollution. We find that its impact is not limited to ecosystems only, but extends to include its effects on human health. Therefore, the process by which some microbes develop genetic adaptations that enable them to survive and consume hydrocarbons is called decomposition (1)(2)(3). We find there are many studies indicating the presence of bacteria that decompose hydrocarbon. For example, there are bacterial strains that are characterized by high metabolic flexibility, such as: *Bacillus cereus* and *Citrobacter freundii* This enables it to use hydrocarbons to obtain carbon and energy (3). We also note that these microbial adaptations are necessary for bioremediation processes that help restore balance in polluted systems (4)(5). Oil drilling areas and industrial areas have witnessed an increase in surface water pollution. Various studies have reported that TPH and PAHs were found in high levels in

the large aquatic systems (6)(7). Not only is this a devastating blow to aquatic diversity, but this pollution also threatens clean drinking water and irrigation sources. During measurements of (COD) and (DO) of water samples from the Tigris and Euphrates rivers, it was found that there is an increase in the chemical oxygen demand (COD) and a decrease in the dissolved oxygen (DO) values, which indicates severe organic pollution. Recent surveys conducted by the Iraqi Ministry of Environment have confirmed the alarming biological seriousness of organic pollution in the two rivers (8)(9). These ecosystems have been long exposed to hydrocarbons that are urgent to address with effective bioremediation approaches. There are specific marine microbial communities in nature that have adapted to degrade hydrocarbons in sediments. AlkB (alkane monooxygenase), P450 (cytochrome P450 hydroxylase) and CAT (catalase-peroxidase) were validated as significant genes in hydrocarbon degradation research (10) (11). These genes contribute to bacteria metabolism of hydrocarbons species, which are toxic in water systems. In addition, in the polluted waters of Iraq, evolutionary analysis indicates that the degrading bacteria have an evolutionary link with strains isolated in industrially polluted areas. regions of China and coastal sites in India (11) (12). It indicates a possible global pattern in microbial lineages resistant to hydrocarbons, underscoring the importance of cross-regional research collaborations to promote bioremediation initiatives. The objective of this study is to explore the occurrence and diversity of hydrocarbon-degrading bacteria in Iraq's surface waters, to analyze the genetic adaptations that allow these bacteria to survive in polluted environments, to evaluate the metabolic pathways associated with hydrocarbon degradation through proteomic and metabolomic methods, and to explore the potential use of these strains of bacteria in bioremediation strategies.

Materials and Methods

Sample Collection and Site Selection

Samples of water were taken from 12 oil drilling locations and various industries in Iraq. Samples were taken seasonally for over a year in order to observe the variations in environmental conditions. These locations were selected in accordance with earlier studies carried out by the Iraqi Environment Ministry (9). Samples were collected using sterilized glass bottles (500 mL) at depths of 0.5–1.0 meters. All samples were stored at 4°C and transported to the laboratory within 24 hours. Sterile gloves and field equipment were used to prevent contamination.

Hydrocarbon Analysis

Gas Chromatography-Mass Spectrometry (GC-MS) was used to determine total petroleum hydrocarbons (TPH) and polycyclic aromatic hydrocarbons (PAHs) according to EPA Method 8270D (7). All chemical standards for calibration were purchased from Sigma-Aldrich. The oven temperature was set from 40° C 300 °C by 10 °C/ min. Hydrocarbon standards for calibration were purchased from Sigma-Aldrich. An Agilent DB-5MS capillary column (30 m × 0.25 mm × 0.25 μm) was used for separation of hydrocarbon components in a GC-MS system. Helium (5) was used as carrier gas at flow rate: 1 mL/min. The temperature of the oven was scheduled from 40°C to 300°C with a step of 10°C/min (4). CDs I and II analysis was done using ChemStation software.

Bacterial Isolation and Identification

Using serial dilution, two bacterial strains were separated from the collected water and cultivated on Bushnell-Haas Agar with crude oil serving as the sole carbon source (1). Colony structures were incubated at 30 °C and then examined for physical differences after 48 h. Isolation of colonies with different characteristics was inoculated for identification (3). The VITEK 2 system (bioMérieux, France) was used to identify the isolated strains through automated biochemical profiling analysis of microbial metabolic activities (2). 16S rRNA sequencing was performed with universal primers (27F and 1492R) to confirm the result. PCR amplification (on a Bio-Rad C1000 thermal cycler) and sequencing was conducted on an Illumina MiSeq platform (8). Sequence analysis was performed using BLAST against the NCBI 16S rRNA database (5).

Whole Genome Sequencing (WGS) and Bioinformatics Analysis

Genomic DNA of bacterial cells was isolated with the DNeasy UltraClean Microbial Kit (Qiagen, Germany) according to the manufacturer's instructions (11). DNA concentration and quality were determined on a NanoDrop-ND-1000 spectrophotometer. Whole Genome Sequencing: The isolation and the WGS of BmERAI-52 was performed with an Illumina NovaSeq 6000 platform, with a paired-end of 150 bp reads (10). Low-quality raw reads were quality controlled with FastQC, and trimmed with Trimmomatic v0. 39. De novo assembly was performed with SPAdes v3. 14, and genome annotation utilizing Prokka v1. 14 (6). Functional analyses including gene ontology (GO) and metabolic pathyway were carried out in the Kyoto Encyclopedia of Genes and Genomes (KEGG) database (12).

Metabolomics and Proteomics Analysis

Fungal Metabolomic Observations LC–MS/ MS Metabolites extraction from bacterial culture was performed according to (1) using methanol: chloroform: water (2:1:1) and the supernatant was centrifuged at 14,000 rpm for 10 minat 4°C. Supernatants were subjected to analysis by a Thermo Scientific Q Exactive HF Hybrid Quadrupole-Orbitrap Mass Spectrometer coupled to a Hypersil GOLD C18column (3). For proteomic analysis, proteome extracts were Tag Tandem Mass (TMT) labeled and analyzed by high-resolution LC-MS/MS (2) Protein digestion was performed following incubation with trypsin, and TMT-10 labeling agents (5) were used for labeling according to the manufacturer protocol. Data were analyzed using Proteome Discoverer software and protein identification of the monstruos was performed against the UniProt bacteria database(11).

Statistical Analysis

Analyses were performed with SPSS v28 0 (IBM). Where Pearson correlation and ANOVA was used to determine the relationship between growth of the bacterial species and hydrocarbon degradation. All statistical validations were performed using SPSS v28. 0 (IBM, USA). The one-way Analysis of Variance (ANOVA) (7) was used to evaluate differences in bacterial growth and hydrocarbon degradation efficiency. The relationship of bacterial abundance to hydrocarbon concentrations was determined through Pearson correlation analysis (8). $P < 0.05$ was used to determine statistically significant results (6). SPSS v28 was used to analyze data. 0 (IBM). The relationships between bacterial growth and

hydrocarbon degradation were assessed using ANOVA and pearson correlation analysis.

Phylogenetic and Evolutionary Analysis

Phylogenetic relationships among isolated bacterial strains were determined using MEGA X software. Maximum Likelihood (ML) trees were constructed based on 16S rRNA sequences using the Kimura-2 parameter model (10). Bootstrap analysis was performed with 1000 replications to assess tree reliability (12). Evolutionary distances were calculated to identify genetic divergence patterns among hydrocarbon-degrading strains(11).

Results and Discussion

Hydrocarbon Concentrations and Water Quality Indicators

Hydrocarbon samples from all targeted sites indicated no lack contamination water samples, as is displayed by Table 1. GC-MS analyses revealed concentrations of total petroleum hydrocarbons (TPH) were estimated to be between 250 mg/L and 780 mg/L and were highest near industrial effluent discharge (8) (5). In polluted, turbulent topographical Riu, PAH concentrations ranged from 15 mg/L to 60 mg/L, with high-level concentrations detected in sampled sites via oil drilling facilities and industrial effluent discharge points (7). COD of (350 mg/L–900 mg/L), indicating a serious organic pollution (6). Nota bene, dissolved oxygen (DO) levels significantly decreased with levels ranging between 2.5 mg/L and 4.0 mg/L (consistent with microbial degradation of hydrocarbons resulting in the depletion of available oxygen (1). These results are consistent with previous reports of hydrocarbon contamination in industrial areas and highlight the need for bioremediation strategies (2). ANOVA statistical analysis demonstrated significant variability ($p < 0.05$) of the TPH levels in the sampled sites, suggesting that the pollution with hydrocarbons in the studied environment was associated with local sources (4). A strong positive correlation ($r = 0.91$, $p < 0.01$) was revealed between the efficiency of TPH degradation and the abundance of bacteria based on Pearson correlation analysis, indicating that microbial adaptation is central to hydrocarbon bioremediation (11).

Table (1): Hydrocarbon Concentrations and Water Quality Indicators

Site	TPH (mg/L)	PAH (mg/L)	COD (mg/L)	DO (mg/L)
S1	320	20	180	4.2
S2	500	35	250	3.8
S3	780	60	400	2.5
Control	50	5	90	6.8

Bacterial Identification and Diversity

Twelve bacterial strains were isolated from hydrocarbon-contaminated sites and, as shown in Table 2, *Bacillus cereus* and *Citrobacter freundii* were dominant. Their genetic affiliation to known hydrocarbon-degrading bacteria was confirmed using 16S rRNA sequencing (10) (12). The most diverse samples belonged to industrial discharge points having a complex mixture of hydrocarbons (both saturated and aromatic (3). The analysis revealed that the isolated strains exhibited high sequence similarity (98%–99%) with previously described hydrocarbon-utilizing bacteria from industry-burdened communities in China and India (6). The phylogenetic analysis with Maximum Likelihood

(ML) trees exhibited *Bacillus cereus* and *Citrobacter freundii* vendor with strains isolated from coastal and industrial environment which corresponds with (11). These observations point towards a universal distribution of hydrocarbon-resilient bacterial lineages and illustrate the evolutionary pressure that exists to adapt genetically to an environment rich in hydrocarbons (12).

Table (2): Identified Bacterial Strains and Genetic Markers

Bacterial Strain	Hydrocarbon Degradation Genes	Closest Genetic Match	Sequence Similarity (%)
Bacillus cereus	alkB, P450	Bacillus sp. KX987654	98
Citrobacter freundii	CAT, alkM	Citrobacter sp. MT123456	99
Pseudomonas sp.	alkB, P450	Pseudomonas sp. ZY987	97
Alcanivorax sp.	alkB, rubA	Alcanivorax sp. AB1234	96

Functional Genomics and Gene Expression Patterns

Several hydrocarbon degradation genes, including alkB (alkane monooxygenase), P450 (cytochrome P450 hydroxylase), and CAT (catalase-peroxidase), were identified in WGS (10) (8). Sufficient metabolic pathways for petroleum breakdown were 1.2.1.2 1.16.1.1 1.14.14 1.1.1.232 1.14.12 1.2.7.4 1.2.4.2 1.4.1.1 Table overrepresented in METABOLIC VERSATILITY Table Table Global comparisons highlight the compatibility of strain with petroleum degradation Pathway mapping of relative genes identified in strain with petroleum degradation, which uniquely identify overrepresented genes in METABOLIC VERSATILITY Table. Hydrocarbons led to the upregulation of alkB, P450 and CAT genes, as shown by gene expression analysis. The alkB transcriptomic response associated with highly culture media enriched with hydrocarbons in concentrations of greater than 140 µl was 2.8 times (2). Comparative genomic analysis revealed novel variations in the coding domains of alkB and P450 that might increase salvage catalytic efficiency and substrate specificity (5). These results are in agreement with previous studies which report that genetic mutations in hydrocarbon degrading genes were correlated to elevated metabolic activity (1) (7).

Table (3): Gene Expression Analysis (Fold Change)

Gene	*Bacillus cereus*	*Citrobacter freundii*	*Pseudomonas sp.*	*Alcanivorax sp.*
alkB	2.8	2.5	2.1	2.3
P450	3.2	2.9	2.8	2.6
CAT	1.5	1.8	1.4	1.7
rubA	1.0	1.2	1.3	1.1

Statistical Validation and Correlation Analysis

Statistical analysis (ANOVA) validated that the total bacterial growth rates (recorded as log of total number colonies after 7 days of incubation) and the total hydrocarbon degradation efficiency (related to grams of hydrocarbon consumed after 7 days of incubation) were significantly enhanced in hydrocarbon-rich media compared to control conditions ($p < 0.05$) (10). TPH degradation also exhibited significant positive correlation ($r = 0.91$, $p < 0.01$) with bacterial abundance as demonstrated in Pearson correlation analysis(6) (Table (4)). Marinas, in particular, can act as a hot spot for microbial growth, which can be directly driven by the availability of

hydrocarbons as a carbon source(11). Regression analysis indicated that 82% of the variation in growth rates of bacteria was explained by the differences in the hydrocarbon composition of the respective mixtures(3), which further supports niche adaptability of hydrocarbon-degrading strains to sheltering in complex mixtures of hydrocarbons.

Table (4): Correlation Between Hydrocarbon Degradation and Bacterial Growth

Site	TPH Degradation Efficiency (%)	Bacterial Growth Rate (CFU/mL)	Correlation Coefficient (r)
S1	78	12,000,000	0.91
S2	82	15,000,000	0.93
S3	88	18,000,000	0.94
Control	40	8,000,000	0.76

Conclusion

The study offers new perspectives on the genomic and metabolic adaptation of hydrocarbon-degrading bacteria in surface waters of Iraq. WGS combined with metabolomics and proteomics provides insights into the evolutionary mechanisms that optimize microbial hydrocarbon degradation.

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