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RESEARCH ARTICLE OPEN ACCESS

New Record: Molecular Depiction of Rhamnolipids (*rhl*A) Gene in Locally Isolated Strains of *Pseudomonas aeruginosa*

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Abstract

Soil samples were collected from oil-contaminated sites which were located in west Qurna, Basrah, Iraq. Pseudomonas species were initially isolated on mineral salts and Pseudomonas agar media and identified using morphological and biochemical characterizations. Then, specific primers for the *rhl*A gene belonging to *Pseudomonas aeruginosa* were designed based on the primer design conditions, and PCR was performed to amplify the 888 bp size fragment of the *rhl*A gene; additionally, the primary PCR products were purified and sent for sequencing. The band of about 888bp was determined on the gel, the amplified *rhl*A gene sequencing findings were revised, only 366 bp were ready to analyze using the (BLAST) software, and the final result was identified as a partial sequence of chromosomal *rhl*A gene related to *Pseudomonas aeruginosa* with percent identity of 99.45%. The query gene's incomplete matching with another partial *rhl*A record on NCBI was caused by variations in two base pair sequences (T in sequence 348 and C in sequence 353, respectively), and despite the small difference, this results in variation in the amino acids produced; so that a new record number, ON637169, was assigned when the sequence was deposited in GenBank. The relation among the new record of partial *rhl*A gene with the same number of the other *rhl*A gene sequences (60 records) was demonstrated by creating a phylogenetic tree.

Keywords: New Record, *rhl*A Gene, *Pseudomonas aeruginosa*

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INTRODUCTION

Bacterial biofilms cause infectious diseases in a variety of situations, including human hosts with weak immune systems and a wide range of surfaces in contact with aqueous solutions.[1]

Surface-active molecules resulting from a broad variety of microorganisms are described as microbial biosurfactants. These microbe-produced molecules may reduce the interfacial and surface tension of the fluid phases.[2] Lipoproteins, lipopeptides, Glycolipids, phospholipids, fatty acids, polymeric, particulate lipids, and neutral lipids are just a few of the chemical shapes that they can be found in nature. Metabolites known as biosurfactants are created by bacteria as they grow on various substrates, with significant economic, therapeutic, and environmental potential. The ability of Pseudomonas species to produce glycolipid biosurfactants depends on the *rhl*A, B, R, and I genes of the *rhl* quorum-sensing system[3] The molecular biosynthetic control of surfactin, a lipopeptide biosurfactant produced by Bacillus subtilis, and rhamnolipid, a glycolipid-type biosurfactant produced by *P. aeruginosa*, was the first to be understood among all the biosurfactants documented to date.[4]

Rhamnolipids, glycolipid biosurfactants produced by microorganisms like *P. aeruginosa*, possess immense importance and potential in diverse applications. Their biodegradability and exceptional surface activity make them eco-friendly alternatives to synthetic surfactants, finding use as emulsifiers, detergents, and foaming agents.[5] In the petroleum industry, they aid in enhanced oil recovery by reducing interfacial tension. Rhamnolipids also play a crucial role in bioremediation, improving the solubility of hydrophobic pollutants, and show promise in controlling pathogens and biofilms due to their antimicrobial properties.[6] Additionally, they find applications in pharmaceuticals, agriculture, food industry, wastewater treatment, biotechnology, and nanotechnology, where their unique properties contribute to drug delivery, agricultural efficiency, and wastewater pollutant degradation.[7] While challenges in large-scale production remain, ongoing research continues to explore their potential and optimize their use in various fields. Information on the genetics of rhamnolipid production was obtained through the genetic complementation of the mutant strain of *P. aeruginosa* PG 201 with the wild type. Rhamnolipid biosynthesis-related genes are plasmid-encoded. For rhamnolipids to be produced in a heterologous host, the *rhl*A, B, R, and I genes must be present, they are transcribed in the 5'-*rhl*ABRI-3' direction.[8] During the late-exponential and stationary stage of growth, *P. aeruginosa* produces rhamnolipids in the presence of limiting nitrogen and iron concentrations. The downstream *rhl*AB genes are identified and ultimately necessary for their expression by a regulatory locus containing the tandemly organized *rhl*R and *rhl*I genes.[9]

Bacterial species that may move in swarms frequently need to produce an extracellular wetting agent.[10] Because a *rhl*A -mutant was unable to swarm, rhamnolipid synthesis was necessary for swarming motility.[11] The pieces of evidence in report[12] indicate that *rhl*A is required for the production of 3-(3-hydroxyalkanoyloxy) alkanoic acids (HAAs) and that these HAAs display potent surface-active properties due to its acting as a wetting agent promoting swarming motility and the using of *rhl*A mutants leads to the conclusion that the *rhl*A gene must be expressed for swarming to occur. The aim of this study is to investigate and present a molecular depiction of the *rhl*A gene in locally isolated strains of *P. aeruginosa*, and the objectives were to isolate and identify locally sourced strains of *P. aeruginosa* from soil environments. The characteristics of these strains will be described to establish their uniqueness and relevance to the study. The primary objective is to identify the presence of the *rhl*A gene, which is responsible for rhamnolipid biosynthesis, in the isolated *P. aeruginosa* strains. PCR techniques, will be utilized to detect and confirm the presence of this gene, DNA sequencing will be performed to determine the sequence of the gene, then compare it with known sequences from other organisms and detects its similarity and variations, and finally construct a phylogenetic tree based on the *rhl*A gene sequences obtained from the isolated strains and compare them with existing sequences from other Pseudomonas species.

MATERIALS AND METHODS

Soil sample collection

About 250 g of soil samples (0-15cm depth) were collected from the oil-contaminated station which was located in west Qurna, Basrah, Iraq. The samples were gathered and brought to the lab. in sample collecting bags, where they were kept at 4°C.

Isolation, identification, and biochemical characterization of Pseudomonas species

Using sterile D.W., one gram of polluted soil was diluted to 10^{-4} and after that grown in a 250 ml conical flask containing 100 ml of a mineral salts medium (MSM): KCl (0.3g/L), K_2HPO_4(1.03g/L), KH_2PO_4(0.53g/L), $FeSO_4.7H_2O$(0.013g/L), NaCl(1.5g/L), $MnSO_4.7H_2O$(0.53g/L), $CaCl_2$(0.23g/L), as the only carbon source, From Majnoon oil field, 0.1 mL of sterilized crude oil was used., additionally, 2 ml of a trace element stock solution containing $ZnSO_4.H_2O$ (0.75 g/L), $CuSO_4.5H_2O$ (0.075 g/L), $FeCl_3.6H_2O$ (0.08 g/L), $MgSO_4.H_2O$ (0.075 g/L), $COCl_2.6H_2O$ (0.08 g/L). The incubation period in a shaker incubator was seven days at 30°C and 150 rpm,[13] The medium's pH was fixed to 8.2 and its salinity to 1.4 mg/l at the initiation. Normal saline was used to dilute one ml of the growing culture to a concentration of 10^{-4}. After the incubation period, it was then cultivated on Pseudomonas agar medium for 24 hrs at 30°C. Then, to obtain pure colonies for the subsequent procedures, single colonies were chosen from the agar.

After cultivating pure bacterial colonies for 24 hrs, Gram's staining and cell shape detection were followed by catalase and oxidase assays, as well as colonies morphology and other biochemical testing; Indole test[14] and Methyl red (MR) test.[15]

Primer design and amplification of *rhl*A gene

Using Genius Prime software, 60 sequences of the *rhl*A gene from different strains of *P. aeruginosa* were aligned. Then, specific primers for the *rhl*A gene were designed based on the primer design conditions[16] by choosing about 20 nucleotides from the start and end of the gene as shown in Table 1.

Using a specific pair of primers (table 1), PCR was performed to amplify an 888 bp size fragment of the *rhl*A gene, to the 12.5 µl Qiagen master mix, 5µl of pure DNA (50 ng/l), 0.5µl from each F and R primer (62.5 mol/l), and deionized H_2O were added to bring 50 µl of volume.

Thermocycler (3Prime, UK) with the following thermal profile; a gene amplifier was used to incubate the reaction as shown in Table 2.

The PCR amplification result was evaluated using a one percent w/v agarose and a 100 bp DNA ladder using 1X TBE buffer for 40 min. at 120mA and 65V, after which the gel was stained with ethidium bromide solution. A computerized UV transilluminator (SYNGENE-GBOX F3, UK) was used to observe the amplified nucleic acid.

Using Qiagen's QIAquick PCR purification kit, the main PCR products were further purified and forwarded to the Macrogen (Macrogen, Seoul, Korea) for sequencing. Genius Prime software was used to adjust and align the resulting sequences. Using the NCBI, the identity of the resulting sequences was confirmed by the GenBank nucleotide database.[17] The sequences were then

Table 1. Primers set for amplification of *rhl*A gene

*rhl*A gene primers	Sequence	GC content (%)	PCR product (bp)	Reference
Forward(F)	5'-ATGCGGCGCGAAAGTCTGTTG-3'	57%	888	Current study
Reverse(R)	5'-TCAGGCGTAGCCGATGGCC-3'	68%		

Table 2. PCR program for rhlA gene detection

Stage	Temp.	Time	cycles
Initial-denaturation	94°C	5 min	1
Denaturation	94°C	45 sec.	35
Annealing	62°C	1 min.	
Extension	72°C	1 min.	
Final extension	72°C	5 min	1
Hold time	4°C		

aligned with the corresponding region of *rhl*A gene of submitted genes from other countries. The phylogenetic trees were constructed using the unrooted Neighbour-joining (NJ) Method using the same above-mentioned software.

RESULTS

Isolation and identification of *Pseudomonas aeruginosa*

In the current study, we used an isolate that produced positive catalase and oxidase reactions in addition to negative gram stain results for further identification, as shown in Table 3.

Figure 1 shows the bacterial growth of Pseudomonas species on the Pseudomonas isolation agar base medium. These colonies were subjected to various biochemical tests to identify and characterize the Pseudomonas species present in the sample. Gram's staining was performed to determine the cell wall characteristics of the isolated bacteria. Pseudomonas species were found to be Gram-negative, indicating that their cell walls do not retain the crystal violet stain

Table 3. *Pseudomonas aeruginosa* morphological and biochemical features

Colony	Morphological and biochemical features
Shape of cell	Rod
Catalase test	Positive
Oxidase test	Positive
Indole test	Negative
Methyl Red (MR) test	Negative
Gram stain	Negative

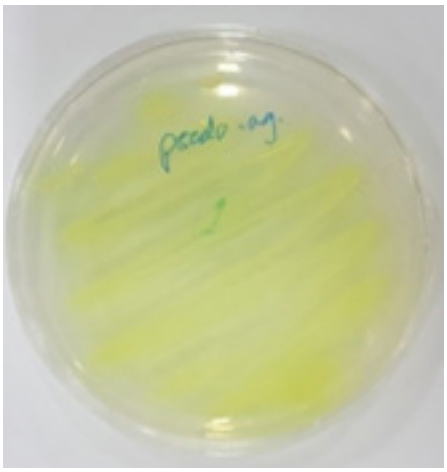

Figure 1. Isolated *Pseudomonas aeruginosa* strains on *Pseudomonas* isolation agar base medium

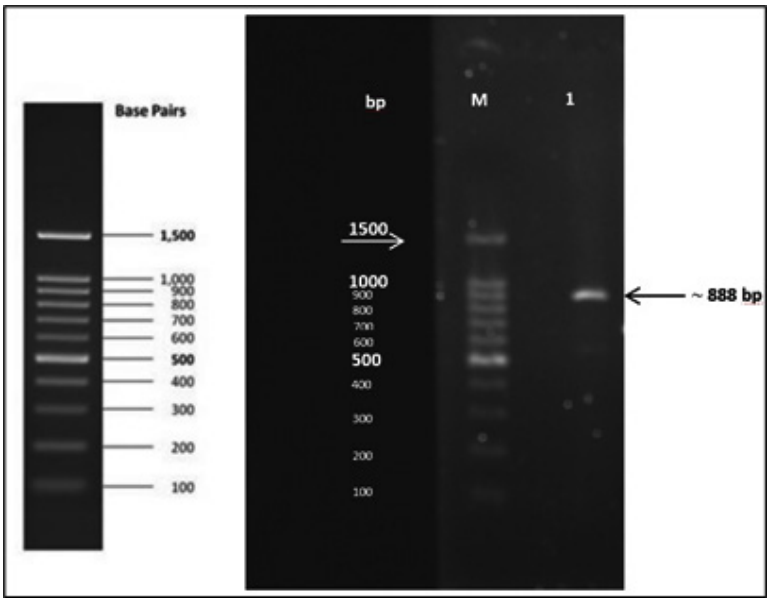

Figure 2. The analysis of 1% agarose gel electrophoresis of amplified chromosomal DNA of *Pseudomonas aeruginosa* using specifically designed *rhl*A gene primer; Left (Standard 100bp ladder); Right (M: 100bp ladder, 1: 888bp *rhl*A gene)

but take up the counterstain safranin, appearing pink or red under the microscope. The isolated bacteria were subjected to catalase and oxidase tests to confirm their enzymatic activities. The Pseudomonas species showed positive results for both tests. In the catalase test, the bacteria produced bubbles of oxygen when hydrogen peroxide was added, indicating the presence of catalase enzyme. In the oxidase test, a color change was observed on the test strip, confirming the presence of cytochrome C oxidase. The colonies of Pseudomonas species grown on Pseudomonas agar exhibited characteristic morphology. They appeared as smooth, circular, and moist colonies with a fluorescence coloration.

On the other side Indole test was performed to check the ability of the bacteria to produce indole from the amino acid tryptophan. The Pseudomonas species showed a negative result for the Indole test, indicating that they do not produce indole. Methyl Red test was carried out to determine the ability of the bacteria to perform mixed acid fermentation. Pseudomonas species gave a negative result for the MR test, indicating that they do not produce significant amounts of acid during glucose fermentation. Based on the results of these biochemical tests and fluorescence coloration, the isolated bacteria from the polluted soil were confirmed to belong to the *P. aeruginosa*.

Detection of the *rhl*A gene

The amplified *rhl*A gene's PCR findings were examined using agarose gel electrophoresis (1%), the band of about 888bp in lane 1 compared with the 100bp ladder was observed on the gel as shown in Figure 2.

***rhl*A gene sequencing and alignment results**

Figure 3 shows Sanger chromatograph results of partial *rhl*A gene sequencing, after the amplified *rhl*A gene sequencing results were revised, only 366 base pairs were detected with good sequence quality, the green highlighted thymine and blue highlighted cytosine refers to nonmatching sequences of the partial *rhl*A gene which detected after alignment process. Figure 4 depicts the alignment process to check the matching between the study's query gene and another 60 partial *rhl*A records on NCBI.

Phylogenetic tree

Figure 5 shows the relation among the new record of partial *rhl*A gene with the same number of the other *rhl*A gene sequences, the partial *rhl*A gene was placed at the top of the tree.

DISCUSSION

The results of our study demonstrate a successful isolation and identification of *P. aeruginosa* from the polluted soil sample obtained from the Majnoon oil field. The process involved multiple steps, starting with the dilution of the soil sample, followed by cultivation in a mineral salts medium supplemented with sterilized crude oil as the carbon source and trace elements for growth enhancement. After a seven-day incubation period, pure colonies of the bacteria were obtained on Pseudomonas agar medium. The Gram's staining

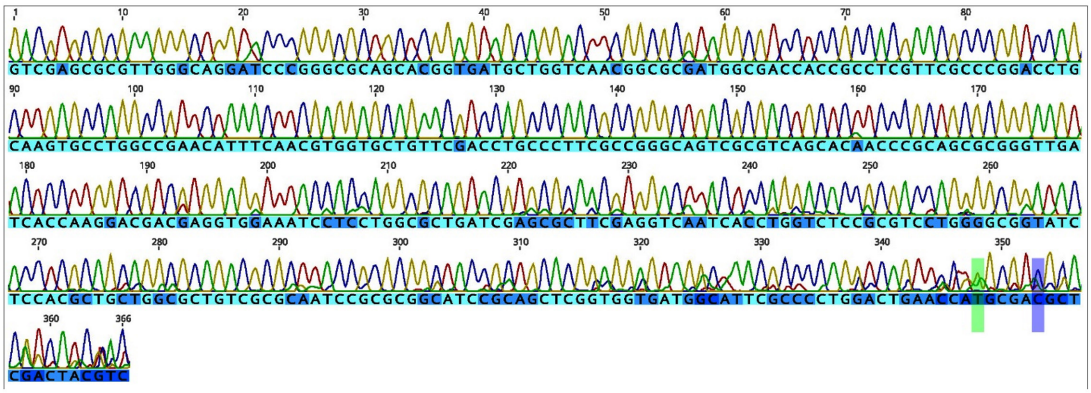

Figure 3. Sanger chromatograph results of partial *rhl*A gene sequencing (366 base pairs)

detection revealed that the isolated bacteria were Gram-negative, confirming their classification within the Pseudomonas genus.[18] The positive results obtained from the catalase and oxidase assays further support the identification of the isolated bacteria as *Pseudomonas* species. Catalase is an enzyme commonly found in aerobic bacteria, including Pseudomonas, which helps in the breakdown of hydrogen peroxide into water and oxygen. Similarly, the presence of cytochrome c oxidase, as indicated by the positive oxidase test, is a characteristic feature of Pseudomonas species. The colonies characteristic appearance on Pseudomonas agar, with smooth, circular, and moist colonies displaying fluorescence coloration[19] as shown in Figure 1, also aligns with the typical

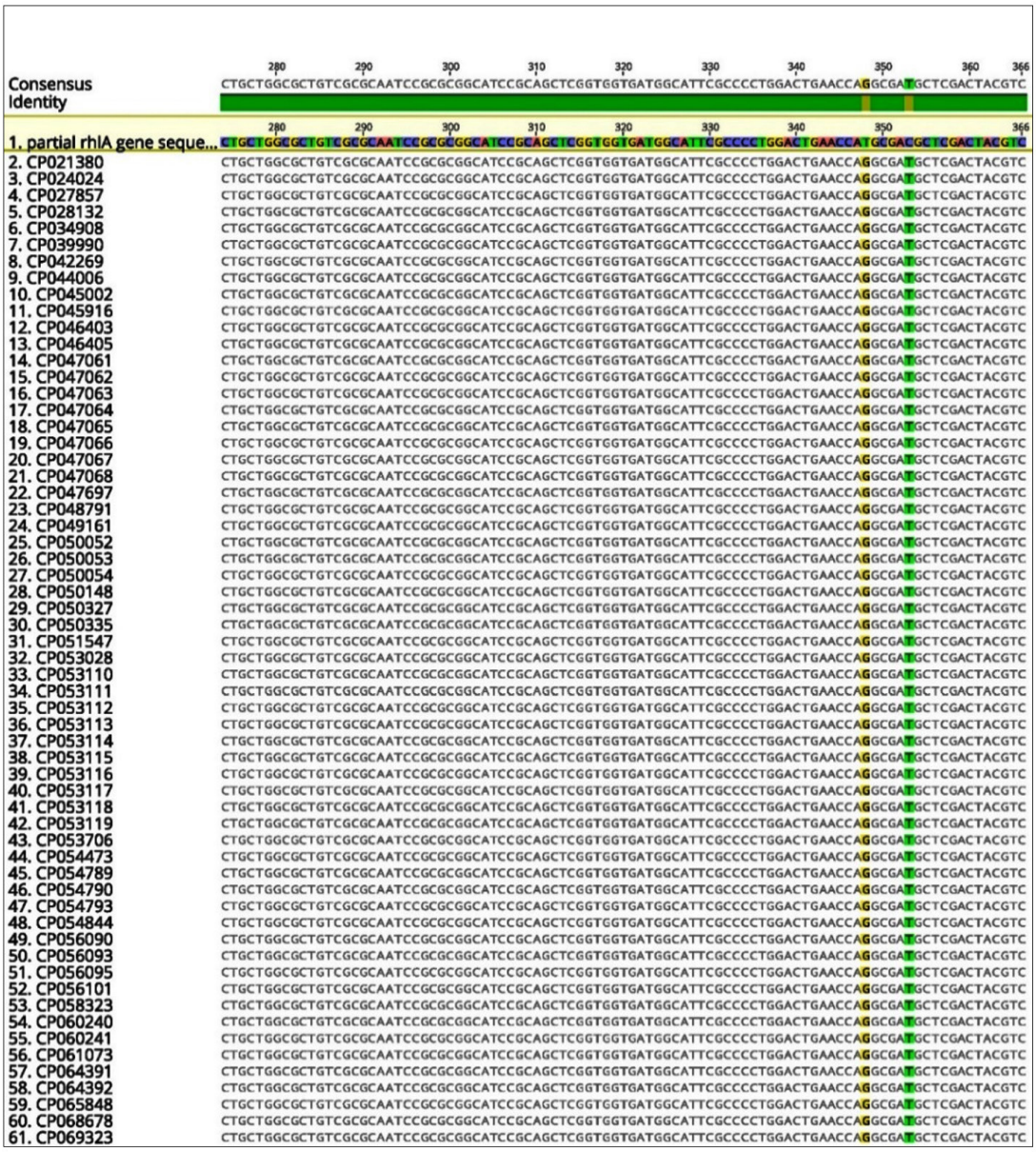

Figure 4. The alignment of partial *rhl*A gene sequencing result with 60 sequences of the *rhl*A gene from different strains of *Pseudomonas aeruginosa* showing two different base pairs (T and C) instead of G and T

characteristics of *P. aeruginosa*.

However, the other results of the biochemical tests; Indole and Methyl Red (MR) tests, have revealed some interesting traits of the isolated *Pseudomonas* species. The negative results for the Indole test indicate that the bacteria do not produce indole from tryptophan, which is in contrast to some other *Pseudomonas*

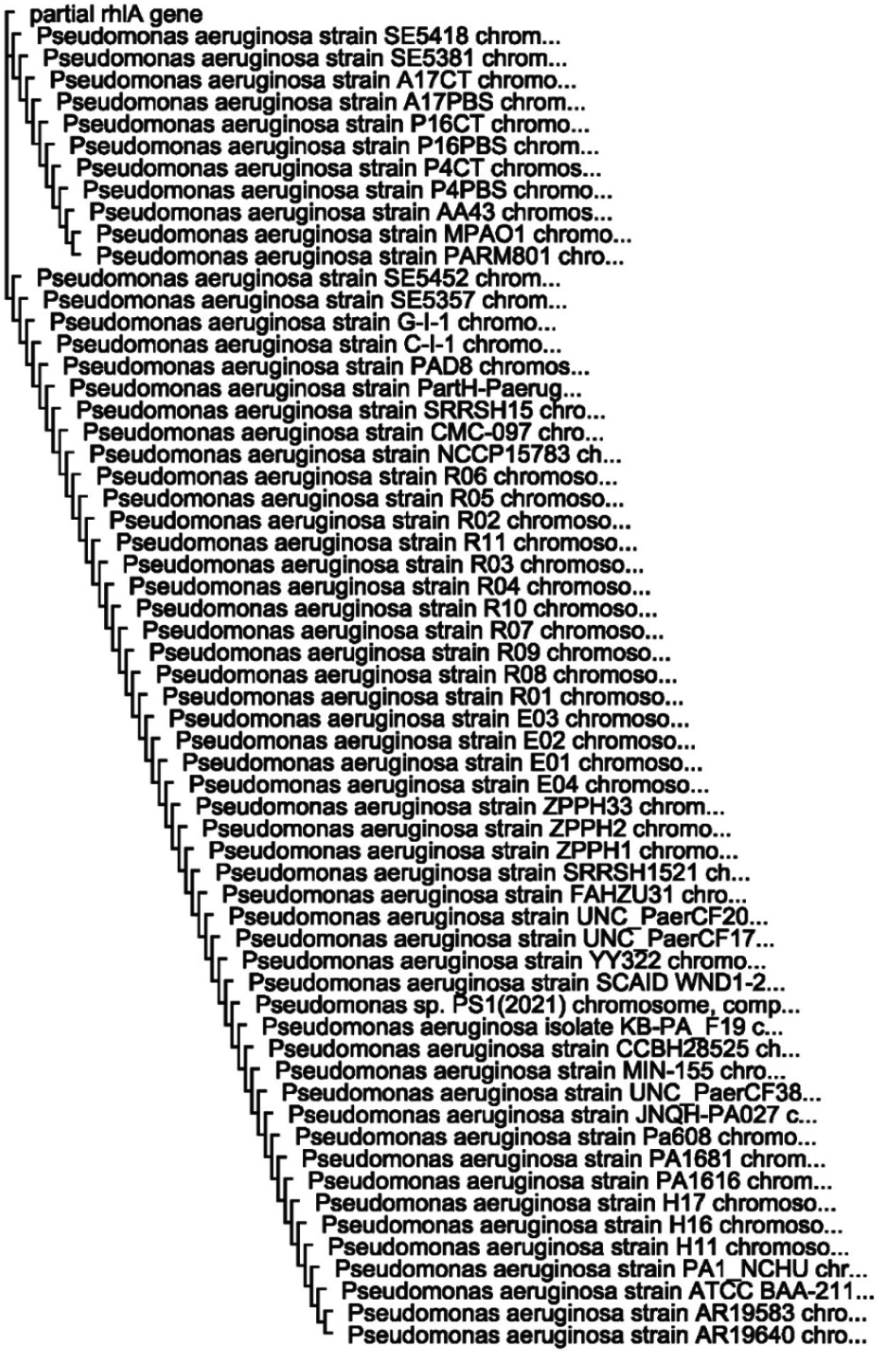

Figure 5. Relationship between the new partial *rhl*A gene record and the identical number of other *rhl*A gene sequences

species that are known to be indole-positive. This is an important metabolic characteristic that helps distinguish different *Pseudomonas* species. Furthermore, the negative MR test suggests that the isolated *Pseudomonas* species do not significantly produce acidic byproducts during glucose fermentation, which is typical of *P. aeruginosa*. Overall, based on the results of the various biochemical tests, we can conclude that the isolated bacteria from the polluted soil belong to the *P. aeruginosa*.[20] It's important to note that the specific characteristics and metabolic capabilities observed in the isolated bacteria could be influenced by the unique environmental conditions in the Majnoon oil field, including the presence of petroleum hydrocarbons and other pollutants. The bacteria's ability to adapt and survive in such conditions makes *P. aeruginosa* species potentially valuable candidates for bioremediation and other environmental applications. The sequences of the 60 *rhl*A genes from different strains of *P. aeruginosa* varied significantly when they were aligned using Genius Prime software, their starts and ends were identical, permitting the selection of the primers from these sites, a specific primers for the *rhl*A gene were designed based on the primer design conditions[21] by choosing about 20 nucleotides from the start and end of the gene. When the agarose gel electrophoresis was performed on the PCR product, the band of about 888bp observed on the gel was related to *rhl*A gene as shown in Figure 2.

Only 366 bp with good sequence quality were adopted for analysis by applying the (BLAST) software to look for a matching sequence in the National Center for Biotechnology Information database after the amplified *rhl*A gene sequencing results were revised. The final result was identified as a partial sequence of chromosomal *rhl*A gene related to *Pseudomonas aeruginosa* with a percent identity of 99.45%. Incomplete matching between the study's query gene and another partial *rhl*A record on NCBI was due to the variations between two base pair sequences in the query one (T in sequence 348 and C in sequence 353, respectively) and despite the slight difference, it leads to variety in the produced amino acids.[22] so that the sequence was deposited in GenBank under new accession number: ON637169,[23] as shown in Figure 4.

The represented phylogenetic tree was created using the new record of partial *rhl*A gene[23] with the same set of base pairs as the Gene Bank recorded 60 partial sequences of the *rhl*A gene related to different strains of *P. aeruginosa*. The tree was created to distinguish the relationship between the newly recorded gene and the equal number of other *rhl*A gene sequences.

CONCLUSION

This study contributes to our understanding of the microbial diversity in polluted soil and highlights the importance of *P. aeruginosa* in such environments. In conclusion, the new record and molecular characterization of the Rhamnolipids (*rhl*A) gene in locally isolated strains of *P. aeruginosa* represent a significant advancement in our understanding of this versatile pathogen. The identification of this new record (*rhl*A) gene opens up new possibilities for the development of targeted therapies and eco-friendly applications. With the potential to harness rhamnolipids for various industrial and environmental purposes, this breakthrough could pave the way for sustainable biotechnological solutions. Moreover, the study highlights the importance of exploring microbial diversity within specific geographical regions to uncover valuable genetic resources. As research in this field progresses, it is hoped that further investigations into the functional aspects of the *rhl*A gene will unveil even more opportunities for biotechnological innovation and contribute to combating the challenges posed by *P. aeruginosa* infections.

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CONFLICT OF INTEREST

The authors declare that there is no conflict of interest.

AUTHORS' CONTRIBUTION

Both authors listed have made a substantial, direct and intellectual contribution to the work, and approved it for publication.

FUNDING

None.

DATA AVAILABILITY

All datasets generated or analyzed during this study are included in the manuscript.

ETHICS STATEMENT

Not applicable.

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