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Utilizing the Adenosine-5'-Phosphosulfate Reductase (*apsA*) Gene for Genetic Profiling of Sulfate-Reducing Bacteria in Oil Fields of Basrah City

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Abstract - This genetic detection study, focusing on the adenosine-5'-phosphosulfate reductase alpha subunit *apsA* gene in water samples in an oil field in Basrah city, southern Iraq, constitutes an important contribution to understanding the distribution and diagnosis of microbiologically influenced corrosion (MIC) bacteria in oil reservoirs. samples of oil produced water were systematically collected from five distinct oil well sites in Basrah city; to facilitate the preparation of sulfate-reducing bacterial growth cultures, various chemical parameters were measured during the study. Primers targeting 678bp of apsA gene were synthesized, followed by PCR amplification and subsequent sequencing of the PCR product. The analysis revealed a high-quality sequence comprising 472 base pairs. Alignment of BLAST results demonstrated a remarkable 99.57% similarity with *Desulfovibrio vulgaris* DP4, confirming the presence of the *apsA* gene in the chromosomal DNA of this bacterium, thereby categorizing it as a sulfate-reducing bacteria (SRB).

استخدام الجين المشفر لإنزيم Adenosine-5'-Phosphosulfate Reductase (*aps*A) للتنميط الوراثي للبكتيريا المختزلة للكبريتات في حقول النفط في مدينة البصرة

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المستخلص – تم جمع عينات الماء المصاحب للنفط بشكل منهجي من خمسة مواقع مختلفة لآبار نفط حقل الرميلة في محافظة البصرة، ولتسهيل تحضير مزارع النمو للبكتيريا المختزلة للكبريتات تم اجراء العديد من القياسات الكيميائية المختلفة خلال الدراسة. تم تصنيع البوادئ التي تستهدف 678 زوج قاعدي من جين *aps*A، وبعد ذلك تم اجراء عملية الـ PCR وبعدها التعرف على تسلسل ناتج عملية الـ PCR. وكُشف عن التسلسلات عالية الجودة التي تضم 472 زوجًا قاعدياً. أظهرت عملية المحاذاة لنتائج الـ BLAST تشابهًا ملحوظًا بنسبة 99.57% مع بكتريا *Desulfovibrio vulgaris* DP4، مما يؤكد وجود جين *aps*A في الحمض النووي الصبغي لهذه البكتيريا، وبالتالي تصنيفها على أنها بكتيريا مختزلة للكبريتات (SRB). تشكل دراسة الكشف الجيني هذه، التي تركز على الجين المشفر لانزيم *apsA* adenosine-5'-phosphosulfate reductase alpha subunit في عينات المياه المصاحب في حقول نفط في مدينة البصرة، العراق، مساهمة مهمة في فهم تواجد وطريقة تشخيص البكتريا المسببة للتآكل (MIC) في مكامن النفط.

الكلمات المفتاحية: البكتريا المختزلة للكبريت، (*apsA*) adenosine-5'-phosphosulfate reductase alpha subunit ، الشجرة الوراثية، حقول النفط ، *Desulfovibrio vulgari*

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Introduction

Metal corrosion is regarded as a major issue affecting oil and gas industry that leading for critically economic and environmental problems. It has been valued that total corrosion cost was exceeded $90 billion per year(Alamri, 2020; Labena *et al.*, 2020) .

Sulfate-reducing bacteria (SRB) are a group of anaerobic bacteria that use sulfate (SO_4^{-2}) as their terminal electron acceptor to break down organic compounds. These bacteria are generally found in environments lacking oxygen and play an important role in the sulfur and carbon cycles. SRB represent a distinct physiological group of microbes, involving of approximately 220 species across 60 genera. (Thakur *et al.*, 2023). The importance of SRB in the environment and oil industry originates from the production of high levels of biogenic H_2S that leads to corrosion and souring in oil fields, decreases oil quality, and threatens workers' health because of its toxicity. Sulfate-reducing bacteria (SRB) are ubiquitous microorganisms known for their significant impact on various industries, particularly the oil and gas sector. These anaerobic bacteria thrive in environments rich in organic matter and sulfate, including oil reservoirs and production facilities. While some SRB species play essential roles in natural biogeochemical cycles, others can cause detrimental effects by leading to souring in oil reservoirs, pipeline corrosion, and hydrogen sulfide (H_2S) production (Etim *et al.*, 2023) .

SRB can be beneficial where they eliminate sulfate and heavy metals, as well as applied in bioremediation of subsurfaces contaminated with toxic metals, polychloroethens, BTEX, trinitrotoluene, many recent studies have reported the possible use of SRB metabolites in enhanced oil recovery (Xu *et al.*, 2020; Zambrano *et al.*, 2023).

Numerous researchers have studied the molecular detection of SRB using phylogenetic markers such as the 16S rRNA gene and functional markers such as the key gene involved in dissimilatory sulfate reduction. Both subunits of the APS reductase are highly conserved (Fritz *et al.*, 2000), and the APS reductase genes have been proposed as a useful phylogenetic marker (Hipp *et al.*, 1997).

The sulfate-reduction pathway in sulfate-reducing bacteria (SRB) involves the catalysis of three crucial enzymes: ATP sulfurylase, APS reductase, and sulfite reductase. In the initial step, sulfate is activated by the ATP sulfurylase enzyme (*sat*, EC 2.7.7.4). This is followed by the conversion of adenosine-50-phosphosulfate (*APS*) to adenosine-monophosphate (AMP) and sulfite through the action of adenylylsulfate reductase (*aps*, EC 1.8.4.9). Subsequently, dissimilatory sulfite reductases (*dsr*, EC 1.8.99.3) further reduce sulfite to hydrogen sulfide (Kushkevych *et al.*, 2020). Within the enzymes involved in the dissimilatory sulfate reduction pathway, *APS* reductase and dissimilatory sulfite reductase serve as appropriate indicators of this process within the environmental context (Fritz *et al.*, 2000; Kushkevych *et al.*, 2020).

Iraq, as one of the world's major oil producers, is not exempt from the challenges posed by SRB in its oil fields. The Basrah region, in particular, is of paramount importance due to its abundant oil resources. Monitoring and understanding the prevalence and genetic characteristics of SRB in this context are crucial to mitigate potential operational and environmental consequences. This study presents a specific study aimed at the genetic detection of SRB bacteria isolated from an oil field in Basrah city, Iraq. By employing molecular techniques related to detection of sulphate reducing bacteria by means of adenosine-5'-phosphosulfate reductase alpha subunit (*apsA) gene*.

Materials and Methods

Samples collection

In this study, produced water samples were systematically acquired from five distinct of AL Rumaila oil wells sites situated within the geographic confines of Basrah governorate during for 3 months (Jan., Feb., Mar.) in 2024, located in the southern region of Iraq. Duplicates of water samples were acquired, sterile glass bottles with a volume of 500mL were used to collect samples and they were completely filled to avoid exposure to air and kept in icebox until transport to the lab. Subsequently, in the laboratory, five bottles were purged with 10% nitrogen (N_2) for biological tests (Xi *et al.*, 2020), while the other duplicated five samples were left untreated with nitrogen gas for chemical tests. All samples were kept at 4℃ until they were utilized.

Chemical parameters analysis

According to ASTM standard (D-19, 1960), various parameters were identified in the water samples, including pH, Conductivity, Total dissolved salts, CO_2, alkalinity and Sulfate.

Isolation and identification of plankton SRB

A volume of one milliliter from water sample was aseptically injected into a nine-milliliter vial containing a SRB (Sulfate-Reducing Bacteria) medium (Table 1), which had been pre-prepared in accordance with the guidelines outlined in NACE TM0194-2004 (TM, 2004). Subsequently, a serial dilution was performed by transferring one milliliter from the initial vial to each of the subsequent six vials, ensuring sterile conditions throughout the process. The injected vials then incubated in 37℃ for 21 days. During the period of incubation, when the culture became visibly turbid and darkened, the cells were subsequently collected through centrifugation at 14,000 rpm for 10 min, the resulting pellets was used for colony PCR, after culture the pellets on solid medium.

(Table 1) The contents of sodium lactate SRB medium (TM, 2004)

Ingredients	Quantities
Sodium lactate solution (60%)	4 ml
Yeast extract	1 gm
Ascorbic acid	1 mg
$Mg(SO_4)_2.7H_2O$	0.2 gm
K_2HPO_4, (anhydrous)	0.01 gm
$FeSO_4(NH_4)_2.6H_2O$	0.2 gm
NaCI	128 gm
Distilled water	1000 ml

Primers and amplification of adenosine-5’-phosphosulfate reductase (*apsA*) gene

The detection of Sulfate-Reducing Bacteria was conducted using PCR primers; APS-F and APS-R, as shown in Table 2. These primers target and amplify the *aps*A gene, which encodes adenosine-5'-phosphosulfate reductase (*aps*A), a key enzyme in sulfate respiration distinctive of

all sulfate-reducing bacteria (Friedrich, 2002; Nasser *et al.*, 2017). The conditions of PCR were as follows: an initial denaturation for 5 min. at 94°C, followed by 35 cycles of 30 sec. at 94°C, 55 sec. at 60°C, 1 min. at 72°C, and a final extension of 7 min. at 72°C. The PCR product was then run on an agarose gel (1%) at 120 V for 45 min.

(Table 2) PCR primers utilized for the amplification of *apsA* gene fragment

Primer name	Sequence	GC content (%)	PCR product (bp)	Reference
APS-F	5'-TGGCAGATVATGATYMACGG-3'	48%	678	(Friedrich, 2002)
APS-R	5'-GCACATGTCGAGGAAGTCTTC-3'	52%		

Chemical parameters

The chemical parameter results are listed in Table 3; pH values ranged from 6.55 to 6.78, and the average value of these results was 6.7, which corresponds to the pH of the prepared culture medium. Total dissolved solids (TDS) values ranged from 123,100 ppm to 134,300 ppm, representing the minimum and maximum values, The TDS of the prepared medium was set at 128 ppm. The chemical parameters concentration of the culture medium were adjusted approximately match their levels in the water samples to minimize any potential physiological stress on the bacteria. (TM, 2004; Ivan, 2019) .

The sulfate value ranged from 11.02 ppm to 37.83ppm, indicating elevated levels that increase the probability of the presence of SRB in this particular environment, the presence and activity of sulfate-reducing bacteria are intricately connected with sulfate levels(Zhang *et al.*, 2022), The presence of sulfate-reducing bacteria (SRB) is typically associated with the concentration of sulfate ions (SO_4^{-2}) in the water environment. These microorganisms flourish in anaerobic conditions and are crucial to the sulfur cycle. They utilize sulfate as a terminal electron acceptor in their metabolic processes, converting it into hydrogen sulfide (H_2S).

The recorded CO_2 concentrations varied between 144 ppm and 224 ppm, the relationship between CO_2 levels and the presence of sulfate-reducing bacteria (SRB) is often intertwined with microbial metabolism, sulfate-reducing bacteria are known to utilize CO_2 as a carbon source during their metabolic processes (Zhao *et al.*, 2023). The availability of CO_2 in an environment can influence the growth and activity of SRB, as it serves as a substrate for their metabolic pathways. In anaerobic conditions, SRB play a crucial role in the sulfur cycle by reducing sulfate (SO_4^{-2}) to sulfide (H_2S). This reduction process is part of their energy-yielding metabolism, and CO_2 can serve as a carbon source in this metabolic pathway. The correlation between CO_2 levels and the presence or activity of SRB is significant in environments where anaerobic conditions prevail, such as in oil reservoirs, high CO_2 levels may indicate conditions favorable for the growth of these bacteria.

(Table 3) Chemical parameters of water samples

Test	PH	Conductivity(ms/cm)	TDS Mg/L	CO_2 PPM	Alkalinity PPM	Sulphate PPM
Station	ASTM-D1293	ASTM-D1125	ASTM D5907	ASTM-D513	ASTM-D1067	HACH METHOD
1	6.55	192.3	123100	196.8	200	37.83
2	6.77	199.1	127400	192	204	23.45
3	6.78	198.3	126900	144	204	11.02
4	6.79	200	128200	139.2	220	34.29
5	6.78	210	134300	224	240	14.93

Characterization of plankton sulfate-reducing bacteria (SRB)

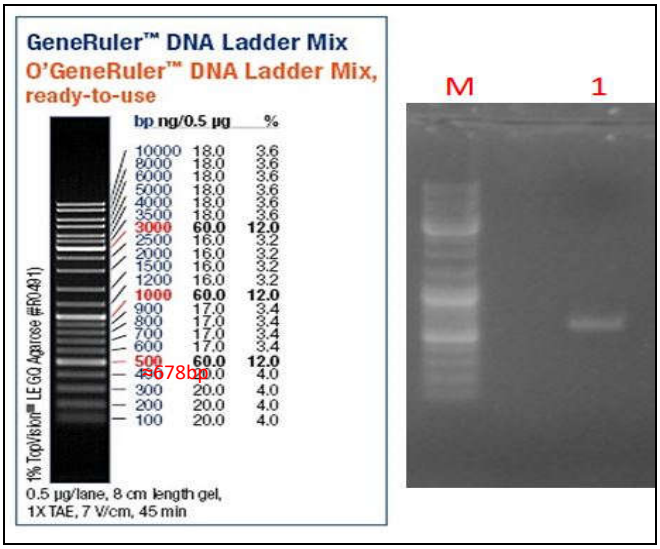
Isolation and Identification:

A positive result is indicated by reduce sulfate (SO_4^{-2}) to hydrogen sulfide (H_2S) which reacts with the iron to form black precipitates of iron sulfide (Figure 1), the black precipitate appears within 7 to 14 days of incubation period and that agree with (Das, 2018; Udowo *et al.*, 2024).

(Figure 1) Black precipitates of iron sulfide

Detection of the (*aps*A) gene

The agarose gel electrophoresis analysis of the amplified *apsA* gene of chromosomal DNA revealed a band of approximately 678bp in lane 1, when compared to the 1kbp ladder, as illustrated in Figure 2, *apsA* gene are located in the chromosomal DNA (Biktasheva *et al.*, 2023).



(Figure 2) Agarose gel electrophoresis image showing fragments of the (*apsA*) gene amplified from the chromosomal DNA

Gene sequencing results

The PCR products of the specific gene were submitted to MACROGEN/Korea's DNA sequencing service at "http://dna.macrogen.com." Subsequently, the Geneious Prime 2019 software version 1.1 (Geneious-Prime, 2024) was employed to review the sequence results, the analysis revealed a sequence of 472 base pairs with high quality. Further analysis involved comparing these sequences to the National Centre for Biotechnology Information Database (NCBI) using the Basic Local Alignment Search Tool (BLAST) at https://blast.ncbi.nlm.nih.gov/Blast.cgi. The alignment findings revealed a 99.57% similarity with *Desulfovibrio vulgaris* DP4, indicating that the (*apsA*) gene is present in the chromosomal DNA of this bacterium, classifying it as a sulfate-reducing bacteria so that in this study, our reliance for the genetic detection of sulfate-reducing bacteria is focused on this specific gene. Employing molecular biological techniques to explore the presence and distribution of bacteria in the environment offers the benefit of directly revealing information about community structure (Ben-Dov *et al.*, 2007).

Phylogenetic Tree

Performing a comparative phylogenetic analysis using 34 nodes and 18 tips of the acquired *apsA* sequences revealed significant sequence conservation in the examined region. As depicted in Figure 4, the *apsA* gene is closely situated to *Desulfovibrio vulgaris*, exhibiting a 99.6% percentage identity with two base pair mismatches. Additionally, it is closely associated with *Nitratidesulfovibrio vulgaris*, showing a 98.1% percentage identity with nine base pair

mismatches, as illustrated in Figure 3,4. The scientific name for both these bacteria is *Desulfovibrio vulgaris* and classified as sulfate-reducing bacteria (Trotter *et al.*, 2023)

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(apsA) gene                CGGGGCGCGGCTTCGTGGCTACGGCTGCGGGTGAACTCGGCATTGCGCTGGAGTGGCGCGGTGAGGGCGTGGACGA
Desulfovibrio vulgaris     ...T.....A.....................................................................
Nitratidesulfovibrio vulgaris ...T.....A.........C........A...........TC....A..........A...C.............
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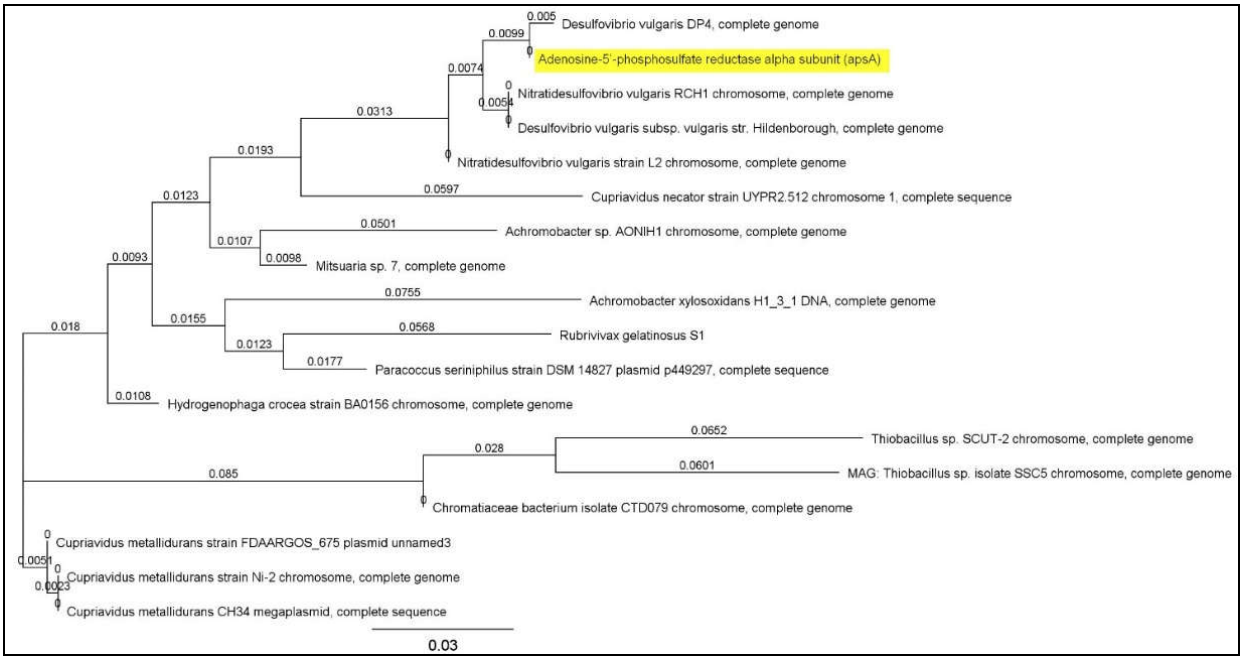
(Figure 3)Alignment of *apsA* gene, *Desulfovibrio vulgaris* and *Nitratidesulfovibrio vulgaris*



(Figure 4) Phylogenetic tree of adenosine-5'-phosphosulfate reductase (*apsA*) gene with 0.03 scale bar

Conclusion

In conclusion, this genetic detection study of SRB bacteria by means of adenosine-5'-phosphosulfate reductase alpha subunit (*apsA) gene* in an oil field in Basrah City, Iraq, represents a significant contribution to the understanding the distribution of microbiologically influenced corrosion (MIC) in oil reservoirs. By illuminating the genetic profiles of these microorganisms, we aim to support the sustainable and efficient exploration, production, and management of oil resources, ultimately contributing to the continued growth of Iraq's vital oil industry.

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