Journal Homepage
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Anbar Journal of Agricultural Sciences
(University of Anbar – College of Agriculture)

Molecular Identification and Antimicrobial Activity of Rhizobacteria Isolated from Plants in The North of Iraq

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Article info

Received: 2024-05-11
Accepted: 2024-12-04
Published: 2025-12-31

DOI-Crossref:
10.32649/ajas.2025.189066

Cite as:
Hussien, E. W., Sultan, R. H., and Edavana, V. (2025). Molecular Identification and Antimicrobial Activity of Rhizobacteria Isolated from Plants in The North of Iraq. Anbar Journal of Agricultural Sciences, 23(2): 946-957.

Abstract

In the winter of 2023, four isolates of rhizobacteria were obtained from the root nodules of several leguminous plants gathered from various agricultural locations in the northern part of Iraq to investigate their antibacterial activity. The application of the liquid fermented filtrate medium of the rhizobacteria ER6 isolate demonstrated an inhibitory impact on the pathogenic bacteria Escherichia coli isolate ER51. At the time of testing the antibacterial activity, the average diameter of inhibition was 16 mm. The ER9 isolate exhibited antibiotic activity against the isolates of Klebsiella pneumoniae (ER54), Bacillus cereus (ER53), and Staphylococcus aureus (ER52). A maximum average inhibition zone of 18 mm was observed against Staphylococcus aureus ER52. Of the four isolates, ER38 and ER14 demonstrated the highest mean zone inhibition against the ER53 Bacillus cereus isolate at 28 and 29 mm, respectively. PCR results from rhizobacterial isolates were sent for sequencing. The ER6 isolate and the Enterobacter quasihormaechei strain WCHEs120003 (accession no. NR_180451.1) were 97% similar while the ER9 isolate and Kosakonia oryzendophytica strain REICA_082 (accession No. NR_125586.1) had 98% homology. The ER14 isolate's homology percentage with Cronobacter turicensis z3032 (accession No. NR_102802.1) was 99% while that between Kosakonia pseudosacchari

strain JM-387 (accession No. NR_135211.1) and ER38 was 98%. The findings show that the rhizobacteria ER6 isolate has potential antimicrobial activity against the three studied pathogenic isolates E. coli ER51, B. cereus ER53 and K. pneumoniae ER54, respectively.

Keywords: DNA sequencing, Rhizobacteria, Antibiotic action, Molecular.

التشخيص الجزيئي والفعالية المضادة المايكروبية للرايزوبكتريا المعزولة من شمال العراق

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الخلاصة

تم عزل أربعة عزلات من الرايزوبكتريا من العقد الجذرية لنباتات بقولية مختلفة جمعت من مناطق زراعية مختلفة من شمال العراق في فصل الشتاء من السنة 2023. تم اختبار العزلات الأربعة للفعالية المضادة للمايكروبات. عند تطبيق الراشح لمزرعة التخمير السائلة للرايزوبكتريا العزلة ER6 أظهرت فعالية مثبطة تجاه العزلة المرضية *Escherichia coli* العزلة ER51. معدل قطر التثبيط وصل الى 16 ملم عند اجراء اختبار الفعالية المضادة للمايكروبات. أظهرت العزلة ER9 فعالية مضادة للمايكروبات تجاه *Staphylococcus aureus* العزلة ER52 و*Bacillus cereus* العزلة ER53 و*Klebsiella pneumoniae* العزلة ER54، حيث لوحظ اقصى معدل منطقة تثبيط تجاه *Staphylococcus aureus* العزلة ER52 والذي بلغ 18 ملم. من بين أربعة عزلات، أظهرت العزلتين ER38 وER14 اقصى معدل منطقة تثبيط تجاه العزلة المرضية *Bacillus cereus* العزلة ER53 والذي بلغ 28 و29 ملم، على التوالي. تم أجراء تفاعل البوليميراز المتسلسل (PCR). أظهرت تسلسلات القواعد النايتروجينية بنسبة 97% للعزلة ER6 مع *Enterobacter quasihormaechei* العزلة WCHEs120003 (رقم انضمام NR_180451.1). تماثل بنسبة 98% بين العزلة ER9 و*Kosakonia oryzendophytica* العزلة REICA_082 (رقم انضمام NR_125586.1). ونسبة تماثل للعزلة ER14 كان 99% مع *Cronobacter turicensis* العزلة z3032 (رقم انضمام NR_102802.1). والتماثل كان بنسبة 98% ما بين العزلة ER38 و*Kosakonia pseudosacchari* العزلة JM-387 (رقم انضمام NR_135211). نستنتج أن الرايزوبكتريا العزلة ER6 تمتلك فعالية مضادة للجراثيم كامنة تجاه ثلاثة

عزلات مرضية مدروسة وهي *E. coli* 51 و *B. cereus* ER53 و *K. pneumoniae* ER53 على التوالي.

كلمات مفتاحية: تحديد تسلسل الحامض النووي الديوكسي رايبوزي، رايزوبكتريا، فعل المضاد الحيوي، جزيئي.

Introduction

According to (8), nitrogen-fixing organisms are responsible for the great bulk of nitrogen fixation activities. Through the enzyme nitrogenase, nitrogen-fixing bacteria biologically transform atmospheric di-nitrogen (N2) into biologically active nitrogen (33). Plant growth-promoting rhizobacteria (PGPR) can promote plant growth in several ways directly through nitrogen fixation or indirectly by increasing nutrient availability, shoot and root development and phytohormone production (12). Gram-negative rhizobacteria inhabit soil. Apart from fixing atmospheric nitrogen, members of the phylum Proteobacteria also supply an extra source of nitrogen that is made available to plants through photosynthesis, hence aiding in the plants' absorption of macronutrients (10).

The process of nodulation in legumes consists of two generally accepted phases. The plant first draws in rhizobacteria by secreting isoflavonoids and flavonoids from its roots. Rhizobacteria produce and secrete nod factors in response to signals received by the *Rhizobium*. These factors then pierce the cuticle of the roots of leguminous plants. This stage, which leads to infection, is indicated by the development of an infection thread that emerges from the division and passes through the root cortex (30). Because it uses the enzyme nitrogenase, which is produced and shielded by legume hemoglobin, to convert atmospheric nitrogen into ammonia, the bacteroid is a modified version of rhizobacteria. It comes from the cells of the host plant and gives the root nodules their pink hue (5). The root nodules are categorized as determinate or indeterminate based on the genes governing their meristematic activity patterns (14). Because it takes 16 molecules of ATP to break down one molecule of N2, fixing nitrogen is an energy-intensive operation. In addition to requiring 12 ATP molecules for the absorption and transport of NH4+, 28 ATP molecules are used as energy (6).

As for rhizobacteria genes, the quantity of genome sequences that are accessible in open databases is continuously rising, even if there are not many whole and completely annotated rhizobacteria genome structures. According to (26), whole genome sequencing can offer fresh perspectives on genetic features that support symbiotic-related functions. These include bacterial adaptation to the rhizosphere, effective competition mechanisms with other bacteria, the capacity to form intricate signaling relationships with legumes, the ability to penetrate roots without inciting plant defenses, and ultimately nitrogen fixation within the host. The rhizobacteria that infect *Ensifer meliloti*, the alfalfa plant, have been found to carry two sizable plasmids known as megaplasmids. The first, the pSymA megaplasmid, is 1410 kb in size while the second, pSymB, is about 1690 kb. They play a significant part in the symbiotic connection and carry genes required for the synthesis of foreign polysaccharides.

The symbiotic genes *fix*, *nif*, and *nod* are found on megaplasmid A. Up to 3690 kb of chromosomes make up the rhizobacteria (21). According to (13), this chromosome

has genes that produce nitrogenous bases, amino acids, antibiotic resistance, and nitrogen fixation. Antimicrobial compounds are generated by microbes in competition with one another for available nutrients and niches. The types of these expelled microorganisms are diverse and include lytic agents (lysozymes), bacteriocins, broad-spectrum non-ribosomal antibiotics, and metabolic products (organic acids) (24). Antibacterial peptides produced by ribosome synthesis and released by bacteria are known as bacteriocins (3). They differ from antibiotics in that they are generated in the ribosome, are active at very low doses, and inhibit organisms closely related to the producer strains (3).

Depending on the target strain's identity, growth conditions, stage of development, and bacteriocin concentration, bacteriocins can be either bacteriostatic or bactericidal, meaning they hinder the growth of the target organism (22). Most bacteria, whether gram-positive or gram-negative, create one or more types of bacteriocins; however, archaea can also manufacture antimicrobials called archaeocins, which resemble bacteriocins (11 and 25). This study shed light on the potential role of local rhizobacterial isolates as antimicrobial agents.

Materials and Methods

Plant samples were collected from various farms in the north of Iraq-Nineveh. They were then examined at the molecular genetics laboratory of the College of Education for Pure Science, University of Mosul.

Rhizobacteria isolation: The four leguminous plants selected for this study were the *Medicago sativa* L. *Vigna unguiculata* L., *Phaseolus vulgaris* L. and *Albizia adoratissima* L collected from several agricultural locations in northern Iraq. Running tap water was used to wash away the soil attached to the roots. Fresh pinkish and mature nodules were selected and immersed in glass beakers containing distilled sterile water for two to four minutes. The root nodules were then transferred and immersed in 70% ethanol. Following that, they were repeatedly cleaned with distilled sterile water to remove any remaining ethanol and alcohol residue, and then submerged for up to 15 minutes in 30% sodium hypochlorite (NaOCl). The root nodules were cleaned with distilled sterile water to remove any remaining sodium hypochlorite (9).

Crushing of the nodules and dilutions were done according to (9). From deletions 0.5 mL were transferred to YEMA medium (agar: 15, K2HPO4: 0.5, MgSO4: 0.2, NaCl: 0.1, mannitol: 10, yeast extract: 0.4) g/l. Spreading was done with an L-shaped glass rod. The plates were then incubated at a temperature of 28 °C (9).

Antimicrobial susceptibility test: Rhizobacterial isolates were cultured in YEM broth medium for 24 h at 28 °C for activation. Then 0.1 mL of fermented culture was transferred into a fresh YEM broth medium and incubated for 24 h at 28 °C. Centrifugation was carried out at 6000 rpm to get free cells suspension. Mueller-Hinton agar plates were prepared by boring 6 mm pores in a Mueller-Hinton agar medium using a sterile cork borer. The wells were filled with 100 µL of free cells suspension for each rhizobacterial isolate as well as 100 µL of normal saline as a negative control. After 24-48 h of incubation at 37 °C, the plates were examined to see whether zones of inhibition had formed around the well (17).

Genomic DNA extraction: The Geneaid Biotech Ltd. genomic DNA isolation kit and instructions were used to recover genomic DNA directly from the rhizobacterial isolates. After measuring the concentration and purity of the genomic DNA, it was stored at -20 °C until needed again. The concentration and purity of the DNA extracted from rhizobacterial isolates were measured using a nanodrop device at a wavelength of 260/280. One microliter of extracted DNA sample was placed on the nanodrop pad to measure the concentration and purity of the sample. The extracted DNA was kept at -20 ºC for future use.

PCR-amplification of 16S rRNA gene: Polymerase chain reaction (PCR) experiments were conducted for the region of the 16S rRNA gene which was amplified using the universal primers 27F (AGAGTTTGATCMTGGCTCAG) and while the backward specialist primer 1522R (AAGGAGGTGATCCARCCGCA) was used for the polymerase chain reaction (PCR) technique (19). PCR was conducted in 20 µl volume reactions using Promega (USA.) provided GoTaq® G2 Green Master Mix containing G2 DNA polymerase, dNTPs, MgCl2, and an appropriate buffer. This master mix was premixed with two dyes (blue and yellow) to help progress the movement of PCR bands. Each 20µl PCR reaction contained the following ingredients: 10µl Master mix, 2µl of 10µM forward primer, 2µl of 10µM reverse primer, variable amount of DNA around 250 ng, and variable amounts of nuclease free water. The components were collected in a 0.2 ml Eppendorf tube and placed in a thermocycler apparatus (Esco Scientific, Singapore).

After denaturation at 95 °C for 5 min. the samples underwent 30 cycles of temperature denaturation at 95 °C for 30 sec., annealing at 55 °C for 30 sec., extension at 72 °C for 1 min., and finally chain elongation at 72 °C for 5 min. The PCR products were then transferred to a 1% (w/v) agarose gel for separation. As advised by the manufacturer, 100 ng of template DNA total was added. For the 16S rRNA gene PCR protocol for the 1% agarose gel, the PCR products were separated and stained with Midori Green Advance DNA stain. A 100-bp DNA marker from New England Biolabs, UK was employed as a molecular weight marker.

Sequencing DNA and searching for homology: Following the PCR of the rhizobacterial isolates 16S rRNA gene, product was forwarded to Psomagen Inc., USA for sequencing. Using the National Center for Biotechnology Information (NCBI) BLAST program, the recovered sequences were compared for similarity to published genes that were in the GenBank.

Results and Discussion

Rhizobacteria isolation: After rhizobacteria were isolated and purified, four isolates, ER6, ER9, ER14 and ER38, from different agricultural areas were obtained. ER6 was recovered from the *Medicago sativa* L. root nodules, ER9 from *Vigna unguiculata* L., ER14 from *Phaseolus vulgaris* L., and ER38 from *Albizia odoratissima* L. (Table 1).

Table 1: Host plants of the isolated rhizobacteria.

Isolation No.	Host plant	Collection area
ER6	*Medicago sativa*	Nimrod/Nineveh
ER9	*Vigna unguiculata* L.	Al-Qayara/Nineveh
ER14	*Phaseolus vulgaris* L.	Yaramja/Nineveh
ER38	*Albizia adoratissima* L.	Erbil/Kurdistan

(20) found that rhizobacteria and non-leguminous plants, including potatoes, have a symbiotic connection. They were able to isolate a strain of rhizobacteria that can fix nitrogen and grow freely in the root areas of potato plants. This isolation was verified by growing the bacteria on a nitrogen-free medium and by looking at its 16S rRNA gene sequence, which revealed that the bacteria formed colonies at the points where the secondary and primary roots joined.

Additionally, 24 isolates of rhizobacteria were obtained from the roots of bean plants in six different places by (4). The isolates displayed normal traits of rhizobial bacteria during phenotypic diagnosis, and it was shown that each one could form nodules in the host plants. Effective rhizobia were found in ivory soils, and the study also showed their ability to form nodules in common bean plants.

Antimicrobial test: Table 2 displays the results of the antibacterial activity. The effect of different filtrates of the liquid culture of the rhizobacteria isolates on the pathogenic bacteria under study varied. The ER6 rhizobacterial isolate's filtered liquid culture demonstrated antimicrobial efficacy against *E. coli* ER51, bacillus cereus ER53, and *Klebsiella pneumoniae* ER54 which exhibited an inhibitory zone having a maximum mean diameter of 30 mm. (Fig.1). However, there was no discernible impact on *Staphylococcus aureus* ER52. With mean diameter inhibition zones of 18, 15, and 15 mm, respectively, the filtered liquid culture of ER9 rhizobacteria showed efficiency against the Staphylococcus aureus ER52, *Bacillus cereus* ER53, and *Klebsiella pneumoniae* ER54 isolates. Only against *Bacillus cereus* ER53 did the rhizobacteria ER14 exhibit antimicrobial action, with a mean diameter inhibition zone of 29 mm. With mean diameter inhibition zones of 28 and 16 mm, respectively, isolate ER38 demonstrated efficacy against *Bacillus cereus* ER53 and *Klebsiella pneumoniae* ER54 (Table 2 and Fig. 1).

(1) examined the antimicrobial activity of 25 rhizobacteria isolates against five distinct pathogenic bacterial species. Among these, the *Ensifer meliloti* MH15 isolate showed the highest rate of inhibition diameter at 25 mm against the pathogenic *E. coli* MJ20 bacteria, while *Ensifer meliloti* MH10 showed the highest rate of inhibition diameter at 27 mm against the B. *cereus* MJ24 bacteria. The isolates that the researcher used had an impact on the S. *aureus* MJ23 bacteria; for example, the isolate *R. leguminosarum* bv. *trifolii* MH3 had the highest rate of inhibition diameter reaching 21 mm, while isolate *Ensifer meliloti* MH9 had 17 mm against the pathogenic bacterium *K. pneumonia* MJ21. On the effect of fermented culture filtrate of rhizobial bacteria isolates against pathogenic bacteria *Klebsiella pneumoniae* AS63, the results of this study differ from those of (31), indicating that half of the isolates in this study had an inhibitory effect against these pathogenic bacteria.

Rhizobium generates antimicrobial substances (AMS) with broad and narrow spectrum antioxidant activity against some pathogenic bacterial species, including

Salmonella typhi, *Escherichia coli*, *Shigella*, *B. cereus*, *S. aureus*, *Proteus mirabilis*, and *Serratia*, according to (15). Several diagnostic studies on these compounds have demonstrated their similarity to bacteriocins. (2) investigated the antimicrobial activity of rhizobial bacteria filtrate culture against the gram-negative *Pseudomonas aeruginosa* bacteria. The results indicated that the inhibition zone induced by the bacteria reached 20 mm, and that the bacteria also had an effect on the pathogenic bacteria *Escherichia coli*, with the inhibition zone reaching 23 mm.

Table 2: Antimicrobial activity of the liquid culture filtrate of isolated rhizobacteria against pathogenic bacteria isolates based on the millimeter-scale inhibition zone.

Isolation No.	*E. coli* ER51	*S. aureus* ER52	*B. cereus* ER53	*K. pneumoniae* ER54
ER6	16*	0.0	12	30
ER9	0.0	18	15	15
ER14	0.0	0.0	29	0.0
ER38	0.0	0.0	28	16

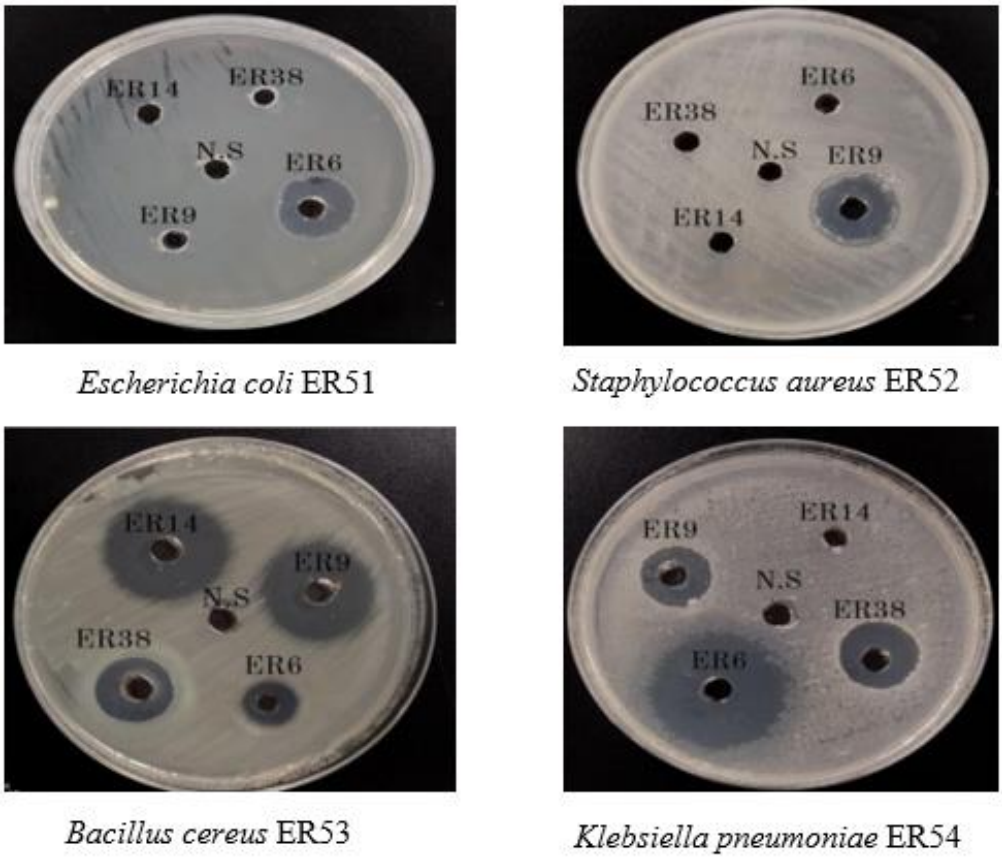

Fig. 1: Filtered liquid culture of rhizobacterial isolates' antimicrobial activity against the pathogenic bacteria under study. (NS): Standard salinity (control)

Extracted DNA purity: In this work, the genomic DNA extracted directly from rhizobacterial isolates ER4, ER9, ER14 and ER38 were found to have purities of between 1.8 - 2.0, which is acceptable according to (28).

Particular 16S rRNA gene polymerase chain reaction method: Results indicated the presence of amplified DNA bands with an estimated size of about 1485 bp. This is the consequence of the general and specific primers of the gene 16S rRNA replicating with

DNA samples of comparable sizes as determined by the DNA ladder (Fig. 2). After obtaining bigger packet sizes of roughly 1500 bp, (16) observed that the results of this study converged with his findings. This is because the nitrogenous bases found in the specialized primers and the genomic DNA of the rhizobia isolates under study have comparable or common sequences. The nitrogenous base sequences in the specialized primers match as a result of this similarity.

The PCR chain reaction is completed by nitrogenous bases found in the genomic DNA of rhizobia isolates that were employed in this study and specialized primer sequences (1522R and 27F) (7). The size of the bands in this gene reached 1200 bp, according to (18), who examined the genetic and phenotypic patterns of eight rhizobacteria isolates obtained from the root nodules of the Arachis hypogaea plant. The findings of this investigation differed from (29), who examined five different species of rhizobacteria and noted DNA bands of 1250 bp in size.

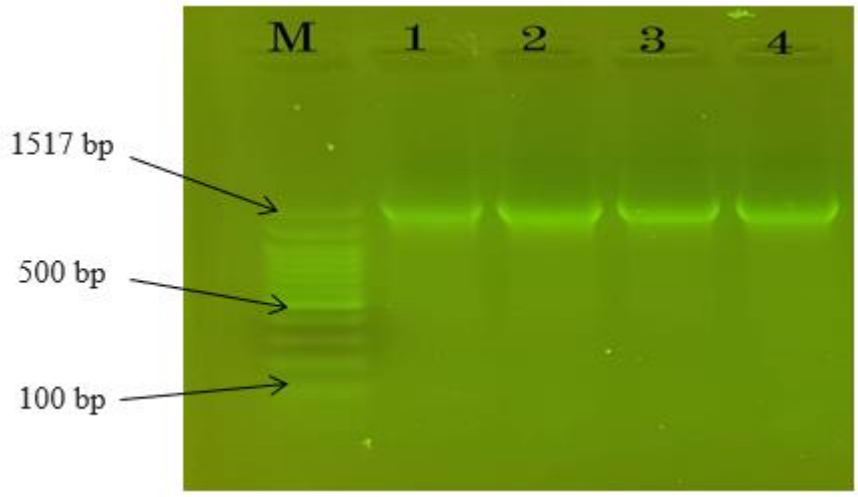

Fig. 2: PCR product content of four selected rhizobacterial isolates electrophoresed in 1% agarose gel. M: Reference marker, ER6, ER9, ER14, ER38, 1 and 2.

Finding the nitrogenous base sequence in a particular PCR product: The following rhizobacterial isolates were identified: ER6, which was isolated from *Medicago sativa* L.; ER9 from *Vigna unguiculata* L.; ER14 from *Phaseolus vulgaris* L.; and ER38 from *Albizia adoratissima* L. The nitrogenous base sequences of the products of the specific PCR of pure DNA for the aforementioned isolates were studied. Genomic DNA was used to perform molecular characterization on the four isolates. DNA fragments of 1300 bp were amplified using PCR of the 16S rRNA gene. The species identity of the isolates was ascertained by sequencing the PCR results using BLAST software.

Through the comparison of 16S rRNA gene sequences with GenBank entries, all four rhizobacterial isolates were recognized to species level, with homology levels ranging from 97% to 99%. The NCBI received the strain sequences of ER6, ER9, ER9, and ER38 and were assigned accession numbers PP70242.1, PP704713, PP708941.1, and PP708935.1, in that order. According to sequences, the ER6 isolate and the *Enterobacter quasihormaechei* strain WCHEs120003 (accession no. NR_180451.1) were 97% similar while it was 98% for ER9 and the *Kosakonia oryzendophytica* strain REICA_082 (NR_125586.1). The ER14 isolate's homology was 99% with the

Cronobacter turicensis strain z3032 (NR_102802.1) and 98% between the *Kosakonia pseudosacchari* strain JM-387 (NR_135211.1) and ER38.

Conclusions

The study showed the rhizobacteria ER6 isolate having potential antimicrobial activity against three of the studied pathogenic isolates.

Supplementary Materials:

Materials were supplemented from the College of Education for Pure Science, University of Mosul.

Author Contributions:

Author 1: Methodology, writing-original draft preparation; Author 2: Methodology, revision; Author 3: review and editing.

Funding:

This research received no external funding.

Institutional Review Board Statement:

The study was conducted in accordance with the protocol authorized by the Department of Biology, College of Education for Pure Science, University of Mosul-Iraq.

Informed Consent Statement:

Not applicable.

Data Availability Statement:

Data available upon request.

Conflicts of Interest:

The authors declare no conflict of interest.

Acknowledgments:

The authors are grateful for the facilities provided for this study by the College of Education for Pure Science, University of Mosul.

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References

1. Al-Jobouri, M. H. (2023). Isolation, biochemical and molecular characterization of local rhizobial isolates and evaluation of antimicrobial activity of crude extract. M.Sc. Thesis, College of Education for Pure Science, University of Mosul, Mosul, Iraq.
2. Almihyawi, R. A., Musazade, E., Alhussany, N., Zhang, S., and Chen, H. (2024). Production and characterization of bacterial cellulose by Rhizobium sp. isolated from bean root. Scientific Reports, 14(1): 10848. https://doi.org/10.1038/s41598-024-61619-w.
3. Arnison, P. G., Bibb, M. J., Bierbaum, G., Bowers, A. A., Bugni, T. S., Bulaj, G., ... and Van Der Donk, W. A. (2013). Ribosomally synthesized and post-translationally modified peptide natural products: overview and recommendations

for a universal nomenclature. Natural product reports, 30(1): 108-160. https://doi.org/10.1039/C2NP20085F.

4. Atsé, M. P. A., N'Gbesso, M. F. D. P., Konaté, I., Coulibaly, N. D., Amani, K., Yéo, K. T., and Butare, L. (2023). Isolation and Authentication of Local Rhizobia Nodulating Common Bean (Phaseolus vulgaris L.) in Different Agro-Ecological Zones of Côte d'Ivoire. Advances in Microbiology, 13(9): 435-447. https://doi.org/10.4236/aim.2023.139028.
5. Brewin, N. J. (1998). Tissue and cell invasion by Rhizobium: the structure and development of infection threads and symbiosomes. In The Rhizobiaceae: Molecular biology of model plant-associated bacteria, 417-429. https://doi.org/10.1007/978-94-011-5060-6_22.
6. Buscot, F. (2005). What are soils?. In Microorganisms in soils: roles in genesis and functions (pp. 3-17). Berlin, Heidelberg: Springer Berlin Heidelberg. https://doi.org/10.1007/3-540-26609-7_1.
7. Bustin, S. A. (2002). INVITED REVIEW Quantification of mRNA using real-time reverse transcription PCR (RT-PCR): trends and problems. Journal of molecular endocrinology, 29: 23-39.
8. Canfield, D. E., Glazer, A. N., and Falkowski, P. G. (2010). The evolution and future of Earth's nitrogen cycle. science, 330(6001): 192-196. https://doi.org/10.1126/science.1186120.
9. Chhetri, T. K., Subedee, B. R., and Pant, B. (2019). Isolation, identification and production of encapsulated Bradyrhizobium japonicum and study on their viability. Nepal Journal of Biotechnology, 7(1): 39-49. https://doi.org/10.3126/njb.v7i1.26950.
10. Compton, K. K., and Scharf, B. E. (2021). Rhizobial chemoattractants, the taste and preferences of legume symbionts. Frontiers in Plant Science, 12: 686465. https://doi.org/10.3389/fpls.2021.686465.
11. Cotter, P. D., Ross, R. P., and Hill, C. (2013). Bacteriocins—a viable alternative to antibiotics?. Nature Reviews Microbiology, 11(2): 95-105. https://doi.org/10.1038/nrmicro2937.
12. De Andrade, L. A., Santos, C. H. B., Frezarin, E. T., Sales, L. R., and Rigobelo, E. C. (2023). Plant growth-promoting rhizobacteria for sustainable agricultural production. Microorganisms, 11(4): 1088. https://doi.org/10.3390/microorganisms11041088.
13. DiCenzo, G. C., MacLean, A. M., Milunovic, B., Golding, G. B., and Finan, T. M. (2014). Examination of prokaryotic multipartite genome evolution through experimental genome reduction. PLoS genetics, 10(10): e1004742. https://doi.org/10.1371/journal.pgen.1004742.
14. Ibáñez, F., Wall, L., and Fabra, A. (2017). Starting points in plant-bacteria nitrogen-fixing symbioses: intercellular invasion of the roots. Journal of Experimental Botany, 68(8): 1905-1918. https://doi.org/10.1093/jxb/erw387.
15. Joseph, M. V., Desai, J. D., and Desai, A. J. (1983). Production of antimicrobial and bacteriocin-like substances by Rhizobium trifolii. Applied and Environmental Microbiology, 45(2): 532-535. https://doi.org/10.1128/aem.45.2.532-535.1983.

16. Jumaah, O. H. (2022). Isolation and characterization of local isolates of rhizobial bacteria and study their antimicrobial activity M.Sc. Thesis, College of Education for Pure Science, Mosul University, Mosul, Iraq.
17. Kalalou, I., Faid, M., and Touhami Ahami, A. (2004). Extending shelf life of fresh minced camel meat at ambient temperature by Lactobacillus dlbrueckii subsp. delbrueckii. Electronic journal of Biotechnology, 7(3): 5-6.
18. Khalid, R., Zhang, X. X., Hayat, R., and Ahmed, M. (2020). Molecular characteristics of rhizobia isolated from Arachis hypogaea grown under stress environment. Sustainability, 12(15): 6259. https://doi.org/10.3390/su12156259.
19. Lane, D. J. (1991). 16S/23S rRNA sequencing. In: Stackebrandt, E., and Goodfellow, M. Eds. Nucleic acid techniques in bacterial systematics. John Wiley and Sons, United Kingdom, 115-175.
20. Naqqash, T., Malik, K. A., Imran, A., Hameed, S., Shahid, M., Hanif, M. K., ... and van Elsas, J. D. (2024). Isolation and characterization of Rhizobium from non-leguminous potato plants: New frontiers in Rhizobium research. Biology and Fertility of Soils, 60(3): 307-325. https://doi.org/10.1007/s00374-024-01800-5.
21. Nelson, M., Guhlin, J., Epstein, B., Tiffin, P., and Sadowsky, M. J. (2018). The complete replicons of 16 Ensifer meliloti strains offer insights into intra-and inter-replicon gene transfer, transposon-associated loci, and repeat elements. Microbial Genomics, 4(5): e000174. https://doi.org/10.1099/mgen.0.000174.
22. Nes, I. F., Brede, D. A., and Holo, H. (2006). The nonlantibiotic heat-stable bacteriocins in Gram-positive bacteria. In Handbook of biologically active peptides, 107-114. https://doi.org/10.1016/B978-012369442-3/50020-9
23. Petit, A., Stougaard, J., Kühle, A., Marcker, K. A., and Tempé, J. (1987). Transformation and regeneration of the legume Lotus corniculatus: a system for molecular studies of symbiotic nitrogen fixation. Molecular and General Genetics MGG, 207(2): 245-250. https://doi.org/10.1007/BF00331585.
24. Riley, M. A. (1998). Molecular mechanisms of bacteriocin evolution. Annual review of genetics, 32(1): 255-278. https://doi.org/10.1146/annurev.genet.32.1.255.
25. Riley, M. A., and Wertz, J. E. (2002). Bacteriocins: evolution, ecology, and application. Annual Reviews in Microbiology, 56(1): 117-137. https://doi.org/10.1146/annurev.micro.56.012302.161024.
26. Sánchez-Cañizares, C., Jorrín, B., Durán, D., Nadendla, S., Albareda, M., Rubio-Sanz, L., ... and Imperial, J. (2018). Genomic diversity in the endosymbiotic bacterium Rhizobium leguminosarum. Genes, 9(2): 60. https://doi.org/10.3390/genes9020060.
27. Savini, V. (Ed.). (2018). Pet-to-man travelling staphylococci: A world in progress. Academic Press.
28. Shen, C. H. (2019). Chapter 6—Extraction and Purification of Nucleic Acids and Proteins. Diagnostic Molecular Biology; Shen, C.-H., Ed.; Academic Press: Cambridge, MA, USA, 143-166.
29. Shihab, M. A., and Alkurtany, E. S. (2023). Isolation and diagnosis of the root nodule bacteria associated with mung bean plant in gypsiferous soil and testing the promotional criterion of isolates. In IOP Conference Series: Earth and

Environmental Science, 1259(1): 012019. DOI: 10.1088/1755-1315/1259/1/012019.

30. Shumilina, J., Soboleva, A., Abakumov, E., Shtark, O. Y., Zhukov, V. A., and Frolov, A. (2023). Signaling in legume–Rhizobia symbiosis. International journal of molecular sciences, 24(24): 17397. https://doi.org/10.3390/ijms242417397.
31. Suliman, A. A. (2021). Isolation and molecular identification of number of rhizobia genera and study their antimicrobial activity against a number of pathogenic bacteria. M.Sc. Thesis, College of Education for Pure Science, Mosul University, Mosul, Iraq.
32. Vincent, J. M. (1970). A Manual for the Practical Study of the Root-Nodule Bacteria. I. B. P. Handbook No. Blackwell Scientific Publication Ltd; Oxford. U.K., 164.
33. Zhang, W., Chen, Y., Huang, K., Wang, F., and Mei, Z. (2023). Molecular mechanism and agricultural application of the NifA–NifL system for nitrogen fixation. International journal of molecular sciences, 24(2): 907. https://doi.org/10.3390/ijms24020907.