Journal of Applied and Natural Science
17(3), 1316 - 1324 (2025)
ISSN : 0974-9411 (Print), 2231-5209 (Online)
journals.ansfoundation.org

Research Article

Role of *Pseudomonas aeruginosa* lipopolysaccharide in controlling bacterial biofilm formation

Safaa Ismail Rashid AL-Obaidi
Department of Biology, College of Education for Pure Science, Mosul University, Mosul, Iraq
Rana Khalid Ahmed AL-Tareea
Department of Biology, College of Education for Pure Science, Mosul University, Mosul, Iraq
Dhuha Jasem Mohammed
Department of Biology, College of Education for Pure Science, Mosul University, Mosul, Iraq
Najwa Ibrahim Khaleel Al-Barhawee*
Department of Biology, College of Education for Pure Science, Mosul University, Mosul, Iraq

*Corresponding author. E-mail: dr.najwa@uomosul.edu.iq

Article Info
https://doi.org/10.31018/jans.v17i3.6697
Received: March 20, 2025
Revised: August 27, 2025
Accepted: September 04, 2025

How to Cite
Al-Obaidi, S.I.R. *et al.* (2025). Role of *Pseudomonas aeruginosa* lipopolysaccharide in controlling bacterial biofilm formation. *Journal of Applied and Natural Science*, 17(3), 1316 - 1324. https://doi.org/10.31018/jans.v17i3.6697

Abstract
Biofilm is a virulence factor used by pathogenic bacteria to facilitate their attachment to the host, thereby facilitating infection. Lipopolysaccharide(LPS) is a large amphipathic molecule found in the outer membrane of Gram-negative bacteria, was extracted from *Pseudomonas aeruginosa* by chloroform-methanol method, lyophilized, and analyzed by gas chromatography-mass spectrometry (GC-MS) and found many chemical compounds like, (11,14-eicosadienoic acid methyl ester, cis-13-Octadecenoic acid, n-Hexadecanoic acid, Octadecanoic acid, and 9,12-octadecadienoyl chloride, (Z, Z), to determine their inhibitory effect at four concentrations (25, 50, 100 and 200 mg/cm3) on biofilm formation in four species of bacteria *Agrobacterium tumefaciens*, *Mesorhizobium cicero, Staphylococcus aureus,* and *Escherichia coli*. It was found that the concentration of 100 mg/cm³ was effective in inhibiting the formation of biofilms in the three pathological species.Nevertheless, it had no inhibitory effect on the formation of biofilms by *M.cicero*. Some genes encoding proteins specific to the adhesion of bacteria to their host were also identified as part of the biofilm mechanism.The results showed that the studied bacteria retained some of these genes and lost others, as evidenced by the appearance of bundles on the agarose gel or not.In *A. tumefaciens*, the celA and celR genes were found to encode cellulose fibres that allow it to adhere to its plant host, *M. cicero*. *M. cicero* and *S.aureus* were found to possess both the NodC and eno genes, but they lost the Nod and ebps genes, respectively. Meanwhile, *E. coli* exhibited a loss of the FimH gene, which is responsible for its association with the pathogenicity.

Keywords: Biofilms, Gas chromatography-Mass spectrometry (GC-MS), Lipopolysaccharide (LPS), Pathogenic bacteria, *Pseudomonas aeruginosa*

INTRODUCTION

Pseudomonas aeruginosa is an aerobic,Gram-negative bacterium, motile bacilli, that grows in a variety of environments and tolerates a wide range of physical conditions. *Pseudomonas aeruginosa* causes acquired infections and damaged host tissue. It has the ability to form a biofilm, which gives it resistance to antibiotics. Lipopolysaccharides (LPS) are considered virulence factors that lead to adhesions and cause tissue damage due to endotoxic activity. LPS is composed of lipid A, which consists of an inner and outer core, both of which contain a structure called 3-deoxy-D-manno-octulosonic acid (KDO) and heptoses. The O-antigen is highly variable and consists of repeating oligosaccharide units (Dardelle *et al.*, 2023). Lipid A is a glucosamine based phospholipid that functions as the hydrophilic anchor to the LPS molecule in the outer membrane of Gram-negative bacteria. It is a very potent pyrogen for which the immune system mounts an immediate response. (Xi *et al.*,2023). Biofilms are groups of cells that are adhered to each other by a group of extracellular polymeric compounds, which consist of exopolysaccharides, proteins, and nucleic acids. These membranes are formed on non-living surfaces, including glass and metals, and may also form on living surfaces, such as human, animal, and plant tissue (Martinet *et al.*, 2024). The biofilm protects the bacteria

from the effects of antibiotics and the host's immune responses. Therefore, it is a crucial virulence factor for the survival of the bacteria, enabling it to colonise the host's cells and tissues, and thereby enhancing their ability to cause disease (Sharma *et al.*, 2023).

Agrobacterium tumefaciens, a Gram-negative bacterium that moves through soil, rapidly forms biofilms and thrives in the rhizosphere, colonizing plant surfaces, is responsible for causing crown gall disease, which results in the growth and development of gall-like tumours on the stems and roots of several economically valuable plant species by transferring T-DNA from the Ti-plasmid into host plant cells (Chauhan *et al.,* 2021). Exopolysaccharide (EPS) controls the process of surface attachment and provides the means for *A. tumefaciens* to switch from free-floating planktonic cells to surface-attached biofilms; as the bacteria spread infection by colonizing and forming biofilms on plant surfaces, new chemicals are needed to inhibit bacterial growth and prevent biofilm development (Jailani *et al.,* 2022). On the other hand, these bacteria possess two genes, *CelR* and *CelR*, which are involved in the construction of cellulose fibres that enable the bacteria to attach to the plant host (Barnhart *et al.,* 2013).

Mesorhizobium ciceri, formerly called *Rhizobium ciceri* , is a soil-endemic, Gram-positive bacterial species isolated from root nodules of chickpea (*Cicer arietinum*) (Isokar *et al*., 2024). The identification of strains of *Mesorhizobium* spp. that possess the *NodD* and *NodC* genes, two naturally occurring genes that encode nodule (Paço *et al.,* 2019). *Staphylococcus aureus* causes superficial lesions, deep-seated and systemic infections, and toxic syndromes (Touaitia *et al*., 2025). It is infection begins with the adhesion of bacteria to host tissues, using special substances called microbial surface components recognizing adhesive matrix molecules (MSCRAMMs) (Berry *et al*., 2022), using specific genes such as *ebpS*, which encodes elastin binding protein, and *eno*, which encodes laminin binding protein (Tuon *et al*2023). *Escherichia coli* live naturally in the human intestine, so it is one of the members of the Enterobacteriaceae family, and it is a motile, Gram-negative bacillus. It is also considered an opportunistic pathogenic bacterium that causes various diseases in humans (Martinez-Medina, 2021; Rahimi *et al*., 2024). It can produce biofilms, which have an important role in facilitating the process of its adhesion to tissues. *Escherishia coli*, the high binding capacity of the substance encoded by the *FimH* gene can lead to increased bacterial attachment to target cells and increase the pathogenicity of these bacteria, and in addition, this gene can be used as a good tool in rapid diagnostic tests for these bacteria (Hojati *et al.,* 2015). In another study, Foroogh *et al.* (2021) demonstrated that the FimH gene is responsible for bacterial pathogenesis and biofilm production, which are crucial virulence factors in uropathogenic *E. coli* strains. The present study aimed to evaluate in vitro the effect of *Pseudomonas aeruginosa* LPS on biofilm formation as a virulence factor for plant and human pathogenic bacteria, both gram-positive and gram-negative, and for bacterial plant symbionts

MATERIALS AND METHODS

Preparation of bacteria

An Isolated, pure culture of *Pseudomonas aeruginosa* was obtained from the laboratories of the College of Science at Mosul University, Department of Biology. Isolated bacteria was cultured in 5 Litres of Brain Heart Infusion broth medium (BHI) (de Sousa *et al*., 2023), incubated at 37°C for 48-72 hours in a shaker incubator. Cultures were centrifuged at 500 rpm for 30 minutes.The bacterial sediments were washed three times by adding 2 mL of 95% ethyl alcohol and shaken well. Then, they were centrifuged at 3000 rpm for 10 minutes. The precipitation cells were kept in closed tubes in a refrigerator at 4-8°C while using (Sali *et al*., 2021).

Bacterial Lipopolysaccharide (LPS) extraction

The bacterial pellet was resuspended in 10% EDTA and then destroyed by sonication (Ultrasonic Omni International UK) at a frequency of 20,000 vibrations per minute for 30 seconds under refrigerated conditions. The sample was refrigerated, centrifuged, and the supernatant was collected in a clean and sterile test tube. One millilitre of the chloroform/methanol mixture (1:2 v/v) was added to the bacterial pellet-EDTA solution, and then it was covered with paraffin oil and shaken for two hours. Three layers were formed; a layer of chloroform and methanol was taken and dried by placing them inside a hood (Grumov *et al*., 2024).

Lyophilization of LPS

Granules of LPS were dried using a Lyophilizer (Alpha-1-2-Lb, plus 19616, Germany) and stored at 4 °C. The purity of LPS was determined using a Gas Chromatography-Mass Spectrometry (GC-MS) 5973 network mass selective detector (USA) (Scaletti *et al*., 2024).

Analysis of LPS by Gas chromatography-mass spectrometry (GC-MS)

Lyophilized LPS was sent to the University of Basrah for chemical analysis using a gas chromatograph coupled to a GC-MS QP210 ULTRA mass spectrometer (Shimadzu, Japan). The compounds were identified based on their retention times in the GC capillary column and then computer-matched to the mass spectra using the NSTA08 library database and GC-MS Solution software (Al-Rubyee and Al-Barhawi, 2022).

Investigate the ability of LPS to prevent the formation of biofilms

The effect of LPS extracted from the cell wall of *Pseudomonas aeruginosa* bacteria was studied at different concentrations, including (0, 25, 50, 100, 200) µg/cm^3 and prepared based on the method of (Shareef,1998) on the formation of biofilms by the bacteria under study (*Agrobacterium tumefaciens* 1, *Agrobacterium tumefaciens* 2, *Mesorhizobium cicero, Staphylococcus aureus,* and *Escherichia coli*) using a micro-wells dish (MTP 96 wells) and based on the gradation in color intensity formed by the crystal violet dye, the results were recorded according to (Ibrahim, 2016).

Molecular tests

Deoxyribonucleic acid (DNA) extraction

DNA was extracted from bacteria using the protocol provided in the MiniPrep™ Kit (Shen, 2025). 50-100 mg of resuspended bacterial cells were added to 200 µL of isotonic phosphate-buffered saline (PBS), with the pH adjusted to approximately 7.4, in a ZR BashingBead™ Lysis tube (0.1 and 0.5 mm). Then, 750 µL of lysis buffer was added to the mixture. The tube was immobilized in a bead beater fitted with a 2-mL tube holder at maximum speed and was run for ≥5 minutes. The ZR BashingBead™ Lysis tube was then centrifuged in a microcentrifuge at 10,000 × g for 1 minute. Up to 400 µL of supernatant was transferred to a Zymo-Spin™ IV spin filter in a collection tube and centrifuged at 7,000 × g for 1 minute. Next, 200 µL of bacterial DNA binding buffer was added to the filtered solution in the collection tube from the previous step. Then, 800 µL of the mixture was transferred to a Zymo-Spin™ IIC column in a collection tube and centrifuged at 10,000 × g for one minute. The supernatant fraction was discarded, and the column was centrifuged again. Afterwards, 200 µL of DNA prewash buffer was added to the Zymo-Spin™ IIC column placed in a new collection tube and centrifuged at 10,000 × g for one minute. The supernatant was carefully discharged from the Zymo-Spin™ IIC column. Then, 500 µL of DNA wash buffer was added to the column, which was then centrifuged at 10,000 × g for one minute. Finally, the contents of the Zymo-Spin™ IIC column were transferred to a clean 1.5-mL microcentrifuge tube. 100 µL of DNA elution buffer was added directly, and the tube was centrifuged at 10,000 × g for 30 seconds to elute the DNA.

Calculating the purity and concentration of extracted DNA

Using the NanoDrop 2000 spectrophotometer to calculate the concentration and purity of each extracted DNA sample. The concentration of DNA was calculated by the instrument from the absorbance at 260 nm according to the Lambert-Beer law. The 260/280 ratio was used as an indicator of the purity of the DNA samples

Table 1. Primers for amplification of different genes

Genes (Bacteria)	Primer	Sequence	Tm (°C)	GC (%)	References
CelR (*Agrobacterium .tumifaciens*)	Forward	5'- GCGGATCCCATATGACGGCGAGAGTTCT -3'	64.8	57.1	Barnhart *et al.*, 2014
	Reverse	5'-CGGATCCTCAGGCCGCGGCGGCCACGACGCG -3'	76.9	80.6	
CelA (*Agrobacterium.tumifaciens*)	Forward	5'- GCGGATCCCATATGAACAAGGCCATCACAGTC -3'	64.8	53.1	
	Reverse	5'-GCGGATCCTCATTTCACGGCTCCGACAGGCTT -3'	68.7	59.4	
NodD (*Mesorhizobium cicero*)	Forward	5'-ATGCGTTTCAAAGGACTTG -3'	51.1	42.1	Paço *et al.*, 2019
	Reverse	5'- TCACAGCGGGGGCAGCCATCC -3'	65.4	70.0	
NodC (*Mesorhizobium cicero*)	Forward	5'- CGACTCGAGAGAGTTCAACTTTC-3'	55.1	47.8%	Rivas *et al.*, 2007
	Reverse	5'- CTCTAATGTACACAAGGCGC-3'	53.5	50.0	
eno (*Staphylococcus aureus*)	Forward	5'- ACGTGCAGCAGCTGACT-3'	57.4	58.8	Tristan *et al.*, 2003
	Reverse	5'- CAACAGCATTCTTCAGTACCTTC-3'	53.8	43.5	
ebps (*Staphylococcus aureus*)	Forward	5'- CATCCAGAACCAATCGAAGAC-3'	53.4	47.6	
	Reverse	5'-CTTAACAGTTACATCATCATGTTTATCTTTG -3'	53.4	29.0	
FimH (*Escherichia coli*)	Forward	5'- TGCAGAACGGATAAGCCGTGG -3'	57.4	58.8	Johnson and Stell, 2000
	Reverse	5'-GCAGTCACCTGCCCTCCGGTA -3'	53.8	43.5	

Table 2. Mixture of the specific interaction for diagnosis genes

Components		Volum (µl)
Taq PCR PreMix kit (i-Taq)		5
Primer	Forward	1 (10 picomols/µl)
	Reverse	1 (10 picomols/µl)
DNA		1.5
Distill water		16.5
Final volume		25µl

Table 3. Optimal Polymerase chain reaction (PCR) conditions for the detection of the *CelR* and *CelA* genes in *Agrobacterium tumifaciens* and *Agrobacterium rhizogenes*

No.	Phase	Tm (°C)	Time) min.)	No. of cycle
1	Initial Denaturation	94	5	1 cycle
2	Denaturation -2	94	1	
3	Annealing	62	1	35 cycle
4	Extension-1	72	1	
5	Extension -2	72	7	1 cycle

(Koetsier and Cantor, 2019).

Agarose gel electrophoresis of DNA

Electrophoresis was performed to determine the DNA fragments after the extraction process or to detect the result of the PCR interaction in the presence of standard DNA, thereby distinguishing the size of the band and the outcome of the PCR interaction on the agarose gel.

Preparation of the agarose gel

According to Sambrook *et al.* (1989), the agarose gel was prepared at 1% condensation by melting (1) g of agarose in (100) ml of previously prepared Tris/Borate/ EDTA (TBE) buffer. The agarose was heated to boiling and then allowed to cool at (45-50) degrees Celsius. The gel was poured into a repared pouring plate ,which had been set up with an agarose support phate , after the comb was secured to create sample-holding holes. The gel was gently poured to avoid air bubbles and allowed to cool for 30minutes. The comb was gently removed from the solid agarose. The plate was secured to its stand in the horizontal electrophoresis unit, which is represented by the tank used in electrophoresis. The tank was filled with Tris-Borate-EDTA (TBE) buffer, which covered the gel surface.

Sample preparation

Three µl of the processor loading buffer (Intron / Korea) was mixed with 5 µl of DNA to be electrotransferred with loading dye, and then the holes of the gel were filled with this mixture. An electric current of 5 v\c^2 has been applied for 30 min until the sample has reached the other side of the gel. The gel was examined by UV-transillumination at 336 nm after being placed in a pool containing 3 µl of red safe nucleic acid staining solution and 500 ml of distilled water.

Diagnosis of gene

Polymerase Chain Reaction (PCR) interaction in the presence of the gene primers:

DNA extracted from the sex bacterial genera was used as a template to amplify the selected genes using the following specific primers (Table 1). The mixture of specific interactions for diagnosing all genes (Table 2). The tubes were then placed in a thermocycler apparatus at the optimum conditions of: *CelR* and *CelA* detection from *A.tumifaciens* and *A.rhizogenes* (Table 3) *, Nod D* and *Nod C* from *Mesorhizobium* (Table 4), *eno* and *ebps* from *S. aureus* (Table 5)*,* and *fim H* from *E. coli*

Table 4. Optimum Polymerase Chain Reaction (PCR) conditions for the detection of the *NodD* and *NodC* genes in *Mesorhizobium cicero*

Nod D				
No.	Phase	Tm (°C)	Time	No. of cycle
1	Initial Denaturation	94	5 (min)	1 cycle
2	Denaturation -2	94	45 (sec)	
3	Annealing	54	45 (sec)	35 cycle
4	Extension-1	72	1 (min)	
5	Extension -2	72	7 (min)	1 cycle
Nod C				
No.	Phase	Tm (°C)	Time(min)	No. of cycle
1	Initial Denaturation	94	5	1 cycle
2	Denaturation -2	94	1	
3	Annealing	48.5	1	35 cycle
4	Extension-1	72	1	
5	Extension -2	72	7	1 cycle

Table 5. Optimum Polymerase Chain Reaction (PCR) conditions for the detection of the *eno* and *ebps* genes in *Staphylococcus aureus*

No.	Phase	Tm (°C)	Time	No. of cycle
1	Initial Denaturation	94	5 (min)	1 cycle
2	Denaturation -2	94	45 (sec)	
3	Annealing	55	45 (sec)	35 cycle
4	Extension-1	72	45 (sec)	
5	Extension -2	72	7 (min)	1 cycle

Table 6. Optimum Polymerase Chain Reaction (PCR) conditions for the detection of the *fimH* gene in *Escherichia coli*

No.	Phase	Tm °C)	Time	No. of cycle
1	Initial Denaturation	94	5 (min)	1 cycle
2	Denaturation -2	94	30 (sec)	
3	Annealing	54	60(sec)	35 cycle
4	Extension-1	72	60(sec)	
5	Extension -2	72	7 (min)	1 cycle

Table 7. Some of the compounds of *Pseudomonas aeruginosa* Lipopolysaccharide (LPS)

Peak#	R.Time	Area	Area%	Name
1	20.710	2869249	37.03	11,14-Eicosadienoic acid, methyl ester
2	20.765	1935402	24.98	cis-13-Octadecenoic acid
3	18.898	778346	10.05	n-Hexadecanoic acid
4	20.968	511954	6.61	Octadecanoic acid
5	23.720	292021	3.77	9,12-Octadecadienoyl chloride, (Z,Z)-

(Table 6). The DNA ladder (100-10000 bp) was used as a marker, and the DNA samples were electrophoresed on a 2% agarose gel for 1.3 hours at 5 vol/cm². Bands were imaged with a Digital camera.

RESULTS AND DISCUSSION

Psuedomonas aeruginosa was identified earlier at the College of Science, University of Mosul, and obtained approximately 0.5 grams per liter of the Brain Heart Nutrient Medium after drying it in the device in the form of a white powder on which a GC-Mass analysis was conducted to ensure its purity,the results of the analysis showed that LPS contained many compounds in different proportions, which are shown in Table 7 and Fig1.

By observing the colour gradations shown in the microtiter plate templates and comparing them with the positive samples (C+) and negative samples (C-) (Fig. 2), different inhibitory effects were observed according to the concentrations of LPS extracted from the cell wall of *P. aeruginosa* on the ability of the studied bacteria to form biofilms, which may be because LPS is an antibacterial substance with a high ability to inhibit the quorum sensing phenomenon, leading to the inhibition of the bacteria's ability to form biofilms (Laekas-Hameder & Daigle, 2024). *M. cicero* was completely resistant to all concentrations of LPS used. This resistance can be explained by the fact that bacteria forming biofilms can evolve resistance mechanisms, such as coding for enzymes that inactivate antimicrobial substances or excreting them using efflux pumps (Ciofu and Tolker-Nielsen, 2019; Dzianach *et al.,* 2019).

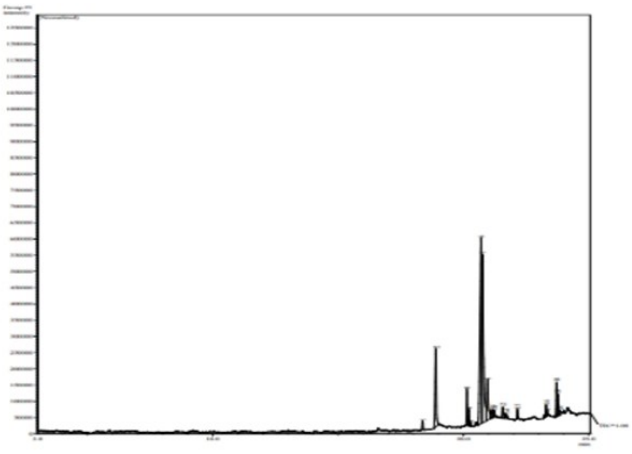

Fig.1.*Cromatography of GC-Mass of Pseudomonas aeruginosa Lipopolysaccharide (LPS)*

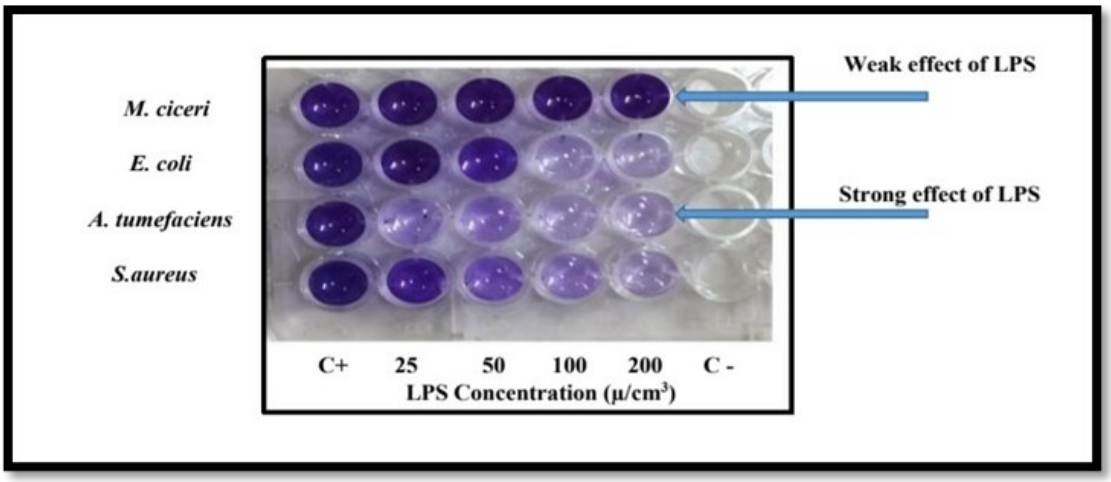

Fig. 2. *Effect on bacterial biofilm formation of four concentrations of Lipopolysaccharide (LPS) Psuedomonas aeruginosa*

Table 8. Concentration and purity of genomic DNA extraction

Bacteria	Conc. (ng/ µl)	Purity (260/280 nm)
Agrobacterium tumifaciens 1	14.7	1.815
Agrobacterium tumifaciens 2	19.4	1.822
Mesorhizobium cicero	23.1	1.894
Staphylococus aureus	21.0	1.815
Escherichia coli	11.2	1.956

Table 9. *Results of Polymerase chain reaction (PCR) genes for different bacteria*

Bacteria	DNA	celR gene	celA gene
Agrobacterium tumifaciens 1	+	+	+
Agrobacterium tumifaciens 2	+	+	+
Bacteria	DNA	*nodD* gene	*nodC* gene
Mesorhizobium cicero	+	-	+
Bacteria	DNA	*eno* gene	*ebps* gene
Staphylococcus aureus	+	+	-
Bacteria	DNA	*fimH* gene	
Escherichia coli	+	-	

Characterization of bacterial isolates according to specific genes

After electrophoresis on a 1% agarose gel for the five samples of genomic DNA, the gel was exposed to ultra-violet radiation at a wavelength of 336 nm in a UV-Transillumination, and five clear DNA bands of large and similar sizes appeared (Fig. 3), consistent with the findings of Al-Barhawi and Ahmed (2022) who studied the polymerase chain reaction and observed seven bands in genomic DNA samples extracted from seven different *Rhizobium* bacterial isolates (*Sinrhizobium meliloti, Sinrhizobium meliloti, Bradyrhizobium elkanii, Rhizobium leguminosarium biovar viciae, Rhizobium leguminosarium biovar phaseoli and Mesorhizobium cicero*, respectively).

The extracted DNA varied in concentration according to the genus and f species bacteria from which it was isolated. Although the purity values were fairly close (1.8-1.9), the concentration was in a range (11-23) ng/µl

Fig. 3. *Gel electrophoresis of genomic DNA extraction from five genera of bacteria*

(Table 8). The ratio of absorbance at (260 and 280) nm was used to assess DNA purity. A ratio of ~ 1.8 is generally accepted as "pure" for DNA. If the ratio is significantly lower (≤1.6), this may indicate the presence of contaminants that absorb strongly at or near 280 nm (Lucena-Aguilar *et al.,* 2016).

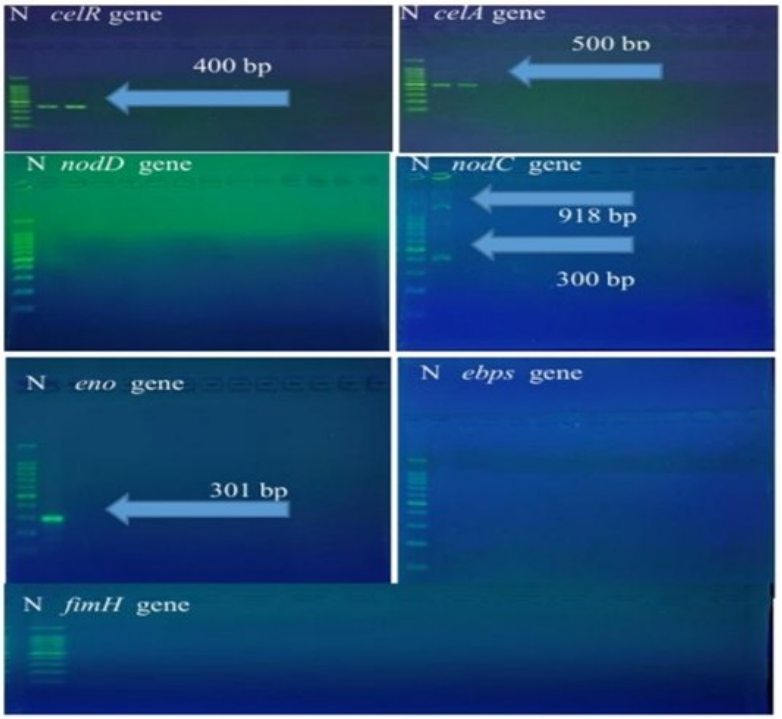

Fig. 4. *PCR products for each genomic DNA extract, with primers specific to the genes required for each bacterial species studied, were electrophoresed on 2% agarose at 5 V/cm² for 1 hour. N: DNA ladder (100)*

In Fig. 4, each gene was amplified using specific primers. It can be observed that bacteria retain some of their genes and lose others within the bacterial genera studied, as also evident in Table 9, which summarizes the results of the gene diagnosis for each bacterial species. This test indicated the ability of the two *Agrobacterium* isolates to produce cellulose fibres that enable them to adhere to their plant host, as evidenced by the amplification of the *celR* and *celR* genes encoding these fiber and their appearance as clear bands on agarose gels. In the bacteria under study, the absence of a band for the *NodD*, *ebps*, and *fimH* genes indicates that it has genes have been lost by deletion, a common method of evolution in bacteria (Bao *et al.*, 2024). It is a mutation in which part of a chromosome or DNA sequence is left behind during DNA replication. Any number of nucleotides can be deleted, from a single base to an entire piece of chromosome, so the term 'lost gene' can be used broadly to refer to the absence of an identified gene and to any allelic variant with loss of function present in a population (Albalat and Cañestro, 2016).

Conclusion

The present study primarily focused on the effect of *Pseudomonas aeruginosa* lipopolysaccharide (LPS) on biofilm formation in a group of human and plant pathogenic bacteria, as well as bacteria coexisting with legumes.The results suggested that *P. aeruginosa* LPS has an inhibitory effect on biofilm formation in *Agrobacterium tumifaciens*, *Staphylococcus aureus*, and *Escherichia coli*, respectively. This had several positive results, including reducing bacterial resistance to antibiotics, improving treatment effectiveness, reducing the spread of infections, and preventing pathogenic biofilm formation in plants. Further studies are needed to investigate the mechanisms responsible for this inhibitory effect and to evaluate the impact of bacterial LPS on pathogenicity and symbiosis, as well as its effect on biofilm formation in *P. aeruginosa*, a virulence factor contributing to antibiotic resistance in this bacterium.

ACKNOWLEDGEMENTS

We are grateful to the University of Mosul for allowing us to conduct this study and have it published in your prestigious journal.

Conflict of interest

The authors declare that they have no conflict of interest.

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