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Short Communication

Expression of *algD* Gene in Single- and Dual-Species Biofilms of *Pseudomonas aeruginosa* and *Staphylococcus aureus* Under Starvation Stress

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ABSTRACT

Dual-species biofilms of *Pseudomonas aeruginosa* and *Staphylococcus aureus* generate difficult-to-treat illnesses. Nutrition stress in biofilms affects physiology, microbial metabolism, and species interactions, impacting bacteria growth and survival. Furthermore, the function of alginate, which is encoded by the *algD* gene, in the production of biofilms has been established. The present study aimed at investigating the impact of starvation on *algD* gene expression in single-species biofilm of *P. aeruginosa* and dual-species biofilms of *P. aeruginosa* and *S. aureus* from hospital sewage. A total of six *P. aeruginosa* and six *S. aureus* isolates were obtained from the microbiology laboratory at the Department of Biology, College of Science, University of Baghdad, Iraq. These isolates are multidrug-resistant and were obtained from various hospital sewage stations in Baghdad city. *P. aeruginosa* and *S. aureus* isolates were co-cultured as single- and dual-species biofilms in full-strength brain heart infusion broth (BHIB) and 1000-fold diluted BHIB. In order to evaluate the level of expression of the *algD* gene in *P. aeruginosa* that had been treated to starvation, the quantitative reverse transcription-polymerase chain reaction (qRT-PCR) was utilized. The results demonstrated that starvation stress significantly ($P< 0.05$) up regulated the expression of *algD* in single-species biofilm (3.117 to 4.532-fold). However, starvation stress down regulated the *algD* expression in dual-species biofilm (0.001 and 0.901-fold). In conclusion, malnutrition up regulated *algD* expression in single-species *P. aeruginosa* biofilms but down regulated it in dual-biofilms. This work helps create biofilm-related disease treatments.

Keywords: *algD*, Dual-species biofilm, *Pseudomonas aeruginosa*, *Staphylococcus aureus*, Starvation .

Introduction

Microbial biofilms are groups of microorganisms that are embedded in an extracellular polymeric matrix and adhered to a living or inert surface in an aquatic environment. that form in response to genotypic and phenotypic characterization. They are mostly encased in a polymeric extracellular matrix that promotes adhesion and cohesion, increasing biofilm resistance to environmental stresses, antimicrobial agents, and nutrient depletion.[1] On the other hand, biofilm is an architecture-complex community that changes dynamically during its existence, based on environmental stress factors such as nutrition.[2] Nutrition stress in a biofilm is a major element impacting physiology, microbial metabolism, and interactions with other species by influencing the development and survival of bacteria within the biofilm.[3] It has been demonstrated that biofilms can be formed at various nutritional concentrations and that they can be created in both rich nutrient and starvation environments.[4]

Due to the fact that bacteria are unicellular animals, they are frequently susceptible to the conditions in their environment, whereas the cell envelope serves as the first line of defense. It is interesting to note that the availability of nutrients influences gene expression, which in turn affects the shape and metabolism of cells.

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The ability of bacteria to adapt to changing environmental conditions has resulted in observations into reactions at both the transcriptional and posttranscriptional levels to environmental perturbations; yet, this phenomenon is still not completely understood.[5]

P. aeruginosa is a human pathogen that causes a variety of infections, such as those of the lungs, urinary tract, burns, and ulcers,[6] recurrent nosocomial infections; moreover, it is one of the primary causes of death in patients with cystic fibrosis.[7] The bacteria can grow on several surfaces, including cystic fibrosis lung mucus plugs, infected catheters, and contact lenses.[8] *P. aeruginosa* biofilm production is a three-step mechanism. It begins with cell adhesion to a surface, then microcolony development, and finally, dispersal and colonize new surfaces. [9] *P. aeruginosa* contains flagella, which allows it to swim in liquid, and type IV pili, which enables it to move across surfaces. [10] When *P. aeruginosa* is cultivated as a biofilm in flow chambers, it usually forms mushroom-shaped multicellular aggregates separated by liquid-filled channels.[11] *P. aeruginosa* has emerged as a model and fundamental bacteria for studying biofilm development.[8]

P. aeruginosa has a biofilm matrix that is primarily made up of exopolysaccharides such alginate, Psl, and Pel. [12] Alginate is chemically composed of β-D-mannuronic acid and α-L-guluronic acid and plays an important role in biofilm firmness and protection. The *algACD* operon in *P. aeruginosa* regulates alginate biosynthesis. Furthermore, alginate is encoded by the *algD* gene, which is a guanosine diphosphate (GDP)-mannose dehydrogenase that converts GDP-mannose to GDP-mannuronic acid. The *algD* gene product regulates alginate production as well as *alg* transcription. Furthermore, *algD* is responsible for the final production of the precursor GDP-mannuronic acid, which is the basic molecule of alginate biosynthesis and polymerization. [13]

One of the most opportunistic microorganisms implicated in the mixed-species biofilm is *P. aeruginosa* and *S. aureus*, which have established a sophisticated network of counter-inhibition, evasion, and subjugation in competition for nutrients and space. They co-occurred in several

biofilm-associated infections, including urinary tract infections and wound and burn infections, increasing the chronicity and severity of the disorders compared to those caused by a single species. *P.aeruginosa* and *S. aureus* are two of the most opportunistic microorganisms implicated in the mixed-species biofilm, which evolved an intricate network of counter-inhibition, evasion, and subjugation in competition for nutrients and space. They both occurred in several biofilm-associated infections, including urinary tract infections, wound, and burn infections, increasing the chronicity and severity of the disorders compared to those caused by a single species.[14]

The vast majority of biofilm investigations have focused on single-species biofilms. However, biofilms in medical and environmental settings commonly consist of multiple species.[15] When multiple bacterial species co-exist in the same biofilm, it can provide numerous benefits to the co-occupants, such as access to chemicals biosynthesized by the co-existing inhabitants or circumvention of host defenses.[16] Despite this, competition among cohabiting bacterial species can be fierce, and bacteria have developed a variety of strategies for disrupting one another.[17] Interspecies interaction frequently leads to a rise in virulence and antibiotic resistance in one form or another.[18] *P. aeruginosa* colonizes the lungs of cystic fibrosis patients after *Staphylococcus aureus* colonization. This dual colonization will result in worsening the disease outcome.[19] Although such interaction between *P. aeruginosa* and *S. aureus* is harmful to the latter, both of them partially derive benefits from this co-existence. For instance, when tobramycin is unable to eliminate the clinical isolates of *P. aeruginosa*, the binding between the *S. aureus* surface protein and the *P. aeruginosa* exopolysaccharide is crucial in determining the configuration of *P. aeruginosa* biofilms, leading to higher tobramycin resistance.[20] Similarly, *S. aureus* and *P. aeruginosa* are considered the most common pathogens in chronic wound infections.[21] *P. aeruginosa* stimulates the formation of biofilm by *S. aureus*. Consequently, the coexistence of *S. aureus* with *P. aeruginosa* increases virulence and drug resistance.[22]

The purpose of the current study was to explore the effect of starvation stress on the expression of the *algD* gene in single-species biofilms of P. aeruginosa and dual-species biofilms of S. *aureus* and *P. aeruginosa* taken from the sewage of a number of different hospitals.

Materials and Methods

Source of bacteria

A total of six *P. aeruginosa* and six *S. aureus* isolates were obtained from the microbiology laboratory at the Department of Biology, College of Science, University of Baghdad, Iraq that were initially collected during June and July, 2022 form the sewage stations in Al-Yarmouk, Baghdad Medical City, and Al-Kindy teaching hospitals in Baghdad, Iraq. These isolates are multidrug-resistant (ceftriaxone, gentamicin, ciprofloxacin, Azithromycin, and tetracycline). The identification was also confirmed using the VITEK 2 compact system (BioMérieux, France).

Microtiter plate assay for single- and dual-species biofilm formation

An aliquot of 200 µl of standard cell suspensions (equal to McFarland turbidity standard No. 0.5) of *P. aeruginosa* and a mixture of *P. aeruginosa* and *S. aureus* (1:1) were prepared in a full-strength brain-heart infusion (BHI) broth. In wells of sterile 96-well flat-shaped bottomed polystyrene microplates, a 1000-fold diluted BHI (BHI/1000) was distributed. Following that, all microplates were covered with lids and incubated aerobically at 37°C for 24 hours. All biofilm experiments were done in triplicate. Bacteria-free brain-heart infusion broth was used as the control in the wells.[23]

Measurement of gene expression using quantitative reverse transcriptase-polymerase chain reaction (qRT-PCR)

The total RNA was isolated through the use of the TRIzol method in accordance with the protocols provided by the manufacturer (Geneaid, Taiwan), and it was kept frozen at -20 degrees Celsius until it was required.

The HiSenScriptTM RH(-) RT PreMix kit (Intron, Korea) was used to produce cDNA. 2 g (1 - 5 l) of RNA was put to the RT premix tube, and the volume was subsequently increased to 20 l with nuclease-free water. All tubes were then incubated at 50°C for 60 minutes and then at 85°C for 10 minutes.

As can be seen in Table 1, the cDNA that was obtained was put to use as a template in the quantitative real-time PCR that was performed using specified primers. Amplification of the 16S ribosomal RNA gene served as the reference gene in this study.[24] After that, RT-PCR was carried out using a Luna Universal qPCR Master Mix kit from New England Biolabs in the United States in conjunction with SYBR green fluorescent dye on a Mic qPCR Cycler manufactured by BioMolecular System in Australia. The PCR program employed for the target and housekeeping genes was as follows: 95°C for 30 s, 40 cycles of 95°C for 5 s, and 60°C for 5 s. A melting curve from 65°C to 95°C at 0.5°C/s was carried out as well. To evaluate the gene expression, the Livak formula was applied.[25] The molecular technique was applied to diagnosis different microorganisms, Some of these bacteria include *Staphylococcus auras*,[26,27] *Clostridium perfringens*,[28] *Brucella melitensis*,[29] *Proteus vulgaris*,[30,31] *Toxoplasma spp* ,[32,33] and SARS-Cov-2.[34]

Table 1: The primers used for measuring gene expression.

Primer name	Sequence (5′ – 3′)	Reference
16S rRNA (housekeeping gene)	F- ACCTGGACTGATACTGACACTGA	29
	R- GTGGACTACCAGGGTATCTAATCCT	
algD	F- GCTCAACCTGTCGCGCTACT	30
	R- GAACTCGCCACCACTTCGTC	

Results and Discussion

In the past several years, research has been done to develop treatments that are more specialized for *P. aeruginosa* infections. These treatments aim to disrupt many distinct pathways, including those that are involved in biofilm development and dispersal, quorum sensing, and iron metabolism. Although the findings are encouraging, it will be difficult to put them into practice because biofilms exhibit a diverse range of lifestyles, compositions, and phenotypes. These characteristics are determined by a number of factors, including the availability of amino acids, carbohydrates, and fatty acids, as well as the presence of other bacterial species. Real-time RT- QPCR data were quantified as Ct values that are inversely related to the amount of starting template. The results of the present study (Table 2) indicated that fasting resulted in a significant increase in *algD* gene expression for all single-species biofilms of *P. aeruginosa*, ranging from 3.117 to 4.532-fold, on the other hand, led to significant downregulation in each and every *P. aeruginosa* dual-species biofilm. According to what is presented in Table 3, the fold change might fall anywhere between 0.001 and 0.901. In conclusion, the lack of nutrients served a multitude of purposes in the P. aeruginosa biofilm. When there was just one species present in the biofilm, it caused the expression of the *algD* gene to increase, but when there were two species present, it caused the expression to drop. Both *S. aureus* and *P. aeruginosa*, which are pathogens that can cause serious illness and even death in humans if they are not treated with antibiotics, have developed multiple strategies to withstand hostile environments and immune system attacks. If these pathogens are not treated with antibiotics, they can cause serious illness and even death in humans.

One of these approaches is the utilization of the production of biofilm as a marker of pathogenicity. [35]

According to the findings that were presented by Obaid and Abdulwahhab [36], the starvation stress significantly increased the amount of biofilm biomass as well as the bacterial count in both mono and mixed biofilm-producing strains. In contrast to what one might anticipate, biofilms grown in co-culture are more resistant to starvation than those developed in monoculture. This is because co-cultured biofilms are grown alongside other organisms. They also discovered evidence that under conditions of hunger stress, the expression of *fnbA* and *pslA* was elevated in both mono- and co-cultured biofilms. In contrast, the expression of both genes was significantly increased when co-cultured biofilm was analyzed in comparison to mono-species biofilm. Alginate decreases the amount of siderophores, 2-heptyl-4-hydroxyquinoline-N-oxide, and rhamnolipids, which are necessary for the effective killing of *S. aureus*. Such studies suggest that alginate overproduction could be a key element in *P. aeruginosa / S. aureus* coinfection.[37]

P. aeruginosa was induced to make pyocyanin, which decreases *S. aureus* survival, when it co-existed with S. aureus in a dual-species biofilm. This induction was caused by the N-acetylglucosamine produced by *S. aureus*.[38] The mucoid *P. aeruginosa* strains overproduce alginate, while non-mucoid strains produce rhamnolipids, antimicrobial siderophores, and 2-heptyl-4-hydroxyquinoline-N-oxide. However, when non-mucoid strains are transformed into mucoid phenotypes, there is a decrease in the production of these substances and a rise in alginate production, resulting in the coexistence of *P. aeruginosa* and *S. aureus*.[37] The interaction of *Staphylococcal* Protein A with Psl and type IV pili of *P. aeruginosa* inhibits the bacteria's ability to form biofilms as well as neutrophil phagocytosis of *P. aeruginosa* .[39] Chew *et al* demonstrated that by producing diguanylate cyclase, *P. aeruginosa* outcompetes *S. aureus* in the multi-species biofilm.[17] The results of this study make it abundantly evident that environmental factors can play a big part in the intricate web of connections that exists between various species. Despite recent advances in diagnosis, there are still significant obstacles to overcome in the study of biofilm growth. These include the high concentration of bacterial cells and biochemical markers required for detection, the absence of data on biomarkers, and the high cost and/or time commitment required by the method.

Table 2: Fold change of *algD* gene expression in single-species biofilms of *Pseudomonas aeruginosa* under starvation stress condition.

Isolate code	Before starvation			After starvation			ΔΔct	Fold change
	16S rRNA	*algD*	Δct	*16S rRNA*	*algD*	Δct		
P1	11.44	16.25	4.81	13.11	15.87	2.76	-2.05	4.141
P2	11.47	16.98	5.51	11.06	14.93	3.87	-1.64	3.117
P3	20.23	38.26	18.03	20.16	38.76	18.6	-2.18	4.532
P4	12.27	20.89	8.62	13.76	20.29	6.53	-2.09	4.257
P5	12.79	16.33	3.54	13.17	14.72	1.55	-1.99	3.972
P6	20.81	38.68	17.87	21.22	37.31	16.09	-1.78	3.434

Table 3: Fold change of *algD* gene expression in dual-species biofilms of *Pseudomonas aeruginosa* under starvation stress condition.

Mixed species	Before starvation			After starvation			ΔΔct	Fold change
	16S rRNA	*algD*	Δct	*16S rRNA*	*algD*	Δct		
P1 + S1	11.44	18.6	7.16	13.11	20.42	7.31	0.15	0.901
P2 + S2	11.47	17.81	6.34	11.06	21.2	10.14	3.8	0.072
P3 + S3	20.23	19.15	-1.08	20.16	20.11	-0.05	1.03	0.490
P4 + S4	12.27	19.15	6.88	13.76	30.13	16.37	9.49	0.001
P5 + S5	12.79	25.03	12.24	13.17	27.83	14.66	2.42	0.187
P6 + S6	20.81	18.22	-2.59	21.22	20.74	-0.48	2.11	0.232

Conclusion

Starvation stress dramatically boosted *alg*D gene expression in single-species biofilms. Starvation decreased dual-species biofilm *alg*D gene expression. Understanding the *algD* gene expression pattern will help design biofilm-related illness treatments. Future study should investigate high-throughput and specific methods for diagnosing *P. aeruginosa* development, which forms biofilms. However, further research into the genetic processes that drive all *P. aeruginosa* biofilm lifestyle cycles may help create therapy strategies that prevent bacterial adhesion and biofilm maturation.

Conflict of Interest

The authors declare no conflict of interest.

Authors' Declaration

The authors hereby declare that the work presented in this article is original and that any liability for claims relating to the content of this article will be borne by them.

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