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Effect of adding exogenous acyl-homoserine lactone signal to *Pseudomonas aeruginosa* premature culture and prediction of the signal binding domain in Rhamnolipids RhlA enzyme

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ABSTRACT

The hierarchy of the quorum-sensing system plays a crucial role in *Pseudomonas (P.) aeruginosa* virulence and the production of important industrial bacterial products like rhamnolipids and proteases. In this study, the effect of adding exogenous acyl-homoserine lactone synthetic signal to the premature culture of *P. aeruginosa* on the production of protease and rhamnolipids was investigated. At the early exponential phase, induction of rhamnolipid production showed a more rapid response than protease production. Prediction of the 3D structure of the acyltransferase RhlA enzyme, which is the first key enzyme in rhamnolipid synthesis, was then done using the I-Tasser program to investigate the possible protein structure that might influence the response to N-acyl-homoserine lactone (AHL) presence. With a good C-score, 3D modeling showed RhlA to have AHL binding pocket where ten ligand binding site residues were elucidated in the protein. Multiple sequence alignment revealed low homology with LuxR proteins. Although conserved residues were depicted from the alignment, they were different from the ligand-binding residues suggesting that AHL binds to RhlA with a different mechanism than LuxR proteins. After further bioinformatics analysis, we found that RhlA binds to AHL in a mechanism similar to the lactonase enzyme. In conclusion, the in silico domain and protein alignment analysis revealed an AHL binding site in the RhlA enzyme protein structure.

Keywords: *acyl-homoserine lactone; P. aeruginosa; I-Tasser.*

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1. Introduction

The quorum-sensing circuit in *P. aeruginosa* represents a complex regulatory system that plays a major role in controlling both genomic and proteomic expression. This control takes place either directly or indirectly **[1, 2, 3]**. There are three central quorum-sensing systems in *P. aeruginosa*, together with establishing a hierarchical network enabling crosstalk between multiple signals **[4]**.

The LasR/LasI as well as her/RhlI quorum sensing systems use N-3-oxo-dodecanoyl-L-homoserine lactone (3-oxo-C12-HSL) and N-butyryl-L-homoserine lactone signals to regulate gene expression **[5]**. These signals drive the expression of virulence genes responsible for

producing various bacterial products, biofilm formation as well as regulating bacterial motility **[6]**. Among the produced bacterial products are rhamnolipids and proteases. Rhamnolipids are biosurfactants that induce hemolysis, necrosis of host macrophages, and polymorphonuclear leukocytes**,** accelerating tissue invasion and damage. This is necessary for the pathogen in the establishment of infection and immune evasion **[7, 8]**. In addition to providing increased fitness and better chances of survival within the host, rhamnolipids can be widely applied in many industries including food, agriculture, petroleum, and bioremediation **[9]**. Similarly, proteases are also important for bacterial virulence and the establishment of infection. Previous studies reported that proteases and lipases are among the major classes of enzymes propelling *P. aeruginosa* pathogenesis. About 2.8% of the *P. aeruginosa* genome is involved in the expression of hallmark protease genes **[10]**. Protease also allows *P. aeruginosa* to use proteins as a nutrient source essential for growth **[11]**. In addition, protease enzymes are valuable commercial enzymes that have biotechnological as well as industrial applications.

The present study aimed to investigate the effect of adding exogenous synthetic AHL signal to *P. aeruginosa* premature culture on rhamnolipids and protease production to provide a better understanding of the complexity of the quorum-sensing system and study the possibility of using this to modulate the production of these bacterial products. Moreover, bioinformatics analysis for domain binding sites in the rhamnolipids key biosynthetic enzyme, rhlA, was investigated to reveal possible ligands affecting the production and whether it has any relationship with the quorum-sensing system in *P.aeruoginosa*.

2. Materials and Methods

2.1. Chemicals and Media

Luria Bertani (LB) broth was obtained from LabM, England. Trichloro acetic acid and Sodium hydroxide were obtained from El-Nasr Chemical Co, Egypt. Azocasein and hexanoyl homoserine lactone (synthetic signal) were products of Sigma-Aldrich, Cairo, Egypt.

2.2. Bacterial isolate

P. aeruginosa clinical isolate was obtained from a urine sample discharged as routine patient care from the Microbiology Lab of Al-Demerdash hospital. Biochemical identification was done followed by confirmation using the Erba kit (Erba Lachema, Czech republic).

2.3. Induction of rhamnolipid and protease production in *P. aeruginosa* premature culture using synthetic signal

A volume of 200 µL of overnight *P. aeruginosa* culture (incubated at 37 °C at 200 rpm) was used to inoculate 20 mL LB broth placed in a 250 mL flask. Hexanoyl homoserine lactone signal was added to a final concentration of 0.1 mM. Control was prepared in the same way without adding the signal molecule. Flasks were incubated at 37 °C at 200 rpm. After 2 hours, 1 mL was collected from both the test and the control, centrifuged to collect the supernatant, and rhamnolipid and protease concentrations were both measured. This was repeated after 4 h of the beginning of incubation and after 18 and 24 h. The optical density of the culture at 600 nm was also measured each time.

2.3.1. Rhamnolipid Production

Rhamnolipid production was assessed using an oil displacement assay **[12]**. In brief, 20 mL of distilled water was overlaid with 20 µL of crude oil then, 12 µL of the collected supernatants were added to the oil surface. The diameter of the oil-free clearing zone formed was measured. The assay was done in triplicates, each time the displacement zone was measured 5 times then, the mean, and the standard deviation was

calculated.

2.3.2. Protease Production

This assay was conducted as described by **[13]** using 2% w/v azocasein as a substrate, 10% trichloroacetic acid to stop the reaction, and 1 M sodium hydroxide for the development of the orange color. The absorbance was measured at 440 nm. Blank was prepared using a plain culture medium instead of the supernatant. The proteolytic activity was then determined using the equation: Y= 0.2221 X + 0.4613, where Y represents the absorbance at 440 nm and X represents the log protease concentration in units/mL.

2.3.3. Statistical analysis

All the experiments were carried out in triplicates and the results were represented as respective average values. Data were analyzed using Graph pad Instant-3 software (Graph Pad Software Inc., USA).

2.4. Prediction of the tertiary structure of RhlA with I-TASSER

Since the protein structure of RhlA was not crystallized until now and subsequently was not deposited into the Protein Data Bank (PDB), we used computational methods to predict the structure of the RhlA enzyme. The amino acid sequence of RhlA of *P. aeruginosa* was retrieved from the National Center of Biotechnology Information (NCBI; https://www.ncbi.nlm.nih.gov/protein/NP_252169.1) with accession code (NP_252169.1). The sequence was then inserted into the I-TASSER website (http://zhanglab.ccmb.med.umich.edu/I-TASSER/,). I-TASSER predicts the protein secondary structure using both the PSSpred algorithm and PSI-BLAST. Moreover, it predicts solvent accessibility, top PDB templates, and ligand binding site prediction using the COACH algorithm **[14, 15]**.

2.5. Multiple sequence alignment with Clustal Omega

Multiple sequence alignment was conducted between 3-(3-hydroxydecanoyloxy) decanoate synthase (RhlA) and LuxR homologs: transcriptional regulators rhlR and lasR of *P. aeruginosa* as well as lactonase sequences obtained from NCBI Genbank Database (http://www.ncbi.nlm.nih.gov). This was carried out using Clustal Omega software (https://www.ebi.ac.uk/Tools/msa/clustalo/).

3. Results

3.1. The effect of synthetic signal on rhamnolipid and protease production of *P. aeruginosa*

Results displayed in **Fig. 1** showed that the incorporation of synthetic hexanoyl homoserine lactone signal in the growth medium of *P. aeruginosa* induced the production of both rhamnolipids (**Fig. 1A**) and protease (**Fig. 1B**) in the premature culture of the test culture relative to the control culture. Induction of rhamnolipids production occurred more rapidly than protease production where a three-fold increase in rhamnolipids production of test isolate relative to the control was observed after only two hours while protease production in test culture was only slightly higher (only 10% higher) than control after two hours.

3.2. Prediction by I-TASSER

I-TASSER program usually begins predicting the protein structure by identifying the top homologs proteins from the PDB like alpha-beta hydrolase. I-Tasser confirmed the belonging of the RhlA enzyme to the alpha/beta hydrolase superfamily with C-score: of 0.23. The tertiary structure is depicted in **Fig. 2.** Then, the ligand-binding sites were predicted for the protein as shown in **Table 1.** RhlA showed the highest

confidence score for binding homoserine lactone (HSL) then (1r)-1,2,2-trimethylpropyl (r)-methyl phosphonate (GD7) then 6-amino-1-methyl-5-(piperidine-1-yl)pyrimidine-2,4(1H,3H)-dione(S07) then methyl 7-{[2-({N-[(2S)-2-hydroxy-3,3-dimethyl-4-(phosphonooxy)butanoyl]-beta-alanyl}amino)ethyl]sulfanyl}-7-oxoheptanoate (ZMK) and finally 4-(3-acetoxymethyl-2-carboxy-8-oxo-5-thia-1-aza-bicyclo[4.2.0]oct-2-en-7-ylcarbamoyl)-1-carboxy-butyl-ammonium (CSC).

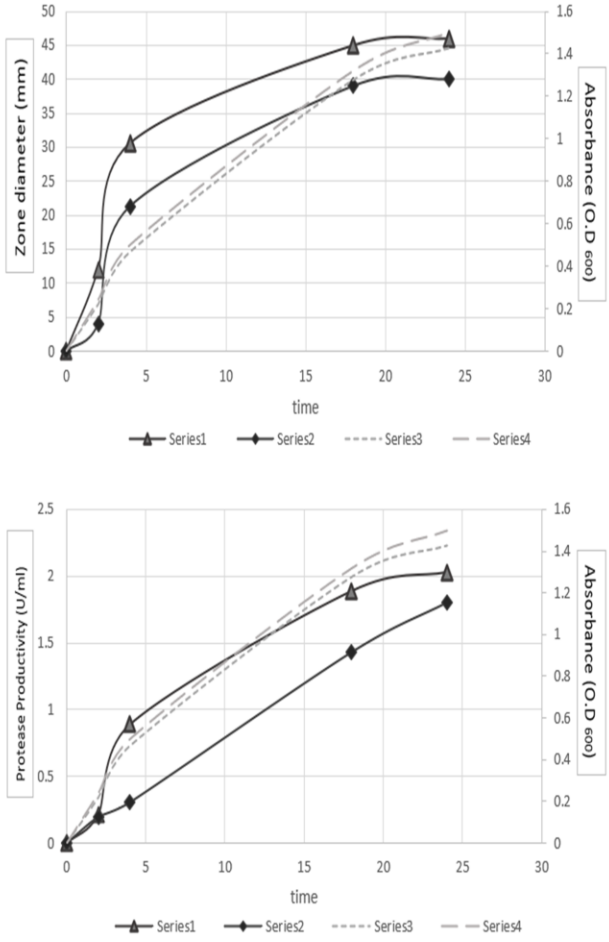

Fig. 1. Effect of synthetic signal on **(A)** rhamnolipid and **(B)** protease production of *P. aeruginosa* where series 1 represents production in induced test culture, series 2 represents production in control culture, series 3 represents growth curve for test culture expressed in terms of measuring the optical density at 600 nm and series 4 represents growth curve for control culture.

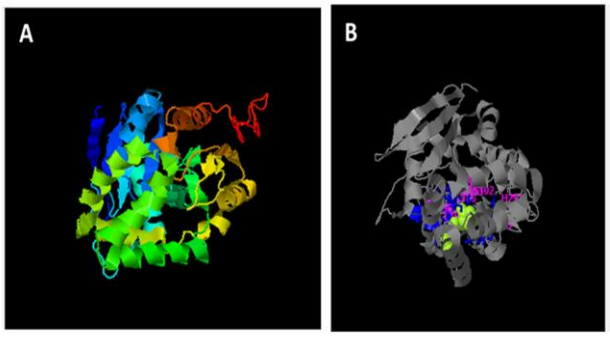

Fig. 2. (A) Rhamnolipid synthase (RhlA) as depicted by I-Tasser. The protein chains are colored from blue at N-terminus to red at the C-terminus. (**B)** The binding site for homoserine lactone in the RhlA enzyme is colored green.

3.3. Multiple sequence alignment

Multiple sequence alignment revealed low homology between RhlA and LuxR proteins where sequence identity with RhlR and LasR did not exceed 19% (**Fig. 3**). Although conserved residues were depicted from the alignment, they were different from the ligand-binding residues suggesting that AHL binds to RhlA with a different mechanism than LuxR proteins. Alignment with AHL lactonases displayed a similarity of approximately 22% and revealed the presence of the conserved hydrophobic Tryptophan and Leucine residues at positions 103 and 197 of RhlA, both previously suggested to be involved in the binding of hexanoyl homoserine lactone to lactonase enzyme as well as the presence of the catalytic Tyrosine residue at the position 225. RhlA also displayed the partial presence of the HXHXDH motif characteristic of

AHL lactonases (**Fig. 4**).

Table 1. The suggested ligand binding site residues according to I-Tasser

Rank	C-score	Cluster size	PDB Hit	Ligand name	Ligand binding site residues
1	0.30	98	4g8bB	HL6	35,36,79,102,103,106,196,197,225,251
2	0.23	40	3f97A	GD7	35,36,38,43,101,102,103,251,252
3	0.03	8	3wkaA	S0G	36,138,223,224,225,251,252
4	0.03	7	4etwA	ZMK	35,36,102,103,135,138,139,142,157,160,161,163,194,197,225,251
5	0.02	7	2vaxH	CSC	35,36,37,42,102,154,161,167,251,252,254,255

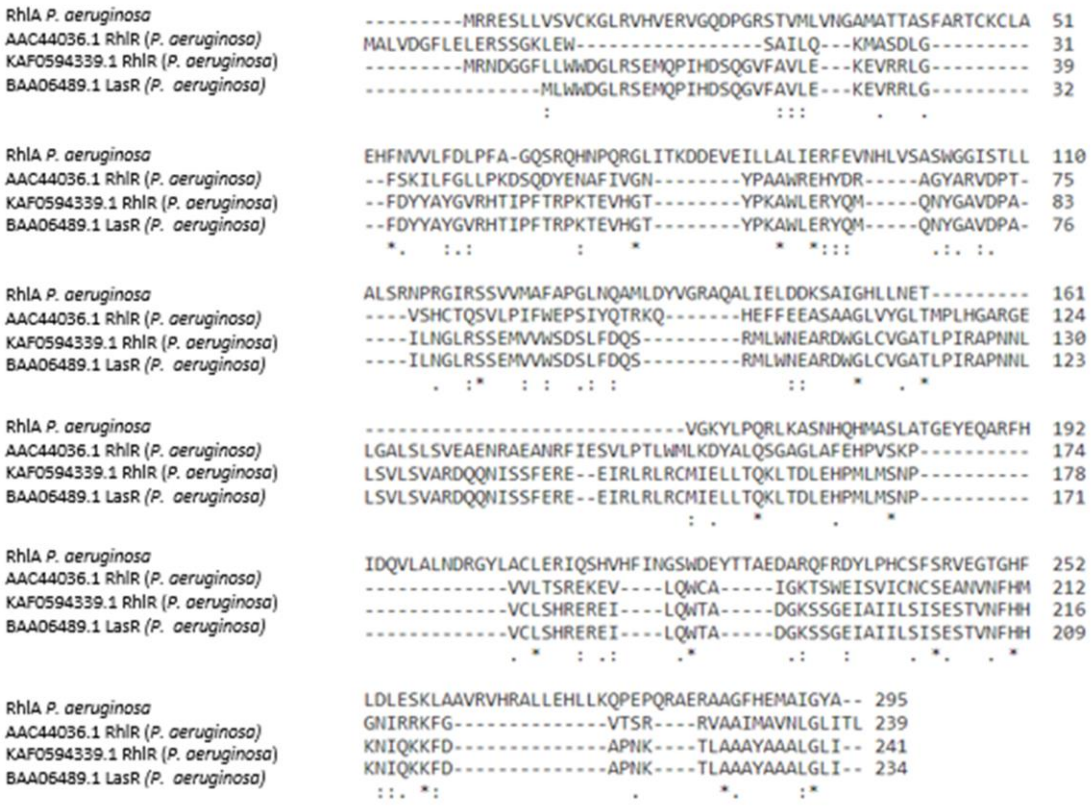

Fig. 3. Alignment of the amino acid sequence of RhlA (Accession code= WP_003113897.1) with LuxR proteins: transcriptional regulator RhlR of *P. aeruginosa* PAO1 (accession code= AAC44036.1), RhlR of *P. aeruginosa* (accession code= KAF0594339.1) and with transcriptional regulator LasR of *P. aeruginosa* (accession code= BAA06489.1) using Clustal Omega.

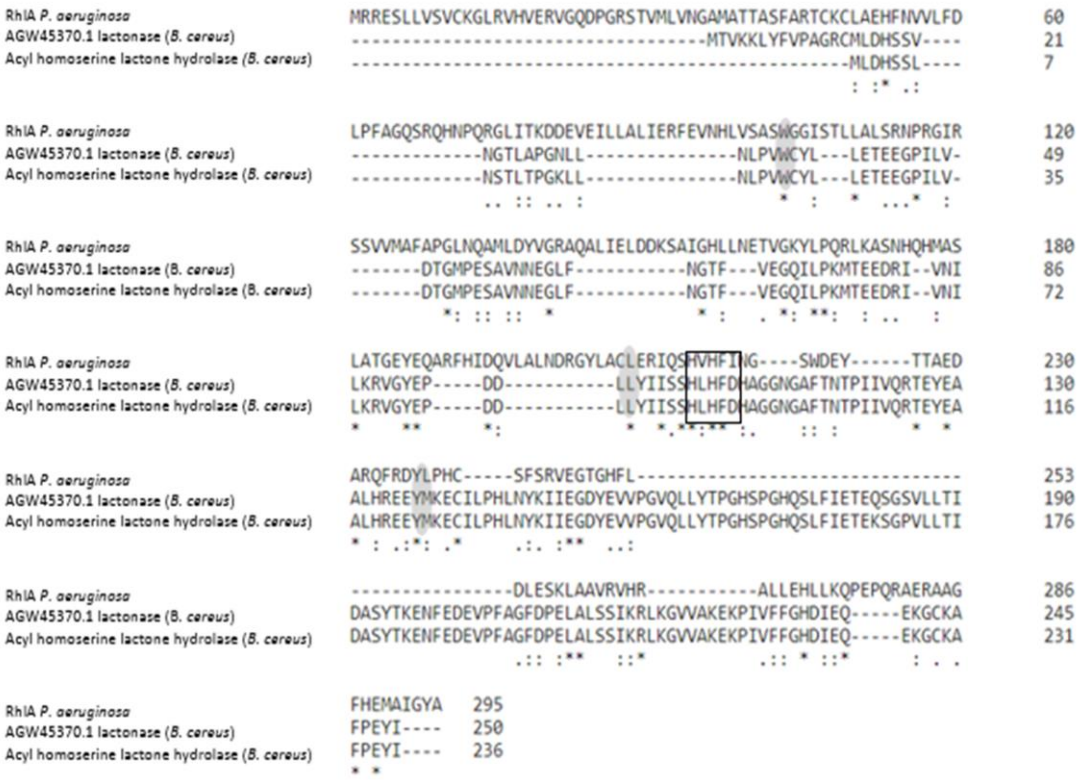

Fig. 4. Alignment of the amino acid sequence of RhlA (Accession code= WP_003113897.1) with N-acyl homoserine lactonase of *Bacillus cereus* (accession code= KF254906) and with N-acyl homoserine lactone hydrolase of *Bacillus cereus* (accession code=KF254908) using Clustal Omega

4. Discussion

Like other pathogens, *P. aeruginosa* encodes virulence determinants that are necessary for establishing the infection within the host and provide it with better chances of survival. Rhamnolipids and protease are among these virulence hallmarks which play a role in maneuvering the host cellular machinery by causing injuries, tissue necrosis, and immune evasion **[16]**. Quorum sensing is responsible for regulating gene expression in the bacterial population. In *P. aeruginosa*, the LasR/LasI and RhlR/RhlI QS systems regulate gene expression by AHL signals. The present study aimed at examining the effect of adding the synthetic signal to the growing *P. aeruginosa* culture on the rhamnolipids and protease production. Results showed that a significant increase in rhamnolipid production was achieved after only two hours of incorporation of AHL signal in the growth medium of *P. aeruginosa*, whereas the production of protease was almost the same as control after two hours and an increase in production was only observed after four hours. This may be attributed to the complexity and multifaceted nature of the quorum-sensing hierarchy in *P. aeruginosa* **[15, 17]**. To make sure that this increase in rhamnolipids production was attributed to the incorporation of synthetic signal in the medium, the growth of the test culture was compared to that of control by measuring the optical density at 600 nm. In

addition to playing an important role in bacterial virulence, rhamnolipids are a class of biosurfactants that can be widely applied in many industries including the synthesis of nanoparticles, food industries, and bioremediation. They are widely utilized as detergents, solubilizers, or emulsifying agents with *P. aeruginosa* being the most competent producer of rhamnolipids **[16, 18]**.

There are three key enzymes for rhamnolipid biosynthesis, RhlA, RhlB, and RhlC, found mostly in *Pseudomonas sp.* with RhlA being the rate-limiting enzyme required for the formation of the precursor hydroxy alkanoic acid (HAA) that constitute the hydrophobic portion of rhamnolipids **[9]**. To understand the possible underlying cause for this result, the 3D structure of RhlA was predicted using the I-Tasser program. This was conducted to investigate the possible differences in the protein structures that might influence the response to AHL presence. With a good C-score, 3D modeling showed RhlA to have AHL binding pocket where ten ligand binding site residues were elucidated in the protein. The usage of computational methods in structure saves a long time of slow experimental crystallization of proteins to identify their structure. However, the choice of the proper software is important for the potential accuracy of the predictive modeling of the protein structure. Since the RhlA enzyme is less than 30% identical to proteins listed in the databases, a threading program should be used. The threading programs' algorithms depend on combining both the homology alignment with other proteins and scratch building of the non-aligned residues **[14, 19]**. I-TASSER program specifically has the added advantage of predicting the ligand-binding sites in the protein structure. This sheds light on the possible usage of the protein in addition to its mechanism of action. In our study, I-Tasser predicted a ligand-binding site in the RhlA enzyme for HSL, and its amino acid sequence was predicted. To the best of our knowledge, this is the first time to predict this pocket for HSL. However, as with all prediction algorithms, its accuracy is questionable and needs experimental validation. Therefore, we decided to make more bioinformatics analyses of this binding site.

Multiple sequence alignment using Clustal omega was then done between RhlA, rRhlR, and LasR. It showed a 19% similarity between RhlA and transcriptional regulators RhlR and LasR and the conserved residues displayed in the alignment were distant from those predicted by the model for the binding of AHL molecules. This result suggested that the binding of the predicted model with AHL molecules occurs differently than the binding of LuxR homologs. Accordingly, the study conducted alignment with AHL lactonases from *Bacillus* isolates for their well-known binding to different AHL molecules **[20]**. Alignment with AHL lactonases displayed a similarity of approximately 22% and revealed the presence of the conserved hydrophobic Tryptophan and Leucine residues at positions 103 and 197 of RhlA, both suggested by the model to be involved in the binding and previously reported to be important in the substrate binding to lactonase enzyme **[21]**. A study on substrate binding mechanism with lactonase enzyme mentioned earlier that a valine hydrophobic residue in position 35 was important for the binding of the hydrophobic acyl chain **[22]**. In the predicted model of RhlA, a hydrophobic glycine residue in position 35 was among the proposed binding sites for AHL molecules. A tyrosine residue at position 225 was also among the suggested ligand binding sites by the predicted model. According to Elias and coworkers, the presence of a tyrosine residue in position 194 of lactonase enzymes played a role in the positioning of the lactone ring of the substrate **[23]**. In a study comparing Aal lactonase with Aii

lactonase, it was reported that Y223 (in AaL) was the equivalent residue toY194 in AiiA **[21]**. We hereby suggest that Y225 in the predicted structure is equivalent to Y223 found in Aal lactonase. Alignment also revealed the conservation of a serine moiety in position 103, suggested by the model to be involved in binding just before the conserved HXHXDH sequence-characteristic of AHL lactonases- which is also partially displayed in the studied sequence.

Conclusion

Induction of rhamnolipid production by synthetic AHL signals in *P. aeruginosa* premature culture occurs faster than induction of protease production. Acyl homoserine lactone molecules bind directly to RhlA as depicted *in silico* modeling. Binding is suggested to be similar to that of AHL with lactonases. Further studies are yet to follow to confirm the binding of AHL to RhlA and determine the possibility of using this to modulate the activity of the enzyme. *P. aeruginosa* quorum-sensing system has a complex hierarchy that yet requires

Declarations

Ethics approval and consent of participation

Not applicable

Consent of publication

Not applicable

Data and materials availability

All data produced or analyzed throughout this study are included in the current manuscript.

Competing interests

No competing interests were found between the authors.

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Authors' contribution

M. Sakr and N. Elsayed designed the study. M. Sakr conducted the experiments and the multiple sequence alignment. N. Elsayed conducted the in-silico modeling. G. El-Housseiny analyzed the data and revised the manuscript. All the authors wrote the manuscript and approved the final manuscript.

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