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Characterization of Protein-Protein Interactions in Quorum-Sensing Receptors From Gram Negative Bacteria

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Characterization of protein-protein interactions in quorum-sensing receptors from Gram-negative bacteria

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Abstract

Pseudomonas aeruginosa is a bacterium capable of causing disease in immunocompromised individuals and individuals with underlying lung disorders. It controls the expression of a subset of its genes through quorum sensing, which is a cell-cell communication system involved in mediating the transition from individual to group behaviors. Group behaviors are cellular processes undertaken by the cell that are beneficial to the collective, such as biofilm formation – a key component of pathogenesis in the human lung. They rely on the production, accumulation, detection, and response to signal molecules called autoinducers. There are two acyl-homoserine lactone autoinducer quorum sensing systems in *P. aeruginosa* that are responsible for the transition to group behaviors: the Las system and Rhl system. The systems contain LasR and RhlR, respectively, which are transcriptional activator proteins known as LuxR-type proteins, as well as LasI and RhlI, which synthesize the respective autoinducers for LasR and RhlR. RhlR directly interacts with another protein called PqsE to control RhlR-dependent transcription. To study whether or not the mechanism of activation between RhlR and PqsE is conserved in other pathogenic bacteria, the interactions of PqsE with LuxR-type receptors from other organisms were assessed. This included SdiA from *Salmonella enterica* and CviR from *Chromobacterium violaceum*. Through a protein pulldown experiment, it was determined that PqsE did not interact with LasR, SdiA, or CviR, and a light assay confirmed these results, indicating that the *P. aeruginosa* PqsE specifically evolved to interact with its paired receptor, RhlR. *Burkholderia cepacia* is a bacterium that is similar to *P. aeruginosa*; it has both PqsE and RhlR homologs (referred to as PqsE and SolR, respectively). With the structure and function of RhlR and PqsE recently determined, we now aimed to determine the function of SolR and PqsE to understand the molecular basis for the evolution of the PqsE-quorum-sensing receptor interactions. A bioinformatics approach was taken using a machine learning program called Alphafold to analyze the structure of SolR and PqsE. It was determined that PqsE from *B. cepacia* does not dimerize like PqsE from *P. aeruginosa*, which altered its ability to interact with its potential binding partner, SolR. Future experiments will focus on biochemical assessment of SolR and PqsE from *B. cepacia*.

Keywords: microbiology, quorum sensing, protein-protein interactions, gene regulation, transcription.

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I'm also incredibly thankful for Dr. Mayerhofer, not only for being the second reviewer of this paper, but for all of the support I've received throughout the years.

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Introduction

Pseudomonas aeruginosa, a bacterium that can cause disease in individuals with underlying lung disorders or weakened immune systems (Centers for Disease Control and Prevention, 2019), utilizes quorum sensing as a means of regulating the expression of a subset of its genes (Albus et al, 1997). Quorum sensing is a form of cell-cell communication that mediates the transition from individual to group behaviors, which can include beneficial processes such as biofilm formation and virulence factor production (Davies et al, 1998). This mechanism relies on the production, detection, and response to signal molecules called autoinducers (Davies et al, 1998). Notably, quorum sensing plays a crucial role in controlling the expression of various virulence factors in pathogenic bacteria (Rutherford and Bassler, 2012).

P. aeruginosa uses two acyl-homoserine lactone autoinducer quorum sensing systems, namely the Las and Rhl systems, which facilitate the transition to group behaviors (Pesci et al, 1997). These systems involve LasR and RhlR, two transcriptional activator proteins, as well as LasI and RhlI, which synthesize the autoinducers necessary for the system's functionality (Pesci et al, 1997).

The quorum sensing processes begins with the synthesis of an autoinducer by a synthase (Seed et al, 1995). As the concentration of the autoinducer increases, it eventually reaches a threshold level, triggering its binding to the corresponding transcription factor receptor (Kiratisin et al, 2002). The receptor-autoinducer complex then binds to specific target promotors and stimulates gene transcription through interaction with RNA polymerase (Kiratisin et al, 2002). Typically, the receptor-autoinducer complex upregulates the transcription of its cognate synthase gene, generating a positive feedback loop where the activation of either LasI or RhlI can lead to increased autoinducer production (Schuster and Greenberg, 2007).

More specifically, the LuxR and LuxI-type receptor and synthase pairs are used to regulate quorum sensing, which are LasR, LasI, RhlR, and RhlI (Gambello and Iglewski, 1991). First is the Las system, where LasI produces the autoinducer N-3-oxo-dodecanoyl-l-homoserine lactone ($3OC_{12}HSL$), which binds to LasR (Pearson et al, 1994), (Seed et al, 1995). Upon binding of the autoinducer, LasR is stabilized and activated as a transcription factor (Kiratisin et al, 2002), which leads to its binding to specific DNA sites and the transcriptional activation of its genes, including RhlI and RhlR (Pearson et al, 1997), (Pearson et al, 1995). Similarly, RhlI synthesizes the autoinducer N-butyryl-homoserine lactone (C_4HSL), which binds to RhlR (McKnight et al, 2000).

The interaction between RhlR and PqsE, another protein within *P. aeruginosa*, has been identified to play a crucial role in controlling the production of both autoinducers and virulence factors (Simanek et al 2022). Given the direct interaction between the two proteins and their involvement in virulence, it was worth exploring if this activation mechanism is also present in other pathogenic bacteria. This study aimed to investigate the possibility of the interactions of PqsE with other LuxR-type proteins, such as SdiA from *Salmonella enterica* and CviR from *Chromobacterium violaceum*. Additionally, this study aimed to determine if the receptors of these proteins possess enzymes analogous to PqsE in their respective organisms that could perform a similar function.

In similarity to *P. aeruginosa*, *Burkholderia cepacia* is another bacterium that contains a homology of PqsE and a protein resembling RhlR, known as SolR, which can interact (Lewenza et al, 1999). Given the structural and functional similarities of RhlR and PqsE in *P. aeruginosa*, this study aimed to investigate the roles of SolR and PqsE in *B. cepacia* to clarify the molecular mechanisms underlying the evolution of the PqsE quorum sensing receptor interaction.

Methods

Cloning E. coli expression vectors:

In this study, three distinct samples were utilized to evaluate the presence of specific DNA fragments. For the first experiment, these samples included JPS375 amplified with primers oJP1438 and oJP1439, JPS375 amplified with primers oJP1462 and oJP1463, and JPS101 amplified with primers oJP1538 and oJP1539. PCR was performed for each sample using a total of 22.5 µL water, 10 µL Q5 buffer, 10 µL Q5 enhancer, 2.5 µL primer one, 2.5 µL primer two, 1 µL DNTPs, 1 µL template, and 0.5 µL Q5 polymerase. The PCR reaction was performed using a gradient to account for the varying annealing temperatures between the templates, specifically 60 ºC for JPS375 and 55 ºC for JPS101. Following PCR, the synthesized DNA fragments were assessed through gel electrophoresis on a 1% agarose gel.

Subsequent to PCR, each sample was subjected to digestion with the appropriate restriction enzymes. Specifically, NdeI and KpnI were used for JPS375, while NdeI and XhoI were used for JPS375. To purify the digested samples, 10 µL of purple loading dye was added, and 60 µL of the mixture was loaded into the wells of a 1% agarose gel, which was then run at 150V for 25 min. The resulting bands at approximately 750bp were excised and transferred to microcentrifuge tubes containing 600 µL QG buffer. After being dissolved, the samples were transferred to filter columns, washed with 600 µL PE buffer, and eluted with 30 µL water. The eluted samples were ligated with 2 µL of T4 buffer and 1 µL of T4 ligase, and transformed using *E. coli* top ten competent cells. Following the growth of bacterial colonies, PCR was performed using these colonies, and the resulting samples were sent for sequencing analysis.

For the second experiment, *B. cepacia* samples were utilized instead, and a range of primers and restriction enzymes were used to repeat the same procedures as described above, including the precise measurements.

PqsE affinity pulldown experiment:

Five different samples, namely JPS102 (pETDuet-*pqsE*), JPS262 (pETDuet-*rhlR*), JPS783 (pETDuet-*cviR*), JPS784 (pETDuet-*lasR*), and JPS804 (pETDuet-*sdiA*) were utilized in this study. The samples were prepared in advance and subsequently sonicated and centrifuged for 15 min. Afterwards, 20 µL of the resulting solution was aliquoted into separate tubes to represent the input fraction. PqsE was added from the first tube into the four other samples, with PqsE lysis buffer included into the PqsE tube to ensure a consistent volume across all tubes. PqsE, being the bait protein and tagged with 6x-Histidine, could be extracted from the solution with any binding partner using Ni-NTA resin. While the volumes in each tube varied due to multiple repetitions, all five tubes had the same volume each time.

Magnetic Ni-NTA resin, along with PqsE lysis buffer and PqsE elution buffer, were utilized to isolate the targeted protein from the solution. Following the application of the magnets, the PqsE lysis buffer was removed and re-added in a volume of 1000 µL three times (with the use of magnets), prior to using PqsE elution buffer. This buffer was applied twice, with each application being 20 µL, and the eluted sample was then collected into separate tubes to represent the output fraction. Subsequently, SDS sample buffer, which contained brilliant blue dye, was added to all ten tubes, including the input fractions. Following this, all ten tubes were heated on a 100 ºC hot plate, and then subjected to SDS-PAGE protein gel electrophoresis.

An identical protocol, with the same measurements as listed above, was used to investigate the interactions of PqsE and SolR from *B. cepacia*, as well as PqsE from *B. cepacia* and RhlR from *P. aeruginosa* as well. The steps were replicated as written above without any modifications.

E. coli luciferase assay:

Using four different prepared samples: JPS 476 (pBADA-*rhlR* PA14 pCS26-p*rhlA-luxCDABE* pACYC-*pqsE*), JPS477 (pBADA-*rhlR* PA14 pCS26-p*rhlA-luxCDABE* pACYC-empty), JPS918 (pBAD24-*sdiA* pCS26-p*rhlA-luxCDABE* pACYC-*pqsE*) and JPS 919 (pBAD24-*sdiA* pCS26-p*rhlA-luxCDABE* pACYC-empty). 200 µL of each sample was mixed with 20 µL of an LB mixture and incubated on a shaker at 37 ºC for three hours. Afterwards, 100 µL of an arabinose solution was added to each sample, followed by 99 µL of each solution being added to eight wells in their respective rows on a light assay plate. For the rows with JPS476 and JPS477, decreasing concentrations of C_4HSL (10mM, 5mM, 2.5mM, 1.25mM, 0.63mM, 0.31mM, 0.16mM, and 0.08mM) were added, while the rows with JPS918 and JPS919 received 3OC_8HSL at the same concentrations. The plate was then incubated on a shaker at the same temperature for an additional three hours before being measured on a fluorometer.

Results

Two sets of samples were submitted for sequencing, but unfortunately, the first set failed to yield any results and the second set did not contain the intended sequence. The images presented below (figure 1) are the outcomes of some of the agarose gel electrophoresis performed as part of the sequencing experiment.

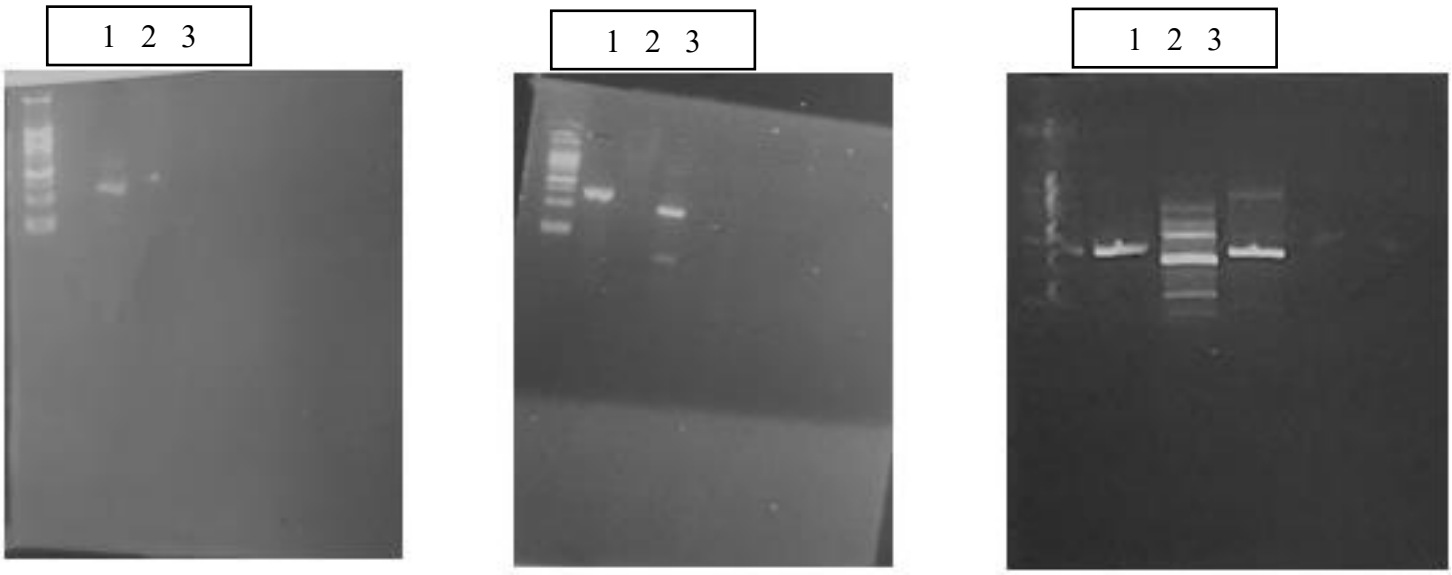

Figure 1: Agarose gel images of *E. coli* expression vectors used for our experiments. The ladder is displayed in the first lane, followed by the samples 1-3. Sample 1 in all images refers to JPS375 with the primers oJP1438 and oJP1439, Sample 2 refers to JPS375 with primers oJP1462 and oJP1463, and Sample 3 indicates JPS101 with primers oJP1538 and oJP1539. In the leftmost image, only lane two shows a band, while the middle image displays bands for all lanes except lane two. The final image on the right illustrates the expected bands in all three lanes for all three samples.

The left image (Figure 1) depicts an unsuccessful PCR test that was not sequenced. Lanes 1 and 3 are empty, despite the expectation of visible bands. In the middle image (Figure 1), although there is no visible sample in lane two, it was not considered a failed attempt as it was expected to show up based on the results from the first image. However, upon sequencing, this set of samples did not contain the intended sequence. The right image (Figure 1) was also sent for sequencing, but no conclusive results were obtained.

Figure 2 illustrates the results of the first affinity protein pulldown experiment between PqsE from *P. aeruginosa* and the other LuxR-type receptors from different bacteria.

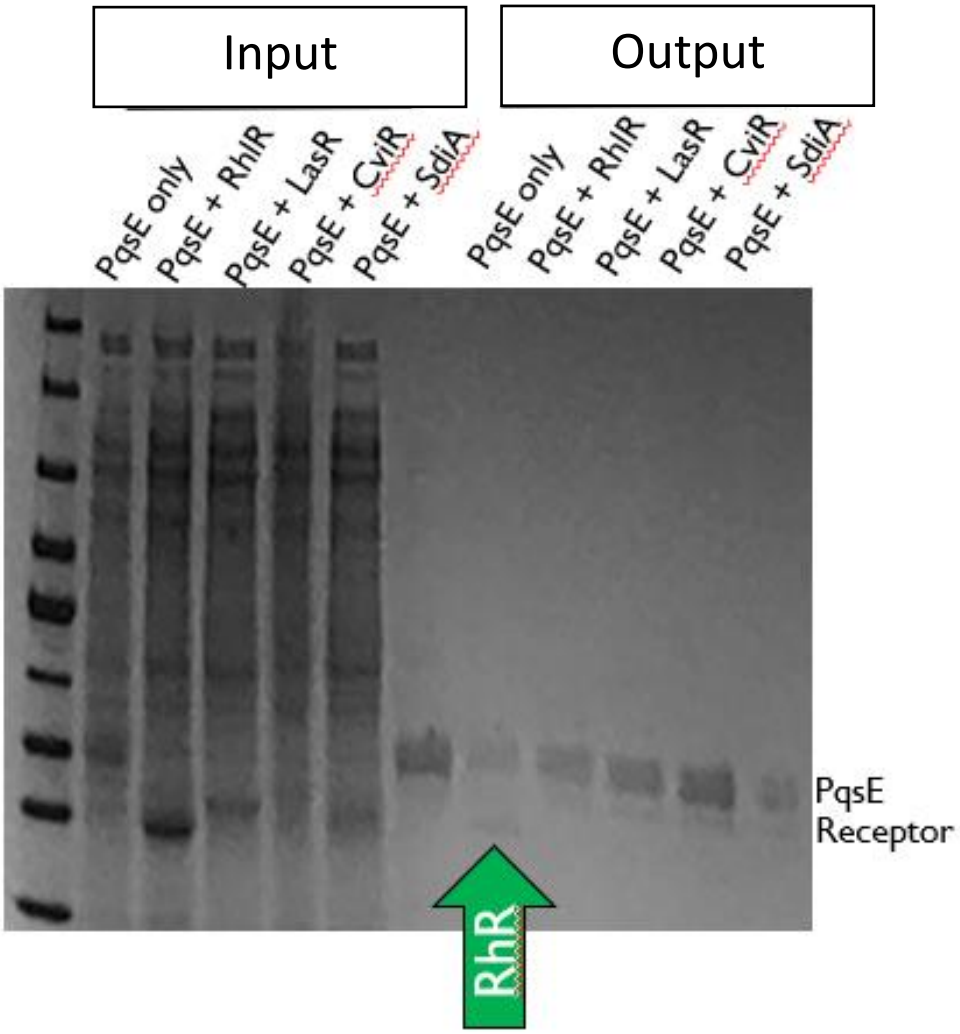

Figure 2: SDS-PAGE protein gel displaying the results of the first affinity protein pulldown experiment. The gel contains the ladder in lane one, followed by five lanes of input tubes and five lanes of output tubes, each labeled appropriately. The bait protein, 6x-His-PqsE, was pulled down by the magnetic Ni-NTA beats, and any bound proteins appeared as bands in the output tubes. The top band present in the output tubes represents the bait protein. The only protein present within the output tubes that showed a band was RhlR, indicating that none of the experimental LuxR-type proteins from other species bound to PqsE.

The SDS-PAGE gel obtained from the protein pulldown experiment suggests that PqsE does not bind to any other receptors not from *P. aeruginosa*. As illustrated in the pulldown section of the gel (Figure 2), PqsE was present in all of the output tubes, but only RhlR was found to bind to it. PqsE did not interact with any of the other receptors, including LasR (which served as the negative control), CviR or SdiA, which were the experimental receptors being tested. While PqsE showed a positive interactions with RhlR, it did not exhibit any interactions with the other experimental receptors.

The results of the *E. coli* luciferase assay are presented in Figure 3, which depicts the relationship between the concentration of autoinducers and the relative light units emitted for each sample.

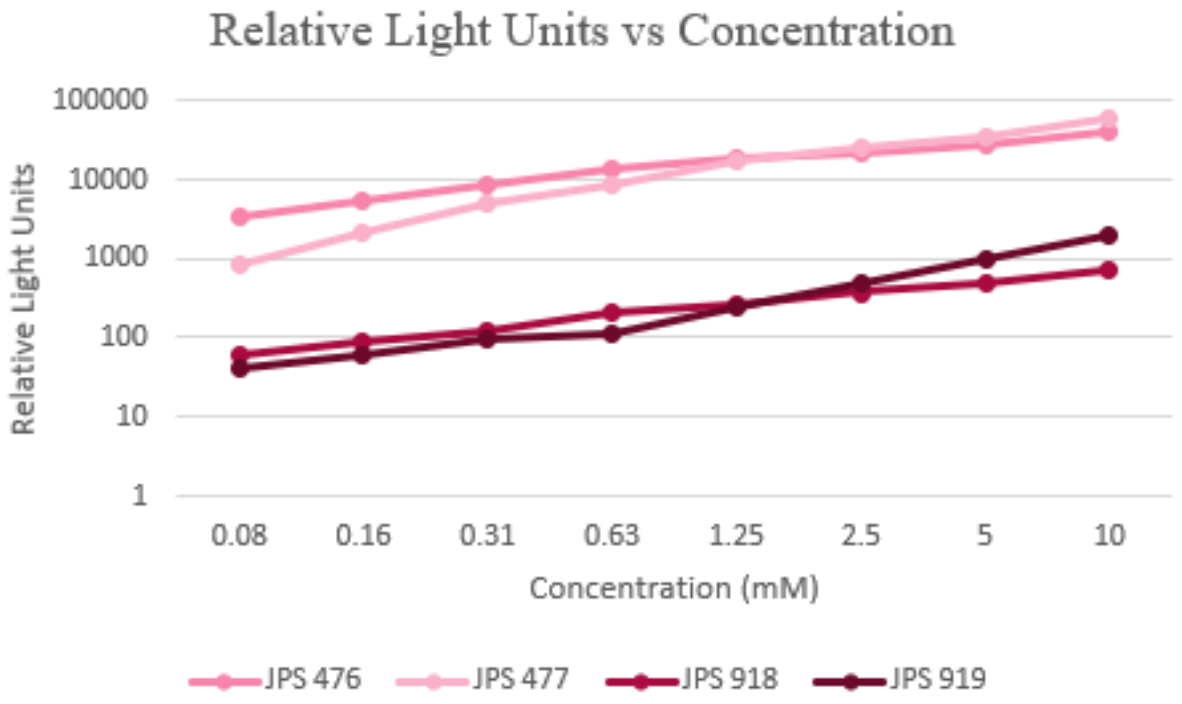

Figure 3: Graphical depiction of *E. coli* luciferase assay. The strains expressing RhlR and RhlR + PqsE are shown in pink lines, while the strains expressing SdiA and SdiA + PqsE are shown in red lines. The graph shows an increase in relative light units with the addition of PqsE for RhlR, indicating that RhlR and PqsE can induce higher levels of transcription and thus, more light. However, this effect is not observed for SdiA and PqsE.

The presence of PqsE leads to a four-to-five-fold increase in relative light units in samples containing RhlR, but not in samples containing SdiA. This indicates that RhlR and PqsE interact and induce higher levels of transcription, resulting in increased luciferase production. In contrast, the difference in relative light units between samples containing SdiA with and without PqsE is negligible, indicating that there is no interaction between SdiA and PqsE, and no enhancement of luciferase production.

Figure 4 displays the agarose gel images of the different stages of the experiment for transforming a plasmid containing a *B. cepacia* gene into *E. coli* competent cells, all of which were unsuccessful. The left image illustrates the digest of a PCR that was run on the agarose gel for purification and later used for a transformation. The middle image contains the same samples as the left image, but employed different primers. The right image depicts a colony PCR where the ligation was found to be unsuccessful after being sent for sequencing. All of the samples in each lane are identical to each other.

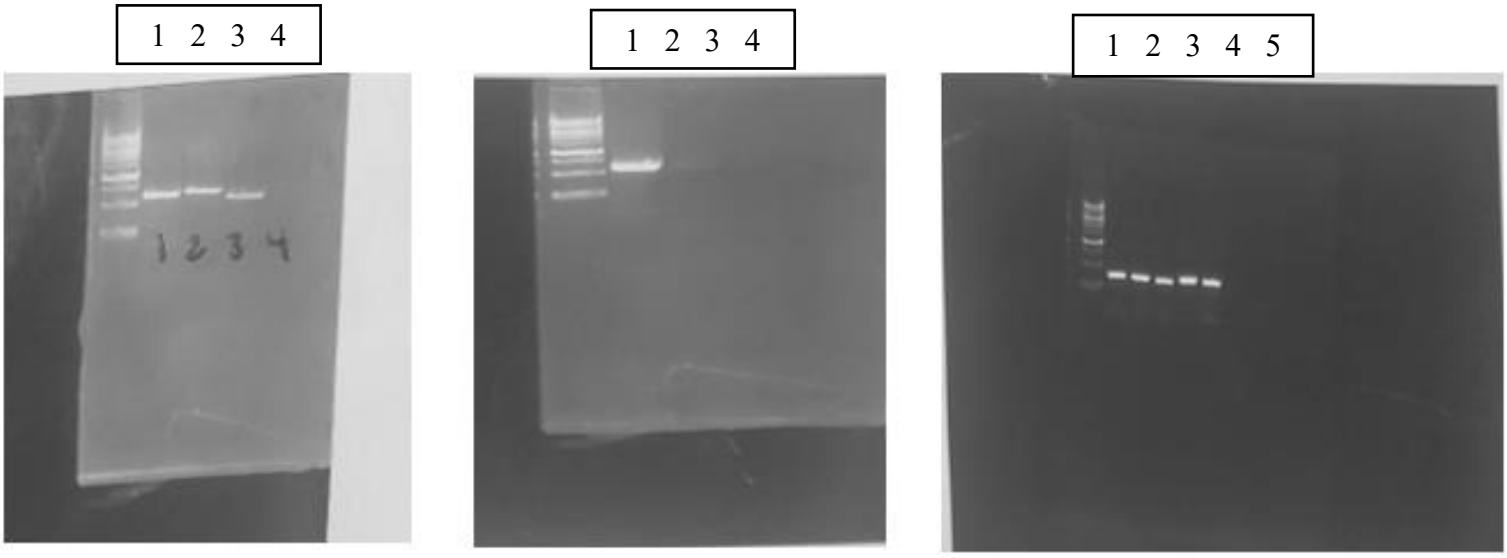

Figure 4: The unsuccessful attempts to transform a gene from *B. cepacia* into *E. coli* competent cells. The image on the left depicts the digest of a PCR, which exhibited visible bands in lanes 1-3, but not in lane 4, despite containing the same sample. This set of samples did not produce colonies during a transformation. Similarly, the middle image used different primers but encountered the same issues, where lanes 2, 3, and 4 showed no visible bands and also failed to

produce colonies during a transformation. The last image on the right is a colony PCR, taken right before being sent for sequencing.

After encountering difficulties with transforming a gene from *B. cepacia*, the decision was made to switch to a bioinformatics experiment instead. The goal was to evaluate the structure of SolR and PqsE within *B. cepacia*. This was accomplished using AlphaFold, a program that can predict the theoretical structure of SolR and PqsE while interacting. Prior to the experiment, it was known that PqsE is found in *P. aeruginosa* and *B. cepacia*, as well as some other closely related species, but that not all of the species have a RhlR homolog. AlphaFold was utilized to determine if the homologs of PqsE and SolR in *B. cepacia* form their complex in the same manner as PqsE and RhlR from *P. aeruginosa*.

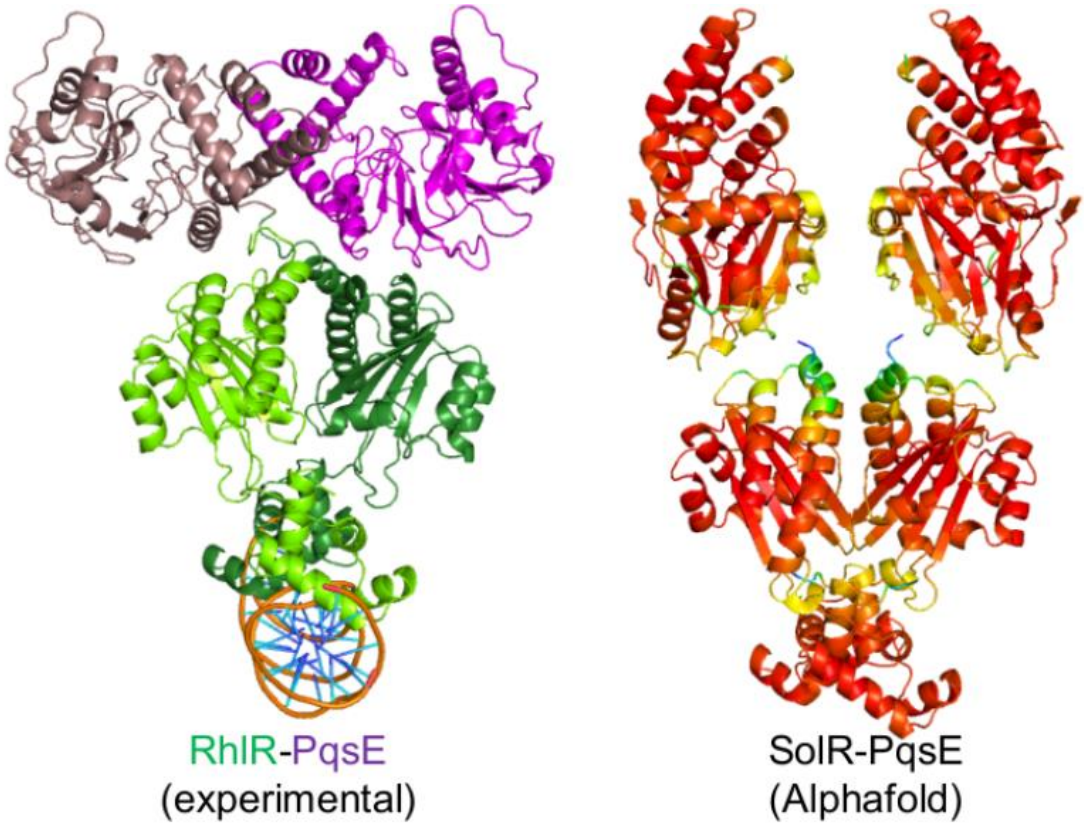

Figure 5: Comparison of the experimentally determined structure of RhlR and PqsE from *P. aeruginosa* by the Paczkowski lab (Simanek et al, 2022) and the predicted structure of SolR and PqsE from *B. cepacia* using a bioinformatics program called AlphaFold. The darker red colors indicate a high confidence in the fold as determined bioinformatically, and the more blue and purple colors indicate a low confidence. While it is known that the binding of PqsE to RhlR forms a dimer, the predicted structure of SolR and PqsE does not show evidence of dimerization.

The predicted structure obtained from AlphaFold revealed that the binding of PqsE to RhlR from *P. aeruginosa* and *B. cepacia* is not the same. In the case of RhlR, PqsE dimerizes upon binding, while in the predicted structure of SolR and PqsE, there is no dimerization.

Figure 6: Amino acid sequences of PqsE from both *P. aeruginosa* and *B. cepacia*. A comparison of the sequences revealed that the region of the PqsE sequence responsible for the dimerization in *P. aeruginosa*, highlighted in orange, is not conserved in the PqsE homolog from *B. cepacia*.

Figure 6 displays a comparison of the amino acid sequence of PqsE from both *P. aeruginosa* and *B. cepacia*. The orange box indicates a highly conserved sequence in both species, while the orange highlighted section denotes the part of the sequence responsible for the dimerization of PqsE when interacting with RhlR. It's noteworthy that this sequence is not conserved in the PqsE homolog that interacts with SolR in *B. cepacia*. As a result, the predicted structure of the PqsE-SolR complex differs from that of the PqsE-RhlR complex, which can be explained by the absence of the dimerization sequence in the PqsE homolog.

Figure 7 depicts the results of SDS-PAGE gel electrophoresis from the second affinity protein pulldown experiment, which involved testing the interactions between SolR and PqsE proteins from *B. cepacia.*

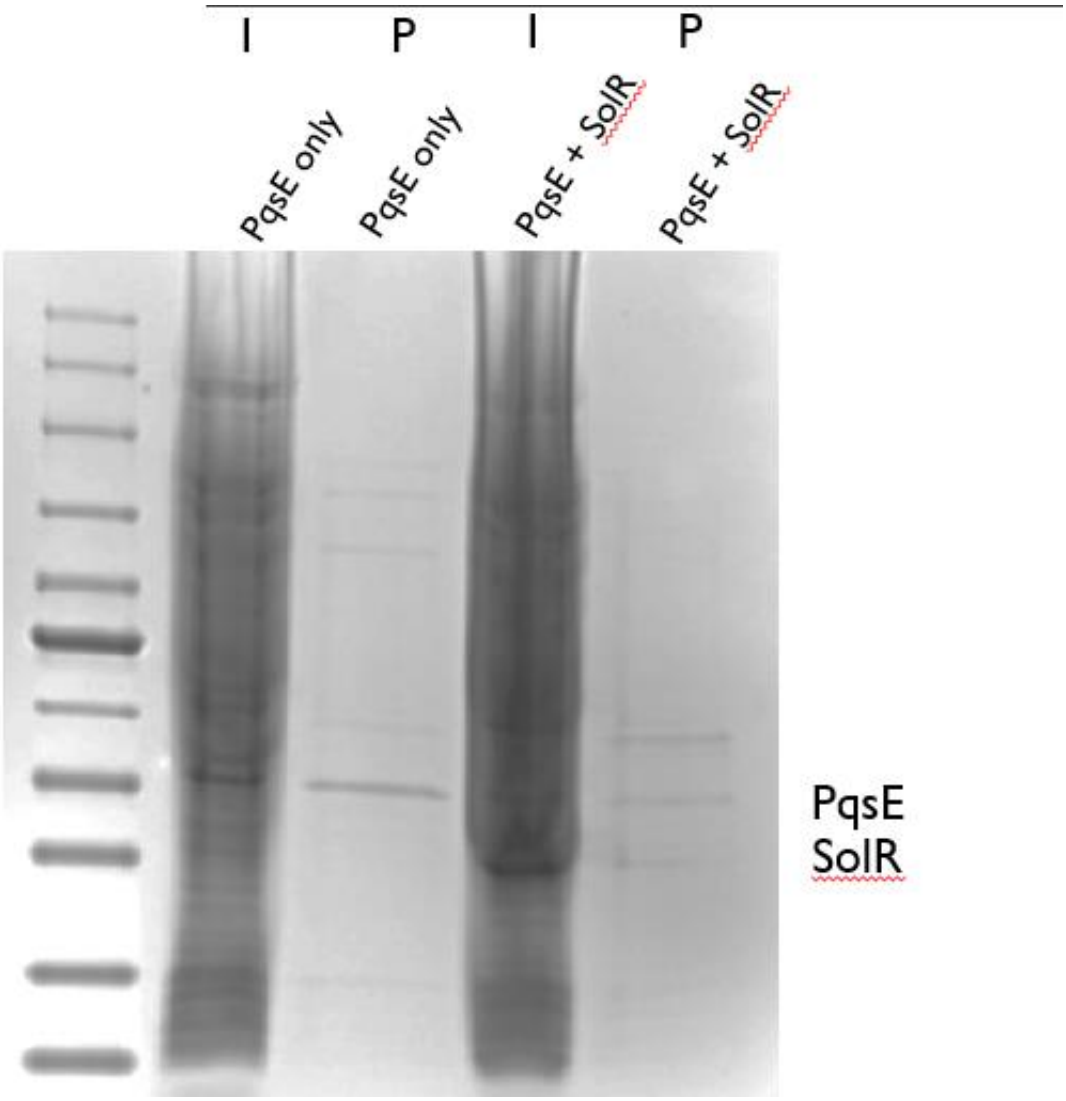

Figure 7: SDS-PAGE image for SolR and PqsE. It depicts the results of the second affinity protein pulldown experiment, where the interactions between PqsE and SolR in *B. cepacia* were analyzed through SDS-PAGE gel electrophoresis. The observed bands suggests that the

respective homologs interact with each other. However, the predicted mechanism of interaction between PqsE and SolR in *B. cepacia* by AlphaFold differs from that in other species.

The results from the second affinity protein pulldown experiment (Figure 7) suggests that the respective homolog of PqsE and SolR from *B. cepacia* do interact, but in a different manner compared to RhlR and PqsE from *P. aeruginosa*. In the second affinity protein pulldown experiment, the interaction between PqsE from *B. cepacia* and RhlR from *P. aeruginosa* was tested as well, and the results are visible in the SDS-PAGE gel image (Figure 8).

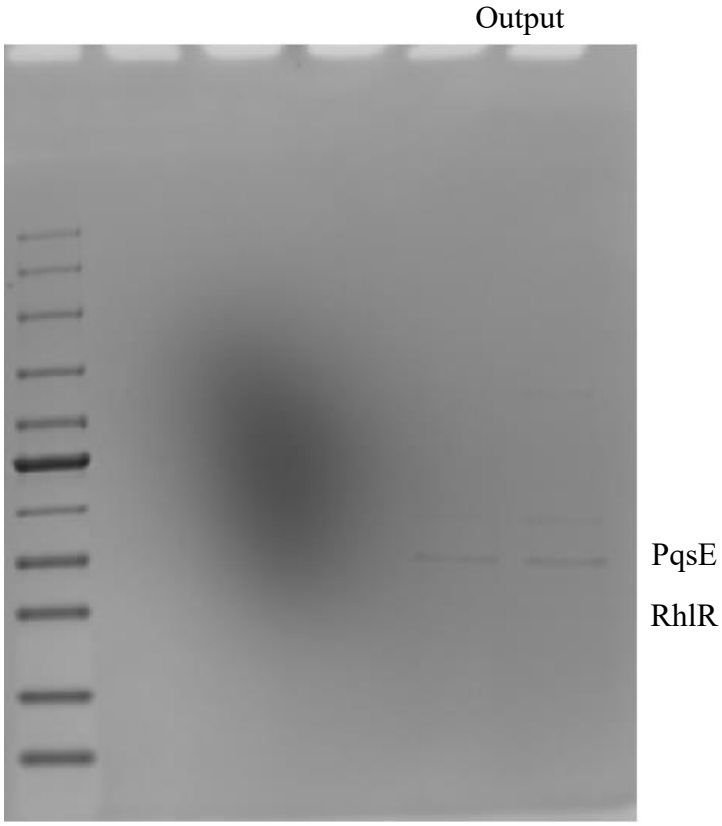

Figure 8: SDS-PAGE gel image for testing the interaction between PqsE from *B. cepacia* and RhlR from *P. aeruginosa*. The SDS-PAGE gel was not loaded with input fractions, so only output fractions are visible in the last two lanes. The absence of input fractions makes it

challenging to draw a definite conclusion. However, only faint bands for PqsE are visible in the output tubes, with no bands present for RhlR. PqsE from *B. cepacia* therefore does not bind with RhlR from *P. aeruginosa*.

The SDS-PAGE gel (Figure 8) indicates that PqsE from *B. cepacia* does not interact with RhlR from *P. aeruginosa*. This is a significant observation as it demonstrates that PqsE cannot interact with just any LuxR-type protein from different bacterial species, and shows a specificity for the LuxR-type protein from its own species. These results suggest an evolved specificity for the interaction between PqsE and LuxR-type proteins.

The image below (Figure 9) depicts the alignment file for SdiA from *S. enterica*, RhlR from *P. aeruginosa*, CviR from *C. violaceum*, and AhyR from *A. hydrophilia*, which indicates the percentage identity of a single amino acid in a group of proteins. However, the image (Figure 9) also suggests that these proteins are considerably different from each other, and therefore, a search for closer homologs is necessary.

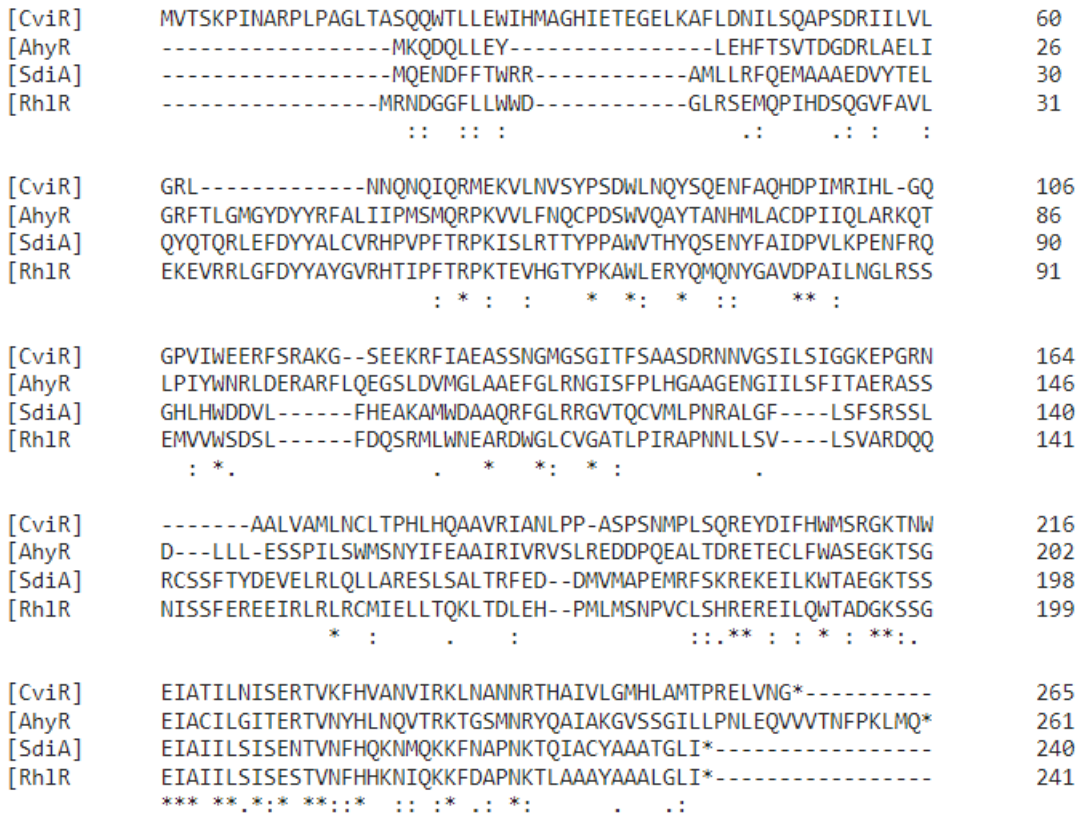

Figure 9: Alignment file for SdiA from *S. enterica*, RhlR from *P. aeruginosa*, CviR from *C. violaceum*, and AhyR from *A. hydrophilia* protein homology. As shown in the image, the sequences are found to be considerably different. Therefore, it would be beneficial to look for closer homologs to RhlR to obtain more accurate results in future studies.

Discussion

PqsE and RhlR directly interact to control the production of autoinducer in *P. aeruginosa*, and this interaction plays a critical role in quorum sensing and virulence of the pathogen (Simanek et al, 2022). To explore whether PqsE can interact with other receptors, SdiA from *S. enterica* and CviR from *C. violaceum* were tested due to their similarity in appearance to RhlR. However, the protein gel results did not show any interaction between PqsE and these receptors. Nonetheless, there are several other receptors with a similar appearance to RhlR that can be further investigated, including those from *A. hydrophilia* and *B. cepacia*, which was looked at due to this fact. Further studies can help reveal whether PqsE can interact with other receptors and how such interactions may impact the virulence and quorum sensing in different bacterial species.

The luciferase assay results provide further evidence that supports the affinity protein pulldown findings that PqsE and RhlR interact directly. In addition, the lack of interaction between PqsE and SdiA or CviR indicates that PqsE may have evolved specificity for interaction with RhlR or closely related homologs. These results also suggest that PqsE may play a specific role in the quorum sensing mechanisms of *P. aeruginosa* and may not have the same function in other bacterial species. Overall, these findings contribute to a better understanding of the molecular mechanisms underlying quorum sensing in *P. aeruginosa*.

Despite the unsuccessful transformation of *B. cepacia* DNA fragments into *E. coli* competent cells, the AlphaFold predictions provided valuable insights into the structure of PqsE when binding to SolR, the RhlR homolog in *B. cepacia*. The prediction showed that PqsE from *B. cepacia* does not dimerize like how PqsE from *P. aeruginosa* does when interacting with RhlR. This observation was confirmed by their DNA sequences. However, the pulldown

experiment confirmed that the homologs do interact, suggesting that the mechanism of interaction may be different in *B. cepacia*. To further understand this mechanism, site-directed mutagenesis could be used to identify the residues required to form the complex.

It was also found that PqsE from *B. cepacia* does not directly interact with RhlR from *P. aeruginosa*. This observation is crucial as it highlights the fact that PqsE has a specific binding affinity towards LuxR-type proteins from its own bacterial species and cannot interact with LuxR-type proteins from other bacterial species. This specificity in the interaction between PqsE and LuxR-type proteins indicates an evolutionarily conserved mechanism that has developed over time. These findings imply that the PqsE-LuxR interaction has undergone selective pressure to maintain specificity, likely due to its crucial role in regulating quorum sensing and virulence in *P. aeruginosa* and related species.

One potential explanation for the difference in interaction between PqsE and SolR in *B. cepacia* and RhlR in *P. aeruginosa* is that there could be unique features in PqsE from *B. cepacia* that allow it to bind to SolR differently, despite having a similar function. Alternatively, PqsE and SolR may still form a dimer or a different complex that was not picked up in the bioinformatics experiment. Further studies are necessary to determine the precise mechanism of interaction between PqsE and SolR in *B. cepacia*.

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