nature portfolio

Peer Review File

PlzR regulates type IV pili assembly in Pseudomonas aeruginosa via PilZ binding

Reviewer #1 (Remarks to the Author):

This manuscript describes Pseudomonas aeruginosa PlzR as an anti-phage protein through an effect on bacterial type IV pilus (T4P). The plzR gene was discovered in a genomic library screen wherein its overexpression led to resistance to the T4P-targeting phage LUZ19. This resistance was shown to be specific to T4P targeting phages because LPS-targeting ones were not affected. It appears that the expression of plzR reduced twitching motility as well as swarming and swimming. Yet no reduction in the phage absorption or piliation was observed. Using the expression of the phage gene (yip) to manipulate c-di-GMP levels in a strain with a plzR promoter fusion, the author concluded that the promoter of plzR is induced by c-di-GMP. PilZ was identified as the binding partner for PlzR by pull-down and two-hybrid experiments. Modeling suggested that certain residues in PilZ and PlzR are critical for their interactions. Mutating these residues reduced or weakened their interactions as detected by two-hybrid systems. The overexpression of a mutant PlzR (Y90A) failed to protect P. aeruginosa from LUZ19 infection. The authors proposed a model wherein the PlzR interacts with PilZ to modulate PilB, thereby affect the function of T4P to influence the infection of T4P-targeting phages. It is also speculated that c-di-GMP upregulates plzR expression to counteract the c-di-GMP binding protein FimX in the regulation of T4P.
The manuscript presents an interesting and noteworthy finding. However, the results in this manuscript are mostly on the preliminary side. Most of the points should be substantiated with more definite or more lines of evidence. The presentation of the figures also leave room for improvement.

Major points:
1) It is unclear how an effect on PilB could lead to non-functional pilus instead of reduced T4P assembly. PilB is well known as the T4P assembly ATPase. If PlzR exerts its function through interactions with PilZ to regulate PilB activity, we would have expected an effect on T4P assembly instead of T4P function. This point should be better substantiated and elaborated.
2) This is related to the 1st point. The piliation levels of plzR deletion and plzR overexpression mutants should be examined quantitatively by EM, immunolabeling or blotting. The phage absorption experiment is crude and only one line of evidence for lack of effect on piliation level. A plzR deletion mutant and complementation experiments should be conducted to demonstrate biological relevance besides overexpression.
3) The pull-down assays were somewhat unconvincing. The interactions between PilZ & PlzR are therefore only examined by two-hybrid systems. Alternative approaches for confirmation would strengthen the argument. The effects of the pilZ mutations on phage infections and motility should be examined besides the plzR mutations.
4) If c-di-GMP regulation is critical for the story, alternative approaches/experiments need to be performed beside the expression of the phage gene yip. The expression of yip has global effects on P. aeruginosa physiology and behavior.

Other points
1) It would be helpful to focus only on PlzR and mention the other two in passing in this manuscript.
2) All illustrations and data presentations could be improved.

Reviewer #2 (Remarks to the Author):

The manuscript by Hendrix et al. describes a protein encoded by Pseudomonas aeruginosa strain PAO1 called PlzR that modulates type IV pili function. Expressing PlzR in trans suppresses twitching motility of PAO1 and prevents infection by pili-dependent phages, but not adsorption of phages to PAO1 cells. The authors characterize PlzR interactions with the type IV pili machinery and find PlzR binds to PilZ. The authors go on to define specific residues required for these interactions. Finally, the authors provide data suggesting that cyclic-di-GMP regulates the expression of plzR. Overall, the authors did a nice job characterizing the function of a previously unknown regulator of type IV pili in P. aeruginosa (Pa). I have some comments and suggestions for the authors to consider in no particular order.

Major:

1. I am not convinced PlzR is a bona fide phage exclusion/resistance system. The authors data could be interpreted as PlzR being a Pa regulator of type IV pili. For example, the use of pili-dependent phages is a great tool to look at pili expression on the cell surface. Just because pili are disabled and the cells become phage resistant does not necessarily make PlzR a phage resistance mechanism, otherwise the same argument could be made for any Pa protein that regulates or is a component of type IV pili.

Additionally, out of all the phage resistance/superinfection exclusion mechanisms I am aware of (most of which are encoded by phages), none of them allow virion adsorption to the host cell. What good is a phage resistance mechanism that allows virions to adsorb to the bacterial host where they would be immediately available to infect as soon as the pili were retracted?

Rather, PlzR seems to couple the regulation of type IV pili to biofilm formation/surface associated growth via cyclic-di-GMP signaling. I suggest re-writing the manuscript around this framework.

2. Fig 2C: I am not seeing any pili in these images, even when I look at where the arrows point. For any conclusions drawn from these images, controls are needed such as a non-pilliated cell (ΔpilA) compared to a hyperpilliated cell (ΔpilT) or gold labeled anti-PilA antibodies.

3. Line 251: Even though the referenced study says PA0721 is a minor capsid protein of Pf4 virions (PMID: 35384225), there is no convincing evidence that this is the case. Structural studies of Pf4 virions did not identify PA0721 as a minor capsid protein (PMID: 32071243). Suggest removing this statement.

Minor:

1. Line 61: Is the “introduction of mutations” an anti-phage strategy? Seems like it is more chance than a deliberate strategy.
2. Lines 266-270: The authors are using the terms “susceptible to adsorption” and “phage sensitivity” interchangeably in these sentences, which makes it hard to follow their logic.

3. Lines 273-4: “inactive yet phage sensitive pili structures” Can the authors re-word so this reads something like “pili retraction is inhibited”?

Response to the reviewers

Throughout the manuscript changes were made to address the comments made by the referees and to more clearly convey the results. Textual changes are colored in red and blue, highlighting the removal and the addition of text, respectively.
A more detailed point-by point response to the referee comments can be found below. Reviewers' comments are in italics. Line numbers mentioned in the report may not coincide with the original line numbers.

Reviewer #1:
> This manuscript describes Pseudomonas aeruginosa PlzR as an anti-phage protein through an effect on bacterial type IV pilus (T4P). The plzR gene was discovered in a genomic library screen wherein its overexpression led to resistance to the T4P-targeting phage LUZ19. This resistance was shown to be specific to T4P targeting phages because LPS-targeting ones were not affected. It appears that the expression of plzR reduced twitching motility as well as swarming and swimming. Yet no reduction in the phage absorption or piliation was observed. Using the expression of the phage gene (yip) to manipulate c-di-GMP levels in a strain with a plzR promoter fusion, the author concluded that the promoter of plzR is induced by c-di-GMP. PilZ was identified as the binding partner for PlzR by pull-down and two-hybrid experiments. Modeling suggested that certain residues in PilZ and PlzR are critical for their interactions. Mutating these residues reduced or weakened their interactions as detected by two-hybrid systems. The overexpression of a mutant PlzR (Y90A) failed to protect P. aeruginosa from LUZ19 infection. The authors proposed a model wherein the PlzR interacts with PilZ to modulate PilB, thereby affect the function of T4P to influence the infection of T4P-targeting phages. It is also speculated that c-di-GMP upregulates plzR expression to counteract the c-di-GMP binding protein FimX in the regulation of T4P.

The manuscript presents an interesting and noteworthy finding. However, the results in this manuscript are mostly on the preliminary side. Most of the points should be substantiated with more definite or more lines of evidence. The presentation of the figures also leave room for improvement.

We would like to thank the reviewer for his/her constructive comments which helped to improve the manuscript. Additional experiments focused on the imaging of T4P displayed on the surface of *P. aeruginosa* cells, and *in vivo* confirmation assays were performed. Moreover, most figures were adapted to include the additional insights and/or improved to streamline the layout.

Specific comments:
1. It is unclear how an effect on PilB could lead to non-functional pilus instead of reduced T4P assembly. PilB is well known as the T4P assembly ATPase. If PlzR exerts its function through interactions with PilZ to regulate PilB activity, we would have expected an effect on T4P assembly instead of T4P function. This point should be better substantiated and elaborated.

We agree with the reviewer that we would expect that PlzR, through its interaction with PilZ and in turn the PilB ATPase, influences T4P assembly. Therefore, as suggested in the following comment, we performed fluorescence microscopy with fluorescently labeled major pilin PilA to visualize the presence and dynamics of the T4P. This showed that indeed no T4P were present on the cell surface upon *plzR* expression. The experiment was performed in triplicate on 50 different cells and compared to standard PAO1 cells, *plzR* deletion mutant cells and PAO1 cells carrying an empty mini-Tn7 transposon mutant cells, which all displayed an average of two pili per cell per 30 sec. This information is included in the text (line 155-163) and in Figures 2B and S3C. Moreover, fluorescence images are attached as supplementary Figure (Fig. S3B). This observation contradicts with previous observation by TEM, where T4P were thought to be detected. However, an expert in the field (Dr. Matthias D. Koch, Texas A&M University, USA) hypothesizes that these were wrongly interpreted artifacts during imaging, as T4P look different (more straight, equal thickness, almost exclusively polar) than those elements observed on the TEM images. The discussion section was corrected accordingly (line 308-329).

2. This is related to the 1st point. The piliation levels of plzR deletion and plzR overexpression mutants should be examined quantitatively by EM, immunolabeling or blotting. The phage absorption experiment is crude and only one line of evidence for lack of effect on piliation level. A plzR deletion mutant and complementation experiments should be conducted to demonstrate biological relevance besides overexpression.

As mentioned in comment 1, fluorescence microscopy with fluorescently labeled major pilin PilA was performed on the *plzR* overexpression mutant, as well as on a *plzR* deletion mutant and two control strains (the PAO1 strain with empty expression construct and the standard PAO1 strain). This experiment allowed to follow the T4P dynamics (including determining the number and length of the T4P displayed per cell per 30 sec), which showed that no T4P were present on the *P. aeruginosa* cell surface upon *plzR* overexpression. The *plzR* deletion

mutant did not show any difference with the control strains. The methodology is included in line 505-518, and the results in line 155-163 as well as in Fig. 2B and Fig. S3B/C. The discussion section was corrected accordingly (line 308-329). Moreover, the adsorption assay was repeated, but instead of just visually determining the presence of non-adsorbed phages in solution, the number of non-adsorbed phages was counted using the double agar method. Moreover, the *P. aeruginosa* infecting phage YuA was used instead of phiKZ, as the latter is predicted to use a second receptor besides T4P to establish its infection which can bias the results. A significant reduced adsorption efficiency was observed in the *P. aeruginosa* strain expressing the *plzR* gene from the mini-Tn7-transposon insert compared to the *P. aeruginosa* with the empty mini-Tn7 transposon construct. This information is included in line 164-170 and Fig. 2C. We also constructed a clean *plzR* deletion mutant using the newly developed CRISPR-Cas3 system by our laboratory (Lammens et al., 2023 *Microbiol. Spectr.*). This mutant strain was used in twitching assays and phage infection assays, for studying both the wild-type PlzR protein (Fig. S3A and Fig. 2C) as well as the PlzR and PilZ mutants (Fig. 5E/5F and Fig. 5C/5D).

3. The pull-down assays were somewhat unconvincing. The interactions between PilZ & PlzR are therefore only examined by two-hybrid systems. Alternative approaches for confirmation would strengthen the argument. The effects of the pilZ mutations on phage infections and motility should be examined besides the plzR mutations.

We used *in vitro* pull-down with purified PlzR as a bait and *P. aeruginosa* cell lysate as an initial interaction screening to identify potential interaction partners of PlzR. The mass spectrometry results clearly detected PilZ and PilB in the elution sample with three and four peptides, respectively. Nevertheless, to reduce false positive hits to a minimum, the mass spectrometry results were also compared with multiple other, previously performed pull-down assays with phage proteins as a bait from our laboratory (e.g. De Smet et al. 2021 *Cell Rep.*; Hendrix et al. 2022 *Cell Rep.*; Van den Bossche et al. 2016 *eLife*), which never detected PilZ and PilB in the elution samples. These *in vitro* pull-downs results were confirmed by an *in vivo* bacterial two-hybrid assay, showing the relevance of our findings by two completely different interaction techniques. Also the bacterial three-hybrid system showed that PlzR and PilB could only be in close proximity in presence of PilZ, further confirming the interaction complex. In addition, the ColabFold models supported these findings, and identified some critical residues in the interaction sites. These specific residues were in turn mutated in the bacterial two-hybrid system, demonstrating that the predicted residues indeed play a role in the interaction strength of the protein-protein interactions. The PlzR^{Y90A} mutant had already been shown to disrupt PlzR's inhibitory activity on phage infection (Fig. 5E). We now demonstrated that it also essential for twitching activity interference (Fig. 5F). Moreover, as suggested in the comment, the PilZ mutants were tested in a *pilZ* deletion mutant strain, and mutant PilZ^{K43A} showed to reduce PlzR's inhibitory effect on twitching motility at lower PlzR concentrations (Fig. 5D). Also the other ColabFold-predicted interacting residues, PilZ^{F52} and PilZ^{W72}, were shown to be essential for PilZ's function (Fig. 5C and 5D). Therefore, we are confident to conclude that the identified interaction complex, PlzR-PilZ-PilB, is true.

4. If c-di-GMP regulation is critical for the story, alternative approaches/experiments need to be performed beside the expression of the phage gene yip. The expression of yip has global effects on P. aeruginosa physiology and behavior.

We disagree with the comment that the PAO1::*yip* strain is no good cell system to study c-di-GMP regulation. Yip was shown to directly bind and activate the c-di-GMP biosynthesis enzyme diguanylate cyclase (DGC) YfiN, resulting in increased c-di-GMP levels in the cells. Diguanylate cyclases (DGC) are generally used to increase the cellular c-di-GMP level to study c-di-GMP-responsive promoters in a reporter-based system. Moreover, we tested the known c-di-GMP-responsive *cdrA* promoter as well as the sequence upstream of the *plzR* gene lacking its predicted promoter as a positive and a negative control, respectively, which confirmed the biological relevance of our results. In addition, our study aimed to confirm a previous observation of an independent group which identified that PA2560/*plzR* is upregulated upon increased c-di-GMP levels along with about 50 other differentially expressed genes, of which many are well-known c-di-GMP regulated genes, including *pel* and *psl* and flagellum and pilus genes. This is true, so we can conclude that two different assays showed that the *plzR* promoter is activated upon increased c-di-GMP levels.

Minor points:
5. It would be helpful to focus only on PlzR and mention the other two in passing in this manuscript.

We adapted the text in the results section (line 102-138) and discussion section (line 291-304) accordingly. Moreover, the phage infection results on the *P. aeruginosa* strain expressing PA0721 was replaced to the supplementary Figure S1D.

6. All illustrations and data presentations could be improved.

We improved the figures and streamlined the layout of the figures.

Reviewer #2:
The manuscript by Hendrix et al. describes a protein encoded by Pseudomonas aeruginosa strain PAO1 called PlzR that modulates type IV pili function. Expressing PlzR in trans suppresses twitching motility of PAO1 and prevents infection by pili-dependent phages, but not adsorption of phages to PAO1 cells. The authors characterize PlzR interactions with the type IV pili machinery and find PlzR binds to PilZ. The authors go on to define specific residues required for these interactions. Finally, the authors provide data suggesting that cyclic-di-GMP regulates the expression of plzR. Overall, the authors did a nice job characterizing the function of a previously unknown regulator of type IV pili in P. aeruginosa (Pa). I have some comments and suggestions for the authors to consider in no particular order.

We thank the reviewer for his/her positive evaluation, and improved the manuscript based on the proposed suggestions.

Specific Points:
1. I am not convinced PlzR is a bona fide phage exclusion/resistance system. The authors data could be interpreted as PlzR being a Pa regulator of type IV pili. For example, the use of pili-dependent phages is a great tool to look at pili expression on the cell surface. Just because pili are disabled and the cells become phage resistant does not necessarily make PlzR a phage resistance mechanism, otherwise the same argument could be made for any Pa protein that regulates or is a component of type IV pili.
Additionally, out of all the phage resistance/superinfection exclusion mechanisms I am aware of (most of which are encoded by phages), none of them allow virion adsorption to the host cell. What good is a phage resistance mechanism that allows virions to adsorb to the bacterial host where they would be immediately available to infect as soon as the pili were retracted?
Rather, PlzR seems to couple the regulation of type IV pili to biofilm formation/surface associated growth via cyclic-di-GMP signaling. I suggest re-writing the manuscript around this framework.

We fully agree with the reviewer that PlzR is essentially a novel identified regulator of T4P, and not a phage resistance determinant in essence. Therefore, we have made substantial changes to the introduction section (line 43-48 and line 73-81) as well as the discussion section (line 285-289, line 373-378, and line 382) to place less focus on the identification of phage resistance determinants and more on the identification of a novel T4P regulator.

2. Fig 2C: I am not seeing any pili in these images, even when I look at where the arrows point. For any conclusions drawn from these images, controls are needed such as a non-pilliated cell (ΔpilA) compared to a hyperpilliated cell (ΔpilT) or gold labeled anti-PilA antibodies.

We would like to refer to comment 1 and comment 2 of reviewer 1 to address this remark.

3. Line 251: Even though the referenced study says PA0721 is a minor capsid protein of Pf4 virions (PMID: 35384225), there is no convincing evidence that this is the case. Structural studies of Pf4 virions did not identify PA0721 as a minor capsid protein (PMID: 32071243). Suggest removing this statement.

We thank the reviewer for noticing this misinterpretation. The statement is removed from the text (line 296)

Minor points:
4. Line 61: Is the "introduction of mutations" an anti-phage strategy? Seems like it is more chance than a deliberate strategy.

Phages pose a selective pressure for bacteria to select for mutations in phage-binding receptors to circumvent phage infection. This is often described as an antiphage mechanism. Nevertheless, we adapted 'anti-phage strategy' to 'bacterial event' (line 63).

5. Lines 266-270: The authors are using the terms "susceptible to adsorption" and "phage sensitivity" interchangeably in these sentences, which makes it hard to follow their logic.

These sentences are removed from the text (line 314-319).

6. Lines 273-4: "inactive yet phage sensitive pili structures" Can the authors re-word so this reads something like "pili retraction is inhibited"?

Since the fluorescence microscopy experiment with fluorescently labeled major pilin PilA showed that T4P assembly is completely blocked upon *plzR* expression, this sentence was removed and the text was rewritten accordingly (line 309-329).

Reviewer #1 (Remarks to the Author):

The focus of this manuscript has notably shifted from an antiphage mechanism to type IV pilus regulation, in response to the criticism from the other reviewer. In response to this reviewer's major comment #1, the narrative concerning the impact of plzR overexpression on piliation levels had undergone a complete reversal. Likewise, the assertion of no effect on phage absorption has been replaced with a claim of significant reduction, directly contradicting the authors' earlier statements (comment #2). I also find the responses to this reviewer's major comments #3 and #4 not satisfactory. Although there is evidence supporting PlzR as a probable regulator of T4P biogenesis, I remain skeptical of its specificity, particularly given the observable effect of the PlzR overexpression on flagellated motility.

Reviewer #2 (Remarks to the Author):

All of my concerns have been addressed. Thank you to the authors for doing the additional experiments (esp the T4P imaging). This will be a nice contribution to the field.

Response to the reviewers

Reviewer #1:
>The focus of this manuscript has notably shifted from an antiphage mechanism to type IV pilus regulation, in response to the criticism from the other reviewer. In response to this reviewer's major comment #1, the narrative concerning the impact of plzR overexpression on piliation levels had undergone a complete reversal. Likewise, the assertion of no effect on phage absorption has been replaced with a claim of significant reduction, directly contradicting the authors' earlier statements (comment #2). I also find the responses to this reviewer's major comments #3 and #4 not satisfactory. Although there is evidence supporting PlzR as a probable regulator of T4P biogenesis, I remain skeptical of its specificity, particularly given the observable effect of the PlzR overexpression on flagellated motility.

We were very thankful for the previous suggestion by reviewer #1 to examine the piliation levels more quantitatively. Our collaboration with Dr. Matthias D. Koch (Texas A&M University, USA), who is an expert in super-resolution microscopic research of *P. aeruginosa*, helped to improve our insights in the working mechanism of PlzR by using fluorescence microscopy with fluorescently labeled PilA. His observations clearly showed the absence of T4P on the *P. aeruginosa* cell surface upon *plzR* expression. The adsorption assay was repeated using another T4P-pili dependent phage, namely phage YuA, as phage phiKZ is known to use other receptors besides T4P to establish its infection (Li et al., 2022. *bioRxiv*).

We agree with the reviewer that PlzR might influence other processes beyond T4P, as flagellum-mediated motility is also affected. We added this remark to the discussion section (line 255-258): "It has to be noted that PlzR overexpression also affects flagellum-dependent motility, suggesting that PlzR might bind additional proteins, similar to PA0721, or an unknown connection between T4P assembly and flagellum function exists".

Reviewer #2:
>All of my concerns have been addressed. Thank you to the authors for doing the additional experiments (esp the T4P imaging). This will be a nice contribution to the field.

We thank the reviewer for his/her positive evaluation.