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

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Type IV pili (T4P) are thin, flexible filaments exposed on the cell surface of gram-negative bacteria and are involved in pathogenesis-related processes, including cell adsorption, biofilm formation, and twitching motility. Bacter-iophages often use these filaments as receptors to infect host cells. Here, we describe the identification of a protein that inhibits T4P assembly in *Pseudo-monas aeruginosa*, discovered during a screen for host factors influencing phage infection. We show that expression of PA2560 (renamed PlzR) in *P. aeruginosa* inhibits adsorption of T4P-dependent phages. PlzR does this by directly binding the T4P chaperone PilZ, which in turn regulates the ATPase PilB and results in disturbed T4P assembly. As the plzR promoter is induced by cyclic di-GMP, PlzR might play a role in coupling T4P function to levels of this second messenger.

A continuous arms race drives the unceasing coevolution between phages and bacteria, in which bacteria develop resistance to protect against phage predation and phages respond by the emergence of counter-strategies^{1–3}. Understanding how bacteria become resistant against phage predation is of interest for example to improve phage-based therapies to treat bacterial infections⁴, and enhance bacteria-based food and biotechnology industries to minimize production losses due to phage contamination⁵.

Phage resistance often develops at the bacterial cell surface, preventing phage adsorption and genome injection^{6,7}. Surface structures widely used as receptor by *Pseudomonas aeruginosa* (*P. aeruginosa*) infecting phages are the type IV pili (T4P)^{8,9}. The T4P are virulence factors involved in twitching motility, cellular adherence, biofilm formation and DNA uptake¹⁰. At least 40 proteins have been identified to be related to T4P functions in *P. aeruginosa*, including both structural pilus components and regulatory proteins^{11,12}. In short, the pili of *P. aeruginosa* consist of major (PilA) and minor (FimU, PilE, PilV, PilW, PilX) pilin subunits which are

assembled and disassembled into long filaments by the cytoplasmic extension ATPase (PilB) and retraction ATPases (PilT and PilU), respectively, and the inner membrane platform protein (PilC) from the motor complex^{13,14}.

Both host- and phage-mediated mechanisms that alter T4P resulting in phage infection inhibition have been reported³. A common bacterial event is the introduction of mutations in the pili genes affecting the proteins or their expression¹⁵. Alternatively, *P. aeruginosa* can glycosylate PilA to prevent the binding of pilus-specific phages¹⁶. Prophages can equip the bacterium with anti-phage mechanisms by modulating the T4P, inducing superinfection exclusion¹⁷. In this regard, the phage-encoded protein Tip encoded by the *P. aeruginosa* prophage D3112 interacts with and blocks the activity of PilB, preventing T4P assembly, twitching motility and T4P-dependent phage infection¹⁸. Homologs of *tip* have also been reported in the closely related *P. aeruginosa* prophages JBD26 and DMS3^{19,20}. The latter gene product additionally targets quorum sensing, which has been annotated anti-quorum-sensing protein 1 or Aqs1¹⁹.

¹Laboratory of Gene Technology, Department of Biosystems, KU Leuven, Heverlee, Belgium. ²Laboratory for Host Pathogen Interactions in Livestock, Department of Biosystems, KU Leuven, Heverlee, Belgium. ³Computational Systems Biology, Department of Microbial and Molecular Systems, KU Leuven, Heverlee, Belgium. ⁴Institute for Sustainable Plant Protection, National Research Council of Italy, IPSP-CNR Headquarter, Turin, Italy. ⁵Department of Biology, Texas A&M University, College Station, TX, USA. ⁶Biomedical Research Institute and Transnational University Limburg, School of Life Sciences, Hasselt University, Diepenbeek, Belgium. ⁷Institute of Biology Leiden, Leiden University, Leiden, The Netherlands. 🖂 e-mail: rob.lavigne@kuleuven.be Screens to detect phage resistance have gained increasing interest^{7,21,22}. Most experimental-based screens are loss-of-function oriented, to identify host genes that are required for or inhibit phage replication, for example, by studying spontaneous phage resistance mutants^{6,22} or using knockout/knockdown mutant libraries²³⁻²⁸. By contrast, gain-of-function screenings which make use of over-expression of single gene or genomic fragment libraries to identify genes that inhibit phage infection have barely been reported and are limited to *Escherichia coli* (*E. coli*)-phage systems^{7,29}. Nevertheless, this approach enables the identification of host factors that confer resistance against phage predation, especially dosage-dependent factors such as regulatory regions or differentially expressed genes⁷.

We here show the results of a genome-wide gain-of-function screen in the model organism *P. aeruginosa* infected with the strictly lytic, T4P-dependent phage LUZ19^{30,31}. *P. aeruginosa* is an opportunistic human pathogen known for its broad antibiotic resistance, and therefore widely studied for phage therapy treatments^{32,33}. A hypothetical *P. aeruginosa* protein was found to prevent phage infection

when overexpressed. It was shown to bind the T4P regulatory protein PilZ, resulting in the inhibition of T4P assembly. Therefore, it was termed PlzR, referring to PilZ regulator.

Results

PlzR prevents infection by type IV pili-dependent phages in P. aeruginosa

A genome fragment library screen in *P. aeruginosa* strain PAO1 infected with phage LUZ19 (Fig. 1A and Fig. S1A) resulted in six fragments that provided the cells a clear reduction in phage sensitivity (Fig. 1B and Fig. S1B, C). One fragment (789,150–793,680 bp) comprised the prophage Pf4 region, which encodes the small prophage peptide PAO721 that was recently identified to induce superinfection exclusion by interacting with the platform protein PilC^{34,35}, validating our screening approach (Fig. S1C, D). In addition, four fragments all matched to a single locus in the *Pseudomonas* genome, which overlapped by 1191 bp (position 2,893,929–2,895,120 bp) encoding two proteins, SrfA and PA2560. Interestingly, the phage resistance phenotype was



Fig. 1 | *P. aeruginosa* genomic fragment library screen to identify genes that induce resistance against phage LUZ19 infection. A Schematic representation of the screening procedure. Host factors that confer resistance against phage infection were identified using a genome fragment library, containing randomly sheared 1.5–5 kb genomic DNA fragments of *P. aeruginosa* strain PAO1 present in the pHERD20T vector, which was first transformed to *P. aeruginosa* PAO1, followed by infection of this recombinant strain with LUZ19. Next, plasmids of selected phage-resistant colonies were isolated and individually transformed to fresh PAO1 cells. The recombinant strains were verified for resistance and the fragments were analyzed by sequencing. Next, the genes of interest were cloned individually into the pHERD20T vector to zoom in on the specific regions. The identified phage resistance determinants were tested against multiple-tailed phages (*Caudoviricetes*).

B Infection curves of phage LUZ19 infecting *P. aeruginosa* cells harboring different PAO1 genome fragments present in the pHERD20T vector. Six plasmids with specific genome fragment, indicated with genomic location, and an empty vector (empty) were tested in both LB with (induced; indicated with full lines) and without (non-induced; indicated with dashed lines) 0.2% L-arabinose. Source data are provided as a Source Data file. **C** Spot assay to evaluate phage resistance in *P. aeruginosa* upon induced expression of PA2560/PIzR against multiple *Caudoviricetes* phages. A 100-fold dilution series of the different phages (indicated at the left) up to 10^{-6} were spotted on a lawn of *P. aeruginosa* PAO1, containing the pHERD20T plasmid with the *plzR* gene, on top of LB agar with or without 0.2% L-arabinose. As a negative control, the empty pHERD20T vector was used.

also observed in the non-induced strains for all four fragments, including the one that contained a reverse complement sequence in the vector compared to the other three fragments, which could be explained by the presence of a promoter in the intergenic region (Fig. 1B and Fig. S1C).

Zooming in on the fragments by cloning the genes individually into the pHERD2OT vector, we identified PA2560 to be responsible for the observed phenotype (Fig. 1C and Fig. S2A). No function could be assigned to this small, 10 kDa peptide which is referred to hereafter as PlzR (PilZ regulator). The observation that the individually cloned gene *plzR* solely caused resistance upon expression induction established the presence of a promoter just upstream of the gene in the genome. Subsequent infection analysis with multiple phages specified the spectrum of resistance for PlzR to T4P-dependent phages, including LUZ19, YuA, phiKZ and LUZ24^{31,36,37}, whereas lipopolysaccharidedependent phages, including 14-1, PEV2 and LKA1^{31,38}, were still able to infect (Fig. 1C). Notably, the effect was transient, since pre-induced strains expressing *plzR* and subsequently infected in absence of induction agent were not resistant to LUZ19 infection anymore (Fig. S2B).

Expression of *plzR* inhibits type IV pili assembly

Based on the spectrum of resistance against T4P-dependent phages, it could be assumed that PIzR causes phage resistance either by modifying or changing the availability of the T4P on the host membrane. This hypothesis was evaluated by measuring the T4P-mediated twitching motility of P. aeruginosa cells on a moist surface of moderate viscosity (1% agar)¹². The motility assay indeed confirmed the absence of active T4P upon *plzR* overexpression, as the diameter of the twitching motility zone decreased from 1.40 (± 0.18) cm to 0.51 (± 0.12) cm upon induction of the PAO1 strain carrying the isopropyl-β-D-1thiogalactopyranoside (IPTG)-inducible plzR gene within the mini-Tn7 transposon (PAO1::plzR) (63% reduction), which was not the case for the empty control (PAO1::E) (Fig. 2A). Moreover, the reduced twitching motility could be confirmed in a *plzR* deletion mutant complemented with the *plzR* gene, showing a reduction of 53% in the diameter of the twitching zone upon *plzR* expression (Fig. S3A). Also, swimming and swarming motilities decreased upon *plzR* overexpression, albeit to a lesser extent, with a 51% and 41% reduction of the diameter of the motility zones, respectively (Fig. 2A).

The effect of PlzR on the T4P dynamics was further studied on single-cell level by fluorescence microscopy (Fig. S3B). For this, a PAO1 mutant strain containing a cysteine point mutation in PilA, PilA^{A86C}, that was then labeled with the thiol-reactive maleimide dye Alexa488-mal was created³⁹. While for the standard strain (PAO1), the *plzR* deletion mutant (PAO1 Δ *plzR*) and the empty control strain (PAO1::E) an average of two pili per cell per 30 s with respective average lengths of 0.73 (±0.31) µm, 0.67 (±0.41) µm and 0.68 (±0.35) µm were observed, no pili could be detected on the PAO1 strain expressing the *plzR* gene from the mini-Tn7 transposon (PAO1::*plzR*) (Fig. 2B and Fig. S3C). This observation points to a complete inhibition of T4P assembly by PlzR.

The lack of T4P on the surface of *P. aeruginosa* cells upon *plzR* expression hints to phage resistance via receptor adsorption inhibition. Indeed, an adsorption assay revealed a significant reduced adsorption efficiency of the T4P-dependent phage YuA on a *plzR* deletion mutant expressing the *plzR* gene from the mini-Tn7 transposon (PAO1 Δ *plzR*::*plzR*) compared to its empty counterpart (PAO1 Δ *plzR*::E) (Fig. 2C).

The promoter of *plzR* is induced by the second messenger cyclic di-GMP

Transcriptional upregulation of the *plzR* gene was previously observed at elevated levels of the intracellular signaling molecule cyclic di-GMP (c-di-GMP)⁴⁰. This is interesting as c-di-GMP signaling is

known to be intertwined with T4P regulation^{41,42}. To study the regulation of *plzR* expression by c-di-GMP, a reporter plasmid was constructed in which the intergenic region upstream of the *plzR* gene is placed upstream of the *msfGFP* reporter gene. In this region, a promoter was predicted with the SAPPHIRE.CNN promoter prediction program (p value = 0.000071)⁴³. Using a P. aeruginosa strain containing the IPTG-inducible vip gene within the mini-Tn7 transposon (PAO1::yip, encoding a phage-encoded diguanylate cyclase YfiN interacting peptide⁴⁴) enabled the correlation of the level of fluorescence to the activity of a given promoter in presence of c-di-GMP. A significantly increased promoter activity was observed for the intergenic region upstream of *plzR*, similar to the known c-di-GMP-responsive cdrA promoter⁴⁵ (Fig. 3). As a control, the intergenic region upstream of the *plzR* gene lacking the predicted promoter did not show increased activity, confirming the presence of a promoter upstream of the *plzR* gene that is regulated by c-di-GMP.

PlzR directly binds the regulatory protein PilZ, which in turn interacts with the ATPase PilB

To search for interaction partners of PlzR within the host, a pull-down was performed using the His-tagged PlzR as bait and crude *P. aeruginosa* PAO1 cell lysate (Fig. 4A). Subsequent ESI-MS/MS analysis of the elution fractions identified PilB and PilZ as potential interaction partners of PlzR (Supplementary Dataset 1). We selected these two proteins for further interaction analysis because of their key role in T4P assembly; the ATPase PilB provides the energy for pili extension, and PilZ is predicted to act as a regulator of T4P assembly⁴⁶.

To confirm the interaction by a complementary interaction approach, the selected targets were tested in a bacterial two-hybrid experiment. For this, the genes of PlzR, PilB and PilZ were all cloned to produce C- and N-terminal fusions to the T18 and T25 domain of the adenylate cyclase of the Bacterial Adenylate Cyclase Two-Hybrid (BACTH)-system (Euromedex). The assay revealed positive hits for PlzR with PilZ, as well as for PilZ with PilB (Fig. 4B). All interactions were reproducible in different vector combinations and specific, showing no notable autoactivations with the empty counterparts (Fig. S4A). Moreover, all three proteins could form homomultimers in our BACTH assay (Fig. S4B).

Although PlzR directly interacts with PilZ, overexpression of *pilZ* could not restore the bacterial motility reduction induced by PlzR (Fig. S5A), nor could it complement the phage infection inhibition (Fig. S5B), showing that the presence of PlzR strongly affects T4P activity in *P. aeruginosa*.

PlzR binds PilZ through a different binding surface than PilB

To test whether PlzR interferes with the PilZ-PilB interaction or mediates interaction with PilZ by a different binding surface than PilB, a bacterial three-hybrid was set up using a newly constructed vector pRSF6032, containing the backbone of the Pseudomonas-E. coli shuttle vector pME6032 in which the ori and replication proteins are swapped with the *ori* of the pRSFDuet-1 vector (Novagen)⁴⁷. Both plzR and pilZ were individually cloned into the pRSF6032 vector, and the plasmid was transformed to the bacterial two-hybrid reporter strain E. coli BTH101 together with the two BACTH plasmids. It should be noted that a modest growth retardation was observed compared to the cells of the bacterial two-hybrid assay due to the presence of a third plasmid and corresponding selection factor. Expression of PlzR did not affect the interaction between PilZ and PilB. Instead, PilZ was able to bring PlzR in close proximity to PilB, resulting in a positive signal in the reporter strain (Fig. 4C). Therefore, we could conclude that both PlzR and PilB can interact with PilZ simultaneously by different binding surfaces, confirming the pull-down results in which both proteins were identified and revealing a novel interaction complex in P. aeruginosa.



Fig. 2 | PlzR inhibits T4P assembly, resulting in reduced motility and disturbed phage adsorption. A Motility assays for *P. aeruginosa* PAO1 cells harboring the *plzR* gene in the mini-tn7T transposon (PAO1::*plzR*) that shows a clear reduction upon 1 mM IPTG induction, which was not the case for the empty control (PAO1::E). Data are presented as mean values \pm SD, *p* values were calculated using the Student's *t*-test (two-sided, *n* = 5 colonies). Source data are provided as a Source Data file. **B** Graphic representation of the length of the T4P displayed on the surface of *P. aeruginosa* cells with fluorescently labeled PilA, observed by fluorescence microscopy. For each condition, three biological replicates of 50 cells were analyzed for 30 s. No pili were observed on *P. aeruginosa* PAO1 cells harboring the *plzR* gene in the mini-tn7T transposon (PAO1::*plzR*) after induction with 1 mM IPTG, in contrast to the PAO1 strain (PAO1), the *plzR* deletion mutant (PAO1*ΔplzR*) and the empty

median and 25%/75% quartiles of the distributions of all measured pili for each sample. Source data are provided as a Source Data file. **C** Adsorption assay of phage YuA on the *P. aeruginosa* PAO1 *plzR* deletion mutant harboring the mini-tn7T transposon with inserted *plzR* gene (PAO1 Δ *plzR*::*plzR*) or empty counterpart (PAO1 Δ *plzR*::E). The number of free phages in solution on time point 20 min compared to 0 min post-infection is displayed as % free phages. This number did not decrease in the strain expressing *plzR* compared to the cell-free control solution (LB), in contrast to the induced empty control PAO1 strain, indicating that no phage adsorption occurred upon *plzR* expression. Data are presented as mean values ± SD, *p* values were calculated using the Student's *t*-test (two-sided, *n* = 3 independent experiments). Source data are provided as a Source Data file.

PilZ residues K43 and F52 are involved in PlzR–PilZ interaction, while PilZ residue W72 plays a key role in PilZ–PilB interaction To map the residues that are important for the PlzR–PilZ and PilZ–PilB interactions, we modeled the interaction complex using ColabFold (Fig. 5A, Zenodo doi: 10.5281/zenodo.11936574). The model confirmed our previous findings that PlzR interacts with PilZ by a different binding surface than PilB. Based on the predicted van der Waals interactions, salt bridges and hydrogen bonds (Supplementary Dataset 2), we predicted that PilZ residues K43 and F52 may be important in the PilZ–PlzR interaction, whereas PilZ residues W72 and M118 are important for the interaction with PilB. PilZ residue F52 had the highest number of predicted interactions with PlzR (9 ± 1 on average), and PilZ residue K43 was the only residue with all types of interactions (in the PlzR:PilZ models) that interacts with PlzR in both the PlzR:PilZ models and the PlzR:PilZ:PilB models. On the other hand, residues W72 and M118 were selected and compared with a previous study that found that the corresponding amino acids in *Xanthomonas citri* are involved in PilZ–PilB interaction⁴⁸.

To experimentally validate these predictions, we mutated the relevant residues individually for PilZ (PilZ^{K43A}, PilZ^{F52A}, PilZ^{W72A} and PilZ^{M118G}) and examined their impact on both interactions by bacterial two-hybrid. All point mutants, except PilZ^{M118G}, showed a reduction in



Fig. 3 | The promoter of *plzR* is induced by the second messenger cyclic di-GMP. A Graphic representation of tested intergenic regions (marked in gray) upstream of genes *plzR* and *cdrA*. The predicted promoter of *plzR* (at position -36 to -81) is marked in black. B Fluorimetric assay to determine the promoter activity of the plzR promoter in the presence of elevated cellular c-di-GMP levels. P. aeruginosa PAO1 strains harboring the yip gene (encoding a phage-encoded diguanylate cyclase YfiN interacting peptide, which induces an increase of c-di-GMP production in the host cell) in the mini-tn7T transposon (PAO1::yip), or the empty counterpart (PAO1::E), supplemented with a reporter plasmid containing the

tested promoter region fused to a *msfGFP* reporter gene were studied in the absence (non-induced) and presence of 1 mM IPTG (induced). As a control, the intergenic region upstream the *plzR* gene lacking the predicted promoter (pPlzR^{Δ45bp}-msfGFP) was tested. Fluorescent measurements were normalized for OD_{600nm} and corrected for background fluorescent levels of the corresponding strain with empty pSEVE421-plasmid. Data are presented as mean values ± SD of four biological replicates, and p values were calculated using the Student's t-test (two-sided, n = 4 colonies). Source data are provided as a Source Data file.

binding strength in the corresponding interactions, as observed by fewer cells exerting β -galactosidase activity (Fig. 5B). Moreover, the specific PilZ mutants did not affect the interaction with the other binding partner, proving that the reduced activity was due to loss of the protein interaction and not instability of the protein. The observation that the PilZ^{M118G} mutant did not affect PilZ–PilB interaction in *P*. aeruginosa indicated that the structural models are only indicative and should be confirmed experimentally.

The PilZ mutants were subsequently examined for interference with the motility and phage infection inhibition by PlzR in P. aeruginosa PAO1. For this, a *plzR* and *pilZ* double deletion mutant was constructed using the CRISPR-Cas3 system⁴⁹, and complemented with the *plzR* gene on the mini-T7 transposon and the *pilZ* gene or variants on the pHERD20T plasmid (PAO1::Δ*plzR*::Δ*pllZ*::*plzR* + *pilZ*^x). Arabinoseinduced production of PilZ^{wt} from the plasmid indeed restored the disrupted T4P functions of the deletion mutant (Fig. 5C, D). This was not the case for complementation with the PilZ^{W72A} mutant, confirming the key role of this residue in PilZ-PilB interaction. Furthermore, the PilZ^{F52A} mutant showed only partial complementation of the phage infection which was completely abolished upon IPTG-induced PIzR production, suggesting that the F52 residue rather plays a role in protein stability or function in P. aeruginosa than PlzR-PilZ interaction. In contrast, the PilZ^{K43A} mutant could restore both tested T4P functions and performed at low PIzR concentration a small, but significant reduced effect on twitching inhibition by PlzR, supporting its role in PlzR-PilZ interaction.

Mutation of PlzR residue Y90 disrupts PlzR-PilZ interaction and abolishes the protein's inhibitory activity on T4P functions

In addition, the ColabFold models enabled us to predict the PIzR residues involved in the PlzR-PilZ interaction, namely, D86 and Y90. The former was selected for participating in different types of interactions, the latter due to its high number of predicted interactions (Supplementary Dataset 2). Bacterial two-hybrid results with both mutants showed that mutation PlzR^{D86A} has only a minor effect on PlzR-PilZ interaction, while PlzR^{Y90A} strongly inhibits the interaction (Fig. 5B). This is consistent with the structural models, as residue Y90 was predicted to be most tightly interacting with PilZ (12 ± 1 predicted interactions on average, versus 3 ± 1 predicted interactions for D86) (Supplementary Dataset 2).

To circle back and determine if the inhibitory effect on phage infection was a direct result of the binding of PlzR to PilZ, we introduced the mutations into the *plzR* expression construct on the mini-T7 transposon and examined the efficiency of infection as well as the twitching motility in the P. aeruginosa cells expressing the mutant proteins. We found that PAO1 cells producing PlzR^{Y90A} could again be infected by phage LUZ19 and twitching motility was restored, similar to the non-induced cells (Fig. 5E, F). This result confirmed that PlzR indeed exercises its activity by directly targeting the T4P regulator PilZ. In contrast, cells expressing PIzR^{D86A} did not show a reduced activity compared to the wild-type PlzR, which can be explained by the only modest interruption of PlzR-PilZ interaction in the bacterial twohybrid results (Fig. 5B).

Discussion

Our gain-of-function screen with a P. aeruginosa PAO1 genomic fragment library in P. aeruginosa PAO1 cells infected with the T4Pdependent phage LUZ19 revealed a novel regulator of T4P. This is consistent with previous phage resistance screens, predominantly detecting phage receptors and their regulators^{7,22,25}. Indeed, apart from this novel T4P regulator, we detected the Pf4 prophage protein PA0721 that was recently found to interact with the T4P platform protein PilC and the methyl-accepting chemotaxis protein receptor Pill, inducing superinfection exclusion^{34,35}. Our results confirmed the broad resistance range of PA0721 against T4P-phages, beyond lysogenic phages, by providing evidence for four more strictly lytic phages belonging to distinct phage clades.

The newly identified regulator of T4P, PA2560 (renamed to PlzR -PilZ regulator), lacked similarity to any known protein or domain. We discovered that PIzR directly interacts with the T4P regulatory protein PilZ, which in turn binds the T4P assembly/extension ATPase PilB (Fig. 6). These interactions lead to the inhibition of T4P assembly. Indeed, when fluorescently labeling the major pilin, no T4P were observed on the *P. aeruginosa* cell surface upon *plzR* expression. This was further confirmed by the observation that T4P-mediated twitching activity was decreased and phage adsorption efficiency by a T4Pdependent phage was reduced upon *plzR* expression in *P. aeruginosa*. Therefore, it can be speculated that the PlzR-PilZ interaction blocks PilB function, resulting in non-piliated *P. aeruginosa* cells⁵⁰. It has to be





and PilB*) fused to the T18 and T25 domain of the adenylate cyclase. Non-fused T25 and T18 domains were used as a negative control (NC), and the leucine zipper of GCN4 was used as a positive control (PC). **C** Bacterial three-hybrid results. The PilZ and PilzR were fused C- (*PilZ) or N-terminally (PlzR*) to the T18 domain, whereas PilB was C-terminally (*PilB) fused to the T25 domain of the adenylate cyclase. Untagged PlzR and PilZ encoded from the pRSF6032 vector were tested to disturb and facilitate the interaction between PilZ-PilB and PlzR-PilB, respectively. For the NC and PC, the empty pRSF6032 vector was tested in combination with the bacterial two-hybrid controls.

noted that PlzR overexpression also affects flagellum-dependent motility, suggesting that PlzR might bind additional proteins, similar to PA0721^{34,35}, or an unknown connection between T4P assembly and flagellum function exists.

Although essential for T4P biogenesis, the precise molecular function of the PlzR's interaction partner PilZ remains to be elucidated. In previous research, it was found that the protein is required for T4P assembly and twitching motility in *P. aeruginosa*⁴⁶. Moreover, structural analysis revealed that, although PilZ homology domains in diverse other proteins have c-di-GMP binding properties, PilZ itself cannot bind this second messenger molecule^{41,51}. Consistent with our findings, in *Xanthomonas axonopodis* pv *citri* (*X. axonopodis* pv *citri*) and *Lysobacter enzymogenes* it was found that PilZ interacts with PilB^{41,52}. Nevertheless, the regulation of this interaction differs between the bacteria. For example, the PilZ in *X. axonopodis* pv *citri* additionally interacts with the regulatory protein FimX, which can bind c-di-GMP, connecting environmental stimuli to pili regulation^{41,53}. However, this interaction appears not to be conserved in *P. aeruginosa*, in which

FimX was found to directly bind PilB but not PilZ^{42,54}. Also, studies with *pilZ* knockout mutants in various bacteria show phenotypical differences as both piliated and non-piliated bacterial surfaces (as well as variations in bacterial motility) have been observed^{46,55,56}. Therefore, it was speculated that the function of PilZ might be controlled by unidentified, external factors, as with FimX in *X. axonopodis* pv *citri*⁴¹. We here provide evidence that *P. aeruginosa* PilZ is indeed regulated by an additional, previously uncharacterized protein, PlzR, and that this interaction influences T4P assembly.

We confirmed that the resistance against phage infection induced by PlzR is due to its direct interaction with PilZ, and that the interaction site differs from those between PilZ and PilB. Establishing ColabFoldbased structural models proved invaluable to further elucidate these interactions. Indeed, by mutating a key residue in PlzR for interaction with PilZ, namely, PlzR^{Y90}, we found that the observed phage infection inhibition and motility reduction are completely abolished. Moreover, the PilZ residues W72 and K43 showed to play an important role in the interaction with PilB and PlzR, respectively. A second residue on



PilZ, F52, might be involved in the interaction with PlzR. However, mutation of this residue also impacts the function of PilZ in *P. aeruginosa*.

PlzR highly impacts T4P functions in *P. aeruginosa*, even at lower concentrations. Indeed, overexpression of PilZ could not restore the bacterial motility reduction induced by PlzR, nor could complement its mechanism of phage infection inhibition. Moreover, PlzR function is transient, meaning that it can readily be switched on and off. This implies that differential expression of the *plzR* gene directly impacts T4P activity in *P. aeruginosa*. For example, a twofold upregulation of the *plzR* gene was previously

detected in response to high c-di-GMP levels⁴⁰. We here confirmed that *plzR* expression can indeed be induced by elevated c-di-GMP levels. As the c-di-GMP binding protein FimX in *P. aeruginosa* does not directly bind PilZ⁵⁴, contrary to some other bacteria, PlzR might add an additional level of T4P regulation for this bacterium. In *P. aeruginosa* it is speculated that at relatively low c-di-GMP levels, the high-affinity c-di-GMP receptor FimX promotes T4P assembly and so increases twitching motility by directly binding PilB⁴². By contrast, our results suggest that PlzR can regulate T4P at higher c-di-GMP levels, influencing PilB activity via binding to its regulator PilZ and thereby inhibiting twitching motility.

Fig. 5 | Specific mutations of residues in PilZ and PlzR disturb the PlzR-PilZ-PilB interaction complex. A Rank 1 ColabFold model of the PlzR-PilZ-PilB interaction complex. The dotted box indicates the region shown magnified at the right. Predicted residues involved in PilZ-PilB interaction (PilZ^{W72} and PilZ^{M118}) and in PlzR–PilZ interaction (PilZ^{K43}, PilZ^{F52}, PlzR^{D86}, and PlzR^{Y90}) are indicated, and their sidechains are shown as sticks. B Bacterial two-hybrid results of PilZ and PlzR mutants. PilZ and its mutants were C-terminally fused (indicated with *PilZ^x) to the T18 domain of the adenylate cyclase, whereas PilB and PlzR and its mutants were C-terminally fused (indicated with *PilB and *PlzRx) to the T25 domain. Combinations with a reduced interaction strength as a result of the mutation are indicated with an asterisk. C Hundredfold serial dilution (100-10-8) of phage LUZ19 dropped on lawns of the P. aeruginosa PAO1 pilZ and plzR double deletion mutant complemented with the IPTG-inducible *plzR* gene on the mini-T7 transposon and the arabinose-inducible *pilZ* gene or variants on the pHERD20T plasmid (PAO1\Delta plzR\Delta pilZ::plzR + pilZ*). The lipopolysaccharidedependent phage PEV2 was dropped in parallel for comparison. D Diameter of the

twitching zone of the *P. aeruginosa* PAO1 *pilZ* and *plzR* double deletion mutant complemented with the IPTG-inducible *plzR* gene on the mini-T7 transposon and the arabinose-inducible *pilZ* gene or variants on the pHERD20T plasmid (PAO1 $\Delta plzR\Delta pilZ$::*plzR* + *pilZ*^{*}). Data are presented as mean values \pm SD and *p* values were calculated using the Student's *t*-test (two-sided, *n* = 5 biological replicates), n.s. = not significant. Source data are provided as a Source Data file. **E** Tenfold serial dilution (10⁻¹-10⁻⁸) of phage LUZ19 dropped on lawns of the *P. aeruginosa* PAO1 *plzR* deletion mutant carrying the *plzR* gene or a mutant (*plzR*^{D86A}, *plzR*^{Y90A}) on the mini-tn7T transposon (PAO1 $\Delta plzR$::*plzR*[×]). The lipopolysaccharide-dependent phage PEV2 was dropped in parallel for comparison. **F** Diameter of twitching zone of the *P. aeruginosa* PAO1 *plzR* deletion mutant complemented with the IPTGinducible *plzR* gene or a mutant on the mini-T7 transposon (PAO1 $\Delta plzR$::*plzR*[×]). Data are presented as mean values \pm SD and *p* values were calculated using the Student's *t*-test (two-sided, *n* = 5 biological replicates). Source data are provided as a Source Data file.



Fig. 6 | **Schematic representation of the hypothesized mode of action of the T4P regulator PlzR.** PlzR binds the T4P chaperone protein PilZ which in turn binds the ATPase PilB, blocking T4P assembly (left panel). This results in reduced twitching motility and disturbed phage adsorption due to the absence of T4P on

the *P. aeruginosa* cell surface. As the expression of PlzR can be induced at elevated levels of the second messenger c-di-GMP, PlzR might function oppositely to the high-affinity c-di-GMP receptor FimX, which is speculated to enhance twitching motility at low c-di-GMP levels by directly binding PilB in *P. aeruginosa* (right panel).

assays and E. coli BL21(DE3) (Thermo Fisher Scientific) for recombinant

Our results show that further investigations regarding the function of hypothetical proteins, even in model strains like *P. aeruginosa* PAO1, are urgently required to elucidate their role and impact during phage infection and beyond. Indeed, the presence of latent regulators of, for example, phage receptors which are expressed under clinical relevant conditions can have a concealed impact on the efficacy of antibacterial treatment, e.g., in phage therapy. Moreover, as T4P are required for full virulence, the interaction between PlzR and PilZ may be an attractive target for the development of novel anti-infectives. This approach has already been successfully exploited for other types of bacterial fimbriae involved in pathogenesis⁵⁷, as well as recently for T4P in *Neisseria meningitides* and *P. aeruginosa*⁵⁸⁻⁶¹.

Methods

Bacterial strains, plasmids and growth conditions

Three *E. coli* strains were used in this study: *E. coli* TOP10 (Thermo Fisher Scientific, Waltham, MA, USA) for cloning procedures, *E. coli* BTH101 (Euromedex, Souffelweyersheim, F) for bacterial two-hybrid

protein expression. The P. aeruginosa strain PAO1 and derivatives were used⁶². Mutant P. aeruginosa PAO1 strains were constructed using the CRISPR-Cas3 system as described by Lammens et al.⁴⁹. The pHERD20T plasmids⁶³ were constructed by restriction-based cloning using Pfu polymerase and restriction enzymes HindIII and EcoRI (Thermo Fisher Scientific). The pUC18-mini-Tn7T-Lac-GW plasmid with plzR gene was constructed and integrated in the PAO1 genome⁶⁴. The pSEVA421 plasmids with msfGFP reporter gene and different intergenic regions upstream of *plzR* or *cdrA* were constructed with SEVAtile shuffling, as described elsewhere⁶⁵. Transformation to *E. coli* was done chemically using a heat shock at 42 °C for 30 s, after the cells were made chemically competent using the rubidium chloride method⁶⁶. Transformation to P. aeruginosa strains was performed via electroporation using the Gene Pulser Xcell[™] system (Bio-Rad, Hercules, CA, USA) at 2.5 kV using a 0.2 cm gap cuvette (Bio-Rad)⁶⁷. Bacterial strains were grown in Lysogeny Broth (LB) at 37 °C supplemented with the appropriate antibiotics. The antibiotics used included ampicillin (Amp; Sigma

Aldrich, St. Louis, MO, USA), kanamycin (Km; Thermo Fisher Scientific), gentamycin (Gm; Thermo Fisher Scientific), tetracyclin (Tc; Sigma Aldrich) and carbenicillin (Cb; Fiers, Kuurne, BE) at a concentration of 100 μ g/ml (Amp¹⁰⁰), 50 μ g/ml (Km⁵⁰), 30 μ g/ml (Gm³⁰), 15 or 60 μ g/ml (Tc¹⁵ for *E. coli*, Tc⁶⁰ for *P. aeruginosa*) and 200 μ g/ml (Cb²⁰⁰), respectively.

Sequences

Nucleic acid and amino acid sequences of PlzR are available on NCBI (Accession numbers NC_002516 and NP_251250, respectively).

Gain-of-function screen in P. aeruginosa

The P. aeruginosa PAO1 strain containing the genome fragment library, which consists of random fragments ranging from 1.5 to 5 kb inserted in a pHERD20T vector under control of a pBAD promotor, was used⁶⁸. In total, 13,920,000 transformants were obtained of which 5,940,000 were predicted to have an insert, yielding a $90 \times$ coverage of the P. aeruginosa genome in all reading frames and orientations (calculated by using the formula derived by Clarke and Carbon)⁶⁹. In parallel with a PAO1 strain containing the empty pHERD20T vector, the strain was grown in 10 ml LB supplemented with Cb²⁰⁰ at 37 °C until an OD_{600nm} of 0.1 was reached, and induced with 0.2% L-arabinose (L-ara). At an OD_{600nm} of 0.3, both strains were infected with phage LUZ19 (MOI = 10) and the OD_{600nm} was measured every 10 min. After 1 h infection, the culture of the infected PAO1 strain containing the fragment library was plated on LB agar supplemented with Cb²⁰⁰ and incubated overnight at 37 °C. In total, 48 colonies were selected and individually checked for sensitivity to LUZ19 infection similar to above-described induction and infection conditions in a 96-well plate (100 µl cultures) using a Bio-Rad 680 Microplate Reader (Bio-Rad). Non-induced, infected cultures were tested in parallel. The plasmids of the positive hits were isolated using the GeneJET Plasmid Miniprep Kit (Thermo Fisher Scientific) and transformed afresh to wild-type P. aeruginosa PAO1 to verify if the phenotype was indeed attributable to the presence of the fragment. For this, both optical density measurements as well as a spot test on double agar plates⁷⁰ were performed. To determine the location of the fragments in the P. aeruginosa genome, the plasmids were Sanger sequenced and the sequencing results were analyzed with Basic Local Alignment Search Tool (BLAST; NCBI71) and the Pseudomonas Genome Database⁷². Individual genes, which were located in the overlap region of the identified fragments, were cloned into the pHERD20T vector for subsequent infection analysis.

In vitro pull-down

To obtain purified PlzR, the gene was fused to a His-tag coding sequence in the pEXP5-NT/TOPO® vector (Thermo Fisher Scientific) following the TA-cloning protocol provided by the manufacturer. Recombinant expression was performed in two 500 ml cultures with exponentially growing *E. coli* BL21(DE3) cells after induction with 1 mM IPTG at 37 °C for 4 h. The protein was purified using a 1 ml HisTrap HP column (Macherey-Nagel, Düren, DE) on an Äkta Fast Protein Liquid Chromatography (FPLC, Cytiva) system, followed by dialysis to storage buffer (50 mM Tris-HCl, pH 7.4, 150 mM NaCl).

For in vitro pull-down⁶⁸, 250 ng PlzR was incubated with 1 ml Ni-NTA Superflow beads (Qiagen, Hilden, DE) in an end-over-end shaker for 1 h at 4 °C. Next, the mixture was centrifuged at $300 \times g$ for 5 min, resuspended in 10 ml pull-down buffer (20 mM Tris pH 7.5, 200 mM NaCl, 20 mM imidazole), and loaded on a prepared 1 mL polypropylene column (Qiagen). Subsequently, filtered *P. aeruginosa* cell lysate was added. The bacterial lysate was obtained by growing *P. aeruginosa* PAO1 in 11 LB until an OD_{600nm} of 0.6, pelleted and resuspended in 40 mL protein A buffer (10 mM Tris pH 8.0, 150 mM NaCl, 0.1% (v/v) NP-40) supplemented with 40 mg HEWL and 24 mg Pefabloc®SC. Cell lysis was done by three freeze-thaw cycles and sonication (40% amplitude, 8 times 30 s with 30 s intervals). Next, three washing steps with pull-down buffer were done on the column. For elution, 500 µl pull-down buffer supplemented with 500 mM imidazole was added, the column was briefly vortexed and incubated for 5 min at room temperature and centrifuged at $1000 \times g$ for 5 min. The elution fractions were analyzed by mass spectrometry analysis. For gel-free trypsin digestion. 10 ul of protein samples was mixed with 25 ul denaturation buffer (50 mM Tris-HCl pH 8.5, 6 M urea, 8.5 mM DTT) and incubated for 1 h at 56 °C in a water bath. After adding 25 µl 100 mM iodoacetamide (Sigma Aldrich) in 50 mM NH₄HCO₃ and 150 µl 50 mM NH₄HCO₃, samples were incubated for 45 min in the dark, followed by the addition of 0.8 µg trypsin (Promega, Madison, WI, USA) and overnight incubation at 37 °C. Mass spectrometry analysis was performed on an Easy-nLC 1000 liquid chromatograph, coupled to a masscalibrated LTQ-Orbitrap Velos Pro via a Nanospray Flex ion source (all Thermo Fisher Scientific) using sleeved 30 µm ID stainless steel emitters. As peptide and protein identification software, the Proteome Discoverer software v.1.4 (Thermo Fisher Scientific) with built-in Sequest search engine and interfaced with an in-house Mascot v.2.5 server (Matrix Science, Boston, MA, USA) were used. Results from both search engines were combined in Scaffold v4.8.9 to produce a more confident estimate of the probabilities of the peptide identifications.

Bacterial two-hybrid and three-hybrid

Bacterial two-hybrid assays were conducted using the BACTH System kit (Euromedex). The P. aeruginosa genes PA2560 (plzR), pilZ and pilB were all cloned into the four vectors (pUT18, pUT18C, pKT25 and pKNT25). Mutants of *pilZ* and *plzR* genes, inserted in, respectively, the pUT18C and pKT25 vectors, were constructed via inverted PCR with two 5'-phosphorylated primers, followed by blunt-end ligation⁷³. Each combination of plasmids was co-transformed to E. coli BTH101 and dilutions of overnight cultures were spotted on synthetic minimal M63 medium (15 mM (NH₄)₂SO₄, 100 mM KH₂PO₄, 1.7 µM FeSO₄, 1 mM MgSO₄, 0.05% (w/v) vitamin B1, 20% (w/v) maltose, 1.5% (w/v) agar) supplemented with Amp¹⁰⁰, Km⁵⁰, 0.5 mM IPTG and 40 µg/ml 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside (X-gal), and plates were incubated for 2 days at 30 °C. As negative controls, the constructs were co-transformed with their empty counterparts. For the bacterial threehybrid, a compatible pRSF6032 vector was constructed, which contained the ori of the pRSFDuet-1 vector (Novagen) within the Pseudomonas-E. coli shuttle vector pME603247. This was achieved by restriction of both pME6032 and pRSFDuet-1 vectors with Xbal and Eco47III, followed by ligation of the RSF ori fragment into the pME6032 backbone. Three hybrid interaction analysis was performed similar to the bacterial two-hybrid, with the exception that Tc¹⁵ was supplemented to the M63 medium and the plates were incubated for 6 days.

Motility assays

To investigate swarming motility, $1.5 \,\mu$ l of an overnight culture of *P. aeruginosa* PAO1 harboring the *plzR* gene in the mini-tn7T transposon (PAO1::*plzR*) or empty counterpart (PAO1::E) was spotted in triplicate on 0.5% (w/v) LB agar plates ($120 \times 120 \,\text{mm}$), whereas swimming motility was evaluated using 0.3% (w/v) LB agar plates. For twitching motility, LB containing 1% (w/v) agar was inoculated with the *P. aeruginosa* PAO1 strain variants using a toothpick by stabbing the plates. The LB agar plates were supplemented with Gm³⁰, and 1 mM IPTG was added for induction. Plates were incubated overnight at 30 °C (swarming) or 37 °C (swimming), or at 37 °C for two nights (twitching). Spreading of bacteria from the inoculation point was measured using a sliding compass.

Phage adsorption assay

The *P. aeruginosa* PAO1 *plzR* deletion mutant strain harboring the *plzR* gene in the mini-tn7T transposon (PAO1 Δ *plzR*::*plzR*) and its

empty counterpart (PAO1Δ*plzR*::E) were grown in LB supplemented with Gm³⁰ and 1 mM IPTG at 37 °C to an OD_{600nm} of 0.3. Next, the cultures were infected at an MOI of 0.01, vortexed, and two 1-ml samples were taken. One sample (t=0) was immediately centrifuged for 5 min at 12,600 × g and diluted 100-fold up to 10⁻⁶. The other sample (t=20) was incubated for 20 min at 37 °C while shaking, centrifuged for 5 min at 12,600 × g and diluted 100-fold up to 10⁻⁶. The dilution series were plated using the double agar method with the *P. aeruginosa* PAO1 wild-type strain to determine the concentration of non-adsorbed phages. As a control, a bacterialfree sample (LB) to check the initial phage concentration was used to compare the adsorption efficiency.

Fluorescence microscopy

Prior to fluorescence microscopy, a P. aeruginosa pilAA86C point mutant was constructed using the CRISPR-Cas3 system⁴⁹, followed by the introduction of the pUC18-mini-Tn7T-Lac-GW plasmid with plzR gene or empty cassette into the mutant strain. Imaging of T4P was performed as described by Koch et al.³⁹. Briefly, overnight cultures were diluted 1:500 and grown in LB supplemented with 1 mM IPTG (and Gm³⁰) for 3 h at 37 °C while shaking. Next, 4.5 µg Alexa Fluor 488 C₅ maleimide dye (Thermo Fisher Scientific) was added to 180 µl cells and incubated for 45 min. The cells were pelleted and resuspended in EZ rich medium. Drops of 1 µl labeled cell suspension were deposited on small 1% (w/v) agarose pads and transferred on glass bottom petri dishes for imaging. Images were taken on an inverted Nikon Ti2 microscope with ×100 NA 1.45 lens and a Hamamatsu FusionBT camera. Videos were taken at a frame rate of 2 Hz for 30 s. The experiment was performed by analyzing three biological replicates of 50 cells/ condition.

Fluorimetric assay to measure promoter activity

To study promoter activity in the presence of increased c-di-GMP levels, a fluorimetric assay was performed⁶⁵. P. aeruginosa PAO1 strains harboring the *yip* gene in the mini-Tn7T transposon (PAO1::*yip*)⁴⁴, or the empty counterpart (PAO1::E) for control, were supplied with the pSEVA421-msfGFP plasmid containing the tested promoter region fused to a msfGFP reporter gene. For each condition, cultures of four biological replicates grown overnight in M9 minimal medium (1x M9 salts, 0.2% citrate, 2 mM MgSO₄, 0.1 mM CaCl₂, 0.5% casein amino acids, supplemented with Gm³⁰ and Sm²⁰⁰) were diluted 40-fold in fresh medium in a 96-well COSTAR® plate (Corning, NY, USA) and incubated for 2 h at 37 °C. Next, 1 mM IPTG was added for induction of the *yip* gene, followed by endpoint measurement of cell growth and fluorescent intensity in the CLARIOstar® Plus Microplate Reader (BMG Labtech, Ortenberg, DE) with software v.5.61. Non-induced cultures were tested in parallel. Cell growth was determined at OD_{600nm}, msfGFP fluorescent intensity at 485 nm (ex)-528 nm (em). All fluorescent measurements were normalized for OD_{600nm} and corrected for background fluorescent levels of the corresponding strain with empty pSEVA421 plasmid. Data analysis was done with the MARS software v.3.41 and Microsoft Excel 2016.

Protein structure modeling

Protein structures were predicted using ColabFold's Alpha-Fold2 mmseqs2 v1.2 notebook (Zenodo doi: 10.5281/ zenodo.11936574)⁷⁴⁻⁷⁶. The PlzR protein model was compared to the contents of the PDB, using DALI-PDB search (webserver, accessed on 14/12/2021⁷⁷). Structures were visualized and inter-protein interactions were determined using ChimeraX v.1.2.5 (tools hbonds and contacts - options restrict ($@C^*$ and distance 3.8⁷⁸). Due to the large size of the PlzR-PilZ-PilB complex, AMBER relaxation and/or increased recycling was not possible. Consequently, PlzR:PilZ models and PlzR:PilZ:PilB1-200 models, containing only the first 200 amino acids of PilB, were used to determine interacting residue pairs. PlzR:PilZ models of lower quality, i.e., lowest ranks and/or poor PAE at the interaction interface, were dropped from the analysis. Residues with many and/or different types of interactions were selected and interactions were verified in the rank 1 PlzR:PilZ:PilB1-200 model. Residues retaining interactions in the trimer were selected for mutational analysis. Determination of residues important for the PilZ-PilB interaction was based on the Llontop model (PDB ID: 7LKO⁴⁸). Briefly, for all mutated PilZ residues, we compared the interactions in the 7LKO model and our rank 1 PlzR:PilZ:PilB1-200 models. When similar, corresponding *P. aeruginosa* residues were retained for mutational analysis.

Reporting summary

Further information on research design is available in the Nature Portfolio Reporting Summary linked to this article.

Data availability

The Genbank accession number of the *P. aeruginosa* PAO1 reference genome is NC_002516. The ColabFold models generated in this study have been deposited in Zenodo under https://doi.org/10.5281/zenodo. 11936574. Data generated in this study are included in the Source Data file provided with this paper. Source data are provided with this paper.

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Author contributions

H.H. conceived and designed research, and wrote the manuscript. H.H., A.I., H.L., L.D., E.V., M.V., E.M.L. and M.B. conducted experiments. J.P.N. performed mass spectrometry analysis. M.D.K., F.H. and A.Y. performed fluorescence microscopy. R.L. supervised the project, and edited and reviewed the manuscript. V.v.N. co-supervised the project and reviewed the manuscript. All authors read and approved the manuscript.

Competing interests

The authors declare no competing interests.

Additional information

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