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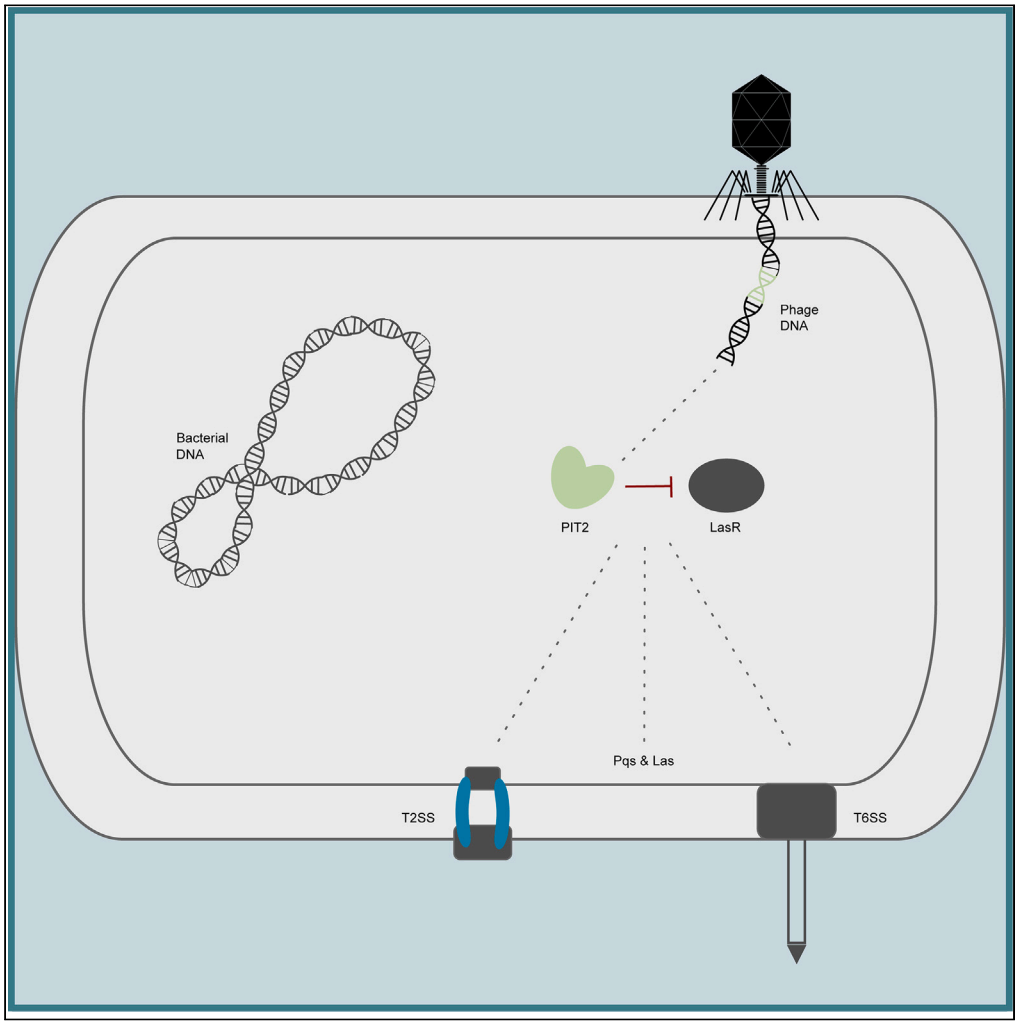

Kaat Schroven, Leena Putzeys, Anne-Laure Swinnen, Hanne Hendrix, Jan Paeshuyse, Rob Lavigne

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Highlights

The *Pseudomonas* phage LMA2 encodes PIT2, a protein that inhibits the T2SS

PIT2 attenuates the PAO1 virulence, demonstrated in *G. mellonella* and HeLa cells

PIT2 interferes with the bacterial QS system through interaction with LasR

This interaction leads to the attenuation of key virulence factors of *P. aeruginosa*

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The phage-encoded protein PIT2 impacts *Pseudomonas aeruginosa* quorum sensing by direct interaction with LasR

Kaat Schroven,[1] Leena Putzeys,[1] Anne-Laure Swinnen,[1,3] Hanne Hendrix,[1] Jan Paeshuyse,[2] and Rob Lavigne[1,4,*]

SUMMARY

In recent decades, there has been a notable increase in antibiotic-resistant *Pseudomonas aeruginosa* isolates, necessitating the development of innovative treatments to combat this pathogen. This manuscript explores the potential of different phage proteins to attenuate virulence factors of *P. aeruginosa*, particularly the type II secretion system (T2SS). PIT2, a protein derived from the lytic *Pseudomonas* phage LMA2 inhibits the T2SS effectors PrpL and LasA and attenuates the bacterial virulence toward HeLa cells and *Galleria mellonella*. Using RNAseq-based differential gene expression analysis, PIT2's impact on the LasR regulatory network is revealed, which plays a key role in bacterial quorum sensing. This discovery expands our knowledge on phage-encoded modulators of the bacterial metabolism and offers a promising anti-virulence target in *P. aeruginosa*. As such, it lays the foundation for a new phage-inspired anti-virulence strategy to combat multidrug resistant pathogens and opens the door for SynBio applications.

INTRODUCTION

Since their discovery, bacteriophages have been the center of attention for the development of novel biotechnological tools and alternative strategies against bacterial pathogens. As natural predators of bacteria, sharing billions of years of co-evolution and adaptation, phages are endowed with an arsenal of factors that can be used to impact and modify the cell.[1] One of the ways phages can hijack their host, emerges from very specific interactions between bacterial and phage proteins. This interference by phage proteins which are produced early in the infection cycle, results in the reprogramming of the metabolism of the bacterial cell in such a way that it benefits the phage for progeny production.[2] However, many of such phage proteins lack homology to known proteins or domains, leaving them as hypothetical proteins. Due to this absence of sequence and even structural similarity to characterized proteins, their function is hard to predict. Consequently, these open reading frames (ORFs) have been coined as the "viral dark matter", gene products of unknown function, and some of them might impact the bacterial cell by yet unrevealed interactions.[3]

One way by which phages may impact the bacterial system, is by modulation of the host's overall virulence. It is known that pathogens owe a large part of their resulting virulence to temperate phages.[4] The phages integrate their DNA into the bacterial genome or reside in the cell as a plasmid.[5] These prophages can endow the emerging bacterial lysogens with additional toxins, modify the bacterial cell envelope or impact bacterial infectivity and cell regulation.[4] The expression of virulence factors is tightly regulated by diverse quorum sensing (QS) systems, a sophisticated bacterial cell-density dependent communication mechanism.[6] In *Pseudomonas aeruginosa*, three major QS systems have been described: two acyl-homoserine-lactone dependent systems (LasIR and RhlIR) and the *Pseudomonas* quinolone signal (PQS) system. These three systems are interconnected in a hierarchal manner, in which the LasIR system serves as the master key, necessary to activate the other two systems.[6] With an estimated 10% of all *P. aeruginosa* genes being regulated by QS, these systems are considered to control a majority of the processes that take place in the cell.[7] Furthermore, the majority of genes that are activated by the QS systems play a role in the expression of several virulence factors. Examples of this include biofilm production, motility, and secretion systems.[8] Another aspect that is regulated by the QS system, is the defense against phage predation. This was reported by Hoque et al.[9] where it was observed that phage receptors were downregulated when the quorum was reached, and by HØyland-Kroghsbo et al.[10] who showed that high cell-densities tend to activate the expression of the CRISPR immune system. Recently, a number of temperate phage-encoded mechanisms that interfere with the bacterial QS system have been described.[11] For example, Aqs1, a phage protein derived from a temperate phage interacts with LasR, the major regulator of the QS system in *P. aeruginosa*.[12] On the other hand, Hendrix et al.[13] showed that the lytic phage LUZ19 exploits

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PqsD, a key enzyme in the PQS biosynthesis pathway. Despite this latest observation, the knowledge on strictly lytic phages influencing bacterial QS systems remains scarce.

This study aims to elucidate phage-derived proteins that attenuate the virulence, and more in particular the T2SS, of *P. aeruginosa*. As such, we identified a protein derived from the lytic *Pseudomonas* phage LMA2 that impacts the T2SS by interference with the bacterial QS system. By interaction with LasR, the transcriptional activator of the three QS systems, LMA2gp01 (renamed as PIT2) attenuates *P. aeruginosa* virulence at several levels, including the inhibition of the type two secretion system (T2SS), *in vitro* and *in vivo*. We further elucidated the impact of PIT2 on the transcriptional landscape and revealed a reduced expression of the genes that are stringently regulated by LasR. To our knowledge, this is the first protein derived from a lytic phage that solely impacts QS of the *P. aeruginosa* cell.

RESULTS

An early produced protein from a lytic *Pseudomonas aeruginosa* phage inhibits the activity of two effectors of the T2SS

To identify phage proteins that impact the T2SS (Figure 1A), a protease IV (PrpL)-based screen was set up. The protease activity was measured by the change in absorbance caused by PrpL using the chromogenic substrate Chromozym PL. The effect of over a hundred early expressed phage proteins from lytic phages on the PrpL activity in the cell supernatants was assessed (Figure S1). The expression of the ORFan LMA2gp01, present in the genome of the *P. aeruginosa* strain PAO1, resulted in a complete abolishment of PrpL protease activity when compared to the control strain (Figure 1B; Student's *t* test, $p < 0.0001$). Moreover, this inhibitory effect could also be observed in the virulent strain PA14 (Figure S2). The phage-derived protein, hereafter referred to as PIT2 (Phage Inhibitor of the T2SS), has a molecular weight of only 11 kDa and is highly conserved among other *Pbunavirus* members. Despite this conservation, neither an accurate predictive protein structure using Collabfold, nor a conserved protein domain by making use of the Pfam database could be obtained.[14] Interestingly, a hundred percent match can be found with a hypothetical protein found in *Staphylococcus aureus* (locus tag HRD08_RS14040). When PIT2 production is induced in the PAO1 cell, no negative effect on the host's fitness is observed (Figure 1C). To test if PIT2 also inhibits the activity of other T2SS effectors, the presence of active elastase A (LasA) in the supernatants was assessed. As it was previously reported that PAO1 is endowed with a LasA inhibitor, degradation of the peptidoglycan cannot be observed for this bacterial strain.[15] Therefore, the staphylolytic activity assay was performed using the clinical strain PA14. Firstly, *pit2* was introduced in the pSTDesR vector containing the RhaRS/P_{rhaBAD} expression system and the vector was electroporated into PA14. The assay revealed that the phage protein PIT2 does prevent peptidoglycan cleavage of *S. aureus* by LasA (Figure 1D). Furthermore, no infection exclusion effects were observed in the propagation host Bo559 when cells producing PIT2 were infected with phage LMA2 (Figure 1E).

PIT2 attenuates the virulence of PAO1 against HeLa cells and *in vivo*

To determine if the observed virulence attenuating properties could also be observed beyond standard *in vitro* settings, two different virulence assays were performed. First, the effect of a *P. aeruginosa* strain producing PIT2 was examined in HeLa cells. To perform this experiment, PAO1 strains were created that express *pit2* constitutively, after genomic introduction of *pit2* downstream of a PEM7 promoter. The virulence of different *P. aeruginosa* strains was determined by incubation of the human cells together with the bacterial strains (Figures 2A and S3). The strains PAO1 and PAO1 carrying an empty construct (PAO1::*empty*) served as negative controls, whereas HeLa cells in growth medium served as positive control and were used to determine the relative viability of the cells. Both negative controls showed an increased virulence toward the human cells (Tucky's test, $p < 0.0001$). In contrast, cells exposed to the *P. aeruginosa* strain producing PIT2 even outcompeted the positive control (Tucky's test, $p < 0.0001$).

To further investigate the effect of PIT2 in an *in vivo* setting, the virulence of the bacterial strains was assessed in *Galleria mellonella*. Data obtained in this model organism enables the accurate assessment of the virulence effect in higher-order mammals. In this experiment, PAO1 with an empty genomic construct served as negative control, whereas phosphate-buffered saline (PBS) buffer was injected as the vehicle control. Administration of the PIT2 producing PAO1 cells resulted in a 100 % survival rate after 24 h, significantly improving the survival of the larvae compared to the PAO1::*empty* strain (Figure 2B) (Log rank test, $p < 0.0001$).

PIT2 alters the transcriptional landscape of the PAO1 host cell

To gain insight in the working mechanism of PIT2 and its effect on overall gene expression in the cell, a differential gene expression experiment was performed using RNA sequencing (RNA-seq). The transcriptional landscape of cells expressing *pit2* was compared to a control, a PAO1 strain harboring an empty construct. For both strains, RNA was sequenced in triplicate for both the uninduced and induced condition to enable differential gene expression analysis.

Principle component analysis clusters the samples of the different conditions, indicating that *pit2* expression leads to a significant change in the PAO1 cell (Figure S4). Upon expression of *pit2*, 73 genes are differentially expressed (threshold values $p_{adj} < 0.0001$ and Log2FoldChange > |2|), of which 71 genes are downregulated and two genes are upregulated (Figure 3A; Table S1). Interestingly, the top 20 of downregulated genes during PIT2 production are all regulated by LasR, either in a direct or indirect way (Figure 3B).

On top of the list of the genes which expression is the most affected, *lasB* and *rsaL* are found (Log2FC = −9.15 and −8.58, respectively, and p_{adj} value < 0.0001) (Table 1). These two genes are directly linked to LasR, via an upstream LasR binding box. RsaL is the negative regulator of the LasIR system and LasB, also known as pseudolysin, an elastase secreted by the T2SS.[16,17] Surprisingly, the expression of *prpL* is the third most affected by PIT2 (Log2FC = −8.33 and p_{adj} value < 0.0001). LasR activates *prpL* expression in an indirect way, via the alternative sigma

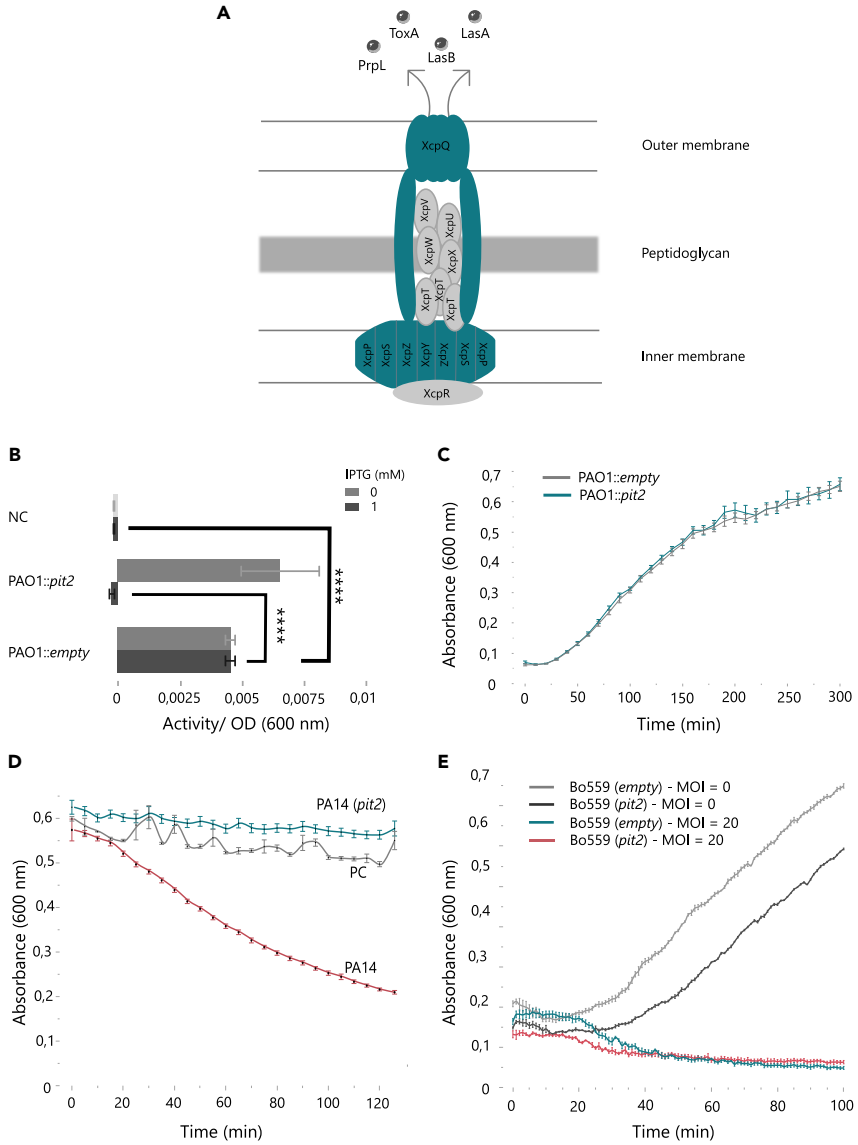

Figure 1. A PrpL-based screen identified PIT2 as an inhibitor of the type two secretion system (T2SS) and its effectors

(A) Schematic overview of the components of the type two secretion system (T2SS) and its effectors.

(B) Normalized activities of PrpL present in the supernatants of different PAO1 strains were determined by making use of the chromogenic substrate Chromozym PL. PAO1::*empty* served as positive control, as PAO1Δ*xcpV* was used as negative control (NC). When production of PIT2 is induced, no PrpL activity is measured anymore (Student's *t* test, **** = $p < 0.0001$).

(C) Growth curves of *P. aeruginosa* control (PAO1::*empty*) and PIT2 producing strain (PAO1::*pit2*). PIT2 production does not hamper the growth of the cells.

(D) Detection of the LasA activity via cleavage of the peptidoglycan of *Staphylococcus aureus* by the different PA14 strains. When PIT2 is present, this activity is blocked and the same absorbance values are observed as in the positive control PC (*S. aureus* culture).

(E) PA Bo559 host cells that express *pit2* did not show any phage infection inhibition mechanisms when infected with LMA2.

factor PvdS, even though *pvdS* itself is not significantly downregulated[18] (Log2FC = −2.19 and p_{adj} value < 0.97). This highlights the strong dependency of the activity of the T2SS, and in particular of PrpL, on the QS system. Moreover the *amb* operon, necessary for the production of a non-proteinogenic toxic compound against prokaryotes and eukaryotes, is significantly downregulated (Log2FC = −6.70 and p_{adj} value < 0.0001).[19] Another gene with downregulated expression is *pqsH* (Log2FC = −4.70 and p_{adj} value < 0.0001). The gene product of *pqsH* is responsible for the hydroxylation of 2-heptyl-4(1H)-quinolone (HHQ) into PQS, a QS signaling molecule that is in its turn controlling the expression of other genes involved in biofilm formation, general virulence, and antimicrobial defense systems.[20] Furthermore, the gene encoding the global positive regulator of the three QS systems, VqsR, has a decreased expression in the PIT2 induced condition (Log2FC = −5.29 and p_{adj} value < 0.0001).[21] Finally, we observed that several genes involved in the T6SS delivery system are downregulated, namely the

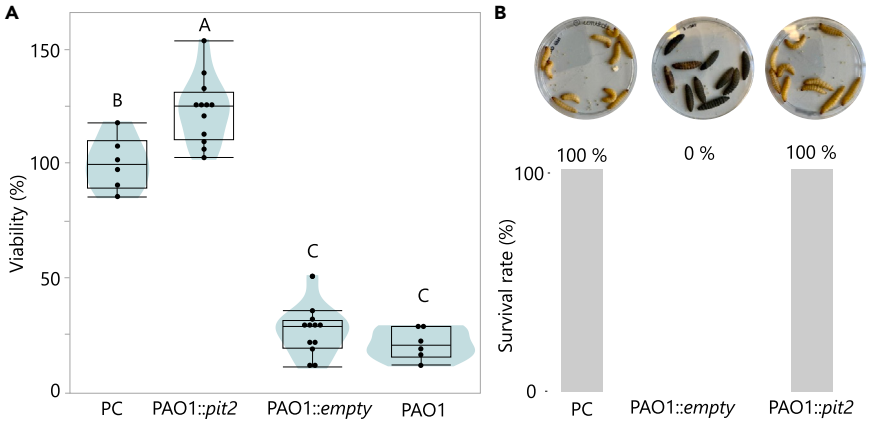

Figure 2. Results of the virulence assays

(A) The viability of HeLa cells in the presence of different PAO1 strains was monitored. The negative controls PAO1 and PAO1::empty are significantly reducing the viability of the human cells compared to the control (PC) (Tucky's test, $p < 0.0001$). The constitutive production of PIT2 in PAO1 results in even a higher viability of the HeLa cells as for the positive control (Tucky's test, $p < 0.0001$). The PC was used to calculate the relative viabilities of the different conditions. See also Figure S3.

(B) Evaluation of *Galleria mellonella* larvae infected with different *P. aeruginosa* strains. Injection of PAO1::*empty* results in a 100 % death of the larvae after 24 h. Production of PIT2 in the PAO1 cells fully rescued the larvae from the effects of the pathogen (Log rank test, $p < 0.0001$). Larvae infected with PBS buffer served as positive control (PC).

effector *tseT* and the regulators *PAAR4, tecT*, and *co-tecT* (Log2FC = −5.45 and p_{adj} value < 0.0001).[22] Together, these findings point to the interference of PIT2 with the regulation of LasR, affecting the expression of the genes controlled by this QS regulator.

The master regulator LasR of the quorum sensing systems is the direct target of PIT2

Based on the observed effect of PIT2 on the downstream regulatory network of LasR and the fact that the expression of *lasR* itself was not downregulated, we hypothesized that PIT2 interacts with LasR. To validate the direct interaction between PIT2 and LasR, a bacterial two-hybrid (B2H) assay was performed. For this, *pit2* and *lasR* were both fused N-terminally and C-terminally to the T18 and T25 domains of the adenylate cyclase of the bacterial adenylate cyclase two-hybrid (BACTH) system. A positive signal was obtained for multiple combinations, confirming the interaction. Moreover, a β-galactosidase-based Miller assay confirmed the interaction and further proves that PIT2 impacts the T2SS by interaction with LasR (Figure 4) (Student's *t* test, $p < 0.0001$).

It was previously shown that another phage protein, Aqs1, interacts with LasR.[12] We therefore aligned the predicted structure of PIT2 with that of Aqs1. As the authors of this manuscript indicated the motif responsible for LasR interaction, we tried to identify the corresponding amino acids present in PIT2. Although the exact amino acid motif (YRDALD) could not be identified, a motif with similar properties was found in PIT2 (FADELQ). In addition, the authors also reported a motif for JBD24-4 (FSDARE), another phage protein with similar properties but unable to interact with LasR. Based on the predicted LasR interaction domain, two conserved residues (first and third residue) could be observed between all three proteins. Therefore, two different point mutations were introduced separately in PIT2 ($PIT2^{F73A}$ and $PIT2^{D75A}$), to identify the amino acids essential for LasR interaction. Remarkably, by mutating aspartate (the third residue of this region) the interaction between LasR and PIT2 was abolished (Figure 5). Conversely, the other mutation ($PIT2^{F73A}$) resulted in an even stronger interaction with LasR, based on the Miller assay.

In addition, a further confirmatory bacterial two-hybrid experiment was performed, in which PIT2 was split into two separate domains, based on the structure predicted by Colabfold (Figure S5). It should be noted that the predicted structure has a very low accuracy and the true domains could therefore vary from the predicted domains in our model. The first domain is predicted to be a β-sheet, whereas the second domain is a probable α-helix and contains the LasR interacting region. However, interactions between LasR and the separate regions could no longer be detected (Figure 5). Therefore PIT2 was split a second time into two different domains. This time the first domain was composed of the predicted β-sheet and the predicted LasR interacting region and the other domain contained the remaining part of the α-helix. The combination of the β-sheet with the predicted interaction domain, gave a positive result (Figure 5).

DISCUSSION

The objective of this study was to elucidate proteins derived from lytic phages that could attenuate the virulence caused by the T2SS. We identified a protein called gp01 from *Pseudomonas* phage LMA2, which efficiently blocked the activity of PrpL and LasA, two T2SS effectors, in the *P. aeruginosa* cell. Therefore, the gene product was renamed to phage inhibitor of the type 2 secretion system (PIT2). Remarkably, this protein inhibited the T2SS without affecting the bacterial cell growth. Additionally, the gene appears to be conserved in other lytic phages belonging to the *Pbunavirus* members and even has a protein counterpart in *S. aureus*. It has been shown previously that *S. aureus* can

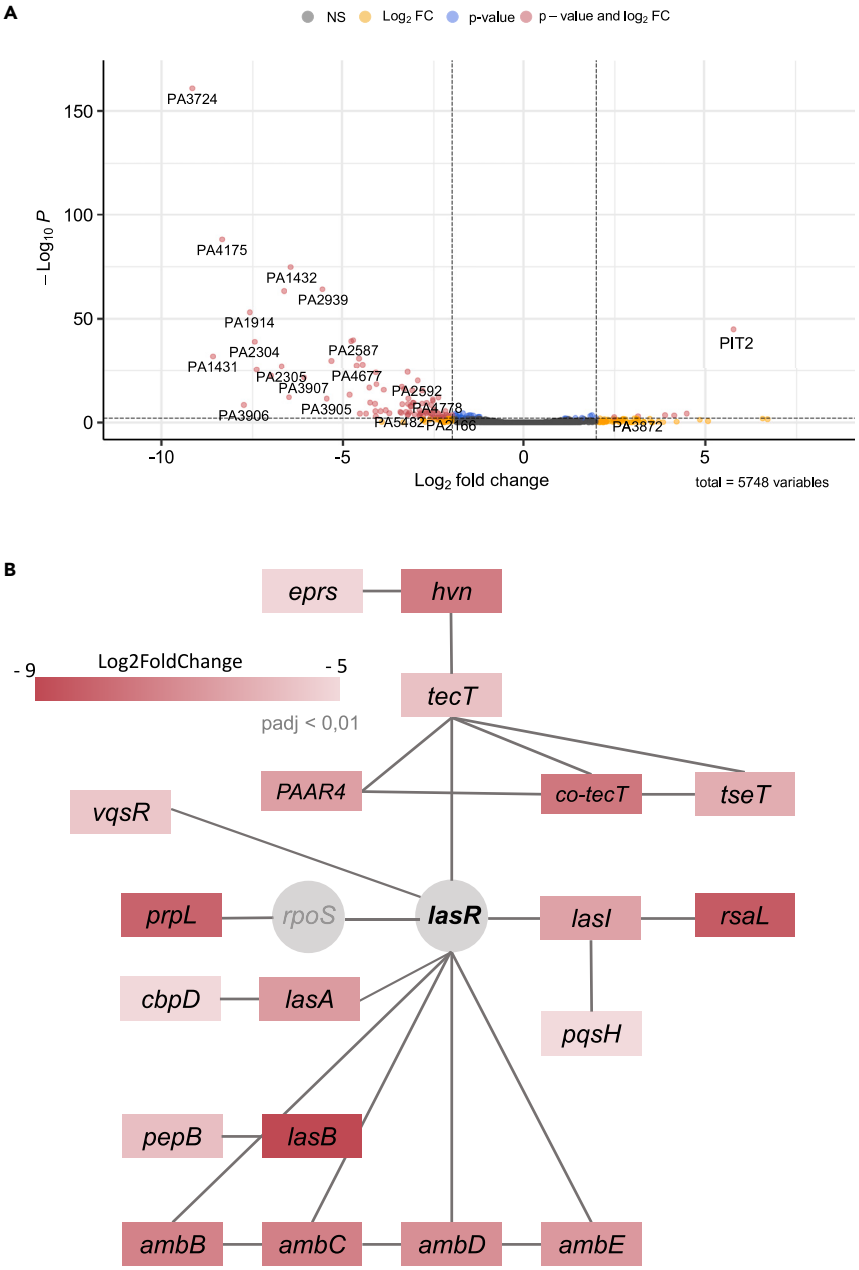

Figure 3. Multiple genes regulated by LasR are downregulated in the presence of PIT2

(A) A volcano plot shows the distribution of the differentially expressed genes by PIT2 production. Numerous genes are being downregulated, when PIT2 production is stimulated.

(B) Network of the Top20 genes downregulated by PIT2 production. The differential expressed genes are interconnected and can all be linked to the global quorum sensing regulator LasR. Genes belonging to the same regulatory pathway are connected by gray lines and genes of the same operon are clustered together. See also Table 1.

modulate the QS activity of *P. aeruginosa*. While no specific proteins have been appointed to this function, this hypothetical protein may play a role in the inhibiting the QS network of *P. aeruginosa*.[23] Hence, it can be speculated that *S. aureus* acquired the gene from a phage.

Furthermore, this study demonstrates that PIT2 successfully rescues *G. mellonella* larvae from an otherwise lethal PAO1 infection. *P. aeruginosa* produces extracellular proteases that aid in the establishment of a successful infection in its host. Notably, PrpL, LasA, LasB, and AprA are involved in the degradation of immune factors. Previous research demonstrated that intrahemocelic injection of those proteases did result in the death of the *G. mellonella* larvae.[24] The increased survival rate observed in the larvae infected by PAO1::*pit2* can be explained by the *in vitro* knowledge of PIT2 and in particular its inhibition of the proteases PrpL and LasA. Furthermore, this attenuation

Table 1. Top20 genes differentially expressed during PIT2 production

Locus tag	Gene name	Log2Fold change	p_{adj}
PA3724	*lasB*	−9.15	1,33E-161
PA1431	*rsaL*	−8.58	1,46E-32
PA4175	*prpL*	−8.33	8,23E-89
PA3906	*co-tecT*	−7.73	5,52E-09
PA1914	*hvn*	−7.57	7,90E-54
PA2304	*ambC*	−7.44	1,62E-39
PA2303	*ambB*	−7.38	3,95E-26
PA2302	*ambD*	−6.98	4,41E-23
PA2305	*ambE*	−6.70	1,26E-27
PA1871	*lasA*	−6.63	6,19E-64
PA3904	*PAAR4*	−6.49	1,16E-12
PA1432	*lasI*	−6.41	2,00E-75
PA3907	*tseT*	−6.08	2,99E-22
PA2939	*pepB*	−5.54	9,09E-65
PA3905	*tectT*	−5.45	4,81E-12
PA2591	*vqsR*	−5.29	2,18E-30
PA3535	*eprS*	−4.82	5,85E-14
PA0852	*cbpD*	−4.75	8,59E-40
PA2587	*pqsH*	−4.70	2,57E-40
PA4677	*hp*	−4.61	4,81E-28

Log2FoldChange > |2| and p_{adj} < 0.0001. With hp = hypothetical protein.

of virulence is also observed in the HeLa cells experiment, suggesting that PIT2 inhibits factors crucial for *P. aeruginosa* colonization in *G. mellonella* and infection of human cells.

The results of this differential gene expression experiment revealed not only a decreased expression of the T2SS effectors but also down-regulation of all three QS systems and other virulence-related genes. During an infection cycle, lytic bacteriophages have a limited time frame for progeny production, necessitating specific and efficient proteins for metabolic host takeover. Previous reports have identified several proteins fulfilling this function. The genomic location of *pit2* suggests that it is transcribed early after infection, highlighting its functional

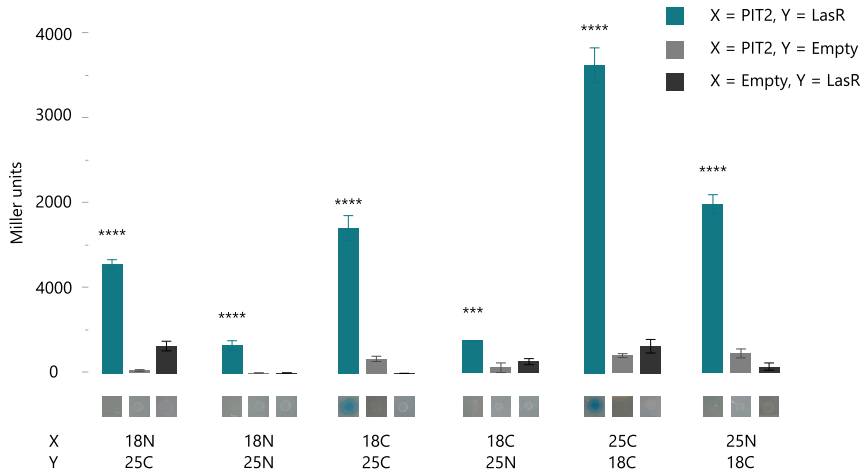

Figure 4. A bacterial two-hybrid assay confirms the interaction between PIT2 and LasR

In the B2H interaction assay, the T25 or T18 domain of the adenylate cyclase CyaA is N-terminally or C-terminally fused to the protein of interest (X or Y). Non-fused T25 and T18 domains served as negative controls. The interactions were tested in a qualitative way via a drop test on minimal medium and quantitatively confirmed by measuring the β-galactosidase activity. A positive interaction between LasR and PIT2 was observed in different combinations (Student's *t* test, *** = p < 0.001 and **** = p < 0.0001).

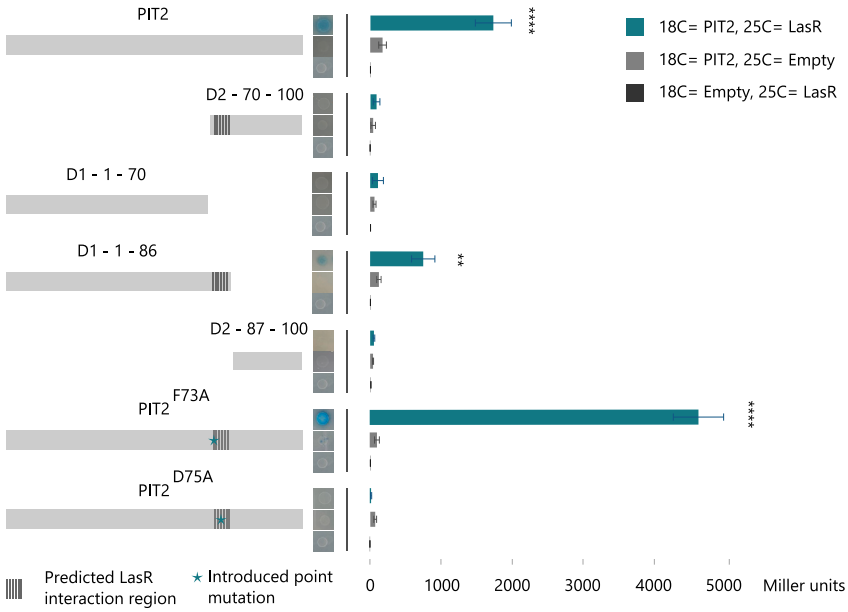

Figure 5. Interaction analysis of LasR with different PIT2 fragments

A bacterial two-hybrid assay in which the T25 of the adenylate cyclase CyaA is N-terminally fused to LasR and the T18 domain is N-terminally fused to fragment of PIT2. The corresponding domains of PIT2 that were tested are shown at the left panel of the figure. Interaction was visualized via a drop test on minimal medium and quantified via β-galactosidase activity measurements. Non-fused T18 and T25 domains served as negative controls. A positive interactions between LasR and PIT2(D1–1-86) is seen when both the predicted β-sheet and LasR interaction domain are present. Mutant fragments $PIT2^{F73A}$ and $PIT2^{D75A}$ resulted in either a stronger observed interaction or absence of interaction, respectively (Student's *t* test, ** = $p < 0.01$ and **** = $p < 0.0001$).

importance. Through interaction with LasR, PIT2 is able to interfere with the bacterial QS network. We might hypothesize that this interaction is advantageous for the phage, given the conservation of the gene among other *Pbunavirus* members. Additionally, other phage proteins like Qst and Aqs1 have been found to modulate the QS network in *P. aeruginosa*, indicating that QS inhibition is a commonly employed strategy by phages.[12,13] On the one hand, it is not surprising that a phage targets a global regulator and in this way significantly impacts the bacterial cell. This is an efficient approach to shut down several metabolic processes and thereby freeing nutrients for progeny production. Conversely, the phage may inhibit the QS network of the bacterial cells to increase the number of phage receptors in the population or inhibit the abortive infection mechanism. Indeed, the type IV pili are known to be common phage receptors and the T2SS has evolved from this system.[25] A recent study showed that upregulation of the QS system increased the expression of phage receptors, leading to higher adsorption and infection rates.[26] In contrast, other studies also reported that an increase of QS molecules resulted in a decrease of phage receptors on the bacterial surface.[9,10] QS signaling may also activate abortive infection of the host cell in an attempt to rescue the bacterial population from predation. Nevertheless, this study did not measure the impact on QS molecule concentration on phage adsorption, so no hard conclusions can be drawn.[12]

Recently, suggestions have been made to combat bacterial infections by targeting bacterial virulence factors. In this regard, PIT2 might serve as a valuable addition to the anti-virulence compound database against *P. aeruginosa*. The phage protein targets the global regulator of the QS network and thus exerts a general effect on the bacterial cell. It should be noted here that the phage protein was able to rescue the *G. mellonella* larvae and HeLa cells from the toxicity caused by PAO1 cells. Nevertheless, there are some indications that LasR might not be an ideal target for therapeutic purposes, as spontaneous *lasR* deletion mutants have been found in chronic *P. aeruginosa* infections.[27] In conclusion, the elucidation of the phage-derived PIT2 protein could initiate the exploration of other phage-derived inhibitors of pathogenic regulators and serve as a basis for orthogonal regulation of bacterial virulence in synthetic biology applications.

Limitations of the study

All assays were performed using an inducible gene expression system in the *P. aeruginosa* strain PAO1. Therefore, all the conclusions are based on a system in which the original phage was not included. A gene knock-out phage would significantly attribute to the biological relevance. However, as PIT2 is the first gene of the viral genome, the generation of a clean deletion of this gene is challenging. Furthermore, we were not able to determine the protein structure of PIT2 via crystallization. The generation of this protein structure would not only help to determine the exact interaction region between LasR and PIT2, but would also contribute in expanding the protein structure database of phage-derived proteins.

STAR★METHODS

Detailed methods are provided in the online version of this paper and include the following:

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SUPPLEMENTAL INFORMATION

Supplemental information can be found online at https://doi.org/10.1016/j.isci.2023.107745.

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AUTHOR CONTRIBUTIONS

K.S., H.H., and R.L. conceived and designed the research and wrote the manuscript. K.S. and A.S. conducted the experiments. L.P. performed the data analysis of the differential gene expression experiment. J.P. provided the instruments and insights about the HeLa experiment. All authors read and approved the manuscript.

DECLARATION OF INTERESTS

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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STAR★METHODS

KEY RESOURCES TABLE

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Bacterial and virus strains		
E. coli TOP10	ThermoFisher Scientific	CAT#C404010
E. coli BTH101	Euromedex	CAT#EUK001
P. aeruginosa PAO1	Stover et al.[28]	N/A
P. aeruginosa Bo559	Pirnay et al.[29]	N/A
P. aeruginosa PAO1 *xcpV*::Tn	Held et al.[30]	PW6207
P. aeruginosa PAO1 mini-Tn7T-LMA2-gp01	Wagemans et al.[31]	N/A
P. aeruginosa PAO1 pBGDes-LMA2-gp01	This paper	N/A
P. aeruginosa PA14 pSTDesR-LMA2-gp01	This paper	N/A
S. aureus ATCC 6538	Pasteur Institute Brussel	N/A
Pseudomonas phage LMA2	Ceyssens et al.[32]	N/A
Chemicals, peptides, and recombinant proteins		
Chromozym PL	Sigma-Aldrich	CAT#10378461001
Critical commercial assays		
BACTH System kit	Euromedex	CAT#EUK001
MTT Cell Growth Assay Kit	Sigma-Aldrich	CAT#CT02
Illumina Stranded Total RNA Prep Ligation Ribo Zero Plus kit	Illumina	CAT#20040525
Deposited data		
Differential gene expression data related to the RNA seq experiment to assess the effect of PIT2 production in PAO1	This paper	GSE227677
Experimental models: Cell lines		
Human: HeLa cells	Prof. Johan Neyts (Rega, KU Leuven)	N/A
Galleria mellonella	Terramania (NL)	N/A
Oligonucleotides		
see Table S2		
Recombinant DNA		
pSTDesR	Lammens et al.[33]	N/A
pBGDes	Lammens et al.[33]	N/A
pUC18-mini-Tn7T-Lac	Choi et al.[34]	N/A
pTNS2	Choi et al.[34]	N/A
pUT18	Euromedex	CAT#EUK001
pUT18C	Euromedex	CAT#EUK001
pUT18C derivatives	This paper	N/A
pN-25	Euromedex	CAT#EUK001
pKT25	Euromedex	CAT#EUK001
Software and algorithms		
ColabFold	Mirdita et al.[14]	N/A
FastQC	Andrews[35]	N/A
Trimmomatic	Bolger et al.[36]	N/A
BWA-MEM aligner	Li and Durbin[37]	N/A

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Continued

REAGENT or RESOURCE	SOURCE	IDENTIFIER
SAMtools	Li et al.[38]	N/A
Integrative Genomics Viewer	Robinson et al.[39]	N/A
FeatureCounts	Liao et al.[40]	N/A
DESeq2 Bioconductor package	Love et al.[41]	N/A
JMP Pro 16	SAS Institute Inc.	N/A

RESOURCE AVAILABILITY

Lead contact

Further information and requests for resources and reagents should be directed to and will be fulfilled by the lead contact, Prof. Rob Lavigne (rob.lavigne@kuleuven.be).

Materials availability

Generated plasmids and strains are available at the Laboratory of Gene Technology, KU Leuven upon request after completion of an MTA. Contact Prof. Rob Lavigne (rob.lavigne@kuleuven.be) for any further information.

Data and code availability

- The raw sequencing data of the RNA-seq based differential gene expression experiment was deposited to the Gene Expression Omnibus (GEO) database. The accession number is listed in the key resources table.
- Any additional information required to reanalyze the data stated in this paper is available upon request. Please contact the lead contact for this.

EXPERIMENTAL MODEL AND STUDY PARTICIPANT DETAILS

Bacterial strains, cells, plasmids and growth conditions

Two *Escherichia coli* strains were used in this study: *E. coli* TOP10 (Thermo Fisher Scientific, Waltham, MA, USA) for all cloning procedures and *E. coli* BTH101 (Euromedex, Souffelweyersheim, FR) for the bacterial two-hybrid assays. The experiments in *P. aeruginosa* were performed in PAO1k, Bo559 and PA14.[28,29,42] A *xcpV* transposon deletion mutant, PW6207, was ordered from the *P. aeruginosa* mutant library.[30] A PAO1 strain with a single-copy integration of the phage gene in the genome under a isopropyl-β-D-1-thiogamactopyranosie (IPTG) - inducible *lac* promoter was created in a previous study.[31] The *Staphylococcus aureus* ATCC 6538 strain (Pasteur Institute Brussel) was used in the LasA detection assay. The plasmids pSTDesR and pBGDes were used for the expression of *pit2* under the RhaRS/P_{rhaBAD} expression system and the PEM7 constitutive promoter, respectively.[33] The HeLa cells used in this research were a kind gift from Professor Johan Neyts, Laboratory of Virology and Chemotherapy (Rega Institute KU Leuven, Belgium).

Bacterial strains were grown in Lysogeny broth (LB) at 37°C and supplemented with the appropriate antibiotics, unless stated differently. The antibiotics used in this study included: ampicillin (Ap100, 100 μg/mL), gentamycin (Gm30, 30 μg/mL), kanamycin (Kan50, 50 μg/mL), spectinomycin (Spec100, 100 μg/mL) and streptomycin (Strep200, 200 μg/mL).

Transformations in *E. coli* were performed in chemically competent cells using the rubidium chloride method[43] followed by a heat shock at 42°C for 30 s. Transformations of plasmids into *P. aeruginosa* were performed via electroporation using the Gene Pulser Xcell system (Bio-Rad, Hercules, CA, USA) at 1.8 kV and a 0.2 cm gap cuvette (Bio-Rad), according to the method described by Choi et al.[44]

Bacteriophages

The *Pseudomonas* bacteriophage LMA2 was amplified on *P. aeruginosa* Bo559 using the soft agar overlay method, followed by PEG8000 precipitation and stored in phage buffer (10 mM Tris- HCl, 10 mM MgSO4, 150 mM NaCl; pH 7.5) at 4°C.[32,45] A dilution series of the phage was spotted on top of a double agar plate to determine the resulting phage titer (PFU/mL).

METHOD DETAILS

Phage infection assay

To investigate the effect of PIT2 on the infectivity of LMA2, the *P. aeruginosa* strain Bo559 harboring the pSTDesR plasmid with *pit2* under control of the RhaRS/P_{rhaBAD} expression system, was grown overnight in LB at 37°C. The following day, the culture was diluted, supplemented with 1 mM rhamnose and grown until an OD_{600nm} of 0.3. Next, the bacterial cultures were infected with different MOI (0 & 20) and 200 μL of the mixed cultures was brought into a 96- well plate. The OD_{600nm} was followed over time for 3 h, at 2 min intervals. As a control, the Bo559 strain carrying an empty pSTDesR plasmid was used. Three biological replicates were measured for each condition.

PrpL detection assay

To measure the PrpL activity in the supernatants, the assay described in Massai et al.[46] was followed. Briefly, bacterial cultures were grown overnight in LB medium. The following day, these cultures were diluted 1000-fold in fresh CAA medium (5% acid hydrolyzed casein, 5 mM K_2HPO_4, 1 mM $MgSO_4$) and 1 mM IPTG was added for induction of the phage gene expression. The next day, the OD_{600nm} was measured, the supernatants were collected and 20 μL was added to 60 μL phosphate buffer and 20 μL Chromozym PL (2 mg/mL). The absorbance was measured for 30 min, every 2 min in the Clariostar Plus microplate reader. The normalized activity was calculated as:

$$\text{Normalized } U = \frac{\text{slope} \times V}{OD_{600} \times v \times \epsilon \times d}$$

With slope the absorbance/minute, V the reaction volume in one well, v the volume of the supernatants used, ϵ the extinction coefficient of *p*-nitroaniline, the path length through one well and OD_{600} the optical density measured of the overnight culture in CAA.

For the PrpL measurements of the PA14 strains, the same procedure was followed, with minor adaptations. The PA14 strains harboring the pSTDesR vector were grown in M9 medium (1× M9 salts, 0.2% citrate, 2 mM $MgSO_4$, 0.1 mM $CaCl_2$) supplemented with 0.5% CAA and to stimulate gene expression, 1 mM rhamnose was added to the diluted cultures. Three biological replicates were measured for each condition.

LasA detection assay

To measure the LasA activity of in the supernatant, an absorbance assay based on Dehbashi et al.[47] was performed. An overnight culture of *S. aureus* was centrifuged and the resulting pellet was resuspended in 0.2 M Tris-HCl solution. Next, this suspension was boiled for 10 min at 95°C and diluted until a final $OD_{600\ nm}$ of 0.8. The overnight cultures of the *P. aeruginosa* strains were centrifuged, the supernatants were collected and filter sterilized. Next, 90 μL of the *S. aureus* solution was mixed with 10 μL *P. aeruginosa* supernatant and the $OD_{600\ nm}$ was measured for 3 h at 5 min intervals in the Clariostar plus microplate reader. Three biological repeats were included in this assay.

In vivo G. mellonella experiment

P. aeruginosa cultures were grown overnight in LB and diluted a 100-fold the next day. When an OD_{600nm} of 0.3 was reached, the bacterial culture was centrifuged (4600 *g*, 10 min) and the pellets were resuspended in sterile Phosphate Buffered Saline (PBS) (137 mM NaCl, 2.7 mM KCl, 8.2 mM Na2HPO4, 1.8 mM KH2PO4; pH 7.4). This bacterial suspension was diluted until 10^{-5} in PBS, representing approximately 1 cell/μL and 10 μL was used to inject the larvae or 10 μL PBS (positive control). Injection took place in the hindmost left pro-leg of the larvae making use of a thin needle (Microfine$^+$ insulin). The injected *G. mellonella* larvae were kept in a Petri dish and placed at 37°C in the dark. The viability was assessed after 24 h. For each bacterial strain, the survival of ten larvae was assessed.

HeLa cell experiment

The virulence of the *P. aeruginosa* strains was assessed against HeLa cells, based on the protocol of Hernandez-Padilla.[48] Cells were cultured in Dulbecco's modified eagle medium (DMEM), supplemented with 10% (v/v) FBS, 1 × (v/v) antibiotic-antimycotic solution, 1 × (v/v) MEM NEAA and 2% (v/v) sodium bicarbonate (7.5%) and incubated at 37°C with 5% CO_2. Upon cell confluency, the cells were seeded in a 96 well plate and incubated overnight. Bacterial overnight cultures were diluted to an OD_{600nm} of 0.3 and centrifuged. The pellets were resuspended in DMEM with 10% (v/v) FBS and 1 × (v/v) MEM NEAA, lacking phenol red. This bacterial suspension was diluted until 10^{-5} and 10 μL of the suspension was added to the human cells, corresponding to approximately 10 bacterial cells. After an incubation period of 15 h, the bacterial cells were removed, the HeLa cells were washed twice with PBS and 10 μL MTT (Sigma Aldrich) was added and incubated for 2 h. Finally, 100 μL solubilization buffer (Sigma Aldrich) was added to dissolve the MTT crystals. The viability of the human cells was assessed by measuring the amount of MTT cleaved into formazan at an optical density of 570 nm in the Clariostar plus microplate reader. In addition, the viability of the exposed HeLa cells was analyzed under the microscope (EVOS FL Auto imaging system, Thermo Fisher Scientific). The experiment was performed with six biological replicates.

Differential gene expression experiment

For RNA extraction, an overnight culture of PAO1::*empty* and PAO1::*pit2* in CAA +150 μM $FeCl_3$ was diluted a 100-fold in 50 mL CAA medium, supplemented with 1 mM IPTG for the induced conditions, to an OD_{600nm} of 2. At that point, 2 mL cells was harvested, 20% ice-cold stop mix solution (95% v/v ethanol, 5% v/v phenol, saturated pH 4.5) was added and the mixture was immediately snap-frozen in liquid nitrogen. Next, samples were thawed on ice and their pellets were collected (20 min, 4°C, 4500 g) for resuspension in 300 μL of a 0.5 mg/mL lysozyme solution in Tris-EDTA buffer (10 mM Tris-HCl, 1 mM EDTA, pH 8). Subsequently, samples were treated via the hot phenol method, followed by ethanol precipitation to extract the RNA. Next, the samples were treated with DNase I and the RNA was further purified using ethanol purification. The absence of genomic DNA was verified by a PCR reaction, using a PAO1-specific primer pair and the quality of the RNA was evaluated by running the samples on the Bioanalyzer (Agilent 2100) using the RNA 6000 Pico Kit. If an RNA integrity number (RIN) of ≥9 was obtained, samples were used for further processing. RNA samples were converted into cDNA using the Illumina Stranded Total RNA Prep Ligation Ribo Zero Plus kit. The Bioanalyzer and the DNA high sensitivity kit (Aligent) were used to verify rRNA depletion and evaluate the average

fragment length, aiming for fragments around 500 bp. Lastly, cDNA samples were paired-end sequenced via Illumina next-generation sequencing (Novaseq 6000). For this, all samples (n = 3 per condition) were pooled together in equimolar amounts to obtain a 25 μL sample of 4 nM. After sequencing, sequencing yields and raw read quality was assessed using FastQC[35] (v0.11.8). The reads were processed using Trimmomatic[36] (v0.39) to remove adapter sequences and low-quality bases. Next, the BWA-MEM aligner[37] (v0.7.17) was used to map the read pairs to the reference genome of PAO1 (NC_002516.2), which was manually altered to carry the pit2 genomic insert in its Tn7 site. The alignment files were converted to BAM files, sorted and indexed using SAMtools[38] (v1.9). Integrative Genomics Viewer (IGV)[39] was used to visually inspect the alignments. Subsequently, the mapped read pairs (fragments) were assigned to the genomic features of PAO1:*pit2* using FeatureCounts[40] (v2.0.1), after which the bacterial rRNA reads were removed *in silico*. The resulting fragment counts were normalized by gene length and the total number of mapped non-rRNA fragments per sample to yield fragments per kilobase of transcript per million mapped fragments (FPKM) values. Principal Component Analysis (PCA) was conducted with the prcomp package in R to evaluate sample clustering. Finally, gene expression analysis was performed using the DESeq2 Bioconductor package in R (v1.30.0).[41] Raw sequencing data were deposited in the GEO database (GSE227677).

Bacterial two-hybrid

Bacterial two-hybrid assays were performed using of the BACTH system kit (bacterial adenylate cyclase two-hybrid system kit, Euromedex). The phage gene *pit2* and *P. aeruginosa* gene *lasR* were both cloned into pUT18, pUT18C, pKTN25 and pKT25. Mutants of *pit2* were only inserted in the pUT18C vector and were constructed via QuickChange ligation. Each plasmid combination was co-transformed into *E. coli* BTH101 and a dilution series (10°–10^{-4}) was spotted on both LB and M63 medium (15 mM $(NH_4)_2SO_4$, 100 mM KH_2PO_4, 1.7 μM $FeSO_4$, 1 mM $MgSO_4$, 0.05% (w/v) vitamin B1, 20% (w/v) maltose, 1.5% (w/v) agar) supplemented with Ap100, Kan50, 1 mM IPTG and 40 μg/mL 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside (X-gal) and incubated for 2–7 days at 30°C. Empty vectors served as negative controls and were co-transformed with their counterparts. To quantify the interactions, a Miller assay was performed in triplicate.[49]

QUANTIFICATION AND STATISTICAL ANALYSIS

The information about the performed statistical test as well as the standard deviations and significance levels (p values) can be found in the figure legends or throughout the text. All calculations were done with the statistical software JMP (JMP Pro 16).