Functional elucidation of antibacterial phage ORFans targeting Pseudomonas aeruginosa

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Summary

Immediately after infection, virulent bacteriophages hijack the molecular machinery of their bacterial host to create an optimal climate for phage propagation. For the vast majority of known phages, it is completely unknown which bacterial functions are inhibited or coopted. Early expressed phage genome regions are rarely identified, and often filled with small genes with no homology in databases (so-called ORFans). In this work, we first analysed the temporal transcription pattern of the N4-like Pseudomonas-infecting phages and selected 26 unknown, early phage ORFans. By expressing their encoded proteins individually in the host bacterium Pseudomonas aeruginosa, we identified and further characterized six antibacterial early phage proteins using time-lapse microscopy, radioactive labelling and pull-down experiments. Yeast two-hybrid analysis gave clues to their possible role in phage infection.

Introduction

Obligately lytic bacteriophages rely heavily on the bacterial metabolism for their replication. From the onset of infection, evolutionary successful phages must establish a favourable environment for phage production and counter or preempt bacterial defence mechanisms (Labrie et al., 2010). Over a billion years of co-evolutionary struggle with an estimated number of $10^{23}$ phage infections per second (Suttle, 2007), phages have evolved an incredible number of highly diverse proteins that either inhibit or adapt bacterial metabolic processes to their own benefit (Hauser et al., 2012). Although not all of these intracellular phage–host interactions are detrimental to the host cell, many of them do indeed lead to cell cycle arrest or even host lethality. In a pioneering study, Liu et al. (2004) explored the antibacterial character of all Staphylococcus phage G1 proteins by expressing them individually in Staphylococcus aureus. Moreover, some of the antimicrobial phage proteins were successfully exploited in drug design by mimicking the effect of the phage protein with a synthetic component.

While therapeutic options to treat Gram-positive bacterial infections have expanded in the last decade, little progress has been made against Gram-negatives (Livermore, 2012). As such, we hypothesized that a novel source of Gram-negative antibacterials might originate from mining the thousands of available sequenced phage genomes. Early phage proteins are of specific interest, as they are responsible for conversion of the host to a phage-oriented metabolism through direct protein–protein interactions. However, two main problems arise. First, the vast majority of phage-encoded proteins remain uncharacterized and show no homolo-
gies to other known proteins, preventing us from predict-
ing their mode of action and bacterial target in silico (Hatfull, 2012). Second, it is often unclear which part of
the phage genome is involved during the early stage of
hijacking the bacterial host, as temporal expression
patterns cannot be readily deduced from the phage
genome sequence alone.

In this article, we address both issues for the N4-related
podoviruses infecting the Gram-negative opportunistic
pathogen Pseudomonas aeruginosa (Ceyssens et al.,
2010). Both phages studied, i.e. LUZ7 and LIT1, share the
core genome and transcriptional scheme of Escherichia
coli-infecting phage N4 (Fig. 1, black arrows). This
scheme is based on the sequential activity of three differ-
ent RNA polymerases (RNAP), the first of which being
a giant (3500 amino acids) single-subunit virion-
encapsulated RNAP (vRNAP) responsible for the early
phage gene products (Falco et al., 1977). Transcription of
the middle genes is carried out by a phage-encoded heterodimeric T7-like RNAP (Willis et al., 2002). Finally,
late genes are transcribed by the bacterial RNAP which is
recruited by the N4 middle gene product gp45 (Cho et al.,
1995; Miller, 1997).

The evolutionary success of this phage genus is
reflected in the conservation of core elements involved in
transcription, DNA replication and virion morphology in at
least 18 phages infecting a broad variety of Gram-negative
bacteria (Fouts et al., 2013). In contrast, no conservation is
present in clusters of small proteins in LUZ7 and LIT1 (Fig.
1A). These small genes of unknown function (encoding
proteins under 250 amino acids) represent 55% of the total
open reading frames in the Pseudomonas N4-like phages,
and are hypothesized to function in Pseudomonas-
specific processes (Ceyssens et al., 2010).

In this article, we mine the genome of these N4-like
Pseudomonas phages for novel antibacterial phage pro-
teins. We maximized the chances to identify such phage
proteins by refocusing the strategy proposed by Liu et al.
(2004) to early expressed proteins. As such we identified
six different inhibitory phage proteins, and performed
protein–protein interaction studies to shed light on their
biological role in phage infection.

Results

Temporal gene transcription analysis with RNA-seq

The majority of phage proteins involved in the conversion
from a pro-host to a pro-viral metabolism are expressed in
the first minutes after injection of the viral genomic DNA
(Roucourt and Lavigne, 2009). Hence, our initial research
focused on the identification of phage genomic regions,
which are transcribed early during infection, by RNA
sequencing. We extracted total RNA from P. aeruginosa
PAO1 cells at 5, 10 and 15 min post infection with N4-like
LIT1 isolate PEV2, which has an eclipse period of about
20 min (Ceyssens et al., 2010) and represents the con-
served overall genome architecture of the two N4-like
viruses in this study. Following rRNA depletion to increase
sequence coverage, RNA-seq was performed on these
samples. Directionality of the reads was maintained
throughout processing, which permits distinguishing
between sense and antisense transcription.

Remarkably, 5 min after infection 26% of the 9.7 × 10⁶
sequenced transcript reads could be uniquely mapped to
the phage genome, replacing host transcripts. This
number increased to 27% of 7.6 × 10⁶ and 56% of
9.4 × 10⁶ after 10 and 15 min respectively (Table S1). An
unexpected complexity was observed when these reads
were mapped to the phage reference genome. Early tran-
scription of the first 50 kb of the LIT1 genome is almost
exclusively performed on the sense strand, even for struc-
tural genes with coding sequences found on the minus
strand, resulting in relatively high abundance of antisense
transcripts for those genes (Fig. 1B).

The RNA-seq results show that the gene encoding for
hypothetical protein gp1 is very strongly expressed early
during infection. Thirty-five per cent of the total gene reads
that map to the phage 5 min post infection align to the gp1
phage genomic region, even though this region comprises
only 255 bp of the 72 kb genome. One of the major
operons present between 28 000 bp and 35 500 bp,
which is responsible for producing the tail proteins,
appears to be transcribed through on the opposite strand,
possibly drowning out any transcription that might leak
through the sense promoter. Middle infection also
appears to show proportionally large amounts of anti-
sense RNA over the region responsible for the proteins
comprising the phage head, including the large vRNAP,
on the same strand. Then, during the late stages of infec-
tion, the phage pregnant cell appears to overwhelm the
antisense transcription of structural gene regions with
sense transcripts (Fig. 1B). Interestingly, transcription
during the middle and late stages of infection is not domi-
nated by genes encoding major virion proteins, but by a
previously non-annotated coding sequence, gp68.1. Tran-
scription of this gene, which lacks similarity to any known
DNA sequence, accounts for 22% of the reads mapping to
the phage genome 10 min post infection and 35% 15 min
post infection respectively.

Screening for inhibitory early phage proteins

Based on the observation that the host spectrum of phage
LUZ7 is approximately twice as broad as the one of LIT1
(Ceyssens et al., 2010), we decided to focus our efforts
on the host-converting proteins of LUZ7. By combining
RNA-seq results with the previously published functional
Fig. 1. (A) Comparative genome annotation of LUZ7 and LIT1 and (B) RNA-seq analysis of LIT1 after *P. aeruginosa* PAO1 infection.

A. An *in silico* comparison of the annotated genomes of the N4-like *Pseudomonas* viruses LUZ7 and LIT1 is displayed. The arrows represent the predicted ORFs and point in the direction of transcription. Genes encoding proteins that share similarity are connected by dashed lines. Structural genes and genes related to N4 (their functional prediction is shown on top) are indicated in yellow and black respectively. All cloned genes are marked in grey, while early proteins with transmembrane domains are green. The identified inhibitory phage proteins are marked in red.

B. RNA-seq read assembly. Reads mapped to the sense and antisense strand are coloured red and green respectively. The blue graphs on top show the relative abundance of each position of the genome normalized by the total amount of sequencing reads in each sample.
genome annotation (Ceyssens et al., 2010), we delineated the genomic region of interest from bp 1 to 11 551 (Fig. 1), corresponding to gene 1 to 33. By only focusing on cytoplasmic uncharacterized proteins lacking any transmembrane domains predicted by TMHMM 2.0 (Krogh et al., 2001) and excluding the phage-encoded heterodimeric RNAP (gp20 and 22), we selected 26 proteins out of the 33 early expressed LUZ7 proteins, indicated as grey- and red-coloured arrows in Fig. 1 and listed in Table S2.

These 26 phage proteins were screened for growth inhibition of \textit{P. aeruginosa} PAO1. First, they were cloned in the \textit{E. coli–P. aeruginosa} shuttle expression vector pUC18-mini-Tn7T-Lac (Choi et al., 2005). Co-transformation of the expression constructs with the pTNS2 plasmid to \textit{P. aeruginosa} PAO1 facilitated stable integration of the expression cassette in the Tn7T site of the \textit{Pseudomonas} genome (Choi et al., 2006). As such, we obtained a single-copy genomic expression construct in \textit{P. aeruginosa} PAO1 for each of the 26 selected phage proteins, under the control of an isopropyl-β-D-1-thiogalactopyranoside (IPTG)-inducible lac promoter.

The growth of the 26 mutant PAO1 strains, each encoding an inducible phage protein, was assessed on solid LB medium in the presence or absence of 1 mM IPTG (Fig. 2A). Interestingly, cell growth was completely abolished when expression of four of these phage proteins was induced (gp7, 8, 14 and 18). Cells expressing gp15 or gp30 were still able to grow but to a lesser extent. As such, 18% of all early expressed proteins showed a growth inhibitory effect on \textit{P. aeruginosa} PAO1. To test the effect under physiological conditions, the assay was repeated on a minimal medium and on artificial sputum medium (Sriramulu et al., 2005), which mimics the sputum of a cystic fibrosis patient where \textit{P. aeruginosa} infections are very common. Interestingly, gp7 only causes toxicity when expression is induced on LB (Fig. S1). This could mean it targets a \textit{Pseudomonas} protein only present under rich growth conditions. For the other mutant PAO1 strains, no clear phenotypic difference could be observed on the various media supplemented with IPTG (Fig. S1). This indicates they impair bacterial growth by interacting with pathways essential under the relevant growth conditions.

In liquid media, the strains expressing gp7, 8, 14, 15 and 30 show a delayed growth compared to the wild-type PAO1 strain, while expression of LUZ7 gp18 generates a normal growth curve (Fig. S2). LUZ7 gp8 is the...
only protein which causes a decrease of density 5–6 h after induction, indicating a bacteriolytic effect.

To further investigate the nature of toxicity, cell growth in the presence of IPTG was monitored in real time using time-lapse microscopy. This revealed various morphologies for the different inhibitory phage proteins (Movies S1–S7, Fig. 2D). Whereas cell growth is only retarded in the presence of the growth-inhibitory proteins gp15 and 30, clear morphological effects were visible after induction of the other proteins. Cells harbouring LUZ7 gp7 failed to divide properly after one correct division, leaving slowly elongating cells with two nucleoids. In contrast, the P. aeruginosa PAO1::LUZ7 gp8 strain starts dividing normally but then stops, leaving two daughter cells sticking to each other and bursting about 4 h after induction. Figure 2D shows the remaining dead cell material. This cell lysis also explains the decreasing density seen in the corresponding growth curve. Finally, LUZ7 gp14 and 18 both cause a filamentous growth type, which correlates with an increasing optical density in liquid media, as observed previously.

**Antibacterial effect on P. aeruginosa PA14 and E. coli MG1655**

Next, we investigated the effect of the phage proteins on bacterial strains which could not be lysed by the phage itself. In a first step, we checked their effect on a virulent isolate of P. aeruginosa, strain PA14 (Rahme et al., 1995) (Fig. 2B). While gp7, gp8 and gp14 displayed the same phenotype in this second host, proteins 15 and 30 depicted slightly less inhibition compared to their PAO1 counterparts. Unfortunately, P. aeruginosa PA14::LUZ7 gp18 mutants could not be generated after several trials, possibly indicating the same detrimental effect as seen in P. aeruginosa PAO1.

Then, we titred the P. aeruginosa PA14 strains on LB lacking IPTG 1 and 3 h after induction of the phage protein early in exponential phase. These results, depicted in Fig. 3, show that gp7 and gp8 are bactericidal for Pseudomonas. Three hours after induction, the initial cell number has reduced with approximately two and three log units respectively. The other proteins (gp14,
gp15 and gp30) only slow down growth when compared to the wild-type strain.

In addition, antibacterial screens were performed in the E. coli Top10 laboratory strain (Invitrogen) (results not shown), which is RecA deficient, and the wild-type E. coli K-12 strain E. coli MG1655 (Blattner et al., 1997) (Fig. 2C). For these screens, the proteins were not expressed from a single-copy cassette integrated in the chromosome, but episomally from a pUC18-mini-Tn7T-Lac construct. Only bacteria expressing LUZ7 gp7 and gp14 show reduced growth compared to the control strain, while all the others grow normally. Furthermore there are no major differences seen between the Top10 and MG1655 strains, indicating that the observed phenotypes are RecA independent (Erill et al., 2007).

In silico analysis of inhibitory phage proteins

In the original genome paper of 2010, no function was attributed to the different inhibitory proteins (Ceyssens et al., 2010). We performed in silico re-analyses of the different inhibitory proteins using BLASTp (Altschul et al., 1990; Gish and States, 1993) and HMMER (Finn et al., 2011) database searches. Gene products 7, 14 and 18 are conserved (amino acid identity > 95%) among Pseudomonas infecting N4-like phages LUZ7, LIT1 and PA26 (Kim et al., 2012) while gp8 is only present in LUZ7 and LIT1. Gp15 and 30, on the other hand, are unique for LUZ7. No significant similarities (e-value < 1 × 10−5) to any other known proteins were found. Subsequently, we searched for conserved domains or protein families using CDART (Geer et al., 2002), CDD (Marchler-Bauer et al., 2013) and Pfam 27.0 (Punta et al., 2012). Only gp18 contains an ELM1 domain (Pfam06258, e-value 9.57 × 10−3). In plants, this domain is involved in mitochondrial fission, by relocating GTPases needed for fission from the cytosol to the mitochondrial fission site (Arimura et al., 2008). Its function in bacteria is still unknown. Finally, similar secondary structures were looked for with Phyre2 (Kelley and Sternberg, 2009) and HHpred (Soding et al., 2005). The Phyre2 search for gp30 showed this protein has a long helical contractile structure with a confidence level of 97.9%.

Impact on bacterial transcription

To test whether the inhibitory proteins affect DNA replication, transcription, or translation, we followed the incorporation of tritium labelled macromolecular precursors in P. aeruginosa cells in presence and absence of the phage proteins. No direct effects on DNA replication or mRNA translation were observed. Conversely, LUZ7 gp8 and gp14 appear to influence 5,6-3H-uridine incorporation (Fig. 4A), indicating a direct effect on host transcription. One hour post induction transcription levels decreased to 35% and 43% of the uninduced samples for gp8 and 14 respectively. To verify if these proteins bind directly to the bacterial RNAP complex during phage infection, or recruit other proteins to this complex, we performed pull-down experiments using P. aeruginosa PAO1 rpoC::PrA and P. aeruginosa PAO1 rpoA::StrepII strains, respectively, encoding a Protein A tag at the C-terminus of the RNAP β′ subunit and a C-terminal StrepII-tag II at the RNAP α subunit. Cells were infected with phage LIT1 isolate PEV2 and harvested 10 min post infection and a pull-down was performed using the respective affinity tags (Fig. 4B). Mass spectrometry analysis of samples with and without phage infection identified all components of the RNAP including the sigma factor σ70 and transcription elongation factor NusA (Table S3). Compared to non-infected cells, three additional sigma factors were purified, namely SigX, σ32 and AlgU. These alternative sigma factors were previously demonstrated to be induced by phage infection or other types of stress (Potvin et al., 2008). However, no phage proteins that might interact with the RNAP were found, indicating no direct interaction between phage proteins, expressed during the early to middle stages of the phage replication cycle, and the host RNAP complex under these experimental conditions.

Yeast two-hybrid (Y2H) interaction assay

To further explore the molecular background causing the inhibitory effects of these proteins, we performed a Gal4p-based yeast two-hybrid (Y2H) analysis (Fields and Song, 1989) in reporter strain Saccharomyces cerevisiae AH109, using the phage-encoded inhibitors as bait against a genomic fragment library of P. aeruginosa PAO1. The results are shown in Table 1. The reproducibility of the Y2H library screening results was validated in an independent Y2H experiment using fresh yeast cells. Moreover, the specificity of the interactions was examined using non-related bait and prey constructs. As the Pseudomonas genome was represented multiple times in the library (Roucourt et al., 2009), identification of overlapping fragments of the same prey protein permitted delineation of the interaction domain.

For LUZ7 gp7, we identified one potential interaction partner in P. aeruginosa, the transcriptional regulator PAO120 (Winsor et al., 2011) (Fig. 5A). By examining the overlapping PAO120 fragments found in different positive colonies, the interaction domain could be delineated from amino acid 74 to 140 of this 228-amino-acid protein (Fig. SB). Inhibitory protein gp8 has a putative interaction with the replicative DNA helicase DnaB and/or the riboflavin metabolism key enzyme RibB. For RibB, the interaction domain spans amino acids 188 to 365 (Fig. 5C).
Fig. 4. 3H-uridine incorporation and RNA polymerase pull-down.

A. Exponentially growing P. aeruginosa PAO1 cells containing an expression cassette of LUZ7 gp8, gp14 or an empty control construct under induced and uninduced conditions were pulse labelled with 3H-uridine for 15 min. Each data point represents the average of three independent samples with standard deviation.

B. Affinity purification of the P. aeruginosa RNA polymerase using a Strep®-tag II at the \( \alpha \) subunit (RpoA) from cells with and without phage infection. The samples were resolved on a 12% SDS-PAGE gel together with a protein size marker. A similar experiment was executed using a Protein A tag at the \( \beta' \) subunit (RpoC). The generated the same pattern as seen for the Strep®-tag II (not shown).

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LUZ7 gp18 putatively interacts with the N-terminal part of PA0657, a probable ATPase from the AAA+ family (Snider et al., 2008). These ATPases are involved in post-translational modifications and protein turnover. Possibly the phage protein interferes with these diverse processes. Finally, Y2H analysis using gp30 as bait revealed a possible interaction with the spermidine acetyltransferase PA4114. Although *P. aeruginosa* also possesses other possible catabolic pathways of spermidine (Dasu et al., 2006), this enzyme plays a role in spermidine degradation in *E. coli* and is essential to counter the toxic effect of spermidine accumulation (Fukuchi et al., 1995; Limswuwun and Jones, 2000). As this suggested that the phage interferes with spermidine breakdown in the cell, we assayed the dependency of N4-like phages on spermidine. By knocking out SpeD, spermidine production in *P. aeruginosa* is abolished (Laber et al., 2004). Intriguingly, we observed that this mutation rendered the bacterial cell resistant to phage infection by the tested N4-like *Pseudomonas* phages (Fig. 6), showing that spermidine is essential in the phage infection cycle, and supporting our Y2H data.

**Discussion**

An important challenge in bacteriophage research is the functional study of the ever-increasing amounts of small, hypothetical genes which are abundantly present in newly sequenced genomes (Hatfull, 2012). We focused on early expressed proteins of N4-like *Pseudomonas* phages. Intriguingly however, in addition to the distinctive early region we expected to find, RNA-seq also revealed a large-scale system of antisense transcription that is likely involved in silencing of translation. In addition, we were able to define previously non-annotated open reading frames as well as transcription start and end sites.

By cloning 26 early LUZ7 proteins in a *P. aeruginosa* expression vector and expressing them individually in *Pseudomonas*, we were able to identify six novel, previously uncharacterized inhibitory proteins, i.e. gp7, 8, 14, 15, 18 and 30 (Table 1). Two different *P. aeruginosa* strains, PA01 and the clinical isolate PA14, display similar phenotypes after expression of the phage proteins. These *Pseudomonas* strains share their core genome, but only differ in their outer membrane and their number of virulence-related genes (Choi et al., 2002; Lee et al., 2006). In addition, none of the antibacterial proteins have a counterpart in any other (N4-like) phage, both suggesting they are specifically tailored to influence the *P. aeruginosa* metabolism. This was also shown in an antibacterial screen in *E. coli*. Only two antibacterial proteins, gp7 and gp14, cause some minor growth inhibition of this second species. Judging by the variety in *Pseudomonas* phenotypes which are provoked by protein production, it is very plausible they act on different metabolic systems in the host bacterium.

To investigate the molecular events underlying the negative effect of the six inhibitory proteins on host growth, we performed radiolabelling and protein–protein interaction experiments. In contrast to many of the established phage–host interactions (e.g. Osmundson et al., 2012; Klimuk et al., 2013), we did not find evidence for a direct interaction between the phage and the bacterial transcription machinery. While the related coliphage N4 activates late transcription by interaction between the late ssDNA-binding protein gp45 and the *E. coli* RNAP β′ subunit (Miller, 1997), no interactions during the early and middle stages of infection were identified between *Pseudomonas* N4-like phages and the *P. aeruginosa* RNAP using both Y2H as pull-down approaches. The observed decrease in incorporation of radioactive labelled uridine as seen in the pulse labelling experiments after induction of gp8 and gp14 might be due to indirect effects caused by stress in the bacteria.

An interesting putative interaction was found between gp8 and RibB. This bifunctional enzyme catalyses the first step in riboflavin metabolism and contains two functional domains, the C-terminal GTP cyclohydrolase II and the N-terminal 3,4-dihydroxy-2-butanoate-4-phosphate synthase (Abbas and Sibiry, 2011). All of the fragments in the screen contain sections of the GTP cyclohydrolase II moiety, with the area of overlap occurring between

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**Table 1. Summary of phenotypic and protein–protein interaction analyses of the inhibitory LUZ7 proteins.**

| Protein | MW (kDa) | *P. aeruginosa* | E. coli | Phenotype | Y2H identification | Functional prediction of the target |
|---------|---------|----------------|--------|-----------|-------------------|-----------------------------------|
| Gp7     | 11.81   | PAO1+/-        | PA14+/-| Division stops | PA0120 Probable transcriptional regulator |
| Gp8     | 9.14    | PAO1+/-        | PA14+/-| Division stops and cells burst | PA4931 Replicative DNA helicase DnaB |
| Gp7     | 11.13   | PAO1+/-        | PA14+/-| Filamentous | PA4054 Riboflavin synthesis enzyme RibB |
| Gp15    | 7.76    | PAO1+/-        | PA14+/-| Delayed growth | PA0657 Probable ATPase of the AAA+ family |
| Gp18    | 9.48    | PAO1+/-        | PA14+/-| Delayed growth | PA4114 Spermidine acetyltransferase |
| Gp30    | 18.30   | PAO1+/-        | PA14+/-|                   |                   |                                    |

a. Growth after induction: –, no growth; +/-, minimal growth; +/-, less growth; +, normal growth; n/a, not available.
amino acid 188 and 365 (Fig. 5C). Inhibition of the enzymatic function of RibB would reduce riboflavin and hence flavin mononucleotide and flavin adenine dinucleotide levels (Ren et al., 2005). Since these cofactors are globally used throughout the bacterial metabolism, enzyme inhibition would therefore have a general impact on nutrient deficiency and would increase oxidative stress (Abbas and Sibirny, 2011) which explains the bactericidal effect of gp8. Moreover, RibB is potentially a good candidate target for a novel antibiotic since Gram-negative bacteria like P. aeruginosa cannot take up riboflavin from the environment, thus relying entirely on endogenously produced riboflavin (vitamin B2), while humans get all of their vitamin B2 from their diet and therefore have no riboflavin biosynthesis pathway and RibB (Kumar et al., 2010).

For gp7, the transcriptional regulator PA0120 was identified as prey. More specifically, the interaction domain was delineated from amino acid 74 to 140 (Fig. 5B). As Fig. 5.

**Fig. 5. Y2H results.**

A. LUZ7 gp7 interacts with *Pseudomonas* protein PA0120, as indicated by the ability to grow on selective SD medium lacking tryptophan, leucine, histidine and adenine. Moreover, the cells become blue when grown on X-α-Gal supplemented medium (right panel). As negative controls the bait was tested for autoactivation using GPA1 and the empty (/) prey vector as prey. Furthermore, the prey was also tested for specificity using GPA1 and the empty bait vector as bait. Presence of bait and prey was assessed on selective medium lacking tryptophan and leucine (left panel).

B. Using LUZ7 gp7 as bait, 10 positive colonies harbouring seven different DNA fragments were found. By analysing the overlap between the different fragments, the interaction domain can be delineated to amino acid 74 to 140 out of this 228-amino-acid-long PA0120 protein.

C. For LUZ7 gp8, seven positive colonies containing five different overlapping ribB fragments were identified. The interaction domain spans amino acids 188 to 365, largely corresponding to the GTP cyclohydrolase II moiety of RibB.
the DNA-binding domain (Punta et al., 2012) ends at position 70, this domain is not necessary to obtain a positive yeast colony, supporting the specificity of the interaction between LUZ7 gp7 and the transcriptional regulator. Moreover, the interaction domain falls in a small part of the ‘FCD’-domain of PA0120. This domain, also present in other transcriptional regulators, binds effector molecules able to influence bacterial transcription (Haydon and Guest, 1991). This makes it plausible that LUZ7 gp7, by binding this domain, changes transcription levels of genes essential or detrimental for efficient phage infection. Furthermore, LUZ7 gp7 was the only protein which caused a different phenotype in Pseudomonas when expressed in bacteria grown on LB, artificial sputum medium or minimal medium. It is possible that the target of LUZ7 gp7, i.e. PA0120, is only expressed in rich medium, thus only leading to toxicity on LB.

Finally, we identified the spermidine acetyltransferase PA4114 as a target for LUZ7 gp30. In E. coli, this protein, which has an N-acetyltransferase domain (Marchler-Bauer et al., 2011), catalyses the first step in spermidine catabolism by transferring the acetyl group from acetyl coenzyme A to this biogenic polyamine. These molecules, which are natural multivalent cationic compounds, are believed to serve as ubiquitous structural components which interact with negatively charged nucleic acid-containing macromolecules (Wortham et al., 2007). As such, spermidine can also be beneficial for all viruses to compactly package their genome in the capsid and to stabilize the viral DNA during the final stage of its ejection in the host cell (Roos et al., 2007; Yu and Schaefer, 2008). Ames et al. already reported in 1958 that some bacteriophage capsids like the one from T4 indeed contain the polyamines putrescine and spermidine (Ames et al., 1958; Ames and Dubin, 1960). This phenomenon could explain the interaction between the inhibitory phage protein LUZ7 gp30 and the spermidine acetyltransferase PA4114. Inhibition of spermidine catabolism by gp30 might indeed be essential to accumulate spermidine to sufficient amounts to neutralize the negatively charged LUZ7 DNA in the mature phage capsids. This is also the first time to observe such a phage mechanism to actively maintain spermidine levels by inhibiting its breakdown. As a side-effect, this spermidine accumulation can lead to Pseudomonas growth inhibition as was reported before in E. coli (Fukuchi et al., 1995; Limsuwun and Jones, 2000). Moreover, in a final experiment we showed that N4-like phage infection is indeed fully dependent on the presence of spermidine in the host cell. This dependency was previously observed for the unrelated Pseudomonas phage JG004 (Garbe et al., 2011), possibly indicating a more common feature for Pseudomonas phages.

In summary, we have mined a virulent P. aeruginosa phage for early expressed, antibacterial proteins. Using protein–protein interaction studies, which still have to be confirmed using complementary techniques, we suggest that these proteins target a variety of processes in the host, which not only learns us more about phage infection but in the future could also lead to innovative antibiotics against Pseudomonas.

**Experimental procedures**

**RNA sampling and RNA-seq analysis**

One 50 ml culture of P. aeruginosa PA01 cells was synchronously infected with LIT1 isolate PEV2 at a multiplicity of infection of 50. At 5, 10 and 15 min after infection, samples intended for RNA sequencing were rapidly suspended in 1/10th volumes of ice-cold stop solution (10% phenol in ethanol) and chilled to inhibit RNA transcription and degradation. Cells were then collected by centrifugation (6000 g) and total RNA was extracted from the resulting cell pellet by vigorously resuspending it in TRIzol (Life Technologies) and performing a classical phenol-chloroform extraction followed by ethanol precipitation. Remaining DNA was removed using TURBO D.Nase (Life Technologies).

DNA-free total RNA was depleted of rRNA using the Ribozero™ Magnetic Kit (Epigence Biotechnologies, Madison, WI). Subsequently, the samples were processed into directional complementary DNA (cDNA) libraries with the TruSeq Stranded mRNA Sample Prep Kit (Illumina, San Diego, CA) and run on an Illumina HiSeq sequencer. For each of our comparative analyses we used the total gene reads mapped to the host (P. aeruginosa PA01) and viral annotated gene features using the CLC Genomics Workbench 6.0.2. software (CLC Bio, Aarhus, Denmark).

**Expression in P. aeruginosa**

All phage genes were cloned in a Gateway entry vector using the pENTR/SD/D-TOPO cloning kit (Invitrogen). Subsequently, the genes were transferred to the E. coli–P. aeruginosa shuttle expression vector pUC18-mini-TnTT-Lac (Choi et al., 2005), which first was made Gateway compatible. Co-transformation of
250 ng of the pUC18-mini-Tn7T-Lac constructs and pTNS2 by electroporation to *P. aeruginosa* PAO1 or PA14 (Choi et al., 2006) allowed single-copy integration of the phage proteins in the *Pseudomonas* genome under the control of an IPTG-inducible lac promoter which was verified using PCR and DNA sequencing (Choi et al., 2005).

*Escherichia coli* and *P. aeruginosa* cells were grown at 37°C in Lysogeny Broth (LB) and on LB, artificial sputum medium (Sirramulu et al., 2005) or M9 minimal medium (Sambrook and Russell, 2001) plates, supplemented with 0.1 mg ml\(^{-1}\) ampicillin, 0.03 mg ml\(^{-1}\) gentamicin and/or 1 mM IPTG, if required. As negative and positive control in the expression experiments, *P. aeruginosa* cells containing an empty vector construct and inhibitory protein gp5 of phage phKMV (J. Wagemans and R. Lavigne, unpubl. data), respectively, were used. Growth curves were generated using a Bioscreen C™ spectrophotometer (Growth Curves USA).

### Time-lapse microscopy

A stationary culture of *P. aeruginosa* PAO1 mutant cells, containing a single-copy expression construct of the different phage proteins was diluted a thousand times and spotted on an LB agar pad containing 1 mM IPTG to induce expression. Subsequently, the growth of a single cell was recorded in real time for 5 h with a temperature controlled (Okolab Ottaviano) Ti-Eclipse inverted microscope (Nikon) equipped with a Ti-CT-E motorized condensor and a CoolSnap HQ2 FireWire CCD-camera. Images were acquired using the NIS-Elements AR 3.2 software (Nikon) as described previously (Cenens et al., 2013).

### Incorporation of \(^3\)H-labelled macromolecular precursors

Stationary cultures of *P. aeruginosa* PAO1, containing the different inhibitory LUZ7 proteins, were diluted 50-fold and grown at 37°C to an OD\(_{600}\) of 0.3. At this point, the culture was labelled for 10 min with \(\text{L-}(4,5\text{-}\text{H})\)-Leucine, \(\text{Methyl-}^{3\text{H}}\)-Thymidine or \(\text{S}-\text{H}^{\text{3}}\text{-H}\)-Uridine (PerkinElmer, Waltham, MA) to a final concentration of 1 μCi ml\(^{-1}\). Subsequently, expression of the phage genes was induced by adding 1 mM IPTG. A non-induced labelled culture was used as a reference. Samples immediately before induction and 10, 30 and 65 min after induction were taken and precipitated in 5% ice-cold trichloroacetic acid (TCA). Using a Filtermate 96 Harvester (Packard), the TCA precipitate was transferred to a Unifilter-96 GF/C (PerkinElmer) and washed four times with 5% TCA, five times with demineralized water and finally washed once with 96% ethanol. After addition of MicroScint 0 (PerkinElmer), the radioactive signal was measured with a TopCount NXT Microplate Scintillation Counter (PerkinElmer). The experiment was repeated in threefold.

### Spermidine dependency assay

To assess phage infection for spermidine dependency, the soft agar [0.7% (w/v) agar] overlay method was used (Sambrook and Russell, 2001). Therefore, a stationary overnight culture (250 μl) of *P. aeruginosa* PAO1 or *P. aeruginosa* PAO1 ΔspeD was mixed with soft agar and poured over a solid LB layer to create a bacterial lawn. Subsequently, a 10\(^{-2}\), 10\(^{-4}\) and 10\(^{-6}\) dilution of phage LUZ7, LIT1 or sterile water was spotted on top of the double agar, after which the plates were incubated at 37°C overnight.

### Pull-down analysis

Affinity purification of the RNAP using a Protein A tag was performed as described previously (Klimuk et al., 2013). For pull-down using the *Strep*-tag II, sample preparation was similar. The obtained soluble fraction of the cell lysate was loaded on a Bio-Rad Poly-Prep® Chromatography column containing 1 ml pre-washed *Strep*-Tactin® Sepharose beads (Titz et al., 2006). Subsequently, the beads were washed five times with 1 ml wash buffer (100 mM Tris HCl pH 8.0, 150 mM NaCl, 1 mM EDTA) and eluted with five times 1 ml elution buffer (100 mM Tris HCl pH 8.0, 150 mM NaCl, 1 mM EDTA, 2.5 mM HABA). The eluted fractions were pooled and concentrated by ultrafiltration (Amicon Ultra 3K, Millipore), heat denatured for 5 min at 95°C and loaded on a 12% SDS-PAGE gel. The Coomassie-stained gel (Simply Blue Safestain; Invitrogen) was cut into slices, which were subjected to trypsin digestion (Shevchenko et al., 1996). Peptides were analysed using electrospray ionization-tandem mass spectrometry (MS/MS) as described previously by Lavigne et al. (2006). The MS/MS data were analysed using Sequest (Thermo Finnigan) against a database containing all *P. aeruginosa* PAO1 proteins and all “stop-to-stop” protein sequences in all six reading frames of phage LIT1.

### Yeast two-hybrid interaction assay

Yeast two-hybrid analysis in *S. cerevisiae* AH109 (James et al., 1996) was performed using a random genomic fragment *P. aeruginosa* PAO1 prey library. Bait proteins were tested for self-activation of the HIS3, ADE2 and MEL1 reporter constructs by transformation (Gietz and Woods, 2002) of the pGBT9 phage gene bait constructs together with an empty pGAD424 prey vector or the unrelated prey Gpa1p (the alpha-subunit of a G protein involved in pheromone signalling in yeast). Subsequently, the bait-containing AH109 cells were transformed with the prey library following the Gietz protocol (Gietz and Schiestl, 2007). Selection of positive colonies was done using synthetic defined minimal medium as described previously (Roucourt et al., 2009).

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Supporting information

Additional Supporting Information may be found in the online version of this article at the publisher’s web-site.

Fig. S1. Antibacterial screens on LB, ASM and MM in the presence and absence of IPTG. Twenty-six mutant P. aeruginosa PA01 strains, each harbouring an individual early LUZ7 phage protein were spotted in triplicate on the rich LB medium, artificial sputum medium (ASM) and minimal medium (MM) with and without IPTG, which induces expression of the phage protein. By comparing the plates, inhibitory proteins can be identified (red boxes). As positive and negative control, another growth inhibitory phage protein found in previous studies and an empty expression cassette was used respectively.

Fig. S2. P. aeruginosa PA01 growth kinetics after induction of phage protein expression. Bacterial growth kinetics were moni-
stored over time by measuring the optical density (OD) of the bacterial culture at 600 nm. Phage protein expression was induced at the start of the experiment. Each data point represents the average of three independent measurements. Standard deviations (not shown on the graph) were less than 0.03 OD units.

**Table S1.** RNA-seq analysis of LIT1-infected *P. aeruginosa* PAO1 5, 10 and 15 min post infection.
**Table S2.** Selection of LUZ7 proteins.
**Table S3.** MS results of a RNAP pull-down with and without LIT1 phage infection.
**Movie S1.** Time-lapse microscopy of negative control.

**Movie S2.** Time-lapse microscopy of *P. aeruginosa* PAO1::LUZ7 gp7 after phage protein induction.
**Movie S3.** Time-lapse microscopy of *P. aeruginosa* PAO1::LUZ7 gp8 after phage protein induction.
**Movie S4.** Time-lapse microscopy of *P. aeruginosa* PAO1::LUZ7 gp14 after phage protein induction.
**Movie S5.** Time-lapse microscopy of *P. aeruginosa* PAO1::LUZ7 gp15 after phage protein induction.
**Movie S6.** Time-lapse microscopy of *P. aeruginosa* PAO1::LUZ7 gp18 after phage protein induction.
**Movie S7.** Time-lapse microscopy of *P. aeruginosa* PAO1::LUZ7 gp30 after phage protein induction.