

Same players, new rules: Redefining the roles of aimP, AimR, and AimX in the arbitrium communication system

Sara Zamora-Caballero

1 Instituto de Biomedicina de Valencia (IBV-CSIC) and CIBER de Enfermedades Raras (CIBERER), 46010 Valencia, Spain

Aisling Brady

2 MRC Centre for Molecular Bacteriology and Infection, Imperial College London, SW7 2AZ, UK

Nuria Quiles-Puchalt

MRC Centre for Molecular Bacteriology and Infection, Imperial College London, SW7 2AZ, UK

Francisca Gallego del Sol

Instituto de Biomedicina de Valencia (IBV-CSIC) and CIBER de Enfermedades Raras (CIBERER), 46010 Valencia, Spain

Javier Mancheño-Bonillo

Instituto de Biomedicina de Valencia (IBV-CSIC) and CIBER de Enfermedades Raras (CIBERER), 46010 Valencia, Spain

Alonso Felipe-Ruíz

Instituto de Biomedicina de Valencia (IBV-CSIC) and CIBER de Enfermedades Raras (CIBERER), 46010 Valencia, Spain

Wilfried J J Meijer

Centro de Biología Molecular Severo Ochoa (CSIC-UAM), Universidad Autónoma, Madrid, Spain

José R Penadés (✉ j.penades@imperial.ac.uk)

MRC Centre for Molecular Bacteriology and Infection, Imperial College London, SW7 2AZ, UK

Alberto Marina (✉ amarina@ibv.csic.es)

Instituto de Biomedicina de Valencia (IBV-CSIC) and CIBER de Enfermedades Raras (CIBERER), 46010 Valencia, Spain

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2 AimX in the arbitrium communication system

3 Sara Zamora-Caballero^{1†}, Aisling Brady^{2,3†}, Nuria Quiles-Puchalt^{2,3†}, Francisca Gallego del
4 Sol¹, Javier Mancheño-Bonillo¹, Alonso Felipe-Ruiz¹, Wilfried J J Meijer⁴, José R Penadés^{2*},
5 Alberto Marina^{1*}

6
7 ¹Instituto de Biomedicina de Valencia (IBV-CSIC) and CIBER de Enfermedades Raras
8 (CIBERER), 46010 Valencia, Spain;

9 ²MRC Centre for Molecular Bacteriology and Infection, Imperial College London, SW7 2AZ,
10 UK.

11 ³Institute of Infection, Immunity and Inflammation, University of Glasgow, Glasgow, G12 8TA,
12 UK

13 ⁴Centro de Biología Molecular Severo Ochoa (CSIC-UAM), Universidad Autónoma, Madrid,
14 Spain.

15
16 [†]These authors contributed equally.

17 *Corresponding authors: Alberto Marina
18 Instituto de Biomedicina de Valencia, Spain
19 e-mail: amarina@ibv.csic.es
20
21 José R Penadés
22 MRC Centre for Molecular Bacteriology and Infection
23 Imperial College London, UK
24 e-mail: j.penades@imperial.ac.uk
25
26
27
28
29
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ABSTRACT

Phages can use a small-molecule communication system, termed arbitrium, to coordinate lysis-lysogeny decisions¹. It was proposed that this system contains three main players. AimR is a transcriptional activator that promotes expression of *aimX*, a small RNA which favours the lytic cycle of the phage by inhibiting the phage master repressor using an uncharacterised *cis*-antisense mechanism. The third player, AimP, is a peptide that accumulates after several rounds of phage infection. When this occurs, AimP binds to AimR inhibiting its function, a process that promotes lysogeny, since *aimX* will not be transcribed. Here we redefined the role for the main players and identified new key ones, explaining the molecular basis of the arbitrium system. Our results demonstrate that AimR is an antiterminator that eliminates the function of a terminator located between *aimP* and *aimX*, which are expressed then as part of a bicistronic operon that initiates in the *aimP* promoter. Importantly, our genetic, biochemical, and structural analyses also demonstrate that *aimX* is not a regulatory small RNA but encodes a peptide, AimX, which promotes the lytic cycle of the phage by binding to the second repressor of the arbitrium system, YopN, eliminating its function. Our results reveal the mechanism by which the arbitrium system controls lysis/lysogeny.

TEXT

Prokaryotes are parasitised by diverse viruses in every explored ecosystem. In bacteria, these viruses (phages) rely on their host's machinery to replicate and produce progeny, a process resulting in lysis of the cell. Importantly, temperate phages possess an alternative stage, termed lysogeny, where the viral DNA can integrate into the host chromosome and becomes dormant. The genetic determinants that govern the lysis-lysogeny decision in temperate phages are well understood in the *Escherichia coli* model phage Lambda. However, less is known for Gram-positive infecting phages (for a review:²). Recently, some key insights were gained following the discovery of the arbitrium system, which allows phages to talk to their progeny. This peptide-based communication system encoded by *Bacillus*-infecting phages allows the virus to coordinate the lysis-lysogeny decision incorporating the population-sensing factor into this outcome¹. This mechanism was first described in phages of the SPbeta group, using phages phi3T and SPβ as models, although it is now clear that the arbitrium system is widespread in nature, being present not only on phages but also on conjugative elements and plasmids³.

The arbitrium system is composed of three main players, all encoded in the phage genome¹. AimP, the arbitrium peptide, is secreted into the medium after phage infection, where it is processed into a mature six-ten amino-acid long peptide that will be internalised by surrounding bacteria via an oligopeptide permease transporter. The second player, AimR, is a transcription factor that in the absence of AimP promotes the expression of the third component, *aimX*, which encodes a small non-coding RNA (sRNA). This sRNA exerts a negative regulatory effect on lysogeny by a mechanism that is vaguely understood. When the AimP peptide concentration is low during the initial stages of the phage infection, the host:phage ratio is high, and the lytic cycle is favoured. On the contrary, after several rounds of phage infection, the amount of AimP increases, promoting lysogeny as a safe solution to allow persistence of both the integrated prophage and the bacterial host¹. Mechanistically,

when AimP accumulates intracellularly, it binds to its cognate AimR, inducing a conformational change that will prevent AimR from binding to its DNA operator, eliminating *aimX* expression and promoting lysogeny⁴. The mechanism by which *aimX* blocks lysogeny is unknown, but it has been proposed that somehow it will inhibit the expression of the master phage repressor^{1,3}, which has recently been identified as YopR for phages belonging to the SPbeta family⁵. It is assumed that in presence of *aimX* the master repressor will not be expressed, or its function will be inactivated, promoting the lytic cycle. By contrast, in the absence of *aimX* expression, the master repressor will be functional, promoting lysogeny^{1,3,6}.

Aside from its role controlling bacterial and phage populations following phage infection, the arbitrium system is also essential during prophage induction⁵⁻⁷. Importantly, one of these studies identified in phage SPβ two additional key players of the system, the aforementioned *yopR* and *yopN*, which are part of a six gene operon (*yopM* to *yopR*; Supplementary Fig. 1) that was postulated to be important for the function of the arbitrium system⁵. By evolving the SPβ Δ*aimR* mutant phage, which cannot form normal plaques after infection, and selecting for evolved phages that could recover this defect by compensatory mutations, we not only identified YopR as the master repressor of the SPβ phage, but also reported that the mutation of *yopN* restored the Δ*aimR* mutation. Moreover, our results also demonstrated that the number of lysogens obtained with the evolved SPβ Δ*aimR* phage carrying the *yopN* mutation were significantly lower than those observed with the SPβ Δ*aimR* phage⁵, suggesting that somehow, YopN acts as an additional repressor that favours lysogenisation. Importantly, introduction of the double Δ*aimR*/Δ*yopN* mutation into the SPβ prophage restored not only the formation of infective phage particles after prophage induction, but also created a phage that was independent of the arbitrium system⁵. In other words, and in order to promote the lytic cycle of the phage, our previous results suggested that instead of performing its role by interacting with the master repressor of the system (YopR), *aimX* could remove YopN's function, promoting the lytic cycle of the phage. This would explain why in absence of YopN, AimR (and therefore *aimX*) functions are unnecessary.

We initiated this study trying to understand how AimR and *aimX* control the entry into the lytic cycle after infection and the exit from the lysogenic cycle after induction. Are both mechanisms similar? We hypothesised that additional layers of control in arbitrium would be involved in the lysogeny/lysis decision to answer this duality. Therefore, understanding how *aimX* controls this process is essential to decipher the molecular mechanism involved in this novel communication system. To our surprise, our results will propose new roles for *aimX* and AimR, as well as an additional function for *aimP*. With these new rules and with the discovery of new additional players involved in the game, our results provide important insights on how the arbitrium system controls lysis/lysogeny decisions in phage communications.

Identification of a key transcriptional terminator essential for the arbitrium system's function.

Since the arbitrium system was discovered using phage phi3T as the model, we repeated the aforementioned strategy with this phage, evolving the phi3T $\Delta aimR$ mutant phage to obtain phages that behaved as the wt. We previously proposed that although phages SP β and phi3T encode different arbitrium systems, being different not just the *aimP*, *aimX* and *aimR* genes but also the *yopM-yopR* like operon (Supplementary Fig. 1), the proteins encoded in the different operons would perform the same functions for their cognate phages⁵. Following this rationale, we expected that the evolved phages carried mutations in either the *phi3T_93* or the *phi3T_97* genes, corresponding to the SP β *yopN* and *yopR* genes, respectively (see Supplementary Fig. 1). Six different evolved phages, from independent experiments, were obtained and sequenced. Consistent with earlier work⁵, we observed a recovery of phage titer following MC induction in all the evolved phages and the plaque morphology of the evolved phages revealed sharp plaques, even sharper than that of the wt (Supplementary Fig. 2a and 2c). Next, we assessed the ability of these evolved phages to form lysogens and found that although capable of, the evolved had significantly reduced their ability to produce lysogens

(Supplementary Fig. 2b). This later result discarded the possibility that these phages carried mutations in the *phi3T* master repressor.

Surprisingly, none of these evolved phages carried mutations in the *phi3T_93* gene either, or in any of the genes encoded in the operon. Instead, all the evolved $\Delta aimR$ mutant phages carried the same mutation in their genomes, located in a non-coding region between *aimP* and *aimX* (Fig. 1a, Supplementary Table S1). Upon closer inspection and using the program ARNold^{8,9}, a putative transcriptional terminator (TT) was identified in this region, upstream of *aimX*. According to ARNold, the mutation specifically affected the stem in the secondary structure (Fig. 1b). Further, and in agreement with this finding, the RNAfold¹⁰ program predicted that the mutation will disrupt the central region of the stem with a considerable difference in the free energy of the thermodynamic ensembles (Fig. 1b). Thus, we hypothesised that the compensatory mutation identified in the evolved phages disrupted the formation of the TT secondary structure, and therefore, will allow the expression of *aimX* directly downstream.

To test this, we firstly confirmed that the TT was functional. To do that, we used a plasmid in which the expression of the β -galactosidase (β -gal) reporter gene was under the control of the P_{Spank} IPTG-inducible promoter. We then cloned the region containing the putative TT between the P_{Spank} promoter and the β -gal gene. As a controls, we generated a derivative plasmid containing the mutated TT, or used the plasmid that only contained the reporter gene under the control of the P_{Spank} promoter (empty plasmid). We then introduced these three plasmids (empty, wt TT or mutated TT) into an ectopic chromosomal locus and measured the expression of the reporter gene in the presence of IPTG. It is important to note here that the cloned 3' region of these constructs contained part of the *aimX* gene, which as previously mentioned is located 3' of the TT. Therefore, in these experiments, *aimX* is transcriptionally fused to the β -gal gene (Fig. 1a). Consistent with our hypothesis, while the presence of the wt TT blocked β -gal (*aimX*) expression, the mutated TT did not (Fig. 1c), confirming that the

evolved phages restored their function just by eliminating the function of this TT, which will allow *aimX* expression from an uncharacterised promoter located upstream *aimX*.

AimR is not a transcriptional activator

It has been postulated that AimR activates *aimX* expression by binding to a specific region (AimR operator) located upstream of the *aimX* gene¹. In fact, this region has been identified, characterised, and the structure of AimR bound to its cognate binding site has been solved^{4,11}. If we assume that this genetic structure is correct, the fact that the ϕ 3T Δ *aimR* mutant restored its functionality by mutating the TT present upstream of *aimX* made sense: in absence of AimR, *aimX* cannot be produced naturally, thus, the mutation of the TT will allow the expression of *aimX* from a transcript that originates upstream of this TT.

However, while all this rationale seemed correct, we were surprised that when we used the ϕ iSITE tool PromoterHunter^{12,13} to analyse the region located after the TT and downstream of the AimR binding site, we could not identify a promoter. To confirm this possibility experimentally, we used a two-plasmid system. In the first plasmid (pJP2750), *aimR* was cloned under the control of the P_{Spank} IPTG-inducible promoter. In the second plasmid, we cloned the region encompassing from the TT to the *aimX* gene, and fused *aimX* to the β -galactosidase reporter. Notice that this region includes the AimR binding site, consisting of 6 bp inverted repeats separated by 25 bp (AAGTTC-25-GAACAT)^{4,11}. We evaluated the expression of the reporter gene in presence or absence of AimR. Surprisingly, either in presence or absence of AimR, no expression of the β -gal reporter was observed, suggesting that there is no promoter in the cloned region. In view of this unexpected result, we hypothesised that in addition to AimR, perhaps other phage-encoded factors (protein or RNA) would be required for *aimX* expression. To test this possibility, we lysogenised the strains carrying the two-plasmid system with ϕ i3T, induced the prophage and measured β -gal expression. Again, we did not observe any expression of the reporter. These results suggested

that AimR induces *aimX* expression by an uncharacterised mechanism, not as a traditional transcriptional activator, and that there is no promoter present for *aimX* in this region.

AimR is an antiterminator that allows expression of *aimX* as a part of a bicistronic transcript that initiates at the *aimP* promoter

These unexpected results, together with the fact that all of the evolved phages carrying the $\Delta aimR$ deletion restored their phenotype by mutating the TT located next to the AimR binding site, raised two immediate questions: *i*) what is the link between AimR and the TT?; and *ii*) where is the origin of the transcript controlled by this TT? Based on our previous results, and to answer the previous questions, we hypothesised that: *i*) AimR is not a transcriptional activator but an antiterminator; and *ii*) the *aimP* promoter originates the transcript that will finish at the TT in the absence of AimR.

To test these hypotheses, we used the same two-plasmid system strategy mentioned before. We generated a set of derivative plasmids containing the region comprised from the middle of the *aimP* gene until the middle of the *aimX* gene, from the *aimP* promoter to the middle of *aimX*, or a derivative plasmid containing from the *aimP* promoter to the *aimX* gene plus the G-C mutation present in the TT of evolved phages (see Fig. 2a for details of the different cloned regions). Remarkably, in absence of AimR, no expression of the β -gal reporter was observed in the plasmids carrying the wt TT, while expression was observed in the plasmid containing the *aimP* promoter with the mutated TT (Fig. 2b). This result not only reaffirms the functionality of the TT, but more importantly, demonstrates that the transcript controlled by the TT initiates at the *aimP* promoter. Further, expression of the β -gal reporter occurred in presence of AimR in the plasmid containing the *aimP* promoter, but not in its absence. This result again confirms that *aimX* expression depends on the *aimP* promoter, but more importantly, suggests that the role of AimR is to act as an antiterminator to prevent termination of expression before *aimX* (Fig. 2b).

To clearly confirm this idea, we hypothesised that the modification of the AimR binding site by specifically mutating the palindromic repeats present in the reporter constructs (see Fig. 2a) would disrupt AimR function as an antiterminator. Indeed, we did not observe expression following modification of the binding site (Fig. 2b), suggesting that AimR binding to its specific binding site is required for antitermination to occur. Similarly, we proposed that modification of the distance between the TT and the AimR binding site will only have an impact on AimR function if it works as antiterminator, but not if AimR works as transcriptional activator. Note that in the latter case AimR would be still able to bind upstream *aimX*, promoting its expression. In support of its role as antiterminator, either the increase (69 bp) or the decrease (8 bp) of the distance between the TT and the AimR binding site abolished AimR function (Fig. 2b).

AimR function is conserved in SPbeta phages

Since the previous results significantly challenged the role assigned to AimR in the arbitrium system, we decided to study AimR function in SP β . In this phage, as occurred with phi3T, the program ARNold also predict a putative TT upstream of AimR binding site, so we used the same two-plasmid system strategy to test AimR function as an antiterminator. Thus, in one of the plasmids we cloned SP β *aimR* under the control of the P_{Spank} IPTG-inducible promoter, while in the other plasmid we fused the region flanking the TT, including the AimR binding site, to the β -gal reporter. To ensure that this region was highly transcribed, we cloned it under the control of the P_{Spank} IPTG-inducible promoter (see scheme in Supplementary Fig. 3a). In support of a conserved role for AimR as antiterminator, the reporter gene was uniquely expressed in the presence of AimR, and when the expression of the transcript was induced by the addition of IPTG (Supplementary Fig. 3b). None of the other conditions tested provided expression of the reporter gene, confirming that the role of AimR in SP β is also to prevent termination of the transcript that originates upstream from the TT.

We also aimed to determine if any cross-interaction occurs between different SPbeta phage arbitrium systems. Using our models SP β and phi3T, we used the same two-plasmid system approach to assess whether the AimR antiterminator from one phage could disrupt the TT from another phage. A reporter construct was generated using the phi3T region from the TT to *aimX* fused to the β -gal reporter gene, but this time the AimR binding site from phi3T was swapped for the sequence encoding the AimR binding site from SP β (see scheme in Fig. 2c). Expression was only observed in the presence of the cognate AimR_{SP β} and not in the presence of AimR_{phi3T} (Fig. 2c). Hence, we suggest that the antiterminator properties of AimR is specific for each individual phage and depends on the interaction of AimR with their cognate DNA binding sites^{4,11}.

To clearly confirm that AimR function is conserved in both phi3T and SP β phages, we hypothesised that it would be possible to obtain some evolved SP β $\Delta aimR$ mutant phages in which the TT localised upstream of the AimR binding site would be mutated. Though we did not find such mutants in the first evolution experiments⁵, we hypothesised that this was due to only analysing 5 mutants. We obtained 8 additional SP β $\Delta aimR$ evolved phages with a restored capacity to form normal plaques on a recipient strain. Importantly, 3 of these mutants contained a single bp mutation in the TT upstream of the AimR binding site (Supplementary Table 1), which inactivated its functionality (Supplementary Fig. 3b).

In summary, our results challenge the current knowledge on how the arbitrium system works. In contrast to the previous understanding our results demonstrate a different role for AimR as antiterminator that prevents transcriptional termination of the mRNA originating from the *aimP* promoter. But also propose an additional one for *aimP*, which in addition to producing AimP provides a long transcript that finishes 3' *aimX*. In other words, *aimP* and *aimX* are part of a polycistronic operon that also contains a TT between the two genes, the activity of which is controlled by AimR.

257 **phi3T *aimX* encodes a small peptide but not an antisense RNA**

258 While it is assumed that *aimX* encodes a small antisense RNA that somehow interferes with
259 the expression of the master phage repressor¹, the fact that *aimX* is part of a long transcript
260 that originates at *aimP* questioned this possibility. Therefore, we searched for additional
261 mechanisms of action, including the possibility that *aimX* encodes a putative peptide.
262 Interestingly, in phi3T genome, a 51 amino acid long ORF, phi3T_91, was annotated in the
263 *aimX* locus. Our *in silico* analysis identified upstream of the putative starting Met codon of this
264 ORF all the elements required for translation, including a putative Shine-Dalgarno like region
265 (Fig. 3a), suggesting that *aimX* encoded for a protein. To test this possibility *in vivo*, we first
266 established a simple approach to test *aimX* function in phi3T. Since the role of *aimR* is to allow
267 *aimX* expression by preventing termination, we hypothesised that the expression of *aimX* from
268 a plasmid in the $\Delta aimR$ mutant prophage would be sufficient to restore prophage induction, a
269 process that is severely affected in the $\Delta aimR$ mutant^{5,7}. Indeed, this was the case, and the
270 phage titres obtained after MC induction of the phi3T $\Delta aimR$ mutant prophage complemented
271 with *aimX* were 1,000X higher than that observed after induction of the $\Delta aimR$ mutant
272 prophage (Fig. 3b). Importantly, the *aimX*-complemented prophage titer was indistinguishable
273 to those obtained after induction of either the wt phi3T prophage or the $\Delta aimR$ mutant phi3T
274 prophage complemented with its cognate AimR (Fig. 3b).

275 Next, to test whether *aimX* encodes a peptide or a regulatory RNA, we repeated the same
276 experiment but now expressing a modified version of *aimX*. In this version, the *aimX* DNA
277 sequence was different (*aimX*^{nt}), but the putative AimX protein encoded by this modified *aimX*
278 gene was the same (Supplementary Fig. 4). As an additional control of this experiment, we
279 expressed the wt *aimX* gene but now introducing a stop codon in the 5th amino acid (L5*) of
280 the coding sequence (*aimX*^{Stop}). In support of the idea that *aimX* encodes a peptide but not a
281 regulatory RNA, only the strain expressing the modified *aimX* gene encoding an identical AimX
282 peptide was able to complement the prophage carrying the $\Delta aimR$ mutation (Fig. 3b).

Finally, to characterise the properties of AimX in more detail, we expressed this peptide heterologously in *E. coli* (fused to a N-terminal histidine tag; His-AimX). The corresponding product was purified yielding a soluble monomeric protein in solution as is shown by size exclusion chromatography coupled with multi-angle light scattering (SEC-MALS) (Supplementary Fig 5a and 5c). Hence, if AimX is a functional peptide in ϕ i3T, how does it perform its regulatory function?

AimX interacts with YopN

To gain further insight into the role of AimX as protein, we looked into the operon located downstream *aimX*. We hypothesised that AimX could perform its function by binding to and inactivating the function of one of the repressors encoded by ϕ i3T, which we anticipated would correspond to the ϕ i3T_93- or ϕ i3T_97- encoded proteins, the functional analogues of the SP β YopN and YopR proteins, respectively (see Supplementary Fig. 1a). Before testing this possibility, we confirmed that *ϕ i3T_93* (*yopN* ^{ϕ i3T}) and *ϕ i3T_97* (*yopR* ^{ϕ i3T}) encode the repressors of the system, and that they behave similarly to SP β YopN and YopR. In support of our hypothesis, deletion of *yopR* ^{ϕ i3T} generated a lytic phage (Supplementary Fig. 6a), confirming that this gene encodes the master repressor for phage ϕ i3T. Further, overexpression of YopR ^{ϕ i3T} or YopN ^{ϕ i3T} in a non-lysogenic strain blocked phage infection and increased lysogenisation (Fig. 4 and Supplementary Fig. 6b), confirming the role of YopN ^{ϕ i3T} as a second repressor of the system.

Next, and since YopR ^{ϕ i3T} and YopN ^{ϕ i3T} seem to be functionally related, we tested whether they interact. We were unable to identify such interaction *in vitro*, using different and complementary approaches including biolayer interferometry (BLI) or native-PAGE (Fig. 5a and Supplementary Fig. 7a). Following the initial assumption that *aimX* could promote the lytic cycle of the phage by interfering by the phage master repressor, we analysed whether the AimX peptide binds to YopR. We were unable to detect the AimX-YopR ^{ϕ i3T} interaction by BLI or native-PAGE (Fig 5b and Supplementary Fig. 7b). We then focus our attention into the

second repressor of the arbitrium system, YopN^{phi3T}, and analysed whether this protein interacted with AimX. Remarkably, the BLI assays showed that AimX binds YopN^{phi3T} with an affinity in the nanomolar range (K_D 184 nM) (Fig 5c and 5g). Importantly, the association (k_{on}) and dissociation (k_{off}) kinetics of the interaction have been observed to be fast (Fig. 5c and 5g), indicating that the binding has a modulatory role. So, when necessary, the AimX-YopN complex can be made and broken very quickly, an type of interaction that we propose is relevant during the phage's decision between lysis or lysogeny.

Structural characterization of AimX-YopN

To shed light into the molecular mechanism of action of AimX by its interaction with YopN and the potential role of such complex, we attempted to solve the three-dimensional structures of both proteins separately or in complex. First, a YopN^{phi3T} version retaining the N-terminal His-tag (His-YopN^{phi3T}) was crystallised yielding crystals that diffracted to atomic resolution (1.9 Å) and allowed to solve the structure using *ab initio* phasing with Arcimboldo Lite¹⁴ (Supplementary Table S2). The aforementioned structure was used to solve the structure by molecular replacement of YopN^{phi3T} without the His-tag, at 2.3 Å (Supplementary Table S2). In both cases, and in agreement with the quaternary organization observed in solution by SEC-MALS analysis (Supplementary Fig 7b and 7c), one dimer was found in the asymmetric unit (Fig. 6a and 6c). Both structures are almost identical (RMSD lower than 0.5 Å for the superposition of the dimer main-chains), with the only exception that the 4 N-terminal residues and the preceding 11 residues corresponding to the His-tag are well ordered and visible in His-YopN^{phi3T} structure (Fig. 6c and 6f). YopN^{phi3T} structures show an all-helices fold composed by a longer N-terminal helix (α 1, residues 9-27) followed by two shorter helices (α 2 and α 3, residues 31-42 and 52-59, respectively) connected by a long unstructured loop (Fig. 6a). For each YopN monomer, α 1 helix is positioned respect to α 2- α 3 helices forming a V-like shape. In the overall dimeric structure, a W-shape is adopted by the positioning of the α 1 helices of each monomer next to each other in a way that seem to be surrounded by the α 2- α 3 helices

from the opposite monomer, resulting in a tight, globular dimer (Fig. 6a). The surface of YopN^{phi3T} dimer, which is mainly negatively charged or neutral, displays two shallow grooves, one across each side of the dimer molecule (Fig. 6b). Interestingly, in the His-YopN^{phi3T} structure, the three N-terminal residues together with part of the preceding His-tag folds as helices, which are accommodated in the dimer grooves (Fig. 6c)

Next, we crystallize the AimX-YopN^{phi3T} complex and solved the structure at 2.2 Å resolution by molecular replacement using YopN^{phi3T} structure as a model. The crystal showed in its asymmetric unit a YopN^{phi3T} dimer that bound two independent AimX molecules (Fig. 6d). Superimposition of the YopN^{phi3T} dimers in the free and AimX-bound forms yielded an extremely low RMSD value (0.47 Å), denoting that YopN presents in solution a competent conformation to bind AimX (Fig. 6f). Interestingly, only a short fragment of AimX (residues 8 to 22), corresponding to 30 % of the protein, is visible in the AimX-YopN^{phi3T} crystal. This AimX fragment folds as a 3-turn helix and is accommodated in the aforementioned shallow grooves present on the sides of YopN^{phi3T} dimer (Fig. 6d and 6e). The main contacts between AimX and YopN are nucleated by the projection of AimX M14 and Y11 in a hydrophobic pocket generated by YopN^{phi3T} W16, M20, L23 and K24, pointing to these hydrophobic interactions as key for YopN-AimX recognition (Fig. 6f and Supplementary Table S3). Additional polar and hydrophobic interactions are mediated by AimX Y11, E13, G17, E20 and M21 with both subunits of the YopN dimer, stabilizing the complex (Supplementary Table 3). Interestingly, AimX binds in a way that is similar to the His-tag in the His-YopN^{phi3T} structure. Indeed, superimposition of His-YopN^{phi3T} and AimX-YopN^{phi3T} showed that the helical part of the His-tag matches the AimX helix and that YopN M1 and F-2 from the His tag replicated the contacts mediated by AimX M14 and Y11, respectively (Fig. 6f and Supplementary Table 3). These observations support that the YopN grooves are properly designed to accommodate a short helix and suggests a transient interaction.

The AimX-YopN^{phi3T} complex indicates that the introduction of the AimX M14 residue in the hydrophobic portion of the YopN^{phi3T} groove must be essential for YopN recognition and

AimX function. Given the hydrophobic environment of the recognition, mutating AimX M14 or the interacting residues in YopN^{phi3T} (M20 and L23) to a charged one should hamper the interaction, preventing AimX function. This would validate the complex structure and confirm the key role of these positions in the AimX-YopN^{phi3T} recognition. To test this possibility, the AimX M14D mutant (AimX^{M14D}) was recombinantly produced and purified, showing similar yield and behaviour in solution than the wt protein (Supplementary Fig. 5a). Importantly, BLI analysis showed no binding between AimX^{M14D} and YopN^{phi3T} (Fig. 5d). Next, we recombinantly produced the YopN^{phi3T} mutants M20D (YopN^{M20D}) and L23D (YopN^{L23D}) and tested their capacity to interact with AimX by BLI. Once again, we observed no binding capacity (Fig. 5e and 5f). Altogether, the *in vitro* results validate the mechanism of AimX-YopN^{phi3T} recognition observed in the 3D structure, which are driven mainly by the insertion of a hydrophobic helix into the YopN^{phi3T} groove.

The YopN^{phi3T}-AimX interaction activates the lytic cycle of the phage, preventing lysogeny

Since our previous results demonstrated that YopN expression promoted lysogeny (Fig. 4), we hypothesised that the interaction with AimX prevented YopN function as repressor, promoting the lytic cycle of the phage. We have previously shown that the expression of the wt AimX was sufficient to restore the defect of the phi3T $\Delta aimR$ prophage (Fig. 3b). Based on this approach we hypothesised that expression of the AimX^{M14D} would not complement the induction of phi3T $\Delta aimR$ prophage because of the incapacity of this AimX mutant to interact with YopN and remove its repression. This was the case (Fig. 3b), confirming that the AimX-YopN^{phi3T} interaction is essential to promote the lytic cycle of the phage.

To additionally support this idea, we used one of the evolved phi3T $\Delta aimR$ mutants carrying the mutation in the TT. This evolved phage is insensitive to AimP and has a reduced ability to produce lysogens (Supplementary Fig. 2b). We postulate that this occurs due to overexpression of AimX from the strong *aimP* promoter, thus preventing YopN^{phi3T} function. To

confirm that the YopN-AimX interaction is key for the lysogeny/lytic decision, we hypothesised that overexpression of YopN in the recipient strain would restore the ability of the evolved phage to produce lysogens, since the overexpression of YopN^{phi3T} from the integrative plasmid would compensate for the overexpression of AimX in the evolved phage. Remarkably, this was the case, and expression of YopN in the recipient cells increased lysogeny of the evolved phages more than 100 times, reaching similar levels to those observed following infection of the wt phi3T prophage (Fig. 4d).

YopN binds to AimX using the same residues required to interact with other partners to promote lysogeny

The fact that the binding to AimX did not change the 3D structure of YopN (Fig. 6f) suggested that AimX does not inactivate YopN's function by modifying its functional conformation, but because AimX competes with another protein to bind to YopN. Binding of YopN to this yet unidentified protein would promote lysogeny, while prevention of the formation of this uncharacterised complex by the AimX-YopN complex would promote the lytic cycle.

To test this hypothesis, we expressed in the recipient cells the YopN^{M20D} or YopN^{L23D} mutant versions, which are unable to bind to AimX (Fig. 5). We hypothesised that while overexpression of YopN in the recipient increases lysogenisation, as previously demonstrated (Fig. 4), overexpression of the YopN^{M20D} or YopN^{L23D} would not, because the inability of these mutants to interact with the additional partner. This was indeed the case and neither overexpression of the YopN^{M20D} or YopN^{L23D} increased lysogenisation, like the wt YopN does (Fig. 4c). To further confirm this hypothesis, we overexpressed wt YopN^{phi3T} and the mutant versions, YopN^{M20D} and YopN^{L23D}, before performing an infection experiment into these strains with wt phi3T. Expression of YopN^{phi3T} blocked the lytic cycle of the phage infect, promoting lysogeny and reducing the phage titre after infection (Fig. 4a). In contrast, and support of our hypothesis, expression of YopN^{M20D} and YopN^{L23D} neither blocked phage infection nor

promoted lysogeny, a behaviour that is indistinguishable to that observed for the phage infecting a strain carrying an empty plasmid (Fig. 4a - 4c).

To rule out that the YopN^{phi3T} M20D or L23D mutations severely affect YopN^{phi3T} structure, and therefore, their function, the SEC analyses of YopN^{M20D} or YopN^{L23D} confirmed that both mutants have similar behaviour in solution than the YopN^{phi3T} wt protein (Supplementary Fig. 5b). Given that none of the mutated residues has impact on the overall YopN^{phi3T} structure, the fact that the YopN^{phi3T} M20D or L23D mutants were unable to promote lysogenisation would confirm that these residues are also key for the interaction with other protein required to promote the lysogenic cycle of the phage.

Expression of yopN^{phi3T}

Our model describing how the arbitrium system works involves a high and constant expression of YopN^{phi3T}. Then, depending on the presence of AimX, YopN^{phi3T} would perform its function or not. Previous transcriptomic analysis confirmed that high levels of yopN^{phi3T} expression were observed after phi3T infection¹⁵. In view of these results, we decided to identify the yopN^{phi3T} promoter, and characterised the region between *aimX* and the first gene of the operon containing yopN^{phi3T}, which corresponds to yopM^{phi3T} (*phi3T_92*) (Supplementary Fig. 1). Interestingly, *in silico* analyses suggested that this region contained a TT at the end of *aimX*, and a putative promoter downstream this TT but upstream yopM^{phi3T}. To characterise the TT located 3' *aimX*, we used the two-plasmid approach once again. We cloned the region from the *aimP* promoter to *aimX*, which was transcriptionally fused to the β-gal reporter (Supplementary Fig. 8a). Next, we repeated the same experiment, but now the cloned DNA was extended to the TT located at the end of *aimX*, fused to the reporter gene. As can be seen in Supplementary Figure 8, the TT present in *aimX* is functional and is not regulated by AimR. This indicates that the polycistronic transcript containing *aimX* finishes at this TT.

Next, we identified and characterised the promoter controlling *yopN* expression. As previously indicated, the phiSITE tool, PromoterHunter, program identified a promoter upstream of *yopM* (Supplementary Fig. 8c). To validate the functionality of this promoter, we transcriptionally fused the region containing this promoter to the β -gal reporter and measured the expression of this reporter in presence or in absence of this region, or in presence of a DNA that had a mutated the putative promoter region (Supplementary Fig. 8c). Our results, summarised in Supplementary Fig. 8d confirm the existence of a strong promoter controlling the expression of the operon that contains the *yopN* gene and that makes its expression independent of *aimP-aimX* operon.

DISCUSSION

It has been previously shown that arbitrium communication systems are highly diverse and are present in numerous phages and in other mobile genetic elements³. In spite of this diversity, all the phage-encoded arbitrium systems present a similar genetic organisation of their lysogeny modules, which is composed by the *aimR-aimP-aimX* module, followed by a gene that encodes the putative master repressor^{1,3}. For some phages, this master repressor is located next to the *aimX* gene, in the opposite direction^{3,6}, while in other phages, including the prototypical phi3T and SP β phages that were used to discovery and characterise the arbitrium system, the master repressor is part of a six gene operon that also contains an additional repressor of the system, YopN⁵.

Independently of their genetic organisation, it has been widely assumed that all the arbitrium systems have a conserved mode of action¹. At the beginning of the infective process, *aimR* and *aimP* are highly expressed. Since at this early stage AimP concentration is low, the apo form of AimR functions as a transcriptional regulator that induces the expression of *aimX*, which blocks lysogeny by directly inhibiting the phage master repressor using an uncharacterised *cis*-antisense mechanism. After several rounds of phage propagation, the amount of AimP peptide will increase, and after binding to AimR, will inhibit its transcriptionally

regulatory activity, decreasing the production of *aimX* and favouring lysogeny. In this work we aimed to shed further light on the aforementioned mechanism of action. However, in doing so, we have fundamentally challenged the roles associated with the main players of the system (see the schematic representation of the new model in Fig. 7). Our results show that AimR is a transcriptional antiterminator. Once the AimR dimer binds to its cognate DNA binding site, it disrupts the functionality of a TT located between the *aimP* and *aimX* genes, which are transcribed as part of a bicistronic operon that initiates at the *aimP* promoter. Hence, in addition to change the role for AimR, we have identified an additional function for the *aimP* gene within the system, namely, to provide expression of *aimX*. In this way the expression of both peptides (AimP and AimX), with antagonistic functions in the lysis/lysogeny decision, is strongly coupled, making easy and fast to change the balance between the phage cycles using AimR as a needle of the scales. Importantly, our results also challenge the role of *aimX*, which in phage phi3T does not work as antisense RNA but as a small peptide that binds to the second repressor of the system, YopN, which is encoded in a six-gene operon that also encodes YopR, the master repressor of the phage⁵. The existence of this second repressor makes completely sense, since the prophages carrying the arbitrium system require activation of the SOS response for induction. Therefore, these phages require a repressor that senses the SOS response (YopR^{phi3T}), and another one that senses AimX (YopN^{phi3T}). Note that we have previously reported that a double *aimR/yopN* SPβ mutant phage was insensitive to the arbitrium but inducible after activation of the SOS response⁵. Based on these results and in the fact that both repressors need to work together to establish lysogeny, it is tempting to speculate here that somehow YopN^{phi3T} and YopR^{phi3T} would interact, although we have not been able to demonstrate such interaction *in vitro*. Another possibility is that YopN^{phi3T} works with other proteins encoded by the six-gene operon, that may either directly or indirectly impact the activity of the master repressor. Note that this scenario is supported by the fact that when we mutate YopN^{phi3T} to avoid interactions with AimX, we also interfere with the ability of YopN^{phi3T} to promote lysogeny. An additional line of evidence supporting this possibility is that different arbitrium systems encode different operons, encoding different proteins that preform

497 identical functions for the phage, as demonstrated here. One can speculate that the reason for
498 that is very simple: as soon as a phage encodes a different AimX, its cognate YopN protein
499 should change to interact with this new AimX. This change will force additional changes in the
500 rest of the proteins encoded by the operon, so at the end they keep interacting in the way the
501 original proteins did, promoting lysogeny. These hypotheses are currently under investigation.

502 Another interesting question that arises from this study is why *aimX* expression depends
503 on a terminator and not a transcriptional activator. Initially, it could be seen as a cleaver
504 strategy to accelerate and secure the switching between the two states, linking, via AimR,
505 signalling and effector molecules with antagonistic functions. Our hypothesis is that this also
506 provides additional layers of control over the system. For example, it could be possible that the
507 long transcript generated from *aimP*, which in presence of AimR would also contain *aimX*,
508 could be processed, which will provide an additional mechanism of control over *aimX*
509 expression. Another interesting possibility relates with the fact that AimR could also control the
510 expression of additional genes, present either in the phage or in the bacterium genome, using
511 this anti-terminator mechanism. Note that we have previously demonstrated the ability of AimR
512 to bind to different AimR binding sites located in the bacterial genome⁴. Therefore, we can't
513 rule out the existence of an AimR regulon, controlled by the anti-terminator activity of AimR,
514 which will act to control the lysis-lysogeny decision in the arbitrium's phages.

515 The arbitrium system was reported as the first demonstration of a system that allowed
516 communication between viruses using a small molecule for lysis-lysogeny decision. However,
517 it is now clear that communication between phages and/or with their host for decision making
518 is a more generalised feature as confirmed by the recent description in *Vibro* phages of quorum
519 sensing receptors that regulate lysis-lysogeny in response to bacterial host inducers, or that
520 some bacterial quorum sensing molecules induce the lytic cycle in different phages^{16,17}. Here,
521 we have redefined the molecular basis of the arbitrium system, by repositing new functions
522 for the classical players and by identifying additional key ones. Our findings add new regulatory
523 layers to the lysis/lysogeny decision indicating that this drastic process for the phage is finely

controlled and must integrate different signals. Since phages and other mobile genetic elements drive bacterial evolution, and since arbitrium systems are widespread in nature³, present in mobile genetic elements that infect both pathogenic and non-pathogenic bacteria, we anticipate that the mechanism reported here would be of high relevance on how virus and other elements communicate, but also how bacteria evolve, becoming more virulent and resistant. We also anticipate that we are just seen the tip of the iceberg in phage communications. It is amazing how such small subcellular organisms have evolved such systems to persist in nature.

METHODS

Bacterial strains and growth conditions

All bacterial strains used in this study are listed in Supplementary Table 4. *B. subtilis* strains were routinely grown at 37°C on LB (Miller) agar plates or in LB (Miller) broth liquid medium shaking at 200 rpm. *E. coli* strains were grown at 37°C on LB (Lennox) agar plates or in LB (Lennox) broth shaking at 180 rpm. When required, antibiotics were utilised at the following concentrations: erythromycin (1 µg ml⁻¹ or 5 µg ml⁻¹), kanamycin (10 µg ml⁻¹), ampicillin (100 µg ml⁻¹) or spectinomycin (100 µg ml⁻¹).

Strain construction

Phages SPβ (NC_001884) and phi3T (KY030782) are used in this study. To generate the *yopR* deletion mutant in phage phi3T, first we introduced into the Δ6 phi3T *phi3T_5::kan* strain a copy of the *phi3T_97* gene under the control of the IPTG inducible promoter P_{spank}. This allowed us to obtain a conditional mutant of the phage repressor (see further information in plasmids and cloning). To ensure expression of YopR^{phi3T}, 1 mM IPTG was added throughout the following steps to generate the mutant. We generated overlapping PCRs containing the erythromycin marker (including the lox sites) and 1 kb of flanking region for the *phi3T_97* gene. These PCRs were transformed into the Δ6 phi3T strain overexpressing YopR^{phi3T} and selected for erythromycin. Once the insertion of the erythromycin cassette was confirmed by PCR and sequencing, the antibiotic resistance cassette was removed as previously described¹⁸. Briefly, plasmid pDR244 was transformed into strains harbouring the loxP-flanked antibiotic resistance cassette with selection for spectinomycin resistance at 30°C to allow for cre/lox-mediated loop-out of the cassette. Transformant colonies were then streaked onto LB plates with 1mM IPTG and incubated overnight at 42°C for removal of the temperature-sensitive plasmid. Resulting strains were screened for plasmid curing (loss of spectinomycin resistance) and the antibiotic resistance cassette (loss of erythromycin resistance). Strains were streaked to single colonies and confirmation of the clean mutant was performed using PCR.

Plasmids and cloning

All plasmids and primers generated in this study are listed in Supplementary Tables 5 and 6. Competent cell preparation and transformation was performed as described¹⁹. Briefly, *B. subtilis* cells were grown in GM1 minimal media to early stationary phase to induce natural competence and 1 µg of DNA (PCR or plasmid) was added, followed by incubation at 37°C for 1 h with shaking at 210 rpm. The culture was centrifuged at 6000 g for 1 min, 800 µl of the supernatant removed, and the pellet re-suspended and plated onto the relevant antibiotic plates. Plates were incubated at 37°C for 24 h. For over-expression in *B. subtilis*, the genes under study were cloned into the *amyE* integration vector pDR110 under the control of the IPTG inducible promoter P_{spank}²⁰. The *yopR*^{phi3T} gene was cloned into a pDR110 where the spectinomycin resistance marker (spec) was replaced by a tetracycline (*tetL*) resistance marker. The pDR110 was digested with EcoRI and AsiSI, and a 6544bp band was gel-extracted and ligated to a PCR amplifying the *tetL* resistance cassette from pDG1515. The reporter plasmid pDG1663²¹ was modified to introduce a new multicloning site to include the following restriction sites: EcoRI-NotI-HindIII-SpeI-NheI-SphI-BamHI. We then cloned into this version of pDG1663 (named pDG1663+) the IPTG inducible promoter P_{spank} using pDR110 plasmid as template, generating pDG1663+ P_{spank}. Cloning was performed after PCR amplification of the appropriate template DNA using primers listed in Table S.

β-galactosidase reporter assays

Strains were grown at 37°C overnight in LB containing the appropriate antibiotics. The strains were diluted 1:100 into fresh LB media supplemented with 0.1 mM MnCl₂ and 5 mM MgCl₂ and grown at 37°C with 210 rpm shaking until reaching OD₆₀₀ 0.2. When needed, 1 mM IPTG was added and cultures were grown for an additional hour before 1 ml of sample was taken and pelleted. OD₅₉₅ values were measured for each sample. To measure β-galactosidase levels, the Miller method was used as described²². Briefly, cells were resuspended in Z buffer (60 mM Na₂HPO₄ 7H₂O, 40 mM NaH₂PO₄, 10mM KCl, 1mM MgSO₄, 50mM β-mercaptoethanol) and permeabilised with lysozyme. Following incubation with ONPG solution

(4.0 mg/ml o-Nitrophenyl- β -D-galactoside in Z buffer), the time taken for samples to change colour was noted and A_{420} readings were taken. Miller units were calculated as $(1000 \times A_{420})/(\text{reaction time (min)} \times \text{OD}_{595})$.

Bacteriophage induction assay

For induction, an overnight culture was diluted 1:100 in LB media supplemented with 0.1 mM MnCl_2 and 5 mM MgCl_2 and then grown at 37°C with 210 rpm shaking until reaching OD_{600} 0.2. This step was repeated twice to ensure the cells were in exponential growth. After the second growth Mitomycin C (MC) at 0.5 $\mu\text{g ml}^{-1}$ was added to the culture. Where experiments were performed to test the complementation of the mutants, 1 mM of IPTG was added at the same time as MC induction. The induced cultures were incubated at 30 °C with 80 rpm shaking for 4 h and then left overnight at room temperature. Following lysis, samples were filtered using 0.2 μm filters and lysates were stored at 4 °C until use.

Bacteriophage titering assay

The number of phage particles contained in the phage lysate of interest was quantified by a titering assay. An overnight culture of the relevant recipient strain (normally *B. subtilis* $\Delta 6$ or with the corresponding integration vector) was diluted 1/100 in LB supplemented with 0.1 mM MnCl_2 and 5 mM MgCl_2 and then grown at 37°C with 210 rpm shaking until reaching OD_{600} 0.2. If needed 0.1 mM IPTG was added. Then, 100 μl of recipient bacteria was infected with 100 μl of serial dilutions of phage lysate in phage buffer (PhB; 1 mM NaCl, 0.05 M Tris pH 7.8, 0.1 mM MnCl_2 , 5 mM MgCl_2) at room temperature for 10 min and 3 ml of phage top agar (LB media supplemented with 0.1 mM MnCl_2 and 5 mM MgCl_2 and 0.7 % agar) at 55 °C was added to the culture-phage mix and immediately poured over phage base agar plates (LB media supplemented with 0.1 mM MnCl_2 and 5 mM MgCl_2 and 1.5% agar). Plaques were counted after overnight growth at 37°C and photographed.

Lysogenisation assays

The number of lysogens were quantified by growing a recipient strain to OD₆₀₀ 0.2. Lysates of interest that contain the kanamycin marker were serially diluted in PhB and 100 µl was added to 1 ml of the recipient bacteria in 12 ml tubes. The mixture was incubated at 37°C for 30 min to allow the phage to infect bacteria. The bacteria-phage mixture was then transferred to 1.5 ml Eppendorf tubes and centrifuged at 6,600 rpm for 1 min. The supernatant was removed, and the bacterial pellet was resuspended in fresh LB broth before plating onto selective antibiotic LB agar plates. Plates were incubated overnight at 37°C. The number of colony forming units (CFU) was calculated.

Infection experiments

Cultures of the indicated strains were grown to OD₆₀₀ 0.2 corresponding to $\sim 3 \times 10^7$ CFU ml⁻¹. If needed 0.1 mM IPTG was added. Then, 15 ml of this culture was infected with the defined phage lysates at an MOI of 1:10 (phage:bacteria) and incubated at 30 °C and 80 rpm. At the indicated timepoints, samples were taken to assess the OD₆₀₀. At the end timepoint, samples were taken to assess the number of lysogens and phage titers.

Statistical analysis

Statistical analysis was performed as indicated in the figure legends. Briefly, phage and lysogenisation titres were log₁₀-transformed and analysed by a One-Way ANOVA followed by a Dunnett's multiple comparisons test using GraphPad Prism version 9.0.0 for Windows, GraphPad Software, San Diego, California USA (www.graphpad.com). The p-values represented in each figure are shown in the figure legends.

Recombinant protein expression and purification

The AimX gene as well as YopR and YopN from phi3T phage were amplified separately using genomic DNA from *Bacillus subtilis* strain 168 as template, adding additional overhangs necessary for cloning (see primers at Supplementary Table 6). Each resulting PCR product was cloned into the pLIC-SGC1 plasmid, which harbours a TEV cleavage site between the N-terminal 6His-tag and the ORF from the protein, using NEBuilder HiFi DNA Assembly master

mix (New England Biolabs) (see plasmids at Supplementary Table 5). After sequence confirmation of each cloning, plasmids were transformed into an E. coli strain BL21 (DE3) RIL (Agilent) for protein overexpression (see strain at Supplementary Table 4). For each construct, a single colony carrying the expression plasmid was grown overnight at 37°C in 50 ml of LB medium supplemented with 50 µL of 100 µg/mL ampicillin solution and 50 µL of 33 µg/mL chloramphenicol solution.

The culture was used to inoculate 1 L of LB medium, containing ampicillin and chloramphenicol, and was grown at 37 °C until an OD at 600 nm between 0.6-0.8 was reached. Then, the temperature was set to 20°C and protein expression was induced with a final concentration of 1 mM of IPTG. The culture was incubated at 20°C for additional 16 hours. Cells were harvested by centrifugation, washed with PBS and frozen at -20°C until used.

Purification of AimX, YopR^{phi3T} and YopN^{phi3T} was carried out following the same procedure. First, pellets were thawed and resuspended in lysis buffer (50 mM Tris pH 8, 500 mM NaCl) and sonicated 10 minutes in ice, with pulses having 1 second on 1 second off to avoid heating the sample. Cell debris was separated from the rest of the sample by centrifugation at 10.000 g for 1 h. The supernatant was filtered using a 0.45 µm filter and loaded into a 1 ml TALON (GE Healthcare) column previously equilibrated with buffer A (50 mM Tris pH 8, 250 mM NaCl), washed and stepwise eluted with buffer B (buffer A with 500 mM imidazole). Fractions containing the purest protein were pooled and digested, if needed, with TEV protease (50:1 molar ratio protein:TEV) while being dialyzed against buffer A supplemented with 1 mM EDTA and 1 mM β-mercaptoethanol. The sample was concentrated and loaded into a Superdex 75 increase 10/300 (GE Healthcare) gel filtration column equilibrated in buffer A. The purest fractions judged by SDS-PAGE were pooled, concentrated and stored at -80°C until use.

Protein crystallisation

All crystals were obtained in the IBV-CSIC crystallogensis facility at 21°C using the sitting-drop vapor-diffusion method. His-YopN^{phi3T} crystals were grown by mixing 0.3 µl protein

solution at 10 mg/mL and 0.3 μ l reservoir solution (0.1 M Tris pH 8, 5% glycerol and 0.6 M Na/K tartrate). YopN^{phi3T} crystals were obtained by mixing 0.3 μ l protein solution at 10 mg/mL and 0.3 μ l reservoir solution (10% PEG8000, 50 mM magnesium acetate and 100 mM sodium acetate). The YopN^{phi3T}-AimX complex crystals were obtained by co-crystallization. The sample was prepared by mixing 65 μ l of YopN at 2 mM and 387 μ l of AimX at 336 μ M (1:1 molar ratio) and concentrating the mixture up to a final volume of 100 μ l. Crystals were obtained by mixing 0.3 μ l of the protein mixture and 0.3 μ l reservoir solution (0.1 M Hepes pH 7.5, 10% PEG8000 and 0.2 M Calcium acetate).

Data collection, structure solution and refinement

Prior to data collection, crystals were flash frozen in liquid nitrogen. For YopN and His-YopN crystals, the corresponding crystallization solution was supplemented with 10% glycerol as cryoprotectant. YopN-AimX complex crystals were frozen in paratone. All the diffraction experiments were carried out at 100 K.

Both, His-YopN^{phi3T} and YopN^{phi3T} (PDB codes 8ANT and 8ANU respectively), diffraction data were collected at Xaloc beamline, ALBA synchrotron in Barcelona, using a wavelength of 0.97925 Å and 0.97918 Å respectively. His-YopN^{phi3T} data reduction was carried out in CCP4i2, whereas YopN^{phi3T} data reduction was carried out in CCP4i. Phases for His-YopN^{phi3T} were obtained by *ab initio* phasing using ARCIMBOLDO LITE¹⁴ as implemented in CCP4i2²³, whereas YopN^{phi3T} phases were obtained by molecular replacement using Phaser as implemented in Phenix²⁴ and His-YopN^{phi3T} as a model. Followed phase determination, model refinement was carried out combining manual building with coot and computational refinement using phenix.refine²⁵. His-YopN^{phi3T} and YopN^{phi3T} final structures have, respectively, a percentage of 99-1 and 96-4 of favoured and allowed Ramachandran positions.

YopN^{phi3T}-AimX (PDB code 8ANV) crystals were collected at Diamond Light Source synchrotron (Oxfordshire, UK), beamline I04, using a wavelength of 0.9795 Å. Data reduction was carried out by the beamline software pipeline. The structure of the complex, solved at 2.2 Å resolution by molecular replacement using YopN^{phi3T} structure as a model and Phaser as

implemented in phenix²⁶. Structure refinement was done combining phenix.refine²⁵ and manual building using coot²⁷. Final structure has 97% and 3% of the residues in favoured and allowed Ramachandran positions respectively.

Binding kinetics analysis

Binding kinetics were measured by biolayer interferometry using the BLITZ system (Forté Bio, PALL) and Ni-NTA biosensors (Sartorius) at room temperature. All the proteins were diluted in binding buffer (25 mM Tris pH 8, 150 mM NaCl, 3 mM Imidazole and 0.005% tween 20). Measurements were carried out by immobilizing AimX at 0,3 mg/mL for 120 seconds (either WT, M14D or G17D) to the biosensors through its N-terminal 6His-tag. After generating a baseline with binding buffer, association and dissociation were monitored for 60 seconds each, using YopN^{phi3T} concentrations (either WT, M20D or L23D) from 1.25 to 0.07813 μ M, obtained by 1:2 serial dilution from the highest concentration.

Size Exclusion Chromatography coupled to Multiangle Light Scattering experiments (SEC-MALS)

20 μ L at 1 mg/mL of YopN^{phi3T} (WT and the different mutants) or AimX constructs were injected into a shodex KW-402.5-4F column, previously equilibrated with 100mM Hepes pH 7,5 and 250 mM NaCl, using a Shimadzu HPLC equipped with a manual injector, at a flow rate of 0,3 ml/min. MALS analysis was performed using a miniDAWN Treos MALS detector followed by an optilab T-rEX and a DLS (Wyatt). Data analysis was performed using Astra software (Wyatt).

Native Polyacrylamide gel electrophoresis (Native-PAGE)

In order to figure out if YopN^{phi3T} and YopR^{phi3T} were interacting, a native polyacrylamide gel electrophoresis experiment (Native-PAGE) was carried out. An 8% Native-PAGE was pre-run for 1 hour prior to loading of the samples. YopR and YopN or YopR and AimX were mixed at a final concentration of 150 μ M each, in buffer 25 mM Tris pH 8 and 250 mM NaCl, and incubated in ice for 1 hour. The equivalent amount of each protein was loaded into the gel

separately, as control. The gel was run for 150 minutes at 100 V at 4°C and stained with coomassie.

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Competing Interests

The authors declare no competing interests.

Contributions

J.R.P, A.M. conceived this study; S.Z-C., A.B., N.Q-P., F.G.S, J.M-B., and A.F-R. performed the experiments S.Z-C., A.B., N.Q-P., F.G.S, J.M-B., A.F-R., W.J.J.M., J.R.P and A.M. processed data. J.R.P, A.M. wrote the manuscript with inputs from all authors.

Data availability

All data are available from the authors upon request. The atomic coordinates of the YopN, His-YopN and AinX-YopX has been deposited with PDB codes 8ANT, 8ANU y 8ANV at <http://www.pdb.org>.

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Figures

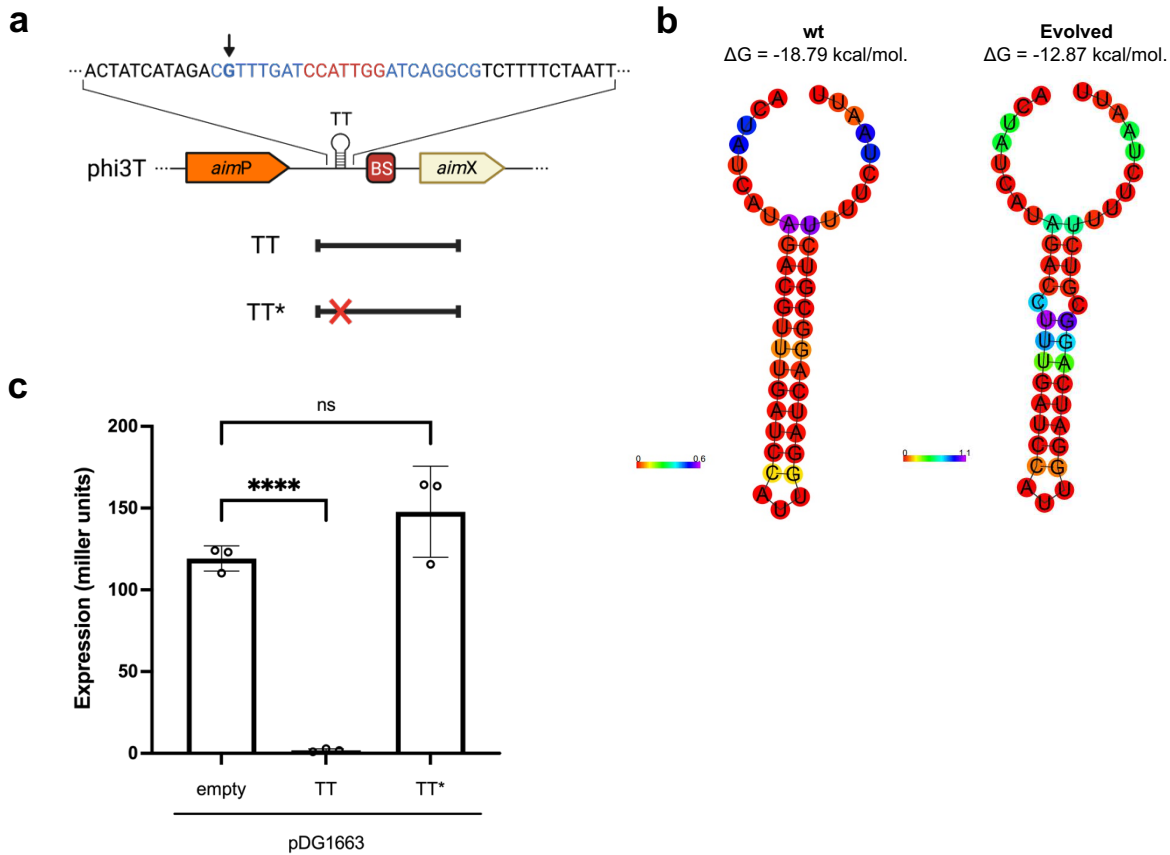


Figure 1 phi3T $\Delta aimR$ evolutions target a novel terminator.

a Schematic representation of the genetic layout where the putative terminator is situated. The DNA sequence at the top shows the predicted terminator and the regions corresponding to the stems (blue) and loop (red) according to ARNold. The mutated base is shown with the black arrow. TT: transcriptional terminator; TT*: mutated TT; BS: AimR binding site. The black blunt-ended lines show the regions cloned into the pDG1663 reporter plasmid. Regions were fused to the β -galactosidase (β -gal) reporter gene and expression was driven by the pSpank IPTG-inducible promoter. **b** Predicted minimum free energy (MFE) secondary structures of the terminator for the wt and evolved sequence according to RNAfold. Colours are representative of positional entropy in the structure. **c** *B. subtilis* strains carrying the respective plasmids were grown to $OD_{600} = 0.2$ and IPTG was added (1 mM) for induction of the pSpank promoter. β -gal expression was measured using the Miller method. Error bars represent the SD of three biological replicates. Adjusted p values indicated were as follows: ****p < 0.0001; ns (not significant).

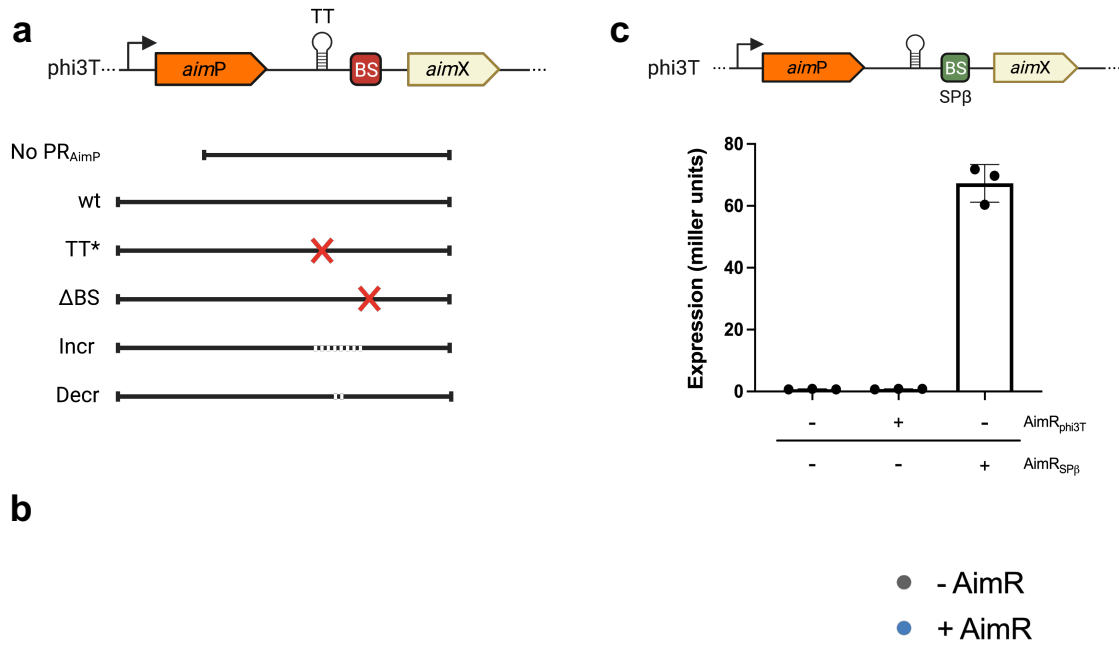


Figure 2 AimR functions as an antiterminator of a transcript that initiates at *aimP*.

a Schematic view of the relevant genetic features of the DNA region upstream from *aimX*. The black arrow indicates the *aimP* promoter. TT: transcriptional terminator. BS: AimR binding site. The black blunt-ended lines show the regions cloned into the pDG1663 reporter plasmid. TT*: evolved transcriptional terminator. ΔBS: mutated BS palindromes. Incr: increased distance between the TT and BS. Decr: decreased distance between the TT and BS. Regions were fused to the β-galactosidase (β-gal) reporter gene. **b** *B. subtilis* strains carrying pDR110 (empty or AimR) and the respective reporter plasmid were grown to OD₆₀₀ = 0.2. IPTG was added (1 mM) for induction of the pSpank promoter in the pDR110 plasmid (empty or AimR). β-gal expression was measured using the Miller method. **c** AimR function is phage-specific. Schematic of the genetic features in the reporter strain used to characterise cross-activity of AimR antitermination for SPβ and phi3T where the binding site has been swapped. *B. subtilis* strains carrying the respective reporter plasmid were grown to OD₆₀₀ = 0.2 and IPTG was added (1 mM) for induction of the pSpank promoter in the pDR110 (empty, AimR_{phi3T} or AimR_{SPβ}) plasmid. β-gal expression was measured using the Miller method. In the schematics, the black arrows indicate promoters. BS: AimR binding site (green: SPβ). Black lollipop symbols represent the putative transcriptional terminators. In all experiments, error bars represent the SD of three biological replicates. Adjusted p values indicated were as follows: ****p < 0.0001; ns (not significant).

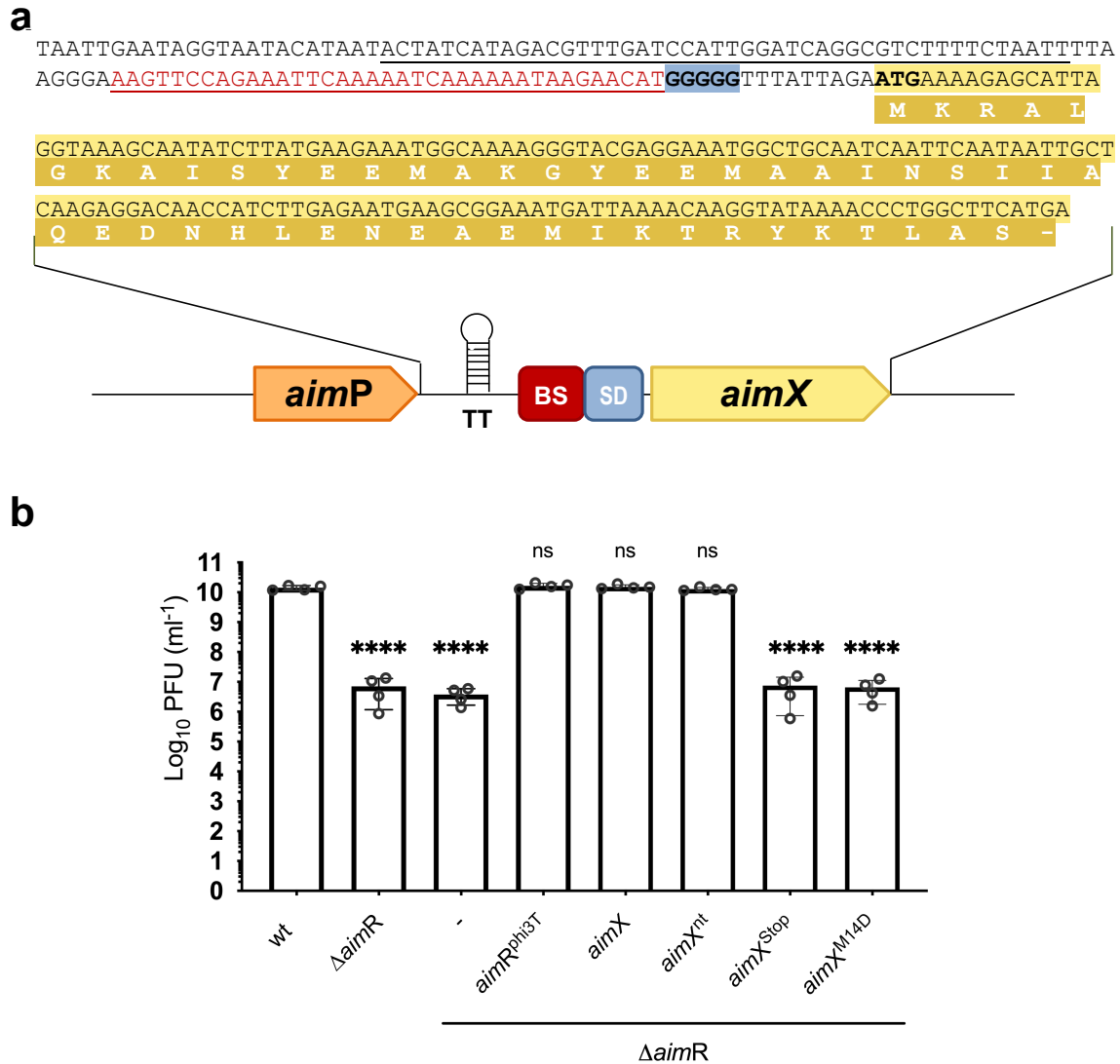


Figure 3. AimX is a peptide.

a Representation of DNA region located between *aimP* and *aimX*. Underlined in black is represented the TT, while underlined in red is represented the AimR binding site (BS). In addition to these regulatory elements, this region contains the elements required for *aimX* translation including the Shine-Dalgarno region (SD), highlighted in blue, and the initial Met. The AimX coding sequence is highlighted in light yellow background with the start codon in bold and the encoded protein sequence is showed below highlighted in dark yellow background. **b** Strains lysogenic for phages phi3T wt, Δ*aimR*, and Δ*aimR* expressing different versions of the *aimX* gene were MC induced (0.5 μg/mL). The number of resulting phages were quantified using 168 Δ6 as the recipient strain. The results are represented as PFUs mL⁻¹. The means and SDs are presented (n = 4). An ordinary one-way ANOVA of transformed data followed by a Dunnett's multiple comparisons test was performed to compare mean differences between titers. Comparisons performed against phi3T Δ*aimR*. Adjusted p values indicated were as follows: ****p < 0.0001; ns (not significant).

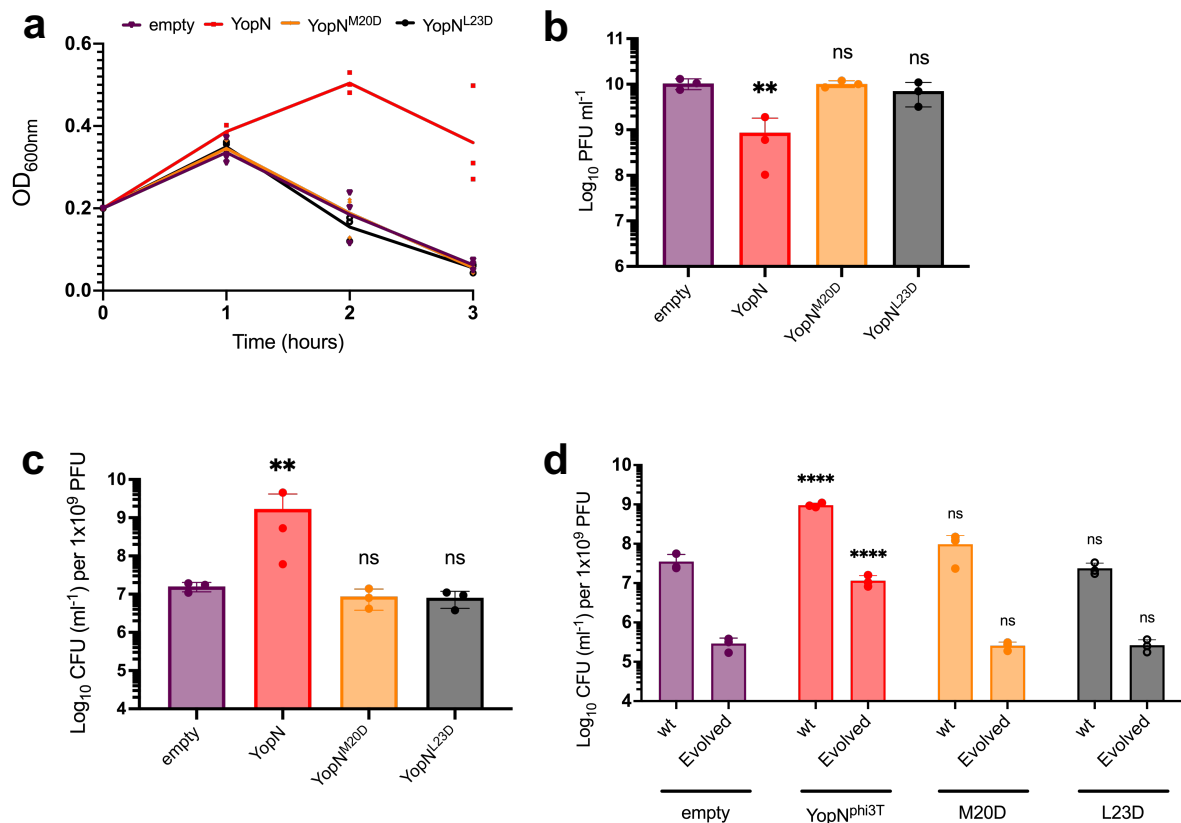


Figure 4. phi3T_YopN promotes lysogeny.

B. subtilis strains either devoid of (purple) or expressing wt YopN^{phi3T} (red), YopN^{M20D} (orange) or YopN^{L23D} (grey) were infected with phage phi3T at an MOI of 1:10 (phage:bacteria). For all infection experiments growth (OD₆₀₀) readings (a), phage titers (b) and the relative number of lysogens (c) were determined. The number of resulting phages (PFUs mL⁻¹) and lysogens (CFUs mL⁻¹) were quantified using 168 Δ6 as the recipient strain. The means and SDs are presented (n = 3). d Strains lysogenic for phi3T wt and evolved ΔaimR phages were MC induced (0.5 μg/mL). The number of resulting lysogens were quantified using 168 Δ6 amyE::Pspank-YopN^{phi3T} (wt/M20D/L23D) as the recipient strain. The results are represented as CFUs mL⁻¹ normalised by PFUs mL⁻¹ and represented as the log CFU of an average phage titer (1×10⁹ PFU mL⁻¹). The means and SDs are presented (n = 3). An ordinary one-way ANOVA of transformed data followed by a Dunnett's multiple comparisons test was performed to compare mean differences between titers. Comparisons performed against phi3T ΔaimR. Adjusted p values indicated were as follows: ****p < 0.0001; ns (not significant).

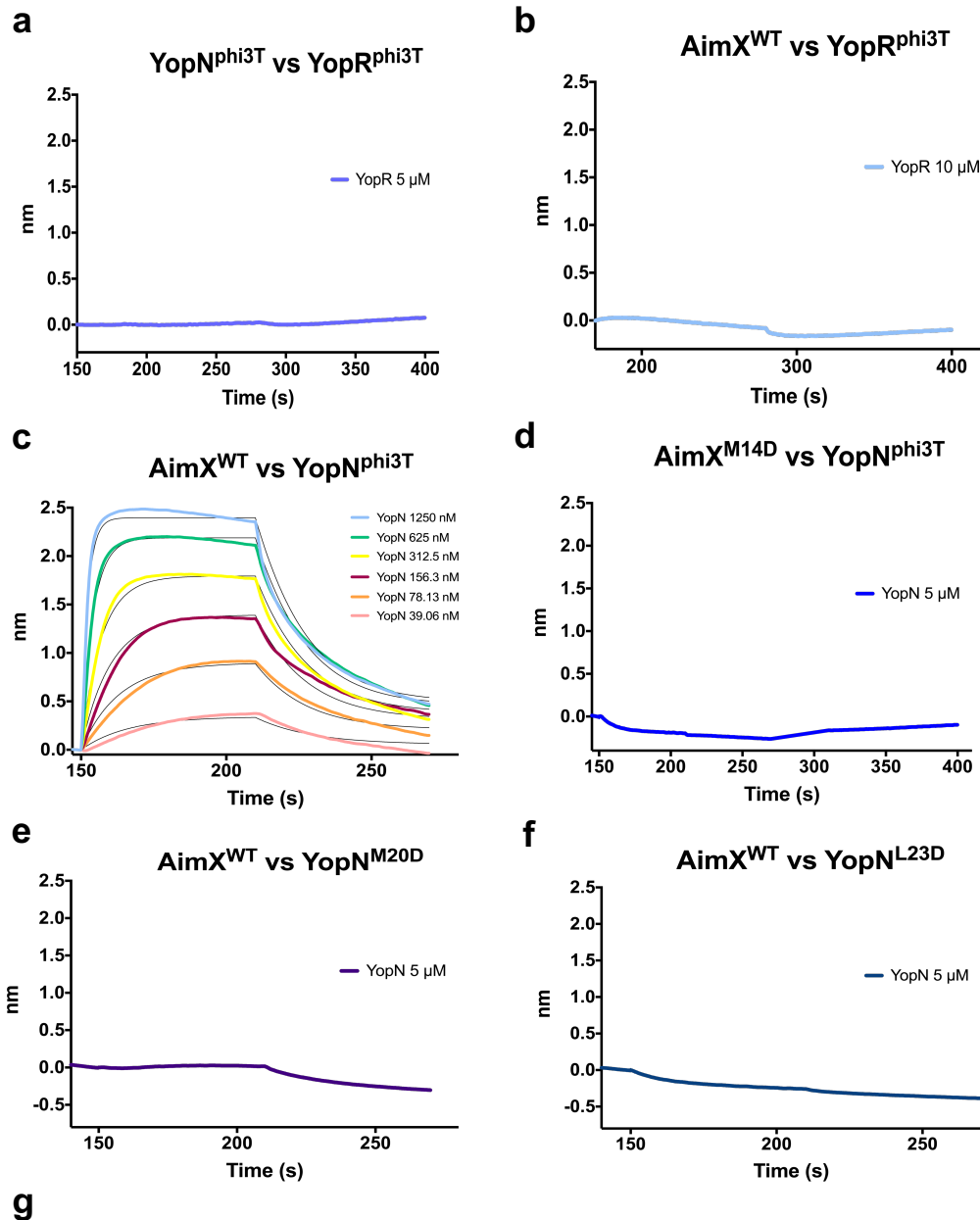


Figure 5. Biolayer interferometry experiments.

a BLI curve for the binding test between YopN^{phi3T} and YopR^{phi3T}. **b** BLI curve for binding test between AimX and YopR^{phi3T}. **c** BLI curve for the binding test between AimX and YopN^{phi3T}. Kinetic curves were monitored using various concentrations in a two-fold dilution series from 1.25 to 0.07813 μ M. Fitting curves are shown in black. **d** BLI curve for binding test between AimX^{M14D} and YopN^{phi3T}. **e** BLI curve for binding test between AimX and YopN^{M20D}. **f** BLI curve for binding test between AimX and YopN^{L23D}. For every experiment, ligand concentration is specified. Note that in panels a,b,d,e and f, only one curve with highly concentrated analyte is depicted to show that there is no binding. **f** Mean values for AimX-YopN^{phi3T} calculated K_D , K_{ON} and K_{OFF} from two independent experiments are shown.

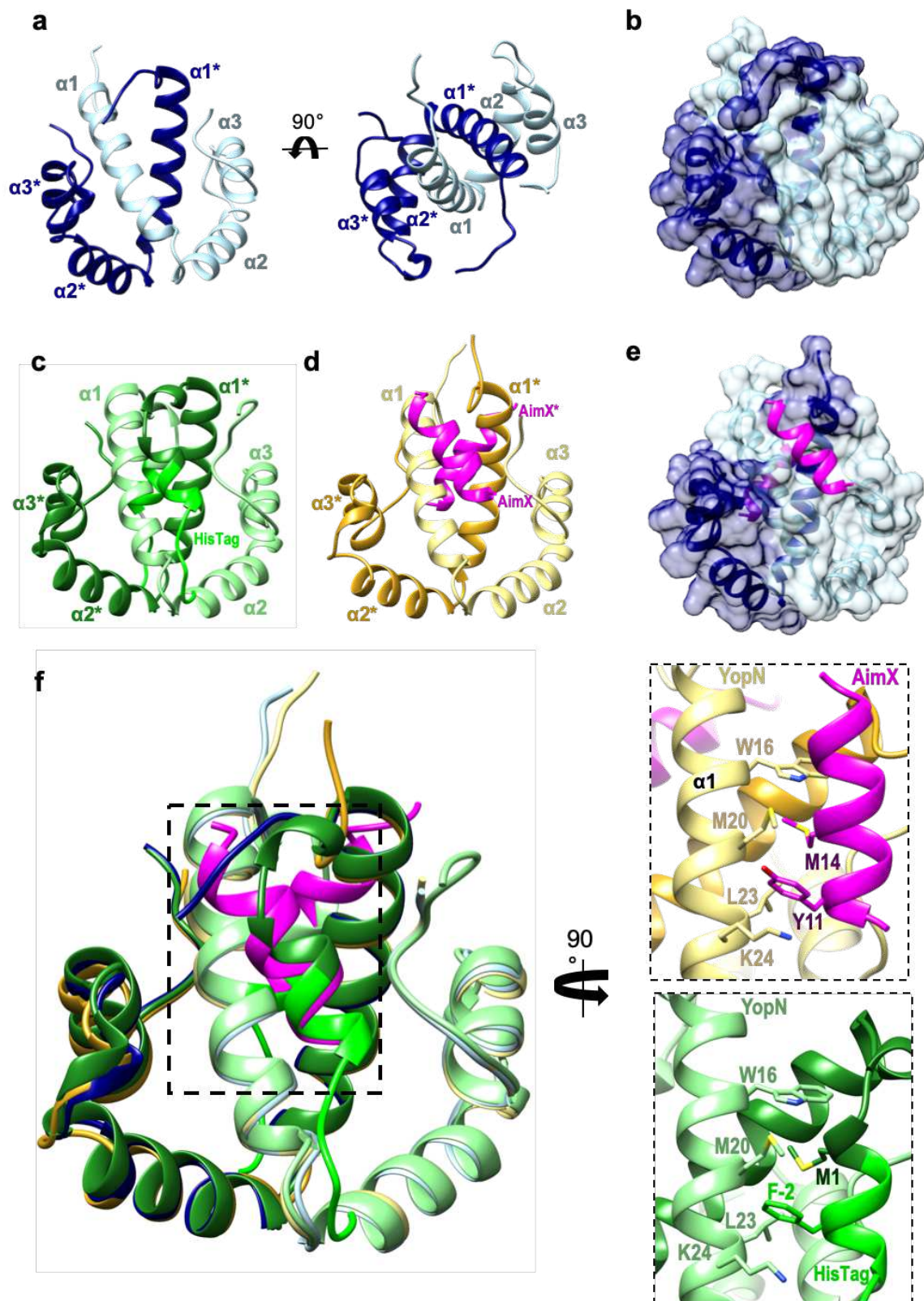


Figure 6. Crystal structures of YopN^{phi3T} and YopN^{phi3T}-AimX^{phi3T} complex. **a** Ribbon representation of YopN^{phi3T} (shades of blue), front and top views respectively. Each YopN monomer is depicted in a different shade of blue and helices have been numbered (* denotes second monomer). **b** Surface representation of YopN dimeric structure following the same colour code as in panel a. **c** Ribbon representation of His-YopN^{phi3T} crystal structure. Each monomer is shown in a different shade of green. Residues belonging to the Histag linker depicted in fluorescent green in both monomers. **d** Ribbon representation of YopN^{phi3T}-AimX^{phi3T} complex with YopN monomers coloured in different shades of yellow and AimXs in magenta. **e** Representation of AimX (magenta) location on the dimeric YopN surface, following the same colour code as in panel b. **f** Structural superimposition of the three crystal structures, following the colour code in panels a, c and d. Zoomed view of the residues involved in the interaction for YopN^{phi3T}-AimX^{phi3T} and for the His-tag linker residues in his-YopN^{phi3T}. Interacting residues are shown in sticks and labelled.

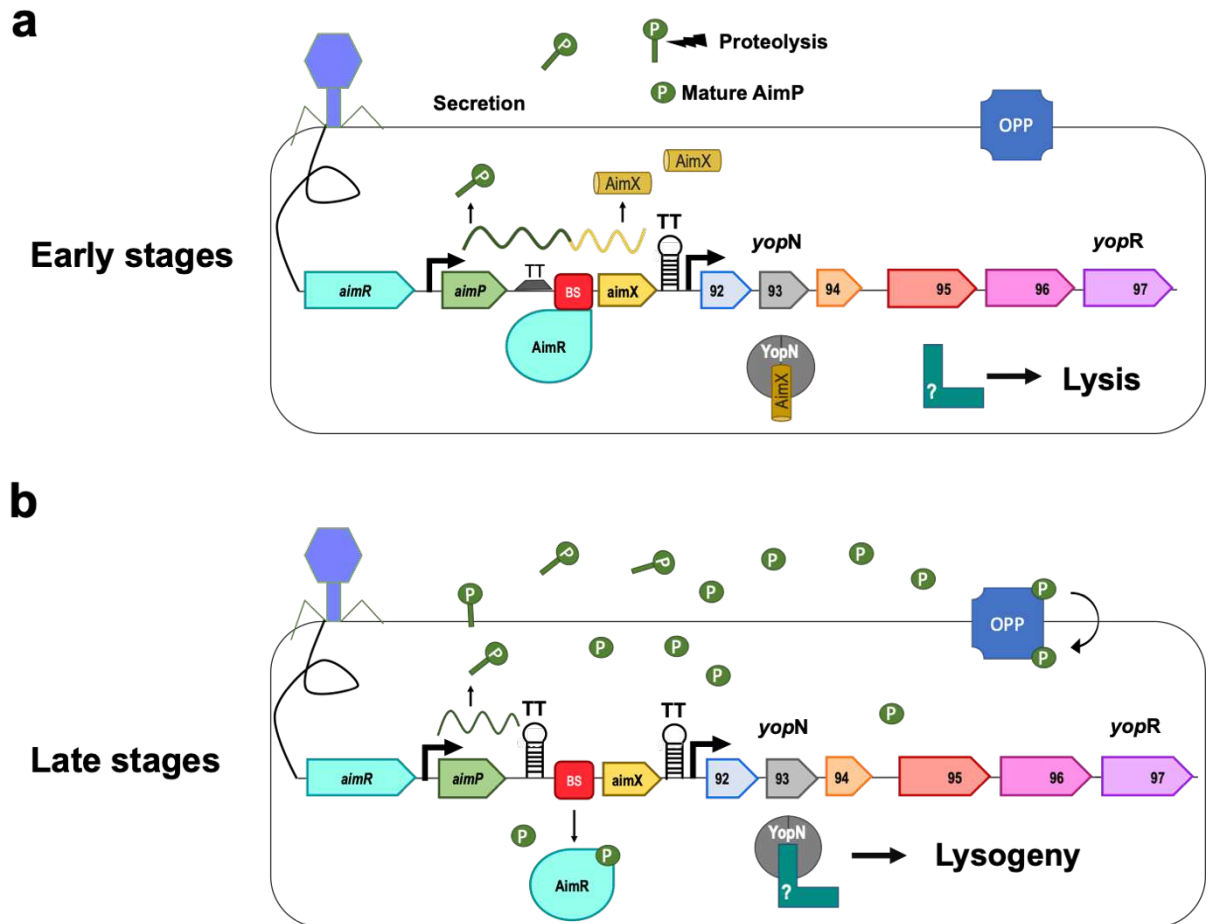


Figure 7. Model for the mechanisms controlling lysis-lysogeny decisions.

a In early stages of the infection, *aimR* gene is being expressed. AimR protein, by binding to the DNA prevents the transcriptional terminator located between *aimP* and *aimX* from having an effect, hence allowing the simultaneous expression of *aimP* and *aimX* genes. AimP will be secreted and processed extracellularly, whereas AimX will remain inside the bacteria and is going to bind YopN. By this binding, AimX is preventing YopN from binding a third protein, promoting the lytic cycle by an unknown mechanism. **b** During late stages of the infection, the mature AimP starts accumulating inside the bacteria thanks to the OPP transporter. Then, when a phage infects the bacterium and starts producing AimR, the later will bind AimP and not to its binding site at the DNA. This will result in an operative transcriptional terminator between *aimP* and *aimX* that is going to prevent the expression of *aimX* from happening. Since there is no AimX, YopN is free to bind a third protein and, by a mechanism that is yet to be described, promote lysogeny.

Supplementary Files

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