



# Widespread Utilization of Peptide Communication in Phages Infecting Soil and Pathogenic Bacteria

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# Widespread utilization of peptide communication in phages infecting soil and pathogenic bacteria

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#### Summary

Temperate phages can adopt either a lytic or lysogenic lifestyle within their host bacteria. It was recently shown that *Bacillus subtilis*-infecting phages of the SPbeta group utilize a peptide-based communication system called arbitrium to coordinate the lysogeny decision. The occurrence of peptide-based communication systems among phages more broadly remains to be explored. Here, we uncover a wide array of peptide-based communication systems utilized by phages for lysogeny decisions. These arbitrium-like systems show diverse peptide codes and can be detected in numerous, genetically distant phage types and conjugative elements. The pathogens *Bacillus anthracis*, *Bacillus cereus* and *Bacillus thuringiensis* are commonly infected by arbitrium-carrying mobile elements, which often carry toxins essential for pathogenicity. Experiments with phages containing these arbitrium-like systems demonstrate their involvement in lysogeny decisions. Finally, our results suggest that the peptide-based decision is executed by an antisense RNA that controls the regulator of the lysogenic state.

#### Introduction

Temperate viruses are viruses capable of two possible life cycles – lytic and dormant (also referred to as "latent" or "lysogenic") (Howard-Varona et al., 2017). The temperate life style is common among viruses that infect bacteria, termed phages. When infecting its host bacterium, a temperate phage must make a choice between replicating in the cell and eventually killing it (lysis) or entering a lysogenic state, in which its genome, termed a prophage, either integrates into the host genome or replicates as an episome (Howard-Varona et al., 2017; Ofir and Sorek, 2018). Prophage-encoded factors protect the host bacterium from further lytic infection by virions of the same phage (Abedon, 2015; Oppenheim et al., 2005).

The lysis-lysogeny decision has been extensively studied for the *Escherichia coli* phage Lambda, and was found to be mainly influenced by the metabolic state of the infected cell and the number of co-infecting phage particles (Oppenheim et al., 2005; Zeng et al., 2010). Genetically, the lysis-lysogeny decision in phage Lambda is controlled by an interplay between the phage-encoded transcriptional regulators CI, CII and Cro, as well as a number of host-encoded factors (Oppenheim et al., 2005; Ptashne, 2004). The master regulator of the lysogenic state is the CI transcriptional repressor, which binds the promoters of the lytic genes and inhibits their expression (Oppenheim et al., 2005).

It was recently shown that *Bacillus* phages of the SPbeta group use a peptide-based communication system termed "arbitrium" to coordinate their lysis–lysogeny decisions (Erez et al., 2017). During lytic infection, each phage produces a measured amount of a communication peptide, which is released into the growth medium and internalized by nearby bacteria. In subsequent infections, progeny phages sense the concentration of this peptide and preferably enter the lysogenic cycle when its concentration is high. Such communication allows the presently infecting phage to evaluate the extent of recent bacterial lysis events by predecessor phage infections, and switch to the lysogenic cycle when the bacterial population dwindles.

The arbitrium communication system has been characterized for phi3T (Erez et al., 2017), a phage from the SPbeta group that infects *Bacillus subtilis*. This system consists of three phage-encoded genes: *aimP*, producing the communication peptide, *aimR*, encoding the intracellular peptide receptor and *aimX*, a non-coding RNA that is a negative regulator of lysogeny. During lytic infection, *aimP* encodes a short protein that is released as a propeptide into the growth medium where it is then further processed by extracellular proteases to produce a 6 amino acid (aa) mature communication peptide. This 6 aa peptide accumulates in the growth medium and is imported by nearby bacteria through the bacterial oligopeptide permease (OPP) channel. In the absence of mature peptides in the cell, AimR binds to the promotor of *aimX* and activates its transcription, resulting in inhibition of lysogeny by AimX through a mechanism yet unknown. When bound by the peptide, AimR dissociates from the DNA, and no longer activates the expression of AimX, leading to lysogeny (Erez et al., 2017).

Close homologs of the arbitrium system were found mainly in genomes of SPbeta-like phages and prophages (Erez et al., 2017), where the system is always organized as an *aimR-aimP-aimX* gene cluster. Interestingly, different phages encode diverse communication peptides that vary in the first three amino acids (for example: the peptide sequence of phage phi3T is SAIRGA, and that of phage SPbeta is GMPRGA). Each phage responds specifically only to its own peptide. Consistently, the structure of the AimR peptide receptor bound to its cognate peptide has recently been determined for phages phi3T and SPbeta, showing high specificity between the peptide and its binding pocket within the receptor (Dou et al., 2018; Gallego del Sol et al., 2019; Wang et al., 2018; Zhen et al., 2019).

While phages from the SPbeta group were shown to frequently encode arbitrium, it is currently unknown how common such communication is in other phages. In addition, the mechanism by which the signal propagates to establish lysogeny, and the role of the AimX non-coding RNA in executing the lysogeny decision, are still unresolved. In this study we found that multiple types of phages and conjugative elements encode arbitrium systems that display a diverse set of peptide codes. We further show evidence that the AimX noncoding RNA controls the master regulator of lysogeny through direct RNA antisense interactions.

#### Results

#### Arbitrium-like systems are common in phages infecting pathogens and soil bacteria

In our previous study (Erez et al., 2017), a BLAST search was used to identify close homologs of the AimR protein within phages and prophages. This search revealed 112 homologs, the majority of which are found within prophages that belong to the SPbeta group of phages. In attempt to identify more distantly related communication systems, we now performed profile-based homology searches using iterative PSI-BLAST (Altschul, 1997) across a database of >60,000 bacterial, archaeal and viral genomes (Methods). This search retrieved 1,180 homologs of AimR, 96% (1135/1180) of them located directly upstream of an *aimP*-like gene that contained an N-terminal signal peptide sequence (Table S1).

The vast majority of these arbitrium-like systems were located in phages and prophages, and these belong to numerous phage groups that vary in genome sizes (23kb-142kb) and architectures (Fig. 1A; Table S1). Apart from SPbeta, these phages include Wbeta, which infects *B. cereus* and *B. anthracis*, and that a variant of which has been widely used for clinical diagnosis of *B. anthracis* infections (Abshire et al., 2005); phage phIS3501, which is utilized by *B. thuringiensis* var israelensis as a genetic switch for the regulation of toxin production (Moumen et al., 2012), and at least 15 other types of phages (Fig. 1A). In addition, 16% of these arbitrium homologs reside within conjugative elements, consistent with previous observations that mobile elements can use peptide communication to regulate their transfer between hosts (Singh and Meijer, 2014).

Overall, mobile genetic elements containing arbitrium and arbitrium-like systems were found within the genomes of 41 species, the vast majority of them represent bacteria from the genus *Bacillus* (Fig. 1B; Table S1). In fact, over 50% (442/782) of all genomes belonging to this genus in the database we analyzed contained at least one arbitrium-carrying prophage or conjugative element, with up to 8 such prophages in a single genome (*B. thuringiensis* YBT-1518). Arbitrium-carrying mobile elements were especially prevalent in the sequenced genomes of the pathogens *B. anthracis* (97%, 89/92), *B. thuringiensis* (89%, 51/57), and *B. cereus* (76%, 167/224). Notably, arbitrium systems are found in plasmids that carry Bt insecticidal toxins in *B. thuringiensis* (Fig. 2A; Fig. S1), as well as in the virulence plasmid pXO2, which is essential for full pathogenicity of *B. anthracis* (Dixon et al., 1999) (Fig. 2B). This implies that peptide-based communication may play a role in regulating horizontal transfer of virulence traits among pathogenic bacteria (see Discussion).

Phylogenetic analysis based on multiple sequence alignment of all AimR homologs showed that they cluster into 10 different clades (Fig. 3). Five of these clades (clades 1,2,3,4 and 9) are found almost exclusively in phages, one clade (5) is specific to conjugative elements, and three clades (6, 7 and 8) can be found both in phages and conjugative elements. An additional clade (colored grey in Figure 3) included predicted AimR homologs that are not found within a mobile element; we consider this clade as a possible non-arbitrium outgroup (see Discussion). The arbitrium systems of phages phi3T and SPbeta localized to clade 2, indicating that the originally described arbitrium systems form only a small subset of the actual genetic diversity of phage communication systems found in nature.

The mature communication peptide of the phi3T and SPbeta phages consists of the Cterminal end of AimP, formed by cleavage of a longer pro-peptide, similar to the Phr quorum sensing peptides of *B. subtilis* (Lazazzera, 2003). We therefore examined the Cterminus of the AimP protein associated with each of the AimR homologs. These Cterminal peptides were characterized by high diversity, with 168 unique sequences found. The peptides corresponding to each of the clades in the AimR tree form distinct sequence signatures, indicating that arbitrium systems from different clades correspond to different peptide codes (Fig. 3). Our experimental data (see below) show that the size of the mature peptide can reach up to 10 aa in some clades (Fig. 3).

#### Arbitrium systems regulate lysogeny in multiple phages

We next examined the location of each arbitrium system within its respective phage genome. Arbitrium systems were frequently found at the edge of the integrated prophage (Fig. 4), and in 70% of the cases (579 of 830 phages), they localized near a phage integrase gene, suggesting that these systems belong to the lysogeny module in most phages (Table S1).

To examine whether arbitrium-like systems from clades other than the phi3T/SPbeta clade also function in guiding phage lysogeny decisions we performed infection experiments

with phage Wbeta, in which we detected an arbitrium-like system from clade 3 (Fig. 5A). The arbitrium system in this phage is located in a genomic region previously defined as the lysogeny control module (Schuch and Fischetti, 2006). If the arbitrium-like system in phage Wbeta indeed guides its lysogeny decision, then we expect that addition of the arbitrium peptide to the growth medium during phage infection will lead to its lysogeny. Based on the observation that the C-terminal 6 aa in the AimP proteins of phages phi3T and SPbeta form the mature, active arbitrium peptide, we synthesized the peptide EIKPGG that represents the last 6 aa of the predicted AimP protein of phage Wbeta (Fig. 5A). *B. cereus* bacteria infected by phage Wbeta in the presence of 1  $\mu$ M of this peptide were completely protected from lysis (Fig. 5B), while shorter forms of the peptide (EIKPG, IKPGG and KPGG) had no effect (Fig. 5C-4E), suggesting that the 6 aa peptide indeed guides Wbeta lysogeny in a highly specific manner. Semi-quantitative PCR using primers spanning the junction of Wbeta integration within the host *B. cereus* genome confirmed that the presence of the 6 aa peptide induces lysogeny as early as 30 minutes following initial infection (Fig. 5F).

We next tested a more distantly related arbitrium-like system from clade 9. This system was found on a prophage that resides within *B. thuringiensis* sv. kurstaki HD-1. This prophage is almost identical to a *Myoviridae* Bacillus phage called Waukesha92 (Sauder et al., 2014) (Fig. 6A). We induced the Waukesha92-like prophage from its original host and found that it can also infect *B. thuringiensis* sv. galleriae BGSC:4G5 (Methods). However, in contrast to the cases of Wbeta, phi3T and SPbeta, a peptide composed of the last 6 aa of the Waukesha92-like AimP protein did not affect its lytic behavior (Fig. 6B).

A closer examination of the AimP of this Waukesha92-like phage revealed a sequence that resembles the consensus cleavage site for peptide maturation encoded immediately before the phi3T and SPbeta mature peptides (Fig. 6A; Fig. S2). This motif is thought to be identified by *Bacillus* extracellular proteases that cleave immediately after it and release the mature short peptide (Erez et al., 2017; Lazazzera, 2003). Interestingly, the position of this predicted motif within the phage AimP suggested that the mature arbitrium peptide in this phage is 10 aa long with the sequence MMSEPGGGGW. Indeed, addition of 1  $\mu$ M of this 10 aa peptide to the growth medium resulted in infection dynamics suggestive of increased lysogeny (Fig. 6C). Shorter versions of the peptide (MSEPGGGGW, 9 aa long; SEPGGGGW, 8 aa; and EPGGGGGW, 7 aa) also induced lysogeny, albeit to a lesser extent (Fig. 6D-5F), possibly due to lower affinity to the cognate AimR. These results show that some arbitrium systems utilize peptides longer than 6 aa, demonstrating diverse peptide codes for these phage communication systems.

Based on these results we predicted the consensus sequences of the mature arbitrium peptides for clades 4-8 (Fig. 3). The arbitrium consensus signatures in these clades resemble the mature arbitrium that we verified in the Waukesha92-like phage that belongs to clade 9. These peptides are characterized by an invariant acidic residue (aspartate or glutamate) followed by proline and glycine. In the Waukesha92-like arbitrium, these conserved residues are found at positions 4-6 of the mature peptide, and we hence predicted the mature peptides of the other clades accordingly. However, we note that these predictions are yet to be verified experimentally, and that the actual mature peptides may

be shorter than what we predicted. In addition, as opposed to most clades in which the peptide size seems to be conserved across the clade, in clade 9 we observed fluctuations in the sizes of the mature peptides, possibly reflecting relaxation of the structural constraints imposed by AimR on the evolution of peptide sizes (Fig. S3).

## The AimX effector transcript

It was previously demonstrated that in phage phi3T, the non-coding RNA AimX functions as a negative regulator of lysogeny (Erez et al., 2017). However, the molecular mechanism by which AimX executes the downstream lysogeny decision is unknown. To search for a cognate *aimX* in phage Wbeta we performed whole transcriptome RNA sequencing (RNA-seq) on total RNA extracted from cells infected by Wbeta in the presence or absence of externally added arbitrium peptide. As in the case of phi3T, the most notable change in expression was that of a transcript encoded immediately downstream to the *aimR-aimP* locus. This transcript showed substantial expression in the absence of arbitrium peptide, but its expression was completely silenced when the medium was supplemented with 1  $\mu$ M of the EIKPGG peptide (Fig. 7). This transcript therefore represents the AimX of Wbeta.

Similar to the case of phi3T, the Wbeta AimX transcript contains a short (40 aa) open reading frame (ORF), followed by a long stretch of non-coding RNA sequence (Fig. 7A). Interestingly, the Wbeta *aimX* non-coding transcript overlaps the phage *cI* repressor gene, which is encoded in the same locus on the opposite DNA strand (Fig. 7A). In the lytic condition (no peptide) the expression of AimX was 15-20 fold higher than that of the phage *cI* repressor, while in the lysogenic condition (with peptide) the expression of the phage repressor dominated (Fig. 7B). The same expression patterns were observed for the Waukesha92-like phage (Fig. S4).

When examining the genomic vicinity of the arbitrium systems we identified in phages and other mobile elements, we found that 80% of these systems were encoded directly upstream to a *cI* repressor-like gene encoded on the opposite strand, similar to the genomic organization of the Wbeta and Waukesha92-like phages (Table S1; also see Fig. 4). This suggests that most of the arbitrium systems execute the lysogeny decision through AimX-mediated regulation of the *cI* repressor, which is the master regulator of lysogeny (Fig. 7C-D; see Discussion).

#### Discussion

In this study we have shown that arbitrium phage communication systems are highly diverse and are present in numerous phages and prophages. We found arbitrium and arbitrium-like systems within phages infecting many bacterial species, most of which are pathogenic and soil Bacilli. While it is possible that communication-based lysis-lysogeny decisions are broadly utilized by phages in nature, curiously homologs of AimR were found only within the phylogenetic premises of the *Bacillales* order. Quorum sensing in this group of bacteria is based on small peptides (Lazazzera, 2003), and the phages seem to have taken advantage of specific characteristics of this quorum sensing, including utilization of the OPP channel and extracellular proteases, for implementing their own

communication system. It is possible that the arbitrium system is limited to phages infecting *Bacillales* because of its dependence on these characteristics. Alternatively, perhaps arbitrium systems are present in phages infecting other bacteria, but these systems have evolved beyond the detection capabilities of homology-based searches due to the rapid evolution of phages (Duffy and Turner, 2008).

In 16% of the cases, arbitrium systems were found to be encoded by conjugative elements. Such elements are known to utilize quorum sensing molecules, including Nacylhomoserine lactones (Fuqua and Winans, 1994; Ramsay et al., 2009) and short peptides (Auchtung et al., 2005; Dunny and Berntsson, 2016; Singh et al., 2013), to control their transfer between bacteria. For example, in the peptide-based communication systems encoded by the *B. subtilis* integrative-conjugative element ICEBs1 (Auchtung et al., 2005) and conjugative plasmid pLS20 (Singh et al., 2013), conjugation is repressed when the peptide accumulates. The peptide is used as a signal that the majority of the population already contains the conjugative element, such that transfer will be repressed when the conjugative element is abundantly present in the nearby bacteria (Auchtung et al., 2005; Singh and Meijer, 2014; Singh et al., 2013). This logic is somewhat similar to that of the arbitrium system, in which lytic propagation of the phage is inhibited upon accumulation of the peptide. We hypothesize that the arbitrium systems that we found on conjugative elements function to serve a similar purpose, especially as these systems are found in the genomic vicinity of genes of the conjugation machinery as well as genes resembling the cI repressor (Table S1; Fig. S1).

Notably, arbitrium systems are especially abundant in phages and mobile elements infecting pathogens such as B. thuringiensis and B. anthracis. B. thuringiensis is a common pathogen of insects, and produces a family of insecticidal proteins called Bt toxins (Schnepf et al., 1998). These toxins play an important biotechnological role, with key crops genetically engineered to express such toxins for protection against insect pests (Romeis et al., 2006). We found several mobile elements in *B. thuringiensis* that carry Bt toxins as well as an arbitrium system (Fig. 2A; Fig S1). It is therefore likely that arbitrium communication is important for controlling horizontal transfer of these plasmids among bacteria. In *B. anthracis*, one of the two key virulence plasmids, called pXO2, encodes an arbitrium system with the predicted peptide KMVGPGGGMG. This plasmid carries genes responsible for synthesis of the antiphagocytic poly-D-glutamic acid capsule that is essential for B. anthracis virulence (Dixon et al., 1999) (Fig. 2B). In the reference strain B. anthracis Ames Ancestor (Ravel et al., 2009), AimR contains a frameshift mutation that likely inactivates it, yet in other *B. anthracis* strains the arbitrium system in pXO2 is intact (Fig. 2B). If indeed the arbitrium system regulates transfer of these virulence plasmids among *B. anthracis* strains, then the arbitrium peptide could be potentially used for limiting such transfer in clinical or field settings.

About 5% of the AimR homologs we detected in this study showed only distant homology to AimR, and localized within the grey-colored clade in the AimR phylogenetic tree (Fig. 3). These homologs are found in diverse bacterial genera, including *Virgibacillus*, *Halobacillus* and *Gracilibacillus*, and most of them are not found within mobile elements (Fig. 3; Table S1). We consider these homologs as possibly encoding bacterial peptide-

based quorum sensing systems that share a distant common ancestry with phage arbitrium systems.

Our transcriptional RNA-seq data (Fig. 7A-B; Fig. S4) may suggest that AimX in the Wbeta and Waukesha92-like phages is a *cis*-antisense RNA transcript that overlaps the transcript of the *cI* repressor. Such *cis*-antisense transcripts are known to silence the genes that they overlap, either by suppressing translation or by stimulating RNA degradation (Sesto et al., 2013). In the lytic condition, transcription of AimX dominates that of the phage *cI* repressor (Fig. 7B), and we hypothesize that through *cis*-antisense regulation, AimX silences the expression of the *cI* repressor. This silencing is, in turn, alleviated in the presence of the arbitrium peptide, which leads to suppression of AimX expression.

These results suggest a more complete model for the arbitrium-mediated lysogeny decision (Fig. 7C-7D). At the first encounter of the phage with a bacterial population, *aimR* and *aimP* are expressed immediately upon infection. AimR functions as a transcriptional regulator and activates the transcription of *aimX*, which blocks lysogeny by directly inhibiting the phage *cI* repressor using a *cis*-antisense mechanism. AimP is released into the medium and processed into the mature arbitrium peptide, which is internalized into neighboring cells via the OPP channel. At later stages, phages will infect cells in which the intracellular concentration of the mature arbitrium peptide is high, and in these cases their AimR binds the arbitrium peptide, thus preventing the expression of *aimX*. Subsequently, the phage repressor will be expressed and will bind the promoters of lytic genes, suppressing their expression and establishing stable lysogeny.

Antisense-based silencing is also known to play an important role in the lysogeny decision of phage Lambda, where, for example, the stability of the *cII* transcript is regulated by a short antisense RNA (OOP) that promotes RNaseIII-mediated degradation (Krinke and Wulff, 1990; Oppenheim et al., 2005; Spiegelman et al., 1972), highlighting a common role for antisense regulation in executing phage lysogeny decisions. Our data imply that most of the arbitrium-carrying phages execute their lysogeny decisions using the *aimX-cI* direct *cis*-antisense regulation due to the similar organization of the lysogeny module in their genomes (*aimR-aimP-aimX-cI*). However, in clade 2 arbitrium systems found in SPbeta-like phages, the *cI* repressor is found elsewhere in the genome, distant from the arbitrium locus. We hypothesize that in these cases, the AimX non-coding RNA regulates the *cI* repressor in *trans*, possibly through a base-pairing mechanism typical to non-coding RNA regulation in bacteria (Waters and Storz, 2009).

Curiously, the AimX transcript also contains, in most cases, a conserved short reading frame (Fig. 7A; Fig. S4). Such a small protein-coding gene is also present on the phi3T AimX transcript, although it is lacking from the SPbeta AimX (Erez et al., 2017). While the role of these small genes is currently unknown, we hypothesize that they may serve as anti-repressors, which are frequently used by diverse phages to sequester the CI repressor protein by binding it and causing it to dissociate from the DNA (Lemire et al., 2011; Susskind and Botstein, 1975). If this hypothesis is correct, then *aimX* encodes two separate functions that together enforce tight regulation of the *cI* repressor, both on the RNA level and on the protein level.

The arbitrium system was initially shown to regulate the lysis-lysogeny decision of phages from the SPbeta group infecting *B. subtilis* (Erez et al., 2017). In the current study we show that such communication systems are highly abundant among phages that infect soil and pathogenic *Bacilli*, utilizing a diverse space of peptide communication codes. Recently, host-derived quorum sensing molecules were shown to regulate the lysogeny decisions of phages infecting *Vibrio cholera* (Silpe and Bassler, 2019), highlighting quorum sensing as an important aspect in phage biology that has so far been overlooked. We anticipate that future studies may expose more roles for small-molecule communication in coordinating group behavior of phages.

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## **Author Contributions**

A.S.A. performed the computational analyses. N.T. performed the experimental assays and their analysis. Z.E. and A.L. assisted in designing experiments and reviewed the manuscript. R.S. supervised the project.

# **Declaration of Interests**

The authors declare no competing interests.

#### **Main Figures**

**Figure 1. Arbitrium systems are found in diverse mobile elements of Bacilli.** (A) Distribution of arbitrium systems in different phages and conjugative elements. Phage genome sequences were clustered to form distinct groups (Methods). A representative phage name is designated for the group in cases where the group contained a previously described phage or phages with strong homology to a known phage (Table S1). PG designates Phage Groups with no strong homology to a known phage. Median genome size for each phage type is in parenthesis. Cases where the AimR is found near genes required for conjugation were designated as conjugative elements. (B) Distribution of arbitrium-carrying mobile elements in host bacteria. Presented are species for which at least two elements with arbitrium systems have been detected.

**Figure 2.** Arbitrium systems on *Bacillus* virulence and insecticidal plasmids. (A-B) Schematic representation of the virulence plasmids pMC95 of *B. thuringiensis* and pXO2 of *B. anthracis*, respectively. The *aimR* gene is colored dark orange; *aimP* is light orange with the putative mature peptide in parenthesis; predicted *cl* repressor dark blue, insectidal Crystal (Cry) genes in burgundy, capsule synthesis genes in purple and conjugation genes in light blue. The bacterial strain and the GenBank accession of the plasmids are specified on the upper left corner. See also Figure S1.

**Figure 3. Phylogenetic tree of 1,180 AimR protein homologs.** Tree visualization was performed using iTOL (Letunic and Bork, 2016). The context of the arbitrium system within the bacterial genome is designated by colored lines in the tree circumference. Bootstrap support values, obtained from 1000 iterations using IQ-TREE (Nguyen et al., 2015), are presented for major branches. Identical or near-identical sequences were collapsed into a single node. The tree is artificially rooted between the grey-colored clade and clades 4-9 for illustrative purposes. Sequence profile of the C-terminus of the respective AimP, representing the predicted mature arbitrium peptide, is presented for each clade. See also Figure S3.

**Figure 4. Arbitrium systems are frequently found in the lysogeny module of diverse phages.** Schematic representation of the integration site of several arbitrium-containing prophages. The *aimR* gene is colored dark orange; *aimP* is light orange with the putative mature peptide in parenthesis; phage integrase light blue, and predicted phage *cl* repressor dark blue. The rest of the phage genes are colored light yellow. Locus tag accessions for the presented *aimR* genes are: BANAU\_2649, HD73\_0378, GBAA0429 and bcere0015\_17290 from top to bottom, respectively. The putative prophage integration site is marked by a dashed line and the prophage length is designated below, with the AimR clade number indicated in parenthesis. The bacterial host strain in which the prophage resides and the scaffold accession are specified to the right of each prophage.

**Figure 5. Arbitrium controls phage Wbeta lysogeny in** *B. cereus.* (**A**) A schematic representation of phage Wbeta genome. Different colors represent functional modules, with the *aimR* homolog colored dark orange, *aimP* light orange, *aimX* green, and the *cl* repressor dark blue. Sequence of the AimP pre-propeptide is indicated, with the signal peptide position predicted using TMHMM (Krogh et al., 2001). (**B-E**) Growth curves of *Bacillus cereus* RSVF1 infected by Wbeta with multiplicity of infection (MOI) of 10. Phages were added at t=0. The sequence of the synthetic peptide supplemented to the medium (1 µM) is indicated above each graph. Data represent an average of three replicates using separate colonies, each with two technical replicates. Error bars are standard deviation of the means. All experiments were performed simultaneously with a single "no peptide, uninfected" and "no peptide, infected" controls. (**F**) Semi-quantitative PCR assay for phage lysogeny during an infection time course of *B. cereus* RSVF1 with phage Wbeta with or without external addition of the synthetic peptide EIKPGG. No DNA, control without

DNA; WT, DNA from uninfected culture; Lysogen, genomic DNA of a Wbeta lysogen; M, DNA ladder with sizes indicated on the right.

**Figure 6. Arbitrium controls lysogeny in a Waukesha92-like phage**. (**A**) A schematic representation of the Waukesha92-like prophage genome. This prophage is found as a circular episome (and is therefore annotated as a plasmid) in the sequenced genome of *B. thuringiensis* sv. kurstaki HD-1. Different colors represent functional modules, with the *aimR* homolog colored dark orange, *aimP* light orange, *aimX* green, and the *cl* repressor dark blue. Sequence of the AimP pre-pro-peptide is indicated, with the signal peptide position predicted using TMHMM (Krogh et al., 2001). (**B-F**) Growth curves of *B. thuringiensis* sv. galleriae BGSC:4G5 infected by the Waukesha92-like phage with MOI of 0.1. Phages were added at t=0. The sequence of the synthetic peptide supplemented to the medium (1  $\mu$ M) is indicated above each graph. Data represent an average of three replicates using separate colonies, each with two technical replicates. Error bars are standard deviation of the means. All experiments were performed simultaneously with a single "no peptide, uninfected" and "no peptide, infected" controls. See also Figure S2.

**Figure 7. The AimX effector transcript overlaps the phage** *cl* **repressor**. (**A**) RNA-seq coverage of the arbitrium locus in phage Wbeta, 20 minutes post infection of *Bacillus cereus* RSVF1 at MOI=10. Top, no peptide supplemented; bottom, with 1  $\mu$ M of synthesized EIKPGG peptide added to the growth medium. (**B**) Ratio between the expression of *aimX* and the *cl* repressor, calculated as the ratio between the RPKM values of the sense and antisense strands. Data for two replicates are shown. (**C-D**) Mechanistic model for the arbitrium-mediated lysis-lysogeny decision via peptide communication controlling *cis*-antisense regulation of the phage *cl* repressor. See also Figure S4

#### **STAR Methods**

#### **Contact for Reagent and Resource Sharing**

Further information and requests for resources and reagents should be directed to and will be fulfilled by the Lead Contact, Rotem Sorek (rotem.sorek@weizmann.ac.il).

#### **Experimental Model and Subject Details**

The strains and phages used in this study are listed in the Key Resources Table. The bacterial strains and media used in this study are: *Bacillus cereus* W ATCC11950, cultivated in brain heart infusion (BHI, BD cat. #237500) at 30°C; *Bacillus cereus* RSVF1 ATCC4342, cultivated in BHI or nutrient broth at 30°C or 37°C (as stated below); *Bacillus thuringiensis* sv. kurstaki HD-1 BGSC:4D1, cultivated in LB at 30°C; and *Bacillus thuringiensis* sv. galleriae BGSC:4G5 (cured of a prophage-like plasmid, see below), cultivated in MMB (LB media supplemented with 0.1 mM MnCl2 and 5 mM MgCl2) at 30°C. The phages used in this study are phage Wbeta and the Waukesha92-like phage.

#### **Method Details**

#### Identification of AimR homologs

AimR homologs were identified using PSI-BLAST version 2.5.0 of BLAST+ (Altschul, 1997; Camacho et al., 2009) with the phi3T phage AimR protein ("GenBank: APD21232.1") as the query sequence. The search was performed in four iterations with an e-value cutoff of 1e-10 against a database of proteins from bacterial, archaeal and viral genomes downloaded from the NCBI FTP site on April 2016 (bacteria and archaea) and May 2018 (viruses) (<u>ftp://ftp.ncbi.nih.gov/genomes/genbank/bacteria/</u>,

ftp://ftp.ncbi.nih.gov/genomes/genbank/archaea/

and

ftp://ftp.ncbi.nih.gov/genomes/genbank/viral/, respectively). The resulting 1327 hits were cross-referenced against the Integrated Microbial Genomes (IMG) database (Chen et al., 2017) using BLASTN to identify genes with 100% identity and 100% query coverage in the same strain. This resulted in 1257 hits for which a corresponding gene was identified in the IMG database. Proteins shorter than 300 amino acids were removed from the dataset, as possibly encoding truncated forms of the AimR protein. Finally, 12 proteins found in viral genomes that were not in IMG were added to our dataset, overall resulting in a final list of 1180 genes that were used for further analyses.

#### Clustering of AimR homologs

Multiple sequence alignment (MSA) of the amino acid sequences of the 1180 AimR homologs was performed using the MAFFT online server (Katoh, 2002; Katoh et al., 2017) version 7 with default parameters. The resulting MSA was used to create a maximum likelihood phylogenetic tree using IQ-TREE (Nguyen et al., 2015) version 1.6.5 including ultrafast bootstrap analysis (Hoang et al., 2018) with 1000 alignments ("-bb 1000") and using the "-m TEST" parameter to test for the best substitution model (Kalyaanamoorthy

et al., 2017). The tree was visualized using the iTOL program (Letunic and Bork, 2016) and rooted between clades 4-9 and the grey clade for display purposes. Clades with an average branch length distance to leaves below  $10^{-6}$  were collapsed.

#### Identification of aimP genes

To identify the putative *aimP* genes associated with each of the *aimR* homologs, we searched for an ORF harboring a putative signal peptide sequence immediately downstream to the *aimR* gene. To this end, the 100 terminal bases of each *aimR* homolog and 500 additional bases downstream to it were extracted. Then, ORFs of 15 or more amino acids were searched within the sequence, including ORFs that begin with alternative start codons (GTG or TTG). The identified ORFs were submitted to SignalP 4.1 (Petersen et al., 2011) using the "gram-positive bacteria organism group" option and a minimal D-cutoff value of 0.3, to identify possible N-terminal signal peptide sequences. If several ORFs with a signal peptide were identified, the one with the highest D-cutoff value was chosen. The C-terminal end of the putative AimP sequences were collected and unique sequences were used to generate a sequence logo using the Weblogo 2.8.2 web server (Crooks, 2004). For 13 of the AimP sequences, which belong to clade 1, an internal peptide (rather than a C-terminal one) was predicted based on homology to the C-terminal end of the rest of the AimP sequences of the clade.

#### The genomic context of arbitrium systems

To check whether the arbitrium systems are located within a prophage, the PHASTER URL API (Arndt et al., 2016) was used. The entire DNA sequence of the contig in which the *aimR* homolog was located was submitted as a query to identify prophage regions, and cases where the *aimR* was included in such a region were recorded. In addition, the genomic neighborhood for each *aimR* homolog was examined using the gene neighborhoods function in IMG (Chen et al., 2017), and the *aimR* was considered to be found in a prophage if it was located next to genes annotated as phage genes. In cases where we were unable to detect phage structural genes and the gene was found near genes annotated as encoding conjugation proteins, the gene was considered to be found in a conjugative element. Genes with the following annotations in IMG were considered as conjugation proteins: Type IV secretory pathway proteins (VirD4, VirB2, VirB4 and VirB6), conjugative transposon proteins (TcpC and TcpE), pilus assembly proteins (CpaB and CpaF), TraG/TraD conjugation proteins or TraM recognition site of TraD and TraG.

To calculate the gene distance of each *aimR* homolog to a phage integrase gene, five neighboring genes from each side of the *aimR* homolog were inspected. An integrase gene was identified as a gene annotated in IMG as an Integrase or Recombinase in one of the following fields: Gene Product Name, COG, Pfam, KEGG Orthology ID, Name or Definition. Putative prophage edges (marking its integration site within the bacterial genome) were identified by genomic comparison to a closely related bacterial strain that lacks the integrated prophage. As the integrase gene is typically present at the edge of the integrated prophage, if a phage integrase was located at the edge of the prophage it was considered the *bona fide* integrase, and others were disregarded.

To identify putative *cI* repressor genes associated with the arbitrium system, three neighboring genes downstream to the *aimR* homolog were inspected. A repressor was identified as a protein of up to 250 aa on the opposite strand of the *aimR* gene that was annotated as a transcriptional regulator in IMG. This includes annotations of helix-turnhelix (HTH), transcriptional regulator, or DNA-binding in one of the following fields: Gene Product Name, COG, Pfam, IMG Term or the gene name of the top hit obtained using a BLASTP search against the nr DB.

#### Phage type determination

Prophage DNA sequences were extracted from the bacterial genomes and sequences longer than 10 kb were taken for further analysis. The extracted prophage sequences, as well as phage sequences that harbor arbitrium, were compared using an "all vs. all" tBLASTX with an e-value of 0.001 and default parameters. Hits with less than 40% query coverage were removed. The query coverage of the top hit for each phage pair was used as a similarity score between the pair, using the best value found between each pair. The phage sequences were then clustered, based on these similarity scores, into groups using the MCL algorithm (van Dongen, 2000; van Dongen and Abreu-Goodger, 2012) version 14-137 with an inflation value of 1.5.

Homology between arbitrium-containing prophages to known phages was searched using BLASTN against a database of viral genomes downloaded from NCBI on May 2018 with an e-value cutoff of 0.05. Hits with over 85% identity and at least 40% query coverage were taken into account for group identity determination. In addition, the "Most Common Phage Name" field in the PHASTER output for each prophage was used if the number of genes (hits) in that field was 18 or more. MCL-derived phage groups for which the majority of the members showed homology to a known group of phages were designated accordingly. Phage groups for which a strong enough hit to a known phage was not found were designated as PG (for Phage Group) with a serial number (Fig. 1A).

# Prophage induction and propagation

*Bacillus cereus* W ATCC11950, containing the Wbeta prophage, was obtained from ATCC. Phage Wbeta was induced by plating the lysogenic strain on a BHI agar plate containing 20 µg/ml phosphomycin, as described previously (Schuch and Fischetti, 2006). After overnight incubation at 30°C, ring-shaped colonies formed. The clearing zones were picked and streaked on a BHI soft agar (0.3%) plate containing *Bacillus cereus* RSVF1 ATCC4342 obtained from ATCC. Plates were incubated overnight at 30°C. Lysis zones were picked into phage buffer (50 mM Tris pH 7.4, 100 mM MgCl2, 10 mM NaCl). Further infection cycles were carried out using a liquid culture grown in nutrient broth (meat extract 1 g/L, peptone 5 g/L, sodium chloride 5 g/L, yeast extract 2 g/L) as described previously (Schuch and Fischetti, 2006) until sufficient titer was achieved.

The Waukesha92-like phage was induced from a liquid culture of *Bacillus thuringiensis* sv. kurstaki HD-1 BGSC:4D1 obtained from the Bacillus Genetic Stock Center (BGSC). The bacteria were streaked on an LB agar plate and a single colony was picked and grown

in liquid LB medium at 30°C. When the bacterial culture reached an OD<sub>600</sub> of 0.3, mitomycin C was added to a final concentration of 0.5 µg/ml. After overnight incubation, the culture was centrifuged for 10 minutes at 3200 g. Supernatant was collected and filtered through 0.2 µm filters (GE Healthcare Life Sciences, Whatman, cat. #10462200). Drop assay was used to test for plaque formation on 0.3% agar plates with MMB containing B. thuringiensis sv. galleriae BGSC:4G5 (obtained from BGSC) cured of a prophage-like plasmid. For curing, a number of *B. thuringiensis* sv. galleriae BGSC:4G5 colonies were picked, and whole genome sequencing was performed to confirm the strain identity and the absence of the prophage-like plasmid ("GenBank: CP010095.1"). A single plaque was picked from the drop assay into 50 µl phage buffer, and passaged two additional times through a single plaque to isolate the phage. Identity of phage was verified using PCR (primers are listed in the Key Resources Table). Phages were then propagated as follows: An overnight starter of the bacteria grown in MMB medium was diluted 1:100 into 2 ml of fresh MMB medium and grown at  $30^{\circ}$ C with shaking until reaching an OD<sub>600</sub> of 0.3. The low-titer phage stock was added to the liquid culture and the culture was incubated for 5 hours with shaking at 30°C. The culture was centrifuged for 10 minutes at 3200 g and the supernatant was collected and then filtered with a 0.2 µm filter. This procedure was repeated until sufficient titer of the phage was obtained. Phage working stocks were stored at 4°C.

#### Synthetic peptides

Lyophilized peptides were ordered from Genscript Corp, with purity ranging from 89.9% to 99.1%. The peptides of phage Wbeta were suspended in 100% DMSO and then gradually diluted with ultrapure water (UPW) to reach 2% DMSO and a peptide concentration of 10 mM. The peptides of the Waukesha92-like phage were suspended in UPW to a peptide concentration of 10 mM. Peptides were further diluted in UPW to obtain working stocks of 100  $\mu$ M.

#### Phage infection dynamics

An overnight starter of the bacteria was diluted 1:100 into 5 ml of medium (MMB medium for *B. thuringiensis* sv. galleriae BGSC:4G5 when infected by the Waukesha92-like phage, and nutrient broth for *B. cereus* RSVF1 ATCC4342 when infected by Wbeta) and incubated at 30°C or 37°C (for *B. thuringiensis* sv. galleriae and *B. cereus* RSVF1, respectively) until reaching an OD<sub>600</sub> of 0.1. The liquid culture was centrifuged for 10 minutes at 3200 g at room temperature, the supernatant was discarded, and the pellet was re-suspended in 0.5 ml of fresh medium. The concentrated bacterial culture was further resuspended in fresh medium at a ratio of 1:9. For the experiments that contained peptides, the synthetic peptide was added at a ratio of 1:100 (final volume) to a final concentration of 1  $\mu$ M. Bacteria were incubated in the presence of the peptide for 1 hour at room temperature prior to infection.

Infection was performed for phage Wbeta at MOI=10 and for the Waukesha92-like phage at MOI=0.1, added in a volume that constituted 10% of the final volume. The OD<sub>600</sub> was measured using a TECAN Infinite 200 plate reader in a 96-well plate at 30°C or 37°C for

10 hours. Throughout the infection experiments, *B. cereus* RSVF1 ATCC4342 was grown in nutrient broth at 37°C and *B. thuringiensis* sv. galleriae BGSC:4G5 was grown in MMB medium at 30°C.

#### Lysogeny assay

For the experiment described in Figure 5F, an overnight culture of *B. cereus* RSVF1 ATCC4342 was diluted 1:100 into fresh nutrient broth and grown with shaking at 37°C until reaching OD<sub>600</sub> of 0.1. The bacterial culture was centrifuged at 3200 g for 10 minutes and the pellet was re-suspended in an equivalent amount of fresh nutrient broth. The culture was subsequently divided into two flasks, one of which was supplemented with the synthetic peptide to reach a final concentration of 1  $\mu$ M. Both flasks were incubated for 1 hour at room temperature with gentle shaking. Following incubation, both liquid cultures were infected with Wbeta at MOI=10. 2 ml was collected from each sample at different time points: 0, 15, 30, 40, and 60 minutes. Each sample was centrifuged immediately for 5 minutes at 3200 g and at 4°C. The pellet was frozen using dry ice and ethanol.

DNA extraction was done using the Qiagen DNeasy blood and tissue kit (cat. #69504). The extracted DNA of each sample was diluted to a final concentration of 8 ng/ $\mu$ l. Semiquantitative PCR was performed using three pairs of primers (Key Resources Table), targeted to amplify the bacterial DNA, the viral DNA and the junction between the integrated phage genome and the bacterial DNA (primers listed below). PCR mix was made using KAPA HIFI HotStart ReadyMix. Annealing temperature was set to 60°C, elongation to 30 seconds and 26 cycles of amplification were performed.

#### <u>RNA-seq</u>

Whole transcriptome RNA sequencing was performed for *B. cereus* RSVF1 ATCC4342 (nutrient broth, 37°C, infected with the Wbeta phage at MOI=10) and *B. thuringiensis* sv. galleriae 4G5 (MMB medium, 30°C, infected with the Waukesha92-like phage at MOI=1). An overnight culture was diluted 1:100 into 40 ml of fresh medium (either nutrient medium or MMB, depending on the bacterium) and grown with shaking until reaching an OD<sub>600</sub> of 0.1. The bacterial culture was centrifuged at 3200 g for 10 minutes and the pellet was resuspended in 40 ml of fresh medium. The culture was then divided into two flasks, one of which was supplemented with the synthetic peptide to reach a final concentration of 1  $\mu$ M. Both flasks were incubated with gentle shaking for 1 hour at room temperature. Then, both liquid cultures were infected with the relevant phage. 5 ml were collected from each sample (at 20 minutes post infection for the Wbeta phage; and at 120 minutes post infection for the Waukesha92-like phage). Each sample was centrifuged immediately for 5 minutes at 3200 g and at 4°C. The pellet was flash frozen using dry ice and ethanol.

RNA extraction was performed as described previously (Dar et al., 2016). Briefly, pellets were re-suspended in 1 ml of RNA protect solution (FastPrep) and lysed by Fastprep homogenizer (MP Biomedicals). RNA was extracted using the FastRNA PRO blue kit (MP

Biomedicals, 116025050) according to the manufacturer's instructions. DNase treatment was performed using the Turbo DNA free kit (Life Technologies, AM2238). RNA was subsequently fragmented using fragmentation buffer (Ambion-Invitrogen, cat. #10136824) at 72°C for 1 min 45 sec. The reactions were cleaned by adding  $\times 2.5$  SPRI beads (Agencourt AMPure XP, Beckman-Coulter, A63881). The beads were washed twice with 80% ethanol and air dried for 5 minutes. The RNA was eluted using H<sub>2</sub>O. Ribosomal RNA was depleted by using the Ribo-Zero rRNA Removal Kit (epicentre, MRZB12424). Strand-specific RNA-seq was performed using the NEBNext Ultra Directional RNA Library Prep Kit (NEB, E7420) with the following adjustments: all cleanup stages were performed using  $\times 1.8$  SPRI beads, and only one cleanup step was performed after the end repair step. Following sequencing on an Illumina NextSeq500, sequenced reads were demultiplexed and adapters were trimmed using 'fastx clipper' with default parameters. Reads were mapped to the phage genome (Gene annotations and sequences were downloaded from "GenBank: ABCZ0200003.1" for the Wbeta prophage within the genome of *B. cereus* W, and "GenBank: CP004871.1" for the Waukesha92-like prophage within the genome of *B. thuringiensis* sv. kurstaki HD-1 BGSC:4D1) by using NovoAlign (Novocraft) v3.02.02 with default parameters, discarding reads that were nonuniquely mapped as previously described (Dar et al., 2016). Reads per gene were calculated for each condition and biological repeat, and normalized using RPKM values.

#### **Quantification and Statistical Analysis**

All of the statistical details of the experiments can be found in the figure legends. Average +/- standard deviation of the mean of biological triplicates is shown throughout unless stated otherwise. For the phylogenetic analysis presented in Figure 3, ultrafast bootstrap support values (Hoang et al., 2018) were calculated based on 1000 iterations using IQ-TREE (Nguyen et al., 2015) and the substitution model was chosen using the "-m TEST" parameter (Kalyaanamoorthy et al., 2017).

#### **Data and Software Availability**

The RNA-sequencing raw data have been deposited in the European Nucleotide Archive (ENA) under the study accession number "ENA: PRJEB31642". Characteristics of arbitrium systems identified in this study are reported in Table S1.

#### Supplemental item titles and legends

**Table S1.** Characteristics of arbitrium systems identified in this study, related to Figure 1 and Figure 3.

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