Supplemental Information

The arbitrium system controls prophage induction

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Figure S1. Model for the mechanism of action of the arbitrium system in phages of the SPβ family, Related to Figure 3. (A) The arbitrium model prior to this study describes that after infection of a SPβ prophage, AimR is being expressed and binds to the operator site promoting expression of the aimX sRNA and promoting the lytic cycle. After AimP accumulates above the threshold levels, it binds to AimR disrupting its binding to the DNA and reducing expression of aimX, leading to lysogeny. (B) Our understanding is that the arbitrium system of SPβ is involved in a more complex mechanism to control prophage induction. The regulator AimR functions to inhibit the phage repressor, YopR, thus promoting prophage induction. Another component of the system is YopN that we hypothesise to promote YopR activity, acting as a negative regulator of prophage induction. We propose that following activation of the SOS response, AimR activates an unknown component that blocks YopN function, thus reducing the activity of YopR and promoting induction.
Figure S2. Analysis of the aimR and aimP deletions in SPβ-type prophages, Related to Figure 5. (A) Lysogenic strains for phage SPβ, SPβ△aimR or SPβ△aimP were MC induced (0.5 μg/ml) and incubated at 30 °C with 80 rpm shaking for 4 h. The lysates were left overnight at room temperature before being photographed. (B) Complementation of the aimR mutant in SPβ. Strains lysogenic for phage SPβ wt, △aimR, △aimR amyE::Pspank and △aimR amyE::Pspank-AimR were MC induced (0.5 μg/ml) and the number of resulting phages were quantified by titering using 168Δ6 as the recipient strain. The results are represented as the plaque forming units (PFUs) mL⁻¹. The means and SDs are presented (n = 5). An ordinary one-way ANOVA of transformed data was performed to compare mean differences between titres. Adjusted p values were as follows: SPβ △aimR* p < 0.0001; △aimR amyE::Pspank *** p < 0.0001; △aimR amyE::Pspank-AimR ns = not significant. (C) Complementation of the aimR mutant in phi3T. Strains lysogenic for phages phi3T wt, △aimR, △aimR amyE::Pspank and △aimR amyE::Pspank-AimR were MC induced (0.5 μg/ml) and the number of resulting phages were quantified by titering using 168Δ6 as the recipient strain. The means and SDs are presented (n = 3). An ordinary one-way ANOVA of transformed data was performed to compare mean differences between titres. Adjusted p values were as follows: SPβ △aimR* p = 0.0205; △aimR amyE::Pspank * p = 0.0049; △aimR amyE::Pspank-AimR * p = 0.0391. (D) Strains lysogenic for phages phi3T wt, △aimR, △aimR amyE::Pspank and △aimR amyE::Pspank-AimR were MC induced (0.5 μg/ml) and the number of resulting phages were quantified by titering using 168Δ6 as the recipient strain. The results are represented as PFUs/mL⁻¹. The means and SDs are presented (n = 3). An ordinary one-way ANOVA of transformed data was performed to compare mean differences between titres. Adjusted p values were as follows: phi3T △aimR* p = 0.0220; △aimR amyE::Pspank * p = 0.0125; △aimR amyE::Pspank-AimR *** p = 0.0005.
Figure S3. Complementation of the aimR mutants in recipient strain, Related to Figure 4 and Figure 5. (A) Strain lysogenic for phage SPβ ΔaimR was MC induced (0.5 μg/ml) and the number of resulting phages were quantified by titering using 168 Δ6 amyE::Pspank (-) or 168 Δ6 amyE::Pspank-AimRSPK (+) as recipient strains. The results are represented as the plaque forming units (PFUs) mL⁻¹. The means and SDs are presented (n = 4). An ordinary one-way ANOVA of transformed data was performed to compare mean differences between titres. Adjusted p values were as follows: column A vs column B ****p = 0.0001; column A vs column C ns = not significant; column A vs column D ****p = 0.0001. (B) Strain lysogenic for phage phi3T ΔaimR was MC induced (0.5 μg/ml) and the number of resulting phages were quantified by titering using 168 Δ6 amyE::Pspank (-) or 168 Δ6 ΔaimR amyE::Pspank-AimRSPK (+) as recipient strains. The results are represented as the plaque forming units (PFUs) mL⁻¹. The means and SDs are presented (n = 3). An ordinary one-way ANOVA of transformed data was performed to compare mean differences between titres. Adjusted p values were as follows: column A vs column B ****p = 0.0001; column A vs column C ns = not significant; column A vs column D ****p = 0.0001. (C) Plaques morphologies produced after titration of the SPβ ΔaimR using 168 Δ6 amyE::Pspank (-) or 168 Δ6 amyE::Pspank-AimRSPK (+) as recipient strains were photographed. (D) Overexpression of AimR does not induce the lytic cycle. Strains lysogenic for phage SPβ amyE::Pspank and SPβ amyE::Pspank-AimRSPK were analysed for their ability to produce phage particles under several conditions: without induction (No MC), with phage induction (+ MC 0.5 μg/ml) and with Pspank induction (+ IPTG 1 mM). The number of resulting phages were quantified by titering using 168 Δ6 as the recipient strain. The results are represented as the plaque forming units (PFUs) mL⁻¹. The means and SDs are presented (n = 3). An ordinary one-way ANOVA of transformed data was performed to compare mean differences between titres. Differences in titer with “No MC” and “+ IPTG” were not significant (ns). The adjusted p value comparing SPβ amyE::Pspank and amyE::Pspank-AimR + MC p = 0.0227.
SPβ ΔaimR lysate was acquired following MC induction of a lysogenic strain carrying the SPβ ΔaimR phage. The lysate was titered using 168 Δ6 as the recipient strain and the resulting cloudy plaques were collected and passaged, as described in the STAR Methods, until wt-appearing plaques were obtained. Created with BioRender.com
Figure S5. Plaque morphology of SPβ wt, ΔaimR, ΔyopN, ΔaimR-yopN and yopR::erm phages, Related to Figure 5 and Figure 6. Strains lysogenic for phage SPβ wt, ΔaimR, ΔyopN and ΔaimR-yopN were MC induced (0.5 μg/ml) and titered using 168 Δ6 as the recipient strain. A strain lysogenic for phage SPβ was transformed with an erythromycin cassette to replace the yopR gene. The resulting strain, supposedly yopR::erm, was MC induced (0.5 μg/ml) and titered using 168 Δ6 as the recipient strain. The resulting plaque morphologies were photographed.
Figure S6. Homology analysis of AimR<sub>SPβ</sub> and AimR<sub>KATMIRA1933</sub>, Related to STAR Methods. AimR sequences from SPβ and KATMIRA1933 were obtained from BLAST. The superposition analysis was made using the PRALINE program. Residues conservancy is depicted by blue to red colours.
Figure S7. Schematic representation of the SPβ-like phages arbitrium and operon genetic layout, Related to Figure 3. Diagram shows the genetic organisation of the arbitrium genes, aimR and aimP, followed by the operon directly downstream. Colours denote putative functions according to BLAST results; orange: arbitrium genes, grey: unknown function, navy blue: HTH_XRE domain, green: integrase domain, purple: ParB domain, light blue: putative repressor. Rotated black line indicates the end/beginning of the contigs containing the genes described for Katmira1933. Created with BioRender.com.
| Strain        | Gene | Mutation                                           |
|--------------|------|---------------------------------------------------|
| JP20762      | yopN | L90S                                             |
| JP20766      | yopN | L46P                                             |
|              | yopQ | T156T                                            |
| JP20769      | yopN | I51* Deletion produces frameshift and stop codon |
| Lytic phage 1| yopR | L140* Deletion produces frameshift and stop codon|
| Lytic phage 2| yopR | L49* Deletion produces frameshift and stop codon |

Table S1. Mutations identified in evolved SPβ ΔaimR phages, Related to Figure 3 and Figure 4.
| Phage/lysogen            | AimR   | AimP   | AimP sequence | Operon genes accession numbers |
|-------------------------|--------|--------|---------------|--------------------------------|
|                         |        |        |               | Gene 1 | Gene 2 | Gene 3 | Gene 4 | Gene 5 | Gene 6 |
| **SPβ**                 | GenBank: NP_389968 | GenBank: NP_389967 | GMPRGA | GenBank: NP_389966 | GenBank: NP_389965 | GenBank: NP_389964 | GenBank: NP_389963 | GenBank: NP_389962 | GenBank: NP_389961 |
| **phi3T**               | GenBank: APD21232 | GenBank: APD21233 | SAIRGA | GenBank: APD21235 | GenBank: APD21236 | GenBank: APD21237 | GenBank: APD21238 | GenBank: APD21239 | GenBank: APD21240 |
| **Bacillus amyloliquefaciens UCMB5033** | GenBank: CDG30054 | *NA | SPSRGA | GenBank: CDG30052 | GenBank: CDG30051 | GenBank: CDG30050 | GenBank: CDG30049 | GenBank: CDG30048 | GenBank: CDG30047 |
| **Bacillus velezensis strain SCGB1** | GenBank: ATC49385 | GenBank: ATC49384 | SIIRGA | GenBank: ATC49382 | GenBank: ATC49381 | GenBank: ATC49380 | GenBank: ATC49379 | GenBank: ATC49378 | GenBank: ATC49377 |
| **Bacillus amyloliquefaciens TA208** | GenBank: AEB23458 | GenBank: AEB23459 | GVVRGA | GenBank: AEB23460 | GenBank: AEB23461 | GenBank: AEB23462 | GenBank: AEB23463 | GenBank: AEB23464 | GenBank: AEB23465 |
| **Bacillus atrophaeus BA59** | GenBank: ATO28982 | GenBank: ATO28981 | GMPRGA | GenBank: ATO28980 | *NA | GenBank: ATO28979 | *NA | GenBank: ATO28978 | GenBank: ATO28977 |
| **Bacillus subtilis KATMIRA1933** | GenBank: WP_033885437 | GenBank: WP_134819006 | GIVRGA | GenBank: WP_033885435 | GenBank: WP_009967507 | GenBank: WP_019712296 | GenBank: WP_033885434 | GenBank: NP_389962.1 | GenBank: WP_003231032 |
| **Bacillus sonorensis L12** | GenBank: WP_051056713 | GenBank: WP_141231111 | GFPRGA | GenBank: WP_006640569 | GenBank: WP_006640568 | GenBank: WP_006640567 | GenBank: WP_006640566 | GenBank: WP_006640565 | GenBank: WP_006640565 |
| **Bacillus licheniformis strain SCDB34** | GenBank: ARC67883 | GenBank: ARC67884 | GFTVGA | GenBank: ARC67885 | GenBank: ARC67886 | GenBank: ARC67887 | GenBank: ARC67888 | GenBank: ARC67889 | GenBank: ARC67889 |

*NA: Not annotated

**Table S2. Genetic composition of the arbitrium-operon region in the different SPβ-like phage families, Related to Figure 3.**
| Strains         | Genotype or description                                                                 | Reference or source |
|-----------------|----------------------------------------------------------------------------------------|---------------------|
| Bacillus subtilis |                                                                                       |                     |
| 168 (1A700)     | trpC2                                                                                  | S1                  |
| Δ6 (1A1299)     | trpC2; ΔSPβ; subclacin 168-sensitive; Δskin; ΔPBSX; Δprophage 1; Δpks::Cm; Δprophage 3; Cm' | S2                  |
| IL26            | phi3T                                                                                  | S3                  |
| BKK20860        | trpC2 ΔaimR::kan                                                                        | S4                  |
| BKE20860        | trpC2 ΔaimR::erm                                                                        | S4                  |
| BKE20850        | trpC2 ΔaimP::erm                                                                        | S4                  |
| BKE20830        | trpC2 Δyop::erm                                                                         | S4                  |
| BKE20790        | trpC2 ΔyopR::erm                                                                        | S4                  |
| JP22770         | trpC2 SPβ ΔaimR                                                                         | This study          |
| JP22771         | trpC2 SPβ ΔaimP                                                                         | This study          |
| JP22776         | trpC2 SPβ ΔaimR; amyE::Pspank                                                         | This study          |
| JP22777         | trpC2 SPβ ΔaimR; amyE::PspankΔaimRGSpij                                               | This study          |
| JP19877         | Δ6 lysogenic SPβ                                                                         | This study          |
| JP19936         | Δ6 lysogenic SPβ ΔaimR                                                                   | This study          |
| JP20866         | Δ6 lysogenic SPβ yokl::kan                                                               | This study          |
| JP22949         | Δ6 lysogenic SPβ yokl::kan ΔaimR                                                        | This study          |
| JP21702         | Δ6 lysogenic SPβ yokl::kan ΔaimP                                                        | This study          |
| JP22950         | Δ6 lysogenic SPβ yokl::kan ΔaimR; amyE::Pspank                                         | This study          |
| JP22951         | Δ6 lysogenic SPβ yokl::kan ΔaimR; amyE::PspankΔaimRGSpij                               | This study          |
| JP21854         | Δ6 lysogenic phi3T                                                                      | This study          |
| JP21870         | Δ6 lysogenic phi3T phi3T_5::kan                                                          | This study          |
| JP22453         | Δ6 lysogenic phi3T phi3T_5::kan ΔaimR                                                   | This study          |
| JP22454         | Δ6 lysogenic phi3T phi3T_5::kan ΔaimP                                                   | This study          |
| JP22518         | Δ6 lysogenic phi3T phi3T_5::kan ΔaimR; amyE::Pspank                                    | This study          |
| JP22519         | Δ6 lysogenic phi3T phi3T_5::kan ΔaimR; amyE::PspankΔaimRGSpij                          | This study          |
| JP20762         | Δ6 lysogenic SPβ ΔaimR; yopN L90S                                                       | This study          |
| JP20766         | Δ6 lysogenic SPβ yokl::kan ΔaimR; yopN L46P; yopQ T156T                                 | This study          |
| JP20769         | Δ6 lysogenic SPβ yokl::kan ΔaimR; yopN A49*                                           | This study          |
| JP22952         | Δ6 lysogenic SPβ ΔyopN                                                                  | This study          |
| JP22953         | Δ6 lysogenic SPβ ΔaimR ΔyopN                                                            | This study          |
| JP21752         | Δ6 lysogenic SPβ yopR::erm                                                               | This study          |
| JP22339         | Δ6 lysogenic SPβ yopR::erm; amyE::PspankΔyopRGSpij                                     | This study          |
| JP19679         | Δ6 amyE::Pspank                                                                         | This study          |
| JP19944         | Δ6 amyE::PspankΔaimRGSpij                                                               | This study          |
| JP22515         | Δ6 amyE::PspankΔaimRGSpij                                                               | This study          |
| JP21941         | Δ6 amyE::PspankΔyopRGSpij                                                               | This study          |
| JP19883         | Δ6 lysogenic SPβ; amyE::Pspank                                                          | This study          |

Table S3. Bacterial strains, Related to STAR Methods.
| Plasmid     | Description                                                                 | Reference or source |
|-------------|------------------------------------------------------------------------------|---------------------|
| pDR244      | *B. subtilis* thermosensitive vector containing Cre recombinase that allows excision of DNA fragments flanked by *loxP* sites | S4                  |
| pDR110      | *B. subtilis* *amyE* integration vector containing IPTG-inducible *P_{spank}*-promoter | S5                  |
| pJP2340     | *aimR*<sub>spi</sub> gene cloned in integration vector pDR110                 | This study          |
| pJP2801     | *aimR*<sub>3T</sub> gene cloned in integration vector pDR111                  | This study          |
| pJP2800     | *yopR*<sub>spi</sub> gene cloned in integration vector pDR110                 | This study          |

Table S4. Plasmids used in this study, *Related to STAR Methods.*
| Mutants | Oligonucleotides | Sequence (5'→3') |
|---------|----------------|-----------------|
| kan marker without loxP | KanR-5m | TTTGATTTTTATGGAATATGTGATATAATGC |
|            | KanR-6c | TCTAGGCTACTAAAAATTACCC |
| erm marker with loxP | ErmR-1m | JAGGCAGAAAGAAGAGAGAAGCAGGAGGAGAGAAAGC |
|            | ErmR-2c | JAGGCTCTTGTGACTGGCTTGGCTGCTGCTGGCCGTATCTGTGCTTCGCTGTCTTGCTGCTTACAAGTCGATATGAGATTTAAG |

| SPβ yokI::kan | Forward Flanking | yokI-5pL | ATCCCTAGTTGATTGATATG |
|              | yokI-1_R | GATATATACCATATTACATTTAAAAATCAACCATCTCCTCTCTTCAGCC |
| Reverse Flanking | yokI-4_F | GGTATAGGTGTTGTTAGCTACCTGAAGATTTAGG |
|              | yokI-3pR | ACTGAAAGACACTCTCTCAAAG |

| phi3T yokI::kan | Forward Flanking | phi3T-1m | GCAGTGTTCGCAACAGAGATTGCC |
|                | phi3T-2c | GATTATATCACATTATCCATTAAAAATCAAAATCATTCTCCTTTCAAGCC |
| Reverse Flanking | phi3T-3m | GGTATAGGTGTTGTTAGCTACCTGAAGATTTAGG |
|                | phi3T-4c | CTCGCTGTAACCTCGCTTC |

| SPβ aimR::erm | AimR-SPB-24mB | CGGCAGATTCCTATAAAGGCGCTGAGATCC |
|              | AimR-SPB-14cS | ACAGGCGAACCAAAATGATTAGGGTCATAAAATAGGC |
| SPβ aimP::erm | AimP-SPB-1mB | CGGCAGATTCGCAAAAGGCGCAAGAAGTGCC |
|              | AimP-SPB-4c | AGTGTGATGACAGAGATAGGGTCATAAAATAGGC |

| SPβ yopN::erm | Spbeta_5_5_F | GTCGAGCACAATCTAAGAAGATTTACCTTCAAG |
|              | YopMN_R | GCCGTCACCTATGGTCTGTCG |

| SPβ yopR::erm | YopR_F | CTTGCTACGAAAGAAGATTTAGG |
|              | YopR_R | CTCGCTTGAACACAGAAGATTTAGG |

| phi3T aimR::erm | Forward Flanking | AimR-phi3T-1m | CGAATGCTGGAAGAAGAATTTCTTAAGATA |
|                | AimR-phi3T-2c | GGTCTCTTCTTTCTGCCTGGCTTTAATTCCAATTTGCTCC |
| Reverse Flanking | AimR-phi3T-3m | GGAAGACGTAGCAAGGTCGATTTACATTTCAAG |
|                | AimR-phi3T-4c | CAAAGCAGTACATGTGTTCCTCCAG |

| phi3T aimP::erm | Forward Flanking | AimR-phi3T-5m | GTGATTTTTGGCAATATGTC |
|                | AimR-phi3T-11c | GCAATATGTTAATGAGCAGAAGCCGAGGATATTGATGGATCTTCAAG |
| Reverse Flanking | AimR-phi3T-11m | GGTTTTGCTCTTAAATATGTGAGGCGCGGCGCGCAGAAGGATTTTGAATTCGATTTCAATTTAGG |
|                | AimR-phi3T-4c | CAAAGCAGTACATGTGGTTCCTCCAG |

| Plasmids | Oligonucleotides | Sequence (5'→3') |
|----------|----------------|-----------------|
| pJP2340  | AimR-SPBeta-1mH | CCCAAGCTGATATGTAAGTATCTATTAG |
|          | AimR-SPBeta-2cS | ACAGGCGAACATATTGTCTCTCCATTAAAAATAGG |
| pJP2750  | AimR-phi3T-9mS | AGGCCTGACCTTTGAATTTCTAGATGAG |
|          | AimR-phi3T-10cSpH | ATAGCATGCGCTTCTTTCAATTTGTCAGAC |
| pJP2800  | YopR_2F | ACAGGCGAACAGGAGTGAGCAGAAAGAAGTG |
|          | YopR_2R | ACATGAGCTGCCATTTTTACAAATAGGACATTAACGAGGAGGAG |

| Southern Blot | Oligonucleotides | Sequence (5'→3') |
|--------------|----------------|-----------------|
| SPβ probe    | SPBeta-1m | GATAGCGTACCAGGAGGTCTCTC |
|              | SPBeta-2c | CTAATGGAGGAGCTGGAGAG |
Supplemental References:

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