

Supplementary Information

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

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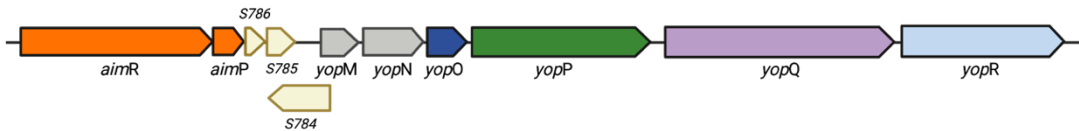
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CONTENTS

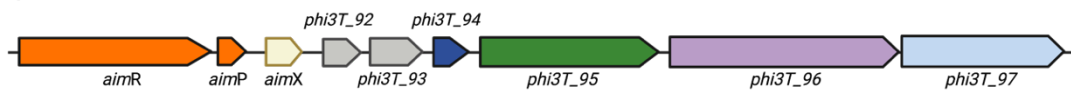
Supplementary Figures	page
Supplementary Figure 1	3
Supplementary Figure 2	4
Supplementary Figure 3	5
Supplementary Figure 4	6
Supplementary Figure 5	7
Supplementary Figure 6	8
Supplementary Figure 7	9
Supplementary Figure 8	10
 Supplementary Tables	
Supplementary Table 1	11
Supplementary Table 2	12
Supplementary Table 3	13
Supplementary Table 4	14
Supplementary Table 5	16
Supplementary Table 6	18
 References	 23

Supplementary Figures

SP β

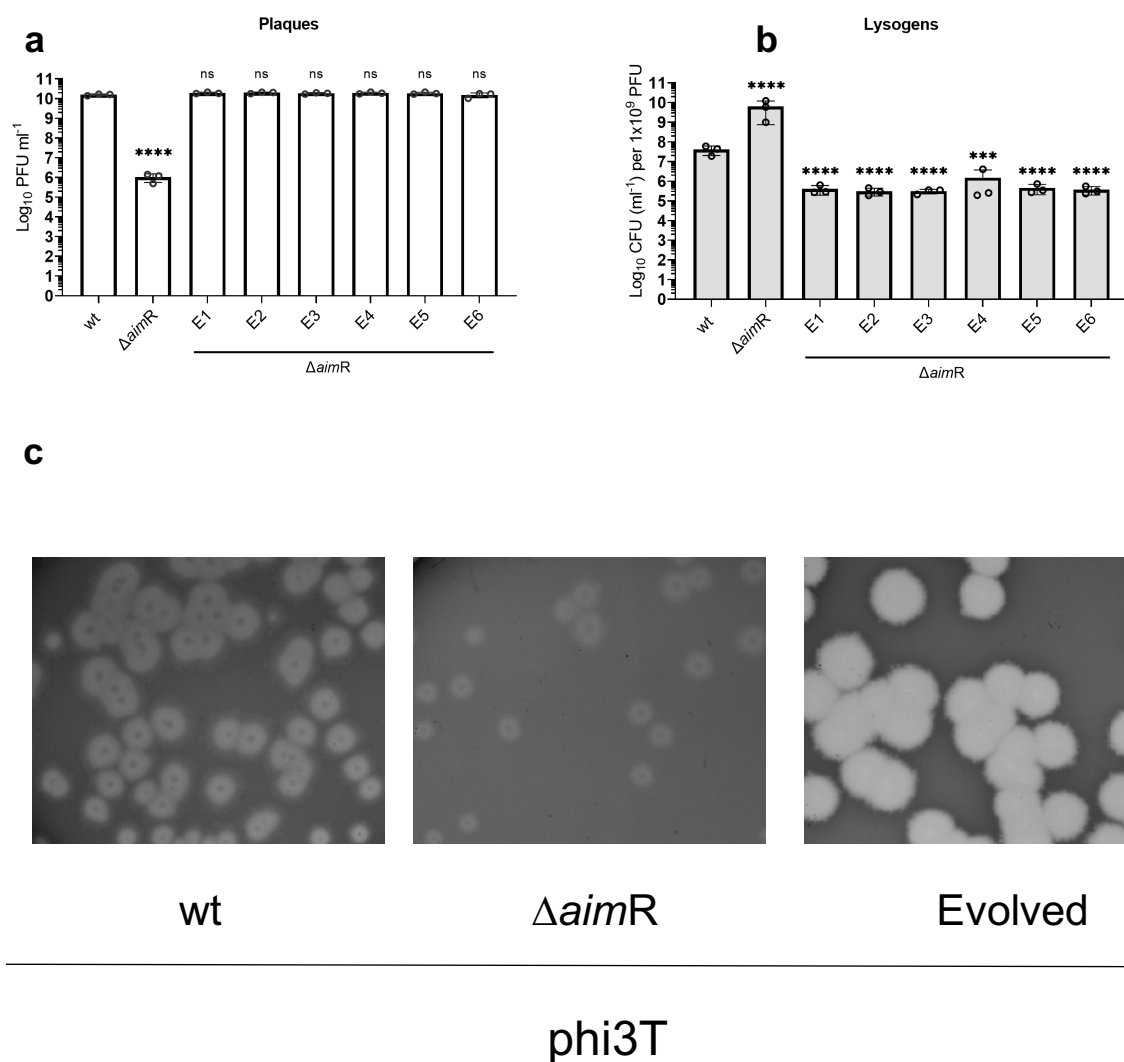


phi3T



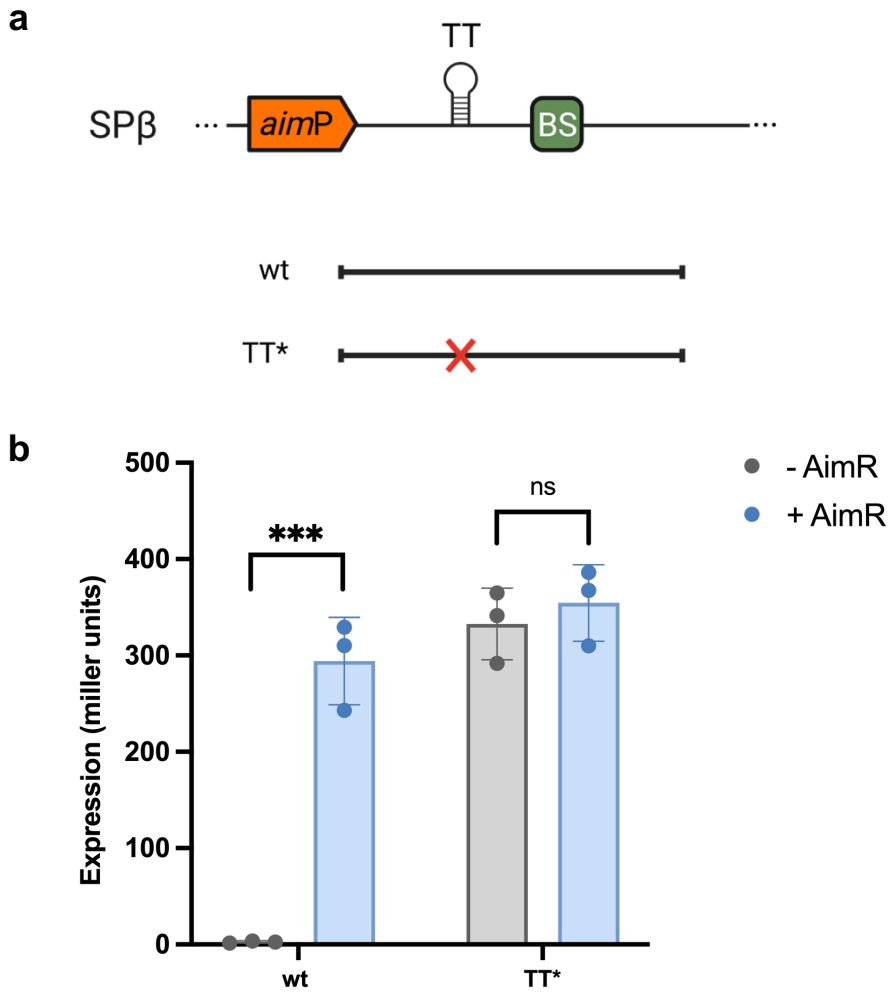
Supplementary Figure 1. Genetic organisation of the phi3T and SP β arbitrium systems and their related operons.

Genetic organisation of the arbitrium genes, *aimR* and *aimP*, followed by the operon directly downstream. Colours denote putative functions according to BLASTP and previously published results; orange, arbitrium genes; light yellow, sRNAs; grey, unknown function; navy blue, HTH_XRE domain; green, integrase domain; purple, ParB domain; light blue, phage repressor.



Supplementary Figure 2. Evolved phi3T *aimR* phages have reduced lysogenisation.

Strains lysogenic for phages phi3T wt, ΔaimR, and evolved aimR phages were MC induced (0.5 μg/mL). **a** 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 = 3). An ordinary one-way ANOVA of transformed data followed by Dunnett's multiple comparisons test was performed to compare mean differences between titers. Adjusted p values were as follows: phi3T ΔaimR ****p < 0.0001; E1- E6 ns (not significant). **b** The number of resulting lysogens were quantified using 168 Δ6 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 followed by Dunnett's multiple comparisons test was performed to compare mean differences in lysogenisation. Adjusted p values were as follows: ****p < 0.0001; E4 ***p = 0.0001. **c** phi3T lysates were tittered using 168 Δ6 as the recipient strain. The resulting plaque morphologies were photographed.



Supplementary Figure 3. AimR is an antiterminator in phage SPβ.

a The schematic shows the genetic features in the region of SPβ DNA cloned into the reporter plasmid under the control of the pSpank promoter. TT*: mutated TT. **b** *B. subtilis* strains carrying pDR110 (empty or AimR) and the reporter plasmids were grown to $OD_{600} = 0.2$. 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. Error bars represent the SD of three biological replicates. Adjusted p values indicated were as follows: ***p = 0.0004; ns (not significant).

a

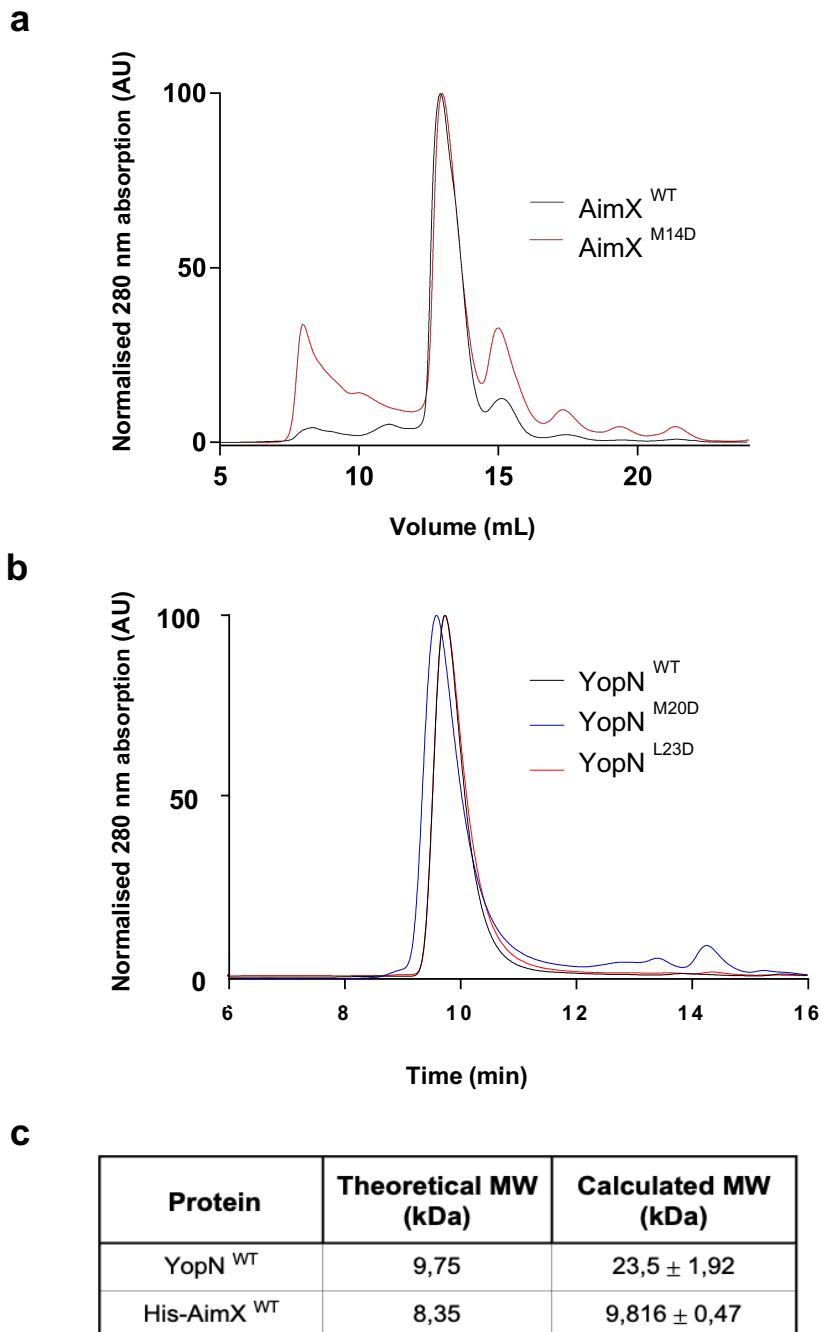
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***** ** ** * ** ** ** **          ** ** ** ***** ** ** ** ** ** ** ** *****
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** ** **      ** ** ** ** ** ** ** ** ** ** ** ** ** ** ** ** ** ** ** ** ** ** ** ** ***** ** **
AGGTATAAAACCCTGGCTTCATGA
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**** ** ** * **      ***
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b

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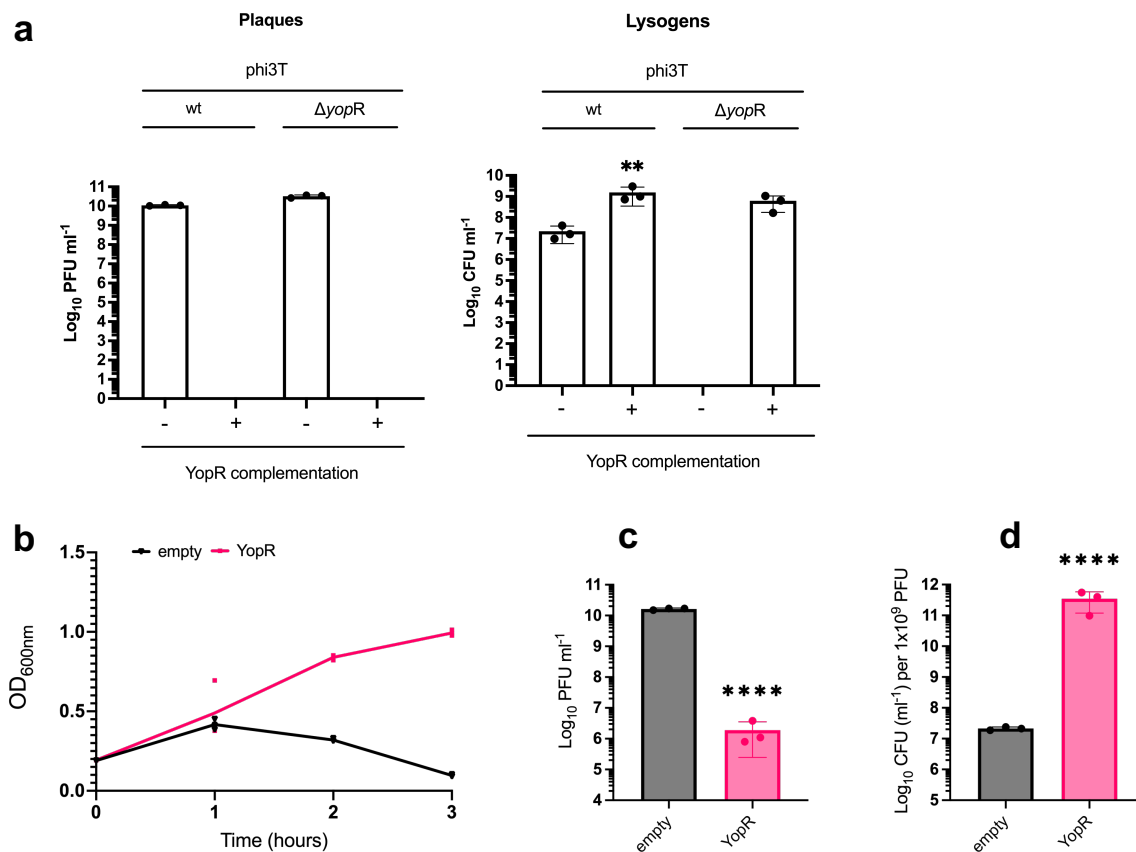
Supplementary Figure 4. Nucleotide and protein sequence comparison between *aimX* and *aimX* variant.

An *aimX* variant was generated by modifying the nucleotide sequence without changing the encoded amino acid sequence. **a** Nucleotide sequence alignment between *aimX* and the *aimX* variant. The upper line shows the wt *aimX* DNA sequence; the lower line shows the *aimX* variant DNA sequence. The nucleotides changed are shown in lower case. Asterisks designate nucleotides conserved between the two. **b** Protein sequence alignment between wt *aimX* and the *aimX* variant. The upper line shows the *aimX* protein sequence; the lower line shows the *aimX* variant protein sequence. Asterisks designate amino acids conserved between the two.



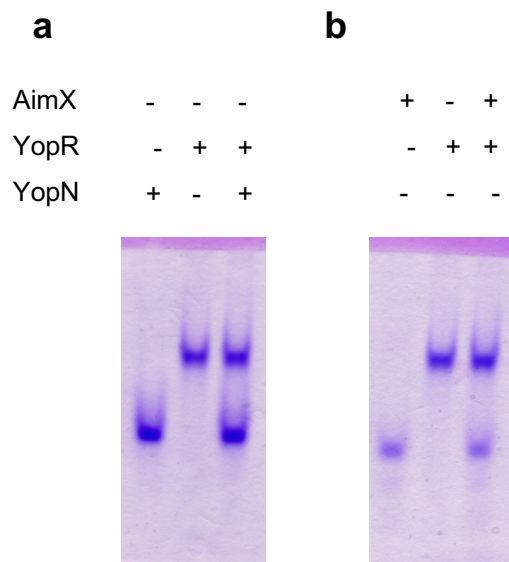
Supplementary Figure 5. Point mutations on AimX^{phi3T} and YopN^{phi3T} do not affect protein behaviour in solution.

a Size exclusion chromatography pattern for AimX^{WT}, AimX^{M14D} and AimX^{G17D} in a Superdex 75 increase column. **b** Size exclusion chromatography pattern for YopN^{phi3T}, YopN^{M20D} and YopN^{L23D} in a Shodex KW-402.5-4F column. **c** Molecular weight for YopN^{phi3T} and AimX^{phi3T} as calculated using SEC-MALS.



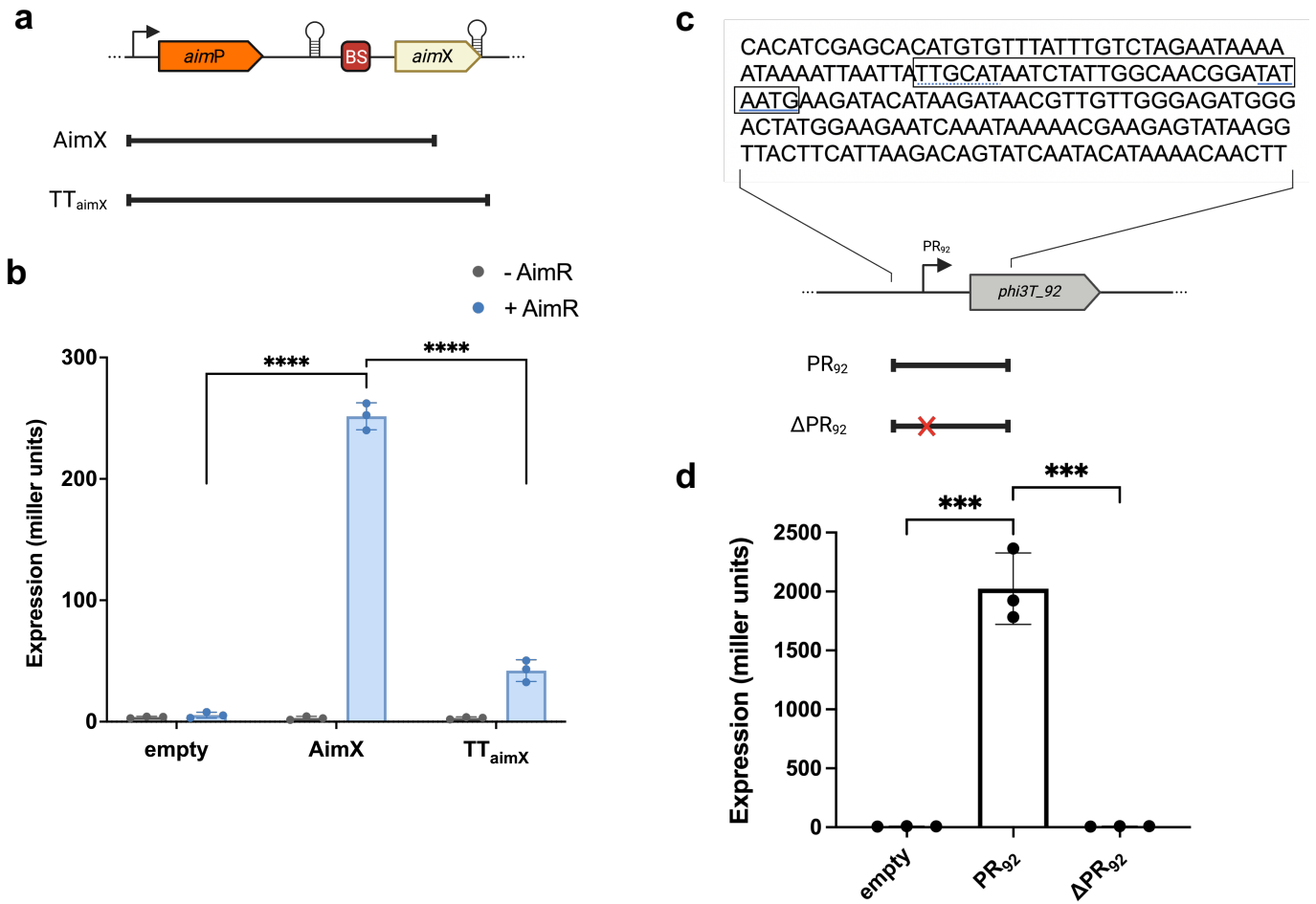
Supplementary Figure 6. Characterisation of phi3T YopR function.

a Lysates containing phages phi3T wt or phi3T $\Delta yopR^{phi3T}$ were used to infect strains 168 $\Delta 6$ or 168 $\Delta 6$ *amyE::Pspank-YopR^{phi3T}*, which overexpress YopR. The number of resulting phages (PFUs mL^{-1}) and lysogens (CFUs mL^{-1}) were then quantified. An ordinary one-way ANOVA of transformed data was performed to compare mean differences between phi3T lysogen titers obtained using 168 $\Delta 6$ or 168 $\Delta 6$ *amyE::Pspank-YopR^{phi3T}* recipient strains (** $p = 0.017$) **b** *B. subtilis* strains either devoid of (purple) or expressing YopR^{phi3T} (red) were infected with phage phi3T at an MOI of 1:10 (phage:bacteria). For all infection experiments OD₆₀₀ readings (**b**), phage titers (**c**) and the relative number of lysogens (**d**) were determined. The number of resulting phages (PFUs mL^{-1}) and lysogens (CFUs mL^{-1}) were quantified using 168 $\Delta 6$ as the recipient strain. The means and SDs are presented ($n = 3$). Adjusted p values indicated were as follows: **** $p < 0.0001$.



Supplementary Figure 7. YopR^{phi3T} does not interact with YopN^{phi3T} or AimX^{phi3T}.

a Native 8% polyacrylamide gel where no presence of YopN^{phi3T}- YopR^{phi3T} complex is detected. First and second lanes correspond to controls where only YopN^{phi3T} or YopR^{phi3T} have been respectively loaded. **b** Native 8% polyacrylamide gel where no presence of AimX^{phi3T}- YopR^{phi3T} complex is detected. First and second lanes correspond to controls where only AimX^{phi3T} or YopR^{phi3T} have been respectively loaded.



Supplementary Figure 8 Expression of the *aimX* and *phi3T_92* genes.

a Schematic of the genetic features in the reporter strains used to characterise the TT downstream of *aimX*. 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. **b** *B. subtilis* strains carrying the respective reporter plasmid were grown to $OD_{600} = 0.2$ and IPTG was added (1 mM) for induction of the pSpank promoter in the pDR110 (empty or AimR) plasmid. β -gal expression was measured using the Miller method. Adjusted p values indicated were as follows: ****p < 0.0001. **c** Schematic of the genetic features in the reporter strains used to characterise the putative promoter upstream of *phi3T_92/yopM^{phi3T}*. The DNA sequence shows the predicted promoter in a box, where dotted and solid blue underlines represent suggested -35 and -10 regions respectively. The black blunt-ended lines show the regions cloned into the pDG1663 reporter plasmid. Regions were fused to the β -gal reporter gene. The putative promoter is represented by the black arrow. A version was designed where 35 and -10 regions were mutated (ΔPR_{92}). **d** *B. subtilis* strains carrying the respective reporter plasmid were grown to $OD_{600} = 0.2$ and β -gal expression was measured using the Miller method. Adjusted p values indicated were as follows: ***p = 0.0003.

Supplementary Tables

Supplementary Table 1. Mutations identified in evolved Δ aimR phages.

Strain	phage	DNA region	nt position (phi3T KY030782)	nt position (SP β NC_001884)	Mutation
JP23123	phi3T	TT between <i>aimP</i> and <i>aimX</i>	70222		G-C
JP23124	phi3T	TT between <i>aimP</i> and <i>aimX</i>	70222		G-C
JP23126	phi3T	TT between <i>aimP</i> and <i>aimX</i>	70222		G-C
JP23127	phi3T	TT between <i>aimP</i> and <i>aimX</i>	70222		G-C
JP23130	phi3T	TT between <i>aimP</i> and <i>aimX</i>	70222		G-C
JP23131	phi3T	TT between <i>aimP</i> and <i>aimX</i>	70222		G-C
JP23775	SP β	TT between <i>aimP</i> and <i>aimX</i>		77686	G-T
JP23776	SP β	Intergenic region <i>yokL-yolA</i>		12942	A-G
	SP β	TT between <i>aimP</i> and <i>aimX</i>		77686	G-T
JP23777	SP β	TT between <i>aimP</i> and <i>aimX</i>		77686	G-T

Supplementary Table 2. Data collection and refinement statistics

	His-YopN ^{Phi3T}	YopN ^{Phi3T}	YopN ^{Phi3T} -AimX
Data collection			
Space group	P 2 ₁	I 2	P 3 ₂ 2 1
Cell dimensions			
<i>a</i> , <i>b</i> , <i>c</i> (Å)	37.5, 50.8, 55.8	46.5, 49.9, 70.5	66.4, 66.4, 85.1
α , β , γ (°)	90, 101.96, 90	90, 107.74, 90	90, 90, 120
Resolution (Å)	54.59-1.9 (1.94-1.9)	40.06-2.31 (2.44-2.3)	57.55-2.2 (2.32-2.2)
<i>R</i> _{merge}	0.079 (0.376)	0.1 (0.212)	0.030 (0.482)
<i>I</i> / σ <i>I</i>	8.0 (1.8)	8.5 (4.4)	7.4 (1.5)
Completeness (%)	98.6 (92.2)	97.1 (96)	99.7 (93.2)
Redundancy	6.0 (5.2)	4.9 (5.1)	18.5 (18.4)
Refinement			
Resolution (Å)	1.9	2.3	2.2
No. reflections	32162	13240	22923
<i>R</i> _{work} / <i>R</i> _{free}	0.20/0.23	0.21/0.26	0.22/0.23
No. atoms	1384	1173	1315
Protein	1319	1134	1280
Ligand/ion	-	2	2
Water	65	37	33
<i>B</i> -factors			
Protein	50.10	38.4	60.30
Ligand/ion	-	45	65.9
Water	52.20	31	61.7
R.m.s. deviations			
Bond lengths (Å)	0.009	0.010	0.014
Bond angles (°)	0.86	1.16	1.38

Supplementary Table 3. Interactions of YopN (dimer) with one molecule of AimX in AimX-YopN structure or with the residues belonging to the linker and Histag in His-YopN structure.

YopN ^{phi3T}		AimX		YopN Linker-Histag	
Chain	Residue	Chain	Residue	Chain	Residue
A	K5	C	E20		
A	Y6	C	E13		
A		C	K16		
A		C	G17		
A		C	E20		
A	Y7	C	E20		
A	W48			A	F-2
A	F51	C	Y18		
B	K13	C	M21		
B	W16	C	M14		
B	W16	C	G17		
B	W16			A	M1
B	K17	C	Y18		
B	H19	C	M14		
B	M20	C	Y11	A	F-2
B		C	M14	A	M1
B		C	A15		
B	L23	C	M14		
B	K24			A	F-2
B	E27			A	V-11
B				A	N-5
B	G61			A	Y-3
B	K62			A	Y-3
B	P63			A	Y-3
B	V64	C	M14		
B	I65	C	E13	A	S0
B				A	M1
B	V67			A	S0

Supplementary Table 4. Strains used in this study

Strain	Genotype or description	Reference or source
<i>Bacillus subtilis</i>		
168 (1A700)	<i>trpC2</i>	¹ ; BGSC
Δ6 (1A1299)	<i>trpC2</i> ; ΔSPβ ; subclacin 168-sensitive; Δ <i>skin</i> ; ΔPBSX; Δprophage 1; Δ <i>pks</i> ::Cm; Δprophage 3; Cm ^r	² ; BGSC
JP21870	Δ6 lysogenic phi3T <i>phi3T_5::kan</i>	³
JP22453	Δ6 lysogenic phi3T <i>phi3T_5::kan</i> Δ <i>aimR</i>	³
JP23123	Δ6 lysogenic phi3T <i>phi3T_5::kan</i> Δ <i>aimR</i> E1	This study
JP23124	Δ6 lysogenic phi3T <i>phi3T_5::kan</i> Δ <i>aimR</i> E2	This study
JP23126	Δ6 lysogenic phi3T <i>phi3T_5::kan</i> Δ <i>aimR</i> E3	This study
JP23127	Δ6 lysogenic phi3T <i>phi3T_5::kan</i> Δ <i>aimR</i> E4	This study
JP23130	Δ6 lysogenic phi3T <i>phi3T_5::kan</i> Δ <i>aimR</i> E5	This study
JP23131	Δ6 lysogenic phi3T <i>phi3T_5::kan</i> Δ <i>aimR</i> E6	This study
JP22949	Δ6 lysogenic SPβ <i>yokl::kan</i> Δ <i>aimR</i>	³
JP23775	Δ6 lysogenic SPβ <i>yokl::kan</i> Δ <i>aimR</i> E1	This study
JP23776	Δ6 lysogenic SPβ <i>yokl::kan</i> Δ <i>aimR</i> E2	This study
JP23777	Δ6 lysogenic SPβ <i>yokl::kan</i> Δ <i>aimR</i> E3	This study
JP23407	Δ6 <i>thrC</i> ::P _{spank}	This study
JP23408	Δ6 <i>thrC</i> ::P _{spank} - transcriptional terminator upstream <i>aimX</i> (TT) - <i>lacZ</i>	This study
JP23409	Δ6 <i>thrC</i> ::P _{spank} - transcriptional terminator upstream <i>aimX</i> mutated version (TT*) - <i>lacZ</i>	This study
JP23748	Δ6 <i>amyE</i> ::P _{spank} ; <i>thrC</i> :: <i>aimP</i> - <i>aimX</i> phi3T - <i>lacZ</i>	This study
JP23728	Δ6 <i>amyE</i> ::P _{spank} ; <i>thrC</i> ::P <i>aimP</i> - <i>aimP</i> - <i>aimX</i> phi3T - <i>lacZ</i>	This study
JP23750	Δ6 <i>amyE</i> ::P _{spank} ; <i>thrC</i> ::P <i>aimP</i> - <i>aimP</i> - <i>aimX</i> phi3T transcriptional terminator mutated (TT*) - <i>lacZ</i>	This study
JP23751	Δ6 <i>amyE</i> ::P _{spank} ; <i>thrC</i> ::P <i>aimP</i> - <i>aimP</i> - <i>aimX</i> phi3T mutant in AimR binding site - <i>lacZ</i>	This study
JP23752	Δ6 <i>amyE</i> ::P _{spank} ; <i>thrC</i> ::P <i>aimP</i> - <i>aimP</i> - <i>aimX</i> phi3T increased distance - <i>lacZ</i>	This study
JP23753	Δ6 <i>amyE</i> ::P _{spank} ; <i>thrC</i> ::P <i>aimP</i> - <i>aimP</i> - <i>aimX</i> phi3T decreased distance - <i>lacZ</i>	This study
JP23755	Δ6 <i>amyE</i> ::P _{spank} <i>aimR</i> _{3T} ; <i>thrC</i> :: <i>aimP</i> - <i>aimX</i> phi3T - <i>lacZ</i>	This study
JP23730	Δ6 <i>amyE</i> ::P _{spank} <i>aimR</i> _{3T} ; <i>thrC</i> ::P <i>aimP</i> - <i>aimP</i> - <i>aimX</i> phi3T - <i>lacZ</i>	This study
JP23757	Δ6 <i>amyE</i> ::P _{spank} <i>aimR</i> _{3T} ; <i>thrC</i> ::P <i>aimP</i> - <i>aimP</i> - <i>aimX</i> phi3T transcriptional terminator mutated (TT*) - <i>lacZ</i>	This study
JP23758	Δ6 <i>amyE</i> ::P _{spank} <i>aimR</i> _{3T} ; <i>thrC</i> ::P <i>aimP</i> - <i>aimP</i> - <i>aimX</i> phi3T mutant in AimR binding site - <i>lacZ</i>	This study
JP23759	Δ6 <i>amyE</i> ::P _{spank} <i>aimR</i> _{3T} ; <i>thrC</i> ::P <i>aimP</i> - <i>aimP</i> - <i>aimX</i> phi3T increased distance - <i>lacZ</i>	This study
JP23760	Δ6 <i>amyE</i> ::P _{spank} <i>aimR</i> _{3T} ; <i>thrC</i> ::P <i>aimP</i> - <i>aimP</i> - <i>aimX</i> phi3T decreased distance - <i>lacZ</i>	This study

Supplementary Table 4. Strains used in this study (continuation)

Strain	Genotype or description	Reference or source
JP23779	$\Delta 6$ <i>amyE</i> ::P _{spank} ; <i>thrC</i> ::P _{spank} - S784 - S785 SP β - <i>lacZ</i>	This study
JP23789	$\Delta 6$ <i>amyE</i> ::P _{spank} ; <i>thrC</i> ::P _{spank} - S784 - S785 SP β transcriptional terminator mutated (TT*) - <i>lacZ</i>	This study
JP23780	$\Delta 6$ <i>amyE</i> ::P _{spank} <i>aimR</i> _{SPβ} ; <i>thrC</i> ::P _{spank} - S784 - S785 SP β - <i>lacZ</i>	This study
JP23790	$\Delta 6$ <i>amyE</i> ::P _{spank} <i>aimR</i> _{SPβ} ; <i>thrC</i> ::P _{spank} - S784 - S785 SP β transcriptional terminator mutated (TT*) - <i>lacZ</i>	This study
JP23806	$\Delta 6$ <i>amyE</i> ::P _{spank} ; <i>thrC</i> ::P _{aimP} - <i>aimP</i> - <i>aimX</i> ϕ i3T binding site <i>AimR</i> _{SPβ} - <i>lacZ</i>	This study
JP23807	$\Delta 6$ <i>amyE</i> ::P _{spank} <i>aimR</i> _{3T} ; <i>thrC</i> ::P _{aimP} - <i>aimP</i> - <i>aimX</i> ϕ i3T binding site <i>AimR</i> _{SPβ} - <i>lacZ</i>	This study
JP23808	$\Delta 6$ <i>amyE</i> ::P _{spank} <i>aimR</i> _{SPβ} ; <i>thrC</i> ::P _{aimP} - <i>aimP</i> - <i>aimX</i> ϕ i3T binding site <i>AimR</i> _{SPβ} - <i>lacZ</i>	This study
JP22518	$\Delta 6$ lysogenic ϕ i3T <i>phi3T_5</i> :: <i>kan</i> Δ <i>aimR</i> ; <i>amyE</i> ::P _{spank}	3
JP22519	$\Delta 6$ lysogenic ϕ i3T <i>phi3T_5</i> :: <i>kan</i> Δ <i>aimR</i> ; <i>amyE</i> ::P _{spank} <i>aimR</i> _{3T}	3
JP23743	$\Delta 6$ lysogenic ϕ i3T <i>phi3T_5</i> :: <i>kan</i> Δ <i>aimR</i> ; <i>amyE</i> ::P _{spank} <i>aimX</i> _{3T}	This study
JP23744	$\Delta 6$ lysogenic ϕ i3T <i>phi3T_5</i> :: <i>kan</i> Δ <i>aimR</i> ; <i>amyE</i> ::P _{spank} <i>aimX</i> _{3T} nucleotide variant	This study
JP23745	$\Delta 6$ lysogenic ϕ i3T <i>phi3T_5</i> :: <i>kan</i> Δ <i>aimR</i> ; <i>amyE</i> ::P _{spank} <i>aimX</i> _{3T} L5*	This study
JP23746	$\Delta 6$ lysogenic ϕ i3T <i>phi3T_5</i> :: <i>kan</i> Δ <i>aimR</i> ; <i>amyE</i> ::P _{spank} <i>aimX</i> _{3T} M14D	This study
JP19679	$\Delta 6$ <i>amyE</i> ::P _{spank}	3
JP23800	$\Delta 6$ <i>amyE</i> ::P _{spank} <i>YopR</i> _{3T}	This study
JP23801	$\Delta 6$ lysogenic ϕ i3T <i>phi3T_5</i> :: <i>kan</i> ; <i>amyE</i> ::P _{spank} <i>YopR</i> _{3T}	This study
JP23811	$\Delta 6$ lysogenic ϕ i3T <i>phi3T_5</i> :: <i>kan</i> Δ <i>yopR</i> ; <i>amyE</i> ::P _{spank} <i>YopR</i> _{3T}	This study
JP23230	$\Delta 6$ <i>amyE</i> ::P _{spank} <i>YopN</i> _{3T}	This study
JP23770	$\Delta 6$ <i>amyE</i> ::P _{spank} <i>YopN</i> _{3T} M20D	This study
JP23771	$\Delta 6$ <i>amyE</i> ::P _{spank} <i>YopN</i> _{3T} L23D	This study
JP23729	$\Delta 6$ <i>amyE</i> ::P _{spank} ; <i>thrC</i> ::P _{aimP} - <i>aimP</i> - <i>aimX</i> - transcriptional terminator downstream <i>aimX</i> - <i>lacZ</i>	This study
JP23731	$\Delta 6$ <i>amyE</i> ::P _{spank} <i>aimR</i> _{3T} ; <i>thrC</i> ::P _{aimP} - <i>aimP</i> - <i>aimX</i> - transcriptional terminator downstream <i>aimX</i> - <i>lacZ</i>	This study
JP23802	$\Delta 6$ <i>thrC</i> ::P _{yopM} _{3T} - <i>yopM</i> _{3T} - <i>lacZ</i>	This study
JP23803	$\Delta 6$ <i>thrC</i> :: Δ P _{yopM} _{3T} - <i>yopM</i> _{3T} - <i>lacZ</i>	This study
<i>Escherichia coli</i>		
BL21-CodonPlus (DE3)-RIL	E. coli B F ⁻ ompT hsdS(r ⁻ m ⁻) dcm ⁺ Tetr gal λ (DE3) endA Hte [argU ileY BB	Agilent

Supplementary Table 5. Plasmids used in this study

Plasmid	Description	Reference or source
pDR244	<i>B. subtilis</i> thermosensitive vector containing Cre recombinase that allows excision of DNA fragments flanked by <i>loxP</i> sites	4
pDR110	<i>B. subtilis amyE</i> integration vector containing IPTG-inducible P_{spank} promoter	5
pDG1515	<i>E. coli</i> BluescriptKS+ plasmid source for tetracycline resistance marker (<i>tetL</i>)	6
pDR110- <i>tetL</i>	<i>B. subtilis amyE</i> integration vector containing IPTG-inducible P_{spank} promoter with <i>tetL</i>	This study
pJP2340	<i>aimR</i> _{SPβ} gene cloned in integration vector pDR110	3
pJP2750	<i>aimR</i> _{3T} gene cloned in integration vector pDR110	3
pDG1663	<i>B. subtilis</i> reporter vector containing β -gal gene, integrates at <i>thrC</i>	7
pDG1663+	<i>B. subtilis</i> reporter vector containing β -gal gene, integrates at <i>thrC</i> . Modified MCS (EcoRI-NotI-HindIII-SpeI-NheI-SphI-BamHI)	This study
pDG1663+ P_{spank}	<i>B. subtilis</i> reporter vector containing β -gal gene, integrates at <i>thrC</i> , containing IPTG-inducible P_{spank} promoter	This study
pJP2808	pDG1663+ P_{spank} - transcriptional terminator upstream <i>aimX</i> phi3T (TT)	This study
pJP2809	pDG1663+ P_{spank} - transcriptional terminator upstream <i>aimX</i> phi3T mutated version (G-C) (TT*)	This study
pJP2810	pDG1663+ <i>aimP</i> - <i>aimX</i> phi3T (No PR <i>aimP</i>)	This study
pJP2811	pDG1663+ <i>PaimP</i> - <i>aimP</i> - <i>aimX</i> phi3T (wt)	This study
pJP2812	pDG1663+ <i>PaimP</i> - <i>aimP</i> - <i>aimX</i> phi3T transcriptional terminator mutated version (G-C) (TT*)	This study
pJP2813	pDG1663+ <i>PaimP</i> - <i>aimP</i> - <i>aimX</i> phi3T mutant AimR binding site (Δ BS)	This study
pJP2814	pDG1663+ <i>PaimP</i> - <i>aimP</i> - <i>aimX</i> phi3T increased distance (Incr)	This study
pJP2815	pDG1663+ <i>PaimP</i> - <i>aimP</i> - <i>aimX</i> phi3T decreased distance (Decr)	This study
pJP2816	pDG1663+ P_{spank} - S784 - S785 SP β (wt)	This study
pJP2817	pDG1663+ P_{spank} - S784 - S785 SP β transcriptional terminator mutated version (G-T) (TT*)	This study
pJP2821	pDG1663+ <i>PaimP</i> - <i>aimP</i> - <i>aimX</i> phi3T AimR binding site SP β	This study
pJP2822	<i>aimX</i> _{3T} gene cloned in integration vector pDR110	This study
pJP2823	<i>aimX</i> _{3T} nucleotide variant gene cloned in integration vector pDR110	This study
pJP2824	<i>aimX</i> _{3T} L5* gene cloned in integration vector pDR110	This study
pJP2825	<i>aimX</i> _{3T} M14D gene cloned in integration vector pDR110	This study
pJP2826	<i>YopR</i> _{3T} gene cloned in integration vector pDR110- <i>tetL</i>	This study
pJP2827	<i>YopN</i> _{3T} gene cloned in integration vector pDR110	This study
pJP2828	<i>YopN</i> _{3T} M20D gene cloned in integration vector pDR110	This study

Supplementary Table 5. Plasmids used in this study (continuation)

Plasmid	Description	Reference or source
pJP2829	<i>YopN</i> _{3T} L23D gene cloned in integration vector pDR110	This study
pJP2830	pDG1663+ <i>PaimP</i> - <i>aimP</i> - <i>aimX</i> - transcriptional terminator downstream <i>aimX</i>	This study
pJP2831	pDG1663+ <i>PyopM</i> _{3T} (PR92)	This study
pJP2832	pDG1663+ <i>PyopM</i> _{3T} mutant in the promoter region (Δ PR92)	This study
pLIC-AimX	AimX with N-terminal His-tag cloned into pLIC SGC1	This study
pLIC-AimXM14D	AimX ^{M14D} with N-terminal His-tag cloned into pLIC SGC1	This study
pLIC-YopR	YopR ^{phi3T} with N-terminal His-tag cloned into pLIC SGC1	This study
pLIC-YopN	YopN ^{phi3T} with N-terminal His-tag cloned into pLIC SGC1	This study
pLIC-YopNM20D	YopN ^{M20D} with N-terminal His-tag cloned into pLIC SGC1	This study
pLIC-YopNL23D	YopN ^{L23D} with N-terminal His-tag cloned into pLIC SGC1	This study

Supplementary Table 6. Primers used in this study

Mutants	Oligonucleotides		Sequence (5'-3')
<i>erm</i> marker with <i>loxP</i>	ErmR-1m		GCAGGCGAGAAAGGAGAGAGAAACGCAAGGAGAGGCACGCGA GGGAGGAAAGGCAGGATACCGTTTCGTATAGCATACATTATACG AAGTTATGAATTC
	ErmR-2c		CGAGGCTCCTGTCACTGCTTCGCTCTGCTTCGGTGTCGTCGC CGTATCTGTGCTCTCTCTACCGTTTCGTATAATGTATGCTATACG AAGTTATCTCGAG
phi3T <i>yopR::erm</i>	Forward Flanking	phi3T_97-1F	CTATAATACTCAACGGGAAGGCAG
		phi3T_97-2R	GTTCTCTCTCCTTTCTCGCCTGCATCTATTTCTCCTTTAGCCTA ACG
	Reverse Flanking	phi3T_97-3F	GCGAAGCAGTGACAGGAGCCTCGTTAATCCCTTGATAGGGATT AATCTTACATAAC
		phi3T_97-4R	GCACTAATTTTCAGCATCTTCTGTTG

Plasmids	Oligonucleotides		Sequence (5'-3')
pDR110- <i>tetL</i>	tetL-1mE		CCGGAATTCATAGTGACGTAAAAAGATTAAATTATTGCTTGG
	tetL-2cA		CCC <u>GCGATCGC</u> CTGTTATAAAAAAAGGATCAATTTTGAACCTCTC
pDG1663+	MCS-3mE		CCGGAATTCGAGCGGCCGCTAAGCTTCTACTAGTGCTAGCGCAT GCAGGATCC
	MCS-4cB		CGCGGATCCTGCATGCGCTAGCACTAGTAGAAGCTTAGCGGCCG CTCGAATTC
pDG1663+ <i>P_{spank}</i>	Pspank-4mE		CCGGAATTCGGTAAATGTGAGCACTCACAATTC
	Pspank-5cNotI		ATAAGAATGCGGCCGCAAGCTTAATTGTTATCCGCTC
pJP2808	AimP-phi3T-10mSpeI		GGACTAGTGTAATTCTTACGGCTTTAGC
	AimX-phi3T-8cB		CGCGGATCCTCCGCTTCATTCTCAAGATGGTTGTCCTC
pJP2809	AimP-phi3T-10mSpeI		GGACTAGTGTAATTCTTACGGCTTTAGC
	AimX-phi3T-8cB		CGCGGATCCTCCGCTTCATTCTCAAGATGGTTGTCCTC
pJP2810	AimP-phi3T-10mSpeI		GGACTAGTGTAATTCTTACGGCTTTAGC
	AimX-phi3T-8cB		CGCGGATCCTCCGCTTCATTCTCAAGATGGTTGTCCTC
pJP2811	AimP-phi3T-11mSpeI		GGACTAGTTATTAAACCTTATCTCTATTTAGGTTTG
	AimX-phi3T-8cB		CGCGGATCCTCCGCTTCATTCTCAAGATGGTTGTCCTC
pJP2812	AimP-phi3T-24mSpeI		GGACTAGTGTTTGACAAATTTGAAAGGAGGTGAGAATATTG
	AimP-phi3T-23cB		CGCGGATCCCCTCTTGAGCAATTATTGAATTGATTGCAGCC

Supplementary Table 6. Primers used in this study (continuation)

Plasmids	Oligonucleotides		Sequence (5'-3')
pJP2813	PCR A	AimP-phi3T-11mSpeI	GG <u>ACTAGT</u> TATTAAACCTTATCTCTATTTAGGTTTG
		AimX-phi3T-5c	TTTTGCCATTTCTTCATAAGATATTGCTTTACCTAATGCTCTTTT CATTCTAATAACCCCCctttttTTATTTTTTGATTTTTGAATTTCTGtttt ttTCCCTTAAAATTAGAAAAGACG
	PCR B (template PCR A)	AimP-phi3T-11mSpeI	GG <u>ACTAGT</u> TATTAAACCTTATCTCTATTTAGGTTTG
		AimX-phi3T-9cB	CGCGGATCCTCCGCTTCATTCTCAAGATGGTTGTCCTCTTGAG CAATTATTGAATTGATTGCAGCCATTTCCTCGTACCCTTTTGCC ATTTCTTCATAAGATATTGCTTTACCTAATGCT
pJP2814	PCR A	AimP-phi3T-11mSpeI	GGACTAGTTATTAAACCTTATCTCTATTTAGGTTTG
		AimP-phi3T-18c	GATTTTTGAATTTCTGGAACCTTCCCTtcatttatcatcatcatctttataatcg atatcgtgatctttataatcgccatcgatctttataatcTAAAATTAGAAAAGACG CCTGATC
	PCR B	AimP-phi3T-14m	GATCAGGCGTCTTTTCTAATTTTAgattataaagatcacgatggcgattataa agatcacgatatcgattataaagatgatgatgataaatgaAGGGAAAGTTCCAGA AATTCAAAAATC
		AimX-phi3T-8cB	CGCGGATCCTCCGCTTCATTCTCAAGATGGTTGTCCTC
	PCR C (template PCR A + PCR B)	AimP-phi3T-11mSpeI	GGACTAGTTATTAAACCTTATCTCTATTTAGGTTTG
		AimX-phi3T-8cB	CGCGGATCCTCCGCTTCATTCTCAAGATGGTTGTCCTC
pJP2815	PCR A	AimP-phi3T-11mSpeI	GGACTAGTTATTAAACCTTATCTCTATTTAGGTTTG
		AimP-phi3T-17c	CCCATGTTCTTATTTTTTTGATTTTTGAATTTCTGGAACCTTAATTA GAAAAGACGCCTGATCCAATGGATCAAACGTCTATGATAGTAT TATG
	PCR B	AimP-phi3T-12m	CATAATACTATCATAGACGTTTGATCCATTGGATCAGGCGTCT TTTCTAATTAAGTTCCAGAAATTCAAAAATCAAAAAATAAGAAC ATGGG
		AimX-phi3T-8cB	CGCGGATCCTCCGCTTCATTCTCAAGATGGTTGTCCTC
	PCR C (template PCR A + PCR B)	AimP-phi3T-11mSpeI	GGACTAGTTATTAAACCTTATCTCTATTTAGGTTTG
		AimX-phi3T-8cB	CGCGGATCCTCCGCTTCATTCTCAAGATGGTTGTCCTC
pJP2816	S785-SPBeta-3mSpeI		GG <u>ACTAGT</u> GCCACGTGGAGCATAAAATCC
	S785-SPBeta-8cB		CGCGGATCCTTAATTAGGGTGTATGTATTTGAAGTTGATC
pJP2817	S785-SPBeta-3mSpeI		GG <u>ACTAGT</u> GCCACGTGGAGCATAAAATCC
	S785-SPBeta-8cB		CGCGGATCCTTAATTAGGGTGTATGTATTTGAAGTTGATC

Supplementary Table 6. Primers used in this study (continuation)

Plasmids	Oligonucleotides		Sequence (5'-3')
pJP2821	PCR A	AimP-phi3T-11mSpeI	GG <u>ACTAGT</u> TATTAACCTTATCTCTATTTAGGTTTG
		AimP-phi3T-19c	GCTCTTTTCATTCTAATAACCCCATCACTAGATGTTATTA AAACCTAATATTTAAGTGATTCCCTTAAATTAGAAAAGACGCCTGA TCC
	PCR B	AimP-phi3T-15m	GGATCAGGCGTCTTTTCTAATTTTAAGGGAATCACTTAAATATT AGGTTTTAATAACATCTAGTGATGGGGTTATTAGAATGAAAA GAGC
		AimX-phi3T-8cB	CGCGGATCCTCCGCTTCATTCTCAAGATGGTTGTCCTC
	PCR C (template PCR A + PCR B)	AimP-phi3T-11mSpeI	GGACTAGTATTAACCTTATCTCTATTTAGGTTTG
		AimX-phi3T-8cB	CGCGGATCCTCCGCTTCATTCTCAAGATGGTTGTCCTC
pJP2822	phi3T_aimX-7F-Sall		ACGCGT <u>CGAC</u> TTTTAAGGGAAAGTTCCAGAAATTC
	phi3T_aimX-8R-SphI		ACATG <u>CATGCT</u> CCGTTGCCAATAGATTATGC
pJP2823	PCR A	phi3T_aimX-12F	gGAaGAtAAtCActtgGAaAAcGAgGCaGAgATGATcAAgACgcggTA cAAgACattaGCgagtTGAGCATATTGAACTAAATAAAATAGACAT TTTAAACACATCG
		phi3T_aimX-8R-SphI	ACATG <u>CATGCT</u> CCGTTGCCAATAGATTATGC
	PCR B (template PCR A)	phi3T_aimX-11F	CgcTgGGgAAgGCgATcagcTAcGAgGAgATGGCcAAgGGtTatGAa GAgATGGCgGCcATaAAcagtATcATaGCgCAGGAaGAtAAtCActtg GAaAAcGAgGCaGAgATGATcAAgA
		phi3T_aimX-8R-SphI	ACATG <u>CATGCT</u> CCGTTGCCAATAGATTATGC
	PCR C (template PCR B)	phi3T_aimX-10F-Sall	ACGCGT <u>CGACT</u> TTTTAAGGGAAAGTTCCAGAAATTCAAAAATCA AAAAATAAGAACATGGGGGTATTAGAATGAAGAGgGCgcTgGG gAAgGCgATcagcTAcGAgGAgATGGCcAAgGG
		phi3T_aimX-8R-SphI	ACATG <u>CATGCT</u> CCGTTGCCAATAGATTATGC
pJP2824	phi3T_aimX-9F-Sall		ACGCGT <u>CGACT</u> TTTTAAGGGAAAGTTCCAGAAATTCAAAAATCA AAAAATAAGAACATGGGGGTATTAGAATGAAAAGAGCATAAT GATAAGCAATATCTTATGAAGAAATGGCAAAAGG
	phi3T_aimX-8R-SphI		ACATG <u>CATGCT</u> CCGTTGCCAATAGATTATGC
pJP2825	PCR A	phi3T_aimX-14F	GCATTAGGTAAAGCAATATCTTATGAAGAAgacGCAAAAGGGTA CGAGGAAATGGCTGCAATC
		phi3T_aimX-8R-SphI	ACATG <u>CATGCT</u> CCGTTGCCAATAGATTATGC
	PCR B (template PCR A)	phi3T_aimX-13F-Sall	ACGCGT <u>CGACT</u> TTTTAAGGGAAAGTTCCAGAAATTCAAAAATCA AAAAATAAGAACATGGGGGTATTAGAATGAAAAGAGCATTAG GTAAAGCAATATC
		phi3T_aimX-8R-SphI	ACATG <u>CATGCT</u> CCGTTGCCAATAGATTATGC

Supplementary Table 6. Primers used in this study (continuation)

Plasmids	Oligonucleotides		Sequence (5'-3')
pJP2826	phi3T_97-8F		ACGCGT <u>CGAC</u> CAATTAATGCTGCTATGAAGAAAAAACTG
	phi3T_97-9R		ACATG <u>CGATGCC</u> ACGATTTCAGCTCATATTTATGTTGG
pJP2827	phi3T_93-9F		ACGCGT <u>CGAC</u> GAGAAGAAATCATAAAGAGCATATGC
	phi3T_93-10R		ACATG <u>CGATGC</u> CTTCATTAAGTCATAACCGTCTAGC
pJP2828	PCR A	phi3T_93-9F	ACGCGT <u>CGAC</u> GAGAAGAAATCATAAAGAGCATATGC
		phi3T_93-15R	GGTTGCTCTAAAAATTTCAAGACAATATCATGCTTTTTCCAGACTTCTTTTTATTTTCC
	PCR B (template PCR A)	phi3T_93-16F	GGAAAATAAAAAAGAAAGTCTGGAAAAAGCATGATATTGTCTTGAAATTTTAGAGCAACC
		phi3T_93-10R	ACATG <u>CGATGC</u> CTTCATTAAGTCATAACCGTCTAGC
	PCR C (template PCR B)	phi3T_93-9F	ACGCGT <u>CGAC</u> GAGAAGAAATCATAAAGAGCATATGC
		phi3T_93-10R	ACATG <u>CGATGC</u> CTTCATTAAGTCATAACCGTCTAGC
pJP2829	PCR A	phi3T_93-9F	ACGCGT <u>CGAC</u> GAGAAGAAATCATAAAGAGCATATGC
		phi3T_93-17R	CCTGGTTGCTCTAAAAATTTATCGACAATCATATGCTTTTTCCAGAC
	PCR B (template PCR A)	phi3T_93-18F	GTCTGGAAAAAGCATATGATTGTCGATAAATTTTAGAGCAACCAAGG
		phi3T_93-10R	ACATG <u>CGATGC</u> CTTCATTAAGTCATAACCGTCTAGC
	PCR C (template PCR B)	phi3T_93-9F	ACGCGT <u>CGAC</u> GAGAAGAAATCATAAAGAGCATATGC
		phi3T_93-10R	ACATG <u>CGATGC</u> CTTCATTAAGTCATAACCGTCTAGC
pJP2830	AimP-phi3T-11mSpeI		GG <u>ACTAGT</u> TATTAACCTTATCTCTATTTAGGTTTG
	AimX-phi3T-10cB		CGCGGATCCACATGTGCTCGATGTGTTTAAATGTC
pJP2831	phi3T_92-17mSpeI		GG <u>ACTAGT</u> CACATCGAGCACATGTGTTTATTTGTC
	phi3T_92-14cB		CGCGGATCCAAGTTGTTTTATGTATTGATACTGTC
pJP2832	PCR A	phi3T_92-18mSpeI	GG <u>ACTAGT</u> ATCGAGCACATGTGTTTATTTGTCTAGAATAAAAAATAA AATTAATTaaaaaaaATCTATTGGCAACGGAaaaaaaaAAGATACAT AAGATAACGTTGTTGGGAGATGGGAC
		phi3T_92-15c	TTTTTATTTGATTCTTCCATAGTCCCATCTCCCAACAACGTTATCTT ATGTATCTTtttttTCCGTTGCCAATAGATtttttAATTAATTTATTTT ATTCTAGACAAATAAAC
	PCR B (template PCR A)	phi3T_92-18mSpeI	GG <u>ACTAGT</u> ATCGAGCACATGTGTTTATTTGTCTAGAATAAAAAATAA AATTAATTaaaaaaaATCTATTGGCAACGGAaaaaaaaAAGATACAT AAGATAACGTTGTTGGGAGATGGGAC
		phi3T_92-16cB	CGCGGATCCAAGTTGTTTTATGTATTGATACTGTCTTAATGAAGTA ACCTTATACTCTTCGTTTTTATTTGATTCTTCCATAGTCCCATCTCC CAACAACGTTATCTT
pLIC-AimX	AimX_FW_pLIC		AGAACCTGTACTTCCAATCCATGAAAAGAGCATTAGGTAAAGC
	AimX_Rv_pLIC		ATCCGTATCCACCTTTACTTTTCATGAAGCCAGGGTTTTATAC
pLIC-AimXM14D	AimX M14D_FW		TTATGAAGAAGATGCAAAAGGGTACGAGGAAATG
	AimX M14D_RV		GATATTGCTTTACCTAATGC
pLIC-YopR	YopR_phi3T_FW		AGAACCTGTACTTCCAATCCATGAGTGAACATACAATGCTG
	YopR_phi3T_RV		ATCCGTATCCACCTTTACTTTTAATCAGCAATCTTTTCTGATTG

Supplementary Table 6. Primers used in this study (continuation)

Plasmids	Oligonucleotides	Sequence (5'-3')
pLIC-YopN	YopN_phi3T_Fw	AGAACCTGTACTTCCAATCCATGACTAACAATAAATATTATACTGAGGAAAA
	YopN_phi3T_RV	ATCCGTATCCACCTTTACTTTCAATGAACAGCTGAGTATTGTTT
pLIC-YopNM20D	YopN M20D_FW	GAAAAAGCATGATATTGTCTTGAAATTTTATAGAGCAAC
	YopN M20D_RV	CAGACTTTCTTTTATTTTCCTC
pLIC-YopNL23D	YopN L23D_FW	TATGATTGTCGATAAATTTTATAGAGCAACCAG
	YopN_L23D_RV	TGCTTTTCCAGACTTTC

References

1. Burkholder, P. R. & Giles, N. H. Induced biochemical mutations in *Bacillus subtilis*. *Am J Bot* **34**, 345–8 (1947).
2. Westers, H. *et al.* Genome engineering reveals large dispensable regions in *Bacillus subtilis*. *Mol Biol Evol* **20**, 2076–90 (2003).
3. Brady, A. *et al.* The arbitrium system controls prophage induction. *Current Biology* **31**, (2021).
4. Koo, B.-M. *et al.* Construction and Analysis of Two Genome-Scale Deletion Libraries for *Bacillus subtilis*. *Cell Syst* **4**, 291-305.e7 (2017).
5. Carniol, K., Ben-Yehuda, S., King, N. & Losick, R. Genetic dissection of the sporulation protein SpoII $\bar{E}$ and its role in asymmetric division in *Bacillus subtilis*. *J Bacteriol* **187**, 3511–20 (2005).
6. Guérout-Fleury, A. M., Shazand, K., Frandsen, N. & Stragier, P. Antibiotic-resistance cassettes for *Bacillus subtilis*. *Gene* **167**, 335–6 (1995).
7. Guérout-Fleury, A. M., Frandsen, N. & Stragier, P. Plasmids for ectopic integration in *Bacillus subtilis*. *Gene* **180**, 57–61 (1996).