Conditional tolerance of temperate phages via transcription-dependent CRISPR–Cas targeting

Gregory W. Goldberg1, Wenyuan Jiang1, David Bikard1† & Luciano A. Marraffini1

A fundamental feature of immune systems is the ability to distinguish pathogenic from self and commensal elements, and to attack the former but tolerate the latter. Prokaryotic CRISPR-Cas immune systems defend against phage infection by using Cas nucleases and small RNA guides that specify one or more target sites for cleavage of the viral genome. Temperate phages include viruses that can integrate into the bacterial chromosome, and they can carry genes that provide a fitness advantage to the lysogenic host. However, CRISPR–Cas targeting that relies strictly on DNA sequence recognition provides indiscriminate immunity both to lytic and lysogenic infection by temperate phages—compromising the genetic stability of these potentially beneficial elements altogether. Here we show that the Staphylococcus epidermidis CRISPR-Cas system can prevent lytic infection but tolerate lysogenization by temperate phages. Conditional tolerance is achieved through transcription-dependent DNA targeting, and ensures that targeting is resumed upon induction of the prophage lytic cycle. Our results provide evidence for the functional divergence of CRISPR-Cas systems and highlight the importance of targeting mechanism diversity. In addition, they extend the concept of ‘tolerance to non-self’ to the prokaryotic branch of adaptive immunity.

Clustered, regularly interspaced, short palindromic repeat (CRISPR) loci and their CRISPR-associated (cas) genes function together as a prokaryotic adaptive immune system which can protect bacteria and archaea from invading genetic elements such as viruses (phages). Repeat elements of the CRISPR locus are intercalated with short ‘spacer’ sequences which typically match phage or plasmid genomes and dictate the targets for immunity on the basis of sequence identity. Active immunity requires transcription of the CRISPR locus, followed by cleavage of the transcript within repeat sequences by Cas endoribonucleases. This liberates small CRISPR RNAs (crRNAs) which specify the target for RNA-guided Cas nucleases that defend the cell from infection by degrading invading genomes. Furthermore, CRISPR-Cas systems can behave in an adaptive fashion through the direct acquisition of new spacer sequences from invading elements, thereby conferring sequence-specific, heritable immunity. On the basis of cas gene content and organization, CRISPR-Cas systems have been classified into three main types (I–III) and at least 12 subtypes (A–K). A growing body of work indicates a common role for these systems in antiviral defence, but the diversity of crRNA processing and targeting mechanisms that have been described suggest the potential for differences in their precise functions in vivo.

It is well established that CRISPR-Cas systems can tolerate ‘self’ spacer elements within the CRISPR locus DNA via sequence discrimination at the flanking repeats. For type I and type II systems, this requires that short sequences which license targeting, known as protospacer adjacent motifs, are absent from the repeat sequences flanking each spacer. For type III systems, targeting is prevented by excessive base pairing between the repeat-derived crRNA tag and its corresponding DNA sequence. Tolerance to ‘non-self’ DNA elements, on the other hand, has yet to be described. Previous reports indicate that active CRISPR–Cas systems and their targets cannot co-exist in the same cell. Thus, CRISPR–Cas targeting that relies strictly on DNA sequence recognition does not offer the flexibility to accommodate genetic elements with ambiguous fitness costs, such as temperate phages. Upon infection, temperate phages can kill the host cell by initiating a lytic cycle, but they may also spare the cell from lysis and establish a lysogenic cycle, typically via repression of lytic genes and integration into the host chromosome as a so-called prophage.

In addition to preventing lysis, lysogenization can result in a variety of phenotypic outcomes which can improve host fitness, for example via expression of non-viral ‘moron’ genes carried on temperate phage genomes. The lysis/lysogen decision is generally governed by a central promoter region which responds to stochastic and environmental factors to control transcription in divergent directions, thereby promoting one or the other infection cycle. Under certain conditions, the prophage can re-initiate a lytic cycle and excise from the chromosome—a process referred to as prophage induction. Commitment to either the lytic or lysogenic cycle does not involve changes in the viral genome sequence. Hence, it is generally accepted that CRISPR–Cas targeting of temperate phages should exclude both infection outcomes; in addition to preventing lysis, CRISPR attack of an integrated prophage target precludes stable lysogenization. Although this appears to be the case for type I-E (ref. 6) and II-A (ref. 16) CRISPR–Cas systems, the potential for tolerance during type III immunity had not been explored.

To investigate the behaviour of type III CRISPR immunity during temperate phage infection, we introduced pGG3, a plasmid carrying the type III-A CRISPR–Cas system of S. epidermidis RP62a (ref. 17), into Staphylococcus aureus RN4220 (ref. 18). This strain is sensitive to the lambda-like temperate phages of S. aureus Newman, a clinical isolate harbouring four heteroimmune prophages (ΦNM1–4) which carry genes that enhance the pathogenicity of their host. We also identified a spacer in one of the CRISPR loci of S. aureus MSHR1132 (ref. 20) with near-perfect identity to a conserved target sequence present in ΦNM1 (Fig. 1a), ΦNM2 and ΦNM4. This spacer, referred to as 32T (Supplementary Table 1), was added to the CRISPR locus of pGG3. Using ΦNM1, we first established that this spacer prevents lytic infection by showing that plaquing efficiency is reduced approximately seven orders of magnitude compared with a strain carrying the pGG3 plasmid without the ΦNM1-targeting spacer (Fig. 1b). We then introduced an erythromycin resistance gene (ermC) into ΦNM1 to facilitate quantification of lysogens that have stably integrated a chromosomal prophage (creating ΦNM1-ErmR). Using this system, we expected to find results consistent with a report describing CRISPR-mediated immunity to lysogenization by phage lambda in E. coli. Surprisingly, we obtained the same efficiency of lysogenization compared with the control strain lacking spacer 32T (Fig. 1c).

To test whether the presence of mismatches between the 32T crRNA and its target was influencing this phenomenon, we engineered spacer 32T* with a perfect match to its target, but obtained the same results (Fig. 1b, c). We next sought to determine whether genetic CRISPR–Cas inactivation is responsible for the apparent tolerance of these lysogens by testing them for sensitivity to ΦNM2. All 14 clones maintained resistance to ΦNM2 mediated by spacer 32T (Extended Data Fig. 1a, d). Finally, we demonstrated that spacer 32T tolerance does not result from genetic alteration of the target phage (Extended Data Fig. 1b, c). Tolerance was also observed for ΦNM4 (Extended Data Fig. 2), demonstrating that the tolerance phenomenon is not specific for the ΦNM1-ErmR phage or its integration.

1Laboratory of Bacteriology, The Rockefeller University, New York, New York 10065, USA. 1Present address: Synthetic Biology Group, Institut Pasteur, 28 Rue du Dr. Roux, 75015 Paris, France.
These results demonstrate that type III-A CRISPR immunity can block lytic infection but tolerate lysogenization without concomitant genetic CRISPR-Cas inactivation or alteration of the phage genome.

To determine whether prophage tolerance is a spacer-specific phenomenon, we designed a variety of spacers with 100% target identity, targeting different regions of the \( \Phi M N1 \) genome on both strands (Fig. 2a). We first tested the ability of each spacer to prevent lytic infection (Fig. 2b). Surprisingly, spacer functionality varied with the predicted transcriptional context of each target sequence. Spacers matching putative lytic genes to the right of the central promoter, which are predicted to be unidirectionally transcribed, were only effective when they targeted the predicted non-template strand (top strand according to our spacer nomenclature). Meanwhile, transcription is predicted to be bi-directional to the left of the central promoter. Spacers targeting this region prevented plaque formation regardless of the strand targeted. This resembled the activity reported for the type III-B CRISPR-Cas system of the archaeon \( \textit{Sulfolobus islandicus} \) \( \textit{REY15A} \), where immunity to plasmid transformation depended on the presence of promoters flanking a target sequence. We thus reasoned that transcription-dependent targeting could explain the discrepancies in spacer functionality.

Indeed, \( \Phi M N1 \) transcription profiles assessed by RNA sequencing of RN4220 cultures 6, 15, 30 and 45 min after infection revealed predominantly unidirectional transcription to the right of the central promoter, while bi-directional transcription was detected to the left of the central promoter (Extended Data Fig. 3).

Further evidence for the transcriptional dependence of type III-A CRISPR-Cas targeting was obtained via the characterization of a spacer 2B CRISPR-escape mutant phage, \( \Phi M N1 \)γ6, exhibiting a clear plaque phenotype characteristic of phages that cannot establish lysogeny (Fig. 2c, inset). Sanger sequencing of the spacer 2B target sequence did not reveal any mutations in the target or flanking sequences (data not shown); thus, we measured the \( \Phi M N1 \)γ6 plaqueing efficiency with other spacers to determine whether it possessed a sequence-independent, general CRISPR-escape phenotype (Fig. 2c). Although most spacers provided immunity against \( \Phi M N1 \)γ6, we identified one additional spacer, 4B, which was escaped by the mutant phage. Both the 2B and 4B spacers target the same strand in the lysogenization operon to the left of the central promoter. Importantly, the two complementary spacers (2T and 4T) targeting the opposite strand of spacers 2B and 4B were not escaped by \( \Phi M N1 \)γ6, indicating that the 2B/4B escape phenotype did not result from changes to the target DNA per se. Consistent with this, we did not observe differences in the \( \Phi M N1 \) and \( \Phi M N1 \)γ6 plaqueing efficiency when targeting the 4B region via Cas9-mediated type II-A CRISPR immunity (Extended Data Fig. 4a), which was shown to cleave double-stranded DNA even in the absence of target transcription. We thus reasoned that the \( \Phi M N1 \)γ6 type III-A CRISPR-escape and clear-plaque phenotypes could result from a localized, unidirectional reduction in transcription, for example, leftward from the central promoters. Indeed, \textit{de novo} sequencing of \( \Phi M N1 \)γ6 revealed a single nucleotide polymorphism in a crucial residue of the leftward –10 element (Fig. 2d) of the central promoters, immediately upstream of the SAPPVI g4 cl-like repressor gene required for lysogenic establishment, and –1700 base pairs (bp) away from the 2B target sequence. Encouraged by this result, we directly assessed \( \Phi M N1 \)γ6 transcription profiles using RNA sequencing, 6 and 15 min after infection.
During lysogeny, we hypothesized that transcription-dependent targets also follow this rule, as bi-directional transcription was detected across target sequences. These findings suggest that transcription across target sequences is a feature of tolerance achieved in the absence of the inducer. CRISPR immunity was only achieved when transcription across the target was induced with hydrodextracycline (ATc) in the presence of an antisense crRNA, regardless of the target's orientation. Once again, we confirmed this finding to be a type-III-specific phenomenon by transforming the strains from Fig. 3c with the spacer 43B-III type-II A CRISPR plasmid targeting the same region (Extended Data Fig. 7b). We corroborated this result by following the growth of spacer 43T transformants in liquid media (Fig. 3e). Upon addition of the inducer, growth was only inhibited for cells with the target in the forward orientation for which spacer 43T produces an antisense crRNA. Importantly, tolerance achieved in the absence of the inducer did not appear to affect growth (Fig. 3e, dotted lines). Finally, having established that type III-A CRISPR-Cas systems can block lytic infection but tolerate lysogenization, we examined the effect of tolerant spacers on prophage induction of ΦNM1 lysogens in culture. Compared with a spacerless lysogen control, the phage titre resulting from spontaneous induction of overnight cultures was significantly lower for lysogens harbouring a tolerant spacer (Fig. 4a). We next followed the growth of cultures induced directly with the DNA-damaging agent mitomycin C (Fig. 4b, solid lines). While the spacerless lysogen control cultures succumbed to prophage induction, the presence of a tolerant spacer prevented lysis.

We report here that type III-A immunity can offer conditional tolerance to ‘non-self’ genetic elements, in this case, temperate phases. This has several important implications for the CRISPR-Cas system and its host population. Tolerance helps ensure the genetic stability of the CRISPR-Cas system, since selective pressure to integrate prophages in the presence of intolerant spacers can drive genetic CRISPR-Cas inactivation (Extended Data Fig. 4c–e)—similar to what occurs during plasmid uptake20.

Figure 3 | Conditional tolerance is achieved via transcription-dependent CRISPR-Cas targeting. a, ΦNM1-Erm<sup>h</sup> lysogenization for additional spacers. C, pGGL3 non-targeting control. b, Transformation of ΦNM1-lysogenic competent cells with CRISPR-Cas plasmids containing different spacers (transformation efficiency is measured as colony-forming units per nanogram of plasmid DNA). C, pGGL3 non-targeting control. c, Integration of the 43T/B ΦNM1 target region into the chromosome of S. aureus. Target sequences (inserted in both forward and reverse orientations) are under the control of the tetracycline-inducible promoter P<sub>_tac</sub>-tac. The 43T/B crRNAs are shown annealing to either the top or bottom strands. d, Transformation of both strains shown in c, as well as an isogenic control strain lacking the target insertion, with CRISPR-Cas plasmids containing spacers 43T or 43B. Transformants were plated on selective plates with or without ATc for induction of the P<sub>_tac</sub>-tac promoter. e, Growth curve of strains shown in d expressing the spacer 43T CRISPR-Cas system, in the presence or absence of ATc addition at the indicated time point (black arrow). Error bars, mean ± s.d. (n = 3).

Figure 4 | Prophage induction is limited by type III-A CRISPR immunity in tolerant lysogens. a, Plate-forming potential (measured in plaque-forming units per millilitre) of supernatants from overnight cultures of ΦNM1 lysogens carrying the tolerant spacer 32T or 43T CRISPR plasmids, or the pGGL3 non-targeting control (C). b, Growth curve of ΦNM1-Erm<sup>h</sup> lysogens or a non-lysoygen control harbouring the pGGL3 or spacer 43T CRISPR plasmids as indicated, with or without the addition of the prophage-inducing agent mitomycin C (MMC) at the indicated time point (black arrow). Error bars, mean ± s.d. (n = 3).
In other words, tolerant spacers ensure that a population can sample potentially beneficial phenotypes that result from prophage integration without compromising their CRISPR-mediated immunity. Tolerance may also be particularly vital for type III systems, which were recently shown to provide immunity despite up to 15 mismatches with their spacer29. Thus, without the potential for phages to evade targeting readily via divergent CRISPR-Cas targeting mechanisms.

Establishes distinct genetic outcomes resulting from immunity to temperate phages via divergent CRISPR-Cas targeting mechanisms.

The requirement for transcription across target sequences during type III-A immunity contrasts with the transcription-independent targeting reported for type I (refs 27) and type II (refs 22, 23) CRISPR-Cas systems. Given the temporal pattern of target transcription observed during the phage lytic cycle, it might be expected that CRISPR targeting of late genes would not provide immunity if the cell’s survival is already compromised at the onset of targeting. Indeed, we observed some differences in spacer effectiveness when infecting cells in liquid culture at very high multiplicity of infection (MOI; ~100): spacers targeting late genes were less protective (Extended Data Fig. 8). However, this effect was not pronounced at an MOI of 10, suggesting that the system is generally robust to delays in target transcription, in accordance with what we observed in efficiency of plaquing assays. Consistent with our findings at large, our survey of sequenced staphylococcal type III spacers showed that naturally acquired spacers with known target sequences produced crRNAs complementary to the non-template strand of predicted ORFs in nine out of ten cases (Supplementary Table 2). This bias suggests negative selection on non-functional spacers targeting template strands. Alternatively, type III systems may utilize an unknown mechanism to discriminate template and non-template strands during spacer acquisition. The molecular details of the transcription-dependent targeting mechanism remain unclear. Preliminary experiments indicate that the presence of a transcript provided in trans is not sufficient to license DNA targeting (Extended Data Fig. 9). Consistent with this, induction of transcription across a plasmid-borne target results specifically in loss of the targeted plasmid (Extended Data Fig. 10). Hence, transcription in cis is probably required for DNA targeting. One possibility is that negative supercoiling generated in the wake of a passing transcription bubble could facilitate target DNA melting and improve crRNA recognition27. This hypothesis, however, would not account for the template and non-template strand asymmetry observed in our system. Another possibility is that exposure of the target non-template strand within the transcription bubble is required for annealing of a crRNA21. In this scenario, the observed asymmetry might be explained by occlusion of base-pairing to the template strand by either the nascent transcript or the RNA polymerase28. However, given the small size of the transcription bubble, this explanation may also be incomplete. Alternatively, transcription may be required to activate a targeting mechanism rather than facilitating target recognition or binding per se. In this case, effective targeting could require base-pairing potential between the crRNA and the nascent transcript in cis, which would be absent for crRNAs with complementarity to the template strand. Previous reports29,30 have revealed specific RNA target cleavage activity for type III-(B/C) systems that could be relevant to the transcription-dependent DNA targeting observed in staphylococci. Further experimentation will be needed to clarify these points. In summary, our work expands the repertoire of CRISPR-based immune functions to include a novel capacity for conditional tolerance of foreign elements, and establishes distinct genetic outcomes resulting from immunity to temperate phages via divergent CRISPR-Cas targeting mechanisms.

Online Content Methods, along with any additional Extended Data display items and Source Data, are available in the online version of the paper; references unique to these sections appear only in the online paper.

Received 13 January; accepted 2 July 2014.

Published online 31 August 2014.

1. Belkaid, Y. & Hand, T. W. Role of the microbiota in immunity and inflammation. Cell 157, 121–141 (2014).
2. Barrangou, R. CRISPR-Cas systems and RNA-guided immunity. Wiley Interdiscip. Rev. RNA 4, 261–273 (2013).
3. Sorek, R., Lawrence, C. M. & Wiedenheft, B. CRISPR-mediated adaptive immune systems in bacteria and archaea. Annu. Rev. Biochem. 82, 237–266 (2013).
4. Brüssow, H., Canchaya, C. & Hardt, W. D. Phages and the evolution of bacterial pathogens: from genomic rearrangements to lysogenic conversion. Microbiol. Mol. Biol. Rev. 68, 560–602 (2004).
5. Cumby, N., Davidson, A. R. & Maxwell, K. L. The moron comes of age. Bacteriophage 2, 225–228 (2012).
6. Edgar, R. & Qimron, U. The Escherichia coli CRISPR system protects from lambda lysogenization, lysogens, and prophage induction. J. Bacteriol. 192, 6291–6294 (2010).
7. Brouns, S. J. et al. Small CRISPR RNAs guide antiviral defense in prokaryotes. Science 321, 960–964 (2008).
8. Carte, J., Wang, R., Li, H., Terns, R. M. & Terns, M. P. Cas6 is an endoribonuclease that generates guide RNAs for invader defense in prokaryotes. Genes Dev. 22, 3489–3496 (2008).
9. Makarova, K. S. et al. Evolution and classification of the CRISPR-Cas systems. Nature Rev. Microbiol. 9, 467–477 (2011).
10. Deveau, P. et al. Phage response to CRISPR-encoded resistance in Streptococcus thermophilus. J. Bacteriol. 190, 1390–1400 (2008).
11. Semenova, E. et al. Interference by clustered regularly interspaced short palindromic repeat (CRISPR) RNA is governed by a seed sequence. Proc. Natl Acad. Sci. USA 108, 10098–10103 (2011).
12. Marraffini, L. A. & Sontheimer, E. J. Self versus non-self discrimination during CRISPR RNA-directed immunity. Nature 463, 568–571 (2010).
13. Bilkad, D., Hatoum-Aslan, A., Chen, S., Zhang, F. & Marraffini, L. A. CRISPR interference can prevent natural transformation and virulence acquisition during in vivo bacterial infection. Cell Host Microbe 12, 177–186 (2012).
14. Jiang, W., Bilkad, D., Cox, D., Zhang, F. & Marraffini, L. A. RNA-guided editing of bacterial genomes using CRISPR-Cas systems. Nature Biotechnol. 31, 233–239 (2013).
15. Johnson, A. D. et al. Lambda Repressor and cr--components of an efficient molecular switch. Nature 294, 217–223 (1981).
16. Nozawa, T. et al. CRISPR inhibition of prophage acquisition in Streptococcus pneumoniae. PLoS ONE 6, e19543 (2011).
17. Hatoum-Aslan, A., Samai, P., Maniv, I., Jiang, W. & Marraffini, L. A. A ruler protein in a bacterial immunity. Proc. Natl Acad. Sci. USA 108, 2012109613 (2011).
18. Kresowith, B. N. et al. The toxic shock syndrome exotoxin structural gene is not detectably transmitted by a prophage. Nature 305, 709–712 (1983).
19. Bae, T., Baba, T., Hiramatsu, K. & Schneewind, O. Prophages of Staphylococcus aureus Newman and their contribution to virulence. Mol. Microbiol. 62, 1035–1047 (2006).
20. Holt, D. C. et al. A very early-branching Staphylococcus aureus lineage lacking the carotenoid pigment staphyloxanthin. Genome Biol. Evol. 3, 881–895 (2011).
21. Deng, L., Garrett, R. A., Shah, S. A., Feng, X. & She, Q. A novel interference mechanism by a type IIb CRISPR-Cmr module in Sulfolobus. Mol. Microbiol. 87, 1088–1099 (2013).
22. Gasinuas, G., Barrangou, R., Horvath, P. & Siksnys, V. Cas9-cRNA ribonuclease protein complex mediates specific DNA cleavage for adaptive immunity in bacteria. Proc. Natl Acad. Sci. USA 109, E2579–E2586 (2012).
23. Jinek, M. et al. A programmable dual-RNA-guided DNA endonuclease in adaptive bacterial immunity. Science 337, 816–821 (2012).
24. Marraffini, L. A. & Sontheimer, E. J. CRISPR interference limits horizontal gene transfer in staphylococci by targeting DNA. Science 322, 1843–1845 (2008).
25. Jiang, W. et al. Dealing with the evolutionary downside of CRISPR immunity: bacteria and beneficial plasmids. PLoS Genet. 9, e1003844 (2013).
26. Manica, A., Zebec, Z., Steinkeller, J. & Schleifer, C. Unexpectedly broad target recognition of the CRISPR-mediated virus defence system in the archaeon Sulfolobus solfataricus. Nucleic Acids Res. 41, 10509–10517 (2013).
27. Westra, E. R. et al. CRISPR immunity relies on the consecutive binding and degradation of negatively supercoiled invader DNA by Cascade and Cas3. Mol. Cell 46, 595–605 (2012).
28. Nucler, E. RNA polymerase active center: the molecular engine of transcription. Annu. Rev. Biochem. 78, 335–361 (2009).
29. Hale, C. R. et al. RNA-guided RNA cleavage by a CRISPR RNA-Cas protein complex. Cell 139, 945–956 (2009).
30. Zhang, J. et al. Structure and Mechanism of the CMR Complex for CRISPR-Mediated Antiviral Immunity. Mol. Cell 45, 303–313 (2012).
construction of temperate phages with a selectable marker. Finally, we acknowledge The Rockefeller University Genomics Resource Center core facility for performing the next-generation sequencing in this work. L.A.M. is supported by the Searle Scholars Program, the Rita Allen Scholars Program, an Irma T. Hirschl Award, a Sinsheimer Foundation Award and a National Institutes of Health Director’s New Innovator Award (1DP2AI104556-01).

Author Contributions G.W.G. and L.A.M. designed experiments. Research was done by G.W.G. W.J. constructed the pWJ40 and pWJ153 plasmids and performed the plasmid-curing experiment. D.B. constructed plasmid pDB184, assisted with phage de novo assembly and provided the reads per million normalization script for RNA sequencing data. G.W.G. and L.A.M. wrote the paper.

Author Information Reprints and permissions information is available at www.nature.com/reprints. The authors declare no competing financial interests. Readers are welcome to comment on the online version of the paper. Correspondence and requests for materials should be addressed to L.A.M. (marraffini@rockefeller.edu).
METHODS

Bacterial strains and growth conditions. Cultivation of S. aureus RN4220 (ref. 18), TB4 (ref. 19) and derivative strains was done in TSB media (BD) at 37 °C, except when phase infections were performed, or when otherwise noted (see below). Whenever applicable, media were supplemented with chloramphenicol at 10 μg ml⁻¹ to ensure CRISPR plasmid maintenance. RN4220 strains harbouring pCL55-derived insertion vectors were grown similarly, but kanamycin was provided at 25 μg ml⁻¹ except during re-culture for competent cell preparation. E. coli DH5α was grown in LB Broth (BD) supplemented with kanamycin at 25–50 μg ml⁻¹ to maintain pCL55-derived plasmids. Selection for ΦN1-Merm lysogens with resistance to erythromycin (10 μg ml⁻¹) was only applied during the lysogenization protocol as described below, and, where applicable, during the subsequent ΦNM2 sensitivity assays.

Estimation of phage lysate titres. Serial dilutions were prepared in triplicate and plated on left lawns of RN4220 in HIB-agar (BD) supplemented with 5 mM CaCl₂, (technical replicates). Plates were incubated at 37 °C for 16–24 h after drying at room temperature (25 °C).

DNA preparation and cloning. Plasmid DNA was purified from 2 to 6 ml of E. coli DH5α or S. aureus RN4220 overnight cultures. For preparation from S. aureus cultures, cells were pelletted, re-suspended in 100 μl TSM buffer (50 mM Tris-HCl pH 7.5, 10 mM MgCl₂, 0.5 M sucrose) then treated with 5 μl lysostaphin (2 mg ml⁻¹) at 37 °C for 1.5 h before treatment with plasmid miniprep reagents from Qiagen. Purification used Qiagen or EconoSpin columns.

Cloning used RN4220 electrocompetent cells unless otherwise stated. For most type III CRISPR plasmids, scarless addition of repeat–spacer units to the pGPG parent vector was achieved by ‘round-the-horn’ PCR (ref. 31) followed by blunt ligation, using common primer oGG12 and spacer-specific oligonucleotides listed in Supplementary Table 3. The pGG3 vector was itself constructed by ‘round-the-horn’ PCR using primers L55 and A10 to remove extraneous repeat–spacer elements from the pW330β (ref. 32) CRISPR array. For construction of the remaining type III CRISPR plasmids, a modified parent vector (pGPG–BsaI) was created by introducing a placeholder spacer harbouring two BsaI restriction sites, to facilitate scarless cloning of spacers by replacement with annealed oligonucleotide pairs possessing the motif for both forward and reverse target junctions using primer pairs oGG50/oGG96 and oGG192, W277 and L325.

For construction of pCL55-derived inducible target vectors, cloning used chemically competent DH5α cells. Briefly, the chloramphenicol resistance cassette was first replaced with a kanamycin resistance cassette amplified from strep LAM2023 using primers L484/L485. This was accomplished by ‘round-the-horn’ PCR on the pCL55-ITET parent vector using primers L482/L483, followed by blunt ligation with the PCR-amplified resistance cassette to create the new pKL55-ITET-B parent vector. Directionality of the insertion was verified afterwards by restriction digest with BglII. Modification of the P₆₆₀₆₀ promoter in accordance with PRAB12 (ref. 33) architecture was achieved via two consecutive overlap PCR steps to introduce point mutations using oligonucleotide pairs oGG108/0GG109 and oGG110/oGG111. PCR products were sequenced by ‘round-the-horn’ PCR step and blunt ligation to introduce the downstream operator sequence using oligonucleotides oGG112 and oGG113. The resulting pKL55-ITET–RC12 vector harbouring the P₆₆₀₆₀ modifications was used for downstream manipulations, as well as integration into the RN4220 chromosome to create the ‘Targetless’ control strain. For forward and reverse target insertions, annealed oligonucleotide pairs (oGG124/oGG125 and oGG126/oGG127, respectively) with appropriate overhangs were ligated into the multiple cloning site of pKL55-ITET–RC12, following directional transformation into the pKL55-ITET–RC12 and pE194 (ref. 34) derived plasmid constructed via multiple steps of either ‘round-the-horn’ PCR followed by blunt ligation or Gibson assembly. The full sequence is provided as Supplementary Sequence 1.

Construction of the ΦN1-Merm lysogen was achieved by pKOR allelic exchange35. Homology arms (~1 kb) were amplified from the chromosome of S. aureus RN4220: ΦN1 using primer pairs oGG181/oGG182 and oGG185/oGG186, while the ~1.25 kb ermC resistance cassette was amplified from a pE194 plasmid preparation using primers oGG183 and oGG184. An ~3.25 kb fragment was assembled by SOEing PCR using external primers oGG181 and oGG186 with donase (QuikChange) attB adenylation to allow homologous directional integration into the pKOR vector36. Sequence integrity of the ~3.25 kb insertion was verified by Sanger using primers L29, oGG191, oGG192, W277 and L325.

Preparation of electrocompetent S. aureus cells. S. aureus RN4220, TB4 or derivative strains were grown overnight in TSB medium, diluted 1:100 in fresh medium without antibiotics, then allowed to grow to an attenuation (A₆₀₀ nm) of 0.8–1.0 for RN4220 or 0.7–0.9 for TB4. Measurements were taken using a NanoDrop 2000c Spectrophotometer (Thermo Scientific) and disposable polystyrene cuvettes. After re-culture, cells were pelleted at 4 °C, and two or three washes were performed using chilled, sterile dH₂O or 10% glycerol. Cells were ultimately re-suspended in 1/100th volume of chilled, sterile 10% glycerol and 50 μl aliquots were distributed for storage at −80 °C.

Efficiency of plasmid insertion. High-titre lysates (~10¹² p.f.u. ml⁻¹) of either ΦN1M, ΦNM1y6 or ΦNM2 were serially diluted in triplicate and applied to soft agar lawns of RN4220 strains harbouring CRISPR plasmids, including pGG3 or pDB184 spacerless control lawns infected in parallel (technical replicates). Plates were incubated at 37 °C for 18 h. After incubation, plates were monitored at bench top for up to 24 h to facilitate quantification of plaque-forming units.

Quantification of erythromycin-resistant lysogens. Overnight cultures of RN4220 with respective CRISPR plasmids were inoculated in triplicate from single colonies in HIB medium supplemented with chloramphenicol (biological replicates). After chilling at 4 °C, 1:10 dilutions were prepared in 1 ml fresh HIB supplemented with chloramphenicol and 5 mM CaCl₂. Diluted cultures were infected with ΦN1-Merm at ~MOI 10 and incubated on ice for 30 min. After incubation on ice, cultures were transferred to a 37 °C incubator for 30 min with shaking. Serial dilutions from each culture were then applied to HIB-agar plates supplemented with chloramphenicol, erythromycin and 5 mM CaCl₂ for quantification of lysogenic colony-forming units. In selected cases, type III-A CRISPR locus and target sequence integrity was verified by colony PCR after re-streaking single colonies using primer pairs L6/L50 (CRISPR array) and oGG256/oGG256 (orf 2) or oGG386/oGG386 (orf 32). Where applicable, Sanger sequencing of PCR products was also performed using these primers. When verifying type II lysogenization isolates, the spacer 43B-III target region was amplified using primers oGG233 and oGG234, and the type II CRISPR array was amplified using L448 and W176. The presence of integrated ΦNM1 or ΦNM1-Merm prophages was confirmed by colony PCR using primer pairs oGG191/W277 and oGG206/w276 to amplify the attL and attR junctions, respectively. To estimate the total number of recipient cells, serial dilutions of untreated overnight cultures were plated on TSB- or HIB-agar supplemented with chloramphenicol. ΦNM2 sensitivity assay. High-titre lysate of ΦNM2 (~10¹³ p.f.u. ml⁻¹) was applied to the surface of a pre-dried HIB-agar plate supplemented with 5 mM MgCl₂ and appropriate antibiotics, then allowed to dry for an additional ~30 min at room temperature. Single colonies isolated from Erm lysogeny experiments or CRISPR plasmid transformations were streaked through the ΦNM2-seeded region using a sterile plastic loop then incubated for ~12 h at 37 °C.

Enumeration of plaque-forming units liberated from lysogen cultures. Overnight cultures of either RN4220:ΦNM1-Erm or RN4220:ΦNM1 lysogens harbouring targeting CRISPR plasmids or non-targeting control plasmids were inoculated in triplicate from single colonies in HIB medium supplemented with chloramphenicol (biological replicates). After overnight growth, cells were transferred to 4 °C then pelleted by centrifugation at 469gC for 5 min. Supernatants were filtered, and 100 μl aliquots of each were added to an indicator strain to target strain overnight culture for plating by the soft agar method. After drying at room temperature (25 °C), plates were incubated 18 h at 37 °C.

Screen for lipase-negative ΦNM4 lysogens. An overnight culture of S. aureus TB4 harbouring the spacer 32T CRISPR plasmid was re-cultured to log phase growth in HIB medium supplemented with 5 mM CaCl₂. After measurement of D₆₀₀ nm, cells were treated with ΦNM4 at ~MOI 50. After incubation for 1 h, cells were plated on TSA supplemented with 5% egg yolk emulsion. After ~24 h incubation at 37 °C, approximately 1,000 colonies were inspected for lipase secretion. Two lipase-negative candidates were re-streaked to single colonies, and the presence of an integrated ΦNM4 prophage was confirmed by colony PCR using primers oGG50 and oGG96 to amplify the attL junction.

Phage DNA isolation and deep sequencing. Samples of high titre phage lysates (~10¹⁰ p.f.u. ml⁻¹) were treated with DNase and RNase to a final volume of 150 μl for 1 h at 37 °C. Samples were treated with EDTA (pH 8.0) to a final concentration
of 20 mM, followed by treatment with SDS to a final concentration of 0.5% and 2 μM proteinase K. Samples were incubated for 1 h at 65°C, then subjected to a PCR purification protocol (Qiagen). Paired-end library preparation was performed on purified phage DNA using a Nextera Tagmentation protocol (Illumina), and samples were pooled for multiplexed sequencing on a MiSeq (Illumina). De novo assembly of phage genomes used ABySS. dna

**RNA preparation for reverse transcription PCR and RNA sequencing.** For reverse transcription PCR (RT–PCR), overnight cultures were diluted 1:20 in 25 ml fresh media and grown for 2.5 h at 37°C with shaking. After re-culture, cells were pelleted and washed twice in 1 ml ice-cold TSM buffer, then treated with 3 μl lysostaphin (2 mg ml⁻¹) for 20 min at 37°C in 500 μl TSM buffer. Treated cells were pelleted then re-suspended in 750 μl cold TRIzol Reagent (Life Technologies) after discarding the supernatant. The following chloroform extraction and precipitation was performed according to the manufacturer’s protocol. After resuspension in 24H₂O, samples were treated with Qiagen DNase I for 45 min at 30°C. The RNase Cleanup columns (Qiagen). In some cases, it was necessary to repeat this step a second time to ensure the complete removal of DNA. After cleanup, all samples were again treated with DNase I (Sigma-Aldrich) for 30–45 min at 30°C, before use in the reverse transcription reaction.

For RNA sequencing, overnight cultures were diluted 1:100 in fresh HIB supplemented with chloramphenicol and 500 μM CaCl₂, and grown for 1.5 h (approximately mid-log phase) at 37°C with shaking. Cultures were removed, infected at MOI = 20 then split into 10 ml portions for an additional 6, 15, 30 or 45 min of growth. Immediately following incubation, samples were mixed with 10 ml of a 1:1 acetone/ethanol solution and transferred to ~80°C. The PhM1 lysogen was grown similarly, except without antibiotics, and harvested immediately after the 1.5 h re-culture at 37°C. At least one overnight at ~80°C, samples were thawed on ice and pelleted by centrifugation at 4696g for 10 min. After two washes of 1 ml TE buffer, cells were re-suspended in 1 ml RTL buffer (Qiagen) supplemented with BME, and transferred to 2 ml tubes pre-loaded with ~0.5–1 cm³ of 0.1 mm glass beads (BioSpec). Samples were processed in a Mini-Beadbeater instrument (BioSpec) three times for 10 s at 4,200 oscillations per minute, with 40 s of chilling on ice between runs. After bead-beating, samples were spun down for 2 min at 16,100 g. Supernatant (750 μl) was transferred to a clean tube for mixing with 500 μl 80% ethanol, and the following RNeasy purification was done according to the manufacturer’s protocol (Qiagen). After elution, samples were treated with either RNeasy Cleanup columns. In some cases, it was necessary to repeat this step a second time to ensure the complete removal of DNA. RNA-depleted samples were subsequently generated using the RiboZero Magnetic Kit for bacteria (Epitope), according to the manufacturer’s protocol.

**RT–PCR.** Reverse transcription used M-Mulv Reverse Transcriptase (NEB), with DNA-free total RNA isolated from RN4220 cultures harbouring either the pNes(wt-d) or pNes(wt-i) plasmids as templates for CDNA synthesis. For pNes(wt-d), reverse transcription used either the L8 or L86 primers in two separate 30 μl reactions, alongside mock reactions (-RT enzyme). For pNes(wt-i), the same was done using primers L8 or L87. After incubation, 1 μl of each reaction was used as a template for PCR, with respective primer pairs for each sample.

**Phage transcriptome analysis and visualization.** Reads were aligned to reference genomes using Bowtie and sorted using Samtools. Using a custom script, sorted reads were accessed via Pysam, normalized as reads per million values, and plotted in log scale as the average over consecutive windows of 500 base pairs using matplotlib tools for IPython.

**Transformation assays.** *S. aureus* RN4220 plasmid preparations were dialysed on 0.025 μm nitrocellulose filters (Millipore) then quantified using a NanoDrop 2000c Spectrophotometer (Thermo Scientific). Aliquots (50 μl) of electrocompetent cells were transformed in triplicate with 80 ng dialysed DNA per transformation using a GenePulser Xcell (BioRad) with the following parameters: 2900 V, 25 μF, 100 Ω, 2 mm (technical replicates). After electroporation, cells were immediately re-suspended in TSB to a final volume of 200 μl and recovered at 30°C for 2 h with shaking. Serial dilutions were then prepared before plating with appropriate antibiotics. For reverse CRISPR immunity assays targeting insertion vectors, additional plating in the presence of ATc at a final concentration of 0.5 μg ml⁻¹ was performed in parallel using the same dilutions. Plates were incubated at 37°C for 18–24 h.

**Plate reader growth curves.** For ATc induction experiments, overnight cultures were launched from single colonies in triplicate and diluted 1:200 in TSB broth (biological replicates). After 1 h of growth, ATc was added at a final concentration of 0.5 μg ml⁻¹ and the growth was measured every 5 min. For mitomycin C induction experiments, overnight cultures were launched from single colonies in triplicate and diluted 1:200 in HIB broth (biological replicates). After 1.5 h of growth, mitomycin C was added at a final concentration of 0.5 μg ml⁻¹ where applicable. Measurements were taken every 5 min. For *F. nucleatum* infections, overnight cultures were launched from single colonies in triplicate and diluted 1:200 in HIB supplemented with CaCl₂ 5 mM (biological replicates). After 1 h 25 min of growth, an average of 0.5 μg ml⁻¹ was measured for three representative cultures to estimate MOI. Aliquots were then loaded into 96-well plates along with *F. nucleatum* at the appropriate MOI (10 or 100), where applicable. Measurements were taken every 5 min. For *F. nucleatum* infections, overnight cultures were launched from single colonies in triplicate and diluted 1:200 in HIB supplemented with CaCl₂ 5 mM (biological replicates). An average MOI of 10 on the basis of this value, where applicable. Measurements were taken every 5 min.

**Plasmid-curing assay.** RN4220 cells harbouring both the pGG3 CRISPR-Cas plasmid and the pWJ153 target plasmid were cultured in TSB supplemented with chloramphenicol (10 μg ml⁻¹) to a D₀₅₆₀ nm of 0.45. After splitting the culture into two, transcription across the target was induced for one of the cultures via the addition of ATc to a final concentration of 0.25 μg ml⁻¹. Aliquots of cells were harvested before and after (0) and after (1, 2, 3, 4, 5 and 6 h) the time of induction. After purification of DNA, plasmids were linearized with the common single cutter BamHI and subjected to agarose gel electrophoresis. In parallel, serial dilutions of both cultures were prepared in triplicate for each time point and plated on TSA plates supplemented with chloramphenicol and erythromycin or chloramphenicol alone, for quantification of antibiotic-resistant colony-forming units (technical replicates).

31. Moore, S. D. & Prevelige, P. E. Jr A P22 scaffold protein mutation increases the robustness of head assembly in the presence of excess portal protein. *[J. Virol.](http://dx.doi.org/10.1128/JVI.76.10.10245-10255.2002)* (2002).
32. Hatoum-Aslan, A., Marin, I., Samai, P. & Marraffini, L. A. Genetic Characterization of antiplasmid immunity through a type III-A CRISPR-Cas system. *[J. Bacteriol.](http://dx.doi.org/10.1128/JB.196.8.310-317.2014)* (2014).
33. Helle, L. et al. Vectors for improved Tet repressor-dependent gradual gene induction or silencing in *Staphylococcus aureus*. *[Microbiology](http://dx.doi.org/10.1099/mic.0.043802-0)* 157, 3314–3323 (2011).
34. Hornouchi, S. & Weisblum, B. Nucleotide sequence and functional map of pE194, a plasmid that specifies inducible resistance to macrolide, lincosamide, and streptogramin type B antibiotics. *[J. Bacteriol.](http://dx.doi.org/10.1128/JB.150.8.804-814.1982)* (1982).
35. Bae, T. & Schneewind, O. Allelic replacement in *Staphylococcus aureus* with inducible counter-selection. *[Plasmid](http://dx.doi.org/10.1016/j.plasmid.2006.06.006)* 55, 58–63 (2006).
36. Horton, R. M. In vitro recombination and mutagenesis of DNA: SOEing together tailor-made genes. *[Methods Mol. Biol.](http://dx.doi.org/10.1007/978-1-59745-127-7) 15, 251–261 (1993).*
Extended Data Figure 1 | Characterization of spacer 32T isolates lysogenized with \( \Phi NM1\)-Erm\( ^R \). a. \( \Phi NM2 \) sensitivity assay. Eight randomly selected \( \Phi NM1\)-Ermed lysogen clones were re-streaked through the indicated \( \Phi NM2 \)-seeded region from top to bottom (1–8); C, sensitive \( \Phi NM1\)-Erm\( ^R \) lysogen harbouring the pGG3 control plasmid. b. PCR amplification of the CRISPR array (upper panel) and spacer 32T target region (lower panel) for the strains tested in a. The pGG3 control lysogen (C) lacks a phage-targeting spacer in its CRISPR array. Size markers of 1 kb and 0.5 kb are indicated. All eight PCR products for the target region were sequenced by the Sanger method and no mutations were found (data not shown). c. Plaque-forming potential of filtered supernatants from spacer 32T lysogen overnight cultures inoculated in triplicate. Plaque-forming units were enumerated on soft agar lawns of RN4220 harbouring either the pGG3 control (C) or spacer 32T CRISPR plasmids. Dotted line represents the limit of detection for this assay. d, \( \Phi NM2 \) plaquing efficiency on soft agar lawns of an additional six randomly selected \( \Phi NM1\)-Erm\( ^R \) lysogen clones isolated during infection of RN4220/spacer 32T (9–14); a \( \Phi NM1\)-Erm\( ^R \) lysogen harbouring the pGG3 control plasmid was also tested (-C/L). Plaques efficiency on the non-lysogenic indicator strain harbouring pGG3 is shown for comparison (-C). Error bars, mean ± s.d. (\( n = 3 \)).

a, b, Single experiments performed for 8 of 32 isolates.
Extended Data Figure 2 | Characterization of spacer 32T isolates lysogenized with ΦNM4. a, Visualization of TB4-derived strains grown on egg-yolk agar. Integration of ΦNM4 within the geh locus of TB4 results in strongly reduced lipase secretion, enabling a screen for ΦNM4 lysogenization with spacer 32T. Right-most lanes display two lipase-negative isolates from the lysogenization screen; picture is representative of five technical replicates for each isolate. b, ΦNM2 sensitivity assay. Strains shown in a were re-streaked through the indicated ΦNM2-seeded region from top to bottom. The pGG3 lysogen and spacer 32T non-lysogen in the two left-most lanes serve as sensitive and insensitive controls, respectively. Picture is representative of three technical replicates for each isolate. c, ΦNM2 plaquing efficiency on soft agar lawns of the strains analysed in a and b. 32T(L1) and 32T(L2) refer to the two ΦNM4 lysogens isolated during the spacer 32T egg-yolk screen. Error bars, mean ± s.d. (n = 3).
Extended Data Figure 3 | Visualization of ΦNM1 transcription profiles 6, 15, 30 and 45 min after infection (MOI 20). Rightward and leftward expression values are plotted as blue and fuchsia lines, respectively, in reads per million (RPM). Position of relevant spacer targets are indicated with vertical solid lines. The dotted line with arrowheads marks the position of the central promoter. To improve readability, all curves were smoothened by plotting the average reads per million values over a 500 bp sliding-window. To the left of the central promoter, rightward expression is comparable to leftward expression by 30 min after infection, consistent with the strand-independent targeting observed for this region.
Extended Data Figure 4 | Type II CRISPR-Cas targeting in S. aureus prevents both lytic and lysogenic infection. a, Plaquing efficiency of ΦNM1 and ΦNM1/E on lawns of RN4220 harbouring type II-A CRISPR-Cas plasmids as indicated. The parental vector, pDB184, serves as a non-targeting control. b, ΦNM1-Erm<sup>β</sup> lysogenization of RN4220 harbouring either the spacer 43B-tII, 4B-tII, or non-targeting type II-A CRISPR plasmids. c, ΦNM2 sensitivity assay for seven randomly selected ΦNM1-Erm<sup>β</sup> lysogen clones isolated during infection of RN4220/spacer 43B-tII (1–7). For comparison, a resistant non-lysogen harbouring the spacer 43B-tII plasmid and a sensitive lysogen harbouring the pDB184 plasmid were included as controls (respectively, C<sup>+</sup> and C<sup>−</sup>). Picture represents a single experiment for 7 of 22 isolates. d, ΦNM2 plaquing efficiency on soft agar lawns for an additional six randomly selected ΦNM1-Erm<sup>β</sup> lysogen clones isolated during infection of RN4220/spacer 43B-tII (8–13); a ΦNM1-Erm<sup>β</sup> lysogen harbouring the pDB184 plasmid is also tested (-C/L). For comparison, plaquing efficiency of ΦNM2 on the non-lysogenic indicator strain harbouring pDB184 or the targeting spacer 43B-tII plasmid are also shown (−C/L). Picture represents a single experiment for 6 of 22 isolates. e, Agarose gel electrophoresis of plasmid DNA purified from isolates 8–13 and the parental spacer 43B-tII strain (C). The symbols + or − indicate the presence or absence of treatment with the BamHI restriction enzyme, which produces two bands for the wild-type spacer 43B-tII plasmid: 5367 bp and 3972 bp. Size markers correspond to 10 kb, 3 kb and 0.5 kb bands of the 1 kb DNA ladder from NEB. f, Colony PCR spanning the type II CRISPR array for isolates 8–13. Spacer 43B-tII plasmid DNA was used as a template for the control (C). Size markers of 3 kb and 0.5 kb are indicated. g, Colony PCR spanning the target region for isolates 8–13 and a ΦNM1-Erm<sup>β</sup> lysogen harbouring the pDB184 control plasmid (C). Isolates 10 and 11 harbour identical deletions within the prophage that remove the target region (see below). Size markers of 3 kb and 0.5 kb are indicated. The presence of attL and attR prophage integration arms was also verified independently for each isolate using PCR (data not shown). h, Location of the 16,985 bp deletion identified within the prophage harboured by isolates 10 and 11 (shaded grey box). The location and orientation of the erm<sup>C</sup> insertion cassette is also shown (blue arrow). Deletion was mapped by primer walking. An ~9.1 kb product spanning the deletion was ultimately amplified using primers oGG6 and oGG241, and the deletion junction was sequenced by the Sanger method using oGG245. A perfect 14 bp direct repeat micro-homology flanks the deletion. i, Plaque-forming potential of overnight culture supernatants from isolates 8, 10 and 11. Supernatants were plated by the soft agar method with RN4220 cells harbouring the non-targeting pDB184 control plasmid as an indicator strain. Supernatants were also plated with spacer 43B-tII targeting lawns, yielding no detectable plaque-forming units. Isolate 8 appears to exhibit wild-type levels of spontaneous prophage induction (compare with pGG3 control in Fig. 4a). No plaque-forming units were detected from the supernatants of isolates 10 and 11 whatsoever, presumably resulting from their deletion of genes essential for prophage induction, including the ORF 43 major capsid protein. Dotted line represents the limit of detection for this assay. Error bars, mean ± s.d. (n = 3). e–g, Single experiments for 6 of 22 isolates.
Extended Data Figure 5 | Visualization of transcription profiles for ΦNM1γ6 and the ΦNM1 prophage. Graphical presentation is the same as in Extended Data Figure 3. a, ΦNM1γ6 transcription profiles 6 and 15 min after infection (MOI 20). Comparison with ΦNM1 samples at equivalent time points (Extended Data Fig. 3) reveals a marked decrease in leftward transcription to the left of the central promoter region. We calculated the fold-change in reads per million between ΦNM1 and ΦNM1γ6 samples 15 min after infection. Leftward expression within the region bounded by the start of the genome and the central promoter was reduced 32-fold, while only a fourfold reduction in leftward expression was observed overall. Meanwhile, rightward expression was reduced fourfold both overall and in this region. This suggests an approximately eightfold net reduction in leftward transcription originating from the central promoter. b, ΦNM1 prophage transcription profiles. Strong leftward transcription originates from the central promoter and a few upstream regions, which are presumed to be important for lysogenic maintenance. Rightward transcription was weaker than leftward transcription as expected, but not absent. Given the strength of rightward transcription observed during the lytic cycle (Extended Data Fig. 3), however, this transcription may originate from a subpopulation of cells undergoing prophage induction, rather than the stable lysogen majority.
Extended Data Figure 6 | Detection of transcription across target insertions for the pNes(wt-d) and pNes(wt-i) plasmids. For each target plasmid, reverse transcription was performed in both directions with DNase-treated total RNA from RN4220 cells harbouring the indicated plasmids, using either forward or reverse primers for cDNA synthesis in two separate reactions. PCR was performed on cDNA products, or plasmid DNA templates for control (C) lanes. The symbols + or − indicate the presence or absence of reverse transcriptase enzyme in the reverse transcription reaction mixture used for PCR. Size markers of 500 bp and 100 bp are indicated. Picture is representative of a single technical replicate.
Extended Data Figure 7 | Reverse CRISPR-immunity assays using inverted chromosomal target insertions or type II CRISPR-Cas plasmids. Values represent the average transformation efficiency of three transformations in colony-forming units per microgram of plasmid DNA transformed. ATc, anhydrotetracycline at 0.5 μg ml⁻¹. Dotted lines represent the limit of detection for these assays. a, Reverse CRISPR-immunity assays using inverted target vector insertions and spacer 43T or 43B plasmid DNA. Inversion of the attP motif (Inv-attP⁻) for forward and reverse insertion vectors causes integration in the opposite orientation relative to the chromosomal origin of replication. b, Reverse CRISPR-immunity assays using type II-A CRISPR plasmid DNA to transform strains from Fig. 3b. The pDB184 parent vector serves as a non-targeting control. Error bars, mean ± s.d. (n = 3).
Extended Data Figure 8 | Infection with ΦNM1 in liquid culture. Growth curves of RN4220 cells harbouring the indicated CRISPR plasmids were infected at time zero with ΦNM1 at an MOI of 10 (a) or 100 (b). Growth of uninfected RN4220/pGG3 cultures is also shown (dotted red lines).
Extended Data Figure 9 | Immunity to $\Phi$NM1γ6 in liquid culture is unaffected by the presence of a tolerated chromosomal target. Growth curves of the indicated chromosomal insertion strains from Fig. 3 harbouring either spacer 43T or pGG3 CRISPR plasmids, in the absence (dotted lines) or presence (solid lines) of $\Phi$NM1γ6 addition at an MOI of 10. Black arrow denotes the time of phage addition; no ATc induction is used in this assay. The presence of a chromosomal target for spacer 43T has no discernable effect on culture growth during spacer 43T-mediated immunity to $\Phi$NM1γ6 (compare solid green and blue lines).
Extended Data Figure 10 | Inducible curing of a target plasmid. 

a, Diagram of plasmids used in the plasmid-curing experiment. The pGG3 CRISPR plasmid harbours a single spacer (‘spc1’) targeting a sequence (‘prtspc1’) inserted downstream of the Pxyl/tet*–inducible promoter in pWJ153. 

b, Agarose gel electrophoresis of linearized plasmid DNA purified both from anhydrotetracycline-treated (+ATc) and untreated (−ATc) cultures at the indicated time points. Size markers of 10 kb, 5 kb and 4 kb are indicated. Picture is representative of a single technical replicate.

c, Colony-forming units recovered from cultures analysed in b at each time point. Cells were plated with selection for either Cm<sup>+</sup> colony-forming units (green) or Cm<sup>+</sup>, Erm<sup>+</sup> colony-forming units (blue). Targeting of the pWJ153 plasmid via induction with ATc (filled circles) is accompanied by a severe drop in erythromycin-resistant colony-forming units relative to untreated cultures (open circles). Error bars, mean ± s.d. (n = 3).