Bacteria exploit viral dormancy to establish CRISPR-Cas immunity

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Summary

CRISPR-Cas systems provide prokaryotes with adaptive immunity against foreign genetic elements, including bacteriophages, by recording DNA-based immunological memories of infection called “spacers.” How cells without preexisting immunity survive a rapid lytic infection long enough to acquire a new spacer and utilize it for defense remains a mystery. Here, we show that bacteria exploit the alternative dormant or “lysogenic” lifecycle of temperate phages to establish CRISPR-Cas immunity. During a phage infection, immunization rates are significantly enhanced in the subpopulation of cells entering lysogeny compared to those undergoing lysis. Furthermore, in the absence of a foreign threat, bacteria can acquire spacers targeting prophages residing within the chromosome. In this case, self-targeting by Cas9 promotes curing of the prophage allowing immunized cells to avoid autoimmunity. The preferred acquisition of spacers during the establishment and maintenance of lysogeny may explain why most spacers in natural bacterial isolates target temperate phages.

Introduction

Bacterial CRISPR-Cas adaptive immune systems acquire short DNA-based memories or "spacers" from invading genetic elements, including bacteriophages[1,2]. Spacers are transcribed into CRISPR RNA (crRNA) guides that direct Cas nucleases to cleave or inhibit matching targets during subsequent infections[3-5]. In the classical model of spacer acquisition, new spacers are created in rare surviving cells during the decimation of a bacterial population by a phage undergoing lytic replication[6]. Without pre-existing immunity, how do cells survive a lytic infective cycle long enough to acquire a spacer, transcribe it into a crRNA guide, load the crRNA into Cas9, and then find and cleave all of the rapidly replicating phage targets? While it is formally possible that CRISPR immunization can outpace the lytic cycle, another possibility is that spacer acquisition succeeds in cells infected by "defective" phages that fail to execute the lytic cycle. For example, DNA damage to the phage genome caused by chemical mutagens, anti-phage defense systems, or nucleases involved in bacterial DNA repair can all enhance spacer acquisition and survival[7-10]. Whether cells can be immunized against phages without such defects is not known.

Most natural spacers with known targets are derived from temperate phages[11,12], which upon infection undergo a partially stochastic decision to enter the lytic pathway, resulting in phage replication and cell lysis, or the lysogenic pathway, whereby a repressor silences lytic gene expression and the phage integrates into the bacterial genome as a dormant prophage[13]. Expression of the repressor is maintained in lysogens until cells experience DNA damage, which causes cleavage of the repressor and allows prophages to excise and resume the lytic cycle. Even in the absence of a DNA damaging agent, some cells undergo spontaneous prophage induction events, likely as a result of DNA damage from free radicals or replication errors[14]. These extracellular phage particles can then 'superinfect' uninduced neighbors that are protected from lysis by the lytic repressor of the cognate prophage. Because lysogeny occurs more frequently than spacer acquisition, nearly all spacer acquisition studies utilize obligate lytic phages or mutant temperate phages that are locked in the lytic cycle[2,7,15-20]. It therefore remains unclear whether CRISPR-Cas systems can leverage the lytic repressor to acquire spacers under non-lytic conditions.

One obvious roadblock to acquiring spacers from prophages or superinfecting phages is that cells would be confronted with self-targeting of the cognate prophage by Cas effectors. In the DNA-targeting CRISPR-Cas systems (type I, II, V), spacers that target the chromosome often result in lethal dsDNA breaks[21-25]. While prophage-targeting spacers have been identified in a subset of sequenced genomes[26-29], the spacers are typically nonfunctional due to an incorrect PAM, expression of a prophage-encoded anti-CRISPR protein, or inactivation of the CRISPR-Cas system[30-32]. Even a prophage-targeting "priming" spacer, which induces very low levels of cleavage by Cas effectors, is not well tolerated and can drive loss of the CRISPR-Cas system[33]. Therefore, cells immunized against a prophage would require mutation or removal of the prophage target. In one intriguing study on the human gut commensal *Roseburia intestinalis*, spacers were acquired that matched a prophage present in the parent strain after several weeks of mouse gut colonization, and the corresponding prophage was absent, or "cured," thus avoiding autoimmunity[34]. However, the new spacers were observed only after the prophage had mutated into an irrepressible lytic variant that lysed the majority of the bacterial population. It is therefore unclear if the spacers were generated from the lytic variant, similar to the canonical model, or from the prophage in the days preceding the lytic event.

Here, we study the *Streptococcus pyogenes* CRISPR-Cas9 system in a native and heterologous host and provide the first experimental evidence that temperate phages are the preferred substrates for spacer acquisition through two main routes: first, cells are immunized against temperate phages at higher rates compared to their nearly identical lytic counterparts, suggesting that the lysis-lysogeny decision is intimately linked to CRISPR-Cas immunity. Second, cells are continuously immunized against endemic prophages already

present within a clonal population, greatly expanding the window for memory formation beyond the lytic sweeps of the canonical model. Spacers are acquired during superinfections by phage particles released from spontaneously lysed neighbors. Cas9 can then facilitate "induced curing" of the targeted prophage, enabling cells to avoid auto-immunity. Altogether, we propose a new paradigm for spacer acquisition whereby CRISPR-Cas systems can exploit multiple stages of the temperate phage lifecycle to generate immunological memories under non-lytic conditions. Finally, by antagonizing temperate phages and actively removing prophages, which often carry antibiotic resistance and virulence genes, we highlight new mechanisms through which CRISPR-Cas systems influence bacterial evolution and pathogenesis.

Results

The decision to enter lysogeny enhances CRISPR-Cas immunization rates

We sought to study spacer acquisition from temperate phages in the type II-A CRISPR-Cas system from *S. pyogenes* strain SF370, which encodes the effector Cas9 that is most commonly used in CRISPR-based gene editing technologies. In recent years, the first studies demonstrating spacer acquisition in the native host have employed bacteriophage A1[35,36]. While A1 encodes an integrase and lytic repressor, we were unable to obtain A1 lysogens. We therefore turned to a heterologous system in which the *S. pyogenes* type II-A CRISPR-Cas locus is expressed from a plasmid in *Staphylococcus aureus* strain RN4220 which lacks prophages. This system benefits from highly tractable genetic tools and a large set of well-characterized phages, and it has been used extensively to study the fundamental principles of CRISPR-Cas9 biology[8-10,18,23,37-42].

Spacer acquisition is typically studied by performing an "immunization assay" in which cells are infected with an untargeted phage, plated on soft agar lawns, and the number of surviving colonies that have acquired a new phage-targeting spacer are counted[2]. This assay is not practical for temperate phages, because survival by lysogeny occurs more frequently than spacer acquisition by several orders of magnitude. We therefore engineered the temperate phage ϕNM1 to express the counterselectable gene *pheS*** (ϕNM1-pheS) in order to inhibit the growth of lysogens during plating (Fig. 1a). *pheS*** encodes a mutant phenylalanine synthetase that misincorporates 4-chlorophenylalanine (4CP) into nascent peptides, resulting in growth arrest in the presence of 4CP (Fig. S1a)[43]. We confirmed that ϕNM1-pheS exhibits plaque morphology and spontaneous induction rates comparable to those of ϕNM1 (Fig. S1b) but that growth of lysogens is inhibited in the presence of 4CP (Fig. S1c).

We next created several obligate lytic variants of ϕNM1-pheS similar to those used in most spacer acquisition studies: a nonsense truncation mutant (ϕNM1-pheSγ7) or a missense point mutant (ϕNM1-pheSγ9) of the lytic repressor, or a truncation of the entire lysogenic cassette of the phage (ϕNM1γ8) (Fig. 1a). We verified that lysogeny was undetectable in each lytic mutant (Fig. 1b) while lytic replication was unaffected (Fig. S1d). We next performed a CRISPR-Cas immunization assay on plates containing 4CP in cells expressing a naïve CRISPR-Cas system with a single repeat and no pre-existing spacers. To facilitate the detection of immunization events, we deleted the autorepressor *tracr-L* (*Δtr-L*), resulting in higher rates of CRISPR-Cas expression[41]. Such CRISPR-Cas overexpressing mutants occur naturally and are enriched during phage infections[35]. Strikingly, CRISPR immunization rates against the temperate parent ϕNM1-pheS approached ~1/1000 infected cells, a 5-10-fold increase compared to the lytic mutants (Fig. 1c, S1e-f). Given that lysogeny is chosen in only a minority of cells (~1/100), the change in the overall immunization rate suggests that the relative immunization rate in cells undergoing lysogeny must be higher than those undergoing lysis by several orders of magnitude.

The decision to lysogenize results in silencing of lytic gene expression followed by integration of the phage genome into the bacterial chromosome. To understand whether phage integration plays a role in spacer acquisition, we repeated the spacer acquisition assay in *ΔattB* cells that lack the integration site for ϕNM1-pheS. We confirmed that in *ΔattB* cells, ϕNM1-pheS does not integrate at its typical location nor elsewhere in the genome but instead forms replication-incompetent episomal pseudolysogens that are diluted out over time (Fig. S1g). In *ΔattB* cells, immunization rates remained higher for the temperate parent compared to the lytic mutants (Fig. 1d, S1h), suggesting that prophage integration does not explain the increased immunization rates for ϕNM1-pheS.

We hypothesized that the initiation of lysogeny could enhance immunizations by (i) increasing the rate of spacer acquisition and/or (ii) promoting the survival of cells undergoing spacer acquisition. To distinguish between these possibilities, we measured raw spacer acquisition rates in liquid cultures before the end of the lytic cycle to remove the effects of survival on spacer abundance. At 10, 30 and 45 minutes post-infection, we performed a PCR that enriches for rare spacer acquisition events[18]. Notably, spacer acquisition rates were not elevated in ϕNM1-pheS compared to the ϕNM1-pheSγ7 lytic mutant (Fig. 1e). This supports the model that entry into lysogeny instead enhances the survival of cells that have acquired new spacers by removing the pressure imposed by the lytic cycle.

Prophages are hotspots for spacer acquisition

Cells infected with a novel temperate phage can survive by spacer acquisition or lysogeny. The above results suggest that CRIPSR-Cas immunizations are enhanced in cells that initiate but do not complete lysogeny. We next asked whether fully lysogenized populations can be immunized against resident prophages. We chose to study this question in *S. pyogenes* strain SF370 which harbors one active prophage (370.1) and three inactive prophage-like elements (370.2-4)[44] (Fig. 2a). We also utilized a heterologous system in which the *S. pyogenes* CRISPR-Cas locus is expressed from a plasmid in *S. aureus* strain Newman, which harbors three active prophages (ϕNM1-2, ϕNM4) and one inactive prophage-like element (ϕNM3)[45] (Fig. 2b). Both systems were CRISPR-Cas overexpressers (*Δtr-L*) and contained a naïve CRISPR array with a single repeat.

The strains were passaged eight times, twice daily, and newly acquired spacers were enriched by PCR and subjected to Next Generation Sequencing. Functional spacers, situated upstream from the correct 5'-NGG-3' protospacer adjacent motif (PAM) required for Cas9 cleavage, or total spacers were aligned to the chromosome and plotted according to genome position (Fig. 2c-d; Fig. S2a-d). Consistent with previous reports, many spacers were acquired from the terminus of the bacterial chromosome[9,46]. These represent a snapshot of cells likely undergoing lethal self-targeting events. Strikingly, we observed spacer acquisition hotspots spanning all active prophages in both strains, and in contrast to the terminus, the percentage of prophage-targeting spacers with correct PAMs increased during passaging (Fig. 2g-h). We also observed prophage hotspots in passaged *S. aureus* strains singly lysogenized with the Newman phages ϕNM1 or ϕNM4 or an unrelated phage ϕ11 (Fig. S2e-g). In SF370, the intensity of the 370.1 hotspot was fairly consistent across replicates (Fig. 2c, Fig. S2a), while in *S. aureus*, the hotspot intensities were consistent between the active prophages within a given experiment but more variable when comparing biological replicates (Fig. 2d, Fig. S2d). Taken together, these data indicate that spacers from prophages are enriched within bacterial populations in a manner distinct from the surrounding bacterial chromosome.

We next sought to understand when prophage-targeting spacers are acquired. In both *S. pyogenes* SF370 and *S. aureus* Newman, spacer acquisition hotspots were present only for the prophages that have been previously shown to actively generate infective particles (Fig. 2c-d)[44,45]. We confirmed that both strains produce infective phage particles spontaneously, in the absence of an exogenous DNA damaging agent (Fig. S2h-i), likely due to sporadic instances of DNA damage from intracellular sources[47,48]. We hypothesized that new spacers targeting prophages present in the ancestor of the clonal population could be acquired (i) in a cell undergoing spontaneous prophage induction or (ii) in a superinfected cell following the lysis of a spontaneously induced neighbor. To distinguish between these scenarios, we repeated the passaging experiments in the presence of sodium citrate, a divalent cation chelator that prevents phage adsorption and thus superinfection (Fig. S2j-k)[49]. In both *S. aureus* and *S. pyogenes*, prophage-derived spacers did not increase during passaging in the presence of sodium citrate (Fig. 2e-h), suggesting that spacers are primarily acquired from phages released by neighbors undergoing spontaneous prophage induction.

Lysogenized bacterial populations can be immunized against endemic prophages

Many of the cells in liquid culture with prophage-targeting spacers (Fig. 2c-d) are likely experiencing autoimmunity and cell death, similar to those with spacers targeting the chromosome terminus. We reasoned that if some cells could instead resolve autoimmunity and survive, these prophage-derived spacers could provide the population with immunity to novel lytic threats that share prophage sequence similarity. We therefore sought a means to select for live cells that had been immunized against a prophage present in the ancestor of the clonal population.

Fortuitously, a selection agent arose naturally during passaging of several *S. aureus* Newman cultures in the form of lytic mutants that were not repressed by any of the endogenous prophages and caused lysis of the culture (Fig. S3a-c). Following each instance of lysis, the culture recovered after one or two additional rounds of passaging (Fig. S3b). To understand how these cells survived, we analyzed the CRISPR arrays in one recovered culture by NGS and found a dominant new spacer that targeted a region in the lysogenic cassette of ϕNM1 (Fig. S3d) as well as the irrepressible phages within that culture (Fig. S3b-c). By streaking the recovered culture to single colonies, we found that 2/8 survivors had acquired a ϕNM1-targeting spacer, with the remainder likely representing surface mutants to which the phage cannot adsorb. For the two survivors with an ϕNM1-targeting spacer, we examined the target site by PCR and found that the ϕNM1 prophage had been completely excised from the genome, leaving behind an intact *attB* site (Fig. S3e). These data show that cells can acquire spacers from prophages already established within a clonal population and avoid autoimmunity by curing the targeted prophage, similar to a recent study in *R. intestinalis*[34].

The spacers in survivors of the lytic sweep could have been acquired (i) from the lytic mutant itself during lysis of the culture, similar to the canonical model of spacer acquisition, or (ii) from the spontaneously induced prophages in the passages preceding the generation of the lytic phage. To ask whether functional spacers can be generated from an induced prophage, i.e. from a temperate phage matching a prophage present in a clonal population, we designed a simplified heterologous system in which the *S. pyogenes* CRISPR-Cas system was expressed in *S. aureus* RN4220 cells lysogenized with a single prophage ϕNM1 (Fig. 3a). Cultures were grown to stationary phase and infected with ϕNM1, simulating infection events from spontaneously induced neighbors. Cultures were then challenged with ϕNK4, a lytic mutant from a Newman passaging experiment (Fig. S3b) that is highly similar to ϕNM1 but not repressed by a ϕNM1 lysogen (Fig S3a, Fig. S4a). ϕNK4 was added at a high

MOI of 50 which renders new spacer acquisition from ϕNK4 unlikely and instead favors the selection of cells with preexisting ϕNM1/ϕNK4-targeting spacers (Fig. S4b). Surviving colonies were enumerated and a subset were characterized by PCR to verify spacer acquisition and check for prophage curing. Infections with ϕNM1 resulted in a 4-fold increase in cell survival following selection by ϕNK4 (Fig. 3b, S4c). Of these survivors, 22/24 had acquired spacers (Fig. S4d), and 8 colonies were further characterized to confirm that the ϕNM1 prophage was cured (Fig. S4e). These data suggest that under non-lytic conditions, populations can use their induced endemic prophages as vaccines against future lytic threats.

The *S. pyogenes* passaging experiments did not yield irrepressible phages for use as a selection agent for live cells with prophage-derived spacers. We therefore developed an alternative strategy to ask whether *S. pyogenes* populations can be immunized against endemic prophages under non-lytic conditions. We reasoned that such cells might resolve autoimmunity by curing the targeted prophage, similar to the heterologous system. We therefore sought a means to select for prophage-cured cells, within which we hoped to find live cells that had acquired a spacer from the cured prophage. We used an *S. pyogenes* strain in which prophage 370.1 harbors a streptomycin-sensitivity gene for counter-selection (SF370.1-str)[50]. We confirmed that plating on streptomycin selected for prophage curing events (Fig. S4f). SF370.1-str cells expressing the wild-type or *Δtr-L* CRISPR-Cas system were passaged twice daily for 8 total passages to allow for spacer acquisition and prophage curing. After passage 8, the culture was diluted into streptomycin-containing media and grown for an additional 24 hours to enrich for cells cured of 370.1 (Fig. 3c). Newly acquired spacers from passage 8 and passage 8+streptomycin cultures were enriched by PCR and subjected to NGS. Indeed, 370.1-targeting spacers were not only present but significantly enriched in streptomycin-treated cultures relative to untreated cultures for both *Δtr-L* (Fig. 3d) and wild type (S4g-h), suggesting that prophage spacer acquisition and prophage curing are functionally linked. We next isolated single colonies from the *Δtr-L* passaged and streptomycin-treated culture and examined the CRISPR array and 370.1 locus by PCR. Of 32 streptomycin-resistant colonies, one had acquired a spacer targeting 370.1 (Fig. 3e), confirming the presence of live cells with functional, prophage-targeting spacers in populations of *S. pyogenes* that had not encountered a lytic threat.

Cas9 self-targeting can induce prophage curing

In both *S. aureus* and *S. pyogenes*, we recovered cells that (i) were immunized against a prophage present in the ancestor of the clonal population and (ii) avoided self-targeting by curing the prophage. We envisioned two pathways of prophage immunization and curing. Prophage curing could occur spontaneously as has been observed for several hosts and phages [34,50] ("spontaneous curing"). Spacers could then be acquired from the pool of extracellular, induced prophages without risking self-targeting. Alternatively, spacers could be acquired from the same extracellular pool of induced prophages in cells that still harbor the identical prophage within the chromosome. In this case, we wondered whether the ensuing self-targeting by Cas9 could serve as a DNA damage signal to induce and excise the prophage ("induced curing").

To measure spontaneous and induced curing rates in the heterologous *S. aureus* system, we again used a strain harboring ϕNM1-pheS which allowed us to select for spontaneously cured cells by plating on 4CP. To simulate induced curing, we introduced a low-copy plasmid expressing a non-targeting, chromosomal-targeting, or ϕNM1-targeting spacer from an anhydrotetracycline (aTc)-inducible promoter (Fig. S5a) and a second plasmid expressing Cas9 and *tracrRNA*. Uninduced overnight cultures plated on 4CP resulted in a 3-log decrease in colony-forming units (CFUs) regardless of the uninduced spacer (Fig. 4a, S5b), and we confirmed that the

majority of survivors had been cured of the ϕNM1-pheS prophage by PCR (Fig. S5c). This suggested that ~1/1000 cells were spontaneously cured of ϕNM1-pheS after one overnight growth. We also plated cultures on aTc to measure induced curing rates and observed that the non-targeting spacer had no effect on CFUs while the chromosome-targeting and prophage-targeting spacers showed a similar, roughly 3-log decrease in survival (Fig. 4a, S5b). Survivors of the chromosome-targeting spacer were never cured of the prophage while survivors of the ϕNM1-targeting spacer were mostly cured (Fig. S5c). However, it is unclear if curing occurred spontaneously before expression of the spacer or if it was induced following self-targeting, given that the spontaneous and induced curing rates were similar in this system.

To simultaneously study spontaneous and induced curing in the same *S. pyogenes* cells, we utilized a strain harboring 370.1-str for which spontaneous curing can be measured by plating on streptomycin. To simulate induced curing, we introduced a low-copy plasmid expressing a non-targeting, chromosomal-targeting, or 370.1-targeting spacer, from an aTc-inducible promoter (Fig. S5a). We plated overnight cultures in the presence of streptomycin and found that CFUs decreased by ~10,000-fold (Fig. 4b, S5d). All streptomycin-treated survivors were cured of 370.1 (Fig. S5e), suggesting that ~1/10,000 cells were spontaneously cured, considerably fewer than in the heterologous system. To measure induced curing, we plated the same overnight cultures in the presence of aTc to induce spacer expression. As expected, the non-targeting spacer did not reduce CFUs, while the chromosomal-targeting spacer caused a significant reduction in CFUs with ~1/500 cells surviving (Fig. 4b, S5d). These survivors all maintained the non-targeted 370.1 prophage and survived by introducing escape mutations or chromosomal rearrangements in the target site (Fig. S5e-f). Strikingly, a considerably higher proportion of cells expressing the 370.1-targeting spacer survived targeting (~1/30) (Fig. 4b, S5d), and we verified that most of these survivors had cured 370.1 by PCR (Fig. S5e). This suggests that (i) prophage targeting by Cas9 can indeed lead to high rates of induced curing and (ii) induced curing may be the dominant prophage immunization pathway in *S. pyogenes*, given that the rates of induced curing are more than an order of magnitude higher than those of spontaneous curing.

In the induced curing model, DNA damage from self-targeting could cause excision of the prophage through the canonical prophage induction pathway, which includes activation of RecA, cleavage of the lytic repressor, and expression of an excisionase that reverses the directionality of the integrase. Alternatively, the cleaved prophage could be repaired by host-encoded dsDNA break repair pathways, resulting in recombination or annealing of the prophage *attL* and *attR* sites, thus deleting the prophage and restoring the *attB* site. To distinguish between these possibilities, we deleted *recA* or 370.1 *integrase*, or introduced mutations in the 370.1 lytic repressor predicted to render it non-cleavable (SF370-str^{NC})[51]. We confirmed that all three mutant backgrounds were unable to generate spontaneous nor mitomycin C-induced infectious 370.1 particles (Fig. S5g). In these backgrounds, we measured spontaneous and induced curing rates as before with streptomycin and aTc respectively (Fig. 4c, S5h). Spontaneous curing was reduced nearly 5-fold for the non-cleavable repressor and over an order of magnitude, near the limit of detection, for the *recA* and *integrase* mutants. Similarly, survivors of the 370.1-targeting spacer induced by aTc were reduced by an order of magnitude in all three mutants compared to wild-type, and none demonstrated loss of the 370.1 prophage. These results suggest that prophage-targeting by Cas9 can lead to prophage curing through the canonical, phage-encoded DNA damage-induced excision pathway.

Discussion

Although many natural spacers target temperate phages, no experimental studies have directly examined whether CRISPR-Cas systems can exploit the lysogenic lifecycle. Here, we show that lysogeny provides the CRISPR-Cas machinery with two unique opportunities to acquire spacers (Fig. 4d). First, cells are immunized against temperate phages 5-10-fold more frequently than obligate lytic mutants. Because temperate phages choose lysogeny in only a fraction of infected cells (~1/100 to 1/1000), immunization rates in cells entering lysogeny must be enhanced by several orders of magnitude for the overall rates to be so affected. We show that integration of the prophage is not responsible for this increase, and raw spacer acquisition rates are comparable for both temperate phages and lytic mutants. This suggests that the initiation of lysogeny instead promotes the survival of cells that have acquired new spacers by inactivating the lytic cycle. Prior work has shown that phage particles rendered "defective" by DNA damage similarly enhance CRISPR-Cas immunization rates[7-10]. Even in the absence of DNA damage, wild-type temperate phages may provide cells undergoing spacer acquisition with similar relief from lysis by stochastically triggering their own program for dormancy.

We establish a second pathway enabling immunizations against temperate phages, whereby spacers can be acquired from prophages already established within a clonal population. The substrates for these immunization events are a steady supply of extracellular phages released from cells undergoing spontaneous prophage induction and lysis events. Subsequently, infected neighbors can be immunized through two routes (Fig. 4d). Cells that have previously been spontaneously cured of the cognate prophage can acquire spacers without risking self-targeting and exploiting the lysogeny decision as described above. Alternatively, cells harboring the cognate prophage can acquire spacers during superinfection events. Although the lytic pathway for the incoming phage is immediately silenced, self-targeting of the cognate prophage is lethal in most cells. However, we show that a subpopulation of cells can survive self-targeting by curing the targeted prophage. Similarly, several other groups have demonstrated that ectopic expression of a CRISPR-Cas spacer targeting an integrated mobile genetic element can select for cells in which that element is cured[22,52-54]. However, it is unclear in those cases whether the curing events were caused by self-targeting or had occurred spontaneously and were then selected by self-targeting. By comparing spontaneous and induced curing rates in the same cells using a prophage-encoded counterselectable marker, we show that in *S. pyogenes*, self-targeting actively promotes prophage excision and that this "induced curing" pathway requires the host DNA damage response initiator RecA and the DNA damage-induced prophage excision machinery.

In another study, CRISPR-Cas targeting of a prophage resulted in a fitness cost that selected for CRISPR-Cas deletion mutants instead of prophage curing events[33]. Several methodological differences may account for this disparity, including the authors' use of a type I CRISPR-Cas system and a "priming" spacer that targeted the prophage. Priming spacers contain one or more mismatches in the PAM and/or seed that dramatically reduce Cas effector cleavage, but can instead promote higher levels of spacer acquisition in the vicinity of the target. It is possible that priming spacers do not elicit the levels of DNA damage required for prophage curing, in contrast to the fully matching prophage-targeting spacers we and others have studied. However, by promoting acquisition of additional, fully matching prophage-targeting spacers, priming spacers could lead to prophage curing in a subpopulation of cells. Even if such cells were outnumbered by cells with CRISPR-Cas deletions, a small subpopulation of cured cells with an intact CRISPR-Cas system could enhance the overall fitness of the population if the CRISPR-Cas system is later needed to combat a new phage threat. Whether

CRISPR-Cas systems can drive prophage curing in type I systems, and whether partially matching spacers contribute to prophage curing in type II systems, will require further study.

We propose a new paradigm for spacer acquisition, whereby cells are immunized continuously against resident prophages while the population grows under non-lytic conditions. This greatly expands the window for spacer acquisition beyond the rapid lytic sweeps of the canonical model. It follows that when populations encounter a new temperate phage, a significant number of spacers may be acquired following the initial wave of lysis and establishment of lysogeny, during the many subsequent generations of coexistence between prophage and CRISPR-Cas system. In this way, bacterial populations can sample beneficial prophage genes while generating a subpopulation of cured, immunized cells that can expand when the prophage is no longer useful, or worse, becomes a threat. For example, we show that prophage-derived spacers can protect bacteria from lytic variants of endemic prophages, which we observed during passaging of *S. aureus* Newman cultures and has been observed previously in industrial settings[55,56] and in a study of the human gut commensal *Roseburia intestinalis*[34]. Prophage-derived spacers can also protect cells from novel lytic threats that share some sequence similarity with endemic phages. Furthermore, cells could be readily immunized against phages that share a lytic repressor binding site with a resident prophage but otherwise have little sequence homology. In this case, bacteria could immunize under non-lytic conditions while avoiding much of the lethal self-targeting resulting from endemic prophage immunizations.

In summary, our work shows that CRISPR-Cas systems exploit the temperate phage lifecycle to establish immunological memories, perhaps explaining (i) the bias for temperate phage-targeting spacers in native CRISPR arrays[11] and (ii) the inverse correlation between CRISPR-Cas systems and prophages[57,58]. Whether from a novel temperate phage or an endemic prophage, activity of the lytic repressor enables spacer acquisition to occur under non-lytic conditions, conceptually similar to a vaccine using an attenuated virus. Furthermore, we provide mechanistic insights into the process by which CRISPR-Cas systems actively remove prophages from genomes in a subpopulation of cells. This heterogeneity could allow bacterial populations to sample beneficial genes, which often include antibiotic resistance genes and virulence factors, while providing a mechanism to dispense of prophages that are no longer useful. We anticipate that these findings are generalizable across CRISPR-Cas systems and may apply to other mobile genetic elements.

Resource Availability

Lead Contact

Requests for further information and resources should be directed to and will be fulfilled by the lead contact, Joshua W. Modell, jmodell1@jhmi.edu.

Material Availability

All materials are available upon request without restriction from corresponding author, Joshua Modell.

Data Availability

All data is available upon request from corresponding author, Joshua Modell.

Code Availability

Original code has been deposited on Github and is publicly available at https://github.com/modelllab/keith_etal_2025 as of the date of publication. Further information is available upon request from corresponding author, Joshua Modell.

Acknowledgements

We thank Vince Fischetti for reagents including the PlyC expression vector, Kevin McIver for allelic exchange vectors, and the members of the Molecular Biology & Genetics department and Modell Lab who provided helpful feedback and suggestions. We thank David Mohr and the GRCF High Throughput Sequencing Center for assistance with next-generation sequencing experiments. Funding was provided by a startup package from the Johns Hopkins School of Medicine, NIH NIGMS R35GM142731, the Rita Allen Foundation (90094894), and an NSF Graduate Research Fellowship for NK.

Author Contributions

JWM and NCK designed the research studies. NCK conducted the experiments. RAS assisted with revision experiments and contributed to experimental conceptualization and design. CWE provided *S. pyogenes* strains and reagents.

Declaration of Interests

The authors declare no competing interests.

Figure Captions

Figure 1. Entry into lysogeny enhances CRISPR-Cas immunization rates. a, Diagram of ϕNM1-pheS and lytic derivatives. Genes found in wild-type ϕNM1 are shown in red and pheS** is shown in light blue. int, integrase. xis, excisionase. hyp, hypothetical protein. rep, repressor. **b,** Growth curve of *S. aureus* RN4220 infected with ϕNM1-pheS (WT) or lytic derivatives. Phages were added at an MOI of 10 at T=0. The recovery of the ϕNM1-pheS-infected culture after 24hr indicates lysogeny. **c-d,** CRISPR-Cas immunity assay of *S. aureus* RN4220 WT (c) or *ΔattB* (d) cells expressing the *S. pyogenes Δtr-L* CRISPR-Cas system with a single repeat infected with the indicated phages at MOI=10. Colony-forming units (CFUs) are normalized to the rate of spacer acquisition based on PCR checks from individual colonies shown in Figure S1f and divided by the total number of infected cells. Error bars are standard error. Significance determined by paired t-test (*P < 0.05, **P < 0.01). n=3 biological replicates. **e,** Short timepoint spacer acquisition assay. WT cells expressing the CRISPR-Cas system from Fig. 1c were infected with ϕNM1-pheS or ϕNM1-pheSγ7 at MOI=10. Newly acquired spacers were characterized by NGS at the indicated timepoints post-infection, and the percentage of phage-targeting spacers of all mapped spacers (phage, chromosome, and plasmid) is shown. Error bars are standard error. n=3 biological replicates.

Figure 2. Prophages are hotspots for spacer acquisition. a-b, Schematic of *S. pyogenes* strain SF370 (a) and *S. aureus* strain Newman (b). Prophage elements are denoted by colored segments and active prophages are

indicated with an asterisk. 'R' denotes the type II-A *Δtr-L* CRISPR-Cas locus containing a single repeat within the chromosome (a) or on a plasmid (b). **c-f,** Abundance in reads per million of newly acquired spacers targeting chromosomal DNA (RPM_{chr}) and upstream of a correct 5'-NGG-3' PAM before (Passage 0) and after (Passage 8) passaging of *S. pyogenes* SF370 (c,e) or *S. aureus* Newman (d,f). Media was supplemented with calcium (c,d) or sodium citrate (e,f) during passaging to allow or prevent superinfection, respectively. The location of prophage elements is denoted by colored boxes underneath the x-axes. Dashed line indicates the terminus of the bacterial chromosome. In c and e, representative plots are shown of 2 independent biological replicates. In d and f, representative plots are shown of 3 or 4 independent biological replicates. **g-h,** Quantification of the percentage of spacers targeting prophage DNA for *S. pyogenes* (g) and *S. aureus* (h). Paired treatments for the same biological replicate are represented by the same shape. P-values determined using paired t-test. For g, n=2. For h, n=4 for Passage 0 vs Passage 8 + $CaCl_2$, and n=3 for Passage 0 vs Passage 8 + NaCitrate.

Figure 3. Functional prophage-targeting spacers are acquired in the absence of a lytic threat. a, Potential outcomes of superinfection. A stationary phase culture of a *S. aureus* RN4220::ϕNM1 lysogen expressing the *Δtr-L* CRISPR-Cas locus with a single repeat was split into two separate cultures with one receiving 3 separate superinfections of ϕNM1 at an MOI of ~10. The next day, cultures were challenged with irrepressible ϕNK4 and plated in soft agar to quantify immunized colonies. 'R' and 'S' denote a repeat or an acquired spacer in a CRISPR array, respectively. **b,** Survival of cultures with and without superinfection. CFUs for each replicate are normalized to the rate of spacer acquisition for the surviving population of each experiment shown in Fig. S4d. Error bars are standard error. Significance determined by ratio paired t-test (**P < 0.01). n=3 biological replicates, paired experiments indicated by shape. FC, fold change. **c,** Schematic of passaging experiment for *S. pyogenes Δtr-L* SF370::SF370.1-str, a strain that contains the native 6-spacer CRISPR array and a counter-selectable streptomycin-sensitivity gene within prophage 370.1. After 8 passages, the culture was passaged once in streptomycin-containing media, and survivors were streaked on agar plates. **d,** Abundance (RPM_{chr}) of newly acquired spacers targeting chromosomal DNA after 8 rounds of passaging of *S. pyogenes Δtr-L* SF370::SF370.1-str before (- strep) and after (+ strep) selection in streptomycin-containing media. Prophage 370.1-str is denoted by a red box underneath the x-axis. Dashed line indicates the terminus of the bacterial chromosome. **e**, 1/32 (+ strep) colonies contained a new 370.1-targeting spacer, verified by PCR across the CRISPR array. 'R' and 'S' denote a repeat or an acquired spacer in a CRISPR array, respectively.

Figure 4. Prophage curing occurs through spontaneous and induced pathways. a, Rate of survival for cultures of *S. aureus* RN4220::ϕNM1-pheS harboring a plasmid with the indicated aTc-inducible spacers. Survival on 4CP (grey bars) reflects spontaneous curing events, as nearly all colonies checked by PCR are cured of ϕNM1-pheS (Fig. S5c). Survivors on aTc (white bars) that were cured of ϕNM1-pheS (blue bars) reflect induced curing events. Error bars are standard error. n=3. **b,** Rate of survival for cultures of *S. pyogenes* SF370::SF370.1-str harboring a plasmid with the indicated aTc-inducible spacers. Survival on streptomycin (grey bars) reflects spontaneous curing events, as all colonies checked by PCR are cured of 370.1 (Fig. S5e). Survivors on aTc (white bars) that were cured of 370.1 (red bars) reflect induced curing events. Error bars are standard error. n=4. **c,** Similar to (b) but using cultures of *S. pyogenes* SF370::SF370.1-str with the indicated mutations. *repNC*, non-cleavable 370.1 repressor mutant; *Δint*, in-frame deletion of 370.1 integrase; *ΔrecA*, in-frame deletion of host *recA*. Error bars are standard error. n=4. **d,** Model. Immunizations against novel temperate phages are enhanced during the entry into lysogeny. Cells can also be immunized against prophages present within a

population. Spontaneous prophage induction events create a pool of extracellular phages. Cells that have spontaneously cured the cognate prophage can acquire spacers from extracellular phages without self-targeting, and spacer acquisition is enhanced in cells entering lysogeny. Alternatively, superinfected cells can acquire spacers, resulting in Cas9 self-targeting events that can induce prophage curing.

STAR Methods

Experimental Model Details

Microbes

For *S. pyogenes*, all experiments were performed in *S. pyogenes* M1 GAS strain SF370 and its derivatives. For *S. aureus*, all experiments were performed in *S. aureus* strain Newman, RN4220, or derivatives. Plasmid cloning was performed in *Escherichia coli* DH5a or *S. aureus* RN4220.

Culture Conditions

For *S. pyogenes*: cultures were maintained in Bacto Brain Heart Infusion (BHI) broth or Thy-D (see below), plated on BHI plates, and maintained at 37°C unless otherwise stated. Liquid cultures were grown without shaking. Antibiotics were supplemented at the following concentrations: chloramphenicol (cm), 3µg/mL; spectinomycin (spec) 100µg/mL; streptomycin (strep), 200µg/mL; anyhydrotetracycline (aTc), 0.1µg/mL; kanamycin (kan), 50µg/mL.

For *S. aureus*: cultures were maintained in BHI and plated on BHI plates and maintained at 37°C unless otherwise stated. Liquid cultures were shaken at 220rpm. Antibiotics were supplemented at the following concentrations: chloramphenicol (cm), 10µg/mL; erythromycin (erm), 10µg/mL; spectinomycin (spec) 250µg/mL; 4-chlorophenylalanine (4CP), 5mM; anyhydrotetracycline (aTc), 0.1µg/mL.

For *E. coli*: cultures were maintained in LB broth and plated on LB plates and maintained at 37°C unless otherwise stated. Liquid cultures were grown shaking at 220rpm. Antibiotics were supplemented at the following concentrations: spectinomycin (spec) 50µg/mL.

Todd-Hewitt Dialysate Media (THY-D)

Media for *S. pyogenes* phage infections was made as described previously[35]. Briefly, 30g Bacto Todd Hewitt Broth media and 20g Bacto yeast extract in 100mL deionized water were heated until homogenous and poured into 11 inches of dialysis tubing (Spectra Por S/P 3; 3.5kDa molecular weight cut-off) and sealed. The bag was submerged in deionized water for 1hr at room temperature (RT), then the water was replaced and the media submerged 2 more hours. The bag was then transferred to a beaker with fresh deionized water and dialyzed at 4°C for 16hr. Media was brought to 1L final volume and autoclaved.

Phages

For *S. pyogenes*, Phage A1 was propagated in C13, a derivative of the prophage-cured SF370 strain CEM1Δϕ that lacks spacers 1-5 and stored at 4°C in phage storage buffer (100mM NaCl, 8mM MgSO4, 50mM Tris-HCl [pH 7.5]).
For *S. aureus*, all phages were propagated in RN4220. Phages were concentrated using 100kDa centrifugal filters (Amicon), resuspended in phage storage buffer, and stored at 4°C.

Method Details

Plasmid Construction

Plasmids, oligos, and primers used in this study can be found in supplementary materials.

For Gibson cloning: Gibson assemblies performed as described previously. Briefly, 5µL of equimolar PCR products were combined with 15µL Gibson master mix and incubated at 50°C for 1hr. Gibson reactions were drop dialyzed for >30min prior to electroporation into *S. aureus* but added directly for heat shock transformation into *E. coli* DH5a. Purified plasmids were generated using Spin MiniPrep kit (Qiagen 27104) per the manufacturer's protocol, except with the addition of 100µg/mL of lysostaphin (Ambi Products LLC, LSPN-5) to buffer P1 and incubation at 37°C for >30min prior to addition of P2.

For oligo cloning: parent plasmids containing a single BsaI cloning site were digested for 8-16hr at 37°C with BsaI (NEB) in CutSmart buffer (NEB). Oligos with complementary regions to the BsaI site were separately annealed by incubating with T4 Polynucleotide Kinase and 1x PNK buffer (NEB) at 37°C for 1hr, boiling with the addition of NaCl at a final concentration of 50mM for 5min, then cooling to RT. The insert was combined with the digest and the mixture was ligated with T4 Ligase/buffer (NEB) at RT overnight. The digest was then transformed into recipient strains as described below.

Transformation into *S. aureus*

To prepare aliquots of electrocompetent cells, *S. aureus* cultures at OD600=~1.0 were pelleted at 4°C for 10min at 3200rcf. The pellet was resuspended in 1/10th volume of ice-cold ddH2O and pelleted at RT for 1min at 3200rcf twice for a total of 2 washes. The pellet was resuspended in 1/20th original volume in ice-cold 10% glycerol. Aliquots were stored at -80°C and thawed on ice before use. Plasmids were drop dialyzed in deionized water for >30min and >5ng were mixed with 50µL electrocompetent cells on ice. The cell/plasmid mixture was transferred to a Gene Pulser 0.2cm Cuvette (Bio-Rad 165-2086) and electroporated in an Eppendorf Eporator at 1.8kV. Cells were rescued in 300µL BHI and incubated at 37°C shaking at 220rpm for 1-2hr before plating on appropriate antibiotic selection plates and incubating at 37°C overnight.

Transformation into *S. pyogenes*

To prepare aliquots of electrocompetent cells, *S. pyogenes* cultures at OD600=~0.4 were pelleted at 4°C for 20min at 3200rcf. The pellet was washed with one volume cold 10% glycerol, again with 1/20th volume cold 10% glycerol, and finally resuspended in 1/150th volume 10% glycerol. Cells were stored at -80°C and thawed on ice before use. Plasmids were drop dialyzed in deionized water for >30min and 0.3–1µg were mixed with 50µL electrocompetent cells on ice. The cell/plasmid mixture was transferred to a cold Gene Pulser 0.1cm Cuvette (Bio-Rad 165-2089) and electroporated in a Gene Pulser Xcell (Bio-Rad; 2.5kV/cm, 200Ω, 25µF). Cells were rescued in 900µL BHI and incubated at 37°C for 1-2hr before plating on appropriate antibiotic selection plates and incubating at 37°C overnight.

Colony Lysis

For *S. pyogenes*: colonies were resuspended in 1x PBS with 1µg/mL PlyC and incubated 10min RT and 10min at 98°C. For PCRs, 0.5-1µL of lysate was used as template.

For *S. aureus*: colonies were resuspended in colony lysis buffer (250mM KCl, 5mM MgCl2, 50mM Tris-HCl [pH 9.0], 0.5% Triton X-100) with 100µg/mL lysostaphin and incubated 10min at 37°C and 10min at 98°C. For PCRs, 0.5-1µL lysate was used as template.

Genomic DNA Extractions

S. pyogenes genomic DNA was isolated using the DNeasy Blood and Tissue Kit (Qiagen 69504) following the manufacturers protocol for bacteria, except cell pellets were lysed in 1x PBS supplemented with 1µg/mL PlyC and incubated at RT for >30min before proceeding at the proteinase K digestion step.

Allelic Exchanges

All oligos and plasmids for construction can be found in the supplementary materials.

For *S. pyogenes*: homology arms >500bp surrounding the desired mutation were inserted into the temperature-sensitive plasmid pCRS via Gibson assembly and transformed into *E. coli* DH5a. Constructs were transformed into *S. pyogenes* as described above, except incubations were performed at 30°C to permit plasmid replication and plates were incubated for 36-48hr to allow colonies to form. Single colonies were inoculated into BHI + spec and incubated overnight at 37°C. The next day, cultures were plated on BHI + spec at 37°C and incubated overnight to select for single crossovers. Single colonies were inoculated into BHI and incubated at 30°C overnight to allow for plasmid excision and loss. Cultures were passaged 1:1000 in BHI and incubated at 37°C for ~8hr, then plated on BHI and incubated overnight at 37°C. To screen for double crossovers, 76-152 colonies were patched onto plates with and without spec, and spec-sensitive colonies were further screened for desired mutations via PCR and Sanger sequencing.

For *S. aureus*: homology arms >500bp surrounding the desired mutation were inserted into the temperature-sensitive plasmid pWJ327 or the non-replicative plasmid pWJ244 via Gibson assembly. For pWJ244 derivatives, constructs were transformed and maintained in *E. coli* DH5a prior to transformation into *S. aureus* as described above except using ~1µg DNA. For pWJ327 derivatives, constructs were transformed into *S. aureus* as described

above except incubations were performed at 30°C to permit plasmid replication and plates were incubated for 36-72hr to allow colonies to form. Single colonies were inoculated into BHI + cm and incubated overnight at 37°C. The next day, cultures were plated on BHI + cm at 37°C and incubated overnight to select for single crossovers. Single colonies were inoculated into BHI and incubated at 30°C (for pWJ327) or 37°C (for pWJ244). The next day, cultures were diluted 1:100 and outgrown 1hr at 30°C in BHI + 5mM $CaCl_2$ before the addition of 10µL of a phagemid targeting the allelic exchange backbone (pWJ326). After 2hr at 30°C, cultures were plated on BHI + erm and incubated at 30°C overnight. Single colonies were inoculated into BHI and grown at 37°C overnight to cure phagemid. Cultures were plated on plain BHI and grown overnight. The next day, 76 single colonies were patched onto BHI, BHI + cm, and BHI + erm to ensure plasmid and phagemid loss. Sensitive colonies were screened for desired mutations via PCR and Sanger sequencing.

Phage Engineering

Phages were generated 3 ways. 1) Prophages were generally engineered using allelic exchange as described above. 2) For other phages, serial dilutions of the parent phage stock were dripped on BHI + appropriate antibiotic + 5mM $CaCl_2$ + 0.75% agar plates containing cells harboring a plasmid containing a Type III CRISPR-Cas system targeting the parent phage. Escape mutant plaques were visible after 24hr at 37°C followed by 24hr at RT. Escaper plaques were serially diluted and replaqued on the targeting strain to purify the stock. Phage stocks were generated using escaper plaques and phage gDNA was isolated using the DNeasy Blood and Tissue Kit (Qiagen 69504) following the bacteria protocol with the following modifications: stocks were first treated for 30min with DNase I (NEB) and RNase A (Thermo) in 1x DNase I Buffer (100mM Tris-HCl, 25mM MgCl2, 5mM $CaCl_2$) at 37°C, and then the protocol was resumed beginning at the proteinase K step. Phage genomes were assembled using Nanopore sequencing technology. 3) same as 2 from above except infecting cells harboring a plasmid that contains a Type II CRISPR-Cas system targeting genes of interest.

Passaging Assays

For *S. pyogenes*: strains of interest were streaked from the freezer onto BHI + 10mM $Na_3C_6H_5O_7$ agar plates. The next day, single colonies were inoculated into Thy-D + 4mM $Na_3C_6H_5O_7$ + 2mg/mL $NaHCO_3$ and incubated overnight. The next day, cultures (Passage 0) were diluted 1:50 into 2 cultures: one of identical media and another of Thy-D + 5mM $CaCl_2$ + 2mg/mL $NaHCO_3$. Cultures were grown ~8hr (Passage 1). Cultures were then diluted 1:50 into one volume of identical media for a total of 8 passages in the mornings (day culture ~8hr) and evenings (overnight cultures ~16hr). Aliquots of Passages 0, 4, and 8 were pelleted at 3200rcf at 4°C for 10min before being flash frozen in LN_2 and stored at -80°C for gDNA extraction as described above, as well as frozen in 10% DMSO and stored at -80°C for future analyses.

For *S. aureus*: Same as above, except strains of interest were streaked from the freezer onto BHI + 10mM $Na_3C_6H_5O_7$ agar plates, and Passage 0 was grown in BHI + cm + 4mM $Na_3C_6H_5O_7$, which was split into identical media or BHI + cm + 5mM $CaCl_2$ for Passage 1 and beyond, and instead of gDNA, minipreps were performed as described.

Spacer Acquisition NGS

For single repeat constructs, we utilized an established protocol for enrichment of adapted CRISPR loci (Workman et. Al. 2021). Briefly, miniprepped CRISPR loci from *S. aureus* or gDNA from *S. pyogenes* were used as templates for an enrichment PCR using Phusion DNA Polymerase (Thermo) and a mix of primers: 3 parts primer upstream of the CRISPR array and 1 part each of reverse primers matching the first CRISPR repeat except with the addition of the 3 nucleotides without a perfect match to the last nucleotide of the leader sequence. Products were purified with Ampure XP beads (Beckman Coulter) and used as templates for the addition of Illumina indexes with barcodes by PCR to distinguish samples in multiplexed high-throughput sequencing. For some samples, the spacer-acquired, index ligated amplicons were ran on an agarose gel and extracted to further enrich for adapted loci. Illumina sequencing was conducted with the MiSeq platform. For *S. pyogenes* strains containing the native 6-spacer array, NGS was performed similarly except a forward primer upstream of the CRISPR array and another within the first native spacer were used for PCR, the entire reaction was run on a gel, and ghost bands were extracted from the gel at a size equivalent to a spacer acquisition event. These products were then used for enrichment PCR as described above.

Using this technique, information about the absolute rates of spacer acquisition is lost given that adapted and unadapted loci are amplified at vastly different rates, and we do not typically sequence unadapted bands. However, this technique is able to quantitatively compare relative rates of spacer acquisition between different phages for the same host cell. This is because host cells are constantly acquiring spacers against the resident chromosome and plasmid. Therefore, by quantifying the % of phage targeting spacers, we are essentially comparing new phage-targeting spacers to the number of pre-existing "background" spacers from the chromosome and plasmid that accumulated during the many generations prior to phage infection.

***S. aureus* Phage Plaquing Assays**

Soft agar lawns of *S. aureus* were made by combining 100µL overnight culture with 6mL molten 0.75% agar supplemented with appropriate antibiotics and 5mM $CaCl_2$ and plated on a BHI agar plate with appropriate antibiotics. Plates were dried next to a flame ~30min. 3µL of serial dilutions of phages in phage storage buffer were spotted or dripped onto the plates, allowed to dry, and incubated at 37°C overnight. Plaques were enumerated the following day.

PCR

PCR was performed using Phusion HF DNA Polymerase and 5x Green Phusion HF Buffer (Thermo). Generally, reactions contained 10-50ng plasmid/PCR product or 1-2µL colony lysate for template DNA, 4µL dNTPs (2.5mM), 5µL buffer, 0.5µL of each primer (100µM), 0.5µL polymerase, and brought to 50µL in water. For some PCRs, 10 or 20µL reactions were used, maintaining the same ratio of reagents as described. Cycling was generally performed as follows: 98°C 30s, [98°C 10s, 60-70°C 20s, 72°C 30s/kb product]x34, 10°C hold. For size checks, products were ran on 1-2% agarose gels containing 1:10,000 v/v ethidium bromide.

One-Step Growth Curve Assay

An overnight culture of *S. aureus* RN4220 was diluted to OD=0.1 in BHI supplemented with 5mM $CaCl_2$ and outgrown to OD=0.3. The outgrowth was split into 4 cultures (one for each phage) and infected with ϕNM1-pheS or its lytic derivatives at an MOI of 0.001. The infected cultures were incubated for 10min to allow phage adsorption and injection, then pelleted at 5000xg and washed twice in plain BHI to remove unadsorbed phage. Pelleted cells were resuspended in plain BHI and part of the culture was removed to determine the total number of infected cells by plaquing assay described above. The culture was grown for 2hr with part of the culture being removed, pelleted, and serially diluting the supernatant every 15min to determine the number of phages produced over time as determined by plaquing assay.

Short Time-Point Spacer Acquisition Assay

Overnight cultures of *S. aureus* RN4220 harboring the Δtr-L Type II-A CRISPR-Cas system from *S. pyogenes* with a single repeat in the CRISPR array were diluted to OD=0.1 in BHI supplemented with appropriate antibiotic and 5mM $CaCl_2$ and outgrown to OD=0.4. Cultures were uninfected or infected with ϕNM1-pheS or ϕNM1-pheSγ7 at an MOI of 10 and grown for 45min. At 10, 30, and 45min post-infection, 1mL of each culture was removed, pelleted at 5000xg, and supernatant removed before flash freezing in LN_2. Each culture was miniprepped to extract CRISPR loci and used as templates for Spacer Acquisition NGS as described above.

***S. aureus* Superinfection Vaccination Assay**

Single colonies of strains of interest were inoculated into BHI + 5mM $CaCl_2$ and appropriate antibiotic and grown ~6hr to stationary phase. Each culture was split into two separate cultures, with one receiving 3 separate infections with ϕNM1 or ϕNM1-pheS at MOI=10 separated by 45min, and the other the equivalent volumes of phage storage buffer. After phage/buffer additions, the cultures were incubated overnight. The next morning, each culture was diluted back to OD=0.1 in the same media, outgrown for ~1.5hr, and diluted back to OD=0.4. Each culture was infected with ϕNK4 at MOI=50, shaken 30min at 37°C, and 1mL of the infection was combined with molten 0.75% agar in BHI + 5mM $CaCl_2$ and appropriate antibiotic and plated on agar plates with appropriate antibiotic. Plates were dried next to a flame and grown overnight at 37°C. The next day, colonies were enumerated and randomly chosen colonies were lysed and used as template DNA for spacer acquisition checks. If necessary, randomly chosen colonies were restreaked to single colonies on agar plates with identical additives and grown at 37°C overnight. Randomly chosen surviving colonies were lysed and used as template DNA for downstream PCR checks to determine the percentage of spacer acquired CFUs from each surviving population. To determine CRISPR immunization rates, total surviving CFUs were multiplied by the percentage of spacer acquired CFUs and divided by the total number of infected cells plated based on a conversion factor of OD600 0.4 = 8.0e7 cells per mL.

Spontaneous and Induced Curing Assays

Overnight cultures of all strains were pelleted, resuspended in an equivalent volume of PBS, and serially diluted in PBS by the indicated dilution factor. 3µL of each dilution was spotted or dripped onto agar plates containing necessary antibiotics for plasmid maintenance. For induced curing experiments, aTc was added to the agar for inducing ectopic spacer expression. For spontaneous curing experiments, 4CP or streptomycin was added to the agar for selection of prophage cured cells in *S. aureus* or *S. pyogenes*, respectively. Plates were dried next to a flame and incubated at 37°C overnight. The next day, colonies were enumerated and randomly chosen colonies were restreaked to single colonies on agar plates with identical additives and grown at 37°C overnight. A single colony from each restreak was lysed and used as template DNA for downstream PCR checks. Rates of survival were determined by dividing the number of survivors on experimental plates by the total CFUs of a plated culture (no additive plates).

S. aureus CRISPR Immunity Assay

Overnight cultures of *S. aureus* were diluted back to OD=~0.1 in BHI + 5mM $CaCl_2$ and appropriate antibiotic and outgrown to OD=0.4. Strains were challenged with ϕNM1-pheS or its derivatives at MOI=10, shaken 30min at 37°C, and a subset of the infection was plated in molten BHI + 0.75% agar with identical additives on plates with appropriate antibiotic. Plates were incubated at 37°C overnight and surviving CFUs were enumerated. Randomly chosen surviving colonies were lysed and used as template DNA for downstream PCR checks to determine the percentage of spacer acquired CFUs from each surviving population. To determine CRISPR immunization rates, total surviving CFUs were multiplied by the percentage of spacer acquired CFUs and divided by the total number of infected cells plated based on a conversion factor of OD600 0.3 = 6.0e7 cells per mL.

S. pyogenes Lysogeny Assay

Overnight cultures of recipient *S. pyogenes* cells were diluted 1:10 in Thy-D + 2mg/mL $NaHCO_3$ + 5mM $CaCl_2$ and incubated at 37°C for 1.5hr. The 0.2µm filtered supernatant of an overnight culture in BHI of donor SF370.1-str or its derivatives was mixed with outgrown recipient cells and incubated at 37°C for 15min. The entire infection was then spread onto BHI + kan agar plates and spread evenly with glass beads until absorbed into the agar. Plates were incubated at 37°C overnight, and the next day surviving colonies were lysed and used as templates for PCR checks of successful lysogeny.

S. aureus Growth Curves

A log phase culture of *S. aureus* in BHI was diluted back to OD600=~0.1 in the same media supplemented with 5mM $CaCl_2$ and added to a flat-bottom 96-well plate (Greiner 655180) and incubated at 37°C in a TECAN Infinite F Nano+ with shaking. If necessary, phage ϕNM1-pheS or one of its lytic derivatives was added at MOI=10 prior to incubation. Measurements of OD600 were recorded every 10min for 24hr.

S. pyogenes Growth Curves

A log phase culture of *S. pyogenes* in BHI was diluted back to OD600=~0.1 in the same media supplemented with $CaCl_2$, $Na_3C_6H_5O_7$, or neither at various concentrations and added to a flat-bottom 96-well plate (Greiner 655180) and incubated at 37 °C in a TECAN Infinite F Nano+ without shaking. If necessary, phage A1µ3 was added at MOI=10 prior to incubation. Measurements of OD600 were recorded every 10min for 24hr.

Quantification and Statistical Analyses

Basic quantification and rate calculations for cell-based assays can be found in Supplementary Table 2. Statistical analyses and graph generation were completed in GraphPad Prism v7.4 and R v4.3.0. The statistical tests used, values of *n*, and definitions of significance are listed in figure legends. Formatting of graphs and figures was completed in Adobe Illustrator v23.0.1.

Spacer NGS Analysis

FastQ files were processed with custom Python scripts and aligned to the appropriate parent *S. aureus* or *S. pyogenes* genome using the Burrows-Wheeler Aligner[59]. Alignment files of acquired spacers were converted to bed format and those with no mismatches were aggregated by genomic position. For final RPM values, reads were multiplied by a correction factor accounting for the last base of the spacer (A: 1.812265748, C: 0.267975994, T: 5.640697298, G: 2.467159171) and the number of normalized reads at each genomic position were divided by the total number of reads aligned to the parent genome for that experiment and multiplied by one million using custom R scripts. Last base correction is used to correct for the purposeful omission of a primer that perfectly binds the single repeat in non-adapted plasmids and therefore enriches for newly acquired spacers. RPMs were aggregated in 10 kb bins for visualization unless otherwise stated.

Table S1. Strains, phages, plasmids and oligos used in this work, related to STAR Methods.

Table S2. Rate calculations used in this work, related to STAR Methods.

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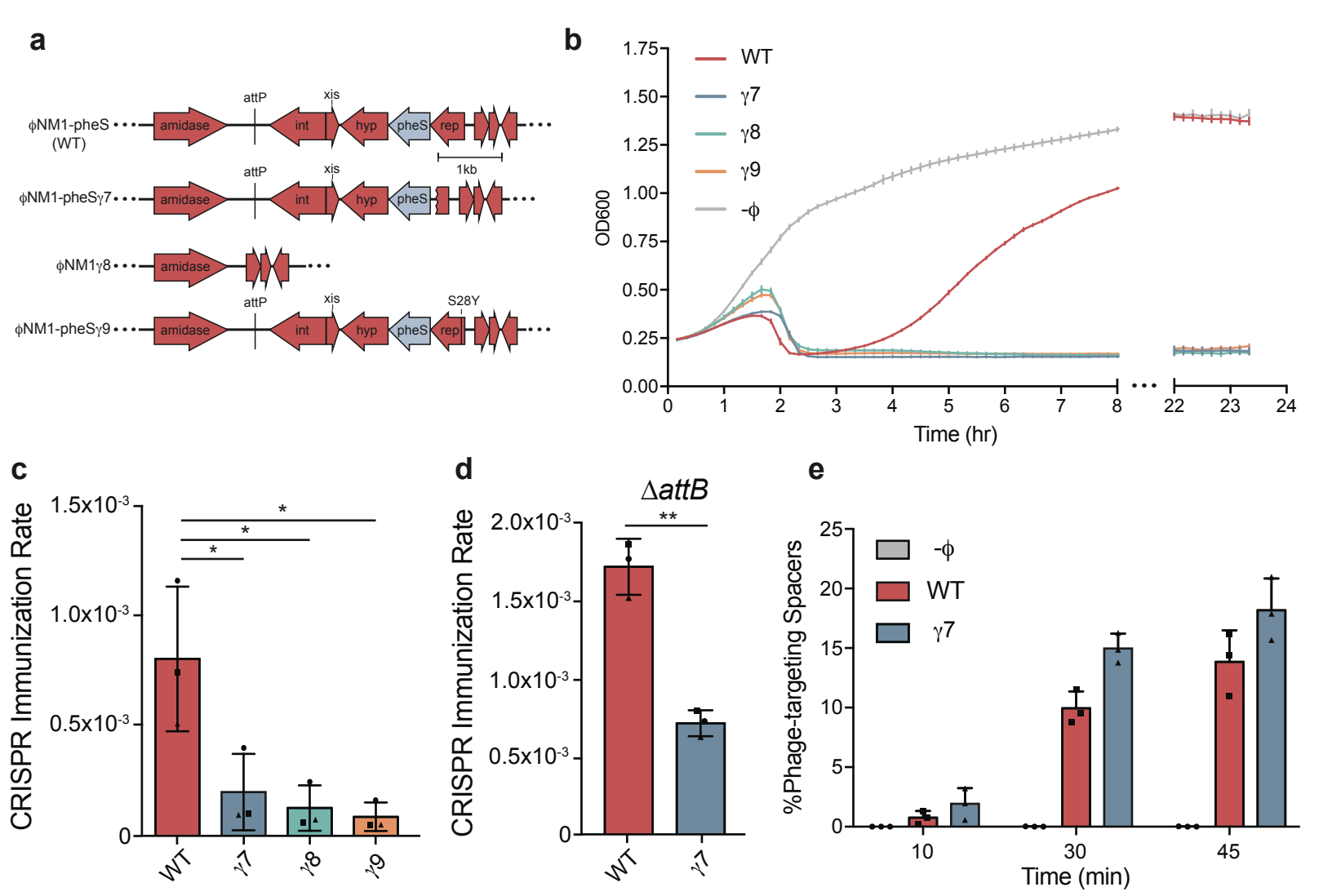
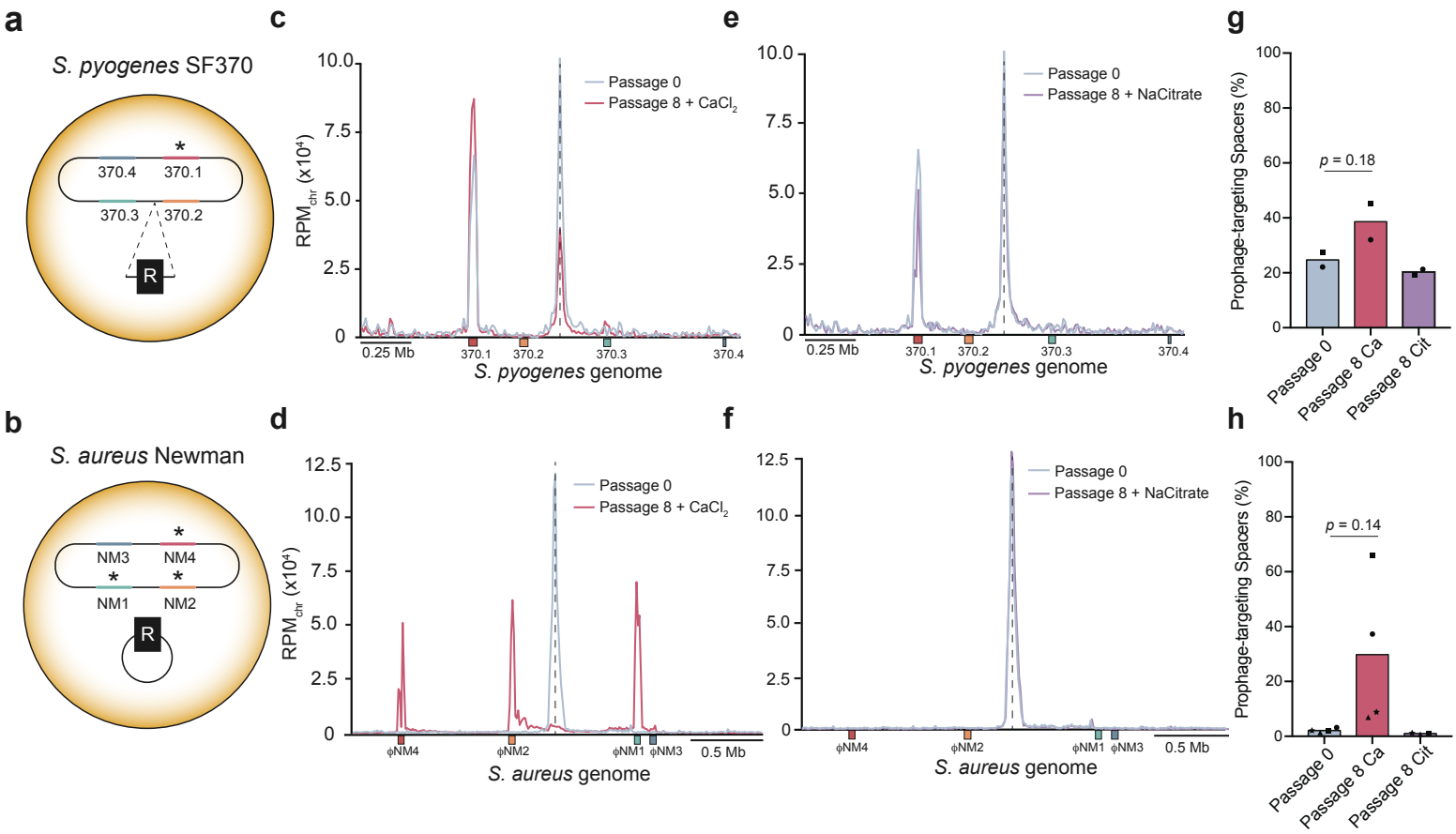
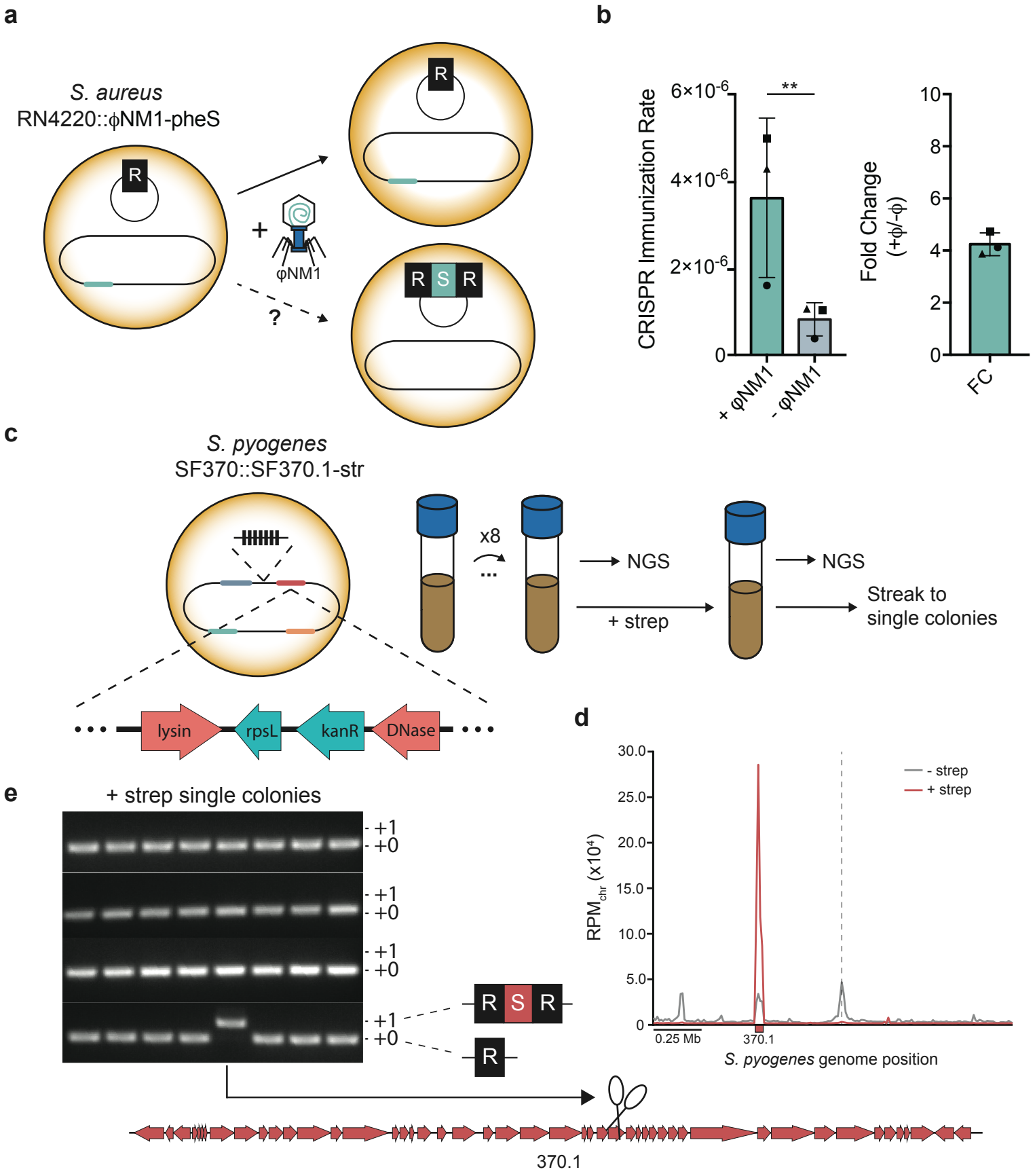
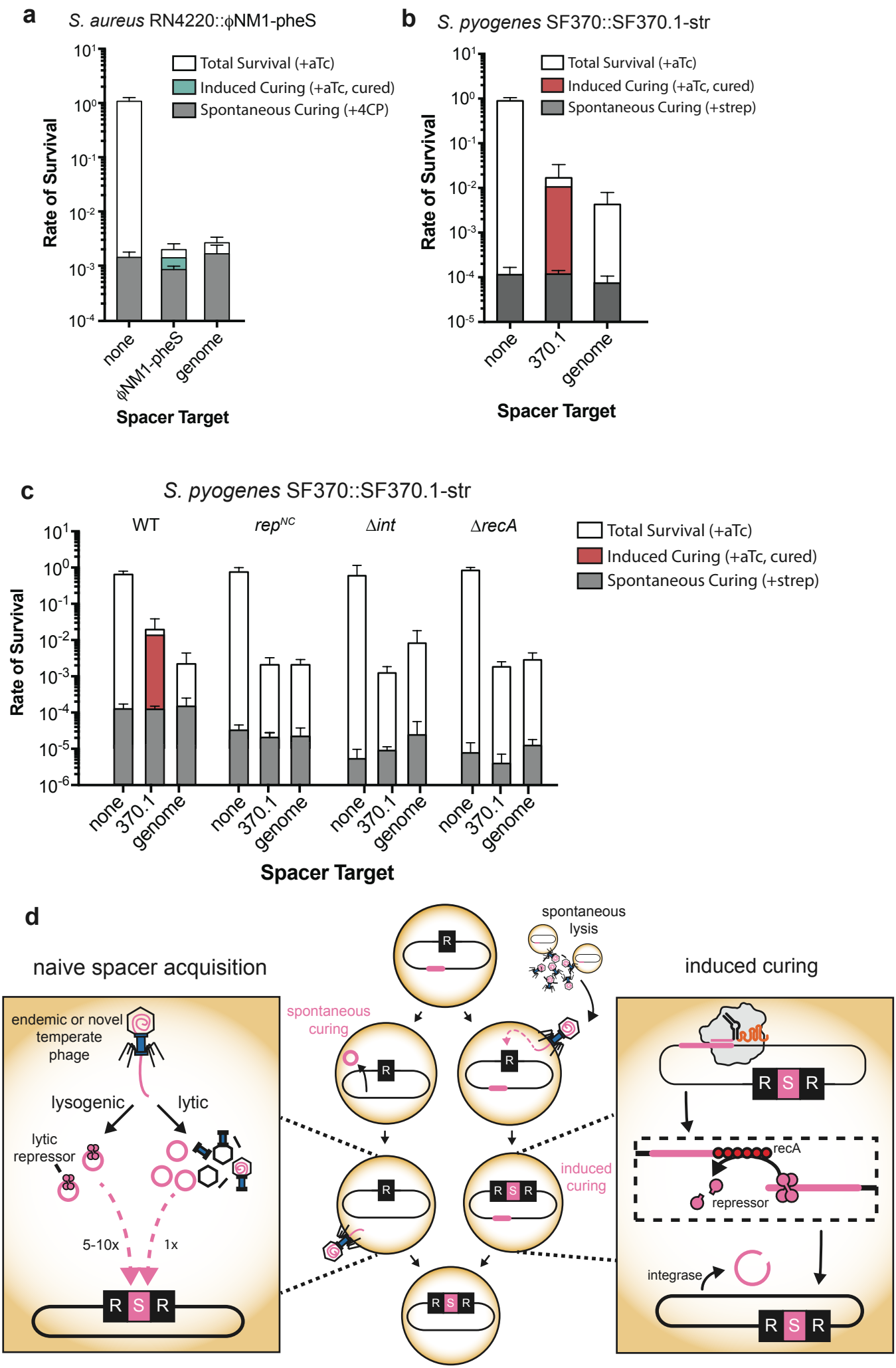
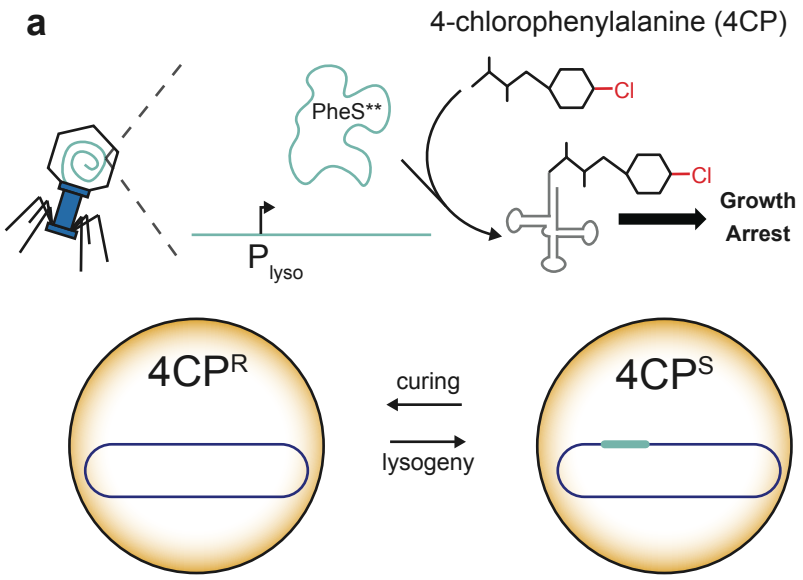
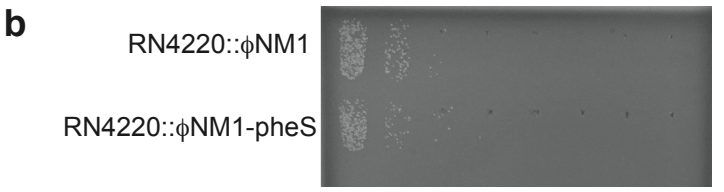

a
attP
xis
ϕNM1-pheS
(WT)
amidase
int
hyp
pheS
rep
1kb
ϕNM1-pheSγ7
ϕNM1γ8
S28Y
ϕNM1-pheSγ9
b
WT
γ7
γ8
γ9
-ϕ
OD600
Time (hr)
c
CRISPR Immunization Rate
1.5x10-3
1.0x10-3
0.5x10-3
0
WT
γ7
γ8
γ9
d
ΔattB
2.0x10-3
1.5x10-3
1.0x10-3
0.5x10-3
e
%Phage-targeting Spacers
Time (min)

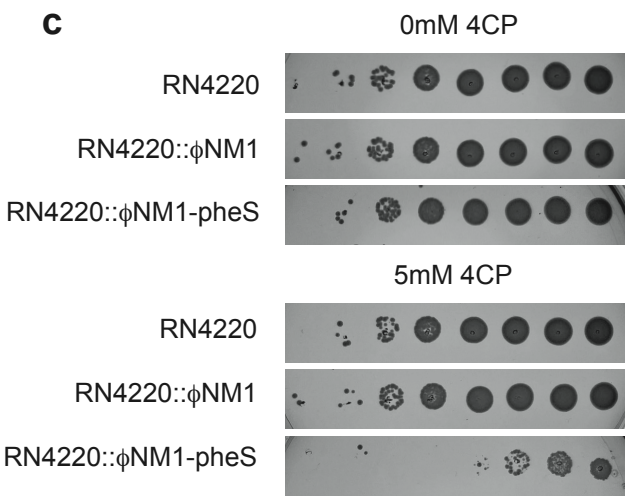

a
S. pyogenes SF370
370.4
370.1
370.3
370.2
R
b
S. aureus Newman
NM3
NM4
NM1
NM2
R
c
RPM$_{chr}$ (x10^4)
Passage 0
Passage 8 + CaCl$_2$
0.25 Mb
370.1
370.2
370.3
370.4
S. pyogenes genome
d
RPM$_{chr}$ (x10^4)
Passage 0
Passage 8 + CaCl$_2$
ϕNM4
ϕNM2
ϕNM1
ϕNM3
0.5 Mb
S. aureus genome
e
Passage 0
Passage 8 + NaCitrate
0.25 Mb
370.1
370.2
370.3
370.4
S. pyogenes genome
f
Passage 0
Passage 8 + NaCitrate
ϕNM4
ϕNM2
ϕNM1
ϕNM3
0.5 Mb
S. aureus genome
g
Prophage-targeting Spacers (%)
p = 0.18
Passage 0
Passage 8 Ca
Passage 8 Cit
h
Prophage-targeting Spacers (%)
p = 0.14
Passage 0
Passage 8 Ca
Passage 8 Cit

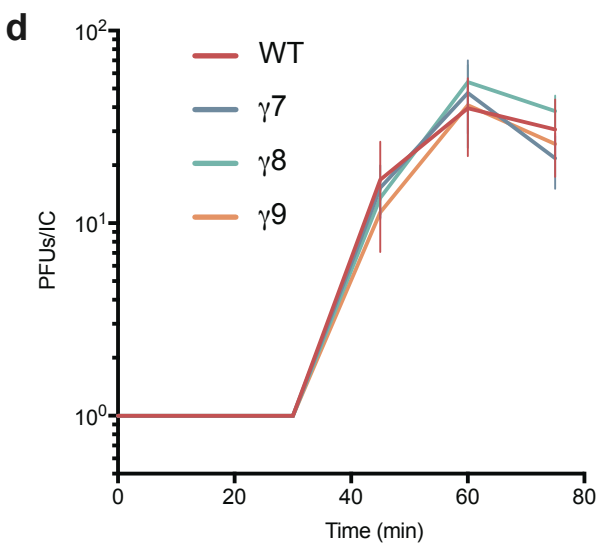

a
S. aureus
RN4220::ϕNM1-pheS
R
+
φNM1
?
R
R S R
b
CRISPR Immunization Rate
6×10-6
4×10-6
2×10-6
0
**
+ φNM1
- φNM1
Fold Change
(+ϕ/-ϕ)
10
8
6
4
2
0
FC
c
S. pyogenes
SF370::SF370.1-str
x8
NGS
+ strep
NGS
Streak to
single colonies
lysin
rpsL
kanR
DNase
d
30.0
25.0
20.0
15.0
10.0
5.0
0
RPMchr (x10⁴)
- strep
+ strep
0.25 Mb
370.1
S. pyogenes genome position
e
+ strep single colonies
+1
+0
+1
+0
+1
+0
+1
+0
R S R
R
370.1

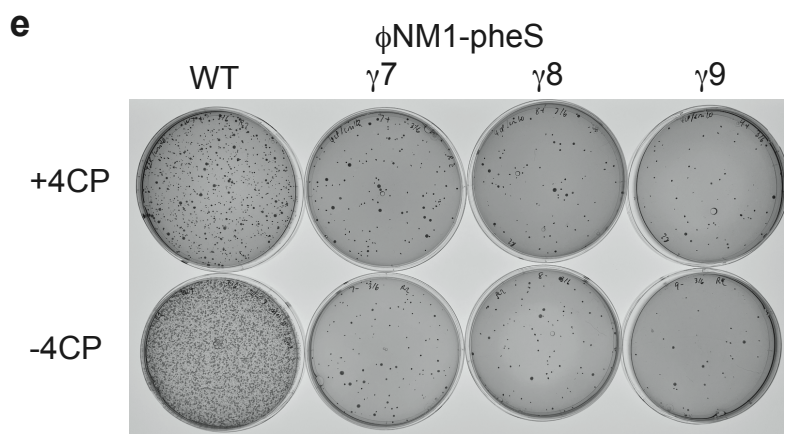

a
S. aureus RN4220::ϕNM1-pheS
Total Survival (+aTc)
Induced Curing (+aTc, cured)
Spontaneous Curing (+4CP)
Rate of Survival
none
ϕNM1-pheS
genome
Spacer Target
b
S. pyogenes SF370::SF370.1-str
Total Survival (+aTc)
Induced Curing (+aTc, cured)
Spontaneous Curing (+strep)
Rate of Survival
none
370.1
genome
Spacer Target
c
S. pyogenes SF370::SF370.1-str
WT
repNC
Δint
ΔrecA
Total Survival (+aTc)
Induced Curing (+aTc, cured)
Spontaneous Curing (+strep)
Rate of Survival
none
370.1
genome
Spacer Target
d
naive spacer acquisition
endemic or novel temperate phage
lysogenic
lytic
lytic repressor
5-10x
1x
R S R
spontaneous lysis
spontaneous curing
induced curing
induced curing
recA
repressor
integrase

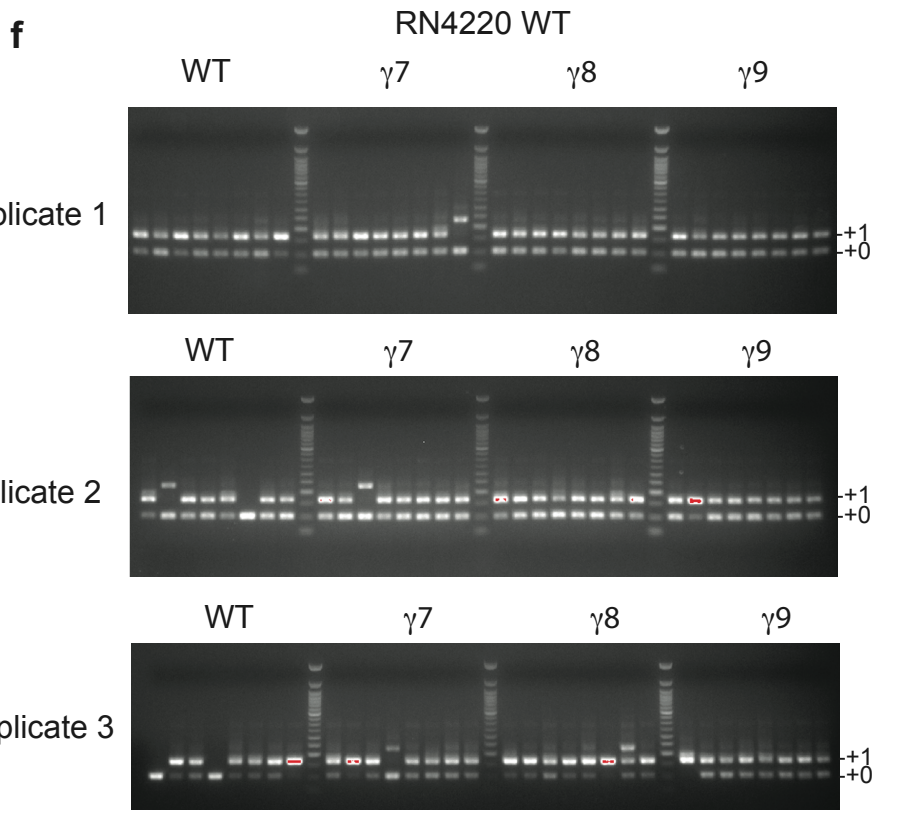

a
4-chlorophenylalanine (4CP)
PheS**
Cl
Cl
Growth
Arrest
P lyso
4CP^R
curing
lysogeny
4CP^S

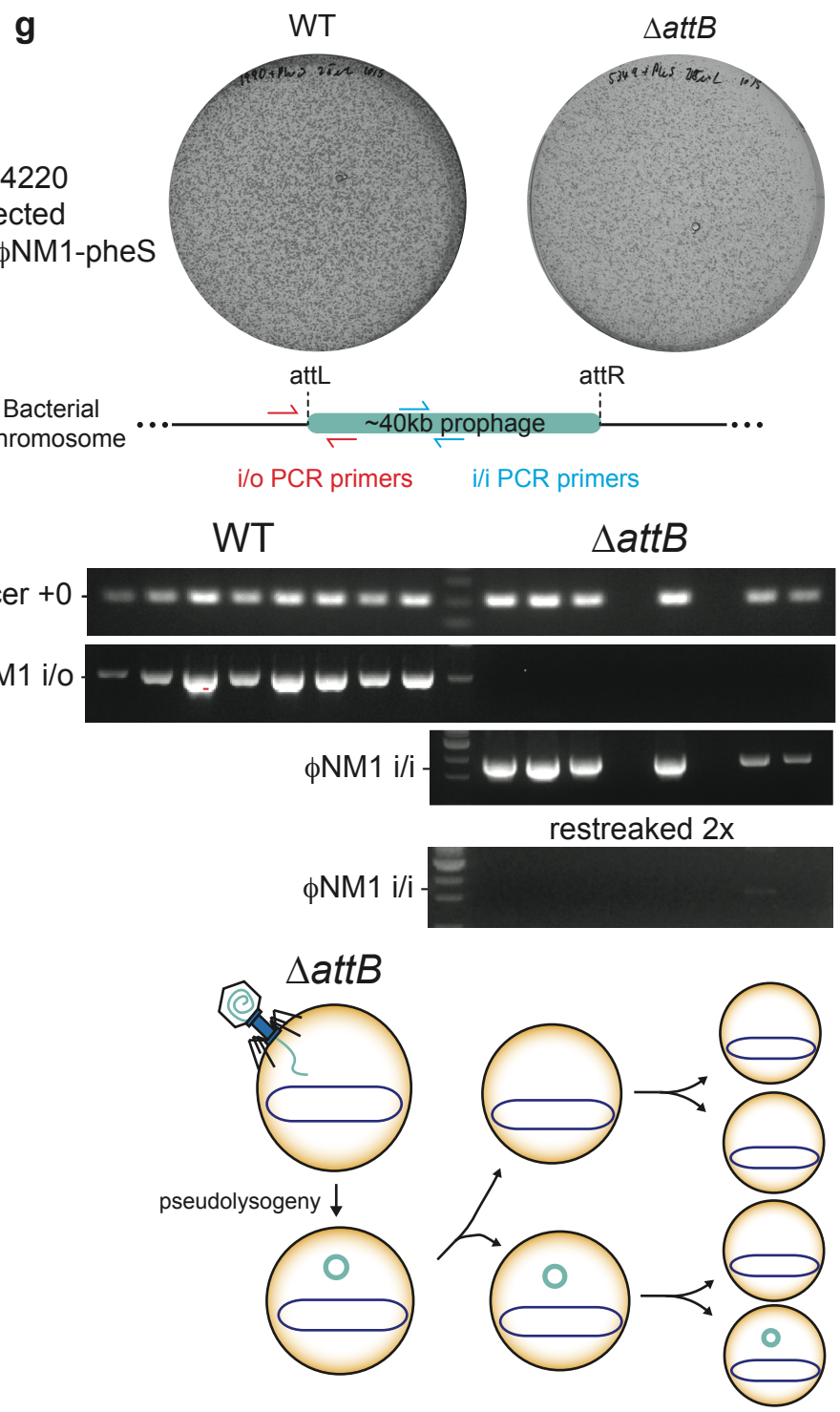

b
RN4220::ϕNM1
RN4220::ϕNM1-pheS

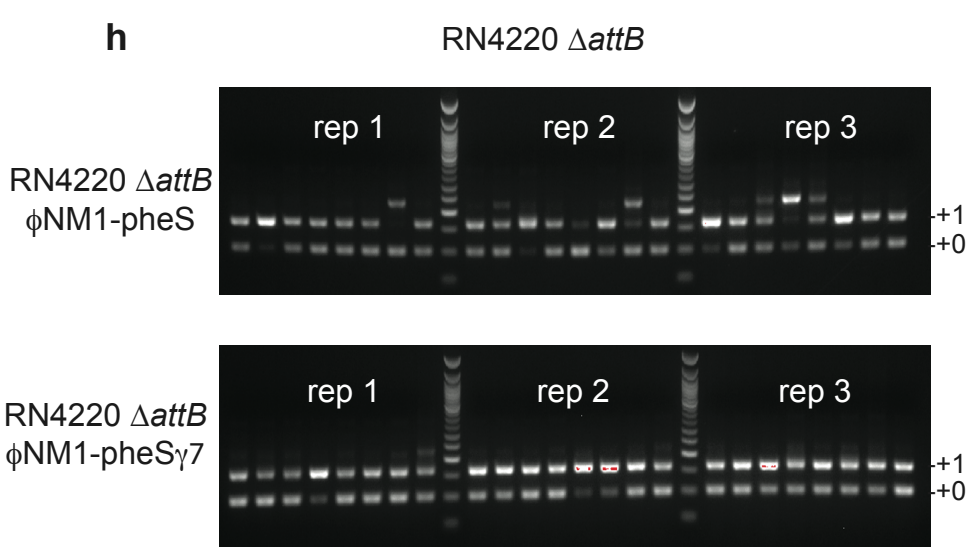

c
0mM 4CP
RN4220
RN4220::ϕNM1
RN4220::ϕNM1-pheS
5mM 4CP
RN4220
RN4220::ϕNM1
RN4220::ϕNM1-pheS

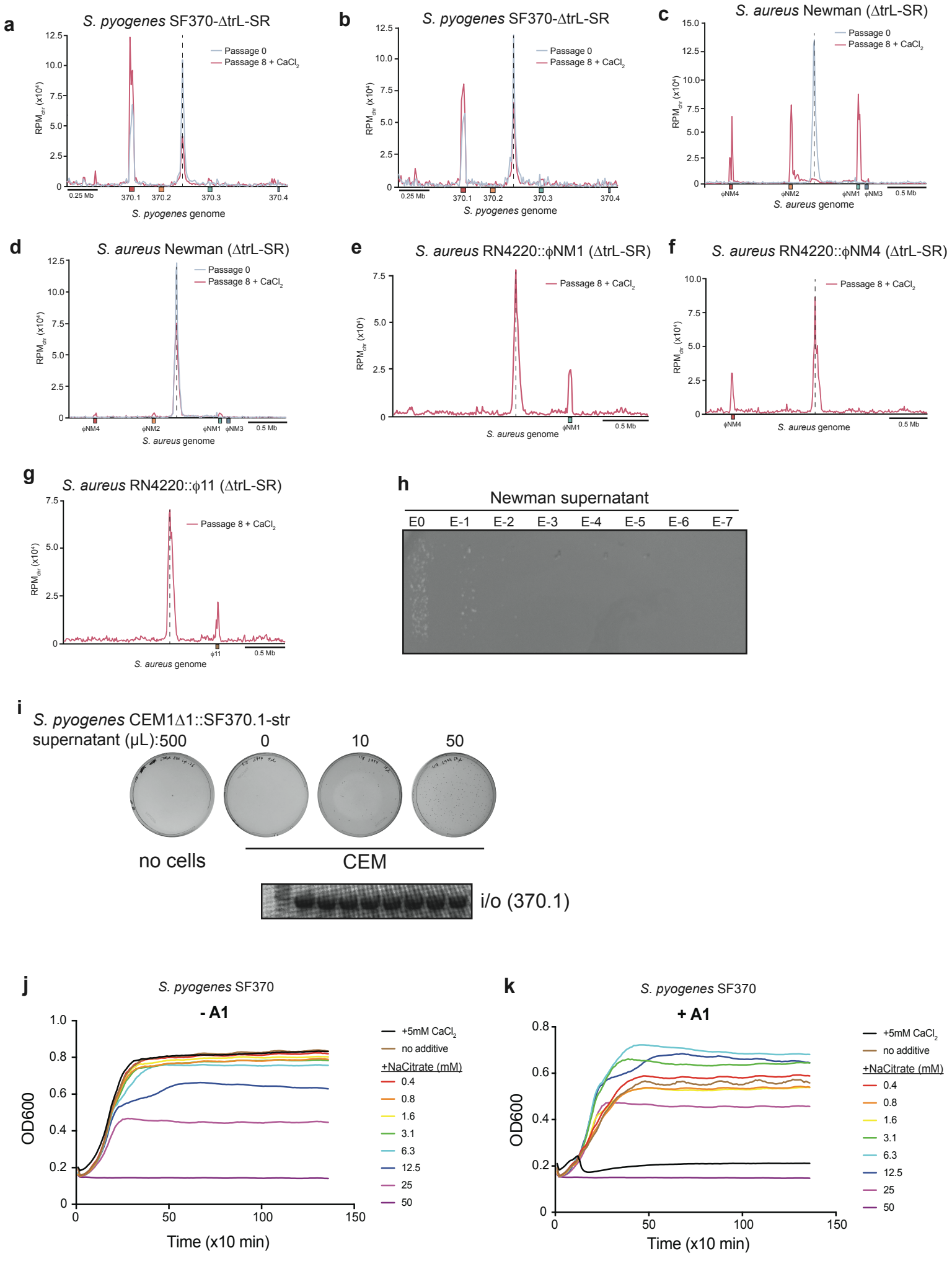

d
WT
γ7
γ8
γ9
PFUs/IC
10^2
10^1
10^0
0
20
40
60
80
Time (min)

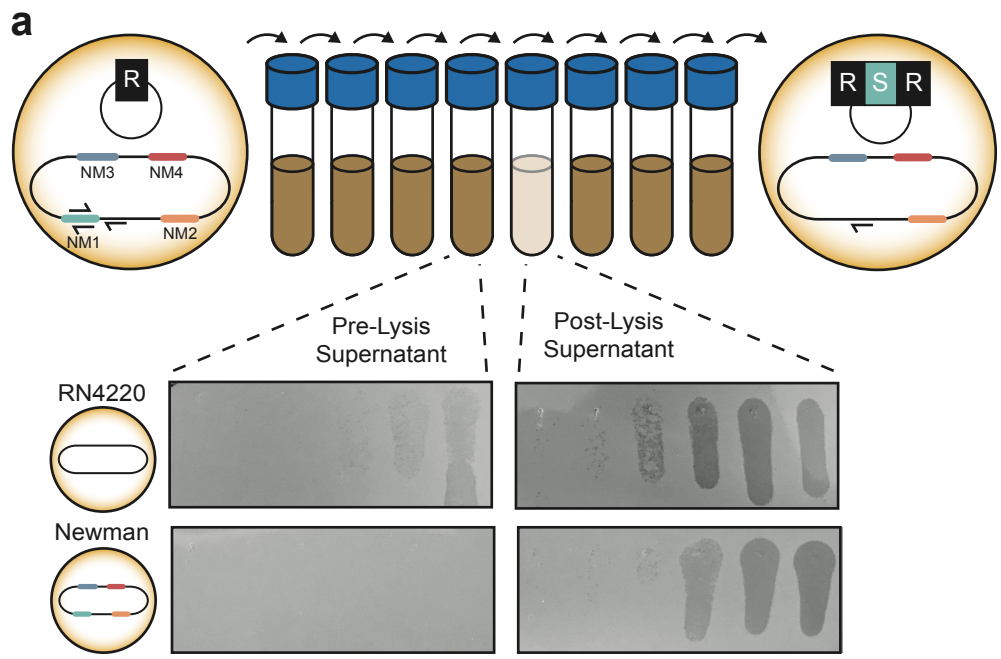

e
ϕNM1-pheS
WT
γ7
γ8
γ9
+4CP
-4CP

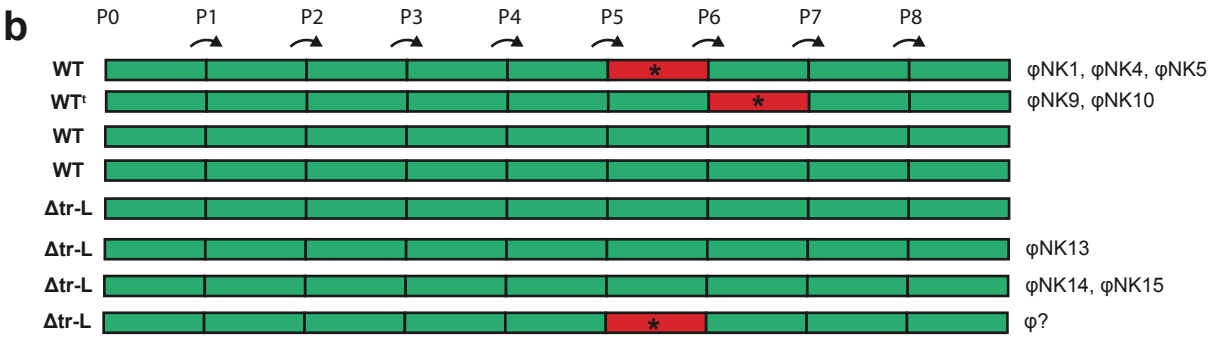

f
RN4220 WT
WT
γ7
γ8
γ9
Replicate 1
+1
+0
Replicate 2
+1
+0
Replicate 3
+1
+0

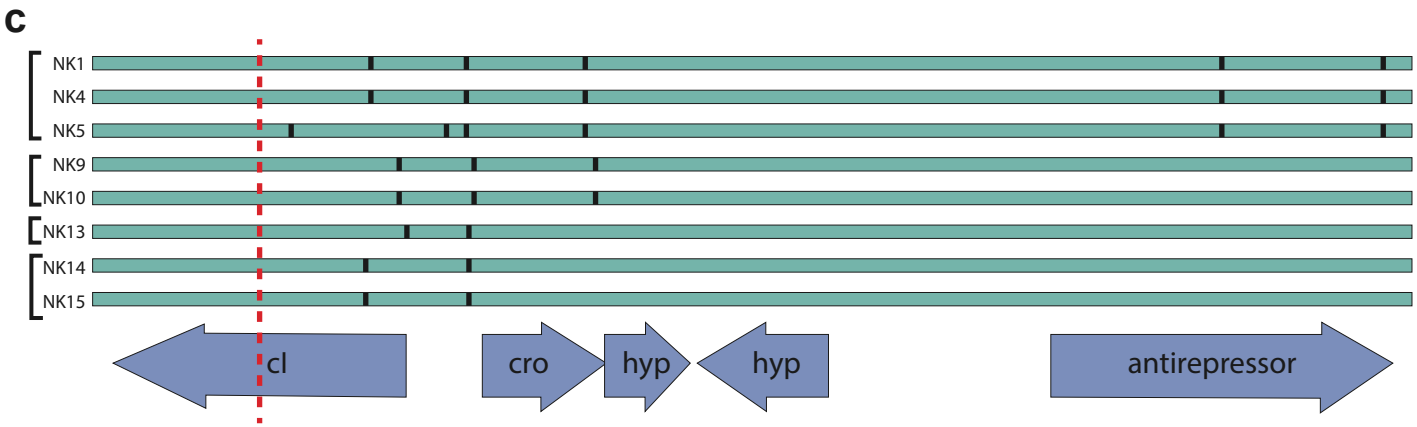

g
WT
ΔattB
RN4220
infected
MOI=10 ϕNM1-pheS
attL
attR
Bacterial
Chromosome
~40kb prophage
i/o PCR primers
i/i PCR primers
WT
ΔattB
spacer +0
ϕNM1 i/o
ϕNM1 i/i
restreaked 2x
ϕNM1 i/i
ΔattB
pseudolysogeny

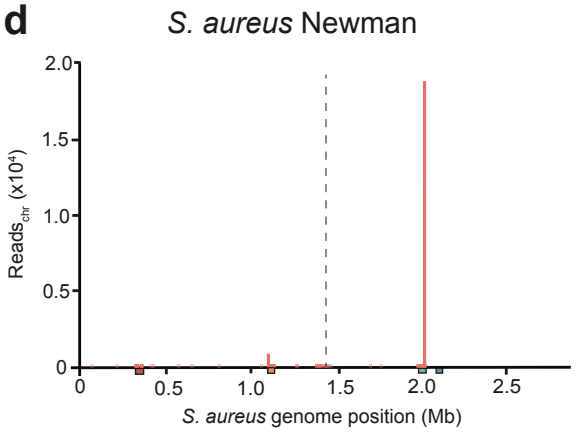

h
RN4220 ΔattB
RN4220 ΔattB
ϕNM1-pheS
rep 1
rep 2
rep 3
+1
+0
RN4220 ΔattB
ϕNM1-pheSγ7
rep 1
rep 2
rep 3
+1
+0

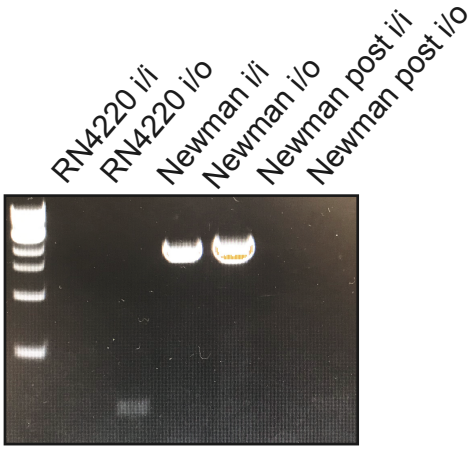

a
S. pyogenes SF370-ΔtrL-SR
Passage 0
Passage 8 + CaCl2
RPMchr (x10⁴)
0.25 Mb
370.1
370.2
370.3
370.4
S. pyogenes genome
b
S. pyogenes SF370-ΔtrL-SR
Passage 0
Passage 8 + CaCl2
RPMchr (x10⁴)
0.25 Mb
370.1
370.2
370.3
370.4
S. pyogenes genome
c
S. aureus Newman (ΔtrL-SR)
Passage 0
Passage 8 + CaCl2
RPMchr (x10⁴)
ϕNM4
ϕNM2
ϕNM1
ϕNM3
0.5 Mb
S. aureus genome
d
S. aureus Newman (ΔtrL-SR)
Passage 0
Passage 8 + CaCl2
RPMchr (x10⁴)
ϕNM4
ϕNM2
ϕNM1
ϕNM3
0.5 Mb
S. aureus genome
e
S. aureus RN4220::ϕNM1 (ΔtrL-SR)
Passage 8 + CaCl2
RPMchr (x10⁴)
ϕNM1
0.5 Mb
S. aureus genome
f
S. aureus RN4220::ϕNM4 (ΔtrL-SR)
Passage 8 + CaCl2
RPMchr (x10⁴)
ϕNM4
0.5 Mb
S. aureus genome
g
S. aureus RN4220::ϕ11 (ΔtrL-SR)
Passage 8 + CaCl2
RPMchr (x10⁴)
ϕ11
0.5 Mb
S. aureus genome
h
Newman supernatant
E0
E-1
E-2
E-3
E-4
E-5
E-6
E-7
i
S. pyogenes CEM1Δ1::SF370.1-str
supernatant (μL):500
0
10
50
no cells
CEM
i/o (370.1)
j
S. pyogenes SF370
- A1
OD600
Time (x10 min)
+5mM CaCl2
no additive
+NaCitrate (mM)
0.4
0.8
1.6
3.1
6.3
12.5
25
50
k
S. pyogenes SF370
+ A1
OD600
Time (x10 min)
+5mM CaCl2
no additive
+NaCitrate (mM)
0.4
0.8
1.6
3.1
6.3
12.5
25
50

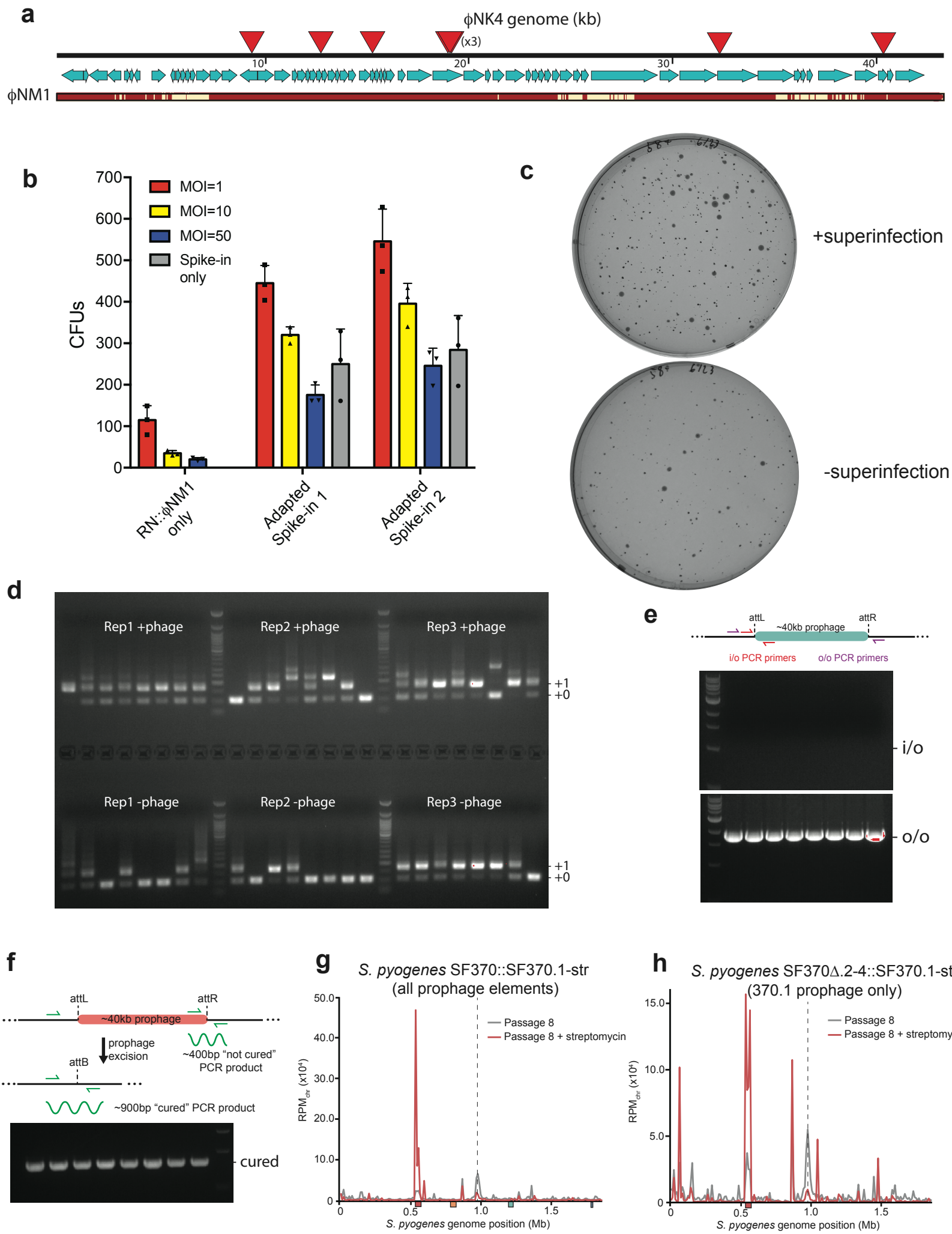

a
R
NM3
NM4
NM1
NM2
R S R
Pre-Lysis Supernatant
Post-Lysis Supernatant
RN4220
Newman

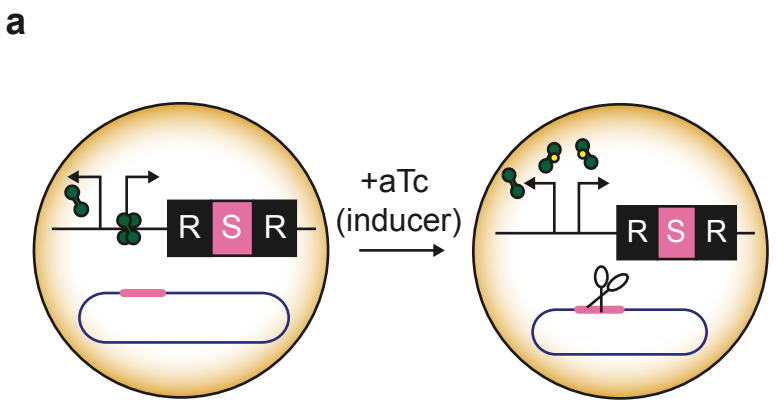

b
P0
P1
P2
P3
P4
P5
P6
P7
P8
WT
WT*
WT
WT
Δtr-L
Δtr-L
Δtr-L
Δtr-L
φNK1, φNK4, φNK5
φNK9, φNK10
φNK13
φNK14, φNK15
φ?

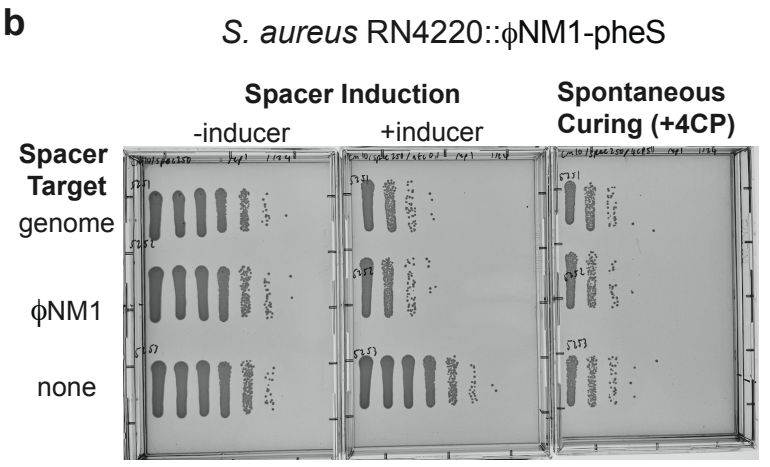

c
NK1
NK4
NK5
NK9
NK10
NK13
NK14
NK15
cI
cro
hyp
hyp
antirepressor

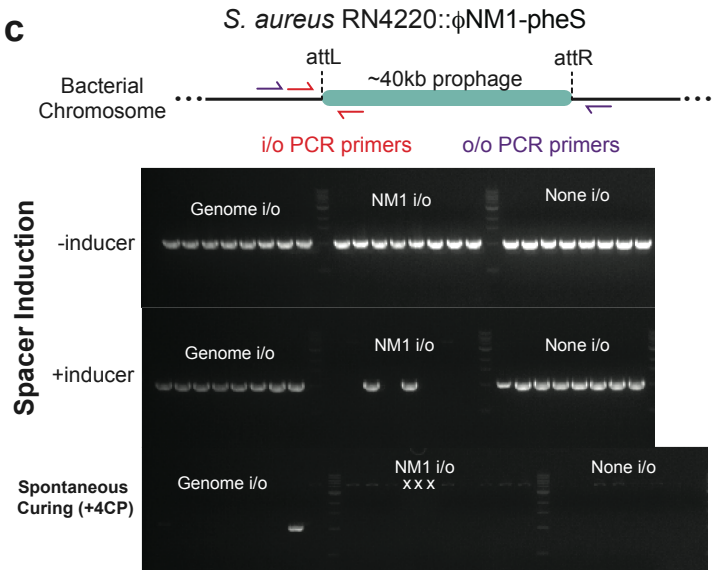

d
S. aureus Newman
Reads chr (x10⁴)
2.0
1.5
1.0
0.5
0
0
0.5
1.0
1.5
2.0
2.5
S. aureus genome position (Mb)

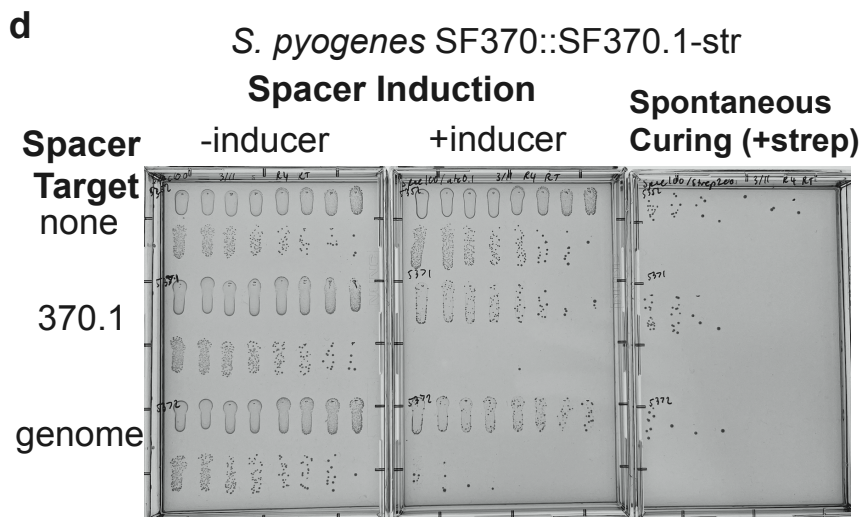

e
RN4220 i/i
RN4220 i/o
Newman i/i
Newman i/o
Newman post i/i
Newman post i/o

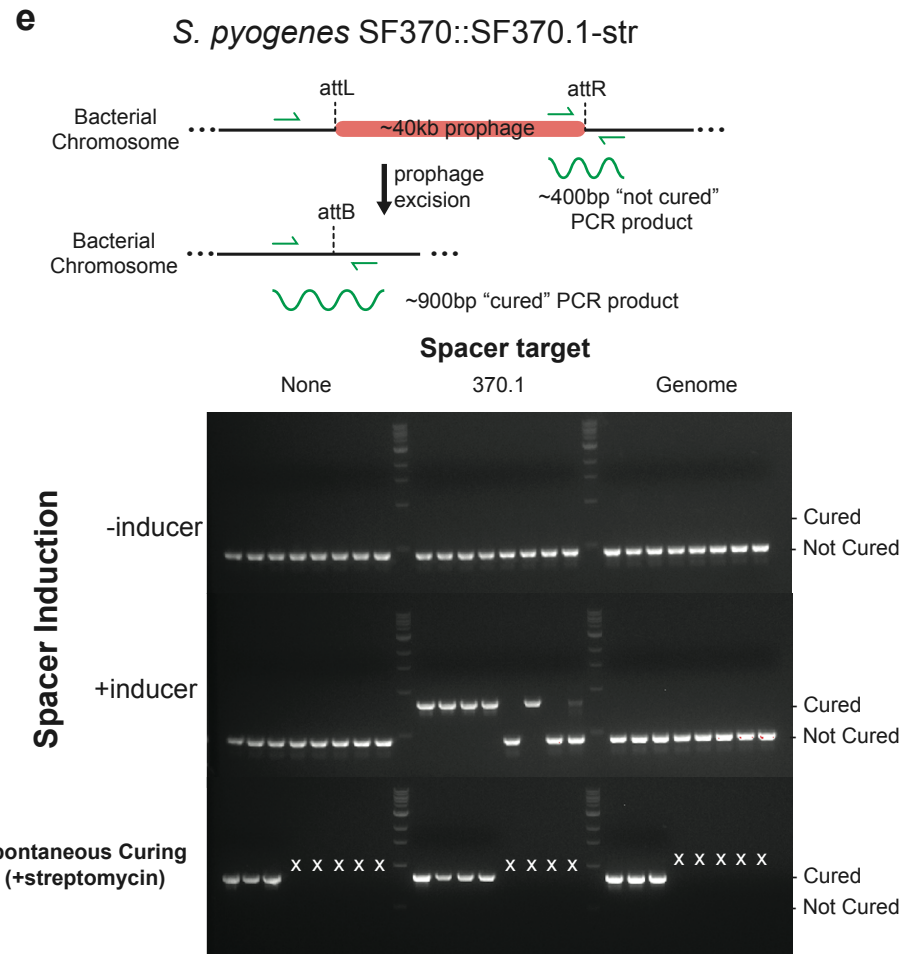

a
ϕNK4 genome (kb)
(x3)
10
20
30
40
ϕNM1
b
CFUs
MOI=1
MOI=10
MOI=50
Spike-in only
RN::ϕNM1 only
Adapted Spike-in 1
Adapted Spike-in 2
c
+superinfection
-superinfection
d
Rep1 +phage
Rep2 +phage
Rep3 +phage
Rep1 -phage
Rep2 -phage
Rep3 -phage
+1
+0
e
attL
attR
~40kb prophage
i/o PCR primers
o/o PCR primers
i/o
o/o
f
attL
attR
~40kb prophage
prophage excision
attB
~400bp "not cured" PCR product
~900bp "cured" PCR product
cured
g
S. pyogenes SF370::SF370.1-str
(all prophage elements)
Passage 8
Passage 8 + streptomycin
RPM_chr (x10^4)
S. pyogenes genome position (Mb)
h
S. pyogenes SF370Δ.2-4::SF370.1-str
(370.1 prophage only)
Passage 8
Passage 8 + streptomycin
RPM_chr (x10^4)
S. pyogenes genome position (Mb)

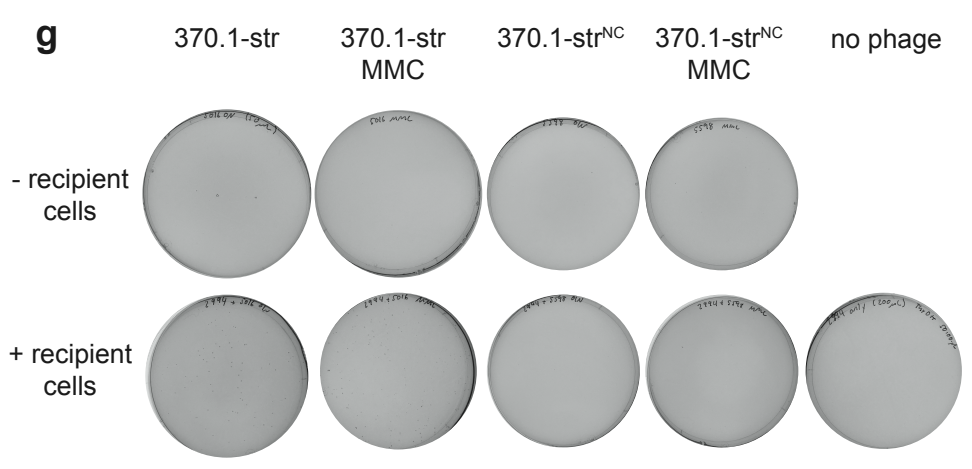












Strain	MMC	kanR colonies?
CEM1Δ1::SF370.1-str	-	+
CEM1Δ1::SF370.1-str	+	++
CEM1Δ1::SF370.1-str(repNC)	-	-
CEM1Δ1::SF370.1-str(repNC)	+	-
CEM1Δ1::SF370.1-str(Δint)	-	-
CEM1Δ1::SF370.1-str(Δint)	+	-
CEM1Δ1::ΔrecA::SF370.1-str	-	-
CEM1Δ1::ΔrecA::SF370.1-str	+	-