CRISPR-Cas Controls Cryptic Prophages

Sooyeon Song[1,2,3*], Ekaterina Semenova[4], Konstantin Severinov[4], Laura Fernández-García[1], Michael J. Benedik[5], and Thomas K. Wood[1*]

[1]Department of Chemical Engineering, Pennsylvania State University,
University Park, Pennsylvania, 16802-4400, USA
Departments of Animal Science[2] and Agricultural Convergence Technology[3], Jeonbuk National University, 587 Baekje-Daero, Deojin-Gu, Jeonju-Si, Jellabuk-Do, 54896, South Korea
[4]Waksman Institute of Microbiology, Rutgers, The State University of New Jersey, Piscataway, NJ 08854
[5]Hamad bin Khalifa University, Education City, Doha, Qatar.

*For correspondence. E-mail songsy@jbnu.ac.kr, tuw14@psu.edu

Tel. (+)1 814-863-4811; Fax (1) 814-865-7846

Running title: **CRISPR-Cas tames cryptic prophages**.

Keywords: CRISPR-Cas, persisters, cryptic prophage

ABSTRACT

The bacterial archetypal adaptive immune system, CRISPR-Cas, is thought to be repressed in the best-studied bacterium, *Escherichia coli* K-12 and thought to be inactive against prophages. Instead, we demonstrate here that the *E. coli* CRISPR-Cas system is active and serves to inhibit its nine defective (i.e., cryptic) prophages. Specifically, compared to the wild-type strain, reducing interfering RNAs (crRNA) from the spacer region due to a *cas2* polar mutation, reduces growth by 40%, increases cell death by 700%, and prevents persister cell resuscitation. Similar results were obtained by inactivating CRISPR-Cas by deleting the entire 13 spacer region (CRISPR array); hence, CRISPR-Cas serves to inhibit the remaining deleterious effects of these cryptic prophages, most likely through CRISPR array-derived crRNA binding to cryptic prophage mRNA and subsequent cleavage of the double-stranded RNA, rather than through cleavage of cryptic prophage DNA; i.e., self-targeting. Consistently, seven of the 13 *E. coli* spacers contain partial matches to the cryptic prophages, and inactivation of CRISPR-Cas increases the level of cryptic prophage CP4-57 and DLP-12 lytic gene transcripts for lysis protein YdfD of Qin and lysis protein RzoD of DLP-12. Also, lysis is clearly seen via transmission electron microscopy when the CRISPR-Cas array is deleted. Therefore, we report the novel results that (i) CRISPR-Cas is active in *E. coli* and (ii) CRISPR-Cas is used to tame cryptic prophages; i.e., unlike with active lysogens, CRISPR-Cas and cryptic prophages may stably exist.

INTRODUCTION

Along with restriction/modification[1] and toxin/antitoxin (TA) systems[2], prokaryotes utilize clustered, regularly-interspaced, short palindromic repeats (CRISPR) and CRISPR-associated (Cas)[3] proteins to combat phages. These systems are interrelated in that we found some Cas proteins and TA systems have a common ancestor; for example, *Sulfolobus solfataricus* Cas2 is structurally similar to antitoxin GhoS of the *Escherichia coli* GhoT/GhoS TA system[4]. In addition, in a manner similar to our discovery[2] that toxins of TA systems inhibit phage by degrading host mRNA after the toxin is activated by the attacking phage when the phage shuts down transcription (e.g., Hok/Sok inhibits T4 phage), some Cas proteins induce host dormancy rather than degrading phage DNA to inhibit phage propagation[5]. Also, TA systems have been found to stabilize CRISPR-Cas systems by making them addictive to the host[6].

Although CRISPR-Cas systems exclude both external lytic and temperate (lysogenic) phages[7], CRISPR-Cas systems of lysogens that target their own integrated prophages decrease long-term fitness and either the cell dies or the prophage is lost[7,8]. Also, the class I-E[3] CRISPR–Cas system of *E. coli* is not related to immunity for external phages[9] and appears to be inactive in the wild-type strain at standard laboratory conditions[10], due to repression by H-NS, although it is functional when induced[11]. To date, the relationship of CRISPR-Cas to cryptic prophages; i.e., those phage remnants that are unable to form lytic particles, has not been investigated.

Up to 20% of a single bacterial genomes may contain stably-integrated phage DNA[12], and for *E. coli,* we discovered that its nine cryptic prophages are not genomic junk but instead encode genes for proteins that increase resistance to sub-lethal concentrations of quinolone and β-lactam antibiotics as well as protect the cell from osmotic, oxidative, and acid stresses (by deleting 166 kb)[13]. Although these cryptic prophages do not enhance the formation of persister cells, a subpopulation of cells that weather extreme stress by entering into a dormant state[14], these phage remnants facilitate the resuscitation of persisters via nutrient sensing[15]. Therefore, the bacterial cell can co-opt the genome of its former parasite to both combat stress[13] as well as to revive from dormancy[15].

Given these active roles of cryptic prophages in the stress response[13] and persister cell resuscitation[15],

we hypothesized here that the native *E. coli* CRISPR-Cas system plays an active role in the regulation of
45 cryptic prophages. We find that the CRISPR-Cas system is required for inhibiting these phage fossils, since, if CRISPR-Cas is inactivated by preventing crRNA production, cells die due to activation of the cryptic prophage lysis proteins YdfD of Qin and RzoD from DLP-12. Hence, we discovered CRISPR-Cas is active in *E. coli* and serves to regulate its former phage foe.

RESULTS

50 **CRISPR-Cas increases growth.** We assayed the importance of CRISPR-Cas in *E. coli* K-12 by testing the individual deletions of the CRISPR-Cas genes on growth in M9 glucose medium (*cas1, cas2, cas3, casA, casB, casC, casD,* and *casE*) and found only the *cas2* deletion, with its kanamycin cassette insertion, had an effect: *cas2* causes 40% slower growth in rich medium compared to the wild-type strain (specific growth rate of 0.79 ± 0.21/h vs. 1.3 ± 0.11/h, respectively). Similarly, in minimal glucose (0.4 wt%) medium,
55 deletion of *cas2* also reduces growth by 33% (0.42 ± 0.02/h vs. 0.62 ± 0.02/h, respectively). We hypothesized that the *cas2* deletion/kanamycin insertion caused a polar effect by reducing spacer production given it is directly upstream of the spacer region (**Fig. S1**). Corroborating these results, inactivation of CRISPR-Cas by eliminating the CRISPR arrays in the isogenic host BW25113 (henceforth Δspacer) also reduced growth by 32% in rich medium. Therefore, the *E. coli* CRISPR-Cas represses some processes that
60 inhibit growth via crRNA, and by this criterion, is active, providing a clear advantage to the host.

CRISPR-Cas increases single-cell resuscitation. Moreover, since CRISPR-Cas increases growth, we tested for its effect on persister cell resuscitation using single-cell microscopy. Persister cell resuscitation is germane in that the dormant cells are highly stressed and have limited resources for their revival via activation of hibernating ribosomes[16-18]; for example, we have shown inhibiting ATP synthesis leads to a
65 5,000-fold increase in persister cell formation[19]. Since we discovered facile means for converting the whole population of cells into persister cells[16,17,19] that has been used by at least 17 independent labs to date with various bacterial species[20], these stressed cells are an excellent model for testing the effects of CRISPR-Cas on *E. coli* physiology.

Here, we found that deletion of *cas2* nearly completely prevents persister cell resuscitation (reduction
70 of 31-fold), while the wild-type cell has 52% resuscitation (**Fig. 1A, Table S1**). Persister cell resuscitation was also prevented (15-fold reduction) in the Δspacer strain (**Fig. 1A, Table S1**). Hence, CRISPR-Cas is active in *E. coli* and plays key roles in its growth and recovery from extreme stress.

CRISPR-Cas prevents cell death by preventing cell lysis. To explore how CRISPR-Cas increases growth (including persister cell resuscitation), we checked for death in resuscitating persister cells of the *cas2*
75 deletion strain using the Live/Dead stain. We found that inactivating CRISPR-Cas leads to a 7-fold increase in death in resuscitating cells (**Fig. 1B, Table S2**). In addition, there were 34-fold more cells termed "ghosts"[21] that lack cytosolic material (**Fig. S2A**) and are likely dead but have intact membranes so are not stained by the propidium iodide dye. Corroborating these results, there was 11-fold and 5-fold more death for stationary- and exponential-phase cells, respectively, when CRISPR-Cas was inactivated via *cas2*.
80 Moreover, for the Δspacer strain, there was 81-fold more cell death in the for stationary-phase cells (**Fig. S3**) and 7-fold more death for resuscitating persister cells (**Fig. 1B, Table S2**). Critically, for the Δspacer strain, waking persister cells clearly show lysis (**Fig. S2A**), and transmission electron microscopy images confirm this lysis with cytosolic materials seen next to lysed cells (**Fig. S2B**). These results indicate inactivating CRISPR-Cas leads to cell death and that the mechanism for cell death is via lysis.

85 **CRISPR-Cas reduces cryptic prophage lysis gene mRNA.** We hypothesized that since CRISPR-Cas systems inhibit some phage before they become lysogens[7,8], the *E. coli* system may be preventing cell death by repressing certain cryptic prophage genes. To test this hypothesis, we first examined the *E. coli* CRISPR-Cas system for spacers related to the nine cryptic prophages. *E. coli* K-12 CRISPR-Cas contains 13 spacers[11,22], each containing 32 or 33 nt[23], between *iap* and *cas2*. Between the 14 29-nt repeat sequences
90 (5'-GTGTTCCCCGCATCAGCGGGGATAAACCG), we found that six of the 13 spacers contain 12 to 16 nt of perfect matches to seven of the nine cryptic prophages (DLP-12, CP4-57, CPS-53, CP4-6, Rac, Qin, and e14) (**Fig. 2A**). In general, spacer lengths vary from 21 to 72 nt[24] with perfect complementarity of 6 to 12 nt[10]. Furthermore, predicted target protospacers in cryptic phage DLP-12, 3'-TTC (consensus CRISPR-

Cas I-E site) and 3'-ATG were identified (**Fig. S4**), supporting rapid and reduced interference in *E. coli*,
95 respectively[25]. Together, these results suggest CRISPR-Cas potentially regulates the *E. coli* cryptic prophages. Note the incomplete CRISPR-Cas in *E. coli* K-12 in between genes *ygcE* and *queE* lacks spacers with sequences that matched the cryptic prophages.

The presence of the cryptic prophage-related targeting spacers suggests that *E. coli* CRISPR-Cas may be preventing expression of cryptic prophage genes and, given the cell phenotype, is suggestive of
100 prophage-encoded lethal genes; hence, we checked the mRNA levels of the cryptic prophage lysis genes encoded by these seven cryptic prophages with spacer matches. Specifically, transcription of *ydfD* (Qin), *hokD* (Qin), *ypjF* (CP4-57), *essD* (DLP-12), and *rzoD* (DLP-12) was checked via quantitative reverse transcription polymerase chain reaction (qRT-PCR). We found that inactivation of CRISPR-Cas via the *cas2* deletion results in a 35-fold increase in *ydfD* mRNA and a 39-fold increase in *rzoD* mRNA in
105 resuscitating persister cells (**Fig. 1C**). RzoD is a putative DLP-12 lysis lipoprotein that we previously showed was toxic through its interaction with toxin Hha[26] of the Hha/TomB TA system[27]. YdfD of Qin cryptic prophage has been shown to lyse cells when induced[28]. Hence, these results show CRISPR-Cas represses at least two *E. coli* proteins RzoD and YdfD that can reduce cell growth.

Excision of DLP-12 is not regulated by CRISPR-Cas. Since inactivation of CRISPR-Cas leads to
110 derepression of the DLP-12 *rzoD* lysis gene, we checked for increased DLP-12 excision with the *cas2* deletion strain using qPCR. We found the *cas2* deletion has little impact on DLP-12 excision (**Table S3**). Given that the DLP-12 toxin gene *essD* is not derepressed also suggests the effect is not due to excision of the prophage. We also tested the effect of CRISPR-Cas on two other cryptic prophages with significant excision (CP4-57 and e14)[13], and also found no effect of deleting *cas2* (**Table S3**). Hence, CRISPR-Cas
115 does not affect cryptic prophage excision.

CRISPR-Cas regulates lytic gene mRNA levels. Since CRISPR-Cas does not affect cryptic prophage excision and is unlikely to degrade cryptic prophage DNA as this would be lethal[7,8], we reasoned that CRISPR-Cas, via crRNA, must be interfering with expression of the mRNA of the lytic genes to prevent production of the lytic proteins. To test this hypothesis, we investigated whether *rzoD* and *ydfD* transcript

120 levels are increased in the *cas2* and Δspacer strain relative to the wild-type after inhibiting transcription via rifampicin. If CRISPR-Cas prevents lytic gene expression by inhibiting lytic transcripts via crRNA, then inactivating CRISPR-Cas should lead to increased *rzoD* and *ydfD* mRNA. In accordance with this hypothesis, *rzoD* mRNA was increased 320% in the Δspacer mutant and 50% in the *cas2* mutant. Moreover, as expected, inactivating CasE increased cell lysis 49-fold compared to the wild-type strain (**Fig. S3, Table**
125 **S2)**, likely by preventing CasE in the CRISPR cascade from properly forming crRNA from pre-crRNA; hence, there is less crRNA to inhibit the cryptic prophage lytic mRNA. Similarly, preventing spacer RNA formation by inactivating Cas2 led to a 10-fold increase in CRISPR array RNA levels in resuscitating persister cells as assayed by qRT-PCR, likely due to a lack of processing of the CRISPR array into crRNA (**Table S3B**). Therefore, CRISPR-Cas prevents cryptic prophage lytic gene expression by interfering with
130 the transcripts from these genes (**Fig. 2B**).

DISCUSSION

Our results reveal a new (non-canonical) role for CRISPR-Cas systems: regulation of phage fossils. The evidence for this includes that inactivating CRISPR-Cas by deleting *cas2* (i) reduces growth by 40%, (ii) nearly eliminates resuscitation from the persister state (**Fig. 2**), (iii) causes ghost cell formation and cell
135 death (**Fig. 1**), (iv) increases mRNA levels of the cryptic prophage lysis genes *ydfD* and *rzoD,* and (v) increases CRISPR array levels by preventing processing into crRNA. Corroborating these results, eliminating the 13 spacers also reduced the growth rate, eliminated persister cell resuscitation, resulted in cell death via lysis, and activated *rzoD.* Given that Cas2 is not required for CRISPR interference, it is likely that the phenotypes produced upon deleting *cas2* are the result of a polar mutation (**Fig. S1**) that inactivates
140 CRISPR-Cas by altering production/processing of the spacers.

Since there is no change in excision of DLP-12 upon inactivating CRISPR-Cas, our results suggest the mechanism for regulating the lysis genes *ydfD* and *rzoD* of the cryptic prophages is via CRISPR-Cas RNA binding to the cryptic prophage mRNA (analogous to RNA interference as seen previously in procaryotic systems with CRISPR-Cas[29]) rather than cleaving excised DNA or directly cleaving the mRNA. This

145 mechanism has been predicted based on bioinformatics with 230 phages, that CRISPR-Cas functions in *E. coli* via endogenous gene expression rather than acting as an immune system[30]. In support of this, the excision rates of the cryptic prophages are so low that there may be insufficient excised DNA to cleave (1 excision in 10^6 cells for CP4-57)[13], and a class I-F CRISPR-Cas system in *P. aeruginosa* uses crRNA to regulate endogenous LasR mRNA to reduce the pro-inflammatory response[31]. Moreover, *E. coli* crRNA
150 have been shown to be activated during infection in mice[31], and the *Salmonella* spp. system is identical to the *E. coli* one (e.g., the repeats are the same), but the spacers are different, which suggests it, too, is for endogenous gene expression.

This new function for CRISPR-Cas with cryptic prophages is likely general and may explain why many species appear to have inactive CRISPR-Cas systems as was previously thought for *E. coli*[10]; i.e., instead
155 of protecting cells from external phages, CRISPR-Cas systems may also control resident cryptic prophages which are prevalent. As additional evidence, we found matches in CRISPR-Cas spacers not only for *E. coli* K-12 (**Fig. 2A**) but also for *Salmonella* spp. and *E. coli* O157:H7 (**Fig. S5**). Critically, our results provide the first example where it is beneficial for the host to have an active CRISPR-Cas system that targets *inactive* integrated phages (i.e., cryptic prophages) since previous reports show targeting *active* temperate
160 phages is deleterious; i.e., either the cell dies or the phage is lost[7,8,32]. Since *E. coli* cryptic prophages like *rac* have been present in its genome for 4.5 million years[33], the active *E. coli* K-12 CRISPR-Cas system is stable with the cryptic prophages; in fact, there has been little change in the *E. coli* spacers for at least 42,000 years[34].

Our results also indicate that, although the cryptic prophages are stable and the cell makes use of the
165 genetic tools encoded by its former foe to combat myriad stresses[13] and to sense nutrients prior to exiting the persister state[15], the source of these tools must be elegantly regulated by CRISPR-Cas since they often harbor deleterious membrane lysis proteins like YdfD and RzoD. Similarly, host Rho has been found recently to silence cryptic prophage toxin/antitoxin systems through transcription termination[35], and H-NS silences cryptic prophages through 65 binding sites[36]. Therefore, phages may be captured by the host, but
170 they must be tamed, and this now includes repression by CRISPR-Cas.

MATERIALS AND METHODS

Bacteria and growth conditions. Bacteria (**Table S4**) were cultured routinely in lysogeny broth[37] at 37ºC, while M9 glucose (0.4 wt%)[38] was used to assay the growth rate and to resuscitate persister cells. pCA24N-based plasmids[39] were retained in overnight cultures via chloramphenicol (30 μg/mL), and kanamycin (50
175 μg/mL) was used for deletion mutants, where applicable. The BW25113 *cas2* and spacer deletion were confirmed by PCR (primers shown in **Table S5**).

Persister cells. Exponentially-growing cells (turbidity of 0.8 at 600 nm) were converted nearly completely to persister cells[16,19] by adding rifampicin (100 μg/mL) for 30 min to stop transcription, centrifuging, and adding LB with ampicillin (100 μg/mL) for 3 h to lyse non-persister cells. To remove ampicillin, cells were
180 washed twice with 0.85% NaCl then re-suspended in 0.85% NaCl. Persister concentrations were enumerated via a drop assay[41].

Single-cell persister resuscitation. Persister cells (5 μL) were added to 1.5% agarose gel pads containing M9 glucose (0.4 wt%) medium [38], and single-cell resuscitation was visualized at 37°C via a light microscope (Zeiss Axio Scope.A1, bl_ph channel at 1000 ms exposure). For each condition, at least two independent
185 cultures were used with 150 to 300 individual cells assessed per culture.

Membrane integrity assay. To determine membrane integrity, the persister cells were analyzed with the LIVE/DEAD BacLight Bacterial Viability Kit (Molecular Probes, Inc., Eugene, OR, catalog number L7012). The fluorescence signal was analyzed via a Zeiss Axioscope.A1 using excitation at 485 nm and emission at 530 nm for green fluorescence and using excitation at 485 nm and emission at 630 nm for red
190 fluorescence.

qRT-PCR. To quantify transcription from the cryptic prophage lytic genes, RNA was isolated from persister cells that were resuscitated by adding M9 glucose (0.4 wt%) medium for 10 min and from exponential cells grown to a turbidity of 0.8. For quantifying transcription from the CRISPR array, RNA was isolated from persister cells that were resuscitated by adding M9 glucose (0.4 wt%) medium for 10
195 min. Samples were cooled rapidly using ethanol/dry ice in the presence of RNA Later. RNA was isolated using the High Pure RNA Isolation Kit (Roche). The following qRT-PCR thermocycling protocol was used

with the iTaq™ universal SYBR® Green One-Step kit (Bio-Rad): 95 ºC for 5 min; 40 cycles of 95 ºC for 15 s, 60 ºC for 1 min for two replicate reactions for each sample/primer pair. The annealing temperature was 60ºC for all primers (**Table S5**).

200 **qPCR**. To quantify prophage excision and the levels of DNA flanking the CRISPR-Cas cleavage sites, total DNA (100 ng) was isolated from exponentially-growing and persister resuscitating cells using an UltraClean Microbial DNA Isolation Kit (Mo Bio Laboratories). Excised cryptic prophage was quantified using primers for each prophage excisionase (**Table S5**) that only yield a PCR product upon prophage excision, and the relative amount of each target gene was determined using reference gene *purM*. The level

205 of cryptic prophage flanking the CRISPR-Cas cleave site was quantified using primers that flank each site (**Table S5**). The qPCR reaction performed using CFX96 Real Time System. The reaction and analysis was conducted using the StepOne Real-Time PCR System (Bio-Rad).

Transmission electron microscopy. For transmission electron microscopy (TEM), the samples were prepared from persister cells that were resuscitated by adding M9 glucose (0.4%) medium for 10 min then

210 washed with 0.85% NaCl. The samples were fixed with buffer (2.5% glutaraldehyde in 0.1M cacodylate buffer, pH 7.4). The negative staining was performed with 2% uranyl acetate for 1 hr, then dehydrated. The sectioned specimens were stained again with uranyl acetate and lead citrate after dehydration and embedded with resin. The TEM image was observed using a Hitachi (H-7605) instrument.

ACKNOWLEDGEMENTS

215 This work was supported by funds derived from the Biotechnology Endowed Professorship at the Pennsylvania State University for TKW and from the National Research Foundation of Korea (NRF) grant from the Korean Government (NRF-2020R1F1A1072397) for SYS. We appreciate the feedback of Dr. Joseph Wade on the PAM sequences. The authors have no conflicts of interest.

REFERENCES

1. Vasu, K. & Nagaraja, V. Diverse Functions of Restriction-Modification Systems in Addition to Cellular Defense. *Microbiol Molec Biol Rev* **77**, 53-72 (2013).
2. Pecota, D. C. & Wood, T. K. Exclusion of T4 Phage by the *hok/sok* Killer Locus from Plasmid R1. *J Bacteriol* **178**, 2044-2050 (1996).
3. Makarova, K. S. *et al.* Evolutionary classification of CRISPR–Cas systems: a burst of class 2 and derived variants. *Nature Rev Microbiol* **18**, 67-83 (2020).
4. Wang, X. *et al.* A Novel Type V TA System Where mRNA for Toxin GhoT is Cleaved by Antitoxin GhoS. *Nature Chem Biol* **8**, 855-861 (2012).
5. Meeske, A. J., Nakandakari-Higa, S. & Marraffini, L. A. Cas13-induced cellular dormancy prevents the rise of CRISPR-resistant bacteriophage. *Nature* **570**, 241-245 (2019).
6. Li, M. *et al.* Toxin-antitoxin RNA pairs safeguard CRISPR-Cas systems. *Science* **372**, eabe5601 (2021).
7. Goldberg, G. W. *et al.* Incomplete prophage tolerance by type III-A CRISPR-Cas systems reduces the fitness of lysogenic hosts. *Nature Commun* **9**, 61 (2018).
8. Edgar, R. & Qimron, U. The *Escherichia coli* CRISPR System Protects from λ Lysogenization, Lysogens, and Prophage Induction. *J Bacteriol* **192**, 6291-6294 (2010).
9. Touchon, M. *et al.* CRISPR Distribution within the *Escherichia coli* Species Is Not Suggestive of Immunity-Associated Diversifying Selection. *J Bacteriol* **193**, 2460-2467 (2011).
10. Shmakov, S. A. *et al.* The CRISPR Spacer Space Is Dominated by Sequences from Species-Specific Mobilomes. *mBio* **8**, e01397-01317 (2017).
11. Pougach, K. *et al.* Transcription, processing and function of CRISPR cassettes in *Escherichia coli*. *Mol Microbiol* **77**, 1367-1379 (2010).
12. Casjens, S. Prophages and bacterial genomics: what have we learned so far? *Mol Microbiol* **49**, 277-300 (2003).
13. Wang, X. *et al.* Cryptic prophages help bacteria cope with adverse environments. *Nat Commun* **1**, 147 (2010).
14. Wood, T. K. & Song, S. Forming and waking dormant cells: The ppGpp ribosome dimerization persister model. *Biofilm* **2**, 100018 (2020).
15. Song, S. *et al. Escherichia coli* cryptic prophages sense nutrients to influence persister cell resuscitation. *Environ Microbiol* **23**, 7245-7254 (2021).
16. Kim, J.-S., Yamasaki, R., Song, S., Zhang, W. & Wood, T. K. Single Cell Observations Show Persister Cells Wake Based on Ribosome Content. *Environ Microbiol* **20**, 2085-2098 (2018).
17. Yamasaki, R., Song, S., Benedik, M. J. & Wood, T. K. Persister Cells Resuscitate Using Membrane Sensors that Activate Chemotaxis, Lower cAMP Levels, and Revive Ribosomes. *iScience* **23**, 100792 (2020).
18. Song, S. & Wood, T. K. Persister Cells Resuscitate via Ribosome Modification by 23S rRNA Pseudouridine Synthase RluD. *Environ Microbiol* **22**, 850–857 (2020).
19. Kwan, B. W., Valenta, J. A., Benedik, M. J. & Wood, T. K. Arrested protein synthesis increases persister-like cell formation. *Antimicrob Agents Chemother* **57**, 1468-1473 (2013).
20. Song, S. & Wood, T. K. Are We Really Studying Persister Cells? *Environ Microbiol Rep* **13**, 3–7 (2021).
21. Cheng, H.-Y. *et al.* Toxin GhoT of the GhoT/GhoS toxin/antitoxin system damages the cell membrane to reduce adenosine triphosphate and to reduce growth under stress. *Environ Microbiol* **16**, 1741-1754 (2014).
22. Militello, K. T. & Lazatin, J. C. Discovery of *Escherichia coli* CRISPR sequences in an undergraduate laboratory. *Biochem Molec Biol Edu* **45**, 262-269 (2017).
23. Brouns, S. J. J. *et al.* Small CRISPR RNAs Guide Antiviral Defense in Prokaryotes. *Science* **321**, 960-964 (2008).

24. Barrangou, R. & Marraffini, Luciano A. CRISPR-Cas Systems: Prokaryotes Upgrade to Adaptive Immunity. *Molec Cell* **54**, 234-244 (2014).
25. Musharova, O. *et al.* Systematic analysis of Type I-E *Escherichia coli* CRISPR-Cas PAM sequences ability to promote interference and primed adaptation. *Mol Microbiol* **111**, 1558-1570 (2019).
26. García Contreras, R., Zhang, X.-S., Kim, Y. & Wood, T. K. Protein Translation and Cell Death: The Role of Rare tRNAs in Biofilm Formation and in Activating Dormant Phage Killer Genes. *PLoS ONE* **3**, e2394 (2008).
27. Marimon, O. *et al.* An oxygen-sensitive toxin–antitoxin system. *Nature Commun* **7**, 13634 (2016).
28. Masuda, H., Awano, N. & Inouye, M. *ydfD* encodes a novel lytic protein in *Escherichia coli*. *FEMS Microbiol Lett* **363** (2016).
29. Mohanraju, P. *et al.* Alternative functions of CRISPR–Cas systems in the evolutionary arms race. *Nature Rev Microbiol* (2022).
30. Bozic, B., Repac, J. & Djordjevic, M. Endogenous Gene Regulation as a Predicted Main Function of Type I-E CRISPR/Cas System in E. coli. *Molecules* **24**, 784 (2019).
31. Li, R. *et al.* Type I CRISPR-Cas targets endogenous genes and regulates virulence to evade mammalian host immunity. *Cell Res* **26**, 1273-1287 (2016).
32. Rollie, C. *et al.* Targeting of temperate phages drives loss of type I CRISPR–Cas systems. *Nature* (2020).
33. Perna, N. T. *et al.* Genome sequence of enterohaemorrhagic *Escherichia coli* O157: H7. *Nature* **409**, 529-533 (2001).
34. Savitskaya, E. *et al.* Dynamics of *Escherichia coli* type I-E CRISPR spacers over 42 000 years. *Molecular ecology* **26**, 2019-2026 (2017).
35. Hafeezunnisa, M., Chhakchhuak, P. I. R., Krishnakumar, J. & Sen, R. Rho-dependent transcription termination regulates the toxin–antitoxin modules of cryptic prophages to silence their expression in *Escherichia coli*. *FEBS Lett* **15**, 2057-2067 (2021).
36. Ishihama, A. & Shimada, T. Hierarchy of transcription factor network in *Escherichia coli* K-12: H-NS-mediated silencing and Anti-silencing by global regulators. *FEMS Microbiol Rev* (2021).
37. Bertani, G. Studies on Lysogenesis .1. The Mode of Phage Liberation by Lysogenic *Escherichia-Coli*. *J Bacteriol* **62**, 293-300 (1951).
38. Rodriguez, R. L. & Tait, R. C. *Recombinant DNA Techniques: An Introduction*. (Benjamin/Cummings Publishing, 1983).
39. Kitagawa, M. *et al.* Complete set of ORF clones of *Escherichia coli* ASKA library (A Complete Set of *E. coli* K-12 ORF Archive): Unique Resources for Biological Research. *DNA Res* **12**, 291-299 (2005).
40. Datsenko, K. A. & Wanner, B. L. One-step inactivation of chromosomal genes in *Escherichia coli* K-12 using PCR products. *Proc Natl Acad Sci USA* **97**, 6640-6645 (2000).
41. Donegan, K., Matyac, C., Seidler, R. & Porteous, A. Evaluation of methods for sampling, recovery, and enumeration of bacteria applied to the phylloplane. *Appl Environ Microbiol* **57**, 51-56 (1991).
42. Pfaffl, M. W. A new mathematical model for relative quantification in real-time RT-PCR. *Nucl Acids Res* **29** (2001).
43. Baba, T. *et al.* Construction of *Escherichia coli* K-12 in-frame, single-gene knockout mutants: the Keio collection. *Mol Syst Biol* **2**, 2006 0008 (2006).
44. Pougach, K. *et al.* Transcription, processing and function of CRISPR cassettes in *Escherichia coli*. *Molec Microbiol* **77**, 1367-1379 (2010).
45. Kitagawa, M. *et al.* Complete set of ORF clones of *Escherichia coli* ASKA library (a complete set of *E. coli* K-12 ORF archive): unique resources for biological research. *DNA Res* **12**, 291-299 (2005).

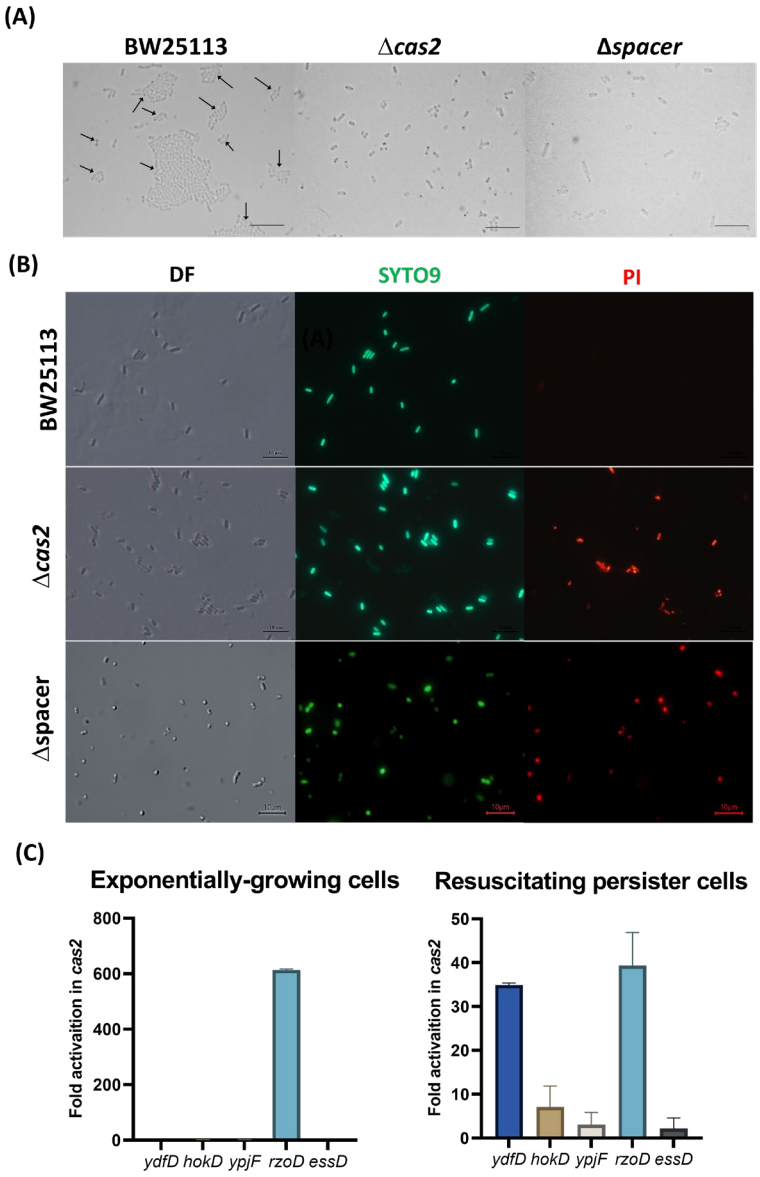


Fig. 1. Inactivating CRISPR-Cas eliminates persister cell resuscitation by activating cryptic prophage lytic proteins, causing cell death. (A) Single cell persister resuscitation for wild-type BW25113, the *cas2* mutant, and the Δspacer mutant after 6 hours on 0.4 wt% glucose minimal medium. Black arrows indicate cells that resuscitate, and the scale bar indicates 10 µm. Cells were observed using light microscopy (Zeiss Axio Scope.A1). Representative results from two independent cultures are shown, and tabulated cell numbers are in **Table S1**. **(B)** LIVE/DEAD staining of resuscitating persister cells shows the *cas2* and Δspacer mutations cause cell death. DF is dark field, SYTO9 is a membrane permeable stain for nucleic acids (green), and PI is propidium iodide, which is a membrane impermeable stain for the nucleic acids of dead cells (red). Tabulated cell numbers are in **Table S2**. **(C)** The *cas2* mutation derepresses cryptic prophage lysis genes *ydfD* (in resuscitating persister cells) and *rzoD* (in both resuscitating persister cells and exponentially-growing cells). Lytic genes from five cryptic prophages were checked by qRT-PCR: *ydfD* (Qin), *hokD* (Qin), *ypjF* (CP4-57), *essD* (DLP-12), and *rzoD* (DLP-12). Error bar indicates standard deviations.

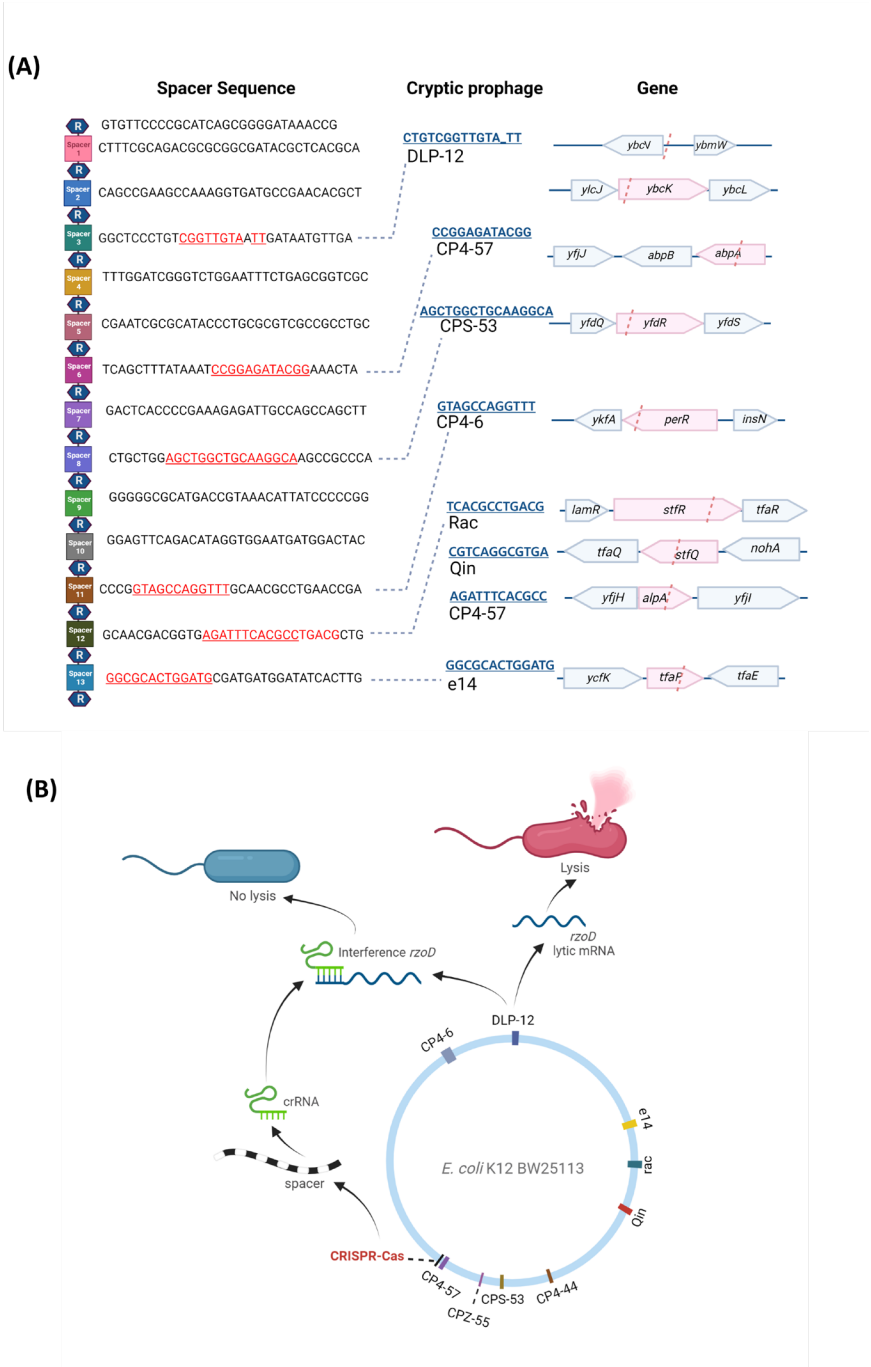


Fig. 2. ***E. coli*** **CRISPR-Cas spacer sequences and lytic gene inhibition mechanism. (A)** The 14 repeat (R, hexagon) and 13 spacer (squares) sequences of the CRISPR-Cas system (from the *iap* to *cas2* part of the *E. coli* genome) showing the cryptic prophage spacer matches (red text) and prophage DNA protospacer sequences (blue text) which includes matches to seven of the nine cryptic prophages (DLP-12, CP4-57, CPS-53, CP4-6, rac, Qin, and e14). Pink highlight and pink dashed lines indicate the spacer positions relative to the cryptic prophage genes. The potential PAM sequences are shown in **Fig. S4**. **(B)** Schematic for the mechanism by which CRISPR-Cas controls cryptic phage lytic genes.

Supporting Information

CRISPR-Cas Controls Cryptic Prophages

Sooyeon Song[1,2,3*], Ekaterina Semenova[4], Konstantin Severinov[4], Laura Fernández-García[1], Michael J. Benedik[5], and Thomas K. Wood[1*]

[1]Department of Chemical Engineering, Pennsylvania State University,
University Park, Pennsylvania, 16802-4400, USA
Departments of Animal Science[2] and Agricultural Convergence Technology[3], Jeonbuk National University, 587 Baekje-Daero, Deojin-Gu, Jeonju-Si, Jellabuk-Do, 54896, South Korea
[4]Waksman Institute of Microbiology, Rutgers, The State University of New Jersey, Piscataway, NJ 08854
[5]Hamad bin Khalifa University, Education City, Doha, Qatar.

*For correspondence. E-mail songsy@jbnu.ac.kr, tuw14@psu.edu

Tel. (+)1 814-863-4811; Fax (1) 814-865-7846

Table S1. Inactivating CRISPR-Cas eliminates persister cell resuscitation on glucose agarose gel pads. Single persister cells were observed using light microscopy (Zeiss Axio Scope.A1). The total number and waking number of persister cells are shown after 6 hours on 0.4 wt% glucose minimal medium. Fold-change in waking is relative to BW25113. These results are the combined observations from two independent experiments (independent culture results separated by "/"), and standard deviations are shown. Sample microscope images are shown in **Fig. 1.**

	Total cells	**Waking cells**	**% waking**	**Fold-change**
BW25113	504/382	199/248	52 ± 18	1
Δ*cas2*	516/677	6/15	1.7 ± 0.7	-30.9
Δspacer	323/174	10/7	3.6 ± 0.7	-14.6

Table S2. Inactivating CRISPR-Cas causes cell death and ghost cell formation. Persister cells were washed with PBS twice, resuscitated by M9 0.4% glucose for 10 min and stained with LIVE/DEAD reagents. Exponential cells were grown to a turbidity of 0.8 (at 600 nm) and stationary cells were grown to turbidity of 2.0. The ghost cells in the persister population were visualized using a Zeiss Axioscope.A1microscope. The results are the combined observations from two independent experiments (independent culture results separated by "/"). The microscope images are shown in **Fig. 1AB** and **Fig. S3**.

	Strains	Total cells	Dead cells	Ghost cells	% Ghost cells	fold-change ghost cells	% dead	fold-change dead cells
Resuscitated	**BW25113**	52/115	2/7	0/1	0.6	1	5.4	1
	Δ*cas2*	142/106	54/38	38/13	20.6	34	37.1	6.9
	Δspacer	161/221	76/76	-	-	-	37.8	7.0
Exponential	**BW25113**	153/147	0/2	-	-	-	0.7	1
	Δ*cas2*	59/40	1/2	-	-	-	3.4	4.9
	Δspacer	95/146	1/5				2.1	3.15
Stationary	**BW25113**	492/123	1/0				0.1	1
	Δ*cas2*	1136/1736	14/18	-	-	-	1.13	11.2
	Δspacer	200/113	19/8				8.26	81.3
	Δ*casE*	208/175	9/10	-	-	-	5.02	49.4

Table S3. CRISPR-Cas does not affect cryptic prophage excision in stationary cells but produces the CRISPR array. (**A**) Fold changes in excision are relative to the wild-type strain as determined by qPCR. (**B**) Fold changes in the CRISPR array are relative to the wild-type strain as determined by qRT-PCR (the Δspacer strain was used as a negative control). Cycle numbers (C_t) are indicated for each sample including that for the target genes as well as that of the house-keeping gene, *purM,* which was used to normalize the data. *cas2* served to inactivate crRNA production due to a polar mutation. Fold changes in the transcription of various targets (i.e., cryptic prophage CP4-57, e14, DLP-12, Spacer3, and Spacer12) with and without *cas2*$^+$ (*cas2*$^+$/*cas2*$^-$) were calculated as described earlier[42]:

$$2^{-(C_{t\ target\_cas2+} - C_{t\ purM\_cas2+})} / 2^{-(C_{t\ target\_cas2-} - C_{t\ purM\_cas2-})}$$

(A) qPCR

Gene	***purM***		**CP4-57**		**e14**		**DLP-12**	
Strain	WT	*cas2*	WT	*cas2*	WT	*cas2*	WT	*cas2*
C_T	9.30 ± 0.13	9.70 ± 0.18	30.45 ± 1.73	30.37 ± 0.68	16.30 ± 0.06	17.45 ± 0.51	30.58 ± 0.49	30.05 ± 0.5
ΔC_T			21.16 ± 1.73	20.67 ± 0.70	7.00 ± 0.15	8.16 ± 0.54	21.29 ± 0.51	20.35 ± 0.53
$\Delta\Delta C_T$				-0.48 ± 0.7		1.16 ±0.54		-0.94 ±0.53
fold				1.40		-2.23		1.92

(B) qRT-PCR

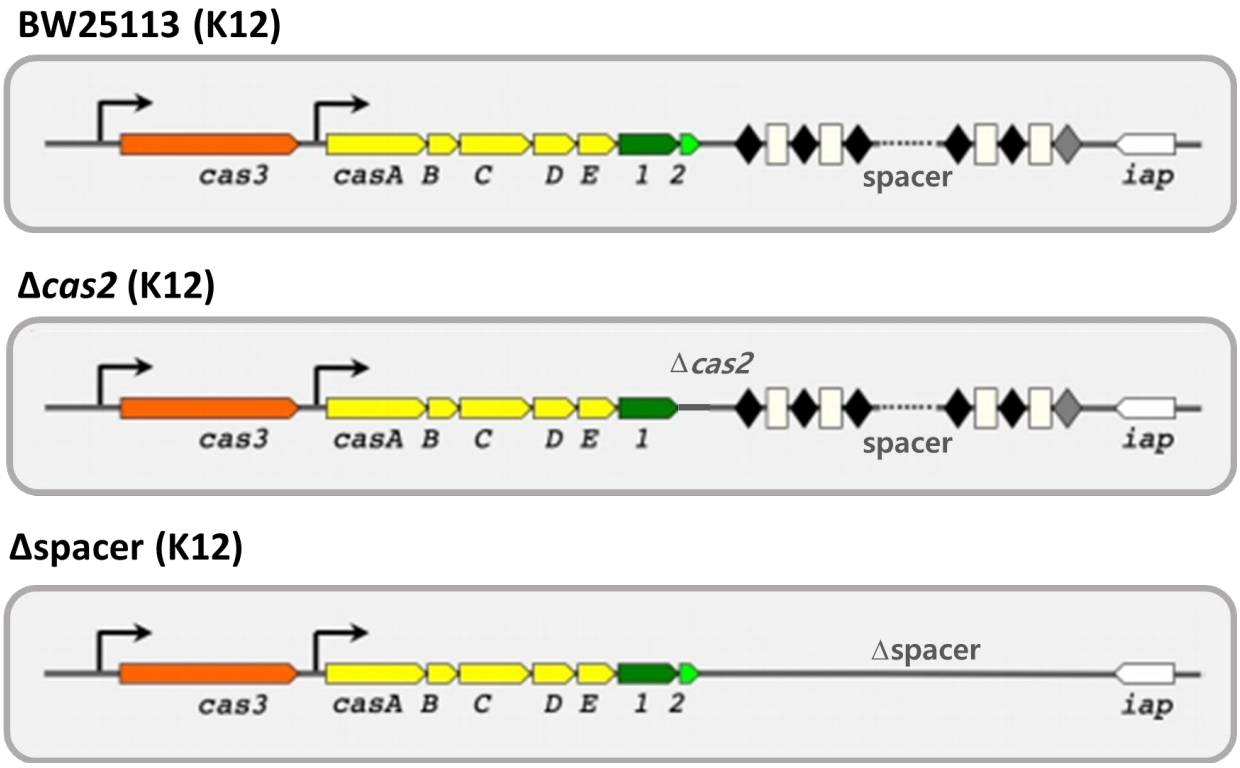
Gene	***purM***			**Spacer3**			**Spacer12**		
Strain	WT	*cas2*	spacer	WT	*cas2*	spacer	WT	*cas2*	spacer
C_T	25.3 ± 0.5	22.2 ± 0.5	22.0 ± 0.2	30.68 ± 1.73	24.35 ± 0.93	-	33.60 ± 1.20	27.12 ± 1.22	-
ΔC_T				5.37 ± 1.08	2.19 ± 1.1	-	8.28 ± 1.31	4.96 ± 1.32	-
$\Delta\Delta C_T$					-3.20 ± 1.51	-		-3.33 ±1.86	-
fold					9.11	-∞		10.02	-∞

Table S4. *E. coli* bacterial strains and plasmids utilized.

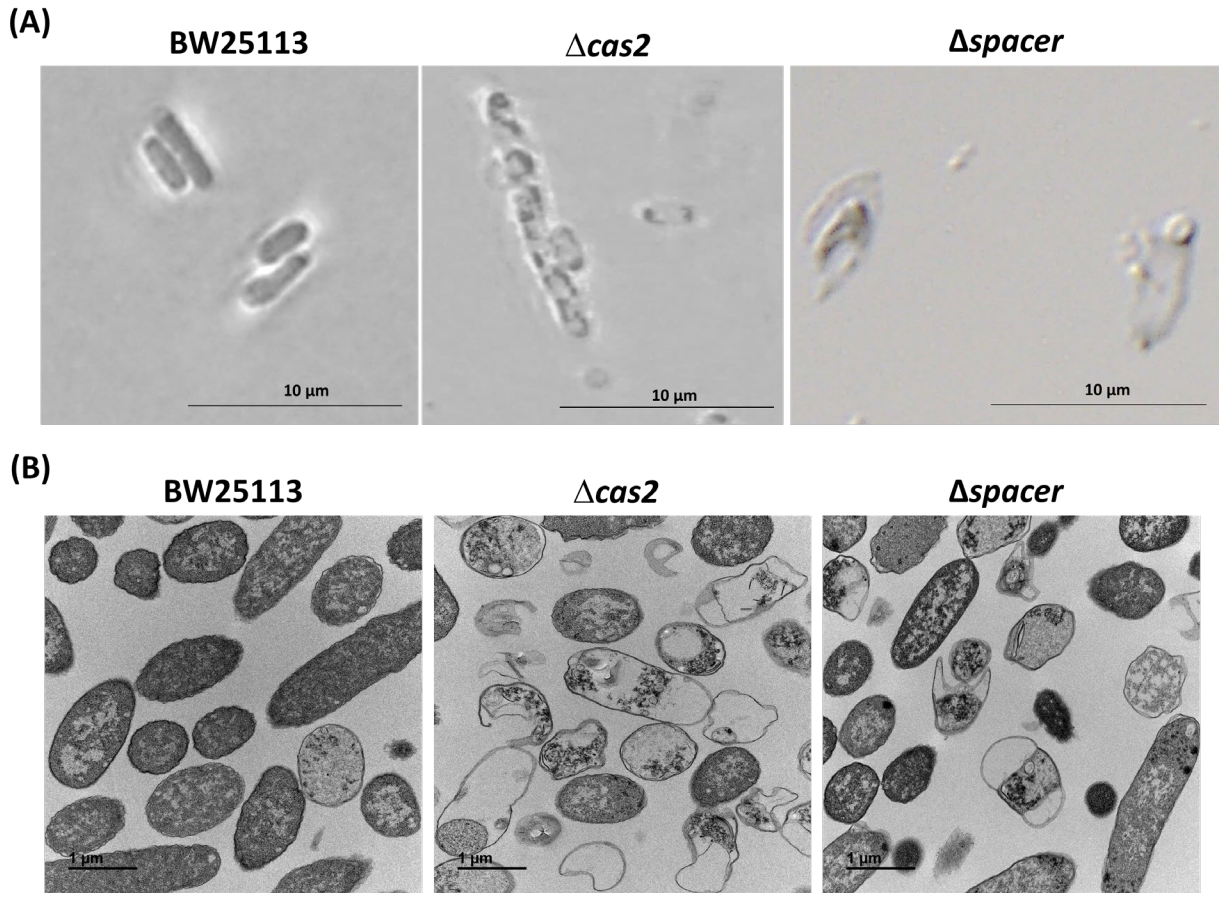
Strains and Plasmids	Features	Source
Strains		
BW25113	*rrnB3* Δ*lacZ4787 hsdR514* Δ(*araBAD*)*567* Δ(*rhaBAD*)*568 rph-1*	43
BW25113 Δ*cas1*	Δ*cas1*, ΩKm^R	43
BW25113 Δ*cas2*	Δ*cas2*, ΩKm^R	43
BW25113 Δ*cas3*	Δ*cas3*, ΩKm^R	43
BW25113 Δ*casA*	Δ*casA*, ΩKm^R	43
BW25113 Δ*casB*	Δ*casB*, ΩKm^R	43
BW25113 Δ*casC*	Δ*casC*, ΩKm^R	43
BW25113 Δ*casD*	Δ*casD*, ΩKm^R	43
BW25113 Δ*casE*	Δ*casE*, ΩKm^R	43
BW25113 Δspacer (also called BW39292)	Δ(13 spacer region), ΩKm^R	44
Plasmids		
pCA24N	Cm^R; *lacI*^{q}	45
pCA24N_*cas2*	Cm^R; *lacI*^{q}, $P_{T5\text{-}lac}$::*cas2*$^+$	45
pKD4	FRT::Kan^R::FRT, Amp^R	40

Table S5. Primers used in this study for qRT-PCR and qPCR. * indicates excision primers.

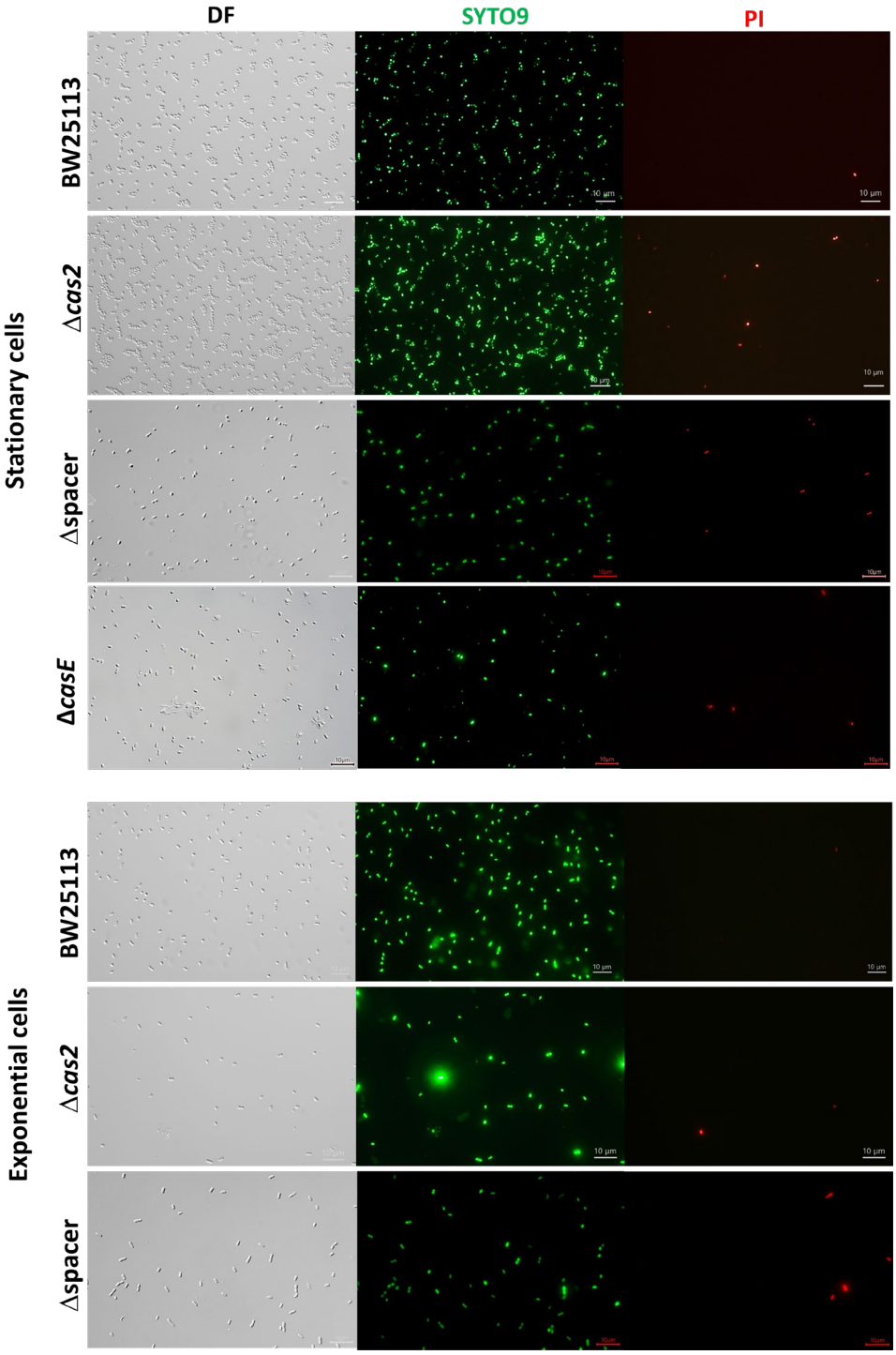
Gene	Sequence (5'-'3)
qRT-PCR	
purM	**F:** CGGTGTTGATATTGACGCGGG
	R: CAGCACGGGTTCACGATATTTTTG
*ydfD (*Qin*)*	**F:** TCAGCATTTGTGCTTGTTCTG
	R: CTGCCGGGATTTCGATATTA
*hokD (*Qin*)*	**F:** GCCCTGATCGTCATCTGTTT
	R: AGCTGTGAAGACAGCGACCT
*ypjF (*CP4-57*)*	**F:** TATGGCCTGACACTGAACGA
	R: GTTGTTGCCACGAAAATCCT
rzoD (DLP-12)	**F:** TGCTCTGCGTGATGATGTTG
	R: TCTCTCTGAGGGTGAAATAATCC
essD (DLP-12)	**F:** CTCACAGTGGGCAGCAATAG
	R: TATTCACCTCTCGCAGCCTT
Spacer3	**F:** GATAAACCGCTTTCGCAGAC
	R: AGAAATTCCAGACCCGATCC
Spacer12	**F:** AACGCCTGAACCGAGAGTT
	R: CTGGGAGTTCTACCGCAGAG
qPCR	
*CP4-57	**F:** AAGCATGTAGTACCGAGGATGTAGG
	R: TATGTCTCCTCACCGTCTGGTCGG
*e14	**F:** GTGCAAACATCGGTGACGAA
	R: TTCAGCAGCTTAGCGCCTTC
*DLP-12	**F:** CAAAAGCCATTGACTCAGCAAGG
	R: CGGATAAGACGGGCATAAATGA
purM 400	**F:** CTGATTGCACTCGGTTCCAG
	R: CGTTTTCACCGTTGGCATTG
Spacer 3-1	**F:** CATGCAATTACAACATCAGGGTA
	R: GCCATTGGTAAAACCTTCCA
Spacer 3-2	**F:** AAATTCCGAAAAAGCTCCTGA
	R: TGCTTTCAAGATTATGGCGTA
Spacer 6	**F:** CACGAAAGCCAGCCTATTCC
	R: CCGCTGTTTCTTTCTCCAGG
Spacer 8	**F:** GCCAGCATAATGAGATCGGC
	R: TTTTACCCAAACTCAGCGCG
Spacer 11	**F:** TGTGCAGTTGTACCAGTGGA
	R: CAACCCAGCAAAGTTTCGGA
Spacer 12-1	**F:** TGTATGTGCCCCGGTGTTAT
	R: GCAGATGAAGGCGCATTACA
Spacer 12-2	**F:** AGCTTTACACCTCGGCTCAT
	R: CCGGAACTCTTGTGTTGGTG
Spacer 12-3	**F:** TCTACAGGGAAAGGACGACC
	R: CTCTGCAACCAAAGTGAACCA
Spacer 13	**F:** ACAACCACTATCGCCCCTTT
	R:GGTAAGGCTGCATTGGGAAG
Verification for cas2 and Spacer	
Cas2	**F:** GAAAATGTACCTCCGCGCTT
	R: CCATCCAAATCTACCGGGGT
Spacer	**F:** GGATCGGGTCTGGAATTTCT
	R: GGGGAACTCGTAGTCCATCA



Supplementary Figure 1. Schematics of the CRISPR-Cas strains used in this study.



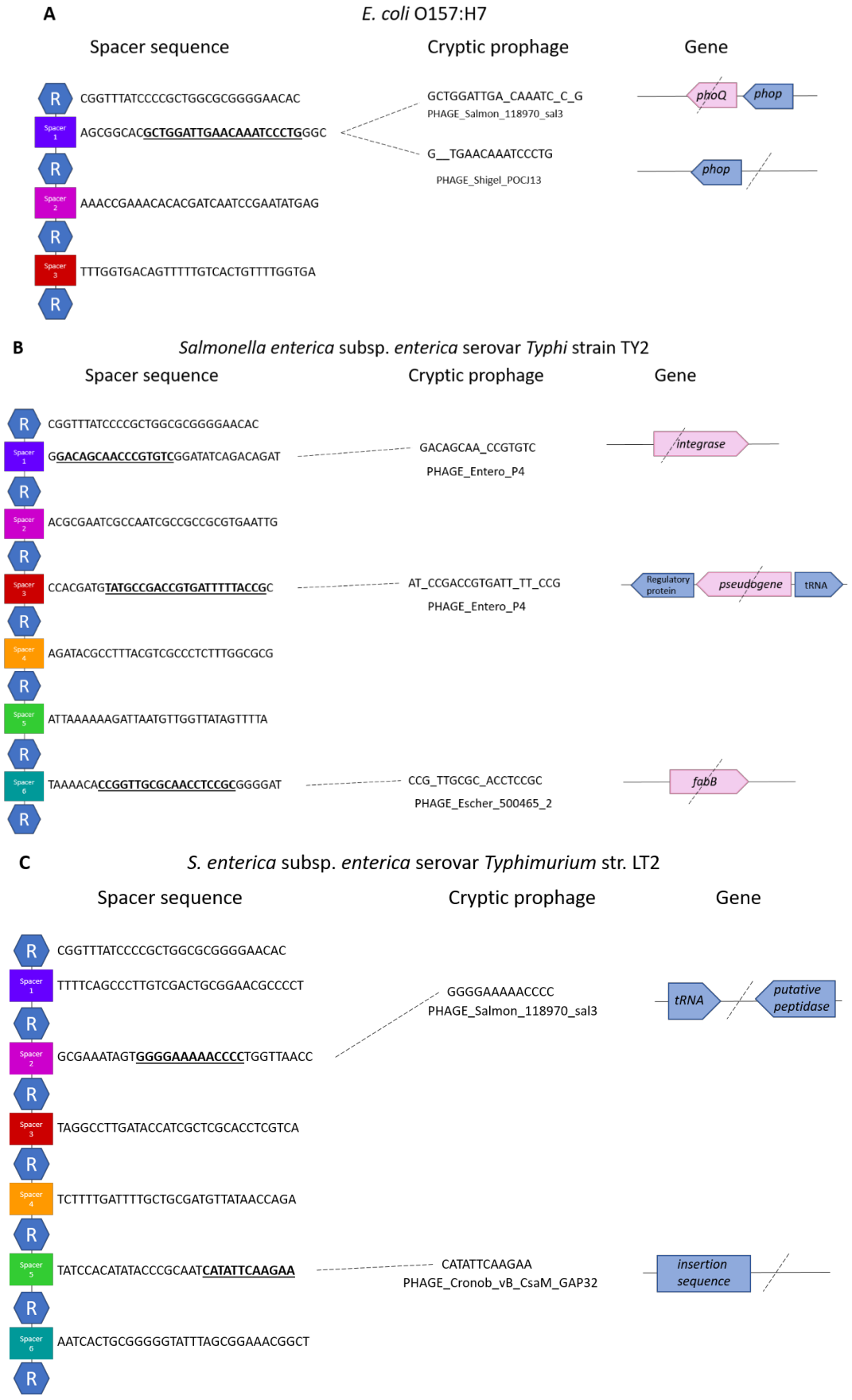
Supplementary Figure 2. CRISPR-Cas prevents cell lysis induced by cryptic phages. (**A**) Single cell persister waking of BW25113 Δ*cas2* and the Δspacer mutant on M9 0.4% glucose agar plates incubated at 37°C for 4 hours. The scale bar indicates 10µm. (**B**) TEM image for persister waking of BW25113 Δ*cas2* and the Δspacer mutant. Persister cells were resuscitated by M9 0.4% glucose for 10 min. One representative image is shown. The scale bar indicates 1 µm.



Supplementary Figure 3. LIVE/DEAD staining of stationary (turbidity 2.0) and exponential (turbidity 0.8) cells shows the *cas2* and the Δspacer mutations cause cell death. DF is dark field, SYTO9 is a membrane permeable stain for nucleic acids (green), and PI is propidium iodide, which is a membrane impermeable stain for the nucleic acids of dead cells (red). Tabulated cell numbers are shown in **Table S2**.

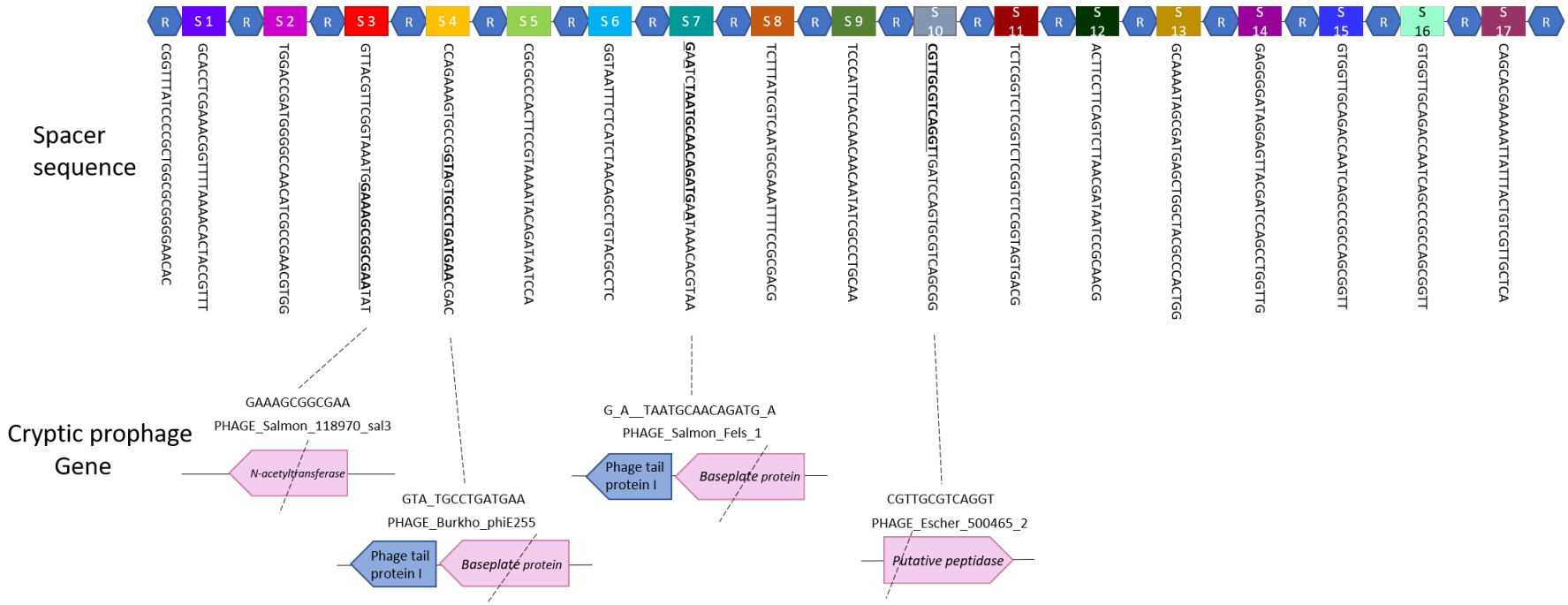
```
Spacer 3:   5'-GGCTCCCTGTCGGTTGTAATTGATAATGTTGA-'3
                     |||||||||||  ||
         3'-ATGTATGTAGACAGCCAACATAAAGGGAGGTCTTA-'5:DLP-12

Spacer 3:   5'-GGCTCCCTGTCGGTTGTAATTGATAATGTTGA-'3
                 ||  |||||||||   |   ||          |
          3'-TTCAGAAGAGACAGCCAAATTACACAGCGGTCTGT-'5:DLP-12

Spacer 6:   5'-TCAGCTTTATAAATCCGGAGATACGGAAACTA-'3
               |    |  |  |   |||||||||||| |  |
         3'-TGTAACGGCAGTCTAACGGCCTCTATGCCCTAGTC-'5:CP4-57

Spacer 8:   5'-CTGCTGGAGCTGGCTGCAAGGCAAGCCGCCCA-'3
               | ||    |||||||||||||||    |
          3'-GACGCCGTGGTCGACCGACGTTTCGTGGCGTACAA-'5:CPS-53

Spacer 11:  5'-CCCGGTAGCCAGGTTTGCAACGCCTGAACCGA-'3
                 | ||||||||||||   ||| | | || ||
         3'-AGTAAGTCATCGGTCCAAAACTTGTGTAGTTCGCG-'5:CP4-6

Spacer 12:  5'-GCAACGACGGTGAGATTTCACGCCTGACGCTG-'3
                   |  | |  ||||||||||||   |  |
          3'-GAGTTAGGTAGGCTGTCTAAAGTGCGGCTCGTAAG-'5:CP4-57

Spacer 12:  5'-GCAACGACGGTGAGATTTCACGCCTGACGCTG-'3
               || |||    | ||   |||||||||||||
          3'-GGTCGCTGCGTATAGTCCCTAGTGCGGACTGCCTA-'5:Rac

Spacer 12:  5'-GCAACGACGGTGAGATTTCACGCCTGACGCTG-'3
               || |||    | ||   |||||||||||||
          3'-GGTCGCTGCGTATAGTCCCTAGTGCGGACTGCCTA-'5:Qin

Spacer 13:  5'-GGCGCACTGGATGCGATGATGGATATCACTTG-'3
               |||||||||||||      |    |  || |
         3'-AGACCGCGTGACCTACATAGTTGGTCAAGGTCACG-'5 :e14
```

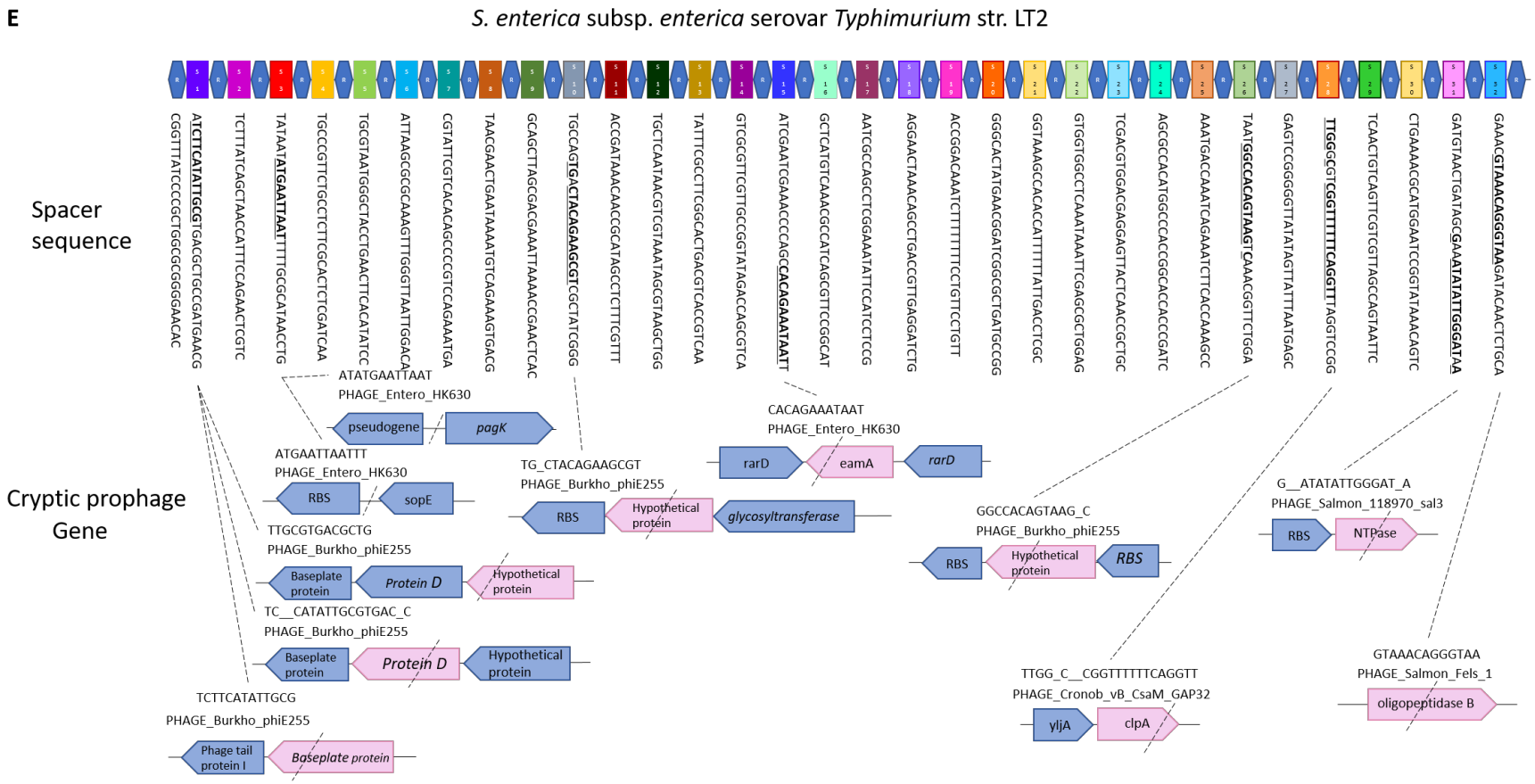
Supplementary Figure 4. Predicted base-pairing interactions between each of the CRISPR spacers (i.e., spacers 3, 6, 8, 11, 12, and 13) and a protospacer within the cryptic phages (DLP-12, CP4-57, CPS-53, CP4-6, CP4-57, Rac, Qin, and e14). Note, some spacers match more than one region within a particular cryptic prophage. The PAMs are indicated by bold/green highlighted text, and yellow highlight indicates active PAMs[25] (3'-TTC is the consensus PAM for *E coli*). The complementary nt between the spacers and cryptic prophage protospacers is indicated by blue text and vertical lines.


A
E. coli O157:H7
Spacer sequence
Cryptic prophage
Gene
CGGTTTATCCCCGCTGGCGCGGGGAACAC
AGCGGCACGCTGGATTGAACAAATCCCTGGGC
AAACCGAAACACACGATCAATCCGAATATGAG
TTTGGTGACAGTTTTTGTCACTGTTTTGGTGA
GCTGGATTGA_CAAATC_C_G
PHAGE_Salmon_118970_sal3
G__TGAACAAATCCCTG
PHAGE_Shigel_POCJ13
phoQ
phop
phop
B
Salmonella enterica subsp. enterica serovar Typhi strain TY2
Spacer sequence
Cryptic prophage
Gene
CGGTTTATCCCCGCTGGCGCGGGGAACAC
GGACAGCAACCCGTGTCGGATATCAGACAGAT
ACGCGAATCGCCAATCGCCGCCGCGTGAATTG
CCACGATGTATGCCGACCGTGATTTTTACCGC
AGATACGCCTTTACGTCGCCCTCTTTGGCGCG
ATTAAAAAAGATTAATGTTGGTTATAGTTTTA
TAAAACACCGGTTGCGCAACCTCCGCGGGGAT
GACAGCAA_CCGTGTC
PHAGE_Entero_P4
AT_CCGACCGTGATT_TT_CCG
PHAGE_Entero_P4
CCG_TTGCGC_ACCTCCGC
PHAGE_Escher_500465_2
integrase
Regulatory protein
pseudogene
tRNA
fabB
C
S. enterica subsp. enterica serovar Typhimurium str. LT2
Spacer sequence
Cryptic prophage
Gene
CGGTTTATCCCCGCTGGCGCGGGGAACAC
TTTTCAGCCCTTGTCGACTGCGGAACGCCCCT
GCGAAATAGTGGGGAAAAACCCCTGGTTAACC
TAGGCCTTGATACCATCGCTCGCACCTCGTCA
TCTTTTGATTTTGCTGCGATGTTATAACCAGA
TATCCACATATACCCGCAATCATATTCAAGAA
AATCACTGCGGGGGTATTTAGCGGAAACGGCT
GGGGAAAAACCCC
PHAGE_Salmon_118970_sal3
CATATTCAAGAA
PHAGE_Cronob_vB_CsaM_GAP32
tRNA
putative peptidase
insertion sequence

D

S. enterica subsp. *enterica* serovar *Typhimurium* str. LT2





Supplementary Figure 5. ***E. coli* O157:H7 and *Salmonella* spp. CRISPR-Cas spacer sequences related to cryptic prophages. (A)** Repeat (R, blue hexagon) and spacer (rectangle) sequences of each CRISPR-Cas system of *E. coli* O157:H7 and *Salmonella* spp. indicating matches with their cryptic prophages. (**A**) *E. coli* O157:H7, (**B**) *S. enterica* subsp. *enterica* serovar *Typhi* strain TY2, and (**C, D, E**) three CRISPR-Cas systems of *S. enterica* subsp. *enterica* serovar *Typhimurium* str. LT2. Pink highlight and dashed lines indicate the spacer positions relative to the cryptic prophage genes.