

# CRISPR-Cas Controls Cryptic Prophages

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**Keywords:** CRISPR-Cas, persisters, cryptic prophage

## ABSTRACT

The bacterial archetypal adaptive immune system, CRISPR-Cas, is thought to be non-functional in the best-studied bacterium, *Escherichia coli* K-12. Instead, we demonstrate here that the *E. coli* CRISPR-Cas system is active and inhibits its nine defective (i.e., cryptic) prophages. Specifically, deactivation of CRISPR-Cas via deletion of *cas2*, which encodes one of the two conserved CRISPR-Cas proteins, reduces growth by 40%, increases cell death by 700%, and prevents persister cell resuscitation; hence, CRISPR-Cas serves to inhibit the remaining deleterious effects of these cryptic prophages. Consistently, seven of the 13 *E. coli* spacers contain matches to the cryptic prophages, and, after excision, CRISPR-Cas cleaves cryptic prophage CP4-57 and DLP-12 DNA. Moreover, we determine that the key genes in these cryptic prophages that CRISPR-Cas represses by cleaving the excised DNA include lysis protein YdfD of Qin and lysis protein RzoD of DLP-12. 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

15 Along with restriction/modification<sup>1</sup> and toxin/antitoxin (TA) systems<sup>2</sup>, prokaryotes utilize clustered, regularly-interspaced, short palindromic repeats (CRISPR) and CRISPR-associated (Cas)<sup>3</sup> proteins to combat phages. These systems are interrelated in that we found some Cas proteins are derived from TA systems; for example, *Sulfolobus solfataricus* Cas2 is structurally similar to antitoxin GhoS of the *Escherichia coli* GhoT/GhoS TA system<sup>4</sup>. In addition, in a manner similar to our discovery<sup>2</sup> that toxins of  
20 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<sup>5</sup>. Also, TA systems have been found to stabilize CRISPR-Cas systems by making them addictive to the host<sup>6</sup>.

Although CRISPR-Cas systems exclude both external lytic and temperate (lysogenic) phages<sup>7</sup>,  
25 CRISPR-Cas systems of lysogens that target their own integrated prophages decrease long-term fitness and either the cell dies or the phage is lost<sup>7,8</sup>. Also, the class I-E<sup>3</sup> CRISPR-Cas system of *E. coli* is not related to immunity for external phages<sup>9</sup> and is thought to be inactive in the wild-type strain<sup>10</sup>, due to repression by H-NS, although it is functional when induced<sup>11</sup>. 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.

30 Up to 20% of bacterial genomes may contain stable phage DNA<sup>12</sup>, 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  $\beta$ -lactam antibiotics as well as protect the cell from osmotic, oxidative, and acid stresses<sup>13</sup>. Although these cryptic prophages do not help a subpopulation of cells weather extreme stress by entering into the dormant state known as persistence<sup>14</sup>, these phage remnants  
35 facilitate the resuscitation of these persister cell via nutrient sensing<sup>15</sup>. Therefore, the bacterial cell can capture the genome of its former parasite to both combat stress<sup>13</sup> as well as to revive from dormancy<sup>15</sup>.

Here we explore the role of the native *E. coli* CRISPR-Cas systems in the regulation of cryptic prophages. We find that the CRISPR-Cas system is required for inhibiting these phage fossils since, if CRISPR-Cas is inactivated, cells die due to activation of the lysis proteins YdfD of Qin and RzoD from

40 DLP-12. Hence, we discovered CRISPR-Cas is active in *E. coli* and serves to regulate its phage fossils.

## RESULTS

**CRISPR-Cas increases growth.** To inactivate CRISPR-Cas in *E. coli*, the *cas2* deletion was chosen since Cas2 is conserved in almost all CRISPR-Cas systems<sup>16</sup> as part of the adaptor complex and serves as the structural scaffold of the Cas1/Cas2 complex which cleaves the source, protospacer DNA, and the CRISPR array<sup>17</sup>. We found the *E. coli* K-12 *cas2* mutant grows 40% slower in rich medium compared to the wild-type strain (specific growth rate of  $0.79 \pm 0.21/h$  vs.  $1.3 \pm 0.11/h$ , respectively). Similarly, in minimal glucose (0.4 wt%) medium, deletion of *cas2* also reduces growth by 33% ( $0.42 \pm 0.02/h$  vs.  $0.62 \pm 0.02/h$ , respectively).

**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<sup>18,19</sup>; for example, we have shown inhibiting ATP synthesis leads to a 5,000-fold increase in persister cell formation<sup>20</sup>. Since we discovered facile means for converting the whole population of cells into persister cells<sup>18-20</sup> that has been used by at least 17 independent labs to date with various bacterial species<sup>21</sup>, these stressed cells are an excellent model for testing the effects of CRISPR-Cas on *E. coli* physiology.

Here, we found inactivation of CRISPR-Cas via *cas2* nearly completely prevents persister cell resuscitation, while the wild-type cell has 52% resuscitation (**Fig. 1A, Table S1**). Hence, CRISPR-Cas is active in *E. coli* and plays key roles in its growth and recovery from extreme stress.

60 **CRISPR-Cas prevents cell death.** To explore how CRISPR-Cas increases growth (including persister cell resuscitation), we checked for death in resuscitating persister cells of the *cas2* 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”<sup>22</sup> that lack cytosolic material (**Fig. S1**) and are likely dead but have intact membranes so are not stained by the propidium iodide

65 dye we use. Corroborating these results, there was 11-fold and 5-fold more death for stationary- and exponential-phase cells, respectively, when CRISPR-Cas was inactivated. These results indicate inactivating CRISPR-Cas leads to cell death.

**CRISPR-Cas increases growth by repressing cryptic prophage genes.** We hypothesized that since CRISPR-Cas systems inhibit some phage lysogens<sup>7,8</sup>, the *E. coli* system may be preventing cell death by repressing some of the 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<sup>11,23</sup>, each containing 32 or 33 nt<sup>24</sup>, between *iap* and *cas2*. Between the 14 29-nt repeat sequences (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<sup>25</sup> with perfect complementarity of 6 to 12 nt<sup>10</sup>. Moreover, we found these spacers cannot be deleted, which shows they are essential for *E. coli* K-12. Together, these results suggest CRISPR-Cas regulates the *E. coli* cryptic prophages.

The presence of the cryptic prophage-related spacers and the cell death seen upon inactivating CRISPR-Cas suggested that *E. coli* CRISPR-Cas suggested may be repressing some of the cryptic prophage lysis genes; hence, we checked for derepression of the cryptic prophage lysis genes in these seven cryptic prophages, specifically *ydfD* (Qin), *hokD* (Qin), *ypjF* (CP4-57), *essD* (DLP-12), and *rzoD* (DLP-12) via quantitative reverse transcription polymerase chain reaction (qRT-PCR). We found that inactivation of CRISPR-Cas via the *cas2* deletion results in activation of *ydfD* by 35-fold and activation of *rzoD* by 183-fold in resuscitating persister cells (**Fig. 1C**). Moreover, *rzoD* was activated 1024-fold in exponentially-growing cells (**Fig. 1C**). RzoD is a putative DLP-12 lysis lipoprotein that we previously showed was toxic through its interaction with toxin Hha<sup>26</sup> of the Hha/TomB TA system<sup>27</sup>. YdfD of Qin cryptic prophage has been shown to lyse cells when induced<sup>28</sup>. Hence, 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 extraordinary derepression of the DLP-12 *rzoD* lysis gene, we checked for increased excision with the *cas2*

deletion strain using qPCR. We found the *cas2* deletion has little impact on DLP-12 excision. We also tested the effect of CRISPR-Cas on two other cryptic prophages with significant excision (CP4-57 and e14)<sup>13</sup>, and also found no effect of deleting *cas2* (**Table S3**). Hence, CRISPR-Cas does not affect cryptic prophage excision.

95 **CRISPR-Cas cleaves DLP-12 and CP4-57 DNA after excision.** Since DLP-12 has significant excision in *E. coli*<sup>13</sup> but inactivation of CRISPR-Cas does not alter its excision, we hypothesized that CRISPR-Cas repressed lysis gene *rzoD* by cleaving DLP-12 DNA after excision. Using qRT-PCR with resuscitating persister cells, we found that the *cas2* deletion leads to 40-fold increase in DLP-12 DNA that includes spacer 3 as well a 184-fold increase in CP4-57 DNA that includes spacer 6 (**Fig. 2B**). Therefore, CRISPR-  
100 Cas actively cleaves excised cryptic prophage DNA to regulate the lysis genes of the captured phage foe.

## DISCUSSION

Our results reveal a new 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 death (**Fig. 1**), (iv) derepresses the cryptic prophage lysis genes *ydfD* and *rzoD*, and (v) increases CP4-57 and DLP-12 cryptic prophage DNA after their excision. Since there is no change in excision of DLP-12 upon inactivating CRISPR-Cas, our results show the mechanism for regulating the lysis genes *ydfD* and *rzoD* of the cryptic prophages is via CRISPR-Cas cleavage of the excised prophages. Supporting this, the *E. coli* K-12 class 1-E CRISPR-Cas system cleaves dsDNA<sup>29</sup>.

110 This new function for CRISPR-Cas is likely general and may explain why many species appear to have inactive CRISPR-Cas systems as was previously thought for *E. coli*<sup>10</sup>; i.e., instead of protecting cells from external phages, CRISPR-Cas systems may also control resident cryptic prophages which are prevalent. 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  
115 targeting *active* temperate phages is deleterious; i.e., either the cell dies or the phage is lost<sup>7,8,30</sup>. Since *E.*

*coli* cryptic prophages like *rac* have been present in its genome for 4.5 million years<sup>31</sup>, the active 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<sup>32</sup>.

Our results also indicate that, although the cryptic prophages are stable and the cell makes use of the genetic tools encoded by its former foe to combat myriad stresses<sup>13</sup> and to sense nutrients prior to exiting the persister state<sup>15</sup>, 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<sup>33</sup>, and H-NS silences cryptic prophages through 65 binding sites<sup>34</sup>. Therefore, phages may be captured by the host, but they must be tamed, and this now includes repression by CRISPR-Cas.

## MATERIALS AND METHODS

**Bacteria and growth conditions.** Bacteria (**Table S4**) were cultured in lysogeny broth<sup>35</sup> at 37°C. pCA24N-based plasmids<sup>36</sup> were retained in overnight cultures via chloramphenicol (30 µg/mL), and kanamycin (50 µg/mL) was used for deletion mutants, where applicable.

**Spacer knockout.** To attempt to delete the CRISPR-Cas spacer region, the one-step inactivation method for single gene deletions<sup>37</sup> was utilized in which primers that included the sequences flanking the spacer region (**Table S5**) were used with plasmid pKD4 to allow for insertion of the kanamycin resistance cassette (1.5 kb) and FRT sites.

**Persister cells.** Exponentially-growing cells (turbidity of 0.8 at 600 nm) were converted nearly completely to persister cells<sup>18,20</sup> 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 washed twice with 0.85% NaCl then re-suspended in 0.85% NaCl. Persister concentrations were enumerated via a drop assay<sup>38</sup>.

**Single-cell persister resuscitation.** Persister cells (5 µL) were added to 1.5% agarose gel pads containing M9 glucose (0.4 wt%) medium<sup>39</sup>, 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 cultures were used with 150 to 300 individual cells used 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 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%) medium for 10 min then washed with 0.85% NaCl and from exponential cells grown to a turbidity of 0.8; 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 °C for 5 min; 40 cycles of 95 °C for 15 s, 60 °C for 1 min for two replicate reactions for each sample/primer pair. The annealing temperature was 60°C for all primers (**Table S5**).

**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 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).

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## FIGURE LEGENDS

- 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 and the *cas2* mutant after 6 hours on 0.4 wt% glucose. Black arrows indicate cells that resuscitate, and the scale bar indicates 10  $\mu$ m. Cells were observed using light microscopy (Zeiss Axio Scope.A1). Representative results from two independent cultures are shown, and tabulated cell numbers are shown in **Table S1**. (B) LIVE/DEAD staining of resuscitating persister cells shows the *cas2* mutation causes 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**. (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).
- Fig. 2. Inactivating CRISPR-Cas increases excised cryptic prophage DNA.** (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). (B) The *cas2* mutation increases cryptic prophage excised DNA that flanks the cleavage sites, as determined by qPCR.

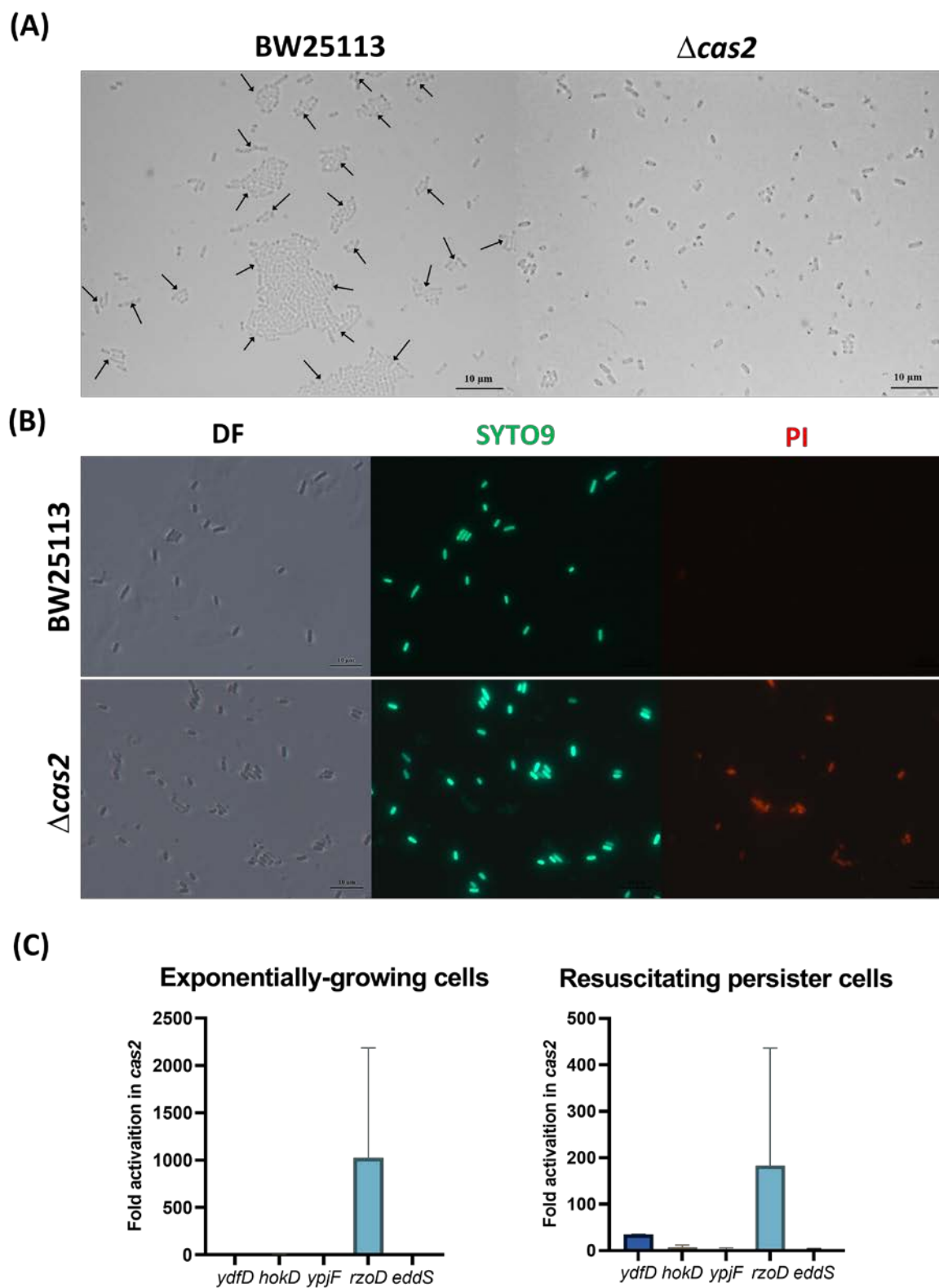


Figure 1

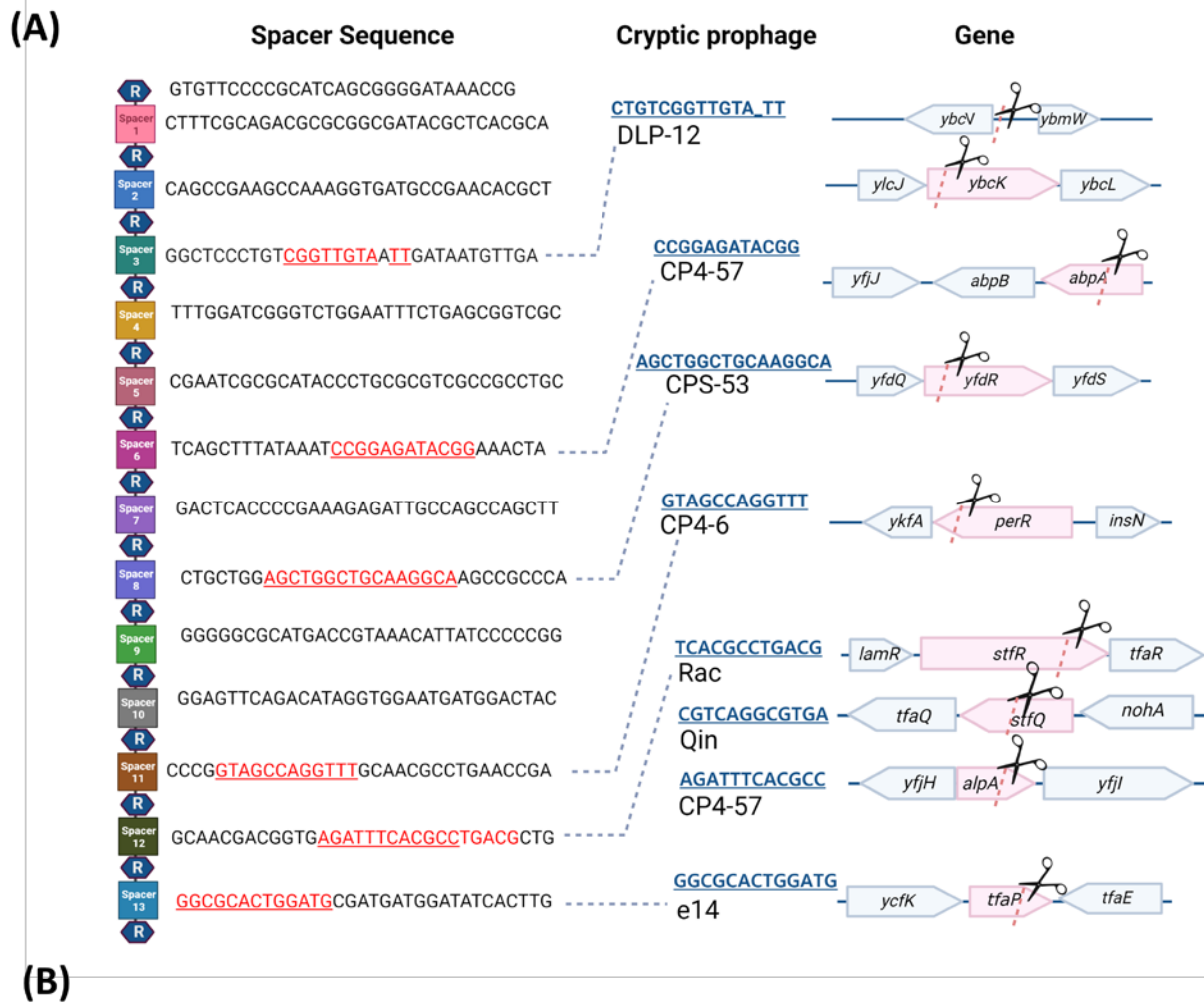


Figure 2

## Supporting Information

### CRISPR-Cas Controls Cryptic Prophages

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**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. 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. The microscope images are shown in **Fig. 1**.

	<b>Total cells</b>	<b>Waking cells</b>	<b>% waking</b>	<b>Fold-change</b>
<b>BW25113</b>	504/382	199/248	52 ± 18	1
<i>Δcas2</i>	516/677	6/15	1.7 ± 0.7	-30.9

**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.A1 microscope. The results are the combined observations from two independent experiments (independent culture results separated by “/”). The microscope images are shown in **Fig. 1B**, **Fig. S1**, and **Fig. S2**.

	Strains	Total cells	Dead cells	Ghost cells	% Ghost cells	fold-change ghost cells	% dead	fold-change dead cells
<b>Resuscitated</b>	<b>BW25113</b>	52/115	2/7	0/1	0.6	1	5.4	1
	<i>Δcas2</i>	142/106	54/38	38/13	20.6	34	37.1	6.9
<b>Exponential</b>	<b>BW25113</b>	153/147	0/2	-	-	-	0.7	1
	<i>Δcas2</i>	59/40	1/2	-	-	-	3.4	4.9
<b>Stationary</b>	<b>BW25113</b>	492/123	1/0				0.1	1
	<i>Δcas2</i>	1136/1736	14/18	-	-	-	1.13	11.2



**Table S3. CRISPR-Cas does not affect cryptic prophage excision in stationary cells.**

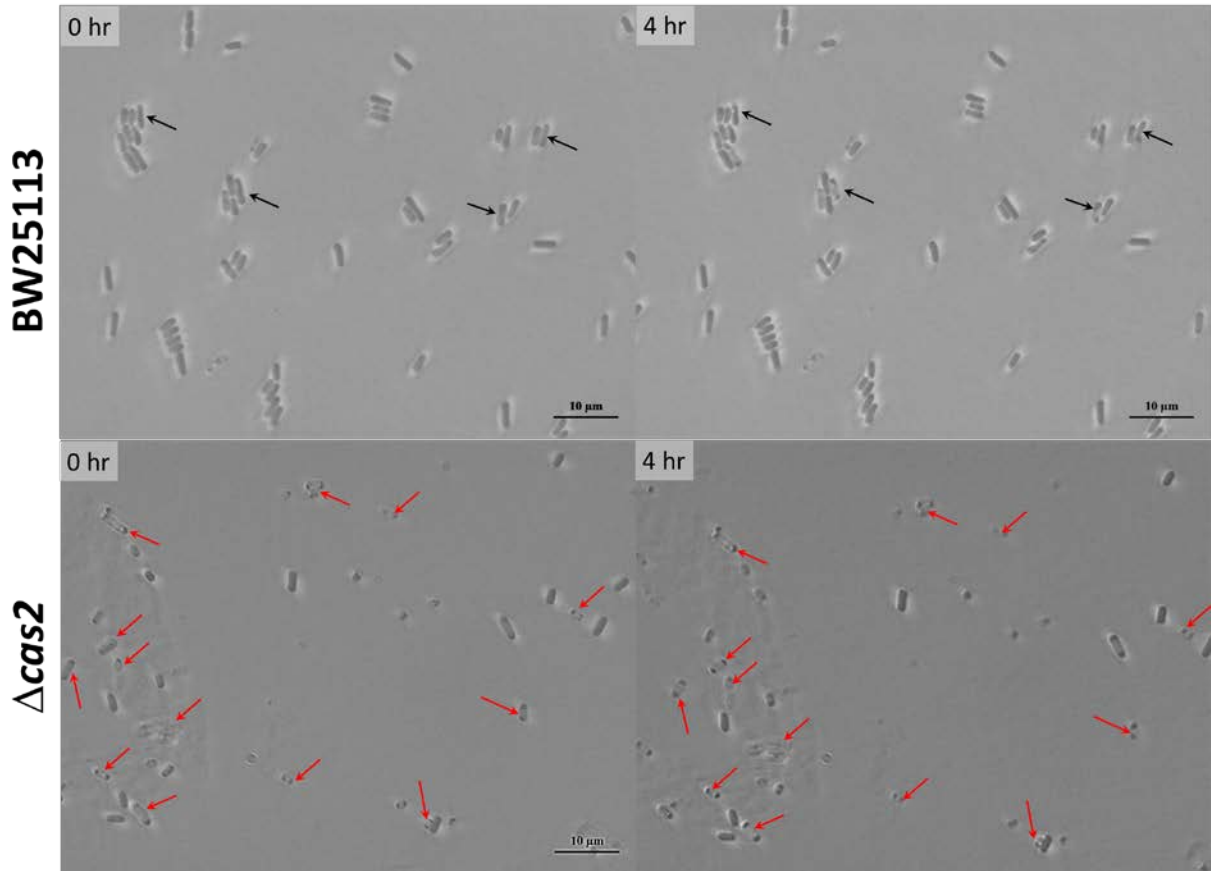
Gene	<i>purM</i> (house keeping gene)		CP4-57		e14		DLP-12	
	WT	Cas2	WT	<i>cas2</i>	WT	<i>cas2</i>	WT	<i>cas2</i>
CT	9.30 ± 0.13	9.70 ± 0.18	30.45 ± 1.73	30.37 ± 0.68	16.30 ± 0.06	17.45 ± 0.51	30.58 ± 0.49	30.05 ± 0.5
ΔCT			21.16 ± 1.73	20.67 ± 0.70	7.00 ± 0.15	8.16 ± 0.54	21.29 ± 0.51	20.35 ± 0.53
ΔΔCT				-0.48 ± 0.7		1.16 ± 0.54		-0.94 ± 0.53
fold				1.40		-2.23		1.92

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

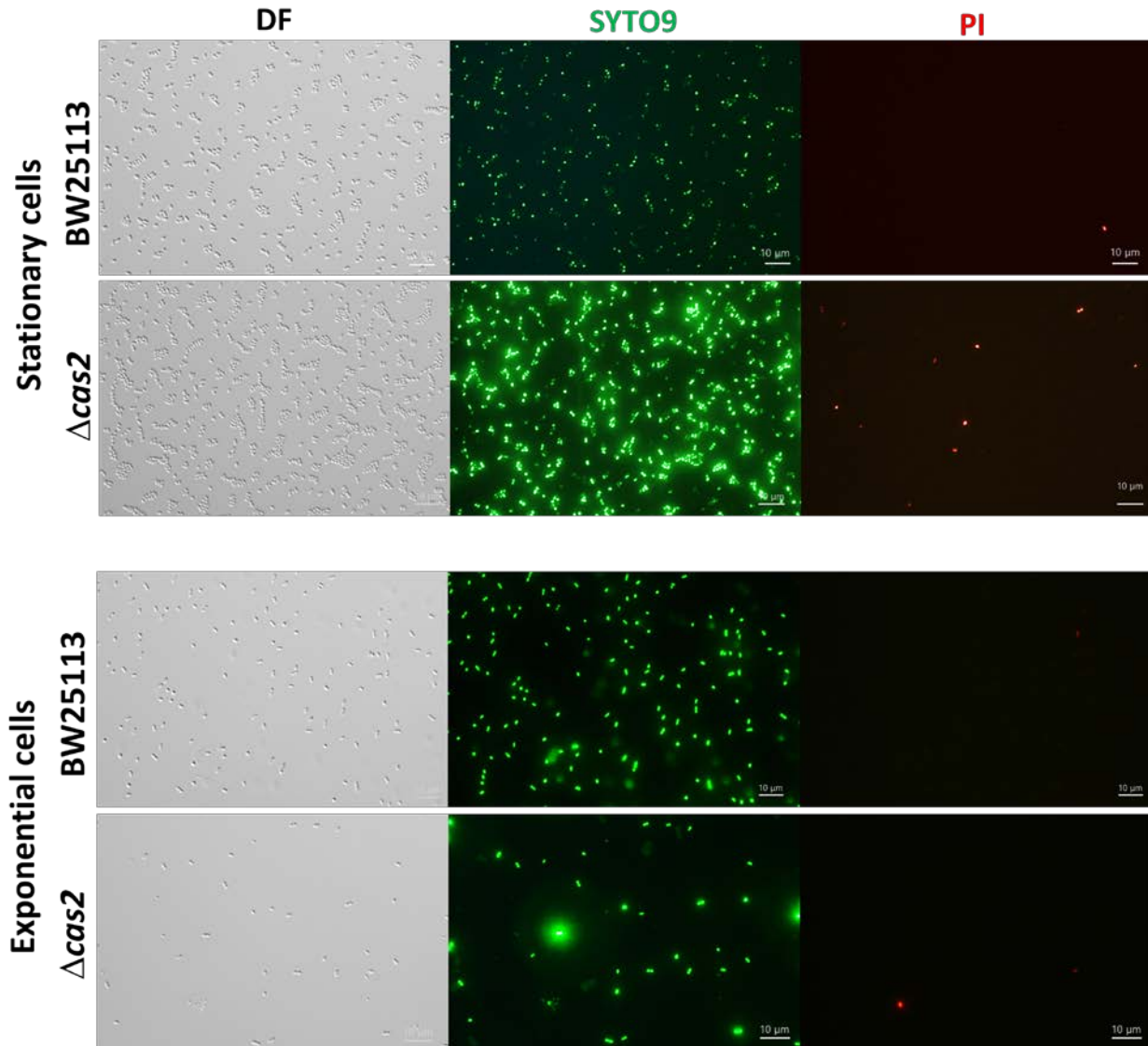
<b>Strains and Plasmids</b>	<b>Features</b>	<b>Source</b>
<b>Strains</b>		
BW25113	<i>rrnB3</i> $\Delta$ <i>lacZ4787</i> <i>hsdR514</i> $\Delta$ ( <i>araBAD</i> )567 $\Delta$ ( <i>rhaBAD</i> )568 <i>rph-1</i>	40
BW25113 $\Delta$ <i>cas2</i>	$\Delta$ <i>cas2</i> , Km <sup>R</sup>	40
<b>Plasmids</b>		
pCA24N	Cm <sup>R</sup> ; <i>lacI<sup>q</sup></i>	41
pCA24N_ <i>cas2</i>	Cm <sup>R</sup> ; <i>lacI<sup>q</sup></i> , P <sub>T5-lac</sub> :: <i>cas2</i> <sup>+</sup>	41
pKD4	FRT::Kan <sup>R</sup> ::FRT, Amp <sup>R</sup>	37

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

Gene	Sequence (5'-3')
<b>Spacer knockout</b>	
DS	<b>F:</b> TTATGCGGATAATGCTACCTCTGGTGAAGGAGTTGGCGAAGGCGTCTTGA GTGTAGGCTGGAGCTGCTTC <b>R:</b> CCCCAGGTAGATTTGGATGGTTAAGGTTGGTGTCTTTTTTACCTGTTTGA CATATGAATATCCTCCTTAG
Spacer	<b>F:</b> GAAGGAGTTGGCGAAGGCGTCTTGA <b>R:</b> GTTGGTGTCTTTTTTACCTGTTTGA
<b>qRT-PCR</b>	
<i>purM</i>	<b>F:</b> CGGTGTTGATATTGACGCGGG <b>R:</b> CAGCACGGGTTACGATATTTTTG
<i>ydfD</i> (Qin)	<b>F:</b> TCAGCATTGTGCTTGTCTG <b>R:</b> CTGCCGGGATTCGATATTA
<i>hokD</i> (Qin)	<b>F:</b> GCCCTGATCGTCATCTGTTT <b>R:</b> AGCTGTGAAGACAGCGACCT
<i>ypjF</i> (CP4-57)	<b>F:</b> TATGGCCTGACTGAACGA <b>R:</b> GTTGTGTCACGAAAATCCT
<i>rzoD</i> (DLP-12)	<b>F:</b> TGCTCTGCGTGATGATGTTG <b>R:</b> TCTCTGAGGGTGAAATAATCC
<i>essD</i> (DLP-12)	<b>F:</b> CTCACAGTGGGCAGCAATAG <b>R:</b> TATTCACCTCTCGCAGCCTT
<b>qPCR</b>	
*CP4-57	<b>F:</b> AAGCATGTAGTACCGAGGATGTAGG <b>R:</b> TATGTCTCCTCACCGTCTGGTCGG
*e14	<b>F:</b> GTGCAAACATCGGTGACGAA <b>R:</b> TTCAGCAGCTTAGCGCCTTC
*DLP-12	<b>F:</b> CAAAAGCCATTGACTCAGCAAGG <b>R:</b> CGGATAAGACGGGCATAAAATGA
<i>purM</i> 400	<b>F:</b> CTGATTGCACTCGGTTCCAG <b>R:</b> CGTTTTACCGTTGGCATTG
Spacer 3-1	<b>F:</b> CATGCAATTACAACATCAGGGTA <b>R:</b> GCCATTGGTAAAACCTTCCA
Spacer 3-2	<b>F:</b> AAATTCCGAAAAGCTCCTGA <b>R:</b> TGCTTTCAAGATTATGGCGTA
Spacer 6	<b>F:</b> CACGAAAGCCAGCCTATTCC <b>R:</b> CCGCTGTTTCTTTCTCCAGG
Spacer 8	<b>F:</b> GCCAGCATAATGAGATCGGC <b>R:</b> TTTTACCCAAACTCAGCGCG
Spacer 11	<b>F:</b> TGTGCAGTTGTACCAGTGGA <b>R:</b> CAACCCAGCAAAGTTTCGGA
Spacer 12-1	<b>F:</b> TGTATGTGCCCGGTGTTAT <b>R:</b> GCAGATGAAGGCGCATTACA
Spacer 12-2	<b>F:</b> AGCTTTACACCTCGGCTCAT <b>R:</b> CCGGAACTCTTGTGTTGGTG
Spacer 12-3	<b>F:</b> TCTACAGGGAAAGGACGACC <b>R:</b> CTCTGCAACCAAAGTGAACCA
Spacer 13	<b>F:</b> ACAACCACTATCGCCCCTTT <b>R:</b> GGTAAGGCTGCATTGGGAAG



**Supplementary Figure 1. Single cell persister waking of BW25113  $\Delta cas2$ .** Persister cell waking of BW25113 and BW25113  $\Delta cas2$  on M9 0.4% glucose agar plates incubated at 37°C for 4 hours. Black arrows indicate waking cells, and red arrows indicate ghost cells. Scale bars indicate 10  $\mu$ m. Representative results from two independent cultures are shown.



**Supplementary Figure 2.** LIVE/DEAD staining of stationary (turbidity 2.0) and exponential (turbidity 0.8) cells shows the *cas2* mutation causes 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**.