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# Cas3 is a limiting factor for CRISPR-Cas immunity in *Escherichia coli* cells lacking H-NS

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## Abstract

**Background:** CRISPR-Cas systems provide adaptive immunity to mobile genetic elements in prokaryotes. In many bacteria, including *E. coli*, a specialized ribonucleoprotein complex called Cascade enacts immunity by “an interference reaction” between CRISPR encoded RNA (crRNA) and invader DNA sequences called “protospacers”. Cascade recognizes invader DNA via short “protospacer adjacent motif” (PAM) sequences and crRNA-DNA complementarity. This triggers degradation of invader DNA by Cas3 protein and in some circumstances stimulates capture of new invader DNA protospacers for incorporation into CRISPR as “spacers” by Cas1 and Cas2 proteins, thus enhancing immunity. Co-expression of Cascade, Cas3 and crRNA is effective at giving *E. coli* cells resistance to phage lysis, if a transcriptional repressor of Cascade and CRISPR, H-NS, is inactivated ( $\Delta hns$ ). We present further genetic analyses of the regulation of CRISPR-Cas mediated phage resistance in  $\Delta hns$  *E. coli* cells.

**Results:** We observed that *E. coli* Type I-E CRISPR-Cas mediated resistance to phage  $\lambda$  was strongly temperature dependent, when repeating previously published experimental procedures. Further genetic analyses highlighted the importance of culture conditions for controlling the extent of CRISPR immunity in *E. coli*. These data identified that expression levels of *cas3* is an important limiting factor for successful resistance to phage. Significantly, we describe the new identification that *cas3* is also under transcriptional control by H-NS but that this is exerted only in stationary phase cells.

**Conclusions:** Regulation of *cas3* is responsive to phase of growth, and to growth temperature in *E. coli*, impacting on the efficacy of CRISPR-Cas immunity in these experimental systems.

**Keywords:** CRISPR-Cas, H-NS, PAM, HtpG, Temperature, *E. coli*

## Background

*Escherichia coli* K-12 utilises Type I-E CRISPR (Clustered Regularly Interspaced Short Palindromic Repeats) loci to gain immunity to invasive DNA such as bacteriophage (“phage”), dependent on activities of Cas (CRISPR-associated) proteins reviewed in [1–5]. CRISPR loci are composed of the AT-rich leader region followed by arrays of sequence repeats separated by spacers that are homologous to sequences of invading DNA (“protospacers”). CRISPR arrays are transcribed into “pre-crRNA” that is further processed into “crRNA” that contains a full or partial spacer sequence reviewed in [1–5]. In *E. coli*, crRNA

assembled into “Cascade” (CRISPR-associated complex for antiviral defence) is targeted to protospacers in “interference” reactions. *E. coli* Cascade comprises five proteins: Cse1 (CasA), Cse2 (CasB), Cas7 (CasC), Cas5 (CasD) and Cas6e (CasE) [6–10]. Interference generates base pairing between crRNA and protospacer DNA in an R-loop, displacing the DNA strand that is not complementary to crRNA [6, 11–15]. This single stranded DNA is degraded by Cas3 helicase-nuclease [16–19].

Cascade catalyses interference R-loops by a sequential process reliant on recognition of protospacer adjacent motif (PAM) sequences located immediately next to a protospacer on the target protospacer DNA [20–23]. CRISPR arrays lack a PAM sequence, helping to prevent targeting of self-DNA by Cascade [24–26]. In *E. coli* K-12 PAM 5′-CTT-3′ is most prevalent (80 %) [22, 26, 27], and

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of 64 possible PAMs, five PAMs are tolerated by Cascade to promote interference [11, 22, 25]. The Cse1 (CasA) subunit of Cascade is important for PAM recognition, and for positioning Cas3 to degrade invader DNA [13, 16, 19]. After PAM recognition, stable R-loop formation requires complementarity between the crRNA-DNA in a “seed” region (8-10 nucleotides from the 5′ end of the crRNA spacer sequence). Upon reaching the end of the protospacer the R-loop becomes locked [28] and by pushing Cse2 dimer it induces repositioning of Cse1 and Cse2 proteins, and conformational change of the whole Cascade complex [29]. After locking of the R-loop, additional PAM verification guides Cas3 binding near the PAM [13, 19, 29] and degradation of the DNA [11, 15, 29].

“Escape” mutations in phage DNA can reduce the stability of *E. coli* Cascade R-loops when they arise in a protospacer seed or a PAM, and correspond to reduced resistance to plaque formation [11, 21–23, 25, 30, 31]. Other mutations in phage DNA protospacers are tolerated for interference (e.g. positions 6, 12, 18, 24, 30) [22]. This is because five Cas7 proteins fold over every sixth nucleotide of the crRNA which are flipped outward and do not participate in DNA recognition [8–10]. Recent results showed that the crRNA spacer sequence also has significant role in helping interference machinery to recognize protospacer with single point mutations within the seed sequence or PAM [32]. Escape mutations that block interference promote rapid acquisition of new spacers from the same target DNA, a process called ‘primed’ adaptation that suggests cross-talk between Cas1-Cas2 DNA capture and alternative binding mode of Cascade that promotes priming [22, 27, 31]. Primed adaptation is a very robust process that tolerates up to 13 mutations either in PAM or protospacer region [22]. Spacers newly acquired during primed adaptation therefore provide elevated protection against invader whose escape mutations were evading robust interference by Cascade [27, 32].

In *E. coli* H-NS (nucleoid-structuring protein) represses transcription of CRISPR and the genes encoding Cascade, Cas1 and Cas2. *E. coli* LeuO de-represses transcription of genes encoding Cascade, by blocking cooperative spreading of H-NS along the promoter of the initially transcribed Cascade gene, *cse1* [33, 34]. In addition to H-NS and LeuO, CRP (cAMP receptor protein) is a transcriptional repressor of *cas* genes in *E. coli* [35, 36]. The CRP binding site in this case overlaps with the LeuO binding site, leading to the proposal that CRP and LeuO compete for binding to the *cse1* promoter depending on the cellular availability of cAMP [35]. In contrast to the apparent complexity of transcriptional regulation of Cascade and CRISPR, factors that control transcription of *E. coli cas3* have not been identified.

Experimental analyses of CRISPR-Cas in *E. coli* can overcome influences of H-NS, LeuO and CRP repression by engineering inducible expression of CRISPR, Cascade and Cas3 from plasmids or their chromosomal loci [27, 37]. Deletion of H-NS ( $\Delta hns$ ), or ectopic over-expression of LeuO in cells with engineered anti- $\lambda$  spacer ( $\lambda T3$ ) into CRISPR, promote CRISPR interference observed as enhanced resistance to phage  $\lambda vir$  [34, 38]. In these studies the protospacer targeted by spacer  $\lambda T3$  crRNA had a non-consensus PAM 5′-CCA-3′ and although resistance to phage  $\lambda vir$  from these strains was effective at 30 °C, we noticed that at 37 °C cells became sensitive to plaque formation. We investigated this further in  $\Delta hns$  cells that had acquired a new spacer ( $\lambda c$ ) targeting protospacer with the consensus PAM 5′-CTT-3′. We report that the effect of temperature on CRISPR immunity in these *E. coli* cells was correlated to expression of Cas3 and its chaperone HtpG (high-temperature protein G). Inducible ectopic expression of Cas3 in the presence, but not absence, of chromosomal *htpG* rescued resistance to phage at 37 °C. Further research will be required to uncover how temperature causes this effect on activity of the *E. coli* CRISPR-Cas system.

## Results

### Temperature-dependent resistance of $\Delta hns$ *E. coli* cells to phage $\lambda vir$ is not caused by PAM sequence variation

Genetic analysis of *E. coli* CRISPR-Cas in previous studies established that H-NS represses transcription of the operon encoding Cascade, Cas1-Cas2 (*casABCDEF12*) and CRISPR locus 2.1 [33–35, 38]. Deleting H-NS ( $\Delta hns$ ) from cells de-repressed transcription, and efficient resistance to  $\lambda vir$  infection at 30 °C was reported when CRISPR of  $\Delta hns$  cells was engineered to contain an anti- $\lambda$  phage spacer sequence ( $\lambda T3$ ) [34, 38]. The importance of the  $\lambda T3$  spacer was highlighted by sensitivity of  $\Delta hns$  cells to  $\lambda vir$  plaque formation compared to  $\Delta hns + \lambda T3$  cells [38]. When repeating these experiments we also observed about 100000 fold elevated resistance of  $\Delta hns + \lambda T3$  cells to  $\lambda vir$  infection at 30 °C, compared to  $\Delta hns$  cells without  $\lambda T3$  spacer (Table 1). However at 37 °C, in otherwise identical assays,  $\Delta hns + \lambda T3$  cells became sensitive to phage (Table 1). Cells with intact H-NS ( $hns^+$ ), with or without  $\lambda T3$ , were sensitive to  $\lambda vir$  infection at both temperatures (Table 1). There was no difference in sensitivity at 37 °C between  $\Delta hns$  cells + or -  $\lambda T3$  spacer. If  $\lambda T3$  spacer was absent from the CRISPR locus 2.1,  $\Delta hns$  cells showed threefold increase in resistance at 30 °C in comparison to 37 °C (Table 1). Therefore using 30 °C temperature of incubation in infectivity assays is an important factor for promoting resistance of  $\Delta hns + \lambda T3$  cells to phage  $\lambda vir$ .

The  $\lambda T3$  spacer sequence engineered into CRISPR used here and in [37, 38] has a nucleotide sequence match with the template strand of  $\lambda$  phage gene lysis R,

**Table 1** *E. coli* cells lacking H-NS show temperature-dependent resistance to phage

strain	genotype	Plaque forming units (PFUs)	
		30 °C	37 °C
BW25113	<i>hns</i> <sup>+</sup>	$3.80 \times 10^{10} \pm 7 \times 10^9$	$4.56 \times 10^{10} \pm 9 \times 10^9$
BW39121	$\Delta hns$	$1.20 \times 10^{10} \pm 1.8 \times 10^9$	$4.23 \times 10^{10} \pm 6.6 \times 10^{10}$
BW39651	<i>hns</i> <sup>+</sup> + $\lambda T3$	$3.66 \times 10^{10} \pm 7.57 \times 10^9$	$4.03 \times 10^{10} \pm 1.2 \times 10^{10}$
BW39671	$\Delta hns$ + $\lambda T3$	$\sim 4 \times 10^5$	$4.16 \times 10^{10} \pm 5.5 \times 10^9$

***λvir***

Cell lawns were infected with phage dilutions (from  $10^{-2}$  to  $10^{-8}$ ) and incubated at either 30 °C or 37 °C. Cells lacking H-NS ( $\Delta hns$ ) or containing H-NS (*hns*<sup>+</sup>) had fully operational CRISPR-Cas systems that were engineered with an anti- $\lambda$  spacer ( $\lambda T3$ ) as indicated. The average of at least three independent experiments are shown

but the PAM sequence (5'-CCA-3', Additional file 1: Figure S1A) deviates from consensus 5'-CTT-3' *E. coli* PAM [20]. Single nucleotide variations in PAMs may disrupt interference in Type I-E, I-F and Type II CRISPR systems by preventing R-loop priming and degradation of invading DNA [21, 22, 25, 29, 30, 39, 40]. Five PAMs, CAT, CTT, CCT, CTC and CTA found previously [11, 22, 25], are utilized by Cascade for robust interference, and ten non-consensus PAMs give a partial resistance phenotype [25]. Therefore, PAM 5'-CCA-3' belongs to the latter group, giving partial resistance to *λvir* as expected from previous data [25]. However, recent findings showed that spacer sequence dictates whether mutant PAM sequences will be tolerated for interference or not [32]. We investigated if the observed major difference in phage resistance of  $\Delta hns$  cells at 30 °C and 37 °C was related to PAM sequence by introducing spacer targeting protospacer with the consensus PAM 5'-CTT-3' into CRISPR. To do this we provoked  $\Delta hns$  +  $\lambda T3$  *E. coli* to acquire a new spacer. One such *E. coli* derivative containing spacer ( $\lambda c$ ) targeting phage *λvir* gene *cII* with a 5'-CTT-3' PAM was selected. The procedure is detailed in the methods and supplementary material. The constructed strain (IIB1039; Table 2) also contains  $\Delta cas1$  mutation as a useful controlling factor to uncouple interference from adaptation, enabling focus on interference reaction only. In phage infectivity assays, *hns*<sup>+</sup>  $\Delta cas1$  +  $\lambda c$  +  $\lambda T3$  cells were sensitive to *λvir* phage at 30 °C and 37 °C, as expected because H-NS represses *cas* genes (Table 3).  $\Delta hns$   $\Delta cas1$  +  $\lambda c$  +  $\lambda T3$  cells showed  $\sim 10^8$  fold increase in resistance at 30 °C compared to *hns*<sup>+</sup>  $\Delta cas1$  +  $\lambda c$  +  $\lambda T3$ , and  $\sim 10^3$  compared to  $\Delta hns$   $\Delta cas1$  +  $\lambda T3$  cells, but remained sensitive to *λvir* infection at 37 °C (Table 3). This showed that  $\Delta cas1$  mutation did not affect interference as expected and confirmed the importance of a consensus PAM for phage resistance in infectivity assays at 30 °C, explained in previous studies by the effect of variable PAMs on efficacy of interference reactions [25, 29]. However, added spacer targeting the consensus PAM 5'-CTT-3' was not able to repeal the temperature dependent resistance of  $\Delta hns$  cells to *λvir* in these assays, which we concluded must be caused by other factor (s).

**Transcription and stability of *cas3* in  $\Delta hns$  cells is limiting for resistance to phage**

We tested if the effect of temperature on phage resistance was influenced by variations in expression of *E. coli* CRISPR-Cas. Robust resistance of  $\Delta hns$   $\Delta cas1$  +  $\lambda c$  +  $\lambda T3$  cells to *λvir* (Table 3) suggested all components of CRISPR-Cas were expressed in sufficient amounts at 30 °C. A previous analysis identified increased transcription of genes encoding Cascade and crRNA in  $\Delta hns$  cells grown to mid-log phase, but no such increase in *cas3* transcripts [34]. We explored if levels of Cas3 RNA or protein in cells might correlate to phage resistance under different temperature conditions of infectivity assays.

By using quantitative PCR (qPCR) we compared *cas3* transcript levels between *hns*<sup>+</sup> and  $\Delta hns$  cells in both mid-log and stationary phases of growth. Relative abundance of *cas3* transcripts was around eight-fold higher in  $\Delta hns$  cells compared to *hns*<sup>+</sup> cells when grown to stationary phase, regardless of the temperature being 30 °C or 37 °C ( $8.99 \pm 3.83$  and  $7.59 \pm 1.59$ ), but remained similar at mid-log phase ( $0.9 \pm 0.56$  and  $2.44 \pm 1.21$  for 30 °C and 37 °C, respectively). This suggested that the growth phase was important for the observed difference in the level of *cas3* transcripts, not the temperature of incubation. As shown in Fig. 1, only  $\Delta hns$  +  $\lambda T3$  cells showed significant difference in *cas3* transcripts depending on the temperature of incubation (marked with different letters d and bc which indicate significant difference in expression values between these two samples ( $p < 0.05$ )). Increased *cas3* transcription at stationary phase at 30 °C or 37 °C was not observed if H-NS was present, and presence of  $\lambda c$  +  $\lambda T3$  spacers had no effect on *cas3* transcript levels (Fig. 1). These results suggest that the sensitivity of  $\Delta hns$  cells to *λvir* plaques at 37 °C is unlikely to be due to lack of *cas3* transcription. We therefore tested if Cas3 protein levels may influence phage sensitivity at 37 °C. The chaperone HtpG was also considered here, because HtpG increases steady-state Cas3 protein levels in *E. coli*, which correlated to stimulation of interference reactions, carried out at 32 °C [41]. We reasoned that sensitivity of  $\Delta hns$   $\Delta cas1$  +  $\lambda c$  +  $\lambda T3$  cells to *λvir* at 37 °C caused by reduced or unstable Cas3 protein might be

**Table 2** List of strains used in this study

Bacterial strain	Relevant genotype	Source or reference
EB304	MG1655, $\Delta cas3::apra$	[44]
BSN22	W3110, $\Delta hns::cat$	[46]
BW25113	$F^- rrnB \Delta lacZ4748 (::rrnB-3) hsdR514 \Delta (araBAD) 567 \Delta (rhaBAD) 568 rph-1 \lambda^-$ Bacterial strains related to BW25113	[47]
BW39121	+ $\Delta hns::kan$	[38]
JW0462	+ $\Delta htpG::kan$	[41]
BW40114	+ $F'$ ( <i>proAB</i> <sup>+</sup> <i>lacI</i> <sup>f</sup> $\Delta$ M15::Tn10) <i>lacUV5-cas3 cat::araBp8-casA</i>	[27]
BW39651	+ $\lambda T3$ spacer	[38]
BW39671	+ $\lambda T3 \Delta hns::kan$	[38]
BW39183	+ $\Delta cas1::kan$	[38]
IIB848	+ $\lambda T3 \Delta cas3::apra$	recombineering using pKD46
IIB870	+ $\lambda T3 \Delta cas3::apra$	P1. IIB848 $\times$ BW39651
IIB965	+ $\lambda T3 \Delta cas1::kan$	P1. BW39183 $\times$ BW39651
IIB966	+ $\lambda T3 \Delta cas1::kan \Delta hns::cat$	P1. BSN22 $\times$ IIB965
IIB969	+ $\lambda T3 lacUV5-cas3 cat::araBp8-casA$	P1. BW40114 $\times$ BW39651
IIB969e	+ $\lambda c$ + $\lambda T3 lacUV5-cas3 cat::araBp8-casA$	Selection of $\lambda^f$ colony with phage acquired spacer
IIB1039	+ $\lambda c$ + $\lambda T3 \Delta cas1::kan$	P1. BW39183 $\times$ IIB969e (selection Km <sup>r</sup> Chl <sup>s</sup> and PCR of the CRISPR-1 region)
IIB1040	+ $\lambda c$ + $\lambda T3 \Delta cas1::kan \Delta hns::cat$	P1. BSN22 $\times$ IIB1039
IIB1043	+ $\lambda c$ + $\lambda T3 \Delta cas1::kan^S$	Removal of kan cassette by pCP20 plasmid
IIB1063	+ $\lambda c$ + $\lambda T3 lacUV5-cas3 cat::araBp8-casA \Delta htpG::kan$	
IIB1065	+ $\lambda c$ + $\lambda T3 \Delta cas1::kan^S \Delta htpG::kan$	P1. JW0462 $\times$ IIB1043
IIB1066	+ $\lambda c$ + $\lambda T3 \Delta cas1::kan^S \Delta htpG::kan \Delta hns::cat$	P1. BSN22 $\times$ IIB1065

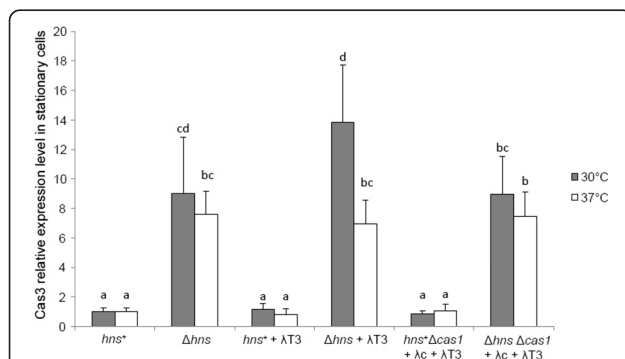
**Table 3** Temperature-dependent resistance of  $\Delta hns$  cells to phage  $\lambda vir$  in the presence of phage acquired spacer

strain	genotype	Plaque forming units (PFUs)	
		30 °C	37 °C
IIB965	$hns^+ \Delta cas1 + \lambda T3$	$4.63 \times 10^{10} \pm 1.6 \times 10^{10}$	$5.8 \times 10^{10} \pm 1.14 \times 10^{10}$
IIB966	$\Delta hns \Delta cas1 + \lambda T3$	$\sim 5 \times 10^5$	$3.35 \times 10^{10} \pm 9 \times 10^9$
IIB1039	$hns^+ \Delta cas1 + \lambda c + \lambda T3$	$3.37 \times 10^{10} \pm 2.5 \times 10^9$	$2.97 \times 10^{10} \pm 9.7 \times 10^9$
IIB1040	$\Delta hns \Delta cas1 + \lambda c + \lambda T3$	$\sim 3 \times 10^2$	$2 \times 10^{10} \pm 6 \times 10^9$

Cell lawns of strains  $hns^+ (\Delta cas1) + \lambda c + \lambda T3$  and  $\Delta hns (\Delta cas1) + \lambda c + \lambda T3$  were infected with phage dilutions (from  $10^0$  to  $10^{-8}$ ) and incubated at either 30 °C or 37 °C. The average of at least three independent experiments are shown

overcome by plasmid expression of HtpG or Cas3. However, we observed that (Fig. 2a) expression of only Cas3 from plasmid restored resistance of  $\Delta hns \Delta cas1 + \lambda c + \lambda T3$  cells to  $\lambda vir$  at 37 °C comparably to at 30 °C, while cells containing empty plasmid vectors (pBAD or pUC19) or HtpG expressing plasmid remained sensitive. These results suggest that endogenous levels of functionally active Cas3 in  $\Delta hns \Delta cas1 + \lambda c + \lambda T3$  cells are too low to be relieved by elevated levels of HtpG at 37 °C. Given the importance of HtpG for phage resistance it was expected that elimination of HtpG from  $\Delta hns \Delta cas1 + \lambda c + \lambda T3$  cells ( $\Delta htpG \Delta hns \Delta cas1 + \lambda c + \lambda T3$ ) would cause sensitivity to phage at both 30 °C and 37 °C (Fig. 2b), compared to phage resistance observed in Fig. 2a. Indeed, plaques were observed at 30 °C in  $\Delta htpG \Delta hns \Delta cas1 + \lambda c + \lambda T3$  cells with or without empty plasmid controls. However, individual plaques were not visible, so the number of PFU is estimation (Fig. 2b). Plasmid expression of Cas3 (pCas3) in

$\Delta htpG \Delta hns \Delta cas1 + \lambda c + \lambda T3$  cells was sufficient for phage resistance at 30 °C (Fig. 2b) indicating that elevated amounts of Cas3 is efficient in phage defence independently of HtpG at 30 °C as shown before [41]. In contrast, elevated levels of Cas3 (pCas3) in  $\Delta htpG \Delta hns \Delta cas1 + \lambda c + \lambda T3$  cells did not rescue phage resistance at 37 °C confirming the importance of HtpG in maintaining functional levels of Cas3 in phage defence. As expected, although plasmid expression of HtpG (pHtpG) in  $\Delta htpG \Delta hns + \lambda c + \lambda T3$  cells rescued phage resistance at 30 °C, it did not at 37 °C (Fig. 2b). Given the known interplay of HtpG and Cas3 in promoting CRISPR interference in *E. coli*, these results suggest that levels of Cas3 protein are the limiting factor for resistance to  $\lambda vir$  phage infection in  $\Delta hns$  cells at 37 °C. Overall, these results show that endogenous levels of Cas3 are expressed in low but sufficient amounts for the CRISPR-Cas mediated immunity in cells lacking HNS grown to stationary phase at 30 °C, but that the levels of functionally active Cas3 becomes limiting at 37 °C and require increased levels of Cas3.

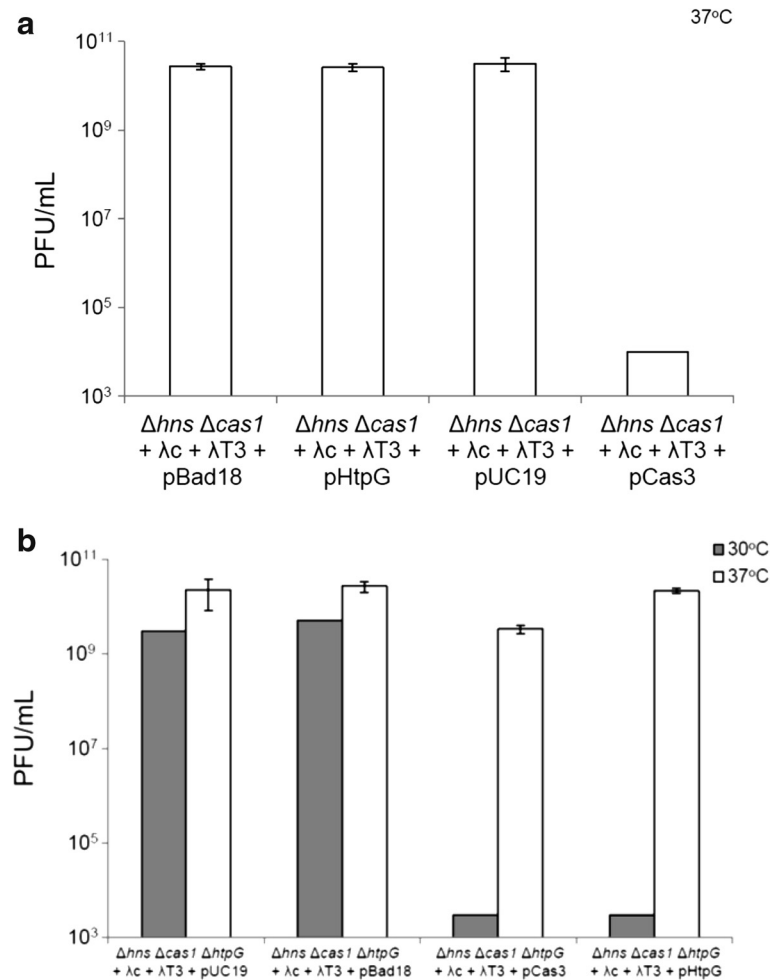


**Fig. 1** Cas3 is transcribed in cells lacking H-NS at both temperatures of incubation in stationary phase of growth. qPCR analysis extended to  $\Delta hns \Delta cas1$  and  $hns^+ \Delta cas1$  cells containing  $\lambda T3$  and/or  $\lambda c$  anti-lambda spacer. Relative expression levels of *cas3* transcripts are measured in cells grown to stationary phase at indicated temperatures of incubation with *groES* as reference gene. Error bars represent normalized error of respective duplicates. Histogram bars marked with different letters (a for  $hns^+$  samples, and b, bc, cd and d for  $\Delta hns$  samples) indicate significant difference between expression values, while samples that share a letter in the notation (e.g. b, bc, cd or d and cd) do not have statistically different expression values. Cas3 expression levels were compared across all samples, and two expression values were considered significantly different as evaluated by One-way ANOVA Duncan Multiple Range post-hoc test ( $p < 0.05$ )

## Discussion

By manipulating the expression of H-NS and CRISPR-Cas in *E. coli* cells we identified that stability or activity of Cas3, with HtpG present, is a limiting factor for resistance to phage  $\lambda vir$  at 37 °C. Our genetic analyses of CRISPR interference at 30 °C agreed with previous studies, by observing robust phage resistance when cells were lacking H-NS repressor ( $\Delta hns$ ), and when an anti- $\lambda$  spacer could target protospacer DNA next to a consensus PAM, 5'-CTT-3'. However, the same assays at 37 °C resulted in a dramatic loss of phage resistance that had not been observed previously. Phage resistance could be restored at 37 °C, to levels comparable with resistance at 30 °C, by inducible expression of *cas3* from plasmids.

Previous analyses of *cas3* transcription sampled only during mid-log growth and showed no difference between  $\Delta hns$  cells compared to  $hns^+$  cells, unlike Cascade genes and crRNA that were much increased in  $\Delta hns$  cells [34]. We measured *cas3* transcripts in mid-log and stationary phase, observing that  $\Delta hns$  cells contained eight-fold more *cas3* transcript compared to  $hns^+$  cells at



**Fig. 2** HtpG is required for resistance to  $\lambda vir$  at 30 °C and 37 °C to prevent Cas3 instability. **a** *E. coli* cell lawns of strain  $\Delta hns \Delta cas1 + \lambda c + \lambda T3$  (IIB1040) transformed with pCas3 and pHtpG expressing plasmids and empty vector controls were infected with phage dilutions (from  $10^0$  to  $10^{-8}$ ) and incubated at 37 °C. Bars represent average and SD of the number of plaque forming units (PFUs) per ml from three independent experiments. **b** *E. coli* cell lawns of strains  $\Delta hns \Delta cas1 + \lambda c + \lambda T3$  (IIB1040) and  $\Delta htpG \Delta hns \Delta cas1 + \lambda c + \lambda T3$  (IIB1066) and IIB1066 transformed with pCas3 and pHtpG expressing plasmids, were infected with phage dilutions (from  $10^0$  to  $10^{-8}$ ) and incubated at either 30 °C or 37 °C. Bars represent average and SD of the number of plaque forming units (PFUs) per ml from three independent experiments

30 °C and 37 °C. This is significant because infectivity assays for measuring resistance of *E. coli* to  $\lambda vir$  use cells in stationary phase. We conclude from this analysis that H-NS regulates expression of the *cas3* gene, as well as Cascade and crRNA, but possibly does so under more specific growth conditions.

Recently it was highlighted that Cas3 requires HtpG chaperone for CRISPR interference assays [41]: Overexpression of HtpG or Cas3 from plasmids in *htpG* deficient cells ( $\Delta htpG \Delta hns$ ) rescued transformation-efficiency at 32 °C. We observed similar interplay of HtpG and Cas3 at 30 °C because plasmid overexpression of either HtpG or Cas3 (pHtpG/pCas3) could restore phage resistance to  $\Delta htpG \Delta hns \Delta cas1 + \lambda c + \lambda T3$  (IIB1066) cells that were otherwise sensitive to phage infection (Fig. 2b). Interestingly, the pCas3 alone did not restore phage resistance to

$\Delta htpG \Delta hns \Delta cas1 + \lambda c + \lambda T3$  cells at 37 °C (Fig. 2b) but did to *htpG*<sup>+</sup>  $\Delta hns \Delta cas1 + \lambda c + \lambda T3$  (IIB1040) cells at 37 °C (Fig. 2a), while pHtpG was unable to sustain phage resistance to any  $\Delta hns$  cells at 37 °C. Thus, HtpG is important for CRISPR-system activity in  $\Delta hns$  cells at 37 °C, but its overexpression from plasmid alone cannot overcome limiting amounts of functionally active Cas3 at 37 °C. Further research will be required to better understand the reasons and mechanisms of Cas3 instability in  $\Delta hns$  cells at 37 °C.

In addition to four  $\sigma^{70}$  (“house-keeping” sigma factor)-promoters in CRISPR-Cas area, two potential  $\sigma^{32}$  (heat-shock sigma factor)-dependent promoters have been mapped within coding regions of *cas7* and *cas1* [42, 43], suggesting another possible link between CRISPR-Cas immunity and heat-shock response. In summary, expression and activity of the CRISPR-Cas system in *E. coli* seem to

be linked to global stress responses, such as H-NS global repressor, heat stress and CRP-cAMP. Perhaps, CRISPR-Cas immunity is designed to become activated during certain phase of growth, at specific environmental habitats and temperature, and instability of the Cas3 may be the mechanism for inactivation of the CRISPR-Cas defence either at inappropriate temperature of incubation or when degradation of foreign DNA is completed.

## Conclusions

We observed that the ability of an *E. coli* CRISPR-Cas system to resist lysis by phage  $\lambda$  was strongly influenced by temperature. Genetic analysis of this effect indicated that sensitivity to phage at 37 °C was caused by limiting amounts of Cas3, rather than effects of PAM sequence variations on Cascade interference reactions. We show that transcription of *cas3* is controlled by H-NS: elimination of H-NS from cells correlated to eight-fold increased levels of *cas3* transcript, specifically in stationary phase growth. At 37 °C, increased expression of *cas3* is required for resistance to  $\lambda$  infection. This suggests that endogenous expression and activity of Cas3 is responsive to signals associated with growth phase and temperature in *E. coli*.

## Methods

### Strains and plasmids

The *E. coli* K-12 strains used in this study are described in the Table 2.

Plasmids used were: pEB526 expressing Cas3 [44], and HtpG expressing plasmid was from pBAD18 plasmid [41].

### Media and general methods

LB broth and agar media (10 g L<sup>-1</sup> bacto-tryptone, 5 g L<sup>-1</sup> yeast extract, 10 g L<sup>-1</sup> NaCl), supplemented with 15 g of agar for solid media. When required appropriate antibiotics were added to LB plates at final concentrations: ampicillin at 100  $\mu$ g/ml, kanamycin at 40  $\mu$ g/ml, apramycin 30  $\mu$ g/ml and chloramphenicol at 15  $\mu$ g/ml. Mutant bacterial strains were made by P1 $\lambda$  transduction and selected for the appropriate antibiotic resistance [45]. When important (for generating IIB969 and IIB1039), the genotype (presence of  $\lambda$ T3 and  $\lambda$ c spacers) of many transductants were screened by colony PCR using the same primers (CRISPR I-R: 5'-GAGATGCAGGCCATCGGA-3' and spacer 4: 5'-GCGACCGCTCAGAAATCCAGACCCGATCCAAA-3') as for spacer acquisition and PCR products were sequenced for confirmation.

### Phage sensitivity assay by plaque formation

Cells were grown to saturation overnight in LB medium supplemented with 0.02 % maltose. LB plates were overlaid with 3.5 ml 0.6 % LB top agar containing 0.2 ml of cells. After solidification, 10  $\mu$ l aliquots of serially diluted phages in 10 mM MgSO<sub>4</sub> were spotted on the surface of

the plate and allowed to soak. Plates were incubated overnight at 30 °C or 37 °C. When required, 1 mM IPTG (isopropyl- $\beta$ -D-thiogalactoside) and 0.2 % L-arabinose were added in plates and top agar. The sensitivity of the cells to infection was represented as the plaque-forming units (PFUs) by counting plaques from several dilutions, and calculating their number per mL.

### Spacer acquisition experiments to generate a consensus PAM for interference

Spacer acquisition was performed according to [27]. Strain IIB969 with *cas* genes fused to inducible promoters and containing the anti-lambda  $\lambda$ T3 spacer in CRISPR locus 2.1 (Table 2) was grown at 37 °C at 200 rpm to log phase in LB medium containing 1 mM IPTG and 0.2 % L-arabinose until OD<sub>600</sub> was 0.4-0.5, and mixed with  $\lambda$ vir lysate at appropriate MOI = 1. Cell-phage mixture was incubated for 15 minutes without agitation at 37 °C. The mixture was then diluted 10 fold with fresh LB medium containing the same inducers and incubated at 37 °C for at least two hours, in most cases overnight. Aliquots were spread on LB plates with IPTG and arabinose and incubated overnight at 37 °C. CRISPR expansion was monitored by PCR using appropriate pairs of primers specific for CRISPR locus 2.1 mentioned above.

Several PCR products of fragments of CRISPR locus 2.1 (Additional file 1: Figure S1B) were sent for sequencing in Macrogen service. One lambda resistant derivative was kept for further research (IIB969e, Table 2). It contained phage-acquired anti- $\lambda$  spacer targeting *lambdaII* gene (called  $\lambda$ c) with consensus PAM 5'-CTT-3' (Additional file 1: Figure S1C).  $\Delta$ *hns* +  $\lambda$ c +  $\lambda$ T3 strain was made by P1 transduction using donor strain  $\Delta$ *cas1*::Km<sup>r</sup> (BW39183) and selecting for *hns*<sup>+</sup> recombinant strain with two extra spacers in the CRISPR locus 2.1 (strain IIB1039; Table 2) and later introducing  $\Delta$ *hns* mutation (strain IIB1040; Table 2). The  $\Delta$ *cas1* mutation was chosen to create  $\Delta$ *hns* +  $\lambda$ c +  $\lambda$ T3 cells because *cas1* gene is not required for interference [37].

### RNA extraction and qPCR

Total RNA was extracted from mid log (OD<sub>600</sub> = 0.4-0.5) and overnight cultures incubated at 30 °C or 37 °C. 1.5 ml of each culture was used and the cell pellet was resuspended in cold 10 mM EDTA and 50 mM sodium citrate and Trizol LS (Invitrogen) was used to extract total RNA following the instructions from the manufacturer. The same amounts of RNA (1  $\mu$ g) was first treated by DNase I, diluted 10 fold and 2  $\mu$ l of each sample (in duplicate) was used as a template for one step amplification reaction using One Step SYBR Prime Script RT-PCR Kit II (Takara Bio. Inc.). The PCR reactions were performed on a 7500 Fast Real Time PCR System (Applied Biosystems) and analysed using 7500 Software

v.2.0.6. (Applied Biosystems). As an internal control the *groES* gene was used. Fold change of the *cas3* gene transcription was calculated using relative quantification with *groES* as endogenous control and *cas3* gene transcript from *E. coli* BW25113 (wild type) abundance as calibrator. All PCR reactions were performed in triplicate. Control PCRs without template were performed to monitor general contamination levels. Results of qPCR ( $\Delta C_t$  values) were analyzed by one-way analysis of variance (ANOVA) using STATISTICA 12.0 (StatSoft Inc, USA) software package. Duncan Multiple Range Test was used for post-hoc analysis. Differences between two sample means were considered statistically significant at  $p < 0.05$ .

Primers used were:

Cas 3-F: 5' -ATCGCGTCAATGTACCCTTC-3'  
 Cas3-R: 5' -TCCAGCCAAAGTAACCCATC-3'  
 groES-F: 5' -CTG GAT CGT CAA GCG TAA AG-3'  
 groES-R: 5' -CAA GGA TAC GGC CAT TGC-3'

## Additional file

**Additional file 1: Figure S1.** Properties of PAM in  $\lambda T3$  spacer and phage acquired spacer (A). Sequence complementarity between  $\lambda T3$  spacer and proto-spacer in lambda with the PAM 5' CCA 3' highlighted in red type. (B) Detection of new spacers acquired in CRISPR locus 2.1. The agarose gel shows products of PCR amplified CRISPR 2.1 from *E. coli* cells IIB969 (lane C "control"), and two phage resistant derivatives after phage challenge of IIB969 (lanes a and b). The PCR fragment size is 723 bp before and 784 bp after spacer acquisition (C).  $\lambda c$  spacer sequence from the strain IIB969e is presented paired with proto-spacer in lambda (targeting gene *chl*) with the PAM 5'-CTT-3' highlighted in red type. (JPG 311 kb)

## Abbreviations

cAMP: cyclic AMP; Cas: CRISPR-associated; Cascade: CRISPR-associated complex for antiviral defence; CRISPR: clustered regularly interspaced short palindromic repeats; CRP: cAMP receptor protein; crRNA: CRISPR-RNA; *E. coli*: *Escherichia coli*; H-NS: nucleoid-structuring protein; HtpG: high-temperature protein G; MOI: multiplicity of infection; OD: optical density; PAM: protospacer adjacent motif; PFU: plaque forming units;  $\lambda$ : lambda.

## Competing interests

The authors declare that they have no competing interests.

## Authors' contributions

KM and IIB performed the experiments and KM performed statistical analyses. IIB and ELB designed the study and analysed the data. IIB supervised experiments and IIB and ELB wrote the manuscript. All authors read and approved the final version of the manuscript.

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## References

- van der Oost J, Westra ER, Jackson RN, Wiedenheft B. Unravelling the structural and mechanistic basis of CRISPR-Cas systems. *Nat Rev Microbiol*. 2014;12:479–92.
- Bhaya D, Davison M, Barrangou R. CRISPR-Cas systems in bacteria and archaea: versatile small RNAs for adaptive defense and regulation. *Annu Rev Genet*. 2011;45:273–97.
- Westra ER, Swarts D, Staals R, Jore MM, Brouns SJ, van der Oost J. The CRISPRs, they are a-changin': how prokaryotes generate adaptive immunity. *Annu Rev Genet*. 2012;46:311–38.
- Wiedenheft B, Sternberg SH, Doudna JA. RNA-guided genetic silencing system in bacteria and archaea. *Nature*. 2012;482:175–82.
- Sorek R, Lawrence CM, Wiedenheft B. CRISPR-mediated adaptive immune systems in bacteria and archaea. *Annu Rev Biochem*. 2013;82:237–66.
- Jore MM, Lundgren M, Van Duijn E, Bultema JB, Westra ER, et al. Structural basis for CRISPR RNA-guided recognition by Cascade. *Nat Struct Mol Biol*. 2011;18:529–36.
- Wiedenheft B, Lander GC, Zhou K, Jore MM, Brouns SJ, van der Oost J, et al. Structures of the RNA-guided surveillance complex from a bacterial immune system. *Nature*. 2011;477:486–9.
- Zhao H, Sheng G, Wang J, Wang M, Bunkoczi G, et al. Crystal structure of the RNA-guided immune surveillance Cascade complex in *Escherichia coli*. *Nature*. 2014;515:147–50.
- Jackson RN, Golden SM, Van Erp PB, Carter J, Westra ER, et al. Crystal structure of the CRISPR RNA-guided surveillance complex from *Escherichia coli*. *Science*. 2014;345:1473–9.
- Mulepati S, Héroux A, Bailey S. Structural biology. Crystal structure of a CRISPR RNA-guided surveillance complex bound to a ssDNA target. *Science*. 2014;345:1779–84.
- Westra ER, Van Erp PB, Künne T, Wong SP, Staals RH, et al. CRISPR immunity relies on the consecutive binding and degradation of negatively supercoiled invader DNA by Cascade and Cas3. *Mol Cell*. 2012;46:595–605.
- Ivančić-Baće I, Howard JA, Bolt EL. Tuning in to interference: R-loops and cascade complexes in CRISPR immunity. *J Mol Biol*. 2012;422:607–16.
- Hochstrasser ML, Taylor DW, Bhat P, Guegler CK, Sternberg SH, et al. CasA mediates Cas3-catalyzed target degradation during CRISPR RNA-guided interference. *Proc Natl Acad Sci U S A*. 2014;111:6618–23.
- Mulepati S, Bailey S. In vitro reconstitution of an *Escherichia coli* RNA-guided immune system reveals unidirectional, ATP-dependent degradation of DNA target. *J Biol Chem*. 2013;288:22184–92.
- Sinkunas T, Gasiunas G, Waghmare SP, Dickman MJ, Barrangou R, et al. In vitro reconstitution of Cascade-mediated CRISPR immunity in *Streptococcus thermophilus*. *EMBO J*. 2013;32:385–94.
- Gong B, Shin M, Sun J, Jung C-H, Bolt EB, et al. Molecular insights into DNA interference by CRISPR-associated nuclease-helicase Cas3. *Proc Natl Acad Sci U S A*. 2014;111:16359–64.
- Beloglazova N, Petit P, Flick R, Brown G, Savchenko A, Yakunin AF. Structure and activity of Cas3 HD nuclease MJ0384, an effector enzyme of the CRISPR interference. *EMBO J*. 2011;30:4616–27.
- Sinkunas T, Gasiunas G, Fremaux C, Barrangou R, Horvath P, Siksnys V. Cas3 is a single-stranded DNA nuclease and ATP-dependent helicase in the CRISPR-Cas immune system. *EMBO J*. 2011;30:1335–42.
- Huo Y, Nam KH, Ding F, Lee H, Wu L, et al. Structures of CRISPR Cas3 offer mechanistic insights into Cascade-activated DNA unwinding and degradation. *Nat Struct Mol Biol*. 2014;21:771–7.
- Mojica FJ, Díez-Villaseñor C, García-Martínez J, Almendros J. Short motif sequences determine the targets of the prokaryotic CRISPR defence system. *Microbiology*. 2009;155:733–40.
- Semenova E, Jore MM, Datsenko KA, Semenova A, Westra ER, et al. Interference by clustered regularly interspaced short palindromic repeat (CRISPR) RNA is governed by a seed sequence. *Proc Natl Acad Sci U S A*. 2011;108:10098–103.
- Fineran PC, Gerritzen MJ, Suárez-Díez M, Künne T, Boekhorst J, et al. Degenerate target sites mediate rapid primed CRISPR adaptation. *Proc Natl Acad Sci U S A*. 2014;111:E1629–38.
- Blosser TR, Loeff L, Westra ER, Vlot M, Künne T, et al. Two distinct DNA binding modes guide dual roles of a CRISPR-Cas protein complex. *Mol Cell*. 2015;58:60–70.



24. Sashital DG, Wiedenheft B, Doudna JA. Mechanism of foreign DNA selection in a bacterial adaptive immune system. *Mol Cell*. 2012;48:606–15.
25. Westra ER, Semenova E, Datsenko KA, Jackson RN, Wiedenheft B, Severinov K, et al. Type I-E CRISPR-Cas systems discriminate target from non-target DNA through base pairing-independent PAM recognition. *Plos Genet*. 2013;9, e1003742.
26. Swarts DC, Mosterd C, Van Passel MW, Brouns SJ. CRISPR interference directs strand specific spacer acquisition. *Plos ONE*. 2012;7, e35888.
27. Datsenko KA, Pougach K, Tikhonov A, Wanner BL, Severinov K, Semenova E. Molecular memory of prior infections activates the CRISPR-Cas adaptive bacterial immunity system. *Nat Commun*. 2012;3:945.
28. Szczelkun MD, Tikhomirova MS, Sinkunas T, Gasiunas G, Karvelis T, et al. Direct observation of R-loop formation by single RNA-guided Cas9 and Cascade effector complexes. *Proc Natl Acad Sci U S A*. 2014;111:9798–803.
29. Rutkauskas M, Sinkunas T, Songailiene I, Tikhomirova MS, Siksnyus V, Seidel R. Directional R-loop formation by the CRISPR-Cas surveillance complex Cascade provides efficient off-target site rejection. *Cell Reports*. 2015;10:1534–43.
30. Deveau H, Barrangou R, Garneau JE, Labonté J, Fremaux C, et al. Phage response to CRISPR-encoded resistance in *Streptococcus thermophilus*. *J Bacteriol*. 2008;190:1390–400.
31. Redding S, Sternberg SH, Marshall M, Gibb B, Bhat P, et al. Surveillance and processing of foreign DNA by the *Escherichia coli* CRISPR-Cas system. *Cell*. 2015;163:854–65.
32. Xue C, Seetharam AS, Musharova O, Severinov K, Brouns SJJ, et al. CRISPR interference and priming varies with individual spacer sequences. *Nucleic Acids Res*. 2015;43:10831–47.
33. Pul Ü, Wurm R, Arslan Z, Geissen R, Hofmann N, Wagner R. Identification and characterization of *E. coli* CRISPR-Cas promoters and their silencing by H-NS. *Mol Microbiol*. 2010;75:1495–512.
34. Westra ER, Pul Ü, Heidrich N, Jore MM, Lundgren M, et al. H-NS-mediated repression of CRISPR-based immunity in *Escherichia coli* K12 can be relieved by transcription activator LeuO. *Mol Microbiol*. 2010;77:1380–93.
35. Yang CD, Chen YH, Huang HY, Huang HD, Tseng CP. CRP represses the CRISPR/Cas system in *Escherichia coli*: evidence that endogenous CRISPR spacers impede phage P1 replication. *Mol Microbiol*. 2014;92:1072–91.
36. Shinkai A et al. Transcription activation mediated by a cyclic AMP receptor protein from *Thermus thermophilus* HB8. *J Bacteriol*. 2007;189:3891–901.
37. Brouns SJ, Jore MM, Lundgren M, Westra ER, Slijkhuis RJ, et al. Small CRISPR RNAs guide antiviral defense in prokaryotes. *Science*. 2008;321:960–4.
38. Pougach K, Semenova E, Bogdanova E, Datsenko KA, Djordjevic M, et al. Transcription, processing and function of CRISPR cassettes in *Escherichia coli*. *Mol Microbiol*. 2010;77:259–72.
39. Almendros C, Guzmán NM, Díez-Villaseñor C, García-Martínez J, Mojica FJ. Target motifs affecting natural immunity by a constitutive CRISPR-Cas system in *Escherichia coli*. *Plos ONE*. 2012;7:e50797.
40. Vercoe RB, Chang JT, Dy RL, Taylor C, Gristwood T, et al. Cytotoxic chromosomal targeting by CRISPR/Cas systems can reshape bacterial genomes and expel or remodel pathogenicity islands. *Plos Genet*. 2013;9, e1003454.
41. Yosef I, Goren MG, Kiro R, Edgar R, Qimron U. High-temperature protein G is essential for activity of the *Escherichia coli* clustered regularly interspaced short palindromic repeats (CRISPR)/Cas system. *Proc Natl Acad Sci U S A*. 2011;108:20136–41.
42. Nonaka G, Blankschien M, Herman C, Gross CA, Rhodius VA. Regulon and promoter analysis of the *E. coli* heat-shock factor; sigma32, reveals a multifaceted cellular response to heat stress. *Genes Dev*. 2006;20:1776–89.
43. Wade JT, Castro Roa D, Grainger DC, Hurd D, Busby SJ, Struhl K, et al. Extensive functional overlap between sigma factors in *Escherichia coli*. *Nat Struct Mol Biol*. 2006;13:806–14.
44. Ivancić-Baće I, Radovčić M, Bočkor L, Howard JL, Bolt EL. Cas3 stimulates runaway replication of a ColE1 plasmid in *Escherichia coli* and antagonises Rnase HI. *RNA Biol*. 2013;10:770–8.
45. Miller JH. *Experiments in Molecular Genetics*. Cold Spring Harbor, NY: Cold Spring Harbor Laboratory Press; 1992.
46. Sonden B, Uhlin BE. Coordinated and differential expression of histone-like proteins in *Escherichia coli*: regulation and function of the H-NS analog StpA. *EMBO J*. 1996;15:4970–80.
47. Datsenko KA, Wanner BL. One-step inactivation of chromosomal genes in *Escherichia coli* K-12 using PCR products. *Proc Natl Acad Sci U S A*. 2000;97:6640–5.

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