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 (Δhns). We present further genetic analyses of the regulation of CRISPR-Cas mediated phage resistance in Δhns E. coli cells.

Results: We observed that E. coli Type I-E CRISPR-Cas mediated resistance to phage λ 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
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 (Δ*hns*), or ectopic over-expression of LeuO in cells with engineered anti-λ spacer (ΔT3) into CRISPR, promote CRISPR interference observed as enhanced resistance to phage λvir [34, 38]. In these studies the protospacer targeted by spacer ΔT3 crRNA had a non-consensus PAM 5′-CCA-3′ and although resistance to phage λvir 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 Δ*hns* cells that had acquired a new spacer (λc) 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 Δ*hns* *E. coli* cells to phage λvir 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 (casABCDE12) and CRISPR locus 2.1 [33–35, 38]. Deleting H-NS (Δ*hns*) from cells de-repressed transcription, and efficient resistance to λvir infection at 30 °C was reported when CRISPR of Δ*hns* cells was engineered to contain an anti-λ phage spacer sequence (ΔT3) [34, 38]. The importance of the ΔT3 spacer was highlighted by sensitivity of Δ*hns* cells to λvir plaque formation compared to Δ*hns* + ΔT3 cells [38]. When repeating these experiments we also observed about 100000 fold elevated resistance of Δ*hns* + ΔT3 cells to λvir infection at 30 °C, compared to Δ*hns* cells without ΔT3 spacer (Table 1). However at 37 °C, in otherwise identical assays, Δ*hns* + ΔT3 cells became sensitive to phage (Table 1). Cells with intact H-NS (*hns*), with or without ΔT3, were sensitive to λvir infection at both temperatures (Table 1). There was no difference in sensitivity at 37 °C between Δ*hns* cells + or - ΔT3 spacer. If ΔT3 spacer was absent from the CRISPR locus 2.1, Δ*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 Δ*hns* + ΔT3 cells to phage λvir.

The ΔT3 spacer sequence engineered into CRISPR used here and in [37, 38] has a nucleotide sequence match with the template strand of λ phage gene lysis R,
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 Δhns 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 Δhns + λT3 E. coli to acquire a new spacer. One such E. coli derivative containing spacer (λc) targeting phage λvir gene cl 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 Δcas1 mutation as a useful controlling factor to uncouple interference from adaptation, enabling focus on interference reaction only. In phage infectivity assays, hns+ Δcas1 + λc + λT3 cells were sensitive to λvir phage at 30 °C and 37 °C, as expected because H-NS represses cas genes (Table 3). Δhns Δcas1 + λc + λT3 cells showed ~10³-fold increase in resistance at 30 °C compared to hns+ Δcas1 + λc + λT3, and ~10⁵ compared to Δhns Δcas1 + λT3 cells, but remained sensitive to λvir infection at 37 °C (Table 3). This showed that Δcas1 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 Δhns cells to λvir in these assays, which we concluded must be caused by other factor(s).

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

| strain          | genotype     | Plaque forming units (PFUs) |
|-----------------|--------------|-----------------------------|
| BW25113        | hns⁺         | 3.80 × 10¹⁰ ± 7 × 10⁹       |
| BW39121        | Δhns         | 1.20 × 10³ ± 1.8 × 10⁹     |
| BW39651        | hns⁺ + λT3   | 3.66 × 10⁹ ± 7.57 × 10⁹    |
| BW39671        | Δhns + λT3   | ~4 × 10⁶                   |
|                 |              | 30 °C                       |
|                 |              | 4.56 × 10¹⁰ ± 9 × 10⁹      |
|                 |              | 37 °C                       |
|                 |              | 4.23 × 10¹⁰ ± 6.6 × 10⁹    |

λvir

Cell lawns were infected with phage dilutions (from 10⁻⁴ to 10⁻⁹) and incubated at either 30 °C or 37 °C. Cells lacking H-NS (Δhns) or containing H-NS (hns⁺) had fully operational CRISPR-Cas systems that were engineered with an anti-λ spacer (λT3) as indicated. The average of at least three independent experiments are shown.

Transcription and stability of cas3 in Δhns 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 Δhns Δcas1 + λc + λT3 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 Δhns 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⁺ and Δhns cells in both mid-log and stationary phases of growth. Relative abundance of *cas3* transcripts was around eight-fold higher in Δhns cells compared to hns⁺ cells when grown to stationary phase, regardless of the temperature being 30 °C or 37 °C (8.99 ± 3.83 and 7.59 ± 1.59), but remained similar at mid-log phase (0.9 ± 0.56 and 2.44 ± 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 Δhns + λT3 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 λc + λT3 spacers had no effect on *cas3* transcript levels (Fig. 1). These results suggest that the sensitivity of Δhns 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 Δhns Δcas1 + λc + λT3 cells to λvir at 37 °C caused by reduced or unstable Cas3 protein might be
| Bacterial strain | Relevant genotype | Source or reference |
|------------------|-------------------|---------------------|
| EB304            | MG1655, Δcas3::apra | [44]               |
| BSN22            | W3110, Δhns::cat   | [46]               |
| BW25113          | F− mmb ΔlacZ4748 ΔrrnBΔlacZ4748 Δ(aarBAD) ΔsdsR314 Δ(srbBAD) Δrp-1 λ− | [47]               |
| BW39121          | + Δhns::kan       | [38]               |
| JWO462           | + ΔhtpG-kan        | [41]               |
| BW40114          | +F′ (proAB− lacZΔM15::Tn10) lacUV5-cas3 cat:araBp8-casA | [27]               |
| BW39651          | + λT3 spacer       | [38]               |
| BW39671          | + λT3 Δhns::kan    | [38]               |
| BW39183          | + Δcas1::kan      | [38]               |
| IIIIB848         | + λT3 Δcas3::apra  | recombineering using pKD46 |
| IIIIB70          | + λT3 Δcas3::apra  | P1, IIB848 × BW39651 |
| IIIIB965         | + λT3 Δcas1::kan   | P1, BW39183 × BW39651 |
| IIIIB966         | + λT3 Δcas1::kan Δhns::cat | P1, BSN22 × IIIIB965 |
| IIIIB969         | + λc + λT3 lacUV5-cas3 cat:araBp8-casA | P1, BW40114 × BW39651 |
| IIIIB969e        | + λc + λT3 lacUV5-cas3 cat:araBp8-casA | Selection of λc colony with phage acquired spacer |
| IIIIB1039        | + λc + λT3 Δcas1::kan | P1, BW39183 × IIIIB969e (selection Km^r Chl^s and PCR of the CRISPR-1 region) |
| IIIIB1040        | + λc + λT3 Δcas1::kan Δhns::cat | P1, BSN22 × IIIIB1039 |
| IIIIB1043        | + λc + λT3 Δcas1::kan^S | Removal of kan cassette by pCP20 plasmid |
| IIIIB1063        | + λc + λT3 lacUV5-cas3 cat:araBp8-casA ΔhtpG:kan | P1, IIB1043 |
| IIIIB1065        | + λc + λT3 Δcas1::kan ΔhtpG:kan | P1, JWO462 × IIIIB1065 |
| IIIIB1066        | + λc + λT3 Δcas1::kan ΔhtpG:kan Δhns::cat | P1, BSN22 × IIIIB1065 |
Table 3  Temperature-dependent resistance of Δhns cells to phage λvir in the presence of phage acquired spacer

| strain  | genotype               | Plaque forming units (PFUs) |
|---------|------------------------|----------------------------|
|         |                        | 30 °C          | 37 °C          |
| IIB965  | hns Δcas1 + λT3        | 4.63 × 10^10 ± 2.5 × 10^9 | 5.8 × 10^10 ± 1.14 × 10^10 |
| IIB966  | Δhns Δcas1 + λT3       | ~5 × 10^9        | 3.35 × 10^10 ± 9 × 10^9 |
| IIB1039 | hns Δcas1 + λc + λT3   | 3.37 × 10^10 ± 2.5 × 10^9 | 2.97 × 10^10 ± 9.7 × 10^9 |
| IIB1040 | Δhns Δcas1 + λc + λT3  | ~3 × 10^9         | 2 × 10^10 ± 6 × 10^9 |

Cell lawns of strains hns (Δcas1) + λc + λT3 and Δhns (Δcas1) + λc + λT3 were infected with phage dilutions (from 10^8 to 10^-5) 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 Δhns Δcas1 + λc + λT3 cells to λvir at 37 °C compared 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 Δhns Δcas1 + λc + λT3 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 Δhns Δcas1 + λc + λT3 cells (ΔhtpG Δhns Δcas1 + λc + λT3) 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 ΔhtpG Δhns Δcas1 + λc + λT3 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 ΔhtpG Δhns Δcas1 + λc + λT3 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 ΔhtpG Δhns Δcas1 + λc + λT3 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 ΔhtpG Δhns Δcas1 + λc + λT3 cells rescued phage resistance at 30 °C, it did not at 37 °C (Fig. 2b).

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 λvir 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 (Δhns), and when an anti-λ spacer could target protospacer DNA next to a consensuus 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 Δhns cells compared to hns+ cells, unlike Cascade genes and crRNA that were much increased in Δhns cells [34]. We measured cas3 transcripts in mid-log and stationary phase, observing that Δhns cells contained eight-fold more cas3 transcript compared to hns+ cells at...
30 °C and 37 °C. This is significant because infectivity assays for measuring resistance of E. coli to λvir 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 (ΔhtpG Δhns) 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 ΔhtpG Δhns Δcas1 + λc + λT3 (IIB1066) cells that were otherwise sensitive to phage infection (Fig. 2b). Interestingly, the pCas3 alone did not restore phage resistance to ΔhtpG Δhns Δcas1 + λc + λT3 cells at 37 °C (Fig. 2b) but did to htpG Δhns Δcas1 + λc + λT3 (IIB1040) cells at 37 °C (Fig. 2a), while pHtpG was unable to sustain phage resistance to any Δhns cells at 37 °C. Thus, HtpG is important for CRISPR-system activity in Δhns 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 Δhns cells at 37 °C.

In addition to four σ70 (“house-keeping” sigma factor)-promoters in CRISPR-Cas area, two potential σ32 (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 λ 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 λ 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\(^{-1}\) bacto-tryptone, 5 g L\(^{-1}\) yeast extract, 10 g L\(^{-1}\)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 μg/ml, kanamycin at 40 μg/ml, apramycin 30 μg/ml and chloramphenicol at 15 μg/ml. Mutant bacterial strains were made by P1 transduction and selected for the appropriate antibiotic resistance [45]. When important (for generating IIB969 and IIB1039), the genotype (presence of λT3 and λc spacers) of many transductants were screened by colony PCR using the same primers (CRISPR I-R: 5′-GAGATCGAGGCTCGAATC-3′ and spacer 4: 5′-GCGAGTTCGAAATTCCA GACCCGAATCCAAA-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 μl aliquots of serially diluted phages in 10 mM MgSO\(_4\) 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-β-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 λT3 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\(_{600}\) was 0.4-0.5, and mixed with λvir 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-λ spacer targeting λII gene (called λc) with consensus PAM 5′-CTT-3′ (Additional file 1: Figure S1C). Δhns + λc + λT3 strain was made by P1 transduction using donor strain Δcas1:: Km\(^{r}\) (BW39183) and selecting for hns\(^{r}\) recombinant strain with two extra spacers in the CRISPR locus 2.1 (strain IIB1039; Table 2) and later introducing Δhns mutation (strain IIB1040; Table 2). The Δcas1 mutation was chosen to create Δhns + λc + λT3 cells because cas1 gene is not required for interference [37].

RNA extraction and qPCR
Total RNA was extracted from mid log (OD\(_{600}\) = 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 μg) was first treated by DNase I, diluted 10 fold and 2 μl 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 (ACT 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.

Primes used were:

Cas 3-F: 5′ -ATCCGCTAATGTACCTTC-3′
Cas 3-R: 5′ -TCCAGCCAAGATACCCATC-3′
groES-F: 5′ -CTG CAT CGT CAA GCG TAA AG-3′
groES-R: 5′ -CAA GGA TAC GGC CAT TG-3′

### Additional file

**Additional file 1: Figure S1.** Properties of PAM in λT3 spacer and phage acquired spacer (A). Sequence complementarity between λT3 spacer and proto-spacer in lambda with the PAM 5′-CCA 3′ highlighted in red color. (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 as endogenous control and λ (2016) 16:28 (2016) 16:28)

### 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; cRNA: CRISPR-RNA; E. coli: Escherichia coli; H-NS: nucleoid-structuring protein; HtpG: high-temperature protein G; MO: multiplicity of infection; OD: optical density; PAM: protospacer adjacent motif; PFU: plaque forming units; λ: 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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