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Research Letter

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Cas3-stimulated runaway replication of modified ColE1 plasmids in *Escherichia coli* is temperature dependent

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One sentence summary: Increased plasmid replication depends on change in ATPase/helicase activity of Cas3 protein which depends on the temperature of incubation.

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ABSTRACT

The clustered regularly interspersed short palindromic repeats (CRISPR)-Cas system constitutes an adaptive immunity system of prokaryotes against mobile genetic elements using a CRISPR RNA (crRNA)-mediated interference mechanism. In Type I CRISPR-Cas systems, crRNA guided by a Cascade complex recognises the matching target DNA and promotes an R-loop formation, RNA-DNA hybrid. The helicase-nuclease Cas3 protein is then recruited to the Cascade/R-loop complex where it nicks and degrades DNA. The Cas3 activity in CRISPR-Cas immunity is reduced in Δ*hns* cells at 37°C for unknown reasons. Cas3 can also influence regulation of plasmid replication and promote uncontrolled ('runaway') replication of ColE1 plasmids independently of other CRISPR-Cas components, requiring only its helicase activity. In this work we wanted to test whether Cas3-stimulated uncontrolled plasmid replication is affected by the temperature in Δ*hns* and/or Δ*htpG* mutants. We found that Cas3-stimulated uncontrolled plasmid replication occurs only at 37°C, irrespective of the genotype of the analysed mutants, and dependent on Cas3 helicase function. We also found that plasmid replication was strongly reduced by the *hns* mutation at 30°C and that Cas3 could interfere with T4 phage replication at both incubation temperatures.

Keywords: CRISPR-Cas; H-NS; HtpG; ColE1; temperature; *Escherichia coli*

INTRODUCTION

Clustered regularly interspersed short palindromic repeats (CRISPR) and CRISPR-associated (*cas*) genes represent a prokaryotic adaptive immune system against foreign genetic elements in bacteria and archaea. CRISPR-Cas immunity operates through targeting of RNA to complementary sequences in foreign DNA or RNA, and is divided into three stages: adaptation, expression and maturation, and interference (Mohanraju *et al.* 2016; Hille *et al.* 2018; Xue and Sashital, 2019). The interference reactions involve the detection of foreign DNA by nucleotide base pairing between CRISPR RNA (crRNA) and target DNA, displacement of the non-target strand to form an R-loop (RNA-DNA heteroduplex), and nucleolytic degradation of the target DNA. In *Escherichia coli*, which has the type I-E CRISPR-Cas system, detection of foreign DNA by crRNA is catalysed by the multi-subunit complex Cascade (CRISPR-associated complex for antiviral defence) and the Cas3 protein is involved in target DNA degradation (Brouns *et al.* 2008; Westra *et al.* 2012). Cas3 is a signature protein in all Type I systems and a crucial part of

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CRISPR defence (Makarova *et al.* 2015 *et al.* 2015). It has an N-terminal HD nuclease and C-terminal superfamily 2 (SF2) helicase activities (Beloglazova *et al.* 2011; Howard *et al.* 2011; Sinkunas *et al.* 2011). Target DNA recognition by Cascade initiates from the protospacer adjacent motif (Mojica *et al.* 2009), which bends the dsDNA and allows spontaneous DNA unwinding and seed-bubble formation (Xiao *et al.* 2017). Upon full R-loop formation, Cascade undergoes conformational change that will recruit Cas3 to introduce a nick at the exposed ssDNA followed by processive target degradation (Westra *et al.* 2012; Mulepati and Bailey 2013; Sinkunas *et al.* 2013; Hochstrasser *et al.* 2014; Loeff, Brouns and Joo 2018; Xiao *et al.* 2018).

The onset of CRISPR interference reactions in *E. coli* is transcriptionally regulated (Westra *et al.* 2010; Patterson, Yevstigneyeva and Finneran 2017). In *E. coli*, the regulatory protein H-NS (nucleoid structuring protein) represses transcription of the CRISPR locus and all *cas* genes (Westra *et al.* 2010; Majsec, Bolt and Ivančić-Baće 2016). However, the ability of Δ*hns* cells to resist phage infection is strongly influenced by temperature. Genetic analysis showed that Δ*hns* cells with an anti-phage spacer were resistant to phage infection at 30°C, but became sensitive at 37°C (Pougach *et al.* 2010; Majsec, Bolt and Ivančić-Baće 2016). The temperature dependence was caused by the limiting amounts of Cas3, rather than by the effects of protospacer adjacent motif sequence variations or *cas3* transcription (Majsec, Bolt and Ivančić-Baće 2016). Another factor that influences resistance to phage attack is high-temperature protein G (HtpG) chaperone. This protein was identified in a genetic screen as a nonessential *E. coli* gene that is essential for CRISPR activity. More specifically, HtpG was shown to maintain functional levels of Cas3 (Yosef *et al.* 2011). Overexpression of HtpG or Cas3 restored phage resistance to Δ*hns* Δ*htpG* cells with an anti-phage spacer at 30°C, but not at 37°C (Majsec, Bolt and Ivančić-Baće 2016). Overall, these findings indicate that increased amounts of Cas3 and the presence of chromosomal *htpG* are required for resistance to phage infection at 37°C..

Interestingly, when Cas3 is overexpressed from the pUC19 plasmid, which has a modified ColE1 origin, it can also induce its uncontrolled runaway replication (Ivančić-Baće *et al* 2013). For this effect, Cas3 ATPase/helicase activity was required, but Cascade complex or protospacer adjacent motif were not. This suggested that the role of Cas3 in plasmid replication is distinct from its crucial role in CRISPR interference (Ivančić-Baće *et al.* 2013). However, plasmid replication and DNA target degradation share one common trait—the R-loop which is formed either by RNAII or by the crRNA from the Cascade complex (Jore *et al.* 2011; Ivančić-Baće, Howard and Bolt 2012; Xiao *et al.* 2017). RNAII is a plasmid-encoded transcript that forms a stable RNA-DNA hybrid (R-loop) at the *ori*. The transcript serves as a primer to initiate replication after processing by RNase HI (Itoh and Tomizawa 1980; Ogawa and Okazaki 1984). A second plasmid-encoded transcript, RNAI, is transcribed from the opposite strand and is antisense to the 5' end of RNAII. By pairing with RNAII, RNAI prevents R-loop formation involving RNA II and reduces plasmid copy number (Eguchi, Itoh and Tomizawa 1991). The RNAI-RNAII interaction is stabilized by plasmid-encoded Rop protein which is deleted in many commercial plasmids (Cesareni, Muesing and Polisky 1982; Camps 2010). Plasmid copy number can be modulated by host genes, such as *pcnB* and *recG,* which can increase or reduce ColE1 plasmid copy number, respectively (Xu, Lin-Chao and Cohen 1993; Fukuoh *et al.* 1997; Vincent, Mahdi and Lloyd 1996). In our previous work, we proposed that Cas3 helicase activity dissociates RNAI from RNAII in a 3' to 5' direction to liberate RNAII and indirectly stimulate plasmid replication (Ivančić-Baće *et al.* 2013).

Here we asked whether the temperature-dependent change in Cas3 activity or the change in amounts of Cas3 that was manifested as reduced CRISPR-Cas immunity at 37°C would be observed in Cas3-induced uncontrolled plasmid replication. We hypothesized that the ability of Cas3 to induce uncontrolled plasmid replication may also be dependent on the temperature of incubation. We also extended our research by asking if Cas3 could interfere with T4 replication and consequently with plaque formation, since R-loops are required for origin-dependent replication of T4 phage.

MATERIALS AND METHODS

E. coli K12 strains and plasmids used in this study are listed in Table 1.

Media and general microbiology methods

Lysogeny broth (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 or 6 g for top agar. When required, appropriate antibiotics were added to LB plates at final concentrations: ampicillin at 100 μg/ml, kanamycin at 40 μg/ml and chloramphenicol at 15 μg/ml. Mutant bacterial strains were made by P1*vir* transduction and selected for the appropriate antibiotic resistance (Miller 1992). To extract plasmids, cell cultures were grown overnight with shaking at 30°C and 37°C and cell density was measured at 600 nm (OD_{600}). Cell viabilities were calculated by plating 100 µl of bacteria cultures that had been serially diluted in 67 mM phosphate buffer (pH = 7.0) onto

Table 1. *Escherichia coli* strains and plasmids used in this study.

Strain	Genotype	Reference
MG1655	F⁻ λ⁻ *ilvG*⁻ *rfb*-50 *rph*-1	Blattner *et al.* 1997
BW39121	BW25113 Δ*hns*::*kan*	Pougach *et al.* 2010
JW0462	BW25113 Δ*htpG*::*kan*	Yosef *et al.* 2011
IIB928	MG1655 Δ*hns*::*kan*	P1.BW39121 x MG1655
IIB1150	MG1655 Δ*htpG*::*kan*	P1.JW0462 x MG1655
IIB1152	MG1655 Δ*htpG*::*kan* Δ*hns*::*cat*	P1.JW0462 x IIB1050
pUC19	Empty plasmid vector	Vieira and Messing 1982
pEB526 (pCas3)	*cas3* in pUC19	Ivančić-Baće *et al.* 2013
pJLH11 (pCas3^{K320L})	*cas3*^{K320L} in pUC19	Ivančić-Baće *et al.* 2013
pJLH18 (pCas3^{K78L})	*cas3*^{K78L} in pUC19	Ivančić-Baće *et al.* 2013

Table 2. Plasmid yields, cell viability and plasmid stability.

Escherichia coli strain	Plasmid	Temperature (°C)[a]	Plasmid concentration (ng/μl)[b]	OD_{600}[c]	Viability of cells per ml (x 10^7)[d]	Amp^R cells (% total)[e]
MG1655 (*wt*)	pUC19	30	61.5 ± 1.35	2.44	594.3 ± 103	104.4 ± 5.8
		37	70.2 ± 13.8	1.89	328.5 ± 43	103.6 ± 3.5
	pCas3	30	86.7 ± 11.2	2.54	445.6 ± 48	93.3 ± 9.7
		37	309.9 ± 73.1	2.34	30 ± 12	97.4 ± 5.6
IIB928 (Δ*hns*)	pUC19	30	29.8 ± 5.7	1.98	295.3 ± 26.3	96.4 ± 9.1
		37	50.3 ± 10.2	2.12	175.0 ± 65	98.3 ± 5.8
	pCas3	30	35.7 ± 5.5	2.28	255.50 ± 60	89.2 ± 11.2
		37	256.3 ± 16.6	2.18	27.0 ± 13	95.8 ± 10.5
IIB1150 (Δ*htpG*)	pUC19	30	54.9 ± 5	2.56	416 ± 104	109.2 ± 9.8
		37	67.5 ± 19.1	2.25	254.0 ± 66	95.2 ± 6.2
	pCas3	30	86.4 ± 14.1	2.52	481.0 ± 44	100.0 ± 13.5
		37	295.9 ± 55.1	2.25	39 ± 18	103.0 ± 18
IIB1152 (Δ*hns* Δ*htpG*)	pUC19	30	34.2 ± 4.2	2.24	295.7 ± 35	103.5 ± 5.5
		37	76.0 ± 5.4	1.92	162.0 ± 18	93.4 ± 5.4
	pCas3	30	30.3 ± 4.8	2.30	296.0 ± 9	92.7 ± 1.9
		37	226.4 ± 25.9	2.24	47.4 ± 12	102.9 ± 14.1

[a]Cells were grown as overnight cultures in 3 ml of LB with antibiotic at 30°C or 37°C for extraction of ColE1 based plasmids.
[b]Plasmid concentration was measured in a Nanovue spectrophotometer (GE Healthcare). Values are means of three independent experiments with standard deviations
[c]Optical density of overnight cultures was determined in a UV/Vis Spectrometer UV-4 (Unicam).
[d]Viable cell number was obtained from the counting colonies after plating 100 μl of serially diluted bacteria on agar without antibiotic. Values are means of three independent experiments with standard deviations.
[e]The percentage of cells retaining plasmid was measured by comparing viable colony counts after plating on agar with and without ampicillin. Values are means of three independent experiments with standard deviations.

LB. The fraction of plasmid-free cells was calculated by counting colonies on plates with and without ampicillin selection (Table 2).

Plasmid DNA yields

Plasmid DNA was extracted from 1.5 ml of overnight cultures using the Wizard plus SV mini-prep kit (Promega) using new, unused columns. Plasmid DNA concentrations were measured from 2 μl aliquots in a Nanovue spectrophotometer (GE Healthcare). Plasmid yields were analysed from at least three independent cultures for each strain.

Phage assay

A quantity of 0.2 ml of cells grown overnight at 37°C were mixed with 3 ml of top agar and was allowed to set. Serially diluted T4 phages were spotted on the surface of the plate and allowed to soak. Plates were incubated overnight at 30°C or 37°C and plaques were counted next day. PFUs represent the number of T4 plaques per millilitre from several dilutions. PFUs were analysed from at least three independent experiments for each strain and represented as dot plot with labelled mean and error bars. R package ggplot2 (Wilkinson, 2011) was used for plotting. Welch's t-test, or unequal variances t-test, was used for statistical analysis with confidence level 95%.

RESULTS AND DISCUSSION

The role of incubation temperature on Cas3-stimulated ColE1 plasmid replication was determined by measuring plasmid yields from overnight cultures grown at 30°C and 37°C as described in the previous sections (Ivančić-Baće *et al.* 2013). The plasmid yields of pCas3 (pEB526; *wt* Cas3 expressed from pUC19) and pUC19 (empty vector control) were compared in *wt*, Δ*hns*, Δ*htpG* and Δ*hns* Δ*htpG* *E. coli* cells (Table 1) in which temperature-dependent CRISPR-Cas immunity has been studied before (Majsec, Bolt and Ivančić-Baće 2016). Results presented in Table 2 show that cell viability and plasmid stability were not affected by Cas3 expression at either temperature, the only exception being a modest decrease in cell viability of cells harboring pCas3 at 37°C (of about 5–10 fold). Hence, we noted some differences in the cell numbers (and consequently cell densities) depending on the genotype, growth temperature and plasmid type. Since cell density of the overnight cultures prior to plasmid extraction can affect plasmid yield, which is the basis for determining the plasmid copy number, we normalized plasmid yields by cell density and plasmid size.

pCas3 is considerably larger (about 2-fold) than pUC19, so to make the yields of these different plasmids comparable, we normalized plasmid concentration of pCas3 to plasmid size by dividing it by two. To account for differences in the cell numbers, the plasmid concentrations (normalized for pCas3 and obtained for pUC19) were then divided by cell density. This gave us comparable values—plasmid copy numbers (Table 3) that supported and confirmed our previous conclusions: Cas3 stimulates replication of ColE1 plasmids at 37°C and induces an approximately 2-fold increase in plasmid copy number compared with empty pUC19 (Table 3). The effect was similar in three genetic backgrounds tested, except Δ*hns* Δ*htpG* where the increase in plasmid copy number of pCas3 was less pronounced.

However, when normalized values of plasmid copy numbers of pCas3 were compared to pUC19 in cells grown at 30°C, we noticed several differences from cells grown at 37°C. First, the plasmid copy number of pCas3 was actually decreased in cells grown at 30°C and second, the decrease was dependent on the cell's background. In *wt* and Δ*htpG* cells the copy number of pCas3 was reduced by ∼60% compared with pUC19, whereas in cells containing Δ*hns* mutation (Δ*hns* and Δ*hns* Δ*htpG* cells) the copy number of pCas3 was further decreased, and the actual

Table 3. Normalized plasmid yields from cells grown with or without IPTG.

Strain	pUC19[a)]	pCas3	pCas3K320L	pCas3K78L
MG1655 (*wt*) 30°C	25.3 ± 0.8	16.4 ± 1.4	12.8 ± 1.5	12 ± 2.1
MG1655 (*wt*) 30°C + IPTG	n.d.	17.8 ± 2.7	11.4 ± 2.7	19 ± 4.9
MG1655 (*wt*) 37°C	33.1 ± 3.3	69.8 ± 8.3	12.3 ± 3.9	43.6 ± 4.7
MG1655 (*wt*) 37°C + IPTG	n.d.	61.0 ± 8.7	10.9 ± 0.6	39.9 ± 2.1
IIB928 (Δ*hns*) 30°C	15 ± 1.6	7.5 ± 1.3	n.d.	n.d.
IIB928 (Δ*hns*) 37°C	25.1 ± 3.5	58.8 ± 5.3	n.d.	n.d.
IIB1150 (Δ*htpG*) 30°C	20.6 ± 1.2	15.5 ± 2.8	n.d.	n.d.
IIB1150 (Δ*htpG*) 37°C	27.1 ± 7.7	66.9 ± 12.8	n.d.	n.d.
IIB1152 (Δ*hns* Δ*htpG*) 30°C	15.7 ± 2.3	6.8 ± 0.9	n.d.	n.d.
IIB1152 (Δ*hns* Δ*htpG*) 37°C	38.5 ± 3.0	51.5 ± 6.4	n.d.	n.d.

[a)]Determined as: plasmid concentration of pUC19/OD_{600} and 0.5 x plasmid concentration of pCas3/OD_{600}.
n.d. indicates where data was not determined

copy number of both plasmids was lower than that of *wt* or *htpG* cells (Table 3). In other words, the plasmid copy number of modified ColE1 plasmids is about 2-fold lower in Δ*hns* cells than in *wt* cells (Table 3). A similar effect, a reduced number of chromosomal origins per cell, has been found in *hns* mutants in comparison to *wt* cells, and the effect was more pronounced at 30°C than 37°C. It has been proposed that the H-NS acted indirectly on initiation of replication by somehow increasing the activity of the DnaA protein, a chromosome replication initiator protein at *oriC*, but the mechanism is unknown (Atlung and Hansen 2002). The indirect effect of *hns* mutation on the reduction of the plasmid copy number at 30°C is very interesting, since this effect may additionally help CRISPR-Cas immunity to eliminate the invading DNA. The CRISPR-Cas system is normally repressed in *E. coli wt* cells and de-repressed in Δ*hns* cells (Westra *et al*. 2010; Majsec, Bolt and Ivančić-Baće 2016) so it may not be a coincidence that CRISPR-Cas system is more efficient in resisting phage infection at a lower temperature of incubation. Perhaps phage replication is also reduced in Δ*hns* cells at 30°C, but this was not shown to our knowledge. Phage λ did form plaques in Δ*hns* cells at 30°C with similar efficiency as at 37°C (Majsec, Bolt and Ivančić-Baće 2016), so another possibility could be that this effect on plasmid or chromosomal replication depends on the type of the replication initiation or the replication in general. Interestingly, reduction in ploidy (origins per cell) in Δ*hns* cells was primarily due to a decreased replication time and growth rate (Atlung and Hansen 2002) and it has been shown recently that a low temperature of incubation promotes CRISPR-Cas immunity in *Pseudomonas aeruginosa*. This is explained by increased levels of CRISPR-Cas components and decreased growth rate. When these two effects are combined, more time is allowed for the CRISPR-Cas proteins to destroy a foreign invading element prior to cell division (Høyland-Kroghsbo, Muñoz and Bassler 2018). Further research will be required to better understand the role of temperature and H-NS in the regulation of plasmid and chromosomal copy number and phage defence.

The plasmid copy number of pUC19 and pCas3 in Δ*htpG* cells was comparable to plasmid copy numbers in *wt* cells at both temperatures of incubation. This implies that lack of HtpG plays no role in modified ColE1 plasmid replication in hns^+ background. A previous study showed that HtpG is important to maintain functional levels of Cas3 in the cells, but the lack of *htpG* could be overcome by increased expression of Cas3 (Yosef *et al*. 2011). Since Cas3 is expressed in large amounts from a high copy number plasmid, such as pUC19, this explains the negligible effect of *htpG* mutation on pCas3 replication.

Next, we wanted to understand which enzymatic activity of Cas3 is responsible for the decrease in the plasmid copy number of pCas3 in cells grown at 30°C. One possibility could be that Cas3 retains the function at 30°C but activity is reduced or that protein levels are lower than at 37°C. To address this, cells expressing *wt* Cas3 (pCas3), ATPase/helicase (pCas3K320L) or nuclease defective (pCas3K78L) Cas3 protein were grown overnight in the presence of 1 mM IPTG to induce expression of these proteins. Plasmid yields were then measured as above, including cell viability and plasmid stability and values were normalized to plasmid copy numbers (Table 3 and 4). Induced expression of pCas3, pCas3K78L or pCas3K320L by IPTG had no effect on cell viability and plasmid stability (Table 4) at any temperature. In neither normalized nor total plasmid yield do induced pCas3 and pCas3K320L appear to increase the level of plasmid at 30°C, with the exception of pCas3K78L, which promoted moderately higher plasmid levels (Table 3 and 4). At 37°C, plasmid levels appear to be decreased with IPTG (Table 3 and 4). Since ATPase/helicase activity is functional in nuclease defective Cas3K78L protein and IPTG-induced expression of Cas3K78L corresponded to increased plasmid copy number of pCas3K78L, this implies that the ATPase/helicase activity of Cas3 is reduced or inhibited at 30°C. This is also consistent with unchanged plasmid copy number of ATPase/helicase mutant pCas3K320L in IPTG-induced cells at 30°C (Table 3). Therefore, decreased plasmid replication of pCas3 at 30°C is probably caused by reduced ATPase/helicase activity of the Cas3 protein.

What could be an explanation for reduced Cas3 ATPase/helicase activity at 30°C and why are plasmid yields of pCas3 much lower at 30°C even in IPTG-induced cells than at 37°C? It is known that chromosomal R-loop formation preferentially occurs at low temperature in *topA* cells due to reduced transcription velocity and uncoupled transcription and translation (Massé and Drolet 1999; Harinarayanan and Gowrishankar 2003). In contrast, increased transcription velocity and a tight coupling of translation and transcription at 37°C reduce the chances of R-loop formation. We showed previously that Cas3 does not dissociate R-loops, but instead promotes R-loops by dissociating RNAI from RNAII to free up RNAII to pair with *ori* (Ivančić-Baće *et al*. 2013). It is possible that increased occurrence of chromosomal R-loops at 30°C (Massé and Drolet 1999) titrates or saturates Cas3 helicase activity (and activities of other host proteins) at the expense of generating RNAII at the *ori*. Another possibility is that removal of RNAI by Cas3 at 30°C is inefficient because interaction between RNAI-RNAII may be stronger or ATP levels lower, but this needs to be tested. However, the latter may be difficult to test since intracellular ATP concentrations were

Table 4. Plasmid yields, cell viability and plasmid stability determined from MG1655, grown with or without IPTG.

Plasmid	Temperature (°C)[a]	Plasmid concentration (ng/μl)[b]	OD_{600}[c]	Viability of cells per ml (x 10^7)[d]	Amp^R cells (% total)[e]
pCas3 + IPTG	30	90.2 ± 16.5	2.54	429 ± 23	80.0 ± 27.6
	37	271.9 ± 28.9	2.2	11.4 ± 0.7	90.3 ± 7.0
$pCas3^{K320L}$	30	64.7 ± 8.3	2.50	535 ± 43	90.3 ± 4.1
	37	61 ± 19.3	2.48	248 ± 6	97.0 ± 1
$pCas3^{K320L}$ + IPTG	30	54.3 ± 8.8	2.53	510 ± 86	99.4 ± 1.9
	37	53.3 ± 3.1	2.45	221 ± 42	104.1 ± 12.5
$pCas3^{K78L}$	30	61.3 ± 10.1	2.53	343 ± 39.7	92.8 ± 0.6
	37	184.2 ± 20	2.16	34.7 ± 10	95.8 ± 10.4
$pCas3^{K78L}$ + IPTG	30	95.7 ± 15.5	2.51	442 ± 62	97.3 ± 30
	37	157.5 ± 16.2	1.88	30.8 ± 12.1	92.5 ± 8.2

[a]Cells were grown as overnight cultures in 3 ml of LB with antibiotic, and with or without 1 mM IPTG, at 30°C or 37°C for extraction of ColE1 based plasmids.
[b]Plasmid concentration was measured in a Nanovue spectrophotometer (GE Healthcare) and Nanodrop (ThermoFisher). Values are means of at least three independent experiments with standard deviations
[c]Optical density of overnight cultures was determined in a UV/Vis Spectrometer UV-4 (Unicam). Values are means of at least three independent experiments with standard deviations.
[d]Viable cell number was obtained from the counting colonies after plating 100 μl of serially diluted bacteria on agar without antibiotic. Values are means of three independent experiments with standard deviations.
[e]The percentage of cells retaining plasmid was measured by comparing viable colony counts after plating on agar with and without ampicillin. Values are means of three independent experiments with standard deviations

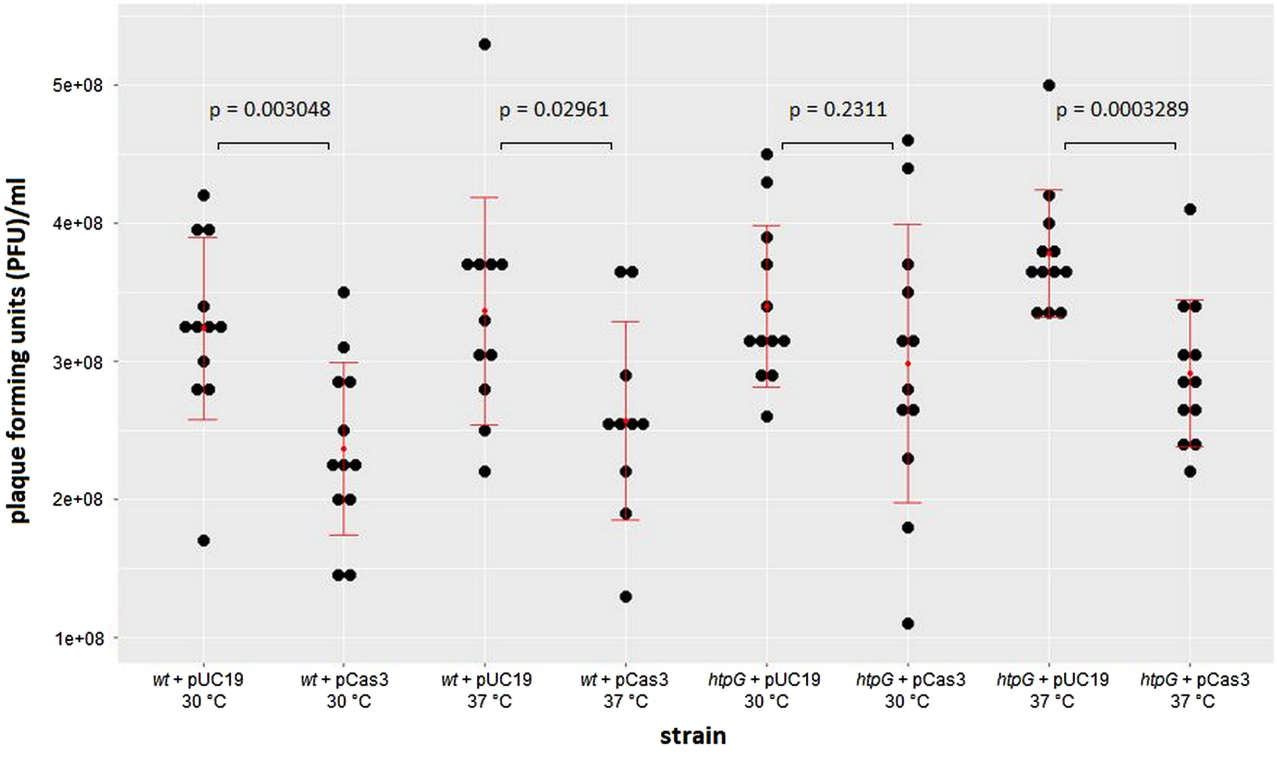

Figure 1. The effect of Cas3 overexpression on T4 infection in cells incubated overnight at 30°C and 37°C. Overnight cultures were infected by T4 phage and incubated at indicated temperatures. Plaques were counted the next day. Dot plot with means, standard errors and P-values are shown. Statistical analysis was set to unequal variance, independent samples with confidence level of 0.95. Cells containing pUC19 and pCas3 were compared at the same temperature of incubation.

shown to be diverse even in a single population of genetically identical cells grown under the same condition. Different levels of ATP, it has been suggested, enable individual cells to adopt different strategies under severe conditions (Yaginuma *et al.* 2014). Single-molecule studies on Cas3 have recently shown that the Cas3 translocation distance can be tuned by different ATP levels suggesting that indeed Cas3 helicase activity may be modulated *in vivo* (Loeff, Brouns and Joo 2018). Decreased ATPase/helicase activity of Cas3 at 30°C may not be sufficient to stimulate ColE1 plasmid replication but it may be sufficient to allow Cas3 to destroy the invading phage DNA after interaction with Cascade complex and R-loop. Another possibility could be that decreasing the plasmid copy number by reducing Cas3 activity at 30°C is a strategy to deal with high copy number plasmids by reducing their demand on DNA metabolism by CRISPR-independent manner, while a combination of nuclease

and helicase activity is required for target-specific CRISPR-Cas mediated DNA degradation. Uncontrolled plasmid replication stimulated by Cas3 at 37°C may be an unfortunate event that cannot be controlled and prevented, because after stimulation of initiation of replication, the next cycles of replication are provoked and result in runaway replication.

We also wondered if overexpression of Cas3 would affect phage replication of a lytic bacteriophage, such as T4. We then examined whether Cas3 overexpressed from the plasmid would influence T4 plaque formation in *wt* and Δ*htpG* cells incubated at 30°C or 37°C after infection. The effect of *hns* mutation was not investigated as it strongly affects plasmid and chromosomal replication at 30°C making interpretation difficult (Atlung and Hansen 2002), and it also upregulates many genes (Atlung and Ingmer 1997). Cells used did not contain anti-T4 spacer in the CRISPR array, so any effect on efficiency of T4 plating is expected to be solely due to Cas3 binding on R-loops that are used to prime leading strand synthesis of phage DNA (reviewed in Kreuzer and Brister, 2010). A modest but statistically significant ($P < 0.05$) decrease in T4 plaque-forming units (PFUs) was observed in *wt* cells at both temperatures and Δ*htpG* cells only at 37°C when overexpressing Cas3 in comparison with those cells with empty vector pUC19 (Fig. 1). The effect was more apparent in the plaque morphology, because plaques were smaller in cells expressing pCas3 (data not shown). Our results suggest that Cas3 can interfere with T4 phage replication and can reduce its PFUs at both temperatures of incubation. The overall effect is modest, but it could be a CRISPR-independent and robust pathway to the temporary inhibition of certain phages that initiate replication from R-loops during early replication, while at the same time building other strategies to more specifically and efficiently destroy phage DNA.

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Conflicts of Interests. None declared

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