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StpA represses CRISPR-Cas immunity in H-NS deficient *Escherichia coli*

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ABSTRACT

Functional CRISPR-Cas systems provide many bacteria and most archaea with adaptive immunity against invading DNA elements. CRISPR arrays store DNA fragments of previous infections while products of *cas* genes provide immunity by integrating new DNA fragments and using this information to recognize and destroy invading DNA. *Escherichia coli* contains the CRISPR-Cas type I-E system in which foreign DNA targets are recognized by Cascade, a crRNA-guided complex comprising five proteins (CasA, CasB, CasC, CasD, CasE), and degraded by Cas3. In *E. coli* the CRISPR-Cas type I-E system is repressed by the histone-like nucleoid-structuring protein H-NS. H-NS repression can be relieved either by inactivation of the *hns* gene or by elevated levels of the H-NS antagonist LeuO, which induces higher transcript levels of *cas* genes than was observed for Δ*hns* cells. This suggests that derepression in Δ*hns* cells is incomplete and that an additional repressor could be involved in the silencing. One such candidate is the H-NS paralog protein StpA, which has DNA binding preferences similar to those of H-NS. Here we show that over-expression of StpA in Δ*hns* cells containing anti-lambda spacers abolishes resistance to λ*vir* infection and reduces transcription of the *casA* gene. In cells lacking *hns* and *stpA* genes, the transcript levels of the *casA* gene are higher than Δ*hns* and similar to *wt* cells overexpressing LeuO. Taken together, these results suggest that Cascade genes in *E. coli* are repressed by the StpA protein when H-NS is absent.

1. Introduction

Escherichia coli K-12 utilises a Type I-E CRISPR-Cas system to gain adaptive immunity against invading DNA such as phages and plasmids. The CRISPR-Cas system in *E. coli* is classified among Class 1 CRISPR-Cas systems (types I, III and IV) characterised by multi-subunit effector proteins [1]. It consists of 8 *cas* genes (*cas1*, *cas2*, *cas3*, and *casABCDE* or *cse1*, *cse2*, *cas7*, *cas5* and *cas6e*) and two CRISPR arrays (CRISPR-1 and CRISPR-2) [2]. CRISPR arrays contain an AT-rich leader region and a series of repeats separated by spacers that originate from invading DNA. Insertion of new spacers forms a record of the infection and provides immunity upon subsequent infection by the same invading element [3]. The immunity pathway can be divided into three stages: adaptation, expression and maturation, and interference [4–6]. During adaptation, which is mediated by Cas1 and Cas2 proteins, small DNA fragments from invading DNA ("protospacers") are acquired and integrated as new spacers into the CRSIPR array [7–9]. In the expression and maturation stage, the CRISPR array is transcribed into a long precursor CRISPR RNA (pre-crRNA) that is further processed into mature short crRNAs. Each crRNA then associates with the multi-subunit complex Cascade (CRISPR associated complex for antiviral defence) and serves as a guide for identification of the matching sequence based on complementarity between crRNA and protospacer DNA [10,11]. In the case of complementarity, an R-loop is formed in which the target strand of the protospacer is paired with crRNA and the non-target strand is displaced [12,13]. During the interference stage, the R-loop recruits the Cas3 nuclease, to cleave and destroy target DNA [13–19].

Three promoters control expression of CRISPR-Cas components: P*cas3* controls expression of the *cas3* gene, P*cas8e* controls expression of the polycistronic *casABCDE12* operon and weak σ^{70} promoters located in leader sequences control expression of CRISPR arrays (Fig. 1A; [20,21]). Transcripts of full-size CRISPR arrays are short lived while processed CRISPR RNAs are stable and their accumulation depends on the presence of the Cas6e endonuclease [21].

Expression of CRISPR-Cas components in *E. coli* is strongly silenced by the repressing activity of the histone-like nucleoid-

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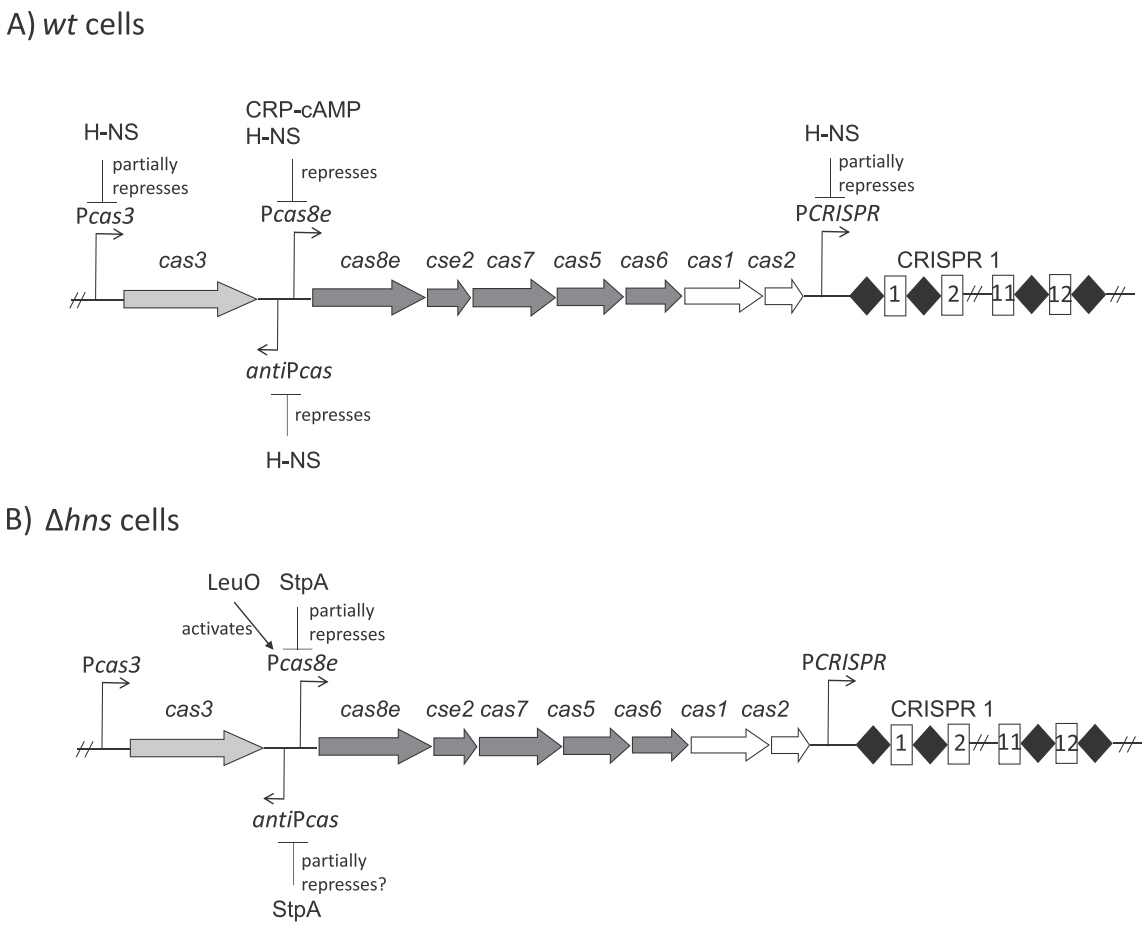

Fig. 1. An overview of CRISPR-Cas Type I-E operon of *E. coli* K-12 showing 8 genes, four promoters and CRISPR-1 array. **(A)** In *wt* cells, repressors H-NS and CRP-cAMP repress *cas8e* and *anti-cas* promoters, and partially repress *cas3* and *CRISPR* promoters. **(B)** In Δ*hns* cells, LeuO and StpA are present at elevated levels which results in partial derepression of *cas8e* and possibly *anti-cas* promoters. P*cas3* is not affected by StpA.

structuring protein (H-NS) [20,21], a global repressor of transcription in many Gram-negative bacteria. H-NS was shown to possess binding affinity for the intergenic region between *cas3* and *casA* (*cse1*) known as IGLB, for **i**nter**g**enic region *ygc**L**-ygc**B***, [20,22]. The two σ^{70}-dependent promoters in IGLB are arranged in divergent orientations, separated by 50 bp [20]. The first of these is P*cas8e,* to which cAMP receptor protein (CRP) and H-NS repressors bind ([23]; Fig. 1A). The second promoter, anti-P*cas*, is also active *in vivo* and it directs a transcript of 150–200 nt that can fold into an intricate secondary structure [20]. Transcription of CRISPR arrays is only partially dependent on H-NS [20], and H-NS also controls expression from P*cas3* in stationary Δ*hns* cells [24]. Repression of transcription is achieved by cooperative spreading of H-NS along AT-rich DNA creating DNA stiffening and DNA-protein bridges [25]. Overall, due to repression by H-NS, the CRISPR-Cas system does not provide protection against phage infection in *wt* cells [21,26]. This is why *in vivo* studies of the CRISPR-Cas system in *E. coli* rely either on heterologous expression of *cas* genes from plasmids in BL21-AI [10] or on *E. coli* K-12 strains with a deleted *hns* gene, expressed *leuO* or native promoters replaced with inducible promoters [6].

Repression of H-NS can be relieved by many proteins; among them, the best characterised is an H-NS antagonist, LeuO, a transcription factor from the LysR family [26]. Many LeuO-activated loci are H-NS-repressed, and a significant overlap of co-regulation of LeuO targets by H-NS has been found (78% in *E. coli* and 40% in *Salmonella enterica*) [27,28]. The H-NS paralog StpA protein (isolated as suppressor of td phenotype A) overlaps with H-NS in DNA binding regions [29,30]. H-NS and StpA inhibit transcription of each other, while the *leuO* gene itself is repressed by H-NS and StpA in *E. coli* and *S. enterica* [31–33]. Therefore, *stpA* is poorly expressed in *wt* cells and its transcription is induced in the *hns* mutant [30]. Expression of StpA can be naturally induced by growth in minimal medium, which is dependent on the leucine responsive regulatory protein (Lrp). Also, increase in medium osmolarity and temperature activates transcription of StpA, while carbon starvation represses it [34]. Interestingly, Lrp protein represses CRISPR in *S.* Typhi but no role for it in regulation in *E. coli* was observed [20,35]. StpA is considered to be a backup of H-NS which can repress transcription of one third of genes when H-NS is absent [36–38]. The StpA protein also has an RNA chaperone activity [39] and inactivation of *stpA* and *hns* genes affects growth rate and cell division [37].

Ectopic plasmid expression of LeuO can relieve H-NS repression of *cas* genes transcription by preventing the spreading of H-NS to the promoter region [26]. However, in cells overexpressing *leuO* the transcript abundances of the Cascade genes were higher than in Δ*hns* cells, which led to the proposal that StpA could repress transcription of *cas* genes in the absence of H-NS [26]. StpA has been shown to have higher binding affinity for P*cas8e* and leader sequence *in vitro* than H-NS [20,38], but its inactivation did not induce transcription of *cas* genes *in vivo,* contrary to the inactivation of H-NS [20]. In this work, we found that StpA protein represses the expression of *cas* genes in cells lacking H-NS. We show

that ectopic expression of StpA protein in Δ*hns* cells containing anti-lambda spacers abolished resistance to λ*vir* infection. Levels of *casA* transcripts corresponded to the presence or absence of StpA protein in Δ*hns* cells. We also found that *wt* cells containing anti-lambda spacers and overexpressing *leuO* are resistant to λ*vir* infection if incubated at 30 °C after infection. Interestingly, in *leuO*-overexpressing cells ectopic expression of StpA could not abolish resistance to λ*vir* infection. The possible role of StpA in regulation of CRISPR-Cas immunity in *E. coli* is discussed.

2. Materials and methods

2.1. Strains and plasmids

The *E. coli* K-12 strains used in this study are described in Table 1.

Plasmids used were: pKEDR13 expressing LeuO from pACYCneo [40], and pMR5 expressing StpA from pBad-HisA (this work).

2.2. 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 and 6 g for top agar. When required, appropriate antibiotics were added to LB plates at final concentrations: ampicillin at 100 μg/ml, kanamycin at 50 μg/ml, apramycin 30 μg/ml, streptomycin 50 μg/ml and chloramphenicol at 15 μg/ml. Mutant bacterial strains were made by P1*vir* transduction and selected for their appropriate antibiotic resistance [42]. The *stpA* gene was cloned into pBad-HisA (Invitrogen) using a NEB assembly cloning kit (NEB Biolabs) and the primers:

stpA_pBad_F 5′ tgcagctggtaccatatgggatATGTCCGTAATGTTACAAAG 3′, stpA_pBad_R 5′ gccaaaacagccaagcttcgTTAGATCAGGAAATCGTCG 3′

2.3. Phage sensitivity assay by plaque formation (interference)

This assay was done according to Majsec et al. [24]. Cells transformed with appropriate plasmids were grown to saturation overnight in LB medium at 37 °C with 0.2% maltose. LB plates were overlaid with 3 ml of 0.6% LB top agar containing 0.2 ml of cells. After solidification, 10 μl aliquots of serially diluted λ*vir* 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 to overnight cultures, plates and top agar. The sensitivity of the cells to infection was represented as the plaque-forming units (PFUs) by plaques from several dilutions being counted and their number per ml calculated.

2.4. RNA extraction and qPCR

Total RNA was extracted from mid log (OD_{600} = 0.4–0.5) cultures incubated at 37 °C according to Majsec et al. [24] with minor changes. 1.5 ml of each culture was used and the cell pellet was resuspended in cold 10 mM EDTA and 50 mM sodium acetate followed by the addition of 10% SDS. Trizol LS (Invitrogen) was used to extract total RNA according to the instructions from the manufacturer. The same amounts of RNA (1 μg) were first treated by DNase I, diluted 1000-fold and 2 μl of each sample (in duplicate) was used as a template for one step amplification reaction using Luna® Universal One-Step RT-qPCR Kit (New England Biolabs). 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* and *casA* gene transcription was calculated using relative quantification with *groES* as an endogenous control and *cas3* gene transcript abundance from *E. coli* BW25113 (wild type) as calibrator. All PCR reactions were performed in 6 replicates. Control PCRs without template were performed to monitor general contamination levels.

Primers used for *cas3* and *groES* were as published before [24] and for *casA*:

CasA-F: 5′- TCCTCACATTACCTCGTCTT -3′
CasA-R: 5′- GA ATAGACCACGGACAAACC -3′

3. Results

3.1. StpA abolishes resistance to λvir phage infection in Δhns cells and inhibits transcription from the Pcas8e promoter

The effect of StpA on CRISPR-Cas-mediated resistance to λ*vir* phage infection was studied in Δ*hns* Δ*cas1* cells that contain two anti-lambda spacers. λT3, which was engineered into CRISPR, targets the proto-spacer with non-consensus 5′-CCA-3′ PAM [21], while λc is a naturally acquired spacer that targets the proto-spacer with consensus 5′-CTT-3′ PAM [24]. Mutations in *hns* and *cas1* genes serve to activate the CRISPR-Cas immune system and uncouple interference from adaptation [24]. As shown before [24], Δ*hns* Δ*cas1* + λT3 + λc cells (IIB1040) showed a ~10^6-fold increase in resistance to λ*vir* infection at 30 °C, but were sensitive to λ*vir* infection at 37 °C (Table 2). The exact number of individual plaques at 30 °C could not be determined because individual plaques were not formed.

We next tested if resistance to λ*vir* infection would be abolished by the expression of H-NS paralog StpA. StpA is expressed in Δ*hns* cells but is degraded by the Lon protease *in vivo* [43]. To increase the endogenous levels of StpA protein, we cloned the *stpA* gene into a medium copy number plasmid pBad-HisA to prevent the excessive expression that is reported to be lethal [37,44]. When repeating

Table 1
List of strains used in this study.

Bacterial strain	Relevant genotype	Source or reference
BL21-AI	*E. coli* B F$^-$ *ompT gal dcm lon hsdS$_B$*($r_B^- m_B^-$) [*malB*$^+$]$_{K\text{-}12}$(λ^S) *araB*::*T7RNAP-tetA*	Invitrogen
BW25113	F$^-$ *rrnB* Δ*lacZ4748*(::*rrnB-3*) *hsdR514* Δ(*araBAD*)*567* Δ(*rhaBAD*)*568 rph-1* λ^-	[41]
Bacterial strains related to BW25113		
IIB969	+ λT3 *lacUV5-cas3 cat*::*araBp8-casA*	[24]
IIB1042	+ λc + λT3 *lacUV5-cas3 cat*::*araBp8-casA*	IIB969 that acquired λc spacer
JW0462	+ Δ*stpA750*::*kan*	*E. coli* stock centre
IIB1043	+ λc + λT3 Δ*cas1*::FRT	[24]
IIB1040	+ λc + λT3 Δ*cas1*::*kan* Δ*hns*::*cat*	[24]
IIB1297	+ λc + λT3 Δ*cas1*::FRT Δ*hns*::*cat*	Removal of kan by pCP20
IIB1325	+ λc + λT3 Δ*cas1*::FRT Δ*hns*::*cat* Δ*stpA750*::*kan*	P1. JW0462 x IIB1297

Table 2
StpA overexpression abolishes resistance to λ*vir* infection in Δ*hns* cells at 30 °C, but not *hns*$^+$ cells with inducible promoters.

bacterial strain[a]	PFU/ml 30 °C	PFU/ml 37 °C
IIB1040	~10^3	$1.79 \times 10^9 \pm 1.3 \times 10^9$
IIB1040 + pBad	~10^3	$4.42 \times 10^9 \pm 2 \times 10^9$
IIB1040 + pStpA	$9.85 \times 10^8 \pm 4.4 \times 10^8$	$6.32 \times 10^9 \pm 3.1 \times 10^9$
IIB1042	~10^3	~10^3
IIB1042 + pBad	~10^3	~10^3
IIB1042 + pStpA	~10^3	~10^3

[a] *E. coli* cell lawns of strains Δ*hns* Δ*cas1* + λc + λT3 (IIB1040) and IIB1042 with inducible promoters *lacUV5-cas3 cat*::*araBp8-casA* + λc + λT3 transformed with pStpA and pBad were infected with λ*vir* phage dilutions (from 10^0 to 10^{-7}) and incubated with L-arabinose at 30 °C or 37 °C. Numbers represent average number and SD of plaque forming units (PFUs) per ml from three independent experiments.

these experiments using plasmid pBad-HisA as an empty vector control and pMR5 plasmid to express *stpA*, we observed that expression of StpA abolished resistance to λ*vir* infection at 30 °C, while cells containing empty vector remained resistant (Table 2).

We reasoned that this effect is caused by StpA binding to P*cas8e* and repressing transcription. Therefore, to better understand this effect, total RNA was isolated from the strains grown to mid-exponential phase at 37 °C: *E. coli* BW25113, Δ*hns* Δ*cas1* + λT3 + λc cells transformed with an empty vector pBad-HisA or the StpA-expressing plasmid, and Δ*hns* Δ*stpA* Δ*cas1* + λT3 + λc cells. Comparison of the *cas3* and *casA* (chosen as a representative gene of the *casABCDE12* operon) genes transcription abundance by the quantitative PCR (qPCR) analysis showed that *cas3* displayed a modest increase (about 2.5-fold) of transcription in Δ*hns* Δ*cas1* + λT3 + λc cells with an empty vector pBad-HisA. No significant increase in *cas3* transcription in Δ*hns* Δ*stpA* Δ*cas1* + λT3 + λc cells was observed, while ectopic expression of StpA in Δ*hns* Δ*cas1* + λT3 + λc cells slightly decreased the expression of *cas3* (Fig. 2A). On the other hand, transcription abundance of the *casA* gene changed significantly depending on the genetic background. The abundance of *casA* transcripts was around 50-fold higher in Δ*hns* cells with pBad-HisA than in *wt* cells, much higher than reported before [26]. When StpA was expressed from a plasmid in Δ*hns* cells, the amounts of the *casA* transcripts strongly decreased but remained about 5-fold higher than in *wt* cells (Fig. 2B). These results showed that StpA preferably binds and regulates transcription from the P*cas8e* promoter so we focused further only on the *casA* transcript analysis and extended our research to *wt* cells overexpressing *leuO* from a plasmid. When LeuO was not induced by IPTG, *casA* displayed a 5-fold increase in transcription abundance (Fig. 2B). After induction of *leuO* expression, the levels of *casA* transcripts strongly increased to about 165-fold, which is slightly less than observed before (Fig. 2B). To our surprise, *casA* displayed an even stronger increase in transcript abundance, of about 250-fold in Δ*hns* Δ*stpA* Δ*cas1* cells, comparable to the *casA* transcript abundance in *leuO*-expressing cells from the previous study [26]. The possible reason for the observed discrepancy in *casA* transcript abundance between our and the previous study might be due to the different strains and primers used. Considering all the evidence, StpA appears to prevent transcription of the *casA* gene from the P*cas8e* promoter in Δ*hns* cells and complete derepression of *casA* (i.e. Cascade genes) can be achieved only after simultaneous inactivation of *stpA* and *hns* genes. Compared with Δ*hns* Δ*stpA* cells, lower levels of *casA* transcripts in *leuO*-overexpressing cells could be due to incomplete displacement of H-NS by LeuO in subpopulation of cells.

To support our finding that StpA represses the P*cas8e* promoter, we used another strain (IIB1042) where native P*cas3* and P*cas8e* are replaced with inducible P*lacUV5* and P*araBp8* promoters, respectively. This strain is *hns*$^+$ and contains the same two anti-lambda spacers as previous strains. In the presence of inducers, high resistance levels to λ*vir* infection at 30 °C and 37 °C were observed (Table 2) probably due to higher amounts of all Cas components expressed from inducible rather than endogenous promoters in Δ*hns* cells. However, ectopic expression of StpA in this strain could not abolish resistance to λ*vir* infection at any temperature, suggesting that StpA cannot inhibit CRISPR-Cas-mediated immunity when Cas components are expressed from inducible promoters.

We also predicted that if StpA ectopic expression represses resistance to λ*vir* infection and inhibits the abundance of *casA* transcripts, then deletion of *stpA* gene would improve resistance in Δ*hns* cells as the double mutant has improved abundance of *casA* transcripts. We thus created the mutant Δ*hns* Δ*stpA* Δ*cas1* + λT3 + λc, strain IIB1325, which showed poor and slow growth, as expected (data not shown). When this strain was infected with λ*vir*, the observed resistance was similar to that of a single Δ*hns* mutant, i.e. cells were sensitive to λ*vir* infection at 37 °C and resistant at 30 °C (data not shown). These results are consistent with previous findings, which showed that inactivation of the *hns* gene led to a high level of protection against λ*vir* infection [21,24] and absence of *stpA* seems to have no additional role in this process.

3.2. LeuO induces resistance to λvir infection at lower temperature of incubation

Interestingly, despite very strong derepression of *casABCDE12* genes in *E. coli* λJ3 overexpressing LeuO, the observed resistance to λ*vir* infection was very modest at 37 °C [26]. Since resistance to λ*vir* infection mediated by CRISPR-Cas has been shown to be temperature-dependent [24], we investigated whether the temperature of incubation could influence resistance to λ*vir* infection in cells overexpressing *leuO* from a plasmid. In the plaque assay, sensitivity to λ*vir* infection was observed at 30 °C and 37 °C in Δ*cas1* + λT3 + λc cells without LeuO induction, showing that insufficient amounts of LeuO protein could not efficiently displace H-NS (Fig. 3). After induction of LeuO with IPTG, a strong increase in resistance to λ*vir* infection was observed at 30 °C, but not at 37 °C, which is reminiscent of λ*vir* protection in Δ*hns* cells (Table 2 and Fig. 3). We therefore conclude that CRISPR-Cas-mediated resistance to λ*vir* infection in *E. coli* is also temperature-dependent in Δ*cas1* + λT3 + λc cells overexpressing *leuO*.

We next investigated whether resistance to λ*vir* infection would be abolished by StpA if Δ*cas1* + λT3 + λc cells were simultaneously ectopically expressing StpA and LeuO. Whether LeuO can prevent StpA binding to IGLB is currently unknown, but LeuO has been shown to displace bound StpA in *Salmonella enterica ompS1* [35]. In the plaque assay, Δ*cas1* + λT3 + λc cells remained resistant to λ*vir* infection at 30 °C and sensitive at 37 °C despite induced levels of StpA along with LeuO (Fig. 3). These results suggest that StpA cannot abolish resistance to λ*vir* infection when CRISPR-mediated immunity is induced by elevated levels of LeuO protein in *hns*$^+$ cells.

4. Discussion

In this work, we have studied the possibility that StpA is involved in the regulation of the CRISPR-Cas Type I-E immune system in *E. coli* lacking H-NS. StpA has been proposed as a highly likely candidate because of its high binding affinity for P*cas8e* [20,26,38], its similarity to H-NS and differences in transcript levels of *casABCDE12* genes between Δ*hns* cells and *wt* cells expressing *leuO*. We found that the transcription abundance of *casA* is strongly dependent on the expression of StpA in cells lacking H-NS.

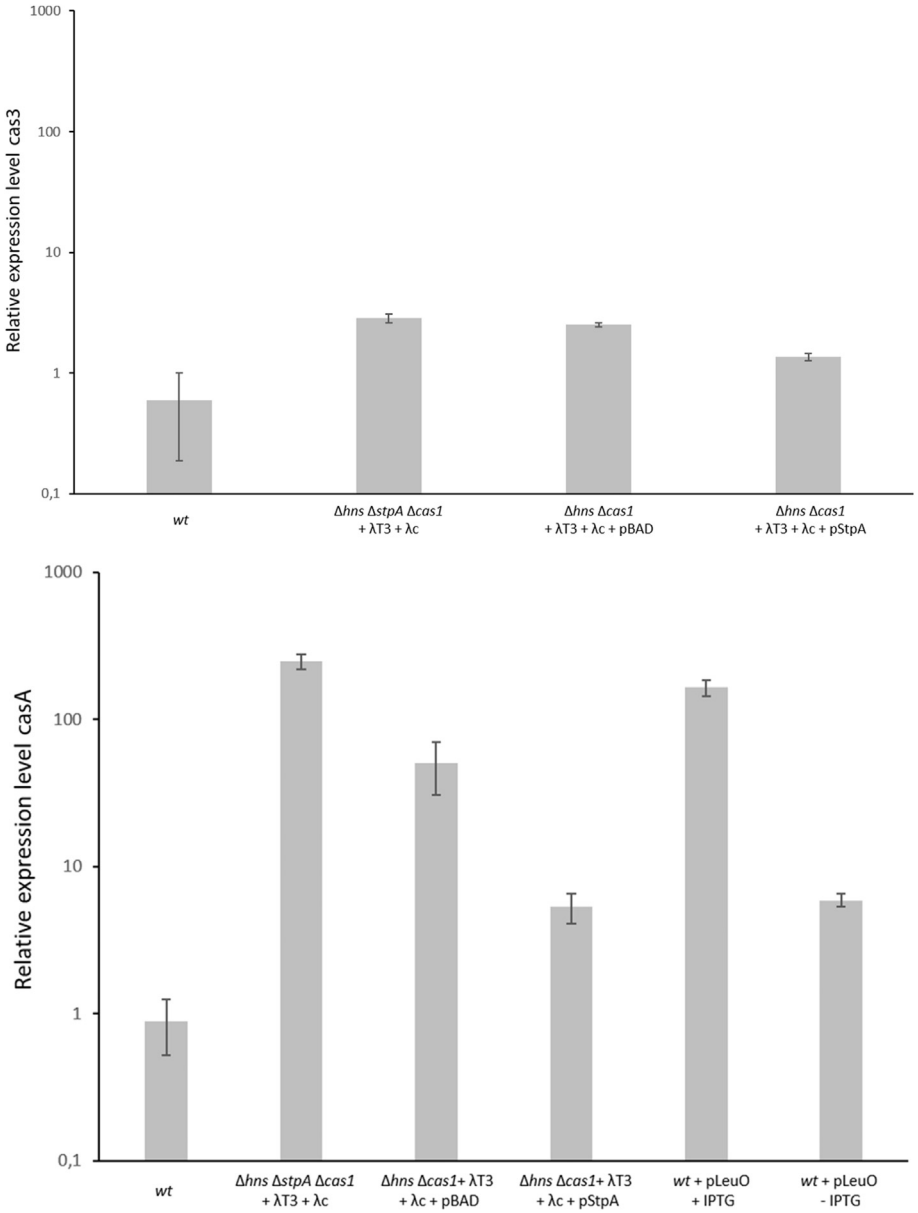

Fig. 2. StpA regulates expression of the *casA* gene. qPCR analysis of (A) *cas3* gene transcript abundance in *E. coli* BW25113 (*wt*), Δ*hns* Δ*cas1* + λc + λT3 cells (IIB1297) transformed with pBad or pStpA and Δ*hns* Δ*stpA* Δ*cas1* + λc + λT3 cells (IIB1325) and (B) *casA* gene transcript abundance in IIB1297 transformed with pBad or pStpA, IIB1325 and *E. coli* BW25113 (*wt*) expressing *leuO* from the plasmid (induced or non-induced with IPTG). Error bars indicate standard deviation.

Transcript levels of the *cas3* gene, on the other hand, did not change significantly under the same experimental conditions (Fig. 2A) indicating that StpA protein shows strong preference for P*cas8e* promoter [20,38]. In Δ*hns* Δ*stpA* cells, the transcript levels of *casA* were much higher than in Δ*hns* cells, and slightly higher than those in *wt* cells overexpressing *leuO* (Fig. 2B). In contrast, in Δ*hns* cells expressing StpA protein from a plasmid, transcription of *casA* was strongly reduced. Overall, these data support the hypothesis that StpA is an additional repressor of the P*cas8e* promoter when H-NS is absent.

These findings are further supported *in vivo* when it was shown that CRISPR-Cas mediated immunity against λ*vir* infection in Δ*hns* cells could be abolished by ectopic expression of StpA at 30 °C (Table 2). However, the same effect was not observed if StpA was expressed in *E. coli* cells containing inducible promoters in front of *cas* genes or cells expressing ectopically both StpA and LeuO (Fig. 3). This indicates again that StpA shows its repressing activity only in cells lacking H-NS. Interestingly, despite different levels of *casA* transcripts in Δ*hns*, Δ*hns* Δ*stpA* cells or *wt* cells overexpressing *leuO* (containing also genomic anti-lambda spacers and Δ*cas1* mutation), the resistance to λ*vir* infection was observed in all three cases, and occurred only at 30 °C. This could mean that sufficient amounts of Cascade protein are synthesized to allow immunity even if transcript levels are modest. It has been estimated recently that the *E. coli* CRISPR system requires about 20 molecules of Cascade complexes to provide 50% protection [45]. Although the number of estimated Cascade complexes is relatively low, it is sufficient to confer CRISPR immunity due to Cascade's fast scanning ability of DNA sequences (30 ms) and movement within the nucleoid and the cytoplasm [45]. The effect of the temperature on the resistance to λ*vir* infection is therefore probably not caused by differences in the amounts of the Cascade complex, but rather the limiting role of

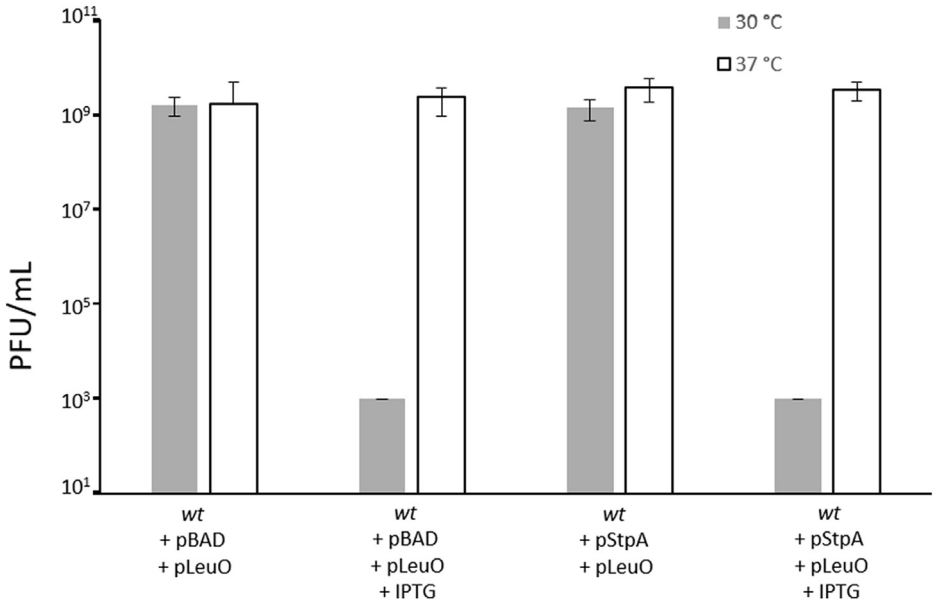

Fig. 3. LeuO overexpression allows resistance to λ*vir* infection at 30 °C, but not at 37 °C. *E. coli* cell lawns of strains Δ*cas1* + λT3 + λc (IIB1043) transformed with pLeuO and pBad, or pLeuO and pStpA were infected with λ*vir* phage dilutions (from 10^0 to 10^{-7}) and incubated with and without IPTG at 30 °C and 37 °C. Bars represent average number and SD of plaque forming units (PFUs) per ml from three independent experiments.

Cas3 [24]. Since expression of *stpA* and *leuO* is negatively regulated by H-NS [37,46], elevated levels of StpA and LeuO are present in Δ*hns* cells ([30]; Fig. 1B). Given that LeuO also positively regulates its own expression [47] and StpA protein becomes unstable and degraded by the Lon protease [43], this implies that the signal from LeuO that induces expression of *cas* genes is expected to be stronger than the negative signal from StpA. In the presence of H-NS, StpA is protected from proteolysis by Lon because it forms a heterodimer StpA:H-NS [43]. Expression of *stpA* from its native promoter is also temperature-regulated, with 2-fold less StpA present at 30 °C than at 37 °C [37]. This additional effect of temperature on StpA levels may further intensify the amplifying signal of LeuO at 30 °C and explain why *casABCDE12* genes are derepressed in Δ*hns* cells despite StpA being expressed and partially repressing their promoter (Fig. 1B). However, in *hns*$^+$ cells ectopically expressing both StpA and LeuO, elevated levels of StpA from the plasmid could not abolish CRISPR-mediated immunity against λ*vir* infection. The most likely explanation is that higher levels of LeuO protein are present in cells when *leuO* is overexpressed from the plasmid than from the chromosome.

In addition to H-NS, StpA and LeuO, another transcriptional repressor of *cas* genes in *E. coli* is CRP (cAMP receptor protein) [23]. The CRP binding site overlaps with the LeuO binding site, which leads to the suggestion that CRP and LeuO compete for binding to the P*cas8e* promoter depending on the cellular availability of cAMP [23]. The levels of CRP are reduced and stringent response is induced under normal growth in Δ*hns* Δ*stpA* cells [48], so one would expect transcription of the *casABCDE12* genes to be further increased, which was observed. Curiously, guanosine tetraphosphate (ppGpp) induces *leuO* expression [49,50] but induction of the stringent response does not induce *cas* genes or the formation of crRNA [26]. Other natural signals that moderately increase LeuO expression in *E. coli* are amino acid starvation and stationary growth phase [27,49,50]. Similar growth requirements for upregulation of LeuO production are found in *S.* Typhimurium [51]. Apparently, there is a very complex regulatory network of the many proteins and environmental factors that affect the expression of numerous genes that regulate one another, making interpretation difficult.

Currently we do not understand why (at least) three repressors are involved in the regulation of the CRISPR-Cas system in *E. coli* and the exact role of StpA. Another H-NS paralogue, the Sfh protein, is encoded by the R27 and pHCM1 plasmids of *S.* Typhimurium. Sfh protein was found to target a subset of H-NS-regulated genes, and just like StpA, it acts as a molecular backup for H-NS [52]. The similarity with StpA stops here because it is suggested that the Sfh protein supplements the depleted population of chromosomally bound H-NS which is temporarily decreased due to H-NS binding onto an acquired high molecular mass AT-rich plasmid. Since decreased H-NS occupancy causes a loss of competitive fitness, Sfh serves to enhance the fitness of bacteria that acquire conjugative plasmids [53]. Additionally, Sfh supports the plasmid establishment by silencing CRISPR transcription [54]. Since StpA is not encoded by the plasmid, an intriguing possibility is that the StpA protein may have a positive role by binding to the anti-P*cas* promoter and inhibiting synthesis of the *anticas* transcript that could prevent translation of Cas3 (Fig. 1B). Divergent promoters and antagonistic protein regulators suggest very tight control of the CRISPR-Cas activity. Complete derepression of *cas* genes is possibly harmful to the host because it could cause autoimmunity if a self-targeting DNA spacer is integrated into the CRISPR array or if that spacer is used in the regulation of endogenous gene expression [55]. Cascade complex could also block DNA replication and cause genome instability [56]. It will be interesting to investigate whether the regulation of the CRISPR-Cas immunity by two similar repressors is present in other species. We know that CRISPR-Cas systems can be regulated through a plethora of regulators such as quorum sensing, CRP (it can act as a repressor or activator depending on the species), RpoN (σ^{54}), membrane stress, dedicated regulators and many others (reviewed in [57]). Despite remarkable progress in understanding the mechanistic details of the CRISPR-Cas activity, knowledge of its regulation is lagging behind. Further research is needed to discover details and interplay of known and unknown regulators and to identify signals that derepress *cas* genes in *E. coli*.

5. Conclusions

This study provides evidence for the first time that StpA is

another repressor involved in regulation of the type I-E CRISPR-Cas system in *E. coli*. When StpA protein is normally expressed from the chromosome due to Δ*hns* mutation, it partially represses the synthesis of *casA* transcript from the P*cas8e* promoter but this does not prevent CRISPR-mediated immunity against λ*vir* infection. However, ectopic expression of StpA protein in Δ*hns* cells containing anti-lambda spacers abolishes resistance to λ*vir* infection and reduces expression of *casA* transcripts. In cells that overexpress *leuO* from the plasmid resistance to λ*vir* infection, just as in Δ*hns* cells, occurs only at 30 °C suggesting that the CRISPR-Cas mediated immunity in *E. coli* is temperature-dependent.

Authors contribution

1. Conception of the work: IIB
2. Experiments, data analysis and interpretation of data: DMitić, MR, DM, and IIB
3. Drafting of the manuscript: IIB
4. Final manuscript approval for submission and publication: all authors

Declaration of competing interest

The authors declare no conflict of interest.

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