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H-NS mediated repression of CRISPR-based immunity in *Escherichia coli* K12 can be relieved by the transcription activator LeuO

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Summary

The recently discovered prokaryotic CRISPR/Cas defense system provides immunity against viral infections and plasmid conjugation. It has been demonstrated that in *Escherichia coli* transcription of the Cascade genes (*casABCDE*) and to some extent the CRISPR array, is repressed by heat-stable nucleoid-structuring (H-NS) protein, a global transcriptional repressor. Here we elaborate on the control of the *E. coli* CRISPR/Cas system, and study the effect on CRISPR-based anti-viral immunity. Transformation of wildtype *E. coli* K12 with CRISPR spacers that are complementary to phage Lambda, does not lead to detectable protection against Lambda infection. However, when an H-NS mutant of *E. coli* K12 is transformed with the same anti-Lambda CRISPR, this does result in reduced sensitivity to phage infection. In addition, it is demonstrated that LeuO, a LysR-type transcription factor, binds to two sites flanking the *casA* promoter and the H-NS nucleation site, resulting in derepression of *casABCDE12* transcription. Over-expression of LeuO in *E. coli* K12 containing an anti-Lambda CRISPR leads to an enhanced protection against phage infection. This study demonstrates that in *E. coli* H-NS and LeuO are antagonistic regulators of CRISPR-based immunity.

Introduction

Invasions by viruses and conjugative plasmids pose a threat to microbial cells. To neutralize selfish DNA elements, bacteria and archaea have developed several defense strategies, such as receptor masking, restriction/modification and abortive infection (Hyman and Abedon, 2010; Labrie *et al.*, 2010). Recently it was discovered that Clustered Regularly Interspaced Short Palindromic Repeats (CRISPR) (Ishino *et al.*, 1987) and CRISPR associated (*cas*) genes (Jansen *et al.*, 2002) form a sophisticated immune system that uses small RNAs to target mobile genetic elements, reviewed by (Horvath and Barrangou, 2010; Karginov and Hannon, 2010; Marraffini and Sontheimer, 2010; van der Oost *et al.*, 2009). CRISPRs consist of repeating sequences of approximately 30 nucleotides that are separated by unique sequences of similar size, called spacers (Mojica *et al.*, 2000). The spacer sequences are commonly derived from phages and plasmids (Bolotin *et al.*, 2005; Mojica *et al.*, 2005; Pourcel *et al.*, 2005), and new spacers can be added to the existing CRISPR array, expanding the invader repertoire (Barrangou *et al.*, 2007), in a process known as CRISPR adaptation. The presence of a spacer matching a viral or plasmid sequence confers resistance to invasion by these elements (Barrangou *et al.*, 2007; Brouns *et al.*, 2008; Marraffini and Sontheimer, 2008). The biochemical pathways underlying CRISPR defense are partially known and involve transcription of the array into a long precursor CRISPR RNA. This precursor is cleaved in the repeat sequences by a Cas endonuclease (CasE in *E. coli* (Brouns *et al.*, 2008), Cas6 in *Pyrococcus furiosus* (Carte *et al.*, 2008)), releasing small crRNAs that serve to guide the defense.

The *cas* genes encode the protein machinery that carries out the various steps of CRISPR defense. Approximately 45 families of *cas* genes have been identified

(Haft *et al.*, 2005) that are classified in eight typical combinations or subtypes named after a representative organism, *e.g.* type E after *E. coli* (Haft *et al.*, 2005).

The type E CRISPR/Cas immune system in *E. coli* K12 is composed of 8 *cas* genes (*cas1*, *cas2, cas3* and *casABCDE*) and a downstream CRISPR locus with type 2 repeats (Kunin *et al.*, 2007) containing 12 spacer-repeat units (CRISPR I) (Fig. 1A). An additional 6 spacer-containing CRISPR (CRISPR II) and a 2 spacer CRISPR (CRISPR III) with type 2 repeats, as well as a CRISPR with type 4 repeats (Kunin *et al.*, 2007) containing 1 spacer repeat unit (CRISPR IV) are located elsewhere on the genome (Diez-Villasenor *et al.*, 2010). In addition to a CRISPR containing an anti-invader sequence, only Cas3 and CasABCDE, forming the protein complex Cascade (CRISPR-associated complex for antiviral defense), are required for CRISPR interference (Brouns *et al.*, 2008). A recent study has demonstrated that in *E. coli* K12 transcription from the *casA* and CRISPR I promoters is repressed by heat-stable nucleoid-structuring protein (H-NS) (Pul *et al.*, 2010), a global repressor of transcription in many Gram-negative bacteria. A microarray study indicates that transcription of *casABC* and *cas2* is elevated in an *E. coli* K12Δ*hns* strain compared to *wt E. coli* K12 (Hommais *et al.*, 2001). In addition, H-NS was shown to possess high binding affinity for the intergenic region between *cas3* and *casA* (Oshima *et al.*, 2006; Pul *et al.*, 2010). H-NS has a preference for binding AT-rich DNA sequences (Navarre *et al.*, 2006). After initial binding of H-NS to high affinity nucleation sites (Bouffartigues *et al.*, 2007; Lang *et al.*, 2007) repression of transcription is mediated by cooperative spreading along the DNA (defined as DNA stiffening (Liu *et al.*, 2010)) and by creating looped structures through formation of DNA-protein-DNA bridges (Dame *et al.*, 2005). Moreover, H-NS acts as a DNA structuring protein (Liu *et al.*, 2010; Stoebel *et al.*, 2008).

Overcoming H-NS mediated repression of *cas* gene transcription may be a key requirement for CRISPR/Cas functionality. Generally, H-NS repression can be relieved by a number of proteins, such as SlyA, VirB and others (Stoebel *et al.*, 2008). One of these proteins is the regulator LeuO (Chen and Wu, 2005; De la Cruz *et al.*, 2007), which belongs to the LysR family of transcription factors (Stoebel *et al.*, 2008) and is found in all proteobacteria, except the δ subdivision (Maddocks and Oyston, 2008). The *leuO* gene maps next to the *leuABCD* operon (Chen *et al.*, 2005; Chen and Wu, 2005; Hertzberg *et al.*, 1980), whose gene products are required for leucine synthesis (Vartak *et al.*, 1991). Recent data indicate that LeuO is involved in regulating transcription of many genes, often as an H-NS antagonist (Shimada *et al.*, 2009; Stoebel *et al.*, 2008). However, since under laboratory growth conditions the genomic *leuO* gene itself is repressed by H-NS (Klauck *et al.*, 1997; Majumder *et al.*, 2001) all LeuO regulation studies make use of plasmid encoded *leuO* under control of constitutive or inducible promoters. In the host environment *leuO* is likely to be induced under certain conditions as for example virulence of *Salmonella enterica* *leuO* mutants is attenuated (Lawley *et al.*, 2006).

A genomic screen for LeuO-binding DNA fragments in *E. coli* K12 revealed 12 gene clusters, including the *casA-cas2* operon (*ygcL*, *ygcK*, *ygcJ*, *ygcI*, *ygcH*, *ygbT*, *ygbF*) (Shimada *et al.*, 2009). When LeuO was over-expressed, increased expression of *casA* and *cas2* was observed in *E. coli* (Shimada *et al.*, 2009), and of *casA* (STY3070) in *Salmonella enterica* serovar Typhi (Hernandez-Lucas *et al.*, 2008). We therefore investigated whether LeuO can mediate H-NS derepression of *cas* gene and CRISPR transcription. In this study we demonstrate that LeuO counteracts H-NS-dependent repression of the *casA* promoter by reorganizing the DNA protein contacts within the transcription initiation region. The resulting change results in increased

transcription of the Cascade genes, the limiting factor for CRISPR-based defense against phage infection in *E. coli* K12.

Results

LeuO activates *cas* gene expression

To study the effect of LeuO on *cas* gene expression, transcript levels of the *E. coli* K12 *cas* genes in mid-exponential growth phase were examined using a DNA microarray approach. RNA samples isolated from a *wt E. coli* K12 strain containing a *leuO* encoding plasmid were compared to RNA isolated from a strain containing the empty vector. In addition, RNA isolated from a Δ*leuO* mutant carrying the empty vector was analyzed. Comparison of *cas* gene transcription levels between the LeuO-expressing strain and the control strain revealed a significant upregulation of transcription of *casABCDE* and *cas1* and *cas2* transcription, showing a gradual decrease from *casA* (65-fold) to *cas2* (5-fold) (Table 1). No change in the transcription level of *cas3* was detected. These results are consistent with a polycistronic transcription of the *casABCDE* and possibly the *cas1*, *cas2* genes, with polar effects for the transcription of the more downstream genes. However, we did not observe significant differences in *cas* gene transcription in the Δ*leuO* mutant compared to the wildtype strain (Table 1), indicating that *leuO* is not expressed under the growth conditions used here.

To verify the observed increase in *cas* gene expression levels, quantitative PCR (qPCR) was performed on total RNA isolated from 3 strains during mid-exponential phase: *wt E. coli*, a Δ*hns* strain and a *wt* strain expressing *leuO* from a plasmid. This analysis showed that *casABCDE* displayed increased transcription in both *hns* knockout and *leuO* expressing strains (Fig. 1B). While the increase in

casABCDE transcripts was modest in *hns* knockout strains, on average 5-fold, the effect of introducing *leuO* was more dramatic, with an average increase of 236-fold after induction of *leuO* expression. An increase in *casABCDE* transcripts was also observed when *leuO* expression was not induced, due to leakage from the P*T5/lac* promoter. The *cas1* and *cas2* genes also displayed increased transcript abundance in *leuO* expressing strains, although at lower levels than *casABCDE*. Consistent with the microarray data, a trend of transcript fold change was observed, with polar effects downstream of *casA*, again suggesting a polycistronic mRNA of the *casABCDE12* operon. Compared to the effect on the other *cas* genes, only a small increase of *cas3* transcription was observed in *leuO* expressing strains.

To further evaluate the effects of H-NS and LeuO on transcription from the *casA* promoter (known as P*cas* (Pul *et al.*, 2010)), RNA samples from *wt* strains expressing *leuO* from a plasmid and strains lacking *hns* were compared by primer extension analysis. No *cas* transcripts were detected in *wt* cells containing an empty expression vector. Transcripts directed from P*cas* were only detected in cells expressing *leuO* from a plasmid or in *hns* knockout strains (Fig. S1), indicating that transcription of the *casABCDE12* operon is tightly controlled by H-NS and LeuO.

LeuO causes increased crRNA abundance

The CRISPR I locus is transcribed in *E. coli* K12 and the transcript is cleaved by the CasE subunit of Cascade into small crRNAs that subsequently remain bound by Cascade (Brouns *et al.*, 2008; Pul *et al.*, 2010). In K12 small crRNAs were virtually undetectable by Northern blot analysis (Brouns *et al.*, 2008) and (Fig. 2A). To investigate whether this was due to too low transcription levels of *casABCDE*, the *wt* strain was transformed with a plasmid encoding the Cascade protein components

under control of an arabinose-inducible promoter. In the *wt* strain expressing *casABCDE* from a plasmid, crRNAs with a length of about 60 nt could be detected. The requirement for plasmid-encoded synthesis of Cascade for detection of small crRNAs indicates that the level of Cascade in *wt E. coli* is insufficient for generating and stabilizing mature crRNAs. Furthermore we analyzed the levels of crRNAs in an *hns* knockout strain and in the *wt* strain expressing *leuO* constitutively from a plasmid. Both deletion of *hns* and over-expression of *leuO* caused significant crRNA accumulation, due to enhanced expression of Cascade in these two strains (Fig. 2B and 2C). The CasA knockout strain (JW2730) serves as a control and marker for the mature crRNA. It was previously shown that a CasA knockout strain generates elevated levels of mature crRNA (Brouns *et al.*, 2008), due to read-through of the downstream *cas* genes from the kanamycin resistance marker containing recombination cassette by which the *casA* gene is replaced (Pougach et al., in press).

Binding of LeuO and H-NS to the DNA sequence upstream of *casA*

The *casA-cas3* intergenic region (here denoted IGLB) contains P*cas*, for which H-NS has strong binding affinity as well as the divergently oriented anti-*cas3* (known as anti-P*cas*) promoter, that is located 80 bp upstream of P*cas* and gives rise to an antisense transcript of unknown function (Fig. 1A and Fig. 3C) (Pul *et al.*, 2010).

Both LeuO and H-NS bind the IGLB fragment, as determined by Electrophoretic Mobility Shift Assay (EMSA) (Fig. 3A, lanes 2-4 and 5-7). Pre-bound LeuO impedes cooperative binding of H-NS to the IGLB fragment (Fig. 3A, lanes 9-11). In line with this, pre-bound H-NS is partly released from the DNA when LeuO is added to the complex (Fig. 3A lanes 12-15). In order to map the binding region of LeuO within the IGLB fragment DNase I footprint analysis was performed. Upon

limited DNase I hydrolysis of the IGLB DNA fragment, H-NS causes an extended footprint (Fig. 3B), as shown before (Pul *et al.*, 2010). In addition, LeuO protects two sites (site 1 and site 2) within the IGLB fragment that flank the high affinity H-NS nucleation site (Fig. 3B and 3C). LeuO site 1 is located 20 bp downstream of P*cas* and LeuO site 2 spans the divergent anti-P*cas* (Fig. 3B and 3C). Interestingly, in the presence of LeuO the extended protection by H-NS is no longer visible (Fig. 3B, compare lanes 2 and 4), indicating that due to LeuO binding the DNA region containing the H-NS high-affinity binding site is no longer protected from DNase I cleavage, in agreement with decreased cooperative binding (Fig. 3A).

In order to analyze the effect of LeuO on RNA polymerase (RNAP) binding to the promoter sites, DNase I footprints were performed in the presence of RNAP and LeuO. Moreover, the effect on transcription initiation and RNAP open complex formation was analyzed by $KMnO_4$ footprints of stable initiation complexes. RNAP binds to the two promoters (P*cas* and anti-P*cas*) (Fig. 4A, lanes 3 and 3', indicated I (P*cas*) and II (anti-P*cas*)) (Pul *et al.*, 2010). Addition of the DNA binding proteins LeuO or H-NS alone does not cause changes in the $KMnO_4$ reactivity (Fig. 4A, lanes 2', 4' and 5'). Binding of LeuO abolishes the spreading of H-NS along the DNA, resulting in a lack of protection by H-NS in the region between positions -160 to -240 (Fig. 4A, compare lanes 2 and 5), as observed before (Fig. 3B). When RNAP binding was studied in the presence of both transcription factors it turned out that the order of addition to the DNA is crucial for the resulting footprint. RNAP binding was only affected when H-NS and/or LeuO were added to the DNA prior to RNAP. While prior binding of H-NS to the IGLB DNA fragment completely abolished RNAP-promoter interaction and open complex formation (Fig. 4A and 4B, compare lanes 3' and 6'), prior binding of LeuO had a repressive effect only on RNAP binding at anti-P*cas* (Fig.

4A and 4B, compare lanes 3' and 7'; Fig. 4E, lane 5'). This can also be seen on the retardation gels (Fig. 4C and 4D), where the DNA/RNAP complex II is lost in the presence of LeuO (Fig. 4C, lane 7). This complex remains stable when H-NS is added last (Fig. 4C, lane 9), in contrast to a sample with only H-NS or where H-NS is added before LeuO (Fig. 4C, lanes 6 or 8, respectively). Moreover, the change in nucleotide reactivities indicates that LeuO binding alters the architecture of the transcription initiation complex at the P*cas* promoter (compare Fig. 4B, lanes 7' with Fig. 4A, line 3' and Fig. 4E, lane 3' with lane 5'). Altogether these data indicate that LeuO plays an important role in the regulation of *casABCDE12* gene expression by antagonizing H-NS-dependent repression of P*cas*.

H-NS and LeuO regulate CRISPR-based immunity against phage infection

The effect of H-NS on CRISPR-based defense against phage infection was analyzed in *wt* and Δ*hns E. coli* strains, grown in Luria Bertani broth. Since none of the spacers of *E. coli* K12 target known bacteriophages, an artificial seven spacer CRISPR (J3) with the native promoter was designed containing one spacer that targets the template strand of the gene encoding the phage Lambda tail protein (J). A non-targeting (N) CRISPR (Brouns *et al.*, 2008) served as a negative control. Introducing the J3 CRISPR reduced the sensitivity to virulent phage Lambda (λ_{vir}) infection 4-fold in the Δ*hns* but not in the *wt* strain (Fig. 5A). Complementation of the Δ*hns* strain reversed the reduction in phage sensitivity, demonstrating that CRISPR-based defense is negatively regulated by H-NS (Fig. 5A). When cells were grown in richer media (2YT) until stationary growth phase, higher resistance levels were observed, up to a 10-fold reduced sensitivity compared to a non-targeting strain (data not shown).

Moreover, plaques were much smaller in the *hns* knockout strains equipped with the J3 CRISPR when using 2YT.

Further evidence that H-NS controls CRISPR-based immunity was obtained using genetically engineered strains (Table S4) in which the genomic CRISPR I locus was replaced by the J3 or a non-targeting CRISPR. *E. coli* K12Δ*CRISPRI::CRISPRJ3* (*E. coli* J3) was fully sensitive to infection by phage Lambda, despite the presence of a genomic J3 spacer (Fig. S2). However, when the dominant negative *hns*^{G113D} mutant was expressed from a plasmid, the sensitivity of *E. coli* J3 to phage λ_{vir} infection was reduced 3.6 fold (Fig. S2). This mutant still forms heterodimers with *wt* H-NS, but does not bind DNA and therefore interferes with H-NS mediated transcriptional repression resulting from the formation of higher-order DNA-protein complexes (Ueguchi *et al.*, 1996). This observation is consistent with the reported finding that expression of *hns* mutant G113D induces transcription from P*cas* in *wt E. coli* (Pul et al., 2010).

When plaque assays were performed in *E. coli* J3 over-expressing *leuO* from a plasmid, a ~6 fold reduced sensitivity to phage λ_{vir} infection was observed (Fig. 5B), demonstrating that LeuO activates CRISPR-based defense in *E. coli*. A 3-fold reduced sensitivity was observed when *leuO* expression was not induced, probably due to leakage from the P*tac* promoter. When *E. coli* J3 cells were grown to stationary phase in rich 2YT medium, an increased resistance level was observed with turbid and very small plaques in the *leuO* over-expressing strains containing a targeting CRISPR (data not shown), whereas plaques in the same strain containing a non-targeting CRISPR were clear and of normal size. Although *cas3* gene expression was not strongly induced when LeuO was expressed from a plasmid (Table 1 and Fig. 1B), the expression of *cas3* was not a limiting factor for resistance, since introduction of a *cas3*

expression plasmid into *E. coli* J3 expressing *leuO* did not lead to elevated resistance levels (Fig. 5B).

When a plasmid expressing *casABCDE12* was introduced in *E. coli* J3, a 2.5-fold reduced sensitivity to phage infection was observed (Fig. 5C), which was not observed when a CRISPR expression vector containing spacer J3 or a plasmid encoding *cas3* was introduced (Fig. 5C), indicating that expression of the genes encoding Cascade (*casABCDE*) is limiting for CRISPR-based defense in *wt E. coli* .

Discussion

The type E CRISPR/Cas system (Cse (Haft *et al.*, 2005)) is present in many proteobacteria and in some actinobacteria, firmicutes and methanogenic archaea. A recent analysis of a collection of natural isolates shows that this CRISPR/Cas subtype occurs in approximately 60% of the *E. coli* strains (Diez-Villasenor *et al.*, 2010). The study presented here provides experimental evidence for regulation of the type E CRISPR/Cas system in *E. coli* K12 by the antagonists H-NS and LeuO. These antagonistic DNA-binding proteins regulate the expression of several genes in *E. coli*, such as the *bgl* operon (utilization of β-glucosides) (Ueguchi *et al.*, 1998), the *yjjQ-bglJ* operon (virulence factor and activator of *bgl*, respectively) (Stratmann *et al.*, 2008) and the *Salmonella enterica* Serovar Typhi *ompS1* gene (outer membrane protein and pathogenicity determinant) (De la Cruz *et al.*, 2007).

We demonstrate that relieving H-NS-mediated repression of *cas* gene transcription is required for CRISPR-based immunity and that derepression is mediated by LeuO through direct binding of DNA sequences upstream of *casA*. The EMSA and footprint results (Fig. 3) support the finding that elevated amounts of LeuO counteract H-NS-mediated repression of *casABCDE12 in vivo*. Moreover, these

data indicate that LeuO-induced activation of transcription from the *casA* promoter (P*cas*) does not simply result from a displacement of bound H-NS, since LeuO cannot facilitate the binding of RNA polymerase when H-NS is pre-bound. Instead, LeuO abrogates the cooperative spreading of H-NS upon binding to the *casA* promoter region.

Interestingly, the transcript levels of the *casABCDE12* operon were higher in cells expressing *leuO* than in *hns* knockout strains (Fig. 1B), suggesting that either LeuO functions as an enhancer of *casABCDE12* transcription, or that derepression in K12Δ*hns* is incomplete. The latter could be due to additional repressors involved in silencing *casABCDE12*, or due to functional redundancy between suppressors of gene transcription. In particular, StpA has been reported to possess high binding affinity for P*cas* (Pul *et al.*, 2010). Although a K12Δ*stpA* strain showed similar *cas* gene transcript levels as a *wt* K12 strain (Pul *et al.*, 2010) it cannot be excluded that StpA-mediated repression of *cas* gene transcription takes place in the absence of H-NS.

Cells expressing *leuO* showed higher resistance levels compared to *hns* knockout strains, due to the higher expression of the *cas* genes and higher abundance of mature crRNA (Fig. 1B and Fig. 2BC). Compared to the CRISPR-based resistance levels to phage infection observed in *Streptococcus thermophilus* (Barrangou *et al.*, 2007) or *E. coli* BL21-AI over-expressing the *cas* genes and the CRISPR (Brouns *et al.*, 2008), the resistance levels of *wt E. coli* over-expressing *leuO* are relatively low. However, at present it is unknown whether a similar level of CRISPR-based immunity can be reached by *wt E. coli*, and if it can, under what conditions.

Although we were able to show that CRISPR-based immunity is activated by overproducing LeuO, the natural growth conditions that induce CRISPR-based defense are still unknown. Our experiments show that a genomic anti-Lambda spacer

alone does not provide resistance to phage infection in *wt E. coli* due to the absence of Cascade. We speculate that *leuO* expression levels under laboratory growth conditions are too low to induce derepression P*cas*, and that phage exposure itself does not activate CRISPR-defense. Unaltered expression of *leuO* and the *cas* genes was also observed during infection with bacteriophage PRD1 (Poranen *et al.*, 2006).

Since H-NS is known to bind DNA of incoming phage or plasmid directly (Navarre *et al.*, 2006; Navarre *et al.*, 2007) this might result in redistribution of H-NS (Dillon *et al.*, 2010; Doyle *et al.*, 2007), allowing expression of the Cascade genes due to decreased local concentrations of the repressor. As *leuO* expression is negatively regulated by H-NS and positively by LeuO itself (Chen *et al.*, 2005; Hommais *et al.*, 2001), this would further amplify the activating signal for *cas* gene transcription.

Interestingly, *leuO* expression levels are induced by the alarmone guanosine tetraphosphate (ppGpp) (Chen *et al.*, 2001; Fang *et al.*, 2000; Majumder *et al.*, 2001). ppGpp is involved in stress signaling cascades leading to the stringent response under nutrient limiting conditions. Since these conditions slow down phage proliferation dramatically (Schrader *et al.*, 1997), bacterial cells may then stand a better chance of surviving phage encounters, hence inducing CRISPR-based defense may be more beneficial. However, induction of the stringent response by amino-acid starvation, e.g. by serine hydroxamate (Tosa and Pizer, 1971) neither increased the transcription from P*cas* nor the formation of mature crRNA (data not shown). Although under laboratory conditions CRISPR-based defense is suppressed, the diversity in spacer content in natural isolates of *E. coli* strongly suggests that the CRISPR/Cas system as a whole is active and functional in natural ecosystems (Diez-Villasenor *et al.*, 2010).

In an independent parallel study, it has been shown that an *E. coli hns* knockout strain containing an anti-Lambda spacer is less sensitive to phage infection

(Pougach et al., in press), in agreement with the data presented here. It seems that the Δ*hns* strain containing the T3 spacer used in (Pougach et al., in press), shows higher levels of resistance than the Δ*hns* strain containing the J3 spacer that was used in this study. The T3 spacer has originally been described (Brouns *et al.*, 2008) as the spacer that confers the highest level of immunity of 8 different spacers tested. In BL21-AI over-expressing the *casABCDE* and *cas3* genes together with either the T3 CRISPR or the J3 CRISPR showed that the T3 CRISPR provides 10-fold more resistance (data not shown), indicating that the observed difference in immunity between (Pougach et al., in press) and this study is most likely resulting from a difference in the efficiency of the spacers used.

Although a number of studies involving H-NS and LeuO have been carried out in *E. coli* and *S. enterica* (Hernandez-Lucas *et al.*, 2008; Hommais *et al.*, 2001; Lucchini *et al.*, 2006; Navarre *et al.*, 2006; Shimada *et al.*, 2009), the outcome of these studies has never been interpreted in the light of CRISPR-based defense. Based on these genome-wide analyses we propose that the expression of the type E (Cse) *cas* genes from *Salmonella enterica* are likely to be regulated by H-NS and LeuO as well. For instance, in *S. enterica* Serovar Typhi transcription of *casA* (STY3070) appears to be affected by H-NS and LeuO (Hernandez-Lucas *et al.*, 2008), despite the poor conservation of the intergenic region between the divergently oriented *cas3* and *casA* genes in this strain. In *S. enterica* Serovar Typhimurium strain LT2 H-NS binding sites were found encompassing the translation start site of the *cas3* gene (Lucchini *et al.*, 2006). Another study showed that in this strain the transcription of *cas3*, *casB*, *casC* and *casD* is elevated in the absence of H-NS (Navarre *et al.*, 2006). Perhaps the *cas* genes are controlled by a single promoter in this strain, since the intergenic region between *cas3* and *casA* is only 12 nucleotides in length.

Altogether, this study provides evidence that the type E CRISPR/Cas system in *E. coli* is regulated by the antagonists H-NS and LeuO, and we propose that this regulatory mechanism is conserved in *S. enterica* as well. The upcoming challenge will be to identify conditions that activate this sophisticated defense system to allow defense against invasion by foreign DNA.

Experimental procedures

Strains

The wildtype *E. coli* K12 W3110 (BW25113) strain and the *E. coli* K12 W3110 derivative Δ*hns* (JW1225) and Δ*casA* (JW2730) from the KEIO collection (Baba *et al.*, 2008), supplied by the American Type Culture Collection (ATCC), and *E. coli* K12 MC4100 (Peters *et al.*, 2003) derivative Δ*hns* (PD32) (Dersch *et al.*, 1993) were used throughout the study.

Gene cloning and recombination

A synthetic recombination cassette was designed corresponding to 400 bp flanking regions on each side of the CRISPR I locus separated by a kanamycin resistance gene flanked by FRT-sites (GAAGTTCCTATACTTTCTAGAGAATAGGAACTTC). The construct contained a *Pst*I site followed by 400 nt of the CRISPR I upstream region of the *E. coli* K12 W3110 genome (2875875-2876274), followed by a *Nco*I site, then the sequence AAACAAAGAATT, a *Kpn*I site, followed by an FRT-site, a *Sph*I site, a kanamycin resistance gene with a sequence corresponding to pJJDuet30 (2186-1276), a *Xho*I site, an FRT site, a *Not*I site, and 395 nt of the CRISPR I downstream region of the *E. coli* K12 W3110 genome (2877225-2877619) (GeneArt AG, Regensburg, Germany). A synthetic CRISPR sequence including leader sequence containing 7 spacers and 8 repeats was used (Table S1) (Geneart AG, Regensburg, Germany). This synthetic CRISPR was cloned between the flanking regions using the *Nco*I and *Kpn*I sites (Fig. S3). The *Nco*I and *EcoR*I sites in the leader and second spacer were used to exchange the first spacer sequence of the CRISPR; the constructs created were named J3 and R44 (Table S1). The other spacers in the CRISPR were sequences with no homology to phage Lambda. These constructs were used as recombination cassettes to

replace the existing CRISPR I locus in the *E. coli* K12 W3110 genome, following a protocol described elsewhere (Datsenko and Wanner, 2000), with minor modifications. For recombination, the sequences were PCR-amplified using primers BG3017 and BG3019 (Table S2) with high fidelity *pfu*-turbo polymerase and transformed by electroporation into *E. coli* K12 W3110 containing pKD46, kindly provided by the ATCC. Transformants were grown at 30°C and plated on LB-Agar supplemented with kanamycin (50 μg ml^{-1}). The pKD46 plasmid has a temperature sensitive origin of replication, and was removed through growth at 37°C (Datsenko and Wanner, 2000). Recombination was validated by PCR and sequencing. The antibiotic resistance cassette was removed using Flp recombinase encoded on plasmid pCP20, and subsequent growth at 37°C, as described (Datsenko and Wanner, 2000). The Δ*leuO* mutant was constructed with the λred-gam system using oligonucleotides T209 and T210 (Table S2), as described (Datsenko and Wanner, 2000). After deletion of *leuO* the resistance cassette used for selection was removed using Flp recombinase encoded on plasmid pCP20 (Datsenko and Wanner, 2000).

Plasmids and Vectors

Plasmid pWUR607 (Tet^R) contains the *casABCDE12* operon, which was PCR-amplified from *E. coli* K12 MG1655 genomic DNA using primers BG2173 and BG2174 (Table S2), and cloned into vector pACYC184 using the restriction sites *EcoR*I and *Nco*I. Plasmid pWUR608 (Cam^R) was constructed by cloning a *cas3* amplicon generated with primers BG2171 and BG2172 (Table S2) into pACYC184 using the restriction enzymes *BamH*I and *Sph*I. In the experiments where a CRISPR was introduced on a plasmid, the pACYCduet-1 vector (Cam^R) (Novagen) was used, using the *Nco*I and *Acc65*I restriction sites. pWUR477 containing the non-targeting

CRISPR (N) was described previously (Brouns *et al.*, 2008). Expression of the CRISPR from this plasmid in K12 was under control of the leader sequence that contains the CRISPR I promoter (Pul *et al.*, 2010). pWUR564 is a derivative of pWUR477 that has the *Nco*I-*Eco*RI fragment (containing the leader sequence up to half of the second spacer) replaced with the *Nco*I-*Eco*RI fragment of construct J3 (Table S1). For expression of *wt hns* and *hns*^{G113D} the previously described pHOP11 and pHM52 plasmids were used, respectively (Pul *et al.*, 2010). The pCA24N plasmid from ASKA(-) clone JW0075 encodes *leuO* behind an P*T5/lac* promoter (IPTG inducible). pKEDR13, encoding *leuO* behind a P*tac* promoter (IPTG inducible), and the control vector pKESK22 were described earlier (Madhusudan *et al.*, 2005; Stratmann *et al.*, 2008). The IPTG inducible *leuO* expression plasmid pNH41 was constructed by cloning the *leuO* amplicon, generated using primers NH329 and NH330 (Table S2), into the 2.2 kb *Xba*I fragment of pZE12-luc, following a previously published protocol (Urban and Vogel, 2007). Plasmid pNH6 contains the *casABCDE* operon (PCR amplified with pre-phosphorylated primer NH193 and primer NH194 (Table S2)) inserted by blunt end and EcoRI cloning into vector pCU01 (pBAD-TOPO vector derivative), as described (Unoson and Wagner, 2008). Plasmid pUC18-IGLB was described before (Pul *et al.*, 2010).

Microarray

E. coli K12 MG1655 was transformed with plasmid pKEDR13 (Kan^R *lacI*^{q} P*tac leuO*) (Stratmann *et al.*, 2008) for expression of LeuO or with control vector pKESK22 (Kan^R *lacI*^{q} P*tac*). Exponential cultures were inoculated from fresh overnight cultures to an OD_{600} of 0.1 in LB supplemented with 25 µg ml^{-1} kanamycin. IPTG was added after 30 min of growth to a final concentration of 1 mM. After additional 60 minutes

the bacteria were harvested using Qiagen RNAprotect and used for RNA isolation using the Qiagen RNeasy MiniKit system. In brief, 1 ml of each culture (OD_{600} between 0.5 and 0.6) was used and processed according to the manufacturer's instructions including an on-column DNase I treatment. RNA quality was assayed by denaturing urea-PAGE and by measuring the ratio of absorption at 260/280 nm in a GeneQuant II spectrophotometer (Amersham). RNA concentration was determined by measuring UV light absorption at 260 nm. The procedure was carried out four times with independent clones.

Synthesis of cDNA (and cRNA) and hybridization of Affymetrix GeneChip® *E. coli* Genome 2.0 microarrays was carried out according to the manufacturer's instructions. In total, four independent RNA samples of each group (wildtype, *leuO* expressing and *leuO* deficient strains) were used. Data analysis was performed using Affymetrix Software. Fluorescence values were normalized to the GeneChip standard reference probes. Differential expression values were calculated as fold-change of *leuO* expressing samples compared to samples of LeuO-deficient control strains.

qPCR analysis of gene expression

qPCR analysis of *cas* gene transcript abundance was performed on cDNA synthesized using High Capacity Reverse Transcription Kit (Applied Biosystems) from RNA extracted by the hot-phenol method (Blomberg *et al.*, 1990) and DNaseI-treated using Turbo DNA-free kit (Ambion). 10 ml samples for RNA extraction were taken at OD_{600} ~0.5 from *E. coli* W3110, *E. coli* W3110 carrying pCA24N (*leuO*) and *E. coli* Δ*hns* (JW1225-2). When LeuO expression was induced, samples were taken 30 min after addition of 0.5 mM IPTG. The qPCR reactions were performed using Power SYBR green PCR master mix (Applied Biosystems) according to manufacturer's

instructions, and primers were designed using Primer Express 3.0 (Applied Biosystems). For the complete list of primers used see Table S3. As an internal control two primer pairs were designed against *casA*. The PCR reactions were performed on a 7300 Real Time PCR System (Applied Biosystems) and analyzed using 7300 System SDS Software 1.3 (Applied Biosystems). Fold-change of *cas* gene transcription was calculated using the relative quantification method with tmRNA as endogenous control and *E. coli* W3110 *cas* gene transcript abundance as calibrators. All PCR reactions were performed in six replicates. Control PCRs without template or without cDNA (produced by standard cDNA synthesis but excluding reverse transcriptase) were performed to monitor general contamination levels and genomic DNA contamination of RNA extracts, respectively.

Northern Blotting

Total RNA was extracted at the OD_{600} indicated using TRIZOL reagent (Invitrogen) according to the manufacturer's protocol. Expression of *casABCDE* from pNH6 was induced at an OD_{600} of 0.5 by adding 0.2% of arabinose for 15 min. 10 µg of total RNA was denatured at 95°C with an equal volume of formamide loading dye, FD (90% formamide, 15 mM EDTA, 0.05% bromophenol blue and 0.05% xylene cyanol), and subsequently separated on an 8% denaturing polyacrylamide gel. A ^{32}P-labeled pUC19 DNA/*Msp*I ladder (Fermentas) was used as size marker. The RNA was electrotransferred to Nylon N+ membranes (GE Healthcare) at 10 V for 15h. Transfer was performed in a BIORAD blotting chamber in 1xTBE buffer at 4°C followed by drying of the membrane and UV-crosslinking. Prehybridization was carried out for 2–4 h at 42°C in 15 ml prehybridization buffer (5x SSC, 5x Dernhardt, 0.05 M sodium phosphate pH 6.7, 1% dextran sulphate, 0.1% SDS) together with 75 µl herring sperm

DNA (20 mg ml^{-1}). Hybridization was carried out overnight at 42°C in the same buffer lacking herring sperm DNA but containing [γ-^{32}P]-ATP-labeled oligonucleotide probe NH30 (Table S2) specific for spacer 2 of the CRISPR1 locus. The probe was labeled with [γ-^{32}P]ATP (40 pmol DNA, 10x kinase buffer, T4 polynucleotide kinase (PNK, Ambion), [γ-^{32}P]ATP) by incubation at 37°C for 45 min. Prior to hybridization, the probe was purified over a G-50 column (GE Healthcare). Membranes were washed once for 20 min at 60°C in 2xSSC, 0.5% SDS and once for 20 min in 0.5x SSC, 0.5% SDS. Signals were quantified in a Molecular Dynamics PhosphorImager model 400S with ImageQuant software version 4.2a (Molecular Dynamics).

Electrophoretic Mobility Shift Assay

E. coli RNAP, LeuO and H-NS were purified according to published procedures (Pul *et al.*, 2010; Stratmann *et al.*, 2008). The IGLB fragment (position -1 to -414, relative to the first nucleotide of the *casA (ygcL)* start codon) was obtained by *EcoR*I/*Hinc*II or *BamH*I/*Sac*I digestion of plasmid pUC18-IGLB. Purified DNA fragments were end-labelled by Klenow (Promega) and [α-^{32}P]-dATP. Binding reactions with the indicated amounts of protein were performed in 50 mM Tris-HCl, pH 7.4, 70 mM KCl, 15 mM NaCl, 1 mM EDTA, 10 mM β-mercaptoethanol at a final heparin concentration of 20 ng μl^{-1}. Complexes were separated on native 5% (w/v) polyacrylamide gels and visualized by autoradiography as described (Pul *et al.*, 2010).

Footprint analyses

DNase I footprinting of free DNA and DNA-protein complexes was performed as described (Pul *et al.*, 2010). Formation of open RNAP-promoter complexes was

analyzed by $KMnO_4$ modification of single-stranded nucleotides within the transcription bubble. 40 µl RNAP-DNA complexes were treated with 160 mM $KMnO_4$ for 2 min at 30°C. The reaction was stopped by addition of 5.3 µl β-mercaptoethanol and 5.3 µl 500 mM EDTA and the samples were ethanol precipitated after phenol/chloroform extraction. Pellets were dissolved in 10% piperidine and incubated at 90°C for 30 min. After two rounds of washing with distilled water followed by lyophilizing, the pellets were dissolved in 50 µl distilled water and precipitated with ethanol. Cleavage products were separated on 10% denaturing polyacrylamid gels and visualized by autoradiography. The following protocol was used in footprint experiments with more than one protein: LeuO or the protein-free buffer, and H-NS or the respective buffer, were incubated with the template DNA for 5 min at 30°C. Next RNAP or the RNAP dilution buffer was added and incubated for another 10 min. Finally heparin was added to a final concentration of 200 ng μl^{-1} with a further incubation at 30°C for 5 min. An aliquot of this solution was loaded on a native gel to verify complex formation and the remaining solution was used for the different footprint experiments.

Primer extension analysis

Primer extension reactions with 25 µg total RNA hybridized to a radiolabeled specific *cas* primer oligonucleotide (5'-ATACAATTAATCTATACATATATTAAGATG-3') were performed with AMV reverse transcriptase (Promega) as described (Afflerbach *et al.*, 1998).

Phage Lambda infection studies

Host sensitivity to phage infection was tested using a virulent phage Lambda (λ_{vir}), as before (Brouns *et al.*, 2008). The host strains for infection were either *wt E. coli* K12 W3110, *E. coli* K12 W3110 Δ*hns*, or the engineered *E. coli* K12 W3110 strains (*E. coli* K12Δ*CRISPRI::CRISPRJ3* and *E. coli* K12Δ*CRISPRI::CRISPRR44*) (Table S4). The sensitivity of the host to infection was calculated as the efficiency of plaquing (the plaque count ratio of a strain containing an anti-Lambda CRISPR to that of the strain containing an non-targeting CRISPR) (Brouns *et al.*, 2008).

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Figure Legends

Fig. 1

LeuO and H-NS regulate *cas* gene expression. **A)** Schematic illustration of the CRISPR/Cas locus in *E. coli* K12 that consists of 8 cas genes (*cas3* (*ygcB*), *casA* (*ygcL* or *cse1*), *casB* (*ygcK* or *cse2*), *casC* (*ygcJ* or *cse4*) *casD* (*ygcI* or *cas5e*) *casE* (*ygcH* or *cse3*), *cas1* (*ygbT*) and *cas2* (*ygbF*)) and a downstream CRISPR locus containing 12 spacers and 13 repeats (CRISPR I). The *cas3,* anti-cas3 (anti-P*cas*), *casA* (P*cas*) and CRISPR I promoter are indicated with an arrow (Pul *et al.*, 2010). **B)** qPCR analysis of *cas* gene transcript abundance in *E. coli* Δ*hns* and *E. coli* W3110 expressing *leuO* (induced or non-uninduced). Fold changes are given as compared to *wt E. coli* W3110 expression levels. Error bars indicate one standard deviation.

Fig. 2

Formation of mature crRNA. **A)** Northern analysis of total RNA from *wt E. coli* K12 and *wt E. coli* K12 + *casABCDE* (pNH6) using the single stranded spacer sequence NH30 (Table S2) as a probe. **B)** Northern analysis as in (A) of total RNA from *E. coli* K12 Δ*hns* and **C)** *wt E. coli* K12 expressing *leuO* from a plasmid (pNH41) with an OD_{600} of 0.5, 1 and 3.0. M, size marker (pUC19/*Msp*I ladder). *E. coli* K12 Δ*casA* (JW2730) serves as a control and marker for mature crRNA.

Fig. 3

H-NS and LeuO binding to the DNA region upstream of *casA* (the IGLB fragment). **A)** Electrophoretic Mobility Shift Assay (EMSA) of the IGLB fragment with LeuO and

H-NS, either alone (lanes 5-7) or with pre-bound LeuO and subsequent addition of H-NS (lanes 8-11) or pre-bound H-NS and subsequent addition of LeuO (lanes 12-15). **B)** DNase I footprint of IGLB in the presence of either H-NS or LeuO or both. LeuO was pre-incubated with the reaction mixture. The 2 main regions protected by LeuO are indicated. The ladder indicates the IGLB coordinates relative to the *casA* start codon, indicated in **C)** the sequence and coordinates of IGLB, with the H-NS and LeuO binding sites indicated with boxes. See also Fig. 4A for footprints showing the LeuO binding region I.

Fig. 4

Effects of LeuO and H-NS on RNA polymerase open complex formation at the IGLB fragment. **A)** and **B)** show either DNase I (lanes 1-14) or $KMnO_4$ (lanes 1'-14') footprint analyses of the non-template strand of IGLB after binding of RNA polymerase, H-NS or LeuO, either individually or in combination. The numbers above the lanes indicate the order of addition of the respective proteins. The *casA* promoter (P*cas*) (I) and the anti-*cas3* promoter (anti-P*cas*) (II) are indicated. The arrowheads indicate the nucleotides within open complexes at the promoter sites. **C)** and **D)** show the binding of RNA polymerase, H-NS or LeuO, either individually or in combination. C) shows Electrophoretic Mobility Shift Assays of the samples in (A) and (B). The numbers above the lanes indicate the order of addition of the respective proteins. D) shows Electrophoretic Mobility Shift Assays of the samples in (E). The positions of the free DNA and complexed DNA are given on the left. The retardation gels (C) and (D) are not standard retardation gels but controls for the footprint complexes to see if no major decomposition has occurred. Because complexes are kept at 30°C for some time under RNAP binding conditions these gels are sometimes

different from standard retardation experiments (e.g. Fig. 3A). **E)** Shows a similar analysis as in (A) and (B) for the template strand of IGLB (DNase I footprint lanes 1-8; $KMnO_4$ footprint lanes 1'-8'). The protein concentrations used are H-NS (1 μM), LeuO (1 μM) and RNA polymerase (50 nM).

Fig. 5

Effect of H-NS and LeuO on CRISPR-based resistance *in vivo*. **A)** A synthetic CRISPR with one spacer (J3) targeting phage Lambda on a plasmid (pWUR564) is introduced in *E. coli* K12Δ*hns*, *E. coli* K12, and complemented *E. coli* K12Δ*hns* expressing *hns* from the pHOP11 plasmid. Phage resistance is monitored by determining the efficiency of plaquing (EOP) after challenge with virulent Lambda phage. **B)** The effects of *leuO* (pKEDR13) and *cas3* (pWUR608) expression on phage resistance is monitored in *E. coli* K12Δ*CRISPRI::CRISPRJ3* (indicated as J3) **C)** The effect of introducing the *casABCDE12* (pWUR607), *cas3* or a CRISPR on phage resistance is monitored in *E. coli* K12Δ*CRISPRI::CRISPRJ3* (indicated as J3).

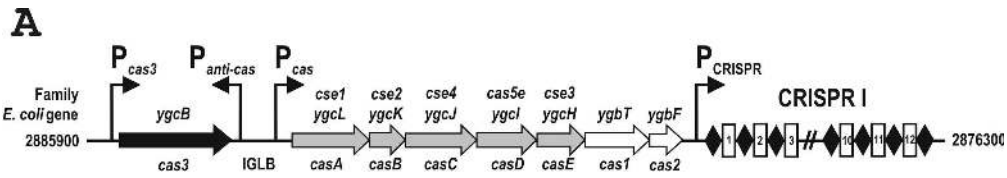
Table 1

Microarray analysis of activation of *cas* genes by LeuO.

Microarray analysis of activation of *cas* genes by LeuO				
	pLeuO / wt		Δ*leuO* / wt	
gene	fold-change[a]	*p* value[b]	fold-change[a]	*p* value[b]
cas3	1.0	n. s. 0.93	1.1	n. s. 0.76
casA	65.4	< 0.05	-1.2	n. s. 0.08
casB	30.0	< 0.05	-1.1	n. s. 0.20
casC	24.8	< 0.05	1.0	n. s. 0.81
casD	17.5	< 0.05	1.1	n. s. 0.68
casE	15.4	< 0.05	-1.2	n. s. 0.31
cas1	8.8	< 0.05	1.2	< 0.05
cas2	5.4	< 0.05	1.1	n. s. 0.34

[a] The fold change of *cas* genes expression was determined by microarray analysis. pLeuO / wt indicates the ratio of *cas* transcripts detected upon overexpression of LeuO (using plasmid pKEDR13) as compared to wildtype *E.coli* K12 (transformed with the empty vector plasmid pKESK22). Δ*leuO* / wt indicates the ratio of *cas* transcripts detected in a Δ$leuO_{FRT}$ mutant as compared to wildtype.

[b] n.s. is not significant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P cas3
P anti-cas
P cas
P CRISPR
Family
E. coli gene
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ygcB
cas3
IGLB
cse1
ygcL
casA
cse2
ygcK
casB
cse4
ygcJ
casC
cas5e
ygcI
casD
cse3
ygcH
casE
ygbT
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CRISPR I
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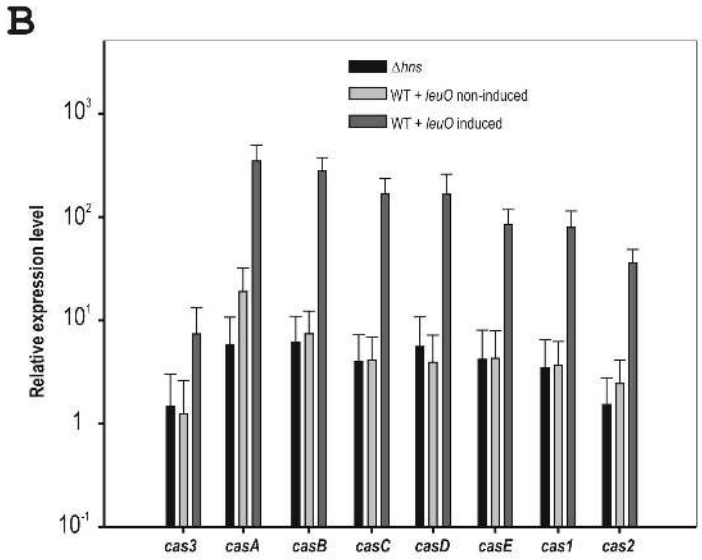
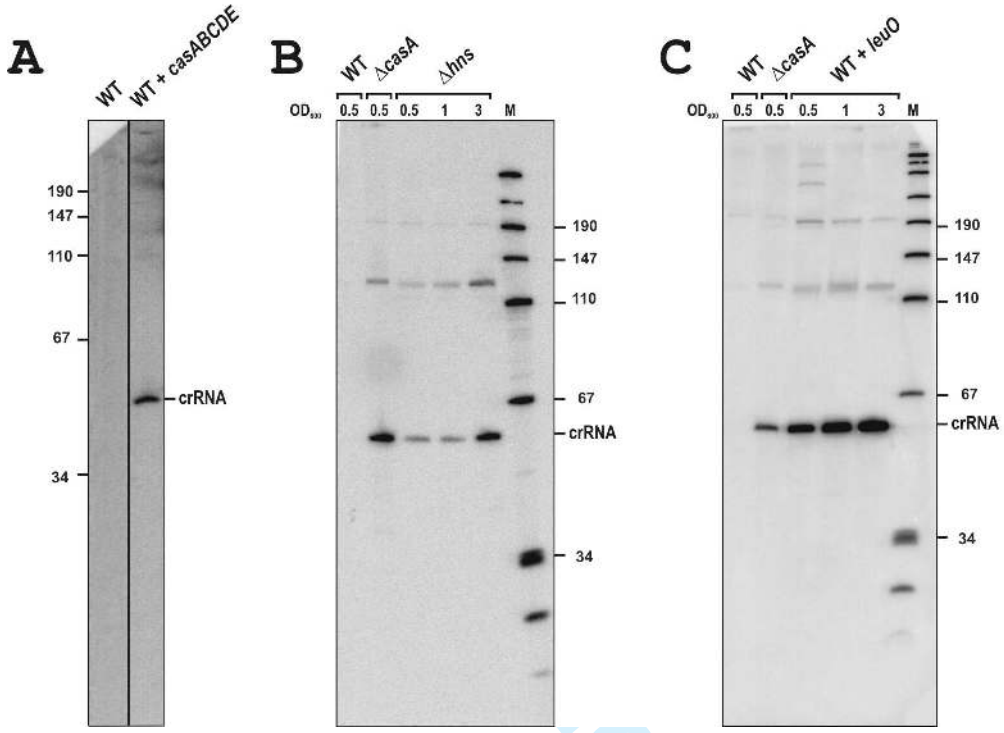
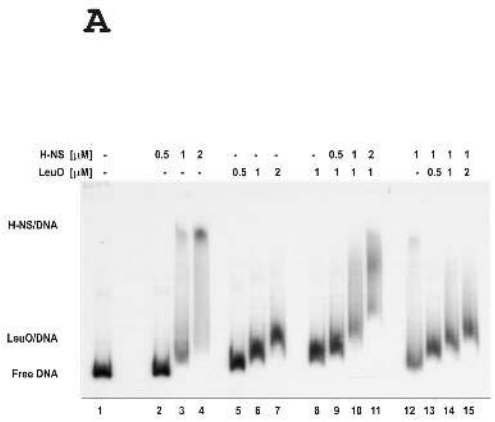
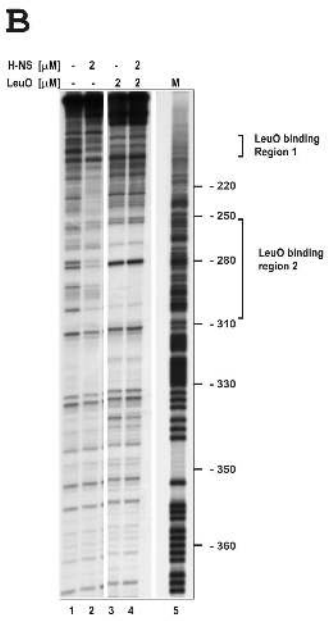

B
Δhns
WT + leuO non-induced
WT + leuO induced
Relative expression level
10^3
10^2
10^1
1
10^-1
cas3
casA
casB
casC
casD
casE
cas1
cas2


A
WT
WT + casABCDE
190
147
110
67
34
crRNA
B
WT
ΔcasA
Δhns
OD600
0.5
0.5
0.5
1
3
M
190
147
110
67
crRNA
34
C
WT
ΔcasA
WT + leuO
OD600
0.5
0.5
0.5
1
3
M
190
147
110
67
crRNA
34





C

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CTTCGGGAAT GATTGTTATC AATGACGATA ATAAGACCAA TAACGGTTTA TCCCTACTTA AGTAGGGAAG GTGCACAATG
GAAGCCCTTA CTAACAATAG TTACTGCTAT TATTCTGGTT ATTGCCAAAT AGGGATGAAT TCATCCCTTC CACGTGTTAC

   -330       -320       -310       -300       -290       -280       -270       -260
TACATCTTCT TTTAATTTCC CGGTATGAGA TTTTATATTC ACAGTATGAA TATTTTATGT AATAAAATTC ATGGTAATTA
ATGTAGAAGA AAATTAAAGG GCCATACTCT AAAATATAAG TGTCATACTT ATAAAATACA TTATTTTAAG TACCATTAAT
                                 LeuO binding region II
                                                    +1 anti-Pcas      +1 Pcas
   -250       -240       -230       -220       -210       -200       -190
TTATAACTAA AAGTTTCTTT AATAATAAAA CGAATAACTT GCAGATTTGA AATGCATGCA TTATTGTCTT TAAACAATTC
AATATTGATT TTCAAAGAAA TTATTATTTT GCTTATTGAA CGTCTAAACT TTACGTACGT AATAACAGAA ATTTGTTAAG
                     H-NS nucleation site
   -170       -160       -150       -140       -130       -120       -110       -100
AACACATCTT AATATATGTA TAGGTTAATT GTATTAAACC AATGAATATA TTTTTGCAGT GAATGTGATT ATTGAATTAA
TTGTGTAGAA TTATATACAT ATCCAATTAA CATAATTTGG TTACTTATAT AAAAACGTCA CTTACACTAA TAACTTAATT
                                 LeuO binding region I
   -90        -80        -70        -60        -50        -40        -30        -20
TTACGCCGTA TTTTTTCTTT GTTTTTACCG ATAACGGAAG TGTGCCGACG TATAGAAATG CAGGAGAAAT GTCGGAGCAT
AATGCGGCAT AAAAAAGAAA CAAAAATGGC TATTGCCTTC ACACGGCTGC ATATCTTTAC GTCCTCTTTA CAGCCTCGTA

   -10        -1
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TACTTCCTCT TGTT
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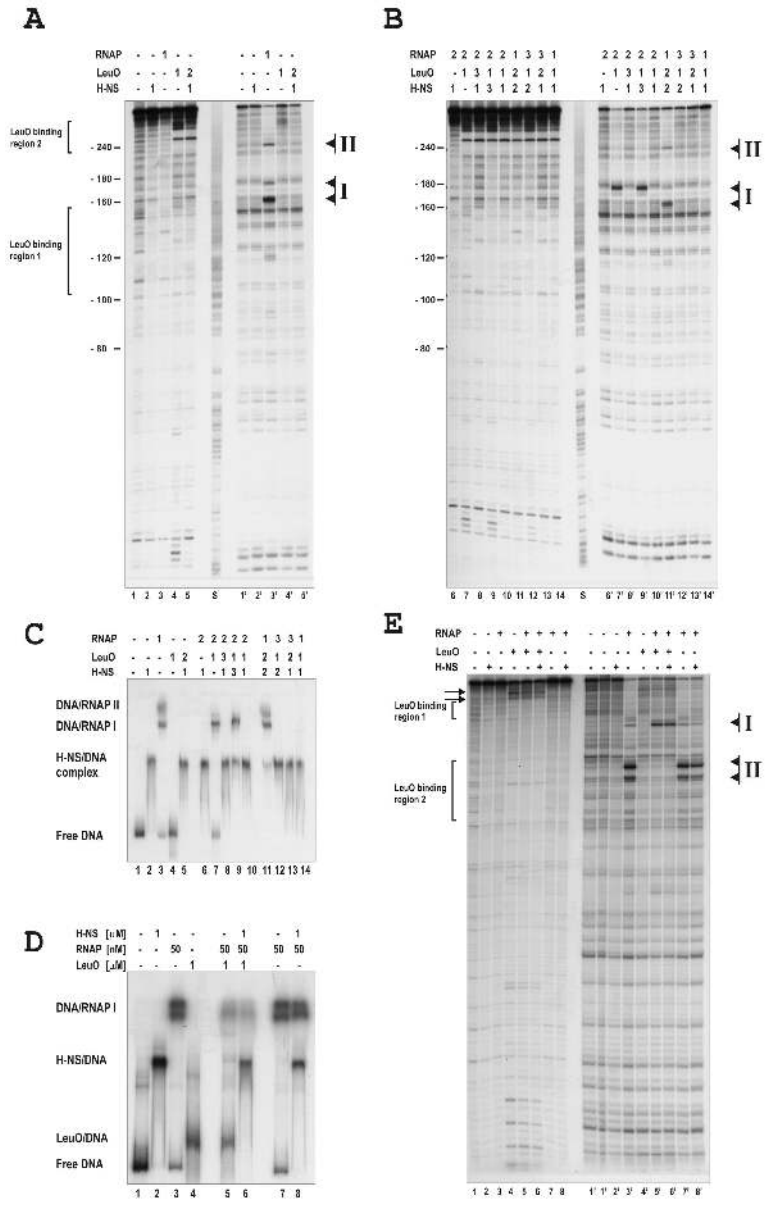
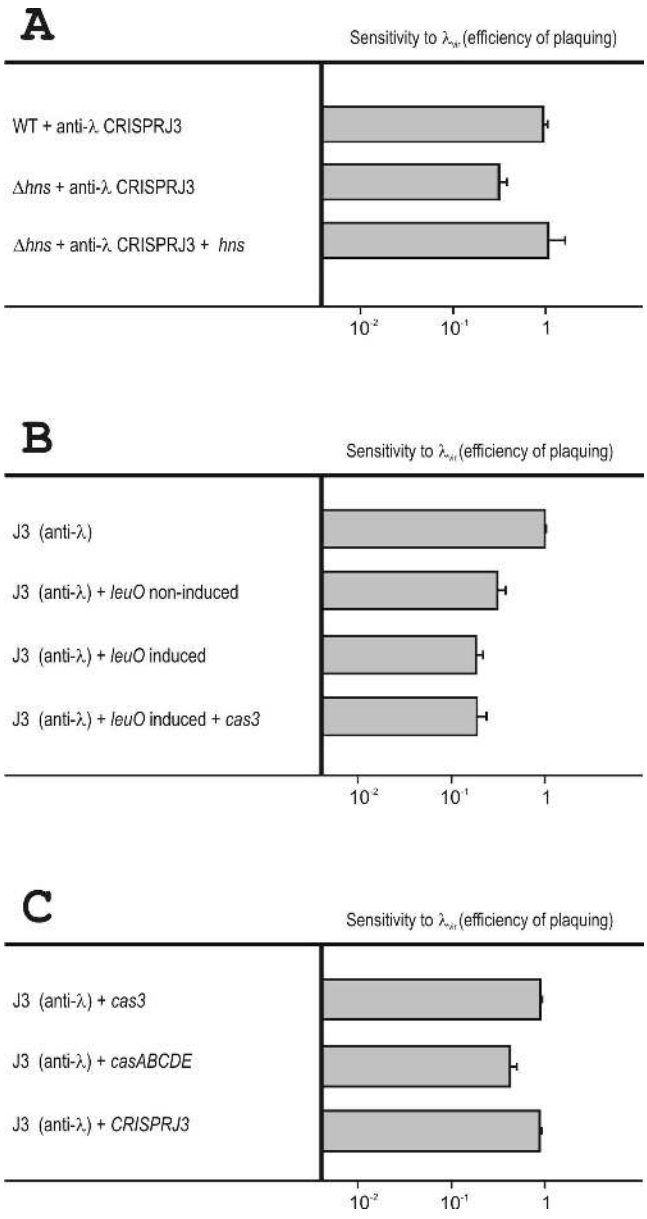

A
RNAP
LeuO
H-NS
LeuO binding region 2
LeuO binding region 1
II
I
B
C
DNA/RNAP II
DNA/RNAP I
H-NS/DNA complex
Free DNA
D
H-NS [nM]
RNAP [nM]
LeuO [µM]
DNA/RNAP I
H-NS/DNA
LeuO/DNA
Free DNA
E


A
Sensitivity to λvir (efficiency of plaquing)
WT + anti-λ CRISPRJ3
Δhns + anti-λ CRISPRJ3
Δhns + anti-λ CRISPRJ3 + hns
10-2
10-1
1
B
Sensitivity to λvir (efficiency of plaquing)
J3 (anti-λ)
J3 (anti-λ) + leuO non-induced
J3 (anti-λ) + leuO induced
J3 (anti-λ) + leuO induced + cas3
10-2
10-1
1
C
Sensitivity to λvir (efficiency of plaquing)
J3 (anti-λ) + cas3
J3 (anti-λ) + casABCDE
J3 (anti-λ) + CRISPRJ3
10-2
10-1
1