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## Ler interdomain linker is essential for anti-silencing activity in enteropathogenic *Escherichia coli*

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### Abstract

Enteropathogenic *Escherichia coli* (EPEC) expresses a type III secretion system (T3SS) required for pathogenesis. Regulation of the genes encoding the T3SS is complex; two major regulators control transcription, the silencer H-NS, and the related H-NS-like protein Ler. Our laboratory is interested in understanding the molecular differences that distinguish the anti-silencer Ler from H-NS, and how Ler differentially regulates EPEC virulence genes. Here, we demonstrate that mutated Ler proteins either containing H-NS  $\alpha$ -helices 1 and 2, missing from Ler, or truncated for the 11 aa C-terminal extension compared with the related H-NS protein, did not appreciably alter Ler function. In contrast, mutating the proline at position 92 of Ler, in the conserved C-terminal DNA binding motif, eliminated Ler activity. Inserting 11 H-NS-specific amino acids, 11 alanines or 6 alanines into the Ler linker severely impaired the ability of Ler to increase *LEE5* transcription. To extend our analysis, we constructed six chimeric proteins containing the N terminus, linker region or C terminus of Ler in different combinations with the complementary domains of H-NS, and monitored their *in vivo* activities. Replacing the Ler linker domain with that of H-NS, or replacing the Ler C-terminal, DNA binding domain with that of H-NS eliminated the ability of Ler to increase transcription at the *LEE5* promoter. Thus, the linker and C-terminal domains of Ler and H-NS are not functionally equivalent. Conversely, replacing the H-NS linker region with that of Ler caused increased transcription at *LEE5* in a strain deleted for *hns*. In summary, the interdomain linker specific to Ler is necessary for anti-silencing activity in EPEC.

### INTRODUCTION

In order for bacterial pathogens to cause disease, they must possess and properly express virulence factors. For the diarrhoeal pathogens enteropathogenic and enterohaemorrhagic *Escherichia coli* (EPEC and EHEC), the causative agents of acute diarrhoea in infants and haemorrhagic colitis, respectively, a type III secretion system (T3SS) delivers effector proteins into the host cell cytosol via an assembled molecular syringe. The alteration of host

cell signalling events leads to the formation of hallmark attaching and effacing (AE) intestinal lesions. EPEC and EHEC bacteria acquired the genes encoding their respective T3SSs by horizontal gene transfer, and by multiple, independent events placing these genes at different tRNA loci, multiple lineages of both pathotypes have arisen (Rumer *et al.*, 2003).

The extent to which EPEC and EHEC bacteria affect their host via their T3SS is profound. At least 39 proteins are injected into the host cell cytosol through this apparatus (Garmendia *et al.*, 2005; Tobe *et al.*, 2006). En route to causing diarrhoea, these effector molecules alter host cell signalling events and loosen tight junctions, leading to cytoskeletal rearrangement and the hallmark pedestals associated with intimate adherence to the intestinal epithelial cell membrane (for review, see Clarke *et al.*, 2003; Kaper *et al.*, 2004). Several of these effector proteins are found within the locus of enterocyte effacement (LEE) encoding the T3SS, but the majority are encoded outside this locus, many within cryptic prophages (Garmendia *et al.*, 2005; Tobe *et al.*, 2006). Specifically, Tir, EspF, EspG, EspH, EspZ and Map are encoded within the LEE, but NleA, NleD, EspG2, EspJ and Cif, for example, are encoded outside the LEE.

Although the mechanism by which the effector molecules encoded outside the LEE are regulated remains mostly unexplained, much information exists regarding control of the LEE pathogenicity islands (PAIs) of EPEC and EHEC (for review, see Mellies *et al.*, 2007a). Among a cadre of regulatory molecules and their perceived environmental inputs, two proteins, Ler and H-NS, have emerged as central in the coordinated expression of the T3SS of these two diarrhoeal pathogens. From genetic and biochemical evidence, H-NS has been shown to silence multiple operons of the EPEC LEE, including *LEE1* (encoding Ler), *LEE2*, *LEE3* and *LEE5* (Bustamante *et al.*, 2001; Haack *et al.*, 2003; Umanski *et al.*, 2002). The nucleoid-associated H-NS protein is thought to bind non-specifically to AT-rich intrinsically curved DNA and in most cases compacts the DNA. However, a specific DNA binding sequence was recently proposed for H-NS (Bouffartigues *et al.*, 2007). Ler, a member of the H-NS family of proteins, relieves transcriptional silencing of the EPEC *LEE2*, *LEE3*, *LEE4* and *LEE5* operons as well as *espG*, *escD* and *map* of the LEE (Bustamante *et al.*, 2001; Elliott *et al.*, 2000; Haack *et al.*, 2003; Li *et al.*, 2004; Mellies *et al.*, 1999; Sánchez-SanMartín *et al.*, 2001; Sperandio *et al.*, 2000; Umanski *et al.*, 2002).

The importance of Ler to EPEC and EHEC pathogenesis is illustrated by the numerous inputs that control *LEE1* transcription. Quorum-sensing signals (Sperandio *et al.*, 1999, 2001), integration host factor (IHF) (Friedberg *et al.*, 1999), Fis (Goldberg *et al.*, 2001), BipA (Grant *et al.*, 2003), PerC and PerC-like molecules (Gomez-Duarte & Kaper, 1995; Iyoda & Watanabe, 2004; Porter *et al.*, 2005), GrIRA (Barba *et al.*, 2005; Deng *et al.*, 2004; Sharp & Sperandio, 2007) and GadX (Shin *et al.*, 2001), as well as a number of environmental cues, including temperature, pH, iron, ammonium and calcium, are known to control Ler expression *in vivo* either directly or indirectly (Beltrametti *et al.*, 1999; Ide *et al.*, 2003; Kenny & Finlay, 1995; Kenny *et al.*, 1997). Thus, multiple regulatory proteins and multiple environmental signals control the AE phenotype of EPEC and EHEC bacteria through the regulation of Ler.

To date, Ler is the only known H-NS-like molecule that relieves transcriptional silencing and our laboratory seeks to understand the mechanism by which Ler functions. Data indicate that Ler stimulates transcriptional activity by disrupting H-NS-dependent nucleoprotein complexes (Bustamante *et al.*, 2001; Haack *et al.*, 2003; Sperandio *et al.*, 2000; Umanski *et al.*, 2002). Ler and the related H-NS molecules of *E. coli* and *Salmonella* are ~15 kDa proteins; Ler and H-NS of *Salmonella* share 24% amino acid identity and 44% amino acid similarity, mostly over their C-terminal DNA-binding domains (Sperandio *et al.*, 2000). H-NS possesses an N-terminal,  $\alpha$ -helical domain, which contains a coiled-coil motif involved in dimerization (Bloch *et al.*, 2003). Substitution of the leucine with a proline at position 33 in  $\alpha$ -helix 3 eliminates DNA binding and dimer formation of H-NS (Ueguchi *et al.*, 1997). Similarly, I20R and L23R mutations within the predicted coiled-coil region of Ler eliminate the ability of this protein to bind to *LEE2* regulatory DNA as well as to relieve transcriptional silencing at this locus (Sperandio *et al.*, 2000).

Though some molecular similarities between H-NS and Ler have been experimentally established, such as the shared coiled-coil domain in their N termini (Sperandio *et al.*, 2000), the precise molecular mechanism of Ler action remains unknown. In this study we investigated the molecular aspects of Ler that distinguish this protein from the related silencer H-NS and enable Ler to increase, as opposed to silence, transcription. We demonstrate that the interdomain linker region of Ler, connecting the N-terminal,  $\alpha$ -helical to the C-terminal, DNA binding domain, is necessary for proper function.

## METHODS

### Bacterial strains, plasmids and phages

The plasmids, strains and phages used in this study are listed in Table 1. Strains were grown at 37 °C with aeration in Luria–Bertani (LB) medium supplemented with the appropriate antibiotic at the following concentrations: chloramphenicol (30  $\mu\text{g ml}^{-1}$ ), kanamycin (50  $\mu\text{g ml}^{-1}$ ), tetracycline (15  $\mu\text{g ml}^{-1}$ ) or ampicillin (100  $\mu\text{g ml}^{-1}$ ). Molecular manipulations of plasmid DNA were performed using standard methods.

### Generation of plasmids containing *lacZ* fusions

The construction of plasmid pKMTIR3 containing EPEC strain E2348/69 *LEE5* regulatory DNA spanning positions –303 to +172 in relation to the transcriptional start site (+1) was described previously (Haack *et al.*, 2003). Plasmids pJLM166 and pJLM172 for constructing the *LEE2-lacZ* (–218 to +670) and *LEE3-lacZ* (–398 to +404) fusions, respectively, were also previously described (Mellies *et al.*, 1999). For plasmid pKHpro1, the regulatory region of *proU* (–398 to +404) was amplified with primers proUregF and proUregR, digested with *EcoRI* and *BamHI*, and cloned into the reporter gene vector pRS551, containing the promoterless *lacZYA* operon (Simons *et al.*, 1987). All oligonucleotide PCR primers for molecular manipulations are listed in Table 2.

### Construction of chromosomal single-copy *LEE-lacZ* and *proU-lacZ* transcriptional fusions

Single-copy chromosomal *lacZ* fusions were constructed by homologous recombination between  $\lambda$ IRS45 and plasmids containing *LEE5*, *proU*, *LEE2* and *LEE3* regulatory

fragments as described by Mellies *et al.* (1999). For strains KH4105, KH4106, KH4102 and KH4103, phage lysate was used to transduce KH4100, a spontaneous *ara*<sup>+</sup> MC4100 mutant, selecting for kanamycin resistance. To generate isogenic *hns* mutants, KH4105, KH4106, KH4102 and KH4103 were transduced with P1 *vir* lysate from strain HN4104, selecting for tetracycline resistance. These mutants were screened for a red appearance on MacConkey agar containing 1% salicin and possessing a mucoid phenotype, confirming deletion of *hns* (Mukerji & Mahadevan, 1997).

### Generation of Ler proteins with altered N and C termini

Plasmid pKLHB01, containing wild-type (wt) *ler*, was generated by PCR amplification of template plasmid pSE1100 (Mellies *et al.*, 1999) with primer pair LerForward and KLHlerwtR containing *Xba*I and *Hind*III sites, respectively. This fragment was directionally ligated into pBAD33 downstream of an arabinose-inducible promoter. Plasmid pKHB1123, containing wt *hns*, was similarly constructed with primers 5hns<sub>swap</sub> and 3hns<sub>swap</sub>, and directionally ligated into pBAD33.

Plasmid pMHB1, encoding the Ler protein with the  $\alpha$ -helices 1 and 2 of H-NS (MSEALKILNNIRT LRAQAREC) fused to its N terminus, was created by PCR mutagenesis using primers NTERMMUT and KLHwtlerR containing *Xba*I and *Hind*III sites, respectively, and the Ler-encoding plasmid pSE1100 as template (Mellies *et al.*, 1999). The amplicon was directionally cloned into the pBAD33 vector (Guzman *et al.*, 1995), containing an arabinose-inducible promoter digested with the same enzymes. Plasmid pKLHB13, in which the 33 base pairs encoding the 11 aa C-terminal extension TEEIIP LKNI are deleted, when comparing Ler to the H-NS protein (see Fig. 1), was created by PCR mutagenesis using primers LerForward and KLHlermutR. As above, the restriction sites *Xba*I and *Hind*III were used to directionally clone the DNA fragment into pBAD33 under the control of the P<sub>BAD</sub> promoter.

Plasmid pLER92A containing a Ler protein with an alanine substituted for the conserved proline at position 92 (position 115 in H-NS) was created by PCR mutagenesis using primers LerForward and 3'lerP115A, with plasmid pSE1100 as template, and directional cloning into the pBAD33 vector using the *Xba*I and *Hind*III restriction sites, as described above.

### Generation of Ler linker insertion mutations

Plasmid pFL08, encoding Ler with an insertion of amino acids ELLNSLA AVKS at position 67, was generated by overlap extension mutagenesis using primer pairs LerForward and ELLF, and KLHlerwtR and ELLR, and plasmid pSE1100 as template (Mellies *et al.*, 1999). The two partially complementary fragments were subjected to a second amplification using LerForward and KLHlerwtR as primers. Plasmids pFL09 and pFL10, encoding Ler with six and eleven alanines, respectively, inserted at position 67, were constructed in an analogous manner, using primers 6AF and 6AR and 11AF and 11AR as the mutagenic primers, respectively. Amplification products obtained in this way were inserted into pBAD33 as *Xba*I/*Hind*III fragments.

## Generation of Ler–H-NS chimeras

For Ler–H-NS chimeras, individual PCR-amplified fragments corresponding to each domain of Ler were generated with the following primer pairs. The LerForward and lerRnar primers were used for the N-terminal fragment, amino acids 7–31, containing the coiled-coil domain defined by mutational analysis (Fig. 1; Sperandio *et al.*, 2000). (For the chimera analyses, the N-terminal methionine at position 7 (accession number AF022236) corresponds to a predicted ribosome-binding site.) Primers lerFnar and lerRsal were used to construct the linker fragment, amino acids 32–78, and lerFsal and KLHlerwtR primers were used for the Ler C-terminal fragment, amino acids 79–123. The LerForward and lerRsal primers were used for the N-terminal and linker fragments, amino acids 7–78, and primers lerFnar and KLHlerwtR for the linker and C-terminal fragments, amino acids 32–123.

For H-NS, analogous fragments were generated with the following primer pairs: 5hnsswap/hnsRnar for the N-terminal fragment, amino acids 1–47; hnsFnar and hnsRsal for the linker fragment, amino acids 48–101; hnsFsal and 3hnsswap for the C-terminal fragment, amino acids 102–138; 5'hnsswap and hnsRsal for the N-terminal and linker fragments, amino acids 1–101; hnsFnar and 3hnsswap for linker and C-terminal fragments, amino acids 49–138. Fragments were cloned into pCR2.1TOPO (Invitrogen) for DNA sequencing.

For chimera C1 (pKHB1527), the *NarI*- and *SalI*-digested linker fragment of H-NS was ligated to the *NarI*-digested N-terminal and *SalI*-digested C-terminal fragments of Ler. For the analogous C2 (pKHB1163) chimera, the *NarI*- and *SalI*-digested linker fragment of Ler was sequentially ligated to the *NarI*-digested N-terminal and *SalI*-digested C-terminal fragments of H-NS. For chimeras C4 (pKHB1047) and C3 (pKHB1083), the *NarI*-digested N-terminal and linker fragment of H-NS and Ler, respectively, were ligated to the *NarI*-digested C-terminal fragment from Ler and H-NS, respectively. Chimeras C5 (pKHB1167) and C6 (pKHB1523) combined the *SalI*-digested linker and N-terminal fragment from Ler and H-NS with the *SalI*-digested N-terminal fragment from H-NS and Ler, respectively. Following ligation of individual domain fragments, the appropriate external primer pairs complementary to either Ler or H-NS were used to amplify the entire chimeric coding region, which was inserted into pCR2.1 TOPO for verification by restriction digestion and DNA sequencing. Chimeric coding regions were then digested with *XbaI*/*HindIII* and inserted into the *XbaI*/*HindIII* site of pBAD33.

## DNA sequencing analysis

All plasmid constructs were confirmed to be correct by DNA sequencing analysis performed at the Vollum Institute at the Oregon Health Sciences University.

## Enzymic assays

Strains KH4105 and KH4115 were transformed with plasmids pKLHB13, pMHB1 and pLER92A, as well as the control plasmids pKLHB01 (containing wt *ler*), pKH1123 (wt *hns*) and pBAD33. Similarly, strains KH4105, KH4115, KH4106 and KH4116 were transformed with plasmids pKHB1527, pKHB1163, pKHB1083, pKHB1047, pKHB1167 and pKHB1523 (chimeras C1–C6). Control plasmids pKLHB01, pKH1123 and pBAD33 were transformed into strains KH4106 and KH4116 as well. Cultures were grown at 37 °C in LB

supplemented with chloramphenicol and either 0.1% arabinose (induced expression of Ler molecules) or 0.2% glucose (repressed expression of Ler molecules) to OD<sub>600</sub> ~0.3–0.5 and then subjected to  $\beta$ -galactosidase assays, as described by Miller (1972).

To measure  $\beta$ -galactosidase activity derived from an E2348/69 *ler* deletion strain, the pKMTIR3 *LEE5-lacZ* fusion plasmid was transformed into SE796 and SE796 containing the following plasmids: pBAD33, pKLHB01, pKH1123, pFL08, pFL09, pKHB1527, pKHB1163, pKHB1047 and pKHB1167. Cultures were grown at 37 °C in LB supplemented with chloramphenicol and ampicillin to maintain the pBAD33 and pRS551-derived vectors, respectively. Assays were performed as described above. Statistical analysis (*t* test) for  $\beta$ -galactosidase assay values in Miller units was performed using JMP software (SAS Institute).

## RESULTS

### The Ler N terminus and C terminus

Ler is predicted to have a single  $\alpha$ -helix in the N-terminal domain [by the NNPREDICT Protein Secondary Structure Prediction software (<http://www.cmpchem.ucsf.edu/~nomi/nnpredict.html>)], whereas the H-NS molecules of *E. coli* and *Salmonella* are predicted to possess three  $\alpha$ -helices in this region. In H-NS,  $\alpha$ -helix 1 spans positions 1–8,  $\alpha$ -helix 2 spans positions 12–19, and  $\alpha$ -helix 3 spans positions 23–47 (Fig. 1; Dorman, 2004; Fang & Rimsky, 2008). N-terminal fragments of H-NS have been shown to exhibit dominant negative effects on transcriptional activity (Ueguchi *et al.*, 1996; Williams *et al.*, 1996), and thus we predicted that placing  $\alpha$ -helices 1 and 2 of H-NS on to the N terminus of Ler would alter its function. To test this prediction, we cloned a DNA fragment encoding a mutated Ler protein containing  $\alpha$ -helices 1 and 2 of H-NS (including the amino acids MSEALKILNNIRT LRAQAREC to position 21) under the control of P<sub>BAD</sub>, the arabinose-inducible promoter in pMHB1. The plasmid was transformed into the K-12-derived strain KH4105 containing a single copy of *LEE5* regulatory DNA, from EPEC strain E2348/69, spanning positions –303 to +172 in relation to the transcriptional start site (Sánchez-SanMartín *et al.*, 2001), fused to the *lacZ* reporter gene (Simons *et al.*, 1987). As expected, control experiments demonstrated that Ler expressed from plasmid pKLHB01 increased transcription at the *LEE5* promoter but not at *proU*, in strains wt and deleted for *hns*, whereas H-NS expressed from plasmid pKHB1123 silenced transcription at both *LEE5* and *proU* (Fig. 2a).

The mutated Ler protein containing the  $\alpha$ -helices 1 and 2 of H-NS expressed from plasmid pMHB1 increased transcriptional activity of the *LEE5-lacZ* fusion in a manner similar to that observed for the wt Ler protein (Fig. 2b), and did not alter expression of the *proU-lacZ* fusion under any of the conditions tested (data not shown). Therefore, we concluded that even gross alteration of the N terminus of Ler did not affect its ability to differentially stimulate transcription of LEE virulence genes.

The C termini of Ler and H-NS contain the core DNA binding motif TWTGXGRXP, where X is any amino acid (Bertin *et al.*, 1999; Dorman *et al.*, 1999). The Ler core DNA binding motif, indicated by the boxed region in Fig. 1, is TWSGVGRQP, a perfect match except for



the serine residue in the third position. The proline at position 116 of H-NS, at the furthest position in the binding motif is highly conserved amongst all H-NS molecules (Dorman, 2004, 2007; Fang & Rimsky, 2008). A P116A mutation abolishes the ability of H-NS to silence transcription *in vivo*, disrupts oligomerization activity (Spurio *et al.*, 1997) and eliminates the ability of the protein to distinguish curved from non-curved DNA (Spurio *et al.*, 1997). We therefore determined whether this conserved proline was necessary for Ler anti-silencing activity, and constructed an analogous mutation in Ler, P92A (Fig. 1). As predicted, this point mutation at the C terminus of the conserved DNA binding motif eliminated the ability of Ler to increase *LEE5-lacZ* transcriptional activity, in strains containing the chromosomal *hns* or deleted for this gene (Fig. 2b). We concluded that the proline in the predicted core DNA binding motif of Ler was necessary for increasing LEE transcriptional activity, and presumably plays an analogous role in both Ler and H-NS.

Ler contains an 11 aa extension on its C terminus that is not present in H-NS (Fig. 1). Based on the prediction that H-NS forms head-to-tail oligomers (Esposito *et al.*, 2002) and the knowledge that Ler binds over an extended region to regulate LEE virulence genes (Haack *et al.*, 2003), we predicted that this 11 aa, C-terminal extension was necessary for Ler to increase LEE transcriptional activity. To test this prediction, we cloned a DNA fragment encoding a mutated Ler protein missing the C-terminal amino acids TEEIIPLKNI under the control of P<sub>BAD</sub>, the arabinose-inducible promoter in pKLHB13, transformed into the K-12-derived strain KH4105 and KH4106, as described above.

In the presence of wt *hns* the truncated Ler protein encoded in the pKLHB13 plasmid increased  $\beta$ -galactosidase activity from the *LEE5-lacZ* fusion to a level similar to that observed for the wt Ler protein, ~1500 Miller units (compare Fig. 2a, b). In the KH4115 strain deleted for *hns*, the truncated Ler protein still increased  $\beta$ -galactosidase activity, from ~100 to 500 Miller units ( $P < 0.0001$ ), but not to ~2000 Miller units as observed for wt Ler under the same conditions (Fig. 2a). The truncated Ler protein in plasmid pKLHB13 did not alter expression of the *proU-lacZ* control fusion under any of the conditions tested (data not shown). We concluded that the 11 aa C-terminal extension found in Ler, but absent from the related H-NS protein, did not contribute to the ability of Ler to increase transcription from the *LEE5-lacZ* fusion, though the level of expression was reduced compared with wt Ler when assayed in a strain deleted for *hns*, ~500 Miller units. As a control, transcriptional activity derived from the *proU-lacZ* fusion increased in the strain deleted for *hns* independently of the presence of arabinose or Ler, and this activity was abolished when H-NS was supplied *in trans* (Fig. 2a). Thus, the 11 aa C-terminal extension might play a role, but is not essential for relieving repression by a non-H-NS factor acting at *LEE5*.

### Non-H-NS factor acting at *LEE5*

Our data indicate that, in addition to H-NS, a second negative-acting factor controls *LEE5* expression at host body temperature, 37 °C (Haack *et al.*, 2003; Fig. 2a). In Fig. 2(a), transcriptional activity derived from the *LEE5-lacZ* fusion strain was modestly de-repressed in the absence of the inducer arabinose, from ~50 to 100 Miller units ( $P = 0.002$ ), when comparing  $\beta$ -galactosidase activity in the presence of wt *hns* with that derived from the *hns* deletion strain. However, the *LEE5-lacZ* reporter gene fusion was still inducible by Ler, to

~2000 Miller units ( $P < 0.0001$ ), in the presence of arabinose in a strain containing an *hns* deletion. Previously published reports investigating Ler and H-NS control of the *LEE5* operon are consistent with these data, demonstrating evidence for a non-H-NS factor acting at *LEE5* in *E. coli* K-12 and EPEC strains (Haack *et al.*, 2003; Umanski *et al.*, 2002). Intriguingly, the mutated Ler protein lacking the 11 aa C-terminal extension in plasmid pKLHB13 exhibited differential ability to increase *LEE5* transcriptional activity depending on whether it was assayed in the presence or absence of the chromosomal *hns* gene (Fig. 2b). Because the mutated Ler protein in pKLHB13 only modestly increased activity in the strain deleted for *hns* in comparison with wt Ler expressed from the pKLHB01 plasmid, this result suggested that the truncated protein was deficient in counteracting the repressing activity of the second, unidentified factor. As a control, it was determined that the recombinant Ler protein expressed from the pKLHB13 plasmid did not affect transcriptional activity from the *proU-lacZ* control fusion in strains either wt for or deleted for *hns* (data not shown). These data indicated that at least two negative-acting factors silence *LEE5*, and that Ler is able to counteract the activities of both H-NS and the unidentified factor to increase transcriptional activity. Again, these data indicated that the unidentified factor, whether it is a protein or a specific nucleoid structure, is not unique to EPEC, but is also found in K-12-derived strains.

### The Ler linker

Stella *et al.* (2005) demonstrated that the H-NS linker is essential for the N-terminal and C-terminal domains to mediate tetramerization, and thus silencing, a first report for a function of the linker besides simply connecting the N-terminal and C-terminal domains. The central linker regions of H-NS and Ler are the least similar regions of the molecules and, coincidentally, 11 aa found in H-NS are missing from the Ler linker region (Fig. 1). We therefore hypothesized that inserting the 11 aa sequence ELLNSLA AVKS from H-NS into position 67 (between amino acids N and V) of the Ler linker would alter Ler function, and more specifically, change Ler into a transcriptional silencer.

We found that the Ler molecule containing the 11 aa H-NS linker sequence (pFL08) was impaired in its ability to increase transcription from the *LEE5-lacZ* fusion in strains wt or deleted for *hns*, but the mutated Ler molecule did not silence *LEE5* transcription (Fig. 3). In the strain deleted for *hns*, the Ler molecule containing the ELLNSLA AVKS insertion modestly increased *LEE5* transcriptional activity, from ~50 to 200 Miller units ( $P < 0.0001$ ). To investigate further we constructed mutated Ler proteins containing either 11 or six alanines inserted into position 67 of Ler. These recombinant proteins were constructed to test whether the spacing between the N terminus and C terminus, irrespective of the specific amino acid sequence of the interdomain linker, was essential for Ler function. With results nearly identical to those for the pFL08 plasmid, inserting either 11 or six alanines (pFL10 and pFL09, respectively) impaired the ability of Ler to increase *LEE5* transcription, with only modest increases in transcription occurring in the strain deleted for *hns* (compare Fig 3 and Fig 2a). These mutated Ler proteins did not decrease transcriptional activity, as observed for the wt H-NS protein acting on the *LEE5* and *proU* fusions in the strain with a deleted chromosomal *hns* gene (Fig. 2a). We concluded that altering the spacing between the N-



terminal and C-terminal domains severely affected the ability of Ler to increase LEE gene expression.

### Replacing the Ler linker and/or C-terminal DNA-binding domain with the corresponding domains of H-NS eliminated Ler anti-silencing activity

Though Ler possesses the coiled-coil domain (Sperandio *et al.*, 2000) and the conserved H-NS DNA binding motif (Fig 1 and Fig 2b) in its N terminus and C terminus, respectively, the modular nature of Ler is not well established experimentally. Therefore, we investigated the contribution of the N-terminal  $\alpha$ -helical, linker region, and C-terminal DNA binding domains of Ler and H-NS in the control of *LEE5* transcriptional activity. Chimeric Ler–H-NS molecules, containing all combinations of the two functional domains and linker regions were constructed. The  $\alpha$ -helical, N-terminal fragments contained the coiled coils and the C-terminal fragments contained the conserved DNA binding motifs of Ler and H-NS, determined by mutational analyses (Sperandio *et al.*, 2000; Ueguchi *et al.*, 1997; Fig. 1). DNA fragments encoding these chimeric proteins were placed under the control of the arabinose-inducible promoter in derivatives of the plasmid pBAD33. The plasmids encoding chimeras, and the wt Ler and H-NS molecules were transformed into the single-copy *LEE5-lacZ* and *proU-lacZ* fusion strains as described above, and  $\beta$ -galactosidase activities were monitored in strains wt and deleted for *hns*. In Fig 4 and Fig 5, domains of the Ler protein are depicted as white rectangles, whereas those of H-NS are depicted as black rectangles.

Expression of chimeras C1, C3 and C6, containing the H-NS linker, H-NS C terminus, and H-NS linker and C-terminal domain, respectively, did not alter transcription at *LEE5* or *proU* promoters under any of the conditions tested (Fig. 4a). These data indicated that the Ler linker and C-terminal domain are critical for anti-silencing activity. Thus, the Ler linker and C terminus are not functionally equivalent to those of H-NS.

### The C2 chimeric protein containing the Ler linker increased LEE gene expression in a strain deleted for *hns*

The opposite of the C1 protein, the C2 protein, exchanged the linker region of H-NS with that of Ler. This chimeric protein did not silence expression from either the *LEE5-lacZ* or the *proU-lacZ* fusions (Fig. 4b). However, the C2 chimera increased transcription 10-fold at *LEE5* in the absence of *hns*, from ~150 to 1500 Miller units ( $P < 0.0001$ ), suggesting that it was still able to disrupt a non-H-NS regulator of *LEE5*. The C2 protein increased *proU-lacZ* expression slightly, less than twofold, in a strain containing an *hns* deletion. These data were consistent with those observed for the C1 protein and our directed mutagenesis of the Ler linker (Fig 3 and Fig 4a), indicating that the Ler and H-NS linker regions are not functionally equivalent.

The C4 protein containing the N terminus and linker of H-NS and the C terminus of Ler increased the expression of the *LEE5-lacZ* fusion sixfold, from ~100 to 600 Miller units ( $P < 0.0001$ ) (Fig. 4b). However, in contrast to C2, the C4 chimeric protein did not affect expression of the *LEE5-lacZ* fusion in a strain containing an *hns* deletion.

Compared with the C4 protein, the C5 chimeric protein contained the N terminus of H-NS and the linker and C terminus of Ler. This protein increased *LEE5-lacZ* expression eightfold

in the presence of *hns*, from ~100 to 800 Miller units ( $P<0.0001$ ) (Fig. 4b). Unlike the C4 protein, however, the C5 chimera also increased *LEE5-lacZ* transcription in a strain deleted for the *hns* gene. Overall, the C5 chimeric protein affected the *LEE5-lacZ* fusion similarly to the wt Ler protein (see Fig. 2a), except that the induced levels of expression were reduced. These data were also consistent with the effect of the pMHB1 construct ( $\alpha$ -helices 1 and 2 of H-NS placed on the N terminus of Ler) on *LEE5* activity (see Fig. 2b), in that both mutated proteins still relieved silencing of LEE transcription in strains wt and deleted for *hns*. Since the only difference between the C4 and C5 chimeras was that C5 contained the linker of Ler, whereas C4 had the linker of H-NS, these data further suggested that the Ler linker is important for interaction with or disruption of the non-H-NS factor acting at *LEE5*.

Interestingly, none of the chimeric proteins appreciably affected expression of the H-NS-regulated *proU* operon. The C4 and C5 proteins increased *proU-lacZ* expression approximately twofold in a strain wt for *hns* (Fig. 4b), whereas wt Ler had no effect on this fusion in either the presence or the absence of *hns* (Fig. 2a). However, the approximately twofold increase in *proU-lacZ* expression in the presence of the C4 and C5 chimeras was far less than the ~15-fold increase in *LEE5-lacZ* expression in the presence of wt Ler (Fig. 2a). Thus, we concluded that though the C4 and C5 chimeras modestly affected *proU-lacZ* expression, most likely due to dominant negative effects on the wt H-NS protein, our analysis did not elucidate a molecular explanation for differential regulation of LEE virulence genes by Ler.

As an important control, we expressed the wt Ler, wt H-NS and chimeric proteins from the arabinose-inducible pBADMyCHISA expression vector in our fusion strains, monitoring *LEE5-lacZ* and *proU-lacZ* activities and protein expression by the anti-Myc epitope antibody (Invitrogen). We observed similar  $\beta$ -galactosidase activities to those of the pBAD33-based constructs presented in Fig 2 and Fig 4, and found that all of the wt and chimeric proteins were visible by immunoblot analysis under growth conditions identical to those used for monitoring activities derived from the *lacZ* fusions (data not shown).

### C2, C4 and C5 chimeric proteins altered expression of the *LEE2* and *LEE3* operons

To ensure that the observed activities associated with the mutated Ler proteins were generalized regulatory phenomena, and not simply specific to the *LEE5* operon of EPEC, we monitored transcriptional activity derived from the EPEC *LEE2* and *LEE3* operons in the presence of the C2, C4 and C5 chimeric proteins. For the C2 chimera containing the linker region of Ler and the N terminus and C terminus of H-NS, expression derived from the *LEE2-lacZ* and *LEE3-lacZ* fusions increased approximately sevenfold ( $P<0.0001$ ) and twofold ( $P<0.0001$ ), respectively, in the strain deleted for the chromosomal *hns* gene (Fig. 5), a similar phenotype to that observed at *LEE5* (Fig. 4b). The C4 protein containing the N terminus and linker of H-NS and the C terminus of Ler increased *LEE2* and *LEE3* expression in the strain wt for *hns* ~10-fold ( $P<0.0001$ ;  $P<0.0001$ ), whereas expression from both of these fusions in the absence of chromosomal *hns* was only modestly affected (Fig. 5). The presence of the C5 chimeric protein increased transcriptional activity derived from the *LEE2-lacZ* fusion in strains with and without *hns* approximately fivefold ( $P=0.0002$ ) and threefold ( $P<0.0001$ ), respectively, a result similar to that observed for the *LEE5-lacZ*

fusion (Fig 4b and Fig 5). The presence of the C5 chimera did not alter expression derived from the *LEE3-lacZ* fusion under any of the conditions tested, but differences in *LEE2* and *LEE3* regulation have been observed elsewhere (Russell *et al.*, 2007). Thus, we concluded that those chimeric proteins with demonstrated effect at the *LEE5* operon functioned similarly at the *LEE2* and *LEE3* operons, with the exception of the C5 chimeric protein not affecting *LEE3* expression (Fig. 5).

### Activity of mutated and chimeric Ler proteins in EPEC

In order to demonstrate regulatory observations in the specific pathotype EPEC, we assayed the effects of the mutated Ler proteins on *LEE5* transcription using the multi-copy *LEE5-lacZ* fusion plasmid pKMTIR3 (Haack *et al.*, 2003). For this analysis, the *ler* deletion strain SE796 with pBAD33-derived vectors expressing Ler, H-NS, the mutated Ler proteins and the empty vector control were transformed with plasmid pKMTIR3. Strains were grown and harvested in conditions identical to those for  $\beta$ -galactosidase assays in K-12-derived strains, except that 0.1% glucose was added to cultures to repress expression of wt *lac* in strain SE796.

As expected, in the presence of the wt Ler protein, *LEE5* transcription increased 20-fold ( $P < 0.0001$ ), whereas the H-NS protein caused a decrease, 0.5-fold ( $P = 0.03$ ), in strain SE796, which is wt for *hns* (Table 3). As presented in Fig. 3, mutated Ler proteins containing either the H-NS sequence ELLNSLA AVKS (pFL08) or six alanines (pFL09) inserted at position 67 of the linker were deficient compared with wt Ler, but were still able to relieve silencing of *LEE5* transcription by 2.1-fold ( $P = 0.002$ ) and 2.6-fold ( $P = 0.001$ ), respectively (Table 3). Consistent with the observed effects of the C1 and C2 chimeras in the K-12-derived strain in the presence of wt *hns* (see Fig. 4a, b), these mutated proteins had negligible effect on *LEE5* transcription, 1.1-fold and 1.0-fold, respectively (Table 3). Surprisingly, the C4 chimeric protein did not show anti-silencing activity towards *LEE5* in strain SE796 (Table 3, Fig. 4b), perhaps due to altered stability in the EPEC strain or interaction with EPEC-specific regulators. The C5 protein caused a 4.6-fold increase in *LEE5* transcription in strain SE796 ( $P = 0.0001$ ), consistent with data presented in Fig. 4(b). We therefore concluded that, as previously reported (Bustamante *et al.*, 2001; Mellies *et al.*, 2007b; Umanski *et al.*, 2002), with the exception of the C4 chimera, Ler-associated regulatory phenomena were similar in EPEC- and K-12-derived strains.

## DISCUSSION

### The Ler linker

In this report we investigated the roles of the Ler N-terminal  $\alpha$ -helical, coiled-coil-containing domain, linker region and C-terminal DNA-binding domain in their ability to increase transcription at the LEE PAI of EPEC. Our investigation focused on the molecular differences between the silencing protein H-NS and the related anti-silencer Ler that allow for opposite functions. The data clearly indicated that the linker region unique to Ler is necessary for function: inserting 11 amino acids specific to the H-NS linker, 11 alanines or six alanines at position 67 severely limited Ler anti-silencing activity (Fig. 3). Additionally, replacing the H-NS linker with the linker of Ler created a chimeric protein (C2) that

increased *LEE* transcription in a strain deleted for the chromosomal *hns* gene (Fig 4b and Fig 5). Combined with evidence of a second, non-H-NS factor presented here and in a previous report (Haack *et al.*, 2003), we propose that the C2 chimera retains the ability to counteract repression by the unknown factor, but has lost the ability to disrupt H-NS-mediated silencing at *LEE* operons. The C5 chimera, containing the linker and C terminus of Ler, relieved silencing of *LEE* genes similarly to the wt Ler protein (Fig 4b and Fig 5), albeit to a lesser extent (Fig. 2a), and the activities of the C2 and C5 chimeric proteins were confirmed in the EPEC strain SE796 (Table 3).

Previous reports have indicated that *LEE* regulatory observations in K-12-derived strains are faithfully reproduced in the EPEC pathotype (Bustamante *et al.*, 2001; Mellies *et al.*, 2007b; Umanski *et al.*, 2002). Here, we made similar observations measuring *LEE5* transcriptional activity on multi-copy plasmids in the E2348/69-derived *ler* deletion strain SE796 as a function of expressed Ler and H-NS molecules (Table 3). Our attempts to construct an EPEC *ler hns* double mutant were unsuccessful, most likely due to disruption of complex regulatory networks within this pathogenic bacterium in the absence of H-NS. Consistently, we observed that *hns* deletion derivatives of the EDL933 *E. coli* serotype O157 : H7 possess growth defects, having a 40% increase in doubling time (*g*), compared with their isogenic parent strains (Torres *et al.*, 2007; our unpublished results). We were able to measure the effects of the mutated Ler proteins in the absence of wt Ler in the EPEC E2348/69 derivative SE796, but were unable to measure effects in the absence of the wt H-NS protein expressed from the chromosomal *hns* locus.

### Interdomain linkers in other transcriptional regulators

Reports from other laboratories have indicated an important role for interdomain linkers in transcriptional regulators. For example, the response regulator OmpR of *E. coli* that controls expression of the outer membrane porins OmpF and OmpC in response to changes in osmolarity contains a linker important for inter-domain communication (Mattison *et al.*, 2002). In OmpR, the C-terminal DNA binding domain is affected by signals mediated by phosphorylation of the N-terminal domain, which are dependent on the interdomain linker (Delgado *et al.*, 1993; Kanamaru *et al.*, 1990). Both OmpR and the related response regulator PhoB contain N-terminal phosphorylation domains and C-terminal DNA binding domains connected by a flexible linker. Even though OmpR and PhoB are closely related members of the OmpR family of response regulators, their interdomain linkers are not interchangeable (Walthers *et al.*, 2003). The OmpR protein requires its linker for activation of the C-terminal winged helix–turn–helix DNA binding domain because the OmpR protein containing the linker of the related PhoB protein is defective in DNA binding and signalling. Thus, the interdomain linkers of related proteins can contribute to differential function of closely related proteins, as OmpR and its cognate sensor kinase EnvZ respond to medium osmolarity, while PhoB and its cognate sensor kinase PhoR respond to extracellular levels of inorganic phosphate. Though Ler and H-NS share N-terminal coiled-coil motifs (Sperandio *et al.*, 2000) and a conserved C-terminal DNA binding motif (Fig 1 and Fig 2b; Bertin *et al.*, 1999; Dorman *et al.*, 1999), The interdomain linker of Ler varies greatly from that of H-NS and partially explains why the activity of Ler is opposite to that of H-NS.

### Ler-H-NS chimeric protein activity

One observation that might explain how the C2, C4 and C5 chimeric proteins altered LEE transcription is that all contained H-NS N-terminal domains, while those without any demonstrable activity possessed the N terminus of Ler (Fig 4 and Fig 5). This observation suggested dominant negative effects with H-NS, or perhaps interactions with other DNA binding/regulatory proteins, since N-terminal fragments of H-NS have been observed to affect transcription in a dominant negative manner (Ueguchi *et al.*, 1996; Williams *et al.*, 1996). The C2 chimera increased *LEE5* transcriptional activity in a strain containing an *hns* deletion, while the C4 chimera increased *LEE5* transcriptional activity in a strain with wild-type *hns* (Fig 4 and Fig 5). Thus, the C4 chimera might interact with H-NS in a dominant negative manner, while the Ler linker region of the C2 chimera functions in the absence of H-NS. We purified the C2, C4 and C5 chimeras and found that their ability to bind *LEE5* regulatory DNA was severely impaired (data not shown). This observation is consistent with the C2, C4 and C5 chimeras altering transcriptional activity via interaction with other proteins as opposed to minor alterations in DNA binding affinities. Though we observed expression of all six chimeric proteins by immunoblot analysis, we were not able to purify the C1, C3 and C6 chimeras (data not shown). Thus, their inactivity might have been due to instability or because they were sequestered within inclusion bodies inside the bacterium, unable to gain access to DNA. Clearly other, yet to be identified regulatory factors also play an important role in regulation of LEE virulence genes.

### Silencing of *LEE5*

Though evidence suggests that Ler does not function as a classical activator proteins at other LEE operons (Bustamante *et al.*, 2001; Umanski *et al.*, 2002), we cannot rule out the possibility that Ler functions as such at *LEE5*, either by recruiting RNA polymerase directly or by altering nucleoid structure. Precedent exists for this type of dual regulatory function, as SlyA of *Salmonella* and a homologue, RovA of *Yersinia*, and ToxT of *Vibrio cholerae* can act as H-NS antagonists as well as classical transcriptional activator proteins (Ellison & Miller, 2006; Heroven *et al.*, 2004; Yu & DiRita, 2002). It is also possible that Ler increases the expression of a yet to be described activator to stimulate *LEE5* transcription. If a non-H-NS, negative-acting protein silencing *LEE5* does exist, a likely candidate is StpA, because its expression increases in an *hns* mutant and there is evidence that it can compensate for the loss of H-NS (Müller *et al.*, 2006).

### Conclusions

Recent work has demonstrated that a major function of H-NS is to silence horizontally acquired segments of DNA, particularly those genes involved in bacterial virulence (Navarre *et al.*, 2006). Anti-silencing, or countersilencing of H-NS leading to gene expression occurs by a variety of mechanisms. Examples of countersilencing include changes in the concentration of H-NS itself (Hansen *et al.*, 2005), direction of transcription by the alternate sigma factors  $\sigma^S$  (Typas *et al.*, 2007), antagonizing H-NS binding or remodelling nucleoprotein structure by specific regulatory proteins such as SlyA (Corbett *et al.*, 2007; Lithgow *et al.*, 2007), changes in local DNA topology (Falconi *et al.*, 1998), and disruption

of silencing by H-NS homologues, which include Ler and the H-NST molecules of EPEC (Haack *et al.*, 2003; Mellies *et al.*, 1999; Williamson & Free, 2005).

Homologues of H-NS, such as Ler, countersilence by either competing for binding sites or interfering with multi-merization (reviewed by Fang & Rimsky, 2008). At the *LEE5* operon of EPEC, Ler and H-NS bind in the same location upstream of the promoter, whereas H-NS also acts downstream at *LEE5* (Haack *et al.*, 2003; our unpublished results). Because Ler possesses a lower  $K_D$  of binding to LEE regulatory DNA than H-NS (Umanski *et al.*, 2002), Ler clearly can compete for binding sites to increase LEE gene expression. However, we do not know whether Ler also affects H-NS multimerization. Additional questions remain. Might the distinct linker region of Ler play a role in disruption of H-NS multimerization? What is the molecular explanation for the greater affinity of Ler for DNA than the related H-NS molecule? Finally, why does Ler regulate LEE virulence genes but not other H-NS-silenced operons such as *proU*, and what other factors contribute to the differential regulation of Ler?

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## Abbreviations

<b>AE</b>	attaching and effacing
<b>EHEC</b>	enterohaemorrhagic <i>E. coli</i>
<b>EPEC</b>	enteropathogenic <i>E. coli</i>
<b>LEE</b>	locus of enterocyte effacement
<b>T3SS</b>	type III secretion system
<b>wt</b>	wild-type

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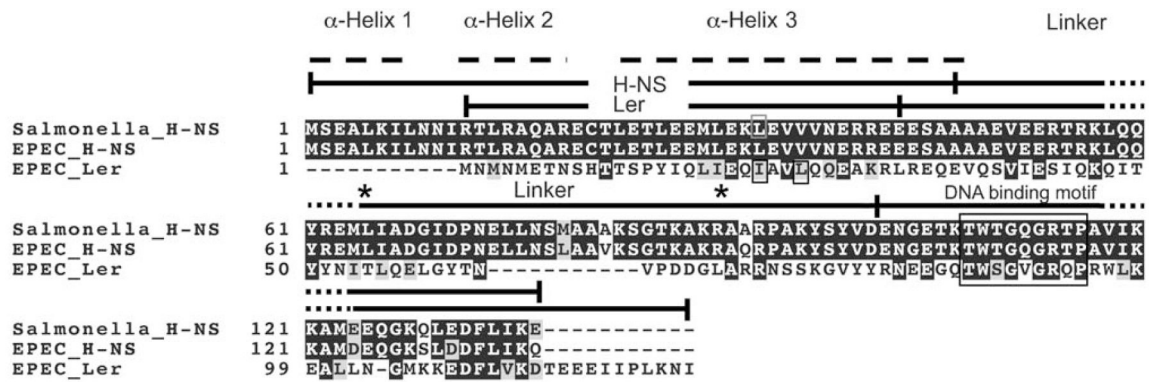


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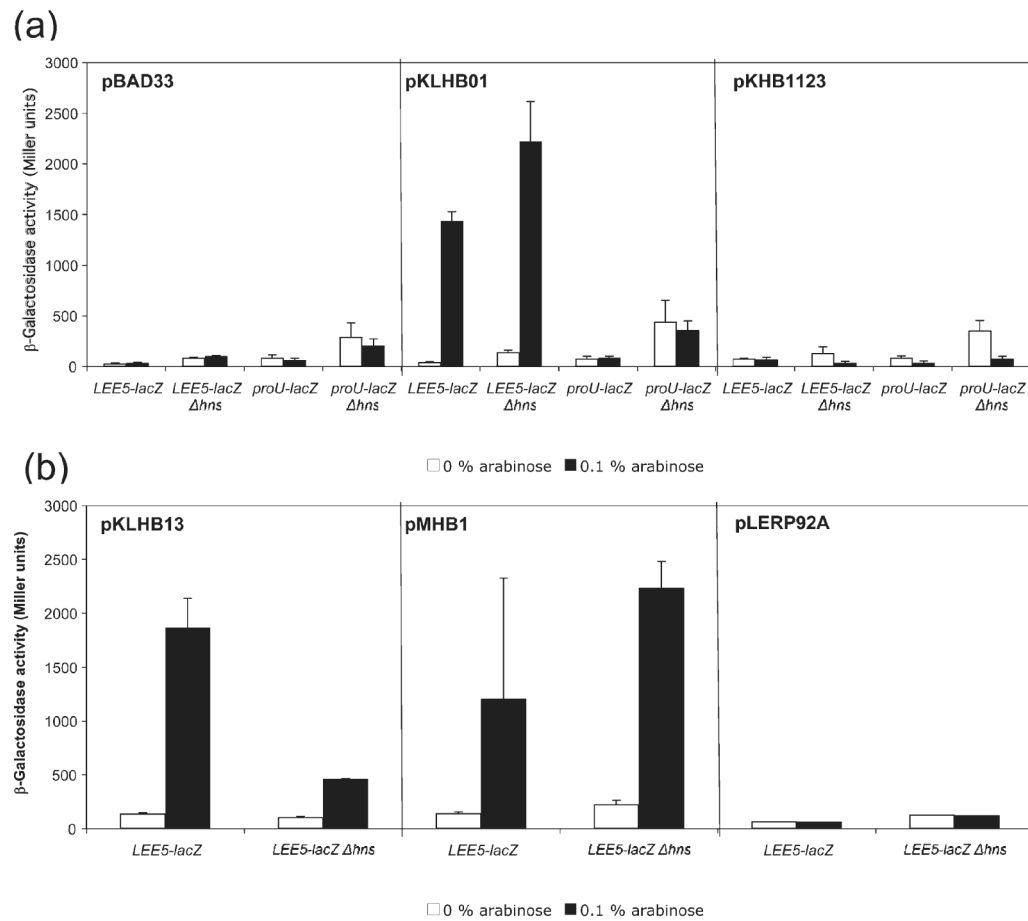
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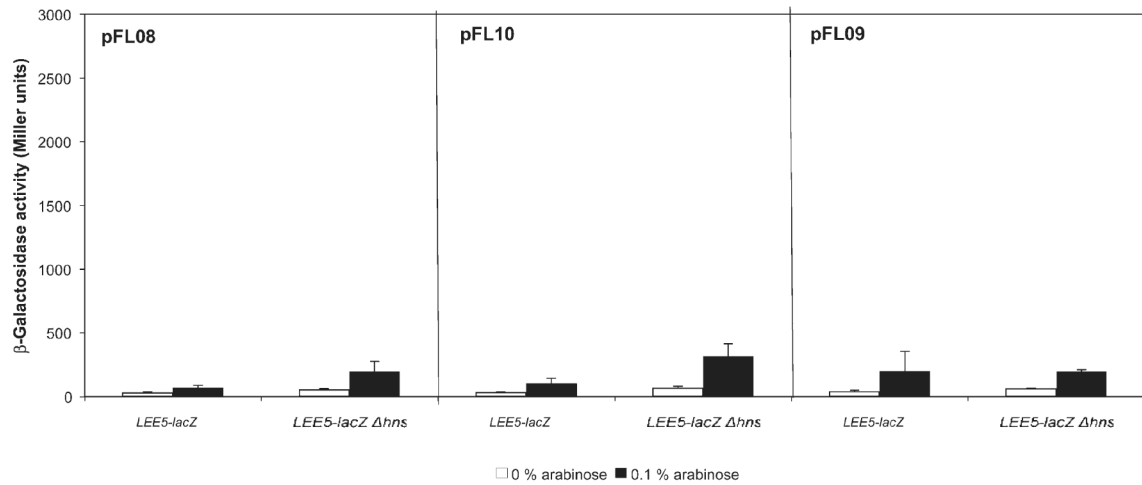
**Fig. 1.**

Sequence alignment of Ler and H-NS. The H-NS amino acid sequences of EPEC strain E2348/69 and *Salmonella typhimurium* are compared to the H-NS-like molecule Ler. Of these proteins, Ler shares greatest amino acid similarity with H-NS of *S. typhimurium* (24% identity, 44% similarity). NMR studies indicate that H-NS is made up of an N-terminal domain containing three  $\alpha$ -helices (indicated by dashed lines; residues 1–64), a structurally undefined linker region required for higher-order oligomerization (residues 65–90), and a C-terminal DNA binding domain (residues 90–137) (Dorman, 2004). Though not experimentally defined, Ler is predicted to share similar structural modularity; Ler is predicted to possess a single  $\alpha$ -helix, instead of three, in the N-terminal region of the protein (see Results). Identity, indicated by black shading, between Ler and H-NS is greater in the C-terminal domain, containing the core DNA binding motif TWTGXGRXP (Bertin *et al.*, 1999; Dorman *et al.*, 1999). Amino acids important for dimerization, found in the N termini of Ler and H-NS, are boxed (Sperandio *et al.*, 2000; Ueguchi *et al.*, 1997). The conserved C-terminal DNA binding motif (Bertin *et al.*, 1999; Dorman *et al.*, 1999) is also boxed. The regions used for the construction of Ler–H-NS chimeras (boundaries indicated by vertical lines) differ from the experimentally determined domains of H-NS (indicated by asterisks) because of technical constraints in molecular cloning protocols. Alignments were created using CLUSTAL\_X (1.83) multiple sequence alignment software, and shading using Boxshade (3.21).

**Fig. 2.**

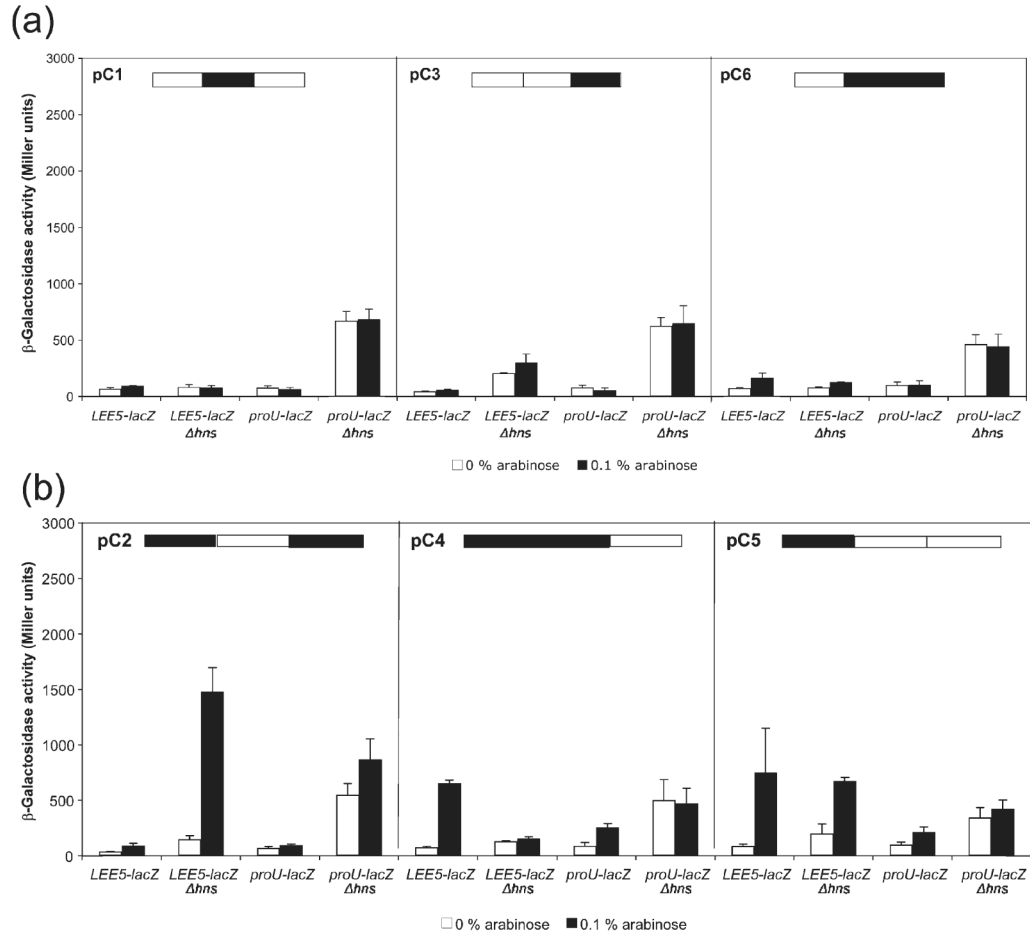
Analysis of the Ler N terminus and C terminus. (a)  $\beta$ -Galactosidase activities were derived from K-12 strains containing *LEE5-lacZ* and *proU-lacZ* fusions in the presence of the pBAD33 vector, wt Ler or wt H-NS proteins, expressed from plasmids pKLHB01 and pKHB1123, respectively, under the control of the arabinose-inducible  $P_{BAD}$  promoter. Plasmids were transformed into *LEE5-lacZ* fusion strains wt for *hns* (KH4105) or deleted for *hns* (KH4115); similarly, the above plasmids were transformed into *proU-lacZ* fusion strains wt for *hns* (KH4106) and deleted for *hns* (KH4116). The values are presented in Miller units and represent the mean of at least two independent assays performed in triplicate. Error bars, SD. (b) Plasmid pKLHB13 encodes a recombinant Ler protein containing a C-terminal truncation, missing the last 11 aa compared with H-NS. Plasmid pMHB1 encodes a protein containing the first 21 aa of H-NS, containing  $\alpha$ -helices 1 and 2, fused to the N terminus of Ler (see Fig. 1). Plasmid pLERP92A contains a mutated Ler protein with the point mutation P92A within the core DNA binding motif.



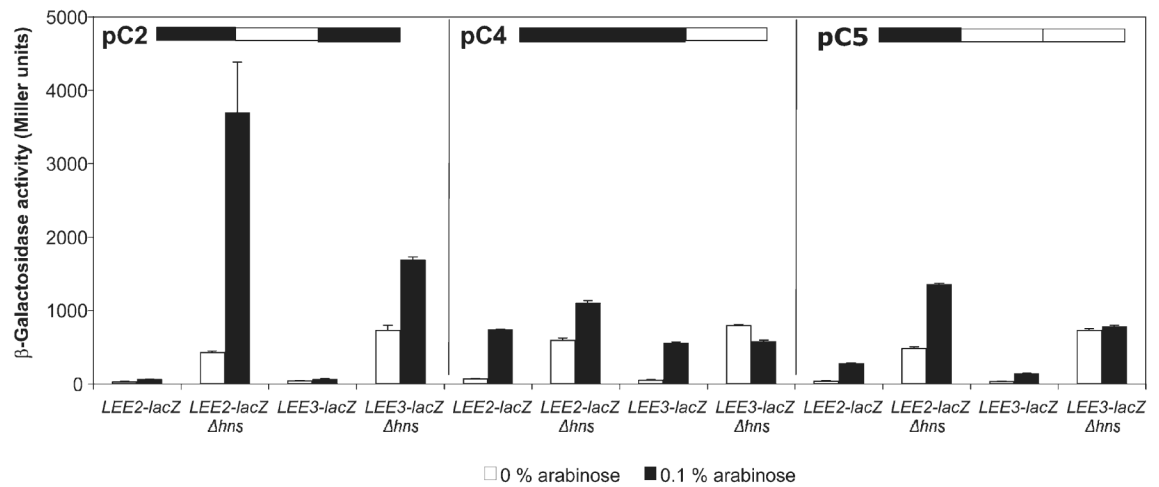


**Fig. 3.**

Inserting the H-NS linker sequence into Ler reduced, but did not eliminate, the ability of Ler to increase *LEE5* activity. β-Galactosidase activities were derived from *LEE5-lacZ* fusions in the presence of Ler proteins containing either the H-NS linker sequence ELLNSLAAVKS (pFL08), 11 alanines (pFL10) or six alanines (pFL09) inserted at amino acid position 67. β-Galactosidase assays were performed as described for Fig. 2.



**Fig. 4.** Analysis of Ler-H-NS chimeric proteins. (a) Replacing the Ler linker and/or C terminus with those of H-NS eliminated Ler and H-NS activity. Chimeric proteins C1, C3 and C6 encoded on plasmids pKHB1527 (pC1), pKHB1083 (pC3) and pKHB1523 (pC6) were transformed into the *LEE5-lacZ* strains wt and deleted for *hns*, KH4105 and KH4115, respectively. Similarly, the three plasmids were transformed into the *proU-lacZ* fusion strains. (b) When paired with the N terminus of H-NS, the linker region of Ler and C-terminal domains conferred distinct effects on *LEE5* transcription; the chimeric proteins C2, C4 and C5 in plasmids pKHB1163 (pC2), pKHB1047 (pC4) and pKHB1167 (pC5), respectively, retained the differential activity of Ler at *LEE5*.  $\beta$ -Galactosidase assays were performed as described for Fig. 2.

**Fig. 5.**

The chimeric proteins C2, C4 and C5 showed similar effects on transcription at *LEE2* and *LEE3* to those for *LEE5*. Chimeric proteins C2, C4 and C5 expressed from plasmids pKHB1163 (pC2), pKHB1047 (pC4) and pKHB1167 (pC5), respectively, were transformed into *LEE2-lacZ* and *LEE3-lacZ* fusion strains, and  $\beta$ -galactosidase activities were determined as described for Fig. 2. The *LEE2-lacZ* fusion strains KH4102 and KH4112 are wt and deleted for *hns*, respectively, while the *LEE3-lacZ* fusion strains KH4103 and KH4113 are wt and deleted for *hns*, respectively.

Table 1

Bacterial strains, plasmids and phages used in this study

Strain, plasmid or phage	Genotype or description	Source or reference
<b>Strains</b>		
DH5 $\alpha$	<i>supE44 (argF-lac)U169 (<math>\Phi</math>80dlac (Z)M15) deoR hsdR17 recA1 endA1 gyrA96 thi-1 relA1</i>	Laboratory stock
MC4100	<i>araD139 (argF-lac)U169 rpsL150 relA1 flbB5301 deoC1 ptsF25 rbsR</i>	Casadaban (1976)
HN4104	MC4100 ( <i>hns tdk adhE oppABCD</i> )118 <i>zch-506</i> : : <i>Tn10</i>	Colonna et al. (1995)
KH4100	MC4100 <i>ara</i> <sup>+</sup>	This study
KH4105	KH4100 $\Phi$ <i>LEE5-lacZ</i> (-303 to +172)	This study
KH4106	KH4100 $\Phi$ <i>proU-lacZ</i> (-398 to +404)	This study
KH4115	KH4105 ( <i>hns tdk adhE oppABCD</i> )118 <i>zch-506</i> : : <i>Tn10</i>	This study
KH4116	<i>KH4106 (hns tdk adhE oppABCD)118 zch-506 : : Tn10</i>	This study
KH4102	KH4100 $\Phi$ <i>LEE2-lacZ</i> (-218 to +670)	This study
KH4112	KH4102 ( <i>hns tdk adhE oppABCD</i> )118 <i>zch-506</i> : : <i>Tn10</i>	This study
KH4103	KH4100 $\Phi$ <i>LEE3-lacZ</i> (-434 to +262)	This study
KH4113	KH4103 ( <i>hns tdk adhE oppABCD</i> )118 <i>zch-506</i> : : <i>Tn10</i>	This study
SE796	E2348/69 <i>ler</i> : : <i>aphA3</i> Km <sup>r</sup>	Elliott et al. (2000)
<b>Plasmids</b>		
pRS551	Promoterless <i>lacZ</i> reporter fusion vector	Simons et al. (1987)
pKMTIR3	<i>LEE5-lacZ</i> (-303 to +172) in pRS551	Haack et al. (2003)
pKHpro1	<i>proU-lacZ</i> (-398 to +404) in pRS551	This study
pJLM166	<i>LEE2-lacZ</i> (-218 to +670) in pRS551	Mellies et al. (1999)
pJLM172	<i>LEE3-lacZ</i> (-434 to +262) in pRS551	Mellies et al. (1999)
pCR2.1 TOPO	Cloning vector	Invitrogen
pBAD33	Arabinose-inducible expression vector Cm <sup>r</sup>	Guzman et al. (1995)
pKLHB01	wt <i>ler</i> in pBAD33	This study
pKHB1123	wt <i>hns</i> in pBAD33	This study
pMHB1	Mutated Ler protein with $\alpha$ -helices 1 and 2 (MSEALKILNNIRTLRAQAREC) of H-NS fused to the N terminus in pBAD33	This study
pKLHB13	Mutated Ler protein with 11 aa, C-terminal extension TEEIIPLKNI deleted in pBAD33	This study
pLERP92A	Mutated Ler protein with alanine substitution for proline at position 92 (position 115 of H-NS) in pBAD33	This study
pFL08	pKLHB01 with residues ELLNSLAAVKS inserted at Ler position 67 in pBAD33	This study
pFL09	pKLHB01 with six alanines inserted at Ler position 67 in pBAD33	
pFL10	pKLHB01 with 11 alanines inserted at Ler position 67 in pBAD33	This study
pKHB1527	Chimera C1 (Ler 7-31 : : H-NS 48-101 : : Ler 79-123) in pBAD33	This study
pKHB1163	Chimera C2 (H-NS 1-47 : : Ler 32-78 : : H-NS 102-137) in pBAD33	This study
pKHB1083	Chimera C3 (Ler 7-78 : : H-NS 102-137) in pBAD33	This study
pKHB1047	Chimera C4 (H-NS 1-101 : : Ler 79-123) in pBAD33	This study
pKHB1167	Chimera C5 (H-NS 1-47 : : Ler 32-123) in pBAD33	This study
pKHB1523	Chimera C6 (Ler 7-78 : : H-NS 48-137) in pBAD33	This study
<b>Phages</b>		

Strain, plasmid or phage	Genotype or description	Source or reference
P1 <i>vir</i>	Generalized transducing phage	Laboratory stock
$\lambda$ RS45	Specialized transducing phage	Simons <i>et al.</i> (1987)

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**Table 2**

Oligonucleotides used in this study

Primer	Sequence (5'-3')*
proUregF	CCGGAATTCCTTTCGCCCGAATGCCGAT
proUregR	CGCGGATCCAGGACTGGAAAGACCATCGCA
LerForward	GCTCTAGACGGAGATTATTTATATGAAATGGA AA CT
KLHlerwR	CCC AAGCTTTTAAATATTTTCAGCGGTATATTTCTTCT
NTERMMUT	GCTCTAGACGGGAGATTATTTATTAIGACGGAAGCACTTAAAAATCTGAACAACATCCGTACTTTCGTGCGCAGGCAAGAGAATGTAATATGGAAACTTAATTCACATACAAACAAGT
KLHlermutR	CCC AAGCTTTTAGTCC TTCACAAGAAAATCTTCTTCT
ELLF	GAACTGCTGAATAGGCTTGGCTGCCGTTAAATCTGTGGCTGATGATGGACTCGCTCGCCCGGAAC
ELLR	AGATTTAAACGGCAGCAAGGCTATTCAGCAGTTCATTAGTATATCCACAGCTCTTGTAAAGGTTAT
6AF	GCTGCAGCAGCGGCTGCAGTGCCTGATGATGGACTCGCTCGCCCGGAAC
6AR	TGCAGCCGCTGCTGCAGCATTAGTATATCCACAGCTCTTGTAAAGGTTAT
11AF	GCTGCAGCGGCAGCTGCTGCAGCGGCTGCAGCTGTGGCTGATGATGGACTCGCTCGCCCGGAAC
11AR	AGCTGCAGCCGCTGCAGCAGCTGCCGCTGCAGCATTAGTATATCCACAGCTCTTGTAAAGGTTAT
3'lerP115A	CCC AAGCTTTTAAATATTTTCAGCGGTATTTTCGTCTTCGGGTGCTTCCACAAGAAAATCTTCTTCCATTCACACAGTGTCTTTTAAAGCCAGCGTGCCTGTCCGGCCAA
lerRnar	GGGGCGCCCAAGTGGCTTTTGTCTTCTGCT
lerFnar	GGGGCGCCCAAGGAAGTTCAAAGTGTAAT
lerRsal	GGGTCCGACGTAGTAAACACCTTTCGATG
lerFsal	GGGTCCGACGAAGAAGGGGCAGACCTGGTC
5hnsswap	GCTCTAGACGGAGATTATTTATATGACGGAAGCACTTAAAAATTTCT
hmsRNar	GGGGCGCCCGGCTTCTTCTTCGGGAC
hmsFNar	GGGGCGCCCGCTGAAGTTGAAGAGCG
hmsRSal	GGGTCCGACGTAGCTATATTTTCGGCGGAC
hmsFSal	GGGTCCGACGAAGAAGGGGCAGACCTGGTC
3hnsswap	CCC AAGCTTTTATTTAGTTGCTTGTATCAGGAAAATC

\* Restriction sites used in cloning are underlined



**Table 3**

Effect of mutated Ler proteins on *LEE5* expression in *EPEC* strain SE796  
 The EPEC E2348/69-derived strain SE796 contains an in-frame deletion of *ler*.

Fusion plasmid	Protein plasmid	Glucose *	Arabinose *	Fold-induction †
–	–	19 (6)	8 (3)	–
pRS551	–	31 (1)	43 (2)	–
pKMTIR3	pBAD33	692 (19)	731 (26)	1.1
pKMTIR3	pKHLB01(Ler)	526 (13)	10 218 (185)	20
pKMTIR3	pKHB1123 (H-NS)	439 (23)	216 (12)	0.5
pKMTIR3	pFL08	752 (19)	1588 (196)	2.1
pKMTIR3	pFL09	717 (74)	1887 (334)	2.6
pKMTIR3	pKHB1527(C1)	996 (68)	1067 (41)	1.1
pKMTIR3	pKHB1163(C2)	1139 (63)	1128 (38)	1.0
pKMTIR3	pKHB1047(C4)	1015 (20)	891 (13)	0.9
pKMTIR3	pKHB1167(C5)	822 (24)	3767 (113)	4.6

\*  $\beta$ -Galactosidase activity derived from the *pKMTIR3 5-lacZ* reporter gene vector in the strain SE796 was monitored in the presence of the listed protein expression and control plasmids under repressing (0.1% glucose) and inducing (0.1% arabinose) conditions. The values are presented in Miller units and represent the mean of representative assays performed in triplicate with SDs in parentheses.

† Represents the ratio of transcriptional activity in the presence of the inducer (arabinose) to that in the presence of the repressor (glucose) for the indicated strains.