Thermoregulated expression of virulence genes in enteropathogenic *Escherichia coli*

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Enteropathogenic *Escherichia coli* (EPEC) causes severe diarrhoea in young children. The locus of enterocyte effacement (LEE) pathogenicity island comprises a cluster of operons encoding a type III secretion system and related proteins that are associated with EPEC virulence. The *LEE1* operon encodes Ler that positively regulates the *LEE2*, *LEE3*, *LEE4*, *LEE5* and *espG* transcriptional units. The LEE operons are repressed at 27 °C and expressed at 37 °C. This paper describes a regulatory cascade of the thermoregulation of LEE operons. *LEE1* including *ler* is repressed by H-NS at 27 °C but not at 37 °C. In contrast, the expression of the *LEE2*, *LEE3*, *LEE4*, *LEE5* and *espG* transcriptional units is repressed by H-NS at both 27 °C and 37 °C. Upon shifting the culture temperature from 27 °C to 37 °C, Ler is synthesized and in turn activates the expression of *LEE2*, *LEE3*, *LEE4* and *espG* by releasing the H-NS mediated repression. In the case of *LEE5*, Ler acts both by alleviating the H-NS mediated repression and by an additional mechanism, as yet to be defined.

Keywords: EPEC, LEE, H-NS, Ler, thermoregulation

INTRODUCTION

Enteropathogenic *Escherichia coli* (EPEC) causes severe diarrhoea in young children. The disease is associated with formation of attaching and effacing (AE) lesions in infected epithelial cells. Formation of AE lesions is involved with localized degeneration of the brush border microvilli and assembly of highly organized pedestal-like actin structures in the epithelial cells beneath intimately attached bacteria (Frankel *et al.*, 1998). EPEC possesses a unique 35·6 kbp pathogenicity island, termed the locus of enterocyte effacement (LEE), that contains genes needed to elicit formation of AE lesions (McDaniel & Kaper, 1997). The LEE consists of 41 ORFs, organized in five major operons, *LEE1* to *LEE5*, and several additional transcriptional units (Elliott *et al.*, 1999, 1998; McDaniel & Kaper, 1997). These genes encode effector proteins and a type III secretion system, which functions as a molecular syringe to translocate effector proteins from the bacterial cytoplasm directly into the cytoplasm of host cells (Knutton *et al.*, 1998; Wolff *et al.*, 1998). The type III secretion system is essential for

AE lesion formation, for colonization of the host and for EPEC virulence.

We previously showed that expression of the LEE-encoded type III secretion system is thermoregulated, being repressed at 27 °C, and expressed at 37 °C (Rosenhine *et al.*, 1996). Thermoregulated expression of virulence genes was reported also in enteroinvasive *E. coli* (Forsman *et al.*, 1992; Jordi *et al.*, 1992) and *Shigella* (Maurelli & Sansonetti, 1988). In some of these cases H-NS (histone-like nucleoid-structuring protein) plays a key role in the repression at the non-permissive temperature. H-NS is a nucleoid-associated protein, frequently involved in the response of enterobacteria to environmental stimuli. In most cases it mediates negative regulation and counteracts a positive regulator (Atlung & Ingmer, 1997). In *E. coli* K-12 H-NS is involved, directly or indirectly, in the regulation of about 5% of the genes (Hommais *et al.*, 2001).

Expression of *LEE2*, *LEE3*, *LEE4*, *LEE5*, *espG* and *orf19* is activated by Ler (LEE-encoded regulator), a distant homologue of H-NS, encoded by the *LEE1* operon (Friedberg *et al.*, 1999; Mellies *et al.*, 1999; Sanchez-SanMartin *et al.*, 2001; Sperandio *et al.*, 2000). Therefore, the decision whether or not to activate expression of *LEE1* is critical for the initiation of a regulatory cascade leading to expression of other LEE operons.

Abbreviations: EPEC, enteropathogenic *Escherichia coli*; GFP, green fluorescent protein; LEE, locus of enterocyte effacement; H-NS, histone-like nucleoid-structuring protein; Ler, LEE-encoded regulator; IHF, integration host factor; Tir, translocated intimin receptor.

Table 1. Bacterial strains and plasmids

Strain or plasmid	Relevant genotype and characteristics	Reference or source
<i>E. coli</i> strains		
EPEC 2348/69	EPEC wild-type, Sm ^r	J. Kaper, Uni. Maryland
SY327 <i>λpir</i>	Used for propagation of pCVD442	Donnenberg & Kaper (1991)
SM10 <i>λpir</i>	<i>thi1 thr leu tonA lacY recA supE</i> (RP4-2 Km ^r <i>tet::Mu</i>)	Donnenberg & Kaper (1991)
DH5α	<i>supE44 ΔlacU169 (φ80 lacZΔM15) hsdR17 recA1 endA1 gyrA96 thi-1 relA1</i>	BRL
<i>E. coli</i> BL21	<i>F' gal met r⁻ m⁻ hsdS λ_{lys} placUV5-T7-gene 1placI^a</i>	Studier & Moffatt (1986)
DF2	E2348/69 <i>ler::kan</i>	Friedberg <i>et al.</i> (1999)
DF3	E2348/69 <i>hns::kan</i>	This study
TU1	E2348/69 <i>ler::kan hns::cat</i>	This study
Plasmids		
pCVD442	A suicide vector containing the <i>sacB</i> gene	Donnenberg & Kaper (1991)
pKD46	Expresses Lambda Red recombinase	Datsenko & Wanner (2000)
pKD3	A template plasmid carrying the <i>cat</i> gene	Datsenko & Wanner (2000)
pBS	Cloning vector	Stratagene
pIR1	pKK177-3 derivative containing <i>gfp mut3</i>	Friedberg <i>et al.</i> (1999)
pUC4K	Contains 1.2 kb <i>kan</i> cassette	Pharmacia
pPD3	A vector expressing T7- <i>hns</i>	Dersch <i>et al.</i> (1994)
pDF6	pBS containing EPEC <i>hns</i> and flanking sequences	This study
pDF7	pBS containing <i>hns::kan</i>	This study
pDF8	pCVD442 containing <i>hns::kan</i>	This study
pTU15	pBS containing <i>hns::cat</i>	This study
pTU1	pIR1 containing <i>LEE1::gfp</i>	This study
pYF4	pIR1 containing <i>LEE2::gfp</i>	Friedberg <i>et al.</i> (1999)
pYF5	pIR1 containing <i>LEE3::gfp</i>	Friedberg <i>et al.</i> (1999)
pTU9	pIR1 containing <i>espG::gfp</i>	This study
pTU10	pIR1 containing <i>LEE4::gfp</i>	This study
pTU11	pIR1 containing <i>LEE5::gfp</i>	This study
pTU14	pACYC184 derivative containing <i>lacI^a</i> and <i>Ptac-ler</i>	This study

LEE1 expression is dependent on activation by Integration Host Factor (IHF) (Friedberg *et al.*, 1999). Additional factors, including Fis, Per and quorum sensing, are involved in modulation of *LEE1* gene expression (Goldberg *et al.*, 2001; Kanamaru *et al.*, 2000; Mellies *et al.*, 1999; Sperandio *et al.*, 1999). Recently it was reported that in the K-12 background, H-NS acts as a negative regulator of *LEE2*, *LEE3* and *orf19* (Bustamante *et al.*, 2001; Sanchez-SanMartin *et al.*, 2001). It was hypothesized that Ler activates gene expression by negating the H-NS-mediated repression (Bustamante *et al.*, 2001; Sanchez-SanMartin *et al.*, 2001). In conclusion, the LEE operons are subject to regulation by multiple factors; some, like IHF, H-NS and Fis are global regulators expressed by K-12 laboratory strains, and others like Ler, Per and perhaps other regulators are specific to EPEC.

The involvement of any of the above factors, including H-NS and Ler, in thermoregulation is not clear. In this communication we describe the role of H-NS in thermoregulated expression of the LEE operons. We report, for the first time, the formation of an EPEC *hns* mutant and an EPEC double mutant of *hns* and *ler*. We used these mutants to analyse the role of *hns* and *ler* in the thermoregulated expression of LEE genes. The genetic

analysis was supported by *in vitro* biochemical analyses using a purified H-NS protein. We show that H-NS is involved in two levels of regulation of the LEE genes. First, it represses the expression of *LEE1* (including *ler*) at 27 °C but not at 37 °C, leading to thermoregulated expression of all other Ler-regulated LEE operons. In addition, H-NS represses expression of the *LEE2*, *LEE3*, *LEE4*, *LEE5* and *espG* operons both at 27 °C and at 37 °C. Upon shifting the culture temperature from 27 °C to 37 °C, the expressed Ler activates the expression of *LEE2*, *LEE3*, *LEE4* and *espG* by releasing the H-NS-mediated repression. In the case of *LEE5*, Ler acts both by antagonizing the H-NS-mediated repression and by an additional mechanism, as yet to be defined.

METHODS

Bacterial strains, culture conditions and oligonucleotide primers. EPEC strains were grown in LB overnight at 27 °C, diluted 1:50 in DMEM and grown to OD₆₀₀ 0.3–0.35 at 37 °C or 27 °C as indicated. The pH of the DMEM was adjusted by adding HEPES buffer (Sigma) at pH 7.4 to a final concentration of 20 mM. When needed IPTG (1 mM) was added 1 h before cultures were harvested. When needed we used 100 µg ampicillin (Amp) ml⁻¹, 40 µg kanamycin (Kan) ml⁻¹, 25 µg chloramphenicol (Cm) ml⁻¹ and 100 µg streptomycin (Str)

Table 2. Oligonucleotides used in this study

Name	Sequence (5′–3′)	Map position 5′ alignment	Accession no.
<i>LEE1</i> and <i>espG</i>			
24R	GCTCTAGACCTTCTTCATTGCGGTAG	4332	AF022236 (LEE)
5R	GCTCTAGATTCTCTGTTTTCTAATGTG	3924	
9F	GGAATTCGTTTATGCAATGAGATCTATC	3740	
27R	GCTCTAGAGAGAGCGTCAGCGAAACG	4183	
14F	GCTCTAGAGGCAACAAGTATCATATAGC	2804	
8R	CGGGATCCGCATGCTTTAATATTTTAAGC	4076	
26F	CGGGATCCAAGCGGATCTGTTTGAGC	2240	
<i>LEE2</i> and <i>LEE3</i>			
30F	GCTCTAGACTGCGTACGCTCAGGAGC	14654	
31R	GCTCTAGACTGCGTACGCTCAGGAGC	15058	
<i>tir</i>			
22F1	GAGTGGATCCCATTACAC	22045	
22R1	GCTCTGAGAGAAGCGTCTTCTGCTC	23814	
<i>LEE4</i> and upstream			
32R	GCTCTAGACAGATCGCTTTGTGCC	30539	
39F	CGGGATCCATGCCATACCAGAGTG	27885	
<i>hns</i>			
hnsF4	CGCAAGCTTCAGTATGCACAGAAGTGG	10273	D90759
hnsR4	CGGGATCCGTTGCGCCGTCTAATCTC	9071	
<i>CIII</i>			
2703	GCGGAATTCGAATTTAGTCTGGATAGC		
2902	GCCGGATCCATGCAATATGCCATTGCAGG		
<i>cat</i>			
cm-pKD3-F	AACTGCAGGTGTAGGCTGGAGCTGCTTC		
cm-pKD3-R	AACTGCAGCATATGAATATCCTCCTTAG

Fig. 1. Schematic presentation of LEE operon-*gfp* transcriptional fusions. An overview (not to scale) of LEE DNA indicating LEE genes (white boxes) and LEE transcriptional units including *LEE1*, *LEE2*, *LEE3*, *LEE4*, *LEE5* and *espG* (Elliott *et al.*, 1999, 1998) is shown. The arrows indicate the direction of transcription. The corresponding *gfp* fusions analysed in this study are shown and their sequence positional units relative to the corresponding transcriptional start sites are indicated. In the case of *espG* where the transcriptional start site is unknown, the indicated positions are relative to the putative translational start site.

ml⁻¹. Bacterial strains and plasmids used in this study are listed in Table 1; the oligonucleotides are listed in Table 2.

Construction of *gfp* transcriptional fusions. DNA fragments were amplified by PCR using specific oligonucleotides (Table 2), digested by *Bam*HI and *Xba*I and cloned into the *Bam*HI and *Xba*I sites of pIR1 to form transcriptional fusions with the *gfp-mut3* gene encoding green fluorescent protein (Cormack *et al.*, 1996) as previously described (Friedberg *et al.*, 1999). Plasmids containing the *gfp* fusions are listed in Table 1. The

amplified fragments including the regulatory regions of the corresponding LEE operons are presented in Fig. 1.

Measurements of gene expression by flow cytometry. Gene expression of bacterial strains containing *gfp* fusions was monitored by flow cytometry as previously described (Friedberg *et al.*, 1999). Cultures grown as indicated above (see Bacterial strains) were centrifuged and suspended in filtered PBS to OD₆₀₀ 0.3. Fluorescence intensity of 10⁴ bacterial-sized particles was acquired and the mean values were presented. Fluorescence measurements were performed with a FACScan cytometer (Becton Dickinson). Data analysis was performed with the program CELLQuest. Experiments were repeated at least three times and the standard error was calculated.

Protein extraction and immunoblot analysis. Bacterial cultures grown as indicated above (see Bacterial strains) were centrifuged and extracted by boiling for 7 min in lysis buffer (1% SDS and 50 mM Tris/HCl pH 7.5), 2 min centrifugation at 14000 g and collecting the supernatant. Protein concentrations of the samples were adjusted and samples were subjected to SDS-PAGE, transferred to nitrocellulose membranes and treated with antibodies as described (Wolff *et al.*, 1998).

Construction of EPEC mutants. To construct an EPEC *hns* mutant, a 1380 bp DNA fragment containing EPEC *hns* gene and flanking regions was amplified by PCR using EPEC DNA

and primers *hnsF4* and *hnsR4* (Table 2). A *Bam*HI/*Hind*III digest of the amplified DNA fragment was cloned into the *Bam*HI/*Hind*III sites of the pBS plasmid to form pBS-*hns* (pDF6). A 1.2 kb DNA fragment containing the *kan* gene cassette was purified as a *Pst*I fragment from pUC4K (Pharmacia), cloned into the *Pst*I site of the *hns* gene in pDF6 to form pDF7 (pBS-*hns::kan*). A *Bam*HI-*Xho*I DNA fragment containing the *hns::kan* DNA was purified from pDF7 treated with Klenow fragment (MBI Fermentas) and cloned into the *Sma*I site of pCVD442 to form pDF8 (pCVD-*hns::kan*), in SY327 λ pir strain. pDF8 was then electroporated into *E. coli* SM10 λ pir. EPEC insertionally inactivated *hns* mutant (designated DF3) was created by allelic exchange of wild-type *hns* with *hns::kan* carried by pDF8, using the sucrose selection method (Donnenberg & Kaper, 1991).

We adopted and modified the one-step inactivation method of chromosomal gene disruption that makes use of the Lambda Red recombinase in *E. coli* K-12 (Datsenko & Wanner, 2000), to operate in EPEC. This system, that enables recombination of linear DNA fragments in the *E. coli* genome (Datsenko & Wanner, 2000), was used to construct an EPEC *ler::kan hns::cat* double mutant. A DNA fragment containing the *cat* gene was amplified from plasmid pKD3 (Datsenko & Wanner, 2000), using primers cm-pKD3-F and cm-pKD3-R. The *cat* fragment was cloned into the *Pst*I site of pDF6 to construct pTU15 (pBS-*hns::cat*). EPEC *ler::kan* (Friedberg *et al.*, 1999) was electroporated with plasmid pKD46 containing the Lambda Red recombinase (Datsenko & Wanner, 2000). This strain was subsequently electroporated with a linear DNA fragment containing *hns::cat*, that was amplified with primers *hnsF4* and *hnsR4*, using pTU15 DNA as a template. Cm^r clones were selected at 37 °C, streaked twice and cm^r amp^s clones that had lost pKD46 were selected. PCR analysis confirmed replacement of the wild-type *hns* gene by *hns::cat* and the formation of EPEC *ler::kan hns::cat* double mutant.

H-NS purification. Native, untagged H-NS was purified to homogeneity from an H-NS-producing clone of *E. coli* BL21/pPD3 as described by Dersch *et al.* (1993). The published procedure was scaled down to 1 l bacterial culture and the purified H-NS was concentrated to 3 mg ml⁻¹ using Centricon YM-10 (Amicon).

DNA band shift assay with H-NS. Three DNA fragments were used: a 186 bp DNA fragment corresponding to the sequence from position -173 to +11 relative to the *LEE1* transcriptional start point (*LEE1* fragment), amplified with primers 9F and 5R; a 395 bp PCR fragment corresponding to sequence from position +117 to -288 nucleotides relative to *LEE2* transcriptional start point (*LEE2-LEE3* fragment), amplified with primers 30F and 31R; and a DNA fragment 171 bp in size containing the λ CIII coding region, amplified with primers 2703 and 2902. The *LEE* fragments and the *CIII* fragment were digested by *Xba*I and *Eco*RI respectively and end-labelled by filling in using Klenow fragment of DNA polymerase I (MBI Fermentas) and [α -³²P]dCTP (3000 Ci mmol⁻¹; Amersham). Binding of H-NS to the end-labelled DNA (3–6 fmol) was performed at 25 °C for 30 min, in 10 μ l binding buffer containing: 50 mM Tris/HCl (pH 7.4), 70 mM KCl, 1 mM EDTA, 10 mM β -mercaptoethanol, 100 μ g bovine serum albumin ml⁻¹, 6% glycerol and 100 ng poly(dI-dC) (Boehringer). At the end of the incubation period the samples were loaded on 4.5% polyacrylamide gel (acrylamide to bisacrylamide, 37.5:1 w/w) in 0.5 $\times$ TBE buffer and electrophoresis was carried out at room temperature. The dried gels were exposed for autoradiography and the results were quantified using a phosphorimager (Fuji) as described.

RESULTS

Expression of *LEE1*, *LEE2*, *LEE3*, *LEE4*, *LEE5* and *espG* is negatively regulated by H-NS in a temperature-dependent manner

We constructed an *hns* mutant of EPEC (*hns::kan*) (see Methods) and tested the role of H-NS in the expression of all major *LEE* transcriptional units. We compared three EPEC strains: wild-type, *hns::kan* mutant and the *hns* mutant complemented by a plasmid encoding H-NS (pDF6). These strains were grown in DMEM at 27 °C or 37 °C to OD₆₀₀ 0.3–0.35 and tested for production of *LEE* encoded proteins using Western analysis with antibodies raised against Ler (*LEE1* encoded), intimin and Tir (translocated intimin receptor; *LEE5* encoded) and EspA, EspB and EspF (*LEE4* encoded) (Fig. 2). At 27 °C, production of these proteins was repressed in the presence of H-NS and derepressed in the absence of H-NS. At 37 °C all the strains expressed these proteins but the expression was somewhat more efficient in the *hns* mutant strain (Fig. 2).

We further tested the role of H-NS in *LEE* regulation by an additional, independent approach. EPEC wild-type and EPEC *hns::kan* mutant were transformed with plasmids containing transcriptional fusions of the *gfp* reporter gene with DNA fragments containing the regulatory sequences of the *LEE1*, *LEE2*, *LEE3*, *LEE4*, *LEE5* and *espG* transcriptional units (Fig. 1). The strains were grown at 37 °C or 27 °C and the level of GFP was

Fig. 2. Expression of *LEE* genes is derepressed in EPEC *hns* null mutant grown at 27 °C. Proteins were extracted from *hns::kan*, *hns::kan/pBS-hns* and EPEC wild-type grown in DMEM at 27 °C or 37 °C. The extracted proteins were applied to SDS-PAGE followed by immunoblot analysis, using anti-Ler, anti-EspA, anti-EspB, anti-EspF, anti-Tir and anti-Intimin antibodies.

Table 3. Thermoregulation of LEE genes

EPEC *hns::kan* mutant and EPEC wild-type containing the indicated *gfp* transcriptional fusions were grown in DMEM at 27 °C or 37 °C and analysed for *gfp* expression using flow cytometry. Each of the fluorescence values is the mean value of three experiments, and the standard error is shown in parentheses. The level of derepression is the ratio of the fluorescence values of *hns::kan* to wild-type.

<i>LEE::gfp</i>	27 °C			37 °C		
	<i>hns::kan</i>	Wild-type	Derepression level	<i>hns::kan</i>	Wild-type	Derepression level
<i>LEE1</i>	396 (24)	23 (1.7)	17.2	261 (14)	126 (8)	2.1
<i>espG</i>	156 (22)	6 (0.14)	26.0	132 (10)	41 (3)	3.2
<i>LEE2</i>	338 (22)	6 (0.43)	56.3	413 (21)	176 (6.5)	2.3
<i>LEE3</i>	239 (21)	9 (0.15)	26.6	114 (10)	89 (7)	1.3
<i>LEE4</i>	641 (30)	39 (3)	16.4	704 (49)	413 (32)	1.7
<i>LEE5</i>	108 (5)	9 (0.5)	12.0	122 (3)	86 (6)	1.4
<i>Ptac</i>	130 (4)	122 (3.6)	1.1	138 (7)	136 (9.5)	1.0

determined (Table 3). Expression of *gfp* by cells grown at 37 °C was similar in EPEC wild-type and *hns* mutant strains, with somewhat higher GFP levels in the *hns* mutant. In contrast, *gfp* expression of wild-type EPEC grown at 27 °C was very low, while the *hns::kan* mutant grown at 27 °C expressed *gfp* efficiently to levels somewhat higher than wild-type levels at 37 °C (Table 3). We observed that in the *hns* mutant background the plasmid copy number is about twofold higher compared with the wild-type level. This could increase the GFP level in the *hns* mutant by twofold. The results with the *gfp* transcriptional fusions are in agreement with the Western analysis shown in Fig. 2. Taken together these results indicate that expression of *LEE1*, *LEE2*, *LEE3*, *LEE4*, *LEE5* and *espG* is negatively regulated by H-NS at the non-permissive temperature of 27 °C.

Ler expressed *in trans* rescues the expression of Ler-activated LEE genes at 27 °C

The above results indicate that the *LEE1*-encoded *ler* is derepressed at 27 °C in EPEC *hns::kan* mutant. Therefore we tested whether the expression of *ler* is sufficient to activate expression of the Ler-activated *LEE2*, *LEE3*, *LEE4*, *LEE5* and *espG* operons at 27 °C in wild-type EPEC. We transformed wild-type EPEC with a plasmid encoding *ler* expressed from the *Ptac* regulated promoter (pTU14). The transformed strain was grown at 27 °C and the effect of *ler* expression on the production of intimin, Tir, EspA, EspB and EspF was tested by Western analysis. The results clearly show that supplying Ler *in trans* at 27 °C is sufficient to activate the production of these proteins (Fig. 3A).

To further confirm these results, EPEC strains containing plasmids encoding *gfp* transcriptional fusions with regulatory regions of *LEE2*, *LEE3*, *LEE4*, *LEE5* or *espG* (see Fig. 1) were transformed with a compatible plasmid encoding *Ptac-ler* (pTU14). We compared *gfp* expression at 27 °C in the absence or presence of Ler

supplied *in trans*. Expression of all of the examined LEE operons in the absence of pTU14 was low, at background levels. In contrast, supplying Ler *in trans* from pTU14 resulted in increased expression of *LEE2*, *LEE3*, *LEE4*, *LEE5* and *espG* by 12-, 7-, 15-, 13- and 7-fold, respectively (Fig. 3B). Expression of *gfp* transcriptional fusions with *tac* or *oxyS* promoters was not affected by Ler (data not shown). These results indicate that expression of Ler is sufficient to induce the expression of *LEE2*, *LEE3*, *LEE4*, *LEE5* and *espG* in wild-type EPEC encoding *hns* and grown at the non-permissive temperature of 27 °C.

Expression of LEE genes in EPEC double mutant inactivated in both *ler* and *hns* genes

To further substantiate the role of H-NS in the regulation of *LEE2*, *LEE3*, *LEE4*, *LEE5* and *espG* without any possible interference of Ler, we constructed an EPEC double mutant inactivated in both *hns* and *ler*. This EPEC mutant was constructed by using the Lambda Red recombinase system that enables recombination of linear DNA into the *E. coli* K-12 genome as described by Datsenko & Wanner (2000) (see Methods). We compared the expression of LEE genes in the background of the following isogenic EPEC strains: wild-type, *ler::kan*, *hns::kan* and *ler::kan hns::cat*. The four strains were grown at 37 °C and analysed by immunoblotting using antibodies raised against EspA, EspB (*LEE4*-encoded proteins), intimin and Tir (*LEE5*-encoded proteins). The EPEC *ler hns* double mutant expressed EspA and EspB at levels comparable to the *hns* mutant. However, this mutant expressed Tir and intimin to levels significantly lower than the *hns* mutant (Fig. 4A). As expected, the EPEC *hns* mutant expressed these proteins at levels comparable to the wild-type EPEC, while the *ler* mutant did not express any of these proteins (Fig. 4A).

We transformed each of the four EPEC strains wild-

Fig. 3. Expression of *ler* in trans rescues the expression of LEE genes in EPEC grown at 27 °C. (A) Immunoblot analysis. Proteins were extracted from EPEC wild-type and EPEC/*ler* (bearing pTU14) grown at 27 °C. The extracted proteins were applied to SDS-PAGE followed by immunoblot analysis using anti-EspA, anti-EspB, anti-EspF, anti-Tir and anti-Intimin antibodies. (B) EPEC wild-type (black columns) and EPEC/*ler* (grey columns) bearing *gfp* fusions of *espG*, *LEE2*, *LEE3*, *LEE4* and *LEE5* were grown at 27 °C and the fluorescence intensity was measured by flow cytometry. The values are the mean of three experiments and the standard error values are shown by error bars.

type, *ler::kan*, *hns::kan* and *ler::kan hns::cat* with plasmids containing the *gfp* transcriptional fusions with the regulatory regions of *LEE1*, *LEE2*, *LEE3*, *LEE4*, *LEE5* or *espG* (see Fig. 1). The bacteria were grown at 37 °C and the level of *gfp* expression was compared (Fig. 4B). Expression of all the LEE operons in the *hns::kan* background was similar to that exhibited by the wild-type EPEC, or somewhat higher, while expression in the *ler::kan* background was strongly inhibited, except for *LEE1* (Fig. 4B). In contrast to *ler::kan*, in the background of *ler::kan hns::cat*, expression of *LEE2*, *LEE3*, *LEE4* and *espG* was completely restored, while expression of *LEE5* was only partially restored (Fig. 4B). As expected, a control using *LEE1* fusion exhibited a similar GFP level in the different background strains.

Fig. 4. Expression of LEE genes in EPEC double mutant inactivated in both *hns* and *ler* genes. (A) Immunoblot analysis. Protein extracts were prepared from EPEC wild-type, *ler::kan*, *hns::kan*, and *ler::kan hns::cat* mutants, grown at 37 °C. The extracted proteins were applied to SDS-PAGE followed by immunoblot analysis using anti-EspA, anti-EspB, anti-Tir and anti-Intimin antibodies. (B) Flow cytometry. EPEC wild-type (dotted columns), *ler::kan* (black columns), *hns::kan* (light grey columns) and *ler::kan hns::cat* (dark grey columns) mutants bearing *gfp* transcriptional fusions of *LEE1*, *espG*, *LEE2*, *LEE3*, *LEE5* and *LEE4* were grown at 37 °C. The expression levels were determined by flow cytometry. The values are the mean of three experiments and the standard error values are shown by error bars.

Taken together, the results shown in Fig. 4 indicate that in the absence of H-NS, Ler is dispensable for full expression of *LEE2*, *LEE3*, *LEE4* and *espG* but it is still needed for efficient expression of *LEE5*. Furthermore, the derepression of *LEE2*, *LEE3*, *LEE4*, *LEE5* and *espG* in the *hns ler* double mutant, in contrast to the repression in the *ler* mutant, suggests that H-NS mediates the repression of each of these transcriptional units.

H-NS binds to the *LEE1* and *LEE2*–*LEE3* promoter regions

We tested whether regulation by H-NS involves its interaction with the regulatory regions of *LEE1* and *LEE2*–*LEE3*. H-NS was purified as described by Dersch

Fig. 5. H-NS binds to the promoter region of *LEE1* and to the overlapping promoter region of *LEE2* and *LEE3*. (A) Purified H-NS protein was subjected to SDS-PAGE followed by Coomassie blue staining. (B) A gel shift assay of the *LEE1* promoter region with H-NS. A DNA fragment corresponding to the sequence from position -173 to $+11$ relative to the *LEE1* transcriptional start point (*LEE1* fragment) was mixed with increasing concentrations (0, 0.191, 0.382, 0.765, 1.531, 3.062, 4.080, 6.125 and 8.160 μ M) of H-NS. (C) Quantification of the gel-shift results with the *LEE1* fragment using a phosphorimager. (D) A gel shift assay of *LEE2* and *LEE3* regulatory region with H-NS. A DNA fragment, corresponding to position $+117$ to -288 relative to *LEE2* transcriptional start point (*LEE2-LEE3* fragment) was mixed with increasing concentrations (0, 0.191, 0.382, 0.765, 1.531, 3.062, 4.080, 6.125 and 8.160 μ M) of H-NS. (E) Quantification of the gel-shift results with the *LEE2-LEE3* fragment using a phosphorimager. (F) A gel shift assay of a λ CIII DNA fragment with H-NS. A 171 bp DNA fragment containing the CIII coding region was incubated with increasing concentrations (0, 1.020, 2.040, 4.080 and 8.160 μ M) of H-NS.

et al. (1993) (Fig. 5A) and used in gel shift assays with three DNA fragments: a fragment that extends nucleotides -173 to $+11$ relative to *LEE1* transcriptional start site (*LEE1* fragment), a fragment containing the overlapping promoter region and the flanking 5' coding regions of *LEE2* and *LEE3*, corresponding to nucleotide positions $+117$ to -288 relative to *LEE2* transcriptional start point (*LEE2-LEE3* fragment), and a control fragment driven from the *CIII* gene of Lambda phage. The *LEE1* and *LEE2-LEE3* fragments formed low-mobility complexes in the presence of H-NS (Fig. 5B, D). However, the formation of a low-mobility complex with the control *CIII* DNA fragment was insignificant and demanded a much higher H-NS concentration (Fig. 5F). This indicates a preferential binding of H-NS to the *LEE1* and *LEE2-LEE3* fragments. To assess the apparent binding affinity, we performed a quantitative analysis of the free and bound DNA in the presence of increasing (1.5 to 2-fold) H-NS concentrations (Fig. 5C, E). A small increase (about 1.5 to 2-fold) in H-NS concentration was sufficient to shift the majority of the *LEE1* DNA, as well as the *LEE2-LEE3* DNA, from a free to a protein-bound form, suggesting a cooperative binding mode of H-NS. The data show an apparent dissociation constant (Kd,

protein concentration that binds 50% of the DNA) of 0.95 μ M and 0.80 μ M with *LEE1* and *LEE2-LEE3* fragments respectively. In both cases the curve shape confirms a cooperative binding mode of H-NS. The binding of H-NS to the LEE promoter regions is consistent with its role as a repressor of each of these LEE genes.

DISCUSSION

In this study we show a regulatory cascade of the thermoregulation of LEE genes encoding the type III secretion system and effector proteins in EPEC. The expression of *ler*, a *LEE1* gene that encodes a positive regulator of most other LEE genes, is subjected to temperature-dependent regulation mediated by H-NS: it is repressed at 27 °C but not at 37 °C. In contrast, all other major LEE operons are repressed by H-NS both at 27 °C and 37 °C. We used two independent techniques to compare wild-type EPEC and the EPEC *hns::kan* mutant: Western analysis and *gfp* transcriptional fusions. Analysis of *gfp* transcriptional fusions carried by a low to medium copy-number plasmid was recently further approved as an accurate and reliable system for monitoring transcriptional regulation in *E. coli* (Kalir *et al.*, 2001). We show that at the non-permissive temperature of 27 °C, H-NS represses the expression of *LEE1*, *LEE2*, *LEE3*, *LEE4*, *LEE5* and *espG*. In contrast, at 37 °C the differences in expression levels between the wild-type and the *hns* mutant were relatively small. By supplying *Ler* *in trans* we demonstrated that ectopic expression of *ler* at 27 °C is sufficient to activate the expression of *LEE2*, *LEE3*, *LEE4*, *LEE5* and *espG* by EPEC wild-type. Taken together, it is evident that H-NS strongly represses *LEE1* at 27 °C, but at 37 °C this repression is inefficient (see Table 3).

However, it was more complicated to draw conclusions regarding the role of H-NS in the regulation of all other LEE operons, since the *LEE1*-encoded *Ler* is a positive regulator of these operons. Therefore, to examine the role of H-NS without the interference of *Ler*, we constructed an EPEC strain mutated in both *hns* and *ler*. The analysis of LEE gene expression in this double mutant was particularly informative. While in the absence of *Ler* at 37 °C the expression of *LEE2*, *LEE3*, *LEE4*, *LEE5* and *espG* operons was repressed, the repression was alleviated upon further inactivation of *hns*. These results indicate that H-NS represses each of these operons regardless of *Ler*. While at 37 °C the H-NS-mediated repression of *LEE1* was inefficient, H-NS efficiently repressed the other operons at 37 °C, including *LEE2*, *LEE3*, *LEE4*, *LEE5* and *espG*.

We further showed that in the absence of H-NS, *Ler* was dispensable for full expression of *LEE2*, *LEE3*, *LEE4* and *espG*. This suggests that *Ler* activates expression of these operons exclusively by preventing the H-NS-mediated repression. However, inactivation of *hns* was insufficient for a complete restoration of *LEE5* expression, in the absence of *Ler*, suggesting that *Ler* activates expression of *LEE5* both by antagonizing the

Fig. 6. A schematic model of the regulatory network of LEE gene expression. At 27 °C, H-NS mediates the repression of all major LEE operons (upper panel). Upon shifting the temperature from 27 °C to 37 °C (lower panel), the expression of *LEE2*, *LEE3*, *LEE4*, *LEE5* and *espG* is still repressed by H-NS, while the expression of *LEE1*, that is activated by IHF and modulated by Fis, Per and quorum sensing (QS), is alleviated. This leads to the production of Ler, which subsequently activates the expression of *LEE2*, *LEE3*, *LEE4*, *LEE5* and *espG*. Ler mediates the activation of these transcriptional units by alleviating the H-NS-mediated repression. The horizontal arrows represent LEE transcriptional units, their heads point in the direction of transcription; white horizontal arrows indicate repressed genes. The striped horizontal arrow represents the temperature-mediated 37 °C-expressed *LEE1* operon; dotted horizontal arrows represent Ler-activated LEE operons. The vertical arrows represent positive regulation of LEE genes; the inverted Ts represent negative regulation of LEE genes.

H-NS mediated repression as well as by an additional mechanism, as yet to be defined.

Based on these results, our current working model, presented in Fig. 6, is as follows. At the non-permissive temperature of 27 °C, H-NS mediates repression of *LEE1*, *LEE2*, *LEE3*, *LEE4*, *LEE5* and *espG*. The physical evidence that H-NS binds to the *LEE1*–*LEE3* regulatory regions supports the speculation that H-NS might act as a direct repressor of these operons. It is yet to be confirmed that this interaction occurs also in the case of the other LEE operons. According to our model, upon shifting the temperature from 27 °C to 37 °C, the expression of *LEE1* is alleviated while the expression of *LEE2*, *LEE3*, *LEE4*, *LEE5* and *espG* is still repressed by H-NS. The production of Ler at 37 °C subsequently activates the expression of *LEE2*, *LEE3*, *LEE4*, *LEE5* and *espG*. Ler mediates the activation of *LEE2*, *LEE3*, *LEE4* and *espG* by alleviating the H-NS-mediated repression. Activation of *LEE5* by Ler involves both antagonizing the H-NS repression and an additional, as yet unknown mechanism, that is independent of H-NS.

The preferential binding of H-NS to the *LEE1* and *LEE2*–*LEE3* promoter regions supports the genetic data that H-NS either alone or together with other factors represses these operons. In the case of the *LEE2*–*LEE3*, the H-NS binding region overlaps with the Ler-binding region (Sperandio *et al.*, 2000). The pattern of H-NS binding to the tested DNA fragments indicates that multiple copies of H-NS bind to the *LEE1* and *LEE2*–*LEE3* promoter regions in a cooperative way, typical to

H-NS binding in other systems (Atlung & Ingmer, 1997).

In conclusion, our results on the opposite role of Ler and HNS in respect to expression of the *LEE2* and *LEE3* operon confirm and substantiate a previous report (Bustamante *et al.*, 2001). In addition we show, for the first time, the opposite role of these two regulators in the case of three additional LEE operons, namely the *LEE4*, *LEE5* and *espG*. More importantly, we describe here for the first time the role of H-NS in temperature-dependent repression of the *LEE1* operon including *ler*. This repression is the basis for the thermoregulated expression of all other LEE operons.

In enteroinvasive *E. coli* and *Shigella*, virulence genes are repressed at 30 °C and expressed at 37 °C (Maurelli & Sansonetti, 1988). H-NS is involved in the thermoregulation of *virF* (Falconi *et al.*, 1998), the first positive activator of a multi-step regulatory cascade leading to expression of the type III secretion system and other virulence effectors (Dorman & Porter, 1998). It was proposed that the physical basis for the thermoregulated expression of *virF* resides in a temperature-dependent structural modification of the *virF* promoter, resulting from a temperature-sensitive curved DNA structure (Falconi *et al.*, 1998). H-NS also mediates repression of the *virB* gene downstream in the *Shigella* regulatory cascade (Tobe *et al.*, 1993). The regulatory cascade in EPEC, whereby the expression of Ler is thermoregulated by H-NS, is apparently analogous to the *Shigella* system. However, in EPEC the role of H-NS is more complex, given its involvement in repression of all other major LEE operons, and because of the activity of Ler as a positive regulator that prevents H-NS-mediated repression of all other LEE operons.

We hypothesize that a temperature-dependent DNA-topology modification of the *LEE1* promoter region might play a role in the thermoregulation of *LEE1*. This hypothesis is supported by our previous data showing that binding of IHF upstream of the *LEE1* promoter is essential for *LEE1* expression (Friedberg *et al.*, 1999). IHF that binds to DNA in specific sites bends the DNA to form nucleoprotein complexes (Rice *et al.*, 1996). The IHF-dependent modification of the DNA curvature might change the accessibility to H-NS in a temperature-dependent manner. It remains to be seen if IHF antagonizes H-NS and thereby alleviates the H-NS mediated repression of *LEE1* at 37 °C. In DNase footprinting experiments H-NS does not show any distinct footprinting, but protects extended DNA sequences of the *LEE1* promoter region, including the IHF binding site (not shown). Interplays, either synergistic or antagonistic, between histone-like proteins, including H-NS and IHF, have been implicated in several unrelated genetic systems (McLeod & Johnson, 2001). For instance, in the case of the early promoter of bacteriophage Mu, it was shown that IHF interacts directly with the transcription initiation complex and in addition alleviates the H-NS mediated repression of the Pe promoter (van Ulsen *et al.*, 1996). In the case of *Shigella flexneri*

virulence genes it was reported that IHF mediates *virB* expression by overcoming the repression exerted by H-NS (Porter & Dorman, 1997).

A finely tuned regulation of the *ler* gene is presumably critical for the timing of LEE gene expression during infection. Fis, PerABC and quorum sensing are also involved in the regulation of *LEE1* (Goldberg *et al.*, 2001; Mellies *et al.*, 1999; Sperandio *et al.*, 1999). The future challenge is to define in EPEC and related organisms the role of all the various regulators and to characterize the interplay that mediates LEE gene expression in response to the host-environmental signals.

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