The flagellar apparatus consists of components that function as a type III secretion system (TTSS). Enteropathogenic and enterohaemorrhagic *Escherichia coli* (EPEC and EHEC, respectively) produce an additional TTSS, which is involved in virulence via the translocation of effector proteins into infected host cells. This system is encoded by the locus of enterocyte effacement (LEE). The authors observed that EPEC and EHEC grown in Dulbecco’s modified Eagle’s medium to the mid- and late-exponential growth phase at 37 °C are non-motile. At the same time these conditions trigger the expression of the LEE-encoded TTSS. Furthermore, it was found that EPEC with an inactivated *ihfA*, which encodes the IHFα subunit of the integration host factor (IHF), becomes hyperflagellated and motile. Similar hypermotility was seen upon inactivation of the *ihfA* of EHEC strains. IHF-mediated repression of the EPEC flagella involves down-regulation of *flhDC*, which encodes a positive regulator of the flagellar regulon. IHF indirectly mediates *flhDC* repression, via a putative EPEC-unique regulator which is not encoded by LEE.

The flagellar apparatus consists of components that function as a type III secretion system (TTSS), facilitating the export of flagellar proteins and flagellar assembly (Aldridge & Hughes, 2002). Enteropathogenic and enterohaemorrhagic *E. coli* (EPEC and EHEC) contain an additional TTSS. This system is encoded by several operons located in the locus of enterocyte effacement (LEE) (McDaniel & Kaper, 1997). The LEE-encoded TTSS mediates the injection of virulence factors into infected mammalian cells. Most of the LEE operons are positively regulated by the Ler regulator, which is encoded by the LEE1 operon (Friedberg *et al.*, 1999; Mellies *et al.*, 1999; Sperandio *et al.*, 2000; Sanchez-SanMartin *et al.*, 2001). Integration host factor (IHF) directly activates the expression of Ler (Friedberg *et al.*, 1999). Additional factors, including Fis, Per and a quorum sensing regulator, are also involved in the modulation of *LEE*1 expression (Goldberg *et al.*, 2001; Kanamaru *et al.*, 2000; Mellies *et al.*, 1999; Sperandio *et al.*, 1999, 2002a, b).

The optimal condition for expression of LEE genes by EPEC is growth of the cells to mid-exponential phase in Dulbecco’s modified Eagle’s medium (DMEM; Sigma) at 37 °C (Rosenshine *et al.*, 1996). We show here that under these conditions EPEC cells are non-motile and do not express flagella. We found that repression of flagellar expression in EPEC involves silencing of *flhDC* by IHF. We further demonstrate that IHF mediates *flhDC* repression indirectly.

**INTRODUCTION**

Flagella play a role in bacterial adaptation to environmental conditions and have often been associated with the virulence of various pathogens (Ottemann & Miller, 1997). The flagellar system of *Escherichia coli* and *Salmonella typhimurium* is encoded by over 40 genes (Aldridge & Hughes, 2002). These genes are organized into several co-regulated operons. The *flhDC* operon encodes FlhD and FlhC, which act as positive regulators of the flagellar regulon. The pattern of *flhDC* expression varies in different bacterial species and strains and is modulated in response to environmental and physiological signals, according to the adaptability characteristics of a given strain. *flhDC* expression is influenced by signals such as variations in temperature and osmolarity (Li *et al.*, 1993; Shi *et al.*, 1993; Shin & Park, 1995), by regulators such as cAMP-catabolite activator protein (CAP), H-NS, HU, DnaK, DnaJ, GrpE, Fis and Lrp, and by a quorum-sensing factor (Silverman & Simon, 1974; Soutorina *et al.*, 1999; Nishida *et al.*, 1997; Shi *et al.*, 1992; Osuna *et al.*, 1995; Hay *et al.*, 1997; Sperandio *et al.*, 2002a, b).

The flagellum consists of components that function as a type III secretion system (TTSS), facilitating the export of flagellar proteins and flagellar assembly (Aldridge & Hughes, 2002). Enteropathogenic and enterohaemorrhagic *E. coli* (EPEC and EHEC) contain an additional TTSS. This system is encoded by several operons located in the locus of enterocyte effacement (LEE) (McDaniel & Kaper, 1997). The LEE-encoded TTSS mediates the injection of virulence factors into infected mammalian cells. Most of the LEE operons are positively regulated by the Ler regulator, which is encoded by the LEE1 operon (Friedberg *et al.*, 1999; Mellies *et al.*, 1999; Sperandio *et al.*, 2000; Sanchez-SanMartin *et al.*, 2001). Integration host factor (IHF) directly activates the expression of Ler (Friedberg *et al.*, 1999). Additional factors, including Fis, Per and a quorum sensing regulator, are also involved in the modulation of LEE1 expression (Goldberg *et al.*, 2001; Kanamaru *et al.*, 2000; Mellies *et al.*, 1999; Sperandio *et al.*, 1999, 2002a, b).

The optimal condition for expression of LEE genes by EPEC is growth of the cells to mid-exponential phase in Dulbecco’s modified Eagle’s medium (DMEM; Sigma) at 37 °C (Rosenshine *et al.*, 1996). We show here that under these conditions EPEC cells are non-motile and do not express flagella. We found that repression of flagellar expression in EPEC involves silencing of *flhDC* by IHF. We further demonstrate that IHF mediates *flhDC* repression indirectly.
**METHODS**

**Bacterial strains, culture conditions and oligonucleotide primers.** The bacterial strains were grown overnight as standing cultures in LB at 37˚C, diluted 1:50 in LB or buffered DMEM, as indicated, and grown to a density of OD600 0.3–0.35. When necessary, IPTG (1 mM) was added 1 h before harvesting the cultures. Antibiotics were added at the following concentrations: ampicillin (Amp), 100 µg ml⁻¹; kanamycin (Kan), 40 µg ml⁻¹; chloramphenicol (Cm), 25 µg ml⁻¹; and streptomycin (Str) 100 µg ml⁻¹. The bacterial strains and plasmids used in this study are listed in Table 1; the oligonucleotides are listed in Table 2.

**Construction of plasmids.** Plasmid pDF12, containing the transcriptional fusion of flhDC<sub>EPEC</sub> with gfp-mut3 (Cormack et al., 1996), was constructed as follows. A DNA fragment starting 415 bp upstream of the putative flhDC<sub>EPEC</sub> translational start point, and 220 bp upstream from a putative translational start frame (Soutourina et al., 1999) including the flhDC<sub>EPEC</sub> coding region, was amplified using primers flh-F5 and flh-R5 with EPEC DNA serving as a template. The amplified DNA fragment was digested with BanHI and XhoI and cloned into the corresponding sites in pRI1 (Friedberg et al., 1999).

Plasmid pDF13, expressing flhDC<sub>EPEC</sub> from the T5-lac promoter, was constructed as follows. A fragment including flhDC<sub>EPEC</sub> was amplified using primers flh-F31 and flh-R1 and cloned into a pACYC184 derivative containing lac<sup>q</sup> and the T5-ribosome-binding site and cloning site derived from the expression vector pQE31 (Qiagen). The fragment was cloned into the BamHI and HindIII sites of the vector, in-frame with the hexahistidine (6×his) tag.

To construct plasmid pDF14, expressing ifhAB from the lac promoter, an AntII–PstI DNA fragment carrying the ifhAB genes expressed from the lac promoter was isolated from plasmid pDRC171 and ligated into the corresponding sites in pACYC177.

**Construction of EPEC lacking the IHF-binding site upstream of LEE1.** A 632 bp DNA fragment was amplified using primers 18F1 and 17R, digested with BanHI and XhoI, and ligated into pBS to form pBS-fragment 1. A 656 bp DNA fragment was amplified by

**Table 1. Bacterial strains and plasmids**

<table>
<thead>
<tr>
<th>Strain/plasmid</th>
<th>Relevant genotype and characteristics</th>
<th>Reference/source*</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Strains</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>E2348/69</td>
<td>EPEC O127: H6 wild-type, Sm&lt;sup&gt;+&lt;/sup&gt;</td>
<td>J. Kaper</td>
</tr>
<tr>
<td>31-6-1(1)</td>
<td>E2348/69 bfpA::Tp5hoA</td>
<td>Donnenberg et al. (1990)</td>
</tr>
<tr>
<td>DF1</td>
<td>E2348/69 ifhA::kan</td>
<td>Friedberg et al. (1999)</td>
</tr>
<tr>
<td>DF2</td>
<td>E2348/69 ler::kan</td>
<td>Friedberg et al. (1999)</td>
</tr>
<tr>
<td>DF4</td>
<td>E2348/69::ΔIHF-BS</td>
<td>This study</td>
</tr>
<tr>
<td>EDL933</td>
<td>EHEC O157:H7 Slt1, Slt2-negative derivative</td>
<td>J. Leong</td>
</tr>
<tr>
<td>85-170</td>
<td>EHEC O157:H7 Slt1-negative derivative</td>
<td>Trizzi et al. (1987)</td>
</tr>
<tr>
<td>N99</td>
<td>F&lt;sup&gt;−&lt;/sup&gt;galK2 rpsL</td>
<td>A. Oppenheim</td>
</tr>
<tr>
<td>N99-A5188</td>
<td>himA82::Tn10</td>
<td>A. Oppenheim</td>
</tr>
<tr>
<td>W3110</td>
<td>F&lt;sup&gt;−&lt;/sup&gt;thyA36 deoC2 IN1</td>
<td>A. Oppenheim</td>
</tr>
<tr>
<td>W3110-YF1</td>
<td>W3110 himA82::tet</td>
<td>Friedberg et al. (1999)</td>
</tr>
<tr>
<td>DH5z</td>
<td>supE44 ΔlacU169 (880 lacZ ΔM15) hsdR17 recA1 endA1 gyrA96 thi-1 relA1</td>
<td>BRL</td>
</tr>
<tr>
<td>SY327&lt;sup&gt;pir&lt;/sup&gt;</td>
<td>thi-1 thr leu tonA lacY recA</td>
<td>M. Donnenberg</td>
</tr>
<tr>
<td>SM10&lt;sup&gt;pir&lt;/sup&gt;</td>
<td>supE (RP4-2 Km&lt;sup&gt;+&lt;/sup&gt; tet::Mu)</td>
<td>M. Donnenberg</td>
</tr>
<tr>
<td><strong>Plasmids</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>pRI1</td>
<td>pKK177-3 derivative containing gfp mut3</td>
<td>Friedberg et al. (1999)</td>
</tr>
<tr>
<td>pDRC157</td>
<td>pBR322 derivative containing P&lt;sub&gt;lac&lt;/sub&gt;-ifhA</td>
<td>A. Oppenheim</td>
</tr>
<tr>
<td>pDRC171</td>
<td>pBR322 derivative containing P&lt;sub&gt;lac&lt;/sub&gt;-ifhA-ifhB</td>
<td>A. Oppenheim</td>
</tr>
<tr>
<td>pCVD442</td>
<td>Suicide vector containing the sacB gene</td>
<td>M. Donnenberg</td>
</tr>
<tr>
<td>pDF5</td>
<td>pACYC184 derivative containing T5-lac-6×his-ler and lac&lt;sup&gt;q&lt;/sup&gt;</td>
<td>Friedberg et al. (1999)</td>
</tr>
<tr>
<td>pDF10</td>
<td>pCVD442 derivative carrying a LEE fragment with a deleted IHF-binding site</td>
<td>This study</td>
</tr>
<tr>
<td>pDF12</td>
<td>pRI1 derivative containing the flhDC::gfp translational fusion</td>
<td>This study</td>
</tr>
<tr>
<td>pDF13</td>
<td>pACYC184 derivative containing T5-lac-6×his-flhDC and lac&lt;sup&gt;q&lt;/sup&gt;</td>
<td>Friedberg et al. (1999)</td>
</tr>
<tr>
<td>pDF14</td>
<td>pACYC177 derivative containing P&lt;sub&gt;lac&lt;/sub&gt;-ifhA-ifhB</td>
<td>This study</td>
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</table>

**Table 2. Oligonucleotides**

<table>
<thead>
<tr>
<th>Name</th>
<th>Sequence</th>
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<tbody>
<tr>
<td>flh-F5</td>
<td>CGGGATCCTGCGACATGTCGGCAACATCCCC</td>
</tr>
<tr>
<td>flh-R5</td>
<td>GCTTCTAGACGCGCTTGATCTCTCTTG</td>
</tr>
<tr>
<td>flh-F31</td>
<td>CGGGATCCAGTGGGGATAAATGCTACCTC</td>
</tr>
<tr>
<td>flh-R1</td>
<td>CCCAGCTTGGCGAGGACCCGTGAGTATCAG</td>
</tr>
<tr>
<td>LEE-16F</td>
<td>CGGGATCCCTTAATGTTTAAAAATATATG</td>
</tr>
<tr>
<td>LEE-17R</td>
<td>GGAATTCCTACTCTGTTAACACGAC</td>
</tr>
<tr>
<td>LEE-18F</td>
<td>CCCAAGCTTGGCGGATATTGTTGTGTTAAC</td>
</tr>
<tr>
<td>LEE-19R</td>
<td>GCTCTAGACTTCGATCGTTATC</td>
</tr>
</tbody>
</table>
PCR using primers 16F and 19R, digested with HindIII and EcoRI, and ligated into pBS-fragment 1 to form pDP9. The insert in pDP9 consists of an EPEC DNA fragment extending from −724 to +540 bp relative to the LEE1 transcription start point, but including a deletion of 20 nt extending from −66 to −87 which is part of an IHF-binding site (Friedberg et al., 1999). The insert was recovered by digestion with XbaI and EcoRI; the fragment ends were filled in using the Klenow fragment and ligated into the SmaI site of pCVD442 to generate pDP10. We used pDP10 to construct an EPEC strain (designated DF4), with a deleted IHF-binding site from −66 to −87 bp upstream to the LEE1 transcription start point. The allelic exchange was carried out as described by Donnenberg & Kaper (1991).

**Measurement 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).

**Protein extraction and immunoblot analysis.** Overnight EPEC cultures were diluted 1:50 in DMEM and grown at 37°C to a density of OD₆₀₀ 0-35-0-4. When necessary, IPTG (1 mM) was added immediately upon inoculation to induce ihfA, or 2-5 h after DME inoculation to induce ler expression. The cultures were centrifuged and the bacteria were lysed by boiling in SDS loading buffer, as previously described (Rosenshine et al., 1996). The protein concentration in the samples was adjusted, and the samples were subjected to SDS-PAGE and transferred to nitrocellulose membranes (AB-S 83; Schleicher & Schuell). Blots were incubated with polyclonal anti-ihfA antibodies (S. Friedberg, unpublished), with secondary anti-rabbit IgG antibody conjugated to alkaline phosphatase (Sigma) and ligated into pBS-fragment 1 to form pDF9. The insert in pDF9 was recovered by digestion with XbaI and EcoRI; the fragment ends were filled in using the Klenow fragment and ligated into the SmaI site of pCVD442 to generate pDF10. We used pDF10 to construct an EPEC strain (designated DF4), with a deleted IHF-binding site from −66 to −87 bp upstream to the LEE1 transcription start point. The allelic exchange was carried out as described by Donnenberg & Kaper (1991).

**Motility tests.** Motility was screened by microscopy and on swarm plates containing gfp fusions was monitored by flow cytometry as previously described (Friedberg et al., 1999).

**Invasion assay.** Invasion of HeLa cells was monitored by the gentamicin protection assay as previously described (Rosenshine et al., 1996). When necessary, IPTG (1 mM) was added.

**Fluorescence microscopy.** HeLa cells were seeded and grown overnight on glass coverslips in 24-well plates containing 1 ml DMEM per well. The cells were then infected with 5 μl of an EPEC culture grown overnight at 37°C in standing cultures of LB. Infection was terminated after 3-5 h and 6 h by fixation of the cells for 30 min in PBS containing 4% paraformaldehyde. The fixed cells were washed with PBS, permeabilized for 5 min with 0.1% Triton X-100 in PBS and washed as before. The actin filaments were stained by overlaying the coverslips with 20 μl (1:100 in TBS) phalloidin-Rhodamine (Sigma). Flagellin was identified using anti-H6(FliC) antiserum (Israeli Ministry of Health; IMH), and secondary anti-rabbit-Alexa-488 conjugated antibody (1:100, Sigma).

**RESULTS**

**IHF represses flagellar expression in EPEC**

EPEC can be ‘pre-activated’ to express the LEE-encoded TTSS by growth in DMEM to mid-exponential phase, at 37°C. Microscopy showed that the activated EPEC cells were typically non-motile. We also found that the EPEC ihfA::kan mutant was highly motile. The motility of the mutant appeared to be constitutive, independent of growth phase or medium. Repression of motility was restored upon transforming the EPEC ihfA::kan with a plasmid encoding the wild-type ihfA allele.

To further validate these observations we grew EPEC strains in DMEM to mid-exponential phase, extracted the bacterial proteins and subjected them to Western blotting, using antibodies raised against flagellar components FlFC, FlFH and FlIA and against the TTSS component EspA. We compared the expression of these proteins in three EPEC strains: wild-type, ihfA::kan, and ihfA::kan containing a plasmid expressing ihfA from the lac promoter (Fig. 1A). The results confirmed previous observations that IHF is required for expression of the TTSS components (Friedberg et al., 1999). In contrast, IHF suppressed production of the flagellar proteins. When we compared the motility of the above three EPEC strains on a swarm plate (Fig. 1B), the results were in agreement with the Western blot analysis. The EPEC ihfA::kan mutant was highly motile and the motility of the strains expressing IHF, either genomic or in trans from a plasmid, was attenuated (Fig. 1B). Electron micrographs of the wild-type cells and the ihfA::kan mutant grown in DMEM showed that the EPEC wild-type cells either do not possess flagella or rarely (about 5% of the bacteria) show a single flagellum. In contrast, the ihfA::kan mutant produced numerous peritrichous flagella (Fig. 1C).

Next, we tested whether similar IHF-mediated repression of the flagella operates in EHEC strains. To this end, we inactivated ihfA in two EHEC O157 isolates: EDL933 and 85-170. Using microscopy of cultures grown in DMEM, the wild-type strains were compared with the mutated strains and with the mutated strains complemented by a plasmid expressing ihfA. The results showed that, like EPEC, EHEC ihfA::kan become hypermotile, whereas the wild-type strain and the complemented mutants were typically non-motile (data not shown).

**IHF does not repress flagellar expression in E. coli K-12 and N99 strains**

We examined whether the role of IHF as a suppressor of flagellar synthesis is unique to EPEC and EHEC or is a general characteristic of E. coli strains. We compared E. coli K-12 W3110 and E. coli N99 with their isogenic ihfA mutants. In contrast to EPEC, the ihfA mutation of W3110 did not result in increased expression of flIC (Fig. 2A) or in increased motility, as seen under the microscope (data not shown) and confirmed by swarm plates (Fig. 2B). In the N99 strain, the ihfA mutation resulted in the opposite effect: i.e. the ihfA mutant exhibited reduced flagellin synthesis (Fig. 2A) and showed reduced motility as compared with wild-type N99, as revealed by microscopy (data not shown) and by swarm plates (Fig. 2B). These findings indicate that IHF does not mediate flagellar repression in E. coli strains W3110 or N99.

**IHF indirectly mediates repression of flhDC**

Based on the results shown in Fig. 1(A), we hypothesized that in EPEC, IHF represses the expression of the flhDC
operon, which encodes the flagellar master regulator. To test this prediction, we constructed a plasmid carrying a transcriptional fusion (designated pDF12 or p\textit{flhDC}EPEC-\textit{gfp}) between the \textit{gfp} reporter gene and an EPEC DNA fragment containing the complete \textit{flhDC} coding region and its upstream region. Introduction of the plasmid into the EPEC wild-type resulted in marked activation of motility, as revealed by microscopy and swarm plates (data not shown). The multiple copy-number of \textit{flhDC}EPEC-\textit{gfp} probably overcame the repression mediated by the chromosomal IHF. To test the effect of IHF on the plasmid-expressed \textit{flhDC}EPEC-\textit{gfp}, we constructed a compatible plasmid based on pACYC177 that encodes the \textit{ihfAB} genes expressed from the \textit{lac} promoter (designated pDF14 or p\textit{ihfAB}).

Introduction of p\textit{ihfAB} into EPEC/p\textit{flhDC}EPEC-\textit{gfp} resulted in strong inhibition of EPEC motility, observed by microscopy (data not shown) and by swarm plates (Fig. 3A). To analyse the role of IHF on \textit{flhDC}EPEC expression, we compared \textit{gfp} expression in EPEC/p\textit{flhDC}EPEC-\textit{gfp} containing or not containing p\textit{ihfAB} (Fig. 3B). The results clearly indicate that in EPEC, supplying IHF in trans strongly repressed expression of \textit{flhDC}EPEC-\textit{gfp}.

It is expected that if IHF represses flagellar expression by direct interaction with the regulatory region of \textit{flhDC}EPEC, the inhibition should also take place in W3110. To test this possibility, we transformed \textit{E. coli} W3110 with p\textit{flhDC}EPEC-\textit{gfp} or with both p\textit{flhDC}EPEC-\textit{gfp} and p\textit{ihfAB} and compared \textit{gfp} expression in these strains. In contrast to EPEC, supplying IHF in trans to W3110 did not affect

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**Fig. 1.** Inactivation of IHF in EPEC induces flagellar expression and motility. (A) Immunoblot analysis. Proteins were extracted from wild-type EPEC, the EPEC \textit{ihfA}::\textit{kan} mutant and the EPEC \textit{ihfA}::\textit{kan} mutant complemented with pDF14 (EPEC \textit{ihfA}::\textit{kan}/\textit{ihfA}). Proteins were analysed by immunoblotting, using antibodies raised against flagellar components (FliC, FliG and FliA) and the LEE TTSS component EspA. (B) Swarm plates were used to test the motility of wild-type EPEC, the EPEC \textit{ihfA}::\textit{kan} mutant and the genetically complemented mutant EPEC \textit{ihfA}::\textit{kan}/\textit{ihfA}. (C) Electron micrographs, negatively stained, of wild-type EPEC and the EPEC \textit{ihfA}::\textit{kan} mutant. Bars, 1 \textmu m.

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**Fig. 2.** IHF does not mediate the repression of motility and flagellin production in \textit{E. coli} strains W3110 and N99. (A) Immunoblot analysis. Proteins were extracted from wild-type and \textit{ihfA}::\textit{kan} mutants of \textit{E. coli} strains W3110 and N99 grown in DMEM to mid-exponential phase. The extracted proteins were subjected to SDS-PAGE followed by immunoblot analysis, using anti-flagellin antibodies. (B) Swarm plates were used to test the motility of W3110 and N99 wild-type and isogenic \textit{ihfA}::\textit{kan} mutants.
IHF represses flagellar expression in EPEC

The LEE genes are not involved in IHF-mediated flagellar repression

The LEE genes are unique to EPEC, EHEC and closely related strains, and their expression is positively regulated by IHF. This raises the possibility that the putative \( flhDC \) repressor of EPEC is encoded by the LEE. To test this hypothesis, we supplied \( ler \) in trans to the EPEC \( ihfA::kan \) mutant, using a plasmid expressing \( ler \) from the \( ptac \) promoter. Expression of the recombinant Ler activated the expression of most of the LEE genes including \( LEE2, LEE3, LEE4, LEE5 \) and the \( espG \) transcriptional units. However, EPEC \( ihfA::kan \) exhibited a high motility and efficient flagellin production even upon expression of the recombinant Ler (Figs 4C and 4A). This indicates that the Ler-regulated LEE genes do not encode the putative \( flhDC \) regulator.

To further exclude the involvement of LEE genes in \( flhDC \) regulation, we constructed a strain (DF4) including a deletion of 20 nt, consisting of the IHF-binding site upstream from the \( LEE1 \) promoter. We confirmed by Western blot analysis, using anti-Ler antibodies, that the mutant does not produce Ler (Fig. 4E). This mutant did not express any of the tested LEE operons, including \( LEE1, LEE2, LEE3, LEE4, LEE5 \) and \( espG \) (data not shown). However, motility was repressed and there was a low level of flagellin production, similar to that of wild-type EPEC (Figs 4A and 4D). The results obtained from the swarm plates were confirmed by microscopy. Cumulatively, these results indicate that the LEE genes are not involved in the repression of flagellar expression.

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Fig. 3. IHF represses the EPEC \( flhDC \) operon in EPEC but not in \( E. coli \) W3110. (A) Motility test. EPEC containing plasmid \( pflhDC^{EPEC}-gfp \) expressing the \( flhDC^{EPEC}-gfp \) fusion, in the absence or presence of plasmid \( pih\text{IAB} \) expressing IHF\( \alpha \) and IHF\( \beta \). (B) Flow cytometry. EPEC/\( pflhDC^{EPEC}-gfp \) and W3110/\( pflhDC^{EPEC}-gfp \) containing or lacking \( pih\text{IAB} \) were grown in DMEM. Fluorescence intensity, which reflects the levels of \( flhDC \) expression, was measured by flow cytometry. The values are presented as means of three experiments, with standard error bars.

Fig. 4. Expression of TTSS LEE genes does not affect flagellin expression and motility of EPEC strains. (A) Immunoblot analysis of proteins extracted from the EPEC strains grown in DMEM to mid-exponential phase: wild-type (lane 1), EPEC/\( pflhDC \) (lane 2), \( ihfA::kan \) (lane 3), \( ihfA::kan/pler \) (lane 4) and a mutant with a deletion in the IHF binding site – \( \Delta \text{IHF-BS} \) (lane 5). The blot was developed with anti-flagellin antibodies. MW, size markers (kDa). (B–D) Relative motility of EPEC strains (strain numbers, indicated in parentheses, correspond to the lane numbers of the EPEC strains used for immunoblot analysis in (A). (E) The \( \Delta \text{IHF-BS} \) mutant is deficient in Ler expression. The mutant and wild-type EPEC, as positive control, were grown to a density of \( OD_{600} \) 0·35–0·4 in DMEM and the extracted proteins were analysed by Western blotting with anti-Ler antibody.
Flagella and the LEE TTSS are both functional upon co-expression

The biological significance of IHF-mediated flagellar repression in EPEC is not clear. One possibility is that the flagellar system is repressed because it interferes with the function of the LEE-encoded TTSS. To test this hypothesis, a plasmid expressing flhDC\textsubscript{EPEC} from the ptac promoter (pDF13 or ptac-flhDC) was constructed and transformed into the EPEC wild-type and into EPEC bfpA::TnphoA strain 36-6-1(1). The latter mutant strain does not express the bundle-forming pili mediating bacterial aggregation (Donnenberg \textit{et al.}, 1992). The two isogenic pairs of strains were used to infect HeLa cells and tested for several functions. These included motility, BFP-mediated aggregation, expression and secretion of EspA, invasion, and formation of actin pedestals. Microscopy indicated that the strains containing ptac-flhDC were highly motile, whereas the corresponding wild-type and bfpA::TnphoA mutant, lacking ptac-flhDC, were non-motile. In contrast, expression of recombinant flhDC had little effect on all the other tested functions (Fig. 5A, B, and data not shown).

Using fluorescence microscopy, we tested whether a single bacterium can simultaneously express flagella and a functional TTSS. Formation of flagellar filaments was used as an indicator of the activity of the flagellar TTSS, and generation of actin pedestals was used as an indicator of functional LEE TTSS. The EPEC bfpA::TnphoA mutant was used to prevent microcolony formation, which complicates the analysis of a single bacterium. We infected HeLa cells with a bfpA::TnphoA strain containing flhDC to drive flagellar expression. Infection was carried out for 3.5 and 6 h and infected cells were fixed and double stained with anti-H6-flagellin antibodies and phalloidin-rhodamine, which stains the actin pedestals. At 3.5 h post-infection most of the bacterial cells attached to the actin pedestals were flagellated (Fig. 5C). This indicates that upon co-expression both the flagellar TTSS and the LEE TTSS are functional.

Interestingly, at 6 h post-infection many of the pedestal-associated bacteria appeared to be non-flagellated, whereas the bacteria associated with HeLa cells, but not with actin pedestals, were mostly flagellated (Fig. 5C). The unattached

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**Fig. 5.** Co-expression of the LEE TTSS and the flagellar TTSS. (A) Expression of EspA by wild-type EPEC and EPEC/ptac-flhDC was compared by immunoblot analysis using anti-EspA antibody. (B) Comparison of the invasiveness of wild-type EPEC, EPEC/ptac-flhDC, EPEC bfpA::TnphoA, bfpA::TnphoA/ptac-flhDC using the gentamicin protection assay. The invasion assays were repeated twice with similar results and the results of one of these experiments are shown. The assays were carried out in quadruplicate. Standard deviation values are shown. (C) HeLa cells were infected with EPEC bfpA::TnphoA/ptac-flhDC, fixed, stained with phalloidin-rhodamine (red) and anti-H6 antibody (green), and analysed by fluorescence microscopy. The fluorescent image (right panels) and the corresponding phase-contrast image (left panels) are shown. White arrowheads point to flagellated bacteria associated with actin pedestals; white arrows (middle panels) point to non-flagellated bacteria associated with actin pedestals; pink arrows point to flagellated bacteria associated with the HeLa cell surface but not with actin pedestals. Bar (below figure), 1 \(\mu\)m.
bacteria were motile and flagellated as well, implying a homogeneous bacterial population. The differential flagellar expression is probably not due to differential flhDC expression, as in these experiments flhDC was expressed from the pTac promoter. The significance of the disappearance of the flagella from EPEC bacteria associated with actin pedestals during late stages of HeLa cell-infection is not clear.

DISCUSSION

In this study we report that expression of flagella in EPEC is repressed by IHF. We further show that IHF represses the flhDC operon, which encodes the master positive regulator of the flagellar regulon. In addition, we demonstrate that IHF-mediated repression of flhDC is unique to EPEC and EHEC and is not found in all E. coli strains. Moreover, the EPEC flhDC operon is repressed in EPEC but not in other E. coli strains. Cumulatively our results indicate that in EPEC, IHF mediates repression of flhDC transcription indirectly via a putative regulator. This putative regulator appears to be unique to EPEC and not present in E. coli K-12. Alternatively, it may be encoded but not expressed in E. coli K-12.

The difference in motility between the non-motile wild-type EPEC and the highly motile EPEC ihfA mutant was particularly enhanced in EPEC grown in DMEM at 37°C to mid-exponential phase. Interestingly, these conditions are optimal for expression of the LEE1 operon, including ler, the positive regulator of the LEE region. These observations, as well as the positive effect of IHF on ler expression, raised the possibility that the putative unique EPEC factor that represses the flagella is encoded by LEE. We examined this possibility using several approaches, but could not find any evidence in favour of this hypothesis. Therefore, we are now using genomic screening to identify the putative EPEC factor that represses flhDC.

The EPEC ihfA mutant fails to induce actin rearrangement upon interaction with epithelial cells, and becomes highly motile. This phenotype is similar to that of the EPEC bipA mutant previously reported by Farris et al. (1998). Therefore, it is conceivable that, like IHF, BipA is also required for expression of the LEE genes and repression of flhDC. It remains to be seen whether IHF and BipA interact in some way to bring about such regulation. A recent report by Girón et al. (2002) suggests that a factor released by the epithelial cells induces flagellar expression in infecting EPEC cells and that the flagella mediate EPEC adherence to epithelial cells. The identity of this putative host factor and its mechanism of function remain obscure. According to our analyses, wild-type EPEC is non-flagellated and non-motile in the absence as well as in the presence of host cells. Perhaps the cell line that we are using does not produce the putative host factor which induces expression of flagella. It is not evident whether IHF, BipA and the putative host factor utilize the same regulatory cascade to regulate flhDC expression.

What is the biological rationale of flagellar repression in EPEC? One possibility is that the flagella interfere with the function of the LEE-encoded TTSS. We tested this concept by constructing an EPEC strain co-expressing the flagella and the LEE-encoded TTSS. Our results indicate that they coexist within the same EPEC cell. Another possibility is that flagellar repression in EPEC acts to lower the level of flagellin, which is a TLR5 ligand (Hayashi et al., 2001), thereby reducing the host’s inflammatory response.

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