

Role of the nucleoid-associated protein Fis in the regulation of virulence properties of enteropathogenic *Escherichia coli*

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Summary

Virulence gene expression in enteropathogenic *Escherichia coli* (EPEC) is governed by a combination of environmental factors and virulence regulators. These factors control the expression of the bundle-forming pili (BFP), intimin, the type III secretion apparatus and the secreted proteins EspA, EspB, EspD and Tir. Expression of the *bfp* genes occurs for a short period in early exponential phase during growth in tissue culture medium. The nucleoid-associated regulator protein, Fis, is also expressed transiently during this period. To determine whether Fis was responsible for the growth phase-dependent expression of *bfp*, *fis* was deleted from the EPEC strain E2348/69S. Paradoxically, the Δ *fis* mutant retained the ability to colonize HEp-2 cells in a characteristic localized adherence pattern, and Fis was found negatively to regulate the expression of BFP. However, the Δ *fis* mutant failed to induce the accretion of filamentous actin, which is associated with attaching and effacing lesions. Using a combination of Western blotting and a novel multiplex primer extension assay (MPEA), we showed that, although the expression of intimin and Tir was not affected, transcription of the LEE4 operon encoding *espADB* and the virulence activator, Ler, were found to be Fis dependent.

Introduction

Enteropathogenic *Escherichia coli* (EPEC) is a common cause of persistent diarrhoea among infants, primarily in developing countries (Levine and Edelman, 1984). EPEC infection of both human gut biopsies and cultured epithelial cells is typified by the formation of characteristic pedestal structures known as attaching and effacing (AE) lesions (Moon *et al.*, 1983; Knutton *et al.*, 1987). AE lesion formation requires the co-ordinated expression of a number of proteins encoded by the chromosomal locus of *enterocyte effacement* (LEE) (Elliott *et al.*, 1998), including EspA, EspB and EspD, which are secreted via the Esc type III secretion system, also encoded by the LEE (Elliott *et al.*, 1998). EspA, B and D are thought to be involved in the translocation of EPEC virulence factors into the host by a mechanism that remains to be fully elucidated (Knutton *et al.*, 1998). These proteins are also required for enterocyte invasion (Donnenberg *et al.*, 1993; Foubister *et al.*, 1994; Kenny *et al.*, 1996). Activation of signal transduction pathways in infected enterocytes results in actin depolymerization and the effacement of microvilli (reviewed by Frankel *et al.*, 1998). A tyrosine-phosphorylated protein, Tir, also encoded by the LEE, is translocated from EPEC into host cells (Kenny *et al.*, 1997), forming a receptor to which the 94 kDa EPEC outer membrane protein intimin (product of the *eae* gene; Jerse *et al.*, 1990) binds. An intimate connection is formed between the host and bacterium, and a complex of cytoskeletal proteins, which includes actin, talin, vinculin and α -actinin, is deposited immediately beneath the point of bacterial attachment (Finlay *et al.*, 1992), forming the characteristic AE lesions that are detectable by the fluorescent actin staining (FAS) test (Knutton *et al.*, 1989). Bieber *et al.* (1998) demonstrated that EPEC infection of adult volunteers also requires the synthesis of bundle-forming pili (BFP). These hair-like surface structures, encoded by the *bfp* operon located on the \approx 80 kb EPEC adherence factor (EAF) plasmid (Girón *et al.*, 1991; Soheli *et al.*, 1996), promote bacterial aggregation and may be involved in initial attachment of bacteria to the mucosal surface of the jejunum.

Puente *et al.* (1996) reported, and we have confirmed, that the *bfp* operon of EPEC is only expressed for a short period during early exponential phase. In this respect, BFP

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synthesis resembles that of the regulator protein Fis (factor for inversion stimulation), which is regarded as a member of the group of histone-like or nucleoid-associated proteins because of its ability to alter DNA topology (Thompson and Landy, 1988; Gille *et al.*, 1991). In batch cultures, Fis is expressed at high levels for a brief period after nutritional upshift, accumulating to 25 000–50 000 dimers per cell at peak levels (Ball *et al.*, 1992). Fis is a transcriptional activator of genes and operons associated with primary metabolism, such as those encoding biosynthetic enzymes, tRNA and rRNA, and it causes a potent upregulation in their expression (Ross *et al.*, 1990; Gonzalez-Gil *et al.*, 1996). This role is believed to be important in ensuring adequate cellular resources for bacteria entering exponential phase after a period of quiescence (Ball *et al.*, 1992). Fis also plays an important role in *oriC*-directed chromosome replication (Gille *et al.*, 1991; Roth *et al.*, 1994) and acts as an enhancer of site-specific DNA inversion and recombination events (reviewed by Finkel and Johnson, 1992). Recently, Fis was shown to regulate levels of DNA supercoiling by repressing *gyrA* and *gyrB* and activating *topA*, the genes encoding DNA gyrase and topoisomerase I respectively (Schneider *et al.*, 1999; Weinstein-Fischer *et al.*, 2000). Fis, which functions as a homodimer, regulates gene expression by binding to and bending gene promoters containing the degenerate consensus sequence GNtYAaWWWtTRaNC (Pan *et al.*, 1996), and interacts with the C-terminal domain of the α -subunit of RNA polymerase (Bokal *et al.*, 1997). As Fis is expressed during early exponential phase and after nutritional upshift, the majority of genes that it influences tend to be expressed at these times, although some genes that are normally expressed at other stages in the growth cycle can be regulated by Fis (Xu and Johnson, 1995). In this paper, we describe experiments initially designed to investigate whether Fis is responsible for the growth phase-dependent expression of *bfp*. Although it was demonstrated that Fis does not regulate the *bfp* operon directly, it does activate transcription of the genes encoding the type III secreted proteins EspA, EspB and EspD via the LEE-encoded virulence regulator, Ler; these secreted proteins are essential for AE lesion formation.

Results

Expression of bfp in E2348/69S grown in DMEM

Expression of EPEC bundle-forming pili *in vitro* depends on a number of environmental and cultural factors. Positive stimulatory factors include early exponential growth at 37°C in tissue culture media such as Dulbecco's modified Eagle medium (DMEM) (Puente *et al.*, 1996; Rosenshine *et al.*, 1996). Negative factors include growth

in rich nutrient media such as Luria–Bertani (LB) broth and temperatures below 37°C (Puente *et al.*, 1996). Northern blot analysis was performed on mRNA from samples of strain E2348/69S taken at intervals during growth in DMEM with a probe directed towards the entire *bfpA* gene (*Experimental procedures*). Figure 1B shows that *bfpA* transcripts appeared early in the exponential growth phase, increased for 2 h, but then declined rapidly while the bacteria were still actively growing in mid-exponential phase. These data are in broad agreement with those of Puente *et al.* (1996), who used chloramphenicol acetyltransferase activity as a reporter of *bfpA* expression to show a modest decline in activity after a peak in early exponential phase.

Expression of fis in E2348/69S grown in DMEM

When considering which regulatory proteins might account for growth phase-dependent expression of the *bfp* operon, we noted that the transcriptional activator Fis is only expressed briefly during early exponential phase, as reported for *Escherichia coli* K-12 (Ball *et al.*, 1992). Figure 2A shows the organization of the *fis* gene in the *E. coli* K-12 genome and includes an upstream gene, *yhdG* (formerly ORF1; Ball *et al.*, 1992), which has been postulated to be involved in nitrogen regulation (Osuna *et al.*, 1995). The promoter for *fis* is located upstream of *yhdG* (Ball *et al.*, 1992). The EPEC *fis* gene was cloned as described in *Experimental procedures* (see Fig. 2B); the 609 bp *KpnI*–*HindIII* restriction fragment used in the construction of pMDG9 was also used as a probe to examine *fis* expression by Northern blot analysis of the RNA samples used to assess temporal regulation of *bfpA* in Fig. 1B. Two hybridizing bands were observed at each time point in wild-type E2348/69S (Fig. 1C): the expected transcript of 1400 bases that also includes the co-transcribed *yhdG* (see Fig. 2A) and a smaller 850 base transcript representing a post-transcriptional cleavage product (Ball *et al.*, 1992). Rapid accumulation of *fis* mRNA was observed 30 min after transferring the overnight LB culture to DMEM, reaching a peak at 60 min, after which levels of *fis* mRNA declined, presumably as a result of dilution during cell growth and instability of the mRNA.

Construction and phenotypic analysis of a fis mutation in E2348/69S

Having confirmed that *fis* and *bfpA* were both expressed with similar kinetics in early exponential phase, we determined the regulatory relationship between the two genes by constructing a defined *fis* deletion mutant (see *Experimental procedures*). The Δ *fis* mutation was introduced into the chromosome of EPEC strain E2348/69S by homologous recombination to give E2348/69S Δ *fis* and

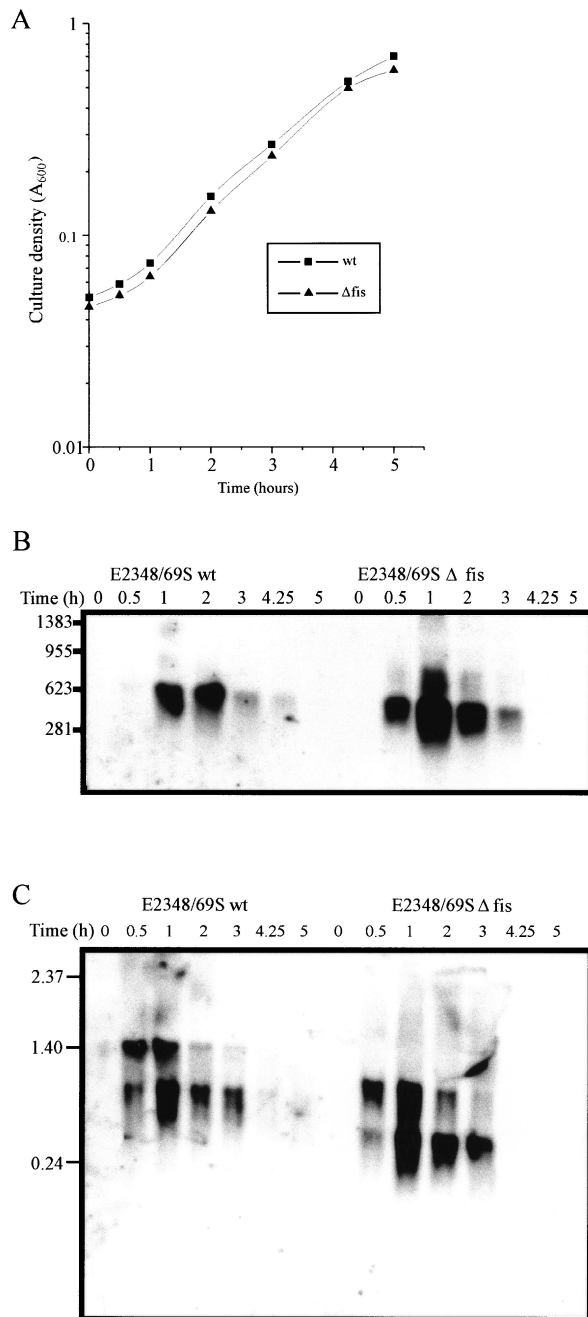


Fig. 1. Growth phase-dependent transcription of *bfpA* and *fis* in EPEC strain E2348/69S and the defined *fis* deletion mutant E2348/69S Δ *fis*. Overnight LB cultures were diluted 1:100 in prewarmed (37°C) DMEM and incubated with shaking at 37°C. Culture densities were measured at intervals (absorbance at 600 nm, A). At each time point, 2.0 absorbance units of cells were harvested, and the RNA was extracted and analysed by Northern blot hybridization to determine transcription of *bfpA* (B) and *fis* (C). The *bfpA* probe was a 632 bp PCR product representing the entire *bfpA* gene, and the *fis* probe was a 609 bp *Kpn*I–*Hind*III fragment used in the construction of pMDG9 (Experimental procedures). Northern blots were performed on several occasions, and reproducible results were obtained.

confirmed by Southern blot analysis (data not shown). Deletion of *fis* did not significantly affect the growth rate of the mutant strain (Fig. 1A). Northern blot analysis of mRNA isolated from samples of strain E2348/69S Δ *fis* taken at intervals during growth in DMEM was performed using the *bfpA* and *fis* probes described above. With the *fis* probe, which carried the 3' portion of *yhdG*, the two hybridizing bands observed were smaller by \approx 300 bp than those in E2348/69S, corresponding to the sequence deleted during construction of the mutation (Fig. 1C). Nevertheless, rapid induction of the *yhdG*- Δ *fis* transcript was observed in E2348/69S Δ *fis*, reaching higher peak levels than in the parent and declining more rapidly so that, by 4.25 h, *yhdG*- Δ *fis* transcripts were no longer detectable. These data are in agreement with those of Ball *et al.* (1992) for *E. coli* K-12. Analysis of the same RNA samples with the *bfpA* probe gave a similar profile of expression to that of *fis* (Fig. 1B). E2348/69S Δ *fis* expressed *bfpA* to a somewhat higher level than the parent strain E2348/69S but, as in the parent, *bfpA* transcription ceased abruptly in mid-exponential phase.

Deletion of fis abolishes the ability of EPEC to induce AE lesion formation and reduces the ability to invade HEp-2 cells

Although *bfpA* transcription was not significantly affected by deletion of *fis*, we wished to determine whether BFP-mediated adherence to cultured cell monolayers was altered in the Δ *fis* mutant. FAS assays on infected HEp-2 cell monolayers indicated that E2348/69S Δ *fis* formed numerous microcolonies, typical of the localized adherence phenotype attributable to BFP, but without the accumulation of high concentrations of filamentous actin characteristic of AE lesions (Fig. 3B). Restoration of *fis* expression by the introduction of pMJ3, a low-copy-number plasmid containing the entire *fis* operon (see Experimental procedures), restored AE lesion formation in this strain (Fig. 3C) to a level comparable with that seen with infection by the *fis*⁺ parent strain E2348/69S (Fig. 3A).

Although EPEC is not generally considered to be an invasive pathogen, directed internalization of EPEC into cultured cell lines has been observed (Rosenshine *et al.*, 1992; Kenny *et al.*, 1996). We assayed the ability of E2348/69S, E2348/69S Δ *fis*, E2348/69S Δ *fis* (pMJ3), UMD864 *espB*⁻ and the K-12 strain, DH5 α , to invade cultured HEp-2 cells. The results shown here represent the mean and standard deviation values obtained from three independent experiments. Although wild-type E2348/69S was found to invade the HEp-2 cells with an efficiency of \approx 10⁵ cfu per monolayer, E2348/69S Δ *fis* demonstrated a reduced ability to invade (6.1% \pm 0.98 of wild-type levels), which was partially restored by

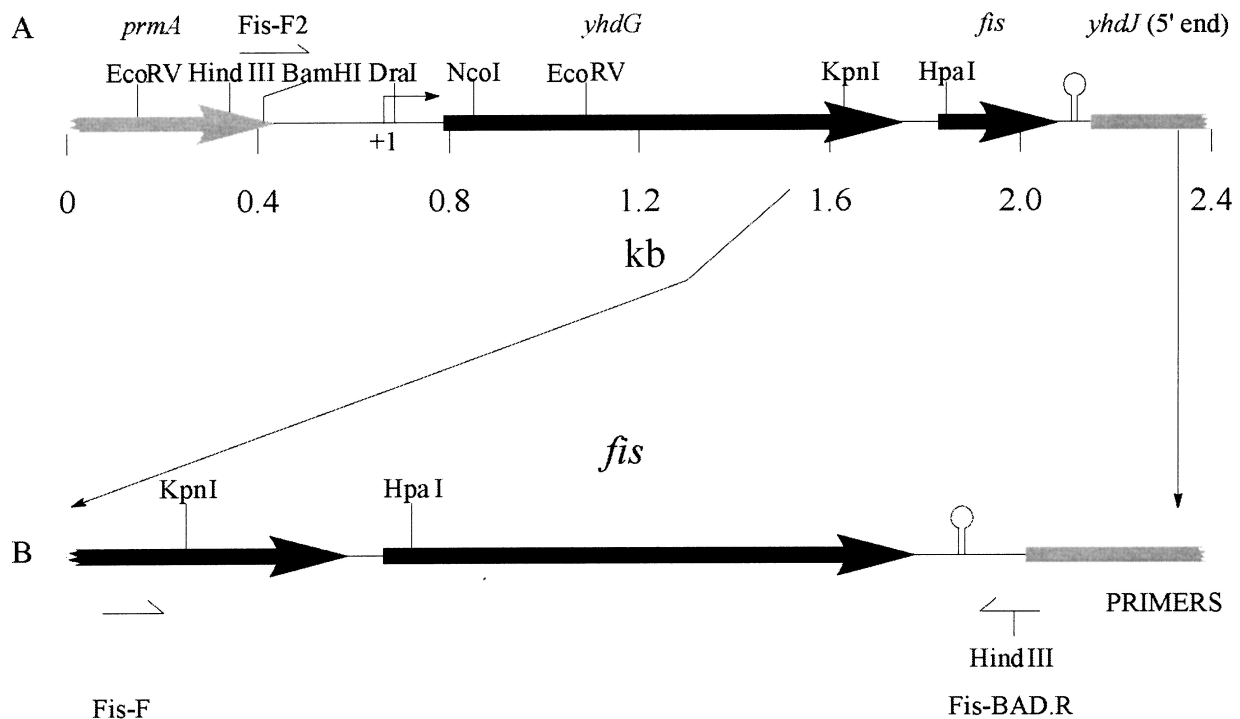


Fig. 2. Organization of the genetic locus encoding *fis* and construction of the Δfis mutation.

A. The *fis* gene is the second of a two-gene operon whose promoter is located upstream of *yhdG*, transcription initiating at +1 (Ball *et al.*, 1992). Also shown is a Rho-independent transcription termination loop downstream of *fis* and the primer site Fis-F2 that was used in conjunction with primer Fis-BAD.R to PCR amplify and clone the entire *fis* operon.

B. The PCR product generated by primers Fis-F and Fis-BAD.R was digested with *KpnI* and *HindIII* and cloned into the cognate sites of pBluescript SK+ to form pMDG9.

transformation with pMJ3 ($52\% \pm 26$ of wild-type levels). In contrast, invasion by the *espB* mutant UMD864 ($0.063\% \pm 0.06$ of wild-type levels) was equivalent to that of the *E. coli* K-12 strain DH5 α ($0.027\% \pm 0.027$ of wild-type levels).

Effects of Δfis on EPEC virulence gene expression

In order to develop a clearer picture of where Fis acts in the regulation of AE lesion formation, we examined whether the expression of a number of key EPEC virulence genes was affected by deletion of the *fis* gene. Intimin (Eae protein) is essential for the process of AE lesion formation (Jerse *et al.*, 1990). Western blot analysis of bacterial proteins using anti-intimin antibody (Fig. 4) identified cross-reacting polypeptides of the predicted molecular weights from wild-type E2348/69S, E2348/69S Δfis and from E2348/69S Δfis (pMJ3). A known intimin-deficient (*eae*) mutant strain, CVD206 (Donnenberg and Kaper, 1991), did not express polypeptides that were recognized by the anti-intimin antibody. These data indicate that intimin expression is not dependent upon Fis.

To investigate the effects of the *fis* mutation upon other virulence genes, we developed a new technique that we have named multiplex primer extension analysis (MPEA),

which enabled us to measure simultaneously the expression of several different genes in the same samples (Fig. 5). Expression of the operon LEE4 encoding the major EPEC-secreted proteins (EspA, EspB and EspD) was found to be significantly reduced in the Δfis mutant. cDNA from wild-type E2348/69S, E2348/69S Δfis and E2348/69S Δfis (pMJ3) was hybridized with a polymerase chain reaction (PCR)-generated probe directed against the 5' end of *espA* (Experimental procedures). Very weak transcription of *espA* was observed in E2348/69S Δfis (Fig. 5A), compared with much stronger signals obtained from either wild-type E2348/69S, or E2348/69S Δfis (pMJ3). Surprisingly, we found that expression of the translocated intimin receptor, Tir, was not reduced in the Δfis mutant when the MPEA blot was hybridized with a PCR-generated probe directed against the 5' end of the *tir* gene. Identical levels of *tir* cDNA transcripts were detected in the Δfis mutant (Fig. 5B) and the wild-type strain E2348/69S. However, we noticed reduced *tir* expression when pMJ3 was introduced into E2348/69S Δfis , suggesting that high levels of Fis can repress this operon.

After recent reports describing the identification of *ler* in the LEE as a transcriptional activator of operons LEE2, LEE3 and LEE4 (Mellies *et al.*, 1999; Sperandio *et al.*, 2000), we decided to test whether *ler* expression is

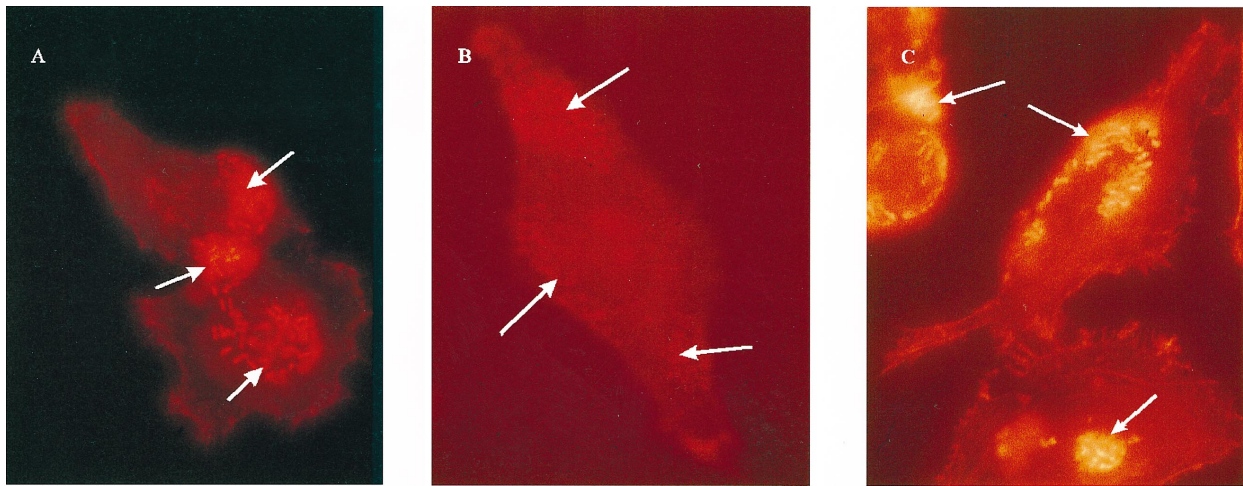


Fig. 3. Fluorescent actin staining (FAS) tests of HEp-2 cells infected with E2348/69S, E2348/69S Δ *fis* and E2348/69S Δ *fis* (pMJ3). Semi-confluent HEp-2 cell monolayers were infected for 3 h with 10^7 cfu of bacteria and then stained for filamentous actin with phalloidin–Texas red. Strong fluorescence associated with the accretion of actin can be visualized around microcolonies (indicated by arrows) of wild-type E2348/69S (A) and of E2348/69S Δ *fis* (pMJ3) (C), but not around the microcolonies of E2348/69S Δ *fis* (B).

affected by the *fis* mutation. We found that *ler* expression was abolished in the *fis* mutant (Fig. 5C) but was fully restored when E2348/69S Δ *fis* was transformed with pMJ3.

There is some uncertainty about whether the promoter for LEE4 is located in the 3'-terminus of *sepL* as in verocytotoxigenic *E. coli* (VTEC) (Beltrametti *et al.*, 1999) or upstream of *sepL* (Mellies *et al.*, 1999). We therefore measured *sepL* expression by MPEA and found that its expression is also dependent upon *Fis* (Fig. 5D). However, its profile of expression differs subtly from that of *espA*; whereas *espA* expression remains constant throughout most of the growth cycle, *sepL* expression gradually increases and then abruptly ceases as the cells approach stationary phase. Northern blot experiments to monitor LEE4 and *tir* expression confirmed the primer extension results presented here (data not shown).

Discussion

When an overnight LB culture of EPEC strain E2348/69S is diluted into prewarmed DMEM, the expression of BFP, as determined by measurements of *bfpA* mRNA, increases rapidly to a maximum in ≈ 2 h and then declines abruptly while the bacteria are still growing logarithmically. We considered two possible regulatory mechanisms for this phenomenon. One explanation is that the accumulation of a quorum-sensing molecule might trigger cessation of *bfp* expression. Quorum sensing plays an important role in virulence regulation in a number of bacterial pathogens (Fuqua and Greenberg, 1998) but, in all cases documented so far, virulence gene expression is activated rather than repressed when bacteria reach a particular cell density. The second possibility is that a

transiently expressed transcriptional activator might be involved in regulation; *Fis* was an obvious candidate as, like BFP, it is expressed in early exponential phase. However, Northern blot analysis of the Δ *fis* mutant demonstrated the same transient modulation of transcription from both *fis* and *bfpA* promoters in early exponential growth phase that is observed in the *fis*⁺ parental strain, indicating that *Fis* is not responsible for the switch-off of production of itself or of BFP. Indeed, maximal expression from both promoters appeared to be greater in the Δ *fis* mutant than in the parent, confirming the observation of Ball *et al.* (1992) that *Fis* negatively regulates its own expression and suggesting that *Fis* may in some way damp-down the expression of the *bfp* operon. When we transformed E2348/69S Δ *fis* with pMJ3 and measured BfpA expression by Western and Northern blotting, the protein and mRNA were barely detectable compared with levels observed in E2348/69S and E2348/69S Δ *fis* (data

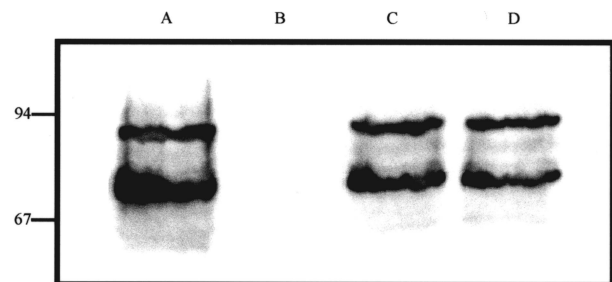


Fig. 4. Effect of the Δ *fis* mutation on intimin expression. Total proteins of EPEC strains growing in DMEM were resolved by SDS–PAGE, blotted onto nitrocellulose and probed with anti-intimin antibodies. Samples were (A) E2348/69S; (B) CVD206 (*eae*); (C) E2348/69S Δ *fis*; and (D) E2348/69S Δ *fis* (pMJ3), and were grown in DMEM to mid-exponential phase. Two reacting bands of 94 and 72 kDa are visible, presumably representing the native protein and a degradation product respectively.

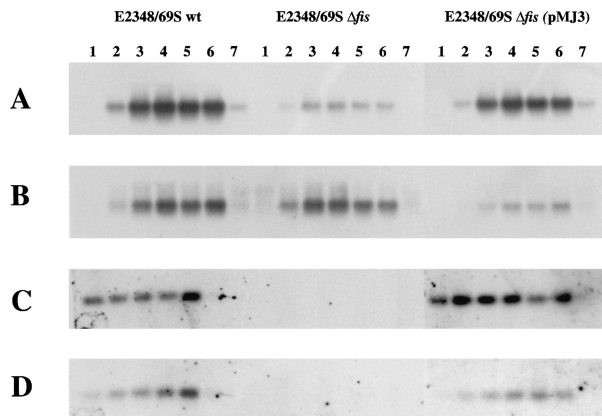


Fig. 5. Multiplex primer extension analysis to monitor expression of several genes simultaneously in the same RNA sample. RNA was extracted from E2348/69S, E2348/69S Δ *fis* and E2348/69S Δ *fis* (pMJ3) grown in DMEM + 0.1% glucose and sampled at 0.5 h (lane 1), 1.5 h (lane 2), 2.5 h (lane 3), 3.5 h (lane 4), 4.5 h (lane 5), 5.5 h (lane 6) and 6.5 h (lane 7). Unlabelled primers were designed to anneal 400–500 bp downstream of the 5' end of the (A) *espA*, (B) *tir*, (C) *ler* and (D) *sepL* transcripts and cDNA synthesized. After degrading the RNA, the cDNA products were electrophoresed and Southern blotted. Fluorescein-dUTP-labelled probes directed towards each cDNA product were hybridized individually with the blot and the signal recorded on X-ray film. Probes were stripped from the blot before hybridizing with the next probe. Essentially identical results were obtained from several independently performed experiments.

not shown). Thus, even when *fis* is cloned into a low-copy-number plasmid such as pWKS30 (5–10 copies per cell), the high levels of Fis that accumulate down-regulate certain genes to an even greater degree than in the wild-type strain carrying a single copy of the gene.

The Δ *fis* mutant formed characteristic localized adherence microcolonies on the surface of infected HEp-2 cells, indicating that expression of BFP and its ability to adhere were not affected. To our surprise, however, adherence was not accompanied by the characteristic induction of AE lesions, as determined by the FAS test. We also observed a marked reduction in the ability of E2348/69S Δ *fis* to invade cultured HEp-2 cells compared with wild-type EPEC. Furthermore, expression of the cloned *fis* gene restored lesion-forming ability to the Δ *fis* mutant and partially restored the invasion potential, indicating that Fis is essential for EPEC virulence. The failure to elicit a positive FAS test reaction is not caused by a failure to produce intimin or the translocated intimin receptor (Tir), which were apparently expressed normally in the Δ *fis* mutant. Rather, our data suggest that it results from a significant reduction in the expression of the secreted proteins, EspABD, which are essential for AE lesion formation (Foubister *et al.*, 1994; Kenny *et al.*, 1996; 1997; Lai *et al.*, 1997). Moreover, as some of these proteins are also necessary for EPEC invasion (Donnenberg *et al.*, 1993; Foubister *et al.*, 1994; Kenny *et al.*, 1996), it was not surprising that the Δ *fis* mutant was impaired for this phenotype. Our observation that the ability of the Δ *fis*

mutant to invade cultured HEp-2 cells was reduced (6% of wild type) rather than abolished (as in a known *espB* mutant, <0.1%) suggests that even low levels of expression of secreted proteins may be sufficient to allow some invasion to occur. It has been reported that a plasmid-encoded virulence regulator, PerA, is required for the transcriptional activation of a number of EPEC virulence genes including *ea*e, *espADB* and *bfp* (Gomez-Duarte and Kaper, 1995; Tobe *et al.*, 1996); however, on account of the differences in the effect of the *fis* mutation upon *espA*, intimin and Bfp expression, we concluded that regulation by Fis does not occur via PerA.

To monitor the expression of several virulence genes simultaneously, we devised a novel and simple assay called multiplex primer extension analysis (MPEA), which involves annealing several unlabelled primers to a single RNA sample. The primers were complementary to the mRNA and designed to anneal \approx 400–500 bp downstream of the transcriptional start sites of the genes to be tested. After conventional Southern blotting, a single transcript was detected at a time and, once the signal had been recorded on X-ray film, the blot was stripped and probed for the next transcript. This method was found to have several advantages over conventional Northern blot analysis. For example, it avoids the laborious precautions necessary when performing Northern blot analysis (to avoid mRNA degradation) and the problems associated with stripping and reprobing, which can be very unreliable. In addition, Northern blot analysis of genes located in operons can result in untidy blots with considerable smearing. A particularly useful feature of MPEA is that the results can be directly superimposed, as the cDNA products are all synthesized from the same RNA sample at the same time. It was reported recently that an ORF at the proximal end of LEE1 encodes a transcriptional activator, Ler, which is required for the expression of LEE2, 3, 4 and the *tir* operon (Mellies *et al.*, 1999). Using MPEA, we were able to demonstrate a greatly reduced level of transcription of the LEE4 operon encoding *espADB* and abolition of *ler* and *sepL* expression in the Δ *fis* mutant. In all cases, expression was restored by the introduction of the complementing plasmid, pMJ3. Conversely, *tir* expression was found to be unaffected in the Δ *fis* mutant. This result is in conflict with recent reports by Friedberg *et al.* (1999) and Elliott *et al.* (2000), who suggested that Ler regulates *tir*. We were also surprised to discover that *espC* was also reported to be Ler-dependent (Elliott *et al.*, 2000), as we observed no reduction in the amounts of EspC secreted in the culture supernatant (data not shown). As our data indicate that *ler* expression is abolished in the Δ *fis* mutant and *tir/espC* remain unaffected, we must therefore conclude that regulation of the *tir* operon and *espC* are subject to additional factors that remain to be identified. The high levels of Fis present

in E2348/69S Δ *fis* (pMJ3) were found to repress *tir* expression but not *espA*, *ler* or *sepL*. This suggests that, at high concentrations, *Fis* can bind to the *tir* promoter and attenuate expression.

In enterohaemorrhagic *E. coli*, *espADB* transcription is initiated at a promoter located in the distal end of the upstream gene *sepL*, giving a 2.8 kb transcript (Beltrametti *et al.*, 1999). In EPEC, on the other hand, it has been proposed that *espADB* transcripts begin at the *sepL* promoter and include the *sepL* gene (Elliott *et al.*, 1998). By Northern blot, we observed two *Fis*-regulated *espADB* transcripts (\approx 5.2 kb and 2.8 kb) when hybridizing with a probe directed against *espB* (data not shown). This suggested that transcription initiates at both promoters in E2348/69S, although the shorter transcript from the promoter located in the distal end of *sepL* appeared to be stronger. This was confirmed by MPEA analysis (see Fig. 5), in which a strong *espA* signal and a comparatively weak *sepL* signal can be seen. Analysis of culture supernatants for the presence of secreted proteins confirmed that deletion of *fis* results in the virtual abolition of EspA, B and D secretion, although, as mentioned above, EspC secretion remained unaffected (data not shown). Unlike the other secreted proteins, EspC is encoded by a gene in a second, recently identified pathogenicity island (Mellies *et al.*, 2001), is not dependent upon the type III Esc secretion system (Kenny and Finlay, 1995) and is not required for AE lesion formation (Stein *et al.*, 1996).

Transcription of the *tir* gene, encoding the translocated intimin receptor, was not affected in the Δ *fis* mutant. It has been reported recently that the closely linked genes *tir*, *cesT* and *eae* form an operon (Elliott *et al.*, 1999). By Western blot, we showed that intimin production in the Δ *fis* mutant appears to be normal (see Fig. 4), and the finding that *tir* expression is also normal (Fig. 5B) corroborates the observation that the promoter of this operon is not *Fis* regulated.

Regulation by *Fis* is likely to reflect the need to coordinate the expression of particular genes with the physiological or nutritional status of the bacteria. In the case of EPEC, it is likely that the complex secretory system that leads to the close contact with intimin can

occur only when bacterial growth conditions are optimal for metabolic activity. It has been shown recently that *Fis* is also essential for expression of the SPI-1 invasion genes in *Salmonella typhimurium* (Wilson *et al.*, 2001), and this phenotype strongly resembles many of the characteristics that we have observed in the EPEC Δ *fis* mutant. It is probable that many, if not all, of the nucleoid-associated proteins will have an impact upon virulence gene expression in EPEC. Indeed, IHF has also been shown to play an important role in activating the expression of *ler* (Friedberg *et al.*, 1999), and we have found that a mutation in *hns*, encoding the global regulator H-NS, has a major effect upon virulence gene expression (unpublished data). We are currently investigating the impact of other nucleoid-associated proteins in virulence gene regulation in enteric pathogens.

Experimental procedures

Bacterial strains, plasmids and growth conditions

Bacterial strains and plasmids used in this study are described in Tables 1 and 2 respectively. Note that all EPEC strains are derivatives of the O127:H6 strain E2348/69 isolated from an outbreak of infantile diarrhoea in Taunton, UK (Levine *et al.*, 1978). Overnight cultures of bacterial strains were grown at 37°C in Luria–Bertani (LB) broth (Sambrook *et al.*, 1989), containing 100 μ g ml⁻¹ ampicillin or 50 μ g ml⁻¹ streptomycin as required. All experiments used DMEM (minus phenol red) containing 0.1% (w/v) glucose (Gibco BRL Life Technologies) except in tissue culture experiments, where DMEM supplemented with 0.45% (w/v) glucose (Gibco BRL Life Technologies) was used. DMEM cultures were inoculated with 1:100 volumes of overnight cultures grown in LB. Plasmids were routinely maintained in *E. coli* strain DH5 α unless they were being specifically introduced into EPEC. SM10 λ *pir* was used to maintain the suicide plasmids pRDH10 and pMDG12.

DNA manipulations and construction of the Δ *fis* mutant

Standard procedures were used in the construction of the plasmids used in this work (Sambrook *et al.*, 1989). The region of the EPEC chromosome containing *fis* was PCR amplified using primers based upon *E. coli* K-12 sequence data. Primers *Fis*-F (5'-ACGTTTCGGAACTGCATGACTT-3')

Table 1. Bacterial strains used in this study.

| | | |
|-------------------------------|---|---------------------------------|
| E2348/69S | Spontaneous streptomycin-resistant derivative of wild-type EPEC E2348/69 O127:H6 (Levine <i>et al.</i> , 1978) | This laboratory |
| E2348/69S Δ <i>fis</i> | E2348/69S with a 303 bp deletion of the entire <i>fis</i> gene | This study |
| CVD206 | E2348/69 <i>eaeA8 nal</i> ^r | Donnenberg and Kaper (1991) |
| UMD864 | E2348/69 Δ <i>espB1</i> | Donnenberg <i>et al.</i> (1993) |
| DH5 α | <i>E. coli</i> K-12 <i>supE44</i> , Δ <i>lacU169</i> (Φ 80 <i>lacZ</i> Δ <i>M15</i>) <i>hsdR17</i> , <i>recA1</i> , <i>endA1</i> , <i>gyrA96</i> , <i>thi-1</i> , <i>relA1</i> | Hanahan (1983) |
| SM10 λ <i>pir</i> | <i>E. coli</i> K-12 <i>thi</i> , <i>thr</i> , <i>leu</i> , <i>tonA</i> , <i>lacY</i> , <i>supE</i> , <i>recA</i> ::RP4-2-Tc::Mu Km ^r λ <i>pir</i> | Miller and Mekalanos (1988) |

Table 2. Plasmids used in this study.

| | | |
|---------------------------|--|-------------------------------------|
| pBluescript SK+ | General purpose cloning vector | Stratagene |
| pUC19 | General purpose cloning vector | Yanisch-Perron <i>et al.</i> (1985) |
| pWKS30 | Low-copy-number cloning vector | Wang and Kushner (1991) |
| pRDH10 | RP4-based suicide vector containing <i>sacB</i> gene for positive selection | Haigh (1999) |
| pMDG9 | pBluescript containing a 609 bp <i>KpnI</i> – <i>HindIII</i> fragment of a PCR product encoding <i>fis</i> and the 3' end of upstream ORF, <i>yhdG</i> | This study |
| pMDG9 Δ <i>fis</i> | As pMDG9 with <i>fis</i> gene deleted by inverse PCR and replaced by a <i>Bgl</i> II restriction site | This study |
| pMDG10 | 305 bp <i>KpnI</i> (blunted)– <i>Bam</i> HI insert from pMDG9 Δ <i>fis</i> , cloned into the <i>Hinc</i> II– <i>Bam</i> HI sites of pUC19 | This study |
| pMDG12 | 330 bp insert from pMDG10 (<i>Sph</i> I– <i>Bam</i> HI) subcloned into the cognate sites of suicide vector pRDH10 | This study |
| pMJ3 | <i>fis</i> operon cloned into the <i>Bam</i> HI– <i>Hind</i> III sites of pWKS30 | This study |

and Fis-BAD.R (5'-TAAGTTCGGCAAGCTTATCACCGT G-3') were used to amplify a 632 bp DNA fragment, the latter introducing a *Hind*III restriction site (Fig. 2B). The PCR product was digested with *Kpn*I and *Hind*III and cloned into the respective sites of pBluescript SK+ to generate plasmid pMDG9. To delete *fis*, inverse PCR (Wren *et al.*, 1994) was performed with primers Δ *fis*-F (5'-TACGGCAAGATCTAATTC AG-3') and Δ *fis*-R (5'-CATAGTTCTGTGATCTTTA-3'), which were designed to anneal to the 3' and 5' ends of *fis*, respectively, facing away from each other. Each primer contained a *Bgl*II restriction site so that the product could be digested with *Bgl*II and ligated to form plasmid pMDG9 Δ *fis*. As pMDG9 Δ *fis* did not contain an *Sph*I site necessary for subcloning into the suicide vector pRDH10, pMDG9 Δ *fis* was digested with *Kpn*I and the ends blunted, followed by digestion with *Bam*HI and cloning of the fragment into the *Bam*HI and *Hinc*II sites of pUC19 to form plasmid pMDG10. This was digested with *Sph*I and *Bam*HI, and the fragment was cloned into the cognate sites of plasmid pRDH10 to form pMDG12, which was transformed into SM10 λ *pir*. To introduce the Δ *fis* deletion into the EPEC chromosome, SM10 λ *pir* pMDG12 was conjugated with E2348/69S according to the method of Blomfield *et al.* (1991), and a single chloramphenicol-resistant merodiploid (E2348/69S::pMDG12) was selected and grown in LB broth to allow resolution of pMDG12 from the chromosome. Resolved merodiploids were identified by their ability to grow on LB agar supplemented with 6% (w/v) sucrose. Deletion of *fis* was determined initially by colony PCR of sucrose-resistant colonies, using PCR primers Fis-F and Fis-BAD.R, and confirmed by Southern blot analysis (data not shown).

Plasmid pMJ3 carrying the *fis* promoter, *yhdG* and the entire *fis* gene was constructed by PCR amplification using Fis-F2 (5'-AAGAAGAGTGGATCCGTATTACC-3') (introduces a *Bam*HI restriction site) and Fis-BAD-R (see above) and cloned into the *Bam*HI–*Hind*III sites of the low-copy-number vector, pWKS30 (Wang and Kushner, 1991). The integrity of pMJ3 was confirmed by sequence analysis.

RNA purification and Northern hybridization

RNA was purified from 2.0 A₆₀₀ equivalents of bacterial culture, using the Promega SV Total RNA isolation system, with an additional preliminary step to weaken the cell walls. Briefly, bacteria were harvested by centrifugation at 4°C; the

bacterial pellet was resuspended in 100 μ l of protoplasting buffer (15 mM Tris-HCl, pH 8.0, 0.45 M sucrose, 8 mM EDTA) containing 80 mg ml⁻¹ lysozyme and incubated on ice for 15 min. The suspension was centrifuged again, and the pellet was resuspended in 425 μ l of lysis reagent (supplied with the kit); subsequent purification steps were as recommended by the manufacturer. Electrophoresis of RNA on 1.2% (w/v) agarose formaldehyde–MOPS gels was performed as described by Sambrook *et al.* (1989). After electrophoresis, the RNA was blotted onto Hybond-N nylon membrane (Amersham International) and UV cross-linked. Uniform transfer and equal loading of RNA onto the membrane were verified by staining with 0.04% (w/v) methylene blue in 0.5 M sodium acetate, pH 5.2, and rinsing in DEPC-treated distilled water. The membranes were prehybridized and hybridized at 65°C (Sambrook *et al.*, 1989) with DNA probes labelled with fluorescein-dUTP and detected using the chemiluminescent Gene Images kit (Amersham International). The light signal was recorded on Hyperfilm MP (Amersham International). The *fis* probe was the 609 bp *Kpn*I–*Hind*III fragment used in the construction of pMDG9 (see Fig. 2A). The *bfpA* probe was a 632 bp PCR product made using the primers *bfpA*-F (5'-TATCCGTGACCTATTAATAC-3') and *bfpA*-R (5'-GATTA CTTCATAAAATATGTAAC-3').

Multiplex primer extension analysis (MPEA)

The primer extension analysis kit manufactured by Promega was used to perform the primer extension reactions. Oligonucleotides (2 μ g) *tir*-R2 (5'-TGCGTATTGAGAATATCA AG-3'), *espA*-R (5'-TTGGGCAGTGGTTGACTCCTT-3'), *LEE1*-R (5'-CCTGCTGTAGAACTGCAA-3') and *sepL*-R (5'-TTCCTTGCCTACCTTTGCG-3') were annealed with 9 μ g of total RNA, and the primer extension reactions were performed as directed by the manufacturer. After synthesis of the cDNA, the RNA was degraded by the addition of 50 ng of RNase A and incubation for 15 min at 37°C. Loading buffer (20 μ l; supplied with kit) was added to each sample, heated at 90°C for 10 min, and the samples were loaded onto a 0.8% (w/v) agarose gel. The cDNA products were blotted onto Hybond-N by conventional Southern blot and hybridized with fluorescein-labelled PCR-generated probes directed towards each cDNA transcript. To synthesize the probes, each primer used in the primer extension reaction (see above) was paired with a complementary primer to generate PCR products of

≈ 400–600 bp. Thus, espA-R was paired with espA-F (5'-CATGGATACATCCAACACTACAGC-3'), tir-R2 was paired with tir-F (5'-GCTGCATACCGTTACGTCAT-3'), sepL-R was paired with sepL-F (5'-CGTGAGTTTCCAATGGCTAATG G-3') and LEE1-R was paired with LEE1-F (5'-TTACCCAAT CACTTACTTATG-3'). The probes were labelled with fluorescein-dUTP and detected with the Gene Images nucleic acid labelling and detection system (Amersham International). The blot was hybridized with one probe at a time, exposed to Hyperfilm MP (Amersham International) overnight, stripped by washing four times in boiling 0.1% (w/v) SDS and hybridized with each of the remaining probes.

FAS tests and invasion assays

The fluorescent actin staining (FAS) test for EPEC AE lesion formation on cultured HEp-2 cells was performed as described by Knutton *et al.* (1989), viewed with a Zeiss Axiomat fluorescence microscope and photographed on Kodak Ektachrome 160T colour reversal film.

For the assessment of EPEC invasion of cultured cells, triplicate wells of 24-well tissue culture trays (Corning) seeded with 10^5 HEp-2 cells were inoculated with $\approx 10^7$ cfu of strains UMD864, DH5 α , E2348/69S, E2348/69S Δ fis or E2348/69S Δ fis (pMJ3). Assay plates were incubated for 3 h at 37°C in 5% CO₂. To determine total bacteria in each culture, the following procedure was used. First, the medium was withdrawn and centrifuged to pellet the extracellular bacteria. Secondly, the cell monolayers were lysed with 250 μ l of 0.5% (w/v) sodium deoxycholate in phosphate-buffered saline (PBS) to release the HEp-2 cell-associated bacteria. The lysates were transferred to the tubes containing the corresponding pelleted extracellular bacteria. To assay intracellular bacteria, monolayers were washed with PBS, incubated for 60 min at 37°C in DMEM containing 250 μ g ml⁻¹ gentamicin and then washed twice more before being lysed as described above. To enumerate bacteria, dilutions were prepared in PBS and plated on LB agar in triplicate.

Western blot analysis of intimin expression

Overnight LB broth cultures of EPEC strains were diluted 1:100 in prewarmed DMEM and incubated for 4.5 h at 37°C in 5% CO₂. One A₆₀₀ unit of each culture was harvested, and the cell pellets were resuspended in SDS-PAGE sample buffer (Laemmli, 1970). Samples were resolved on an 8% SDS-PAGE gel (Laemmli, 1970) and electroblotted onto Schleicher and Schuell 0.2 μ m pore-size nitrocellulose. Intimin was detected using rabbit-derived anti-intimin antibody (Knutton *et al.*, 1997), diluted 1:1000 in TBS (150 mM NaCl, 20 mM Tris-HCl, pH 7.5) containing 0.5% (w/v) bovine serum albumin (BSA; fraction V, Sigma) and 0.05% (v/v) Tween 20. Anti-intimin antibodies were detected with goat-derived anti-rabbit horseradish peroxidase conjugate (Sigma), diluted 1:5000 in TBS containing BSA and Tween 20 (Sigma). The secondary antibody was detected using the Amersham ECL chemiluminescent detection system and recorded on DuPont Cronex medical X-ray film.

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