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Enteropathogenic and enterohaemorrhagic *Escherichia coli* deliver a novel effector called Cif, which blocks cell cycle G_2/M transition

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Summary

Enteropathogenic *Escherichia coli* (EPEC) and enterohaemorrhagic *E. coli* (EHEC) are closely related pathogens. Both use a type III secretion system (TTSS) encoded by the 'locus of enterocyte effacement' (LEE) to subvert and attach to epithelial cells through the injection of a repertoire of effector molecules. Here, we report the identification of a new TTSS translocated effector molecule called Cif, which blocks cell cycle G_2/M transition and induces the formation of stress fibres through the recruitment of focal adhesions. Cif is not encoded by the LEE but by a lambdoid prophage present in EPEC and EHEC. A *cif* mutant causes localized effacement of microvilli and intimately attaches to the host cell surface, but is defective in the ability to block mitosis. When expressed in TTSS competent LEE-positive pathogens, Cif is injected into the infected epithelial cells. These cells arrested at the G_2/M phase displayed accumulation of inactive phosphorylated Cdk1. In conclusion, Cif is a new member of a growing family of bacterial cyclomodulins that subvert the host eukaryotic cell cycle.

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Introduction

Enteropathogenic *Escherichia coli* (EPEC) and enterohaemorrhagic *E. coli* (EHEC) are important causes of infectious diarrhoea, particularly among paediatric populations (Nataro and Kaper, 1998). Although EPEC is a significant health threat in the developing world, EHEC causes sporadic but deadly outbreaks of haemorrhagic colitis and haemolytic–uraemic syndrome in Europe, North America and Japan. EPEC and EHEC are closely related pathogens, which colonize the intestinal mucosa and produce specific attaching-and-effacing (A/E) lesions on gut enterocytes. A/E lesions are characterized by intimate bacterial adhesion, reorganization of host cytoskeletal proteins into pedestal-like structures beneath the adherent bacteria and destruction of the brush border microvilli (Frankel *et al*., 1998; Vallance and Finlay, 2000). These marked cytoskeletal changes seen directly beneath the adherent bacteria are routinely detected *in vitro* through the use of the fluorescent actin-staining (FAS) test (Knutton *et al*., 1989). These phenotypes are dependent on a pathogenicity island named the locus of enterocyte effacement (LEE), which codes for the bacterial outer membrane protein intimin (Jerse *et al*., 1990) and for a type III secretion apparatus (Jarvis *et al*., 1995; McDaniel *et al*., 1995). Type III secretion systems (TTSS) are molecular syringes/needles that inject bacterial virulence factors directly into host cells. These injected effector molecules subvert cellular processes and contribute to disease. Five LEE-encoded effector molecules have been identified so far. The first one, Tir, is transferred into host cells and inserted into the plasma membrane, where it acts as a receptor for intimin (Kenny *et al*., 1997; Deibel *et al*., 1998). The binding of intimin to Tir results in a profound rearrangement of the actin cytoskeleton, which leads to the characteristic pedestal formation beneath adherent bacteria. The second effector, Map, is targeted to mitochondria where it has a membrane potential-disrupting activity (Kenny and Jepson, 2000) and induces filopodia formation (Kenny *et al*., 2002). The third, EspF, is implicated in the disruption of the intestinal barrier function (McNamara *et al*., 2001) and in host cell death (Crane *et al*., 2001). The fourth, EspG, is homologous with VirA, a *Shigella flexneri* protein capable of inducing membrane ruffling through the stimulation of microtubule destabilization (Elliot *et al*., 2001). Finally, the fifth, EspH, modulates

the formation of filopodia and pedestals (Tu *et al*., 2003). These findings have unravelled some of the ways in which pathogenic bacteria exploit host processes from the cell surface and have shed new light on EPEC and EHEC pathogenesis.

In addition to the ability to produce A/E lesions, certain human and animal clinical EPEC isolates trigger an irreversible cytopathic effect (CPE) in epithelial cells. This CPE is characterized by progressive recruitment of focal adhesion plaques leading to the assembly of stress fibres and inhibition of the cell cycle G_2/M phase transition (De Rycke *et al*., 1997; Nougayrede *et al*., 1999; 2001). The cytostatic effect can be summarized as follows: cells progressively accumulate at the 4C and 8C DNA content and do not display signs of mitosis. This cytostatic effect is not functionally related to cytoskeletal rearrangement but is linked to the maintenance of the cyclin-dependent kinase Cdk1, a key effector driving entry into mitosis, in a premitotic tyrosine-phosphorylated state (Nougayrede *et al*., 2001). The ability of EPEC and EHEC to induce both cytoskeletal alterations and to block the G_2/M phase transition depends on a functional LEE type III secretion machinery but not on intimin or Tir (Nougayrede *et al*., 1999; Marchès *et al*., 2000). Therefore, we made the hypothesis that a new TTSS-dependent effector was responsible for the CPE.

In the present study, we identified this novel effector molecule that we have called 'Cif' for cycle inhibiting factor. Upon contact with epithelial cells, the bacteria quickly inject Cif into the host cell. Cif promotes the actin cytoskeleton rearrangement and mediates a G_2 cell cycle arrest characterized by inactive phosphorylated Cdk1. Cif is the first effector molecule not encoded on the LEE but on a lambdoid phage.

Results

Identification of TTSS competent mutants defective for the induction of CPE

In order to identify the EPEC gene(s) coding for the effector molecule(s) involved in the CPE, a collection of 3600 mutants was generated by transposition in the EPEC strain E22. Twenty-six mutants unable to induce large mononucleated cells were identified as described in *Experimental procedures*. Among these 26 mutants, only three independent mutants, named 1F12, 4F6 and 20B8, were still able to induce a FAS positive response. These three FAS^+ CPE^- mutants showed the same adhesion pattern on epithelial cells (Fig. 1A, bottom) and induced the same F-actin accumulation beneath the adherent bacteria as the wild-type EPEC strain E22 (Fig. 1A, top). When tested in the rabbit ileal loop model, these mutants were still able to induce the typical A/E lesions with bacteria adhering intimately to intestinal cells on pedestal-like structures and destruction of surrounding microvilli (not shown).

These mutants were also characterized for their ability to produce a full cytopathic effect. HeLa cells were infected by the mutants and the wild-type E22 strain for 4 h, then washed and incubated in the presence of gentamicin. Seventy–two hours after interaction, the cells were fixed, and stress fibres were visualized by F-actin labelling and focal adhesion by vinculin labelling. Whereas all the cells previously in contact with the wild-type strain E22 showed the typical CPE enlargement associated with F-actin stress fibres and focal adhesion multiplication (Fig. 1B, left), all the cells inoculated with 1F12 (Fig. 1B, right) or 4F6 and 20B8 (not shown), showed a normal F-actin organization and peripheral focal adhesion distribution comparable to non-inoculated control cells (not shown).

In order to check whether these mutants were impaired in their ability to progress in mitosis, we labelled the infected cells with DAPI to visualize chromosome status and with α-tubulin to visualize the mitotic microtubule spindle. No mitotic cells were detected in cells infected with the wild-type strain E22 (Fig. 1C, left). Only interphase cells were seen with large nuclei without the microtubule mitotic spindle. In contrast, observation at the same magnification of cells infected with 1F12 (Fig. 1C, right), 4F6 or 20B8 (not shown) showed the presence of numerous cells in mitosis (3.5% of the cells) with figures of condensed chromosomes and mitotic spindles of the cells in prophase, metaphase, anaphase and telophase (Fig. 1C, arrowheads). Taken together, these results suggest that these three mutants have transposons in genes that are essential for both the long-term cytoskeleton rearrangement and the inhibition of mitosis characterizing the CPE but not the TTSS as they were still FAS^+ and A/E^+.

The CPE mutants 1F12, 4F6 and 20B8 are interrupted in the same 846 bp gene located on a prophage

Analysis of the DNA sequences flanking the transposons in the mutants 1F12, 4F6 and 20B8 (see *Experimental procedures*) showed that the transposons were inserted in the same open reading frame (ORF) of 846 bp encoding a 282-amino-acid protein with a predicted molecular mass of 32 kDa (Fig. 2). We named this ORF *cif* for cycle inhibiting factor. Analysis of the amino acid sequence of Cif revealed no significant matches with well-characterized proteins. However, Cif is homologous (21% identity and 40% similarity) with a putative protein encoded by an ORF in *Burkholderia pseudomallei* strain K96243 (Fig. 3). *B. pseudomallei* is the causative agent of melioidosis, a serious infectious disease of humans and animals that is endemic in subtropical areas. Interestingly, this pathogen

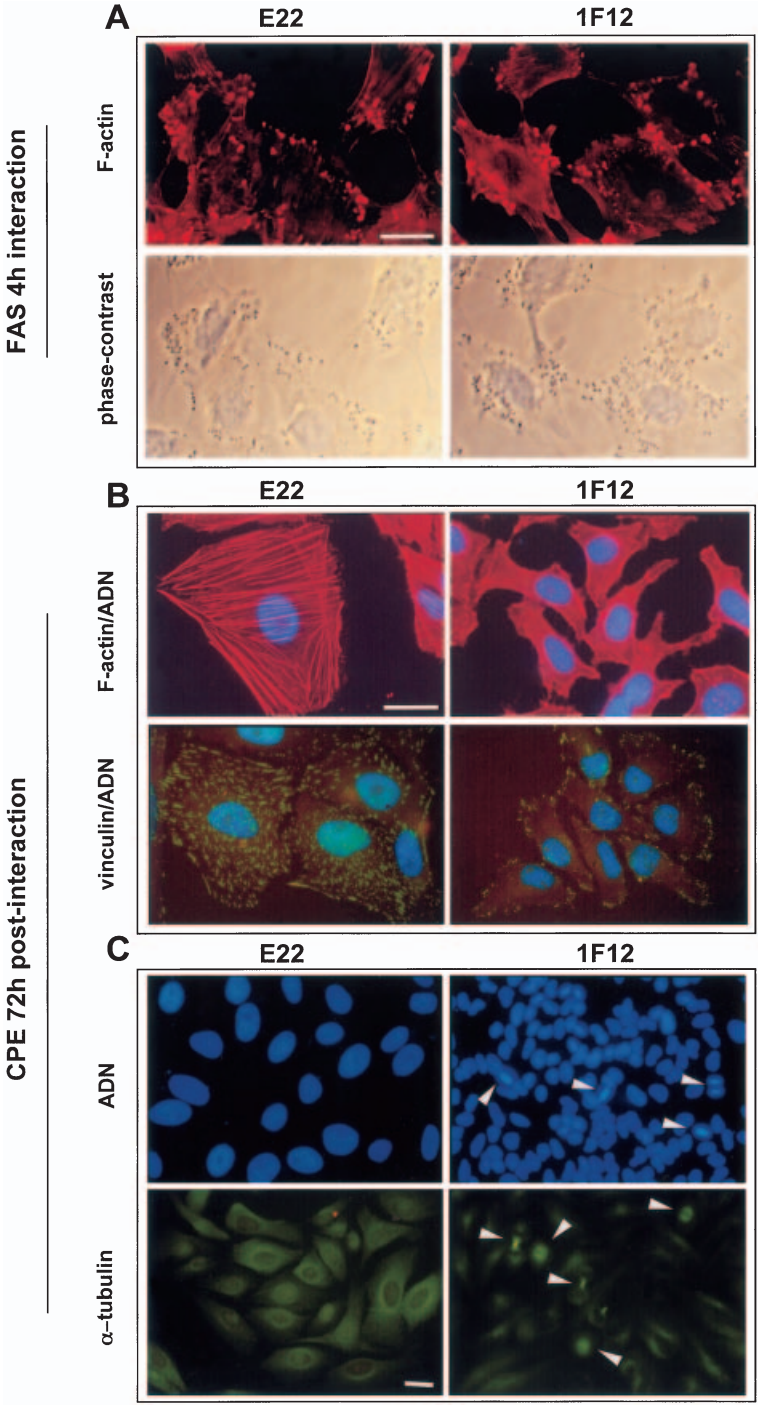

Fig. 1. Analysis of the FAS and CPE responses induced by EPEC strain E22 and transposon insertion mutant 1F12.
A. FAS assay. RK13 cells were infected for 4 h with EPEC E22 or 1F12. At the end of the interaction, the cells were washed with PBS, fixed, permeabilized, and F-actin was stained with rhodamine phalloidin (top). Corresponding phase-contrast micrographs are shown (bottom). Bar represents 20 μm.
B and C. CPE assay. HeLa cells were infected for 4 h with E22 or 1F12. At the end of the interaction, the cells were washed in PBS, and the bacteria were killed with gentamicin. The cell monolayers were incubated further for 3 days. The cells were then washed with PBS, fixed and permeabilized.
B. Nuclei and stress fibres (top) or nuclei and focal adhesion plaques (bottom) were, respectively, stained with DAPI and rhodamine phalloidin or DAPI with anti-vinculin antibody.
C. Dividing cells were detected by staining with DAPI (top) and with anti-α-tubulin antibody (bottom). Arrowheads indicate figures of mitosis. Bars represent 20 μm.

codes for at least three TTSS, two being similar to the Hrc/Hrp type III secretion systems present in *Ralstonia solanacearum* and *Xanthomonas* spp. (Rainbow *et al*., 2002) and the third one being similar to the Inv/Mxi-Spa type III secretion systems of *Salmonella* and *Shigella* (Stevens *et al*., 2002).

To determine the location of *cif* in the EPEC E22 genome, we constructed and screened a genomic DNA library of E22 with a probe corresponding to *cif* (*Experimental procedures*). We selected two overlapping plasmid clones giving a 5265 bp chromosomal fragment encompassing *cif* with 400 bp upstream and 4000 bp downstream flanking DNA regions (Fig. 3). Analysis of the upstream and downstream sequence showed that *cif* is located on a lambda prophage integrated near the Bio operon (at 17.3 min of the K-12 genome) and similar to a

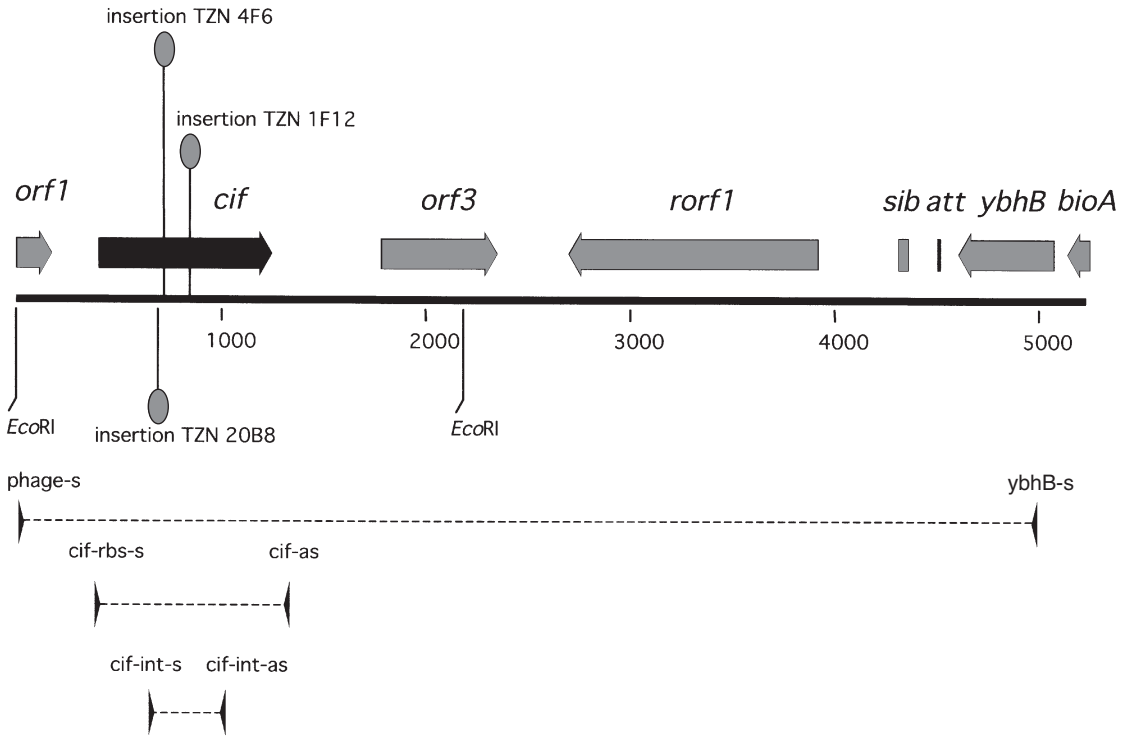

Fig. 2. Physical map and genetic organization of the *cif* locus. The line shows a chromosomal fragment of 5265 bp isolated from EPEC strain E22 (GenBank accession number AF497476). The mini-Tn*5* insertion points of *cif* mutants 20B8, 4F6 and 1F12 are shown. Partial *orf1*, *orf3* and *rorf1* are similar to genes present in lambda phages from EHEC O157:H7 strains EDL933 and Sakai. A putative *cis*-acting *sib* retroregulation site and a conserved lambda *att* site are represented by boxes. Genes *ybhB* and *bioA* are part of the *bio* operon located at 17.3 min in the K-12 chromosome. The positions of the relevant restriction sites are reported. The solid triangles show the positions of the PCR primers used in this study.

cryptic prophage found in EHEC strains of serotype O157:H7.

CPE-negative EPEC and EHEC strains are mutated or deleted for cif

The presence of *cif* in a small collection of EPEC and EHEC strains was checked by polymerase chain reaction (PCR) using two internal primers, cif-int-s and cif-int-as (Fig. 2). We observed an amplicon of the expected size for 12 strains out of the 14 EPEC and EHEC strains tested (Fig. 4A). The two negative strains were the EHEC strains EDL933 and Sakai (O157:H7). This result was consistent with the absence of *cif* in the published genome sequence of both strains. In contrast, the presence of an amplicon for the reference human EPEC strain E2348/69 (O127:H6) was unexpected as we previously observed that this strain was unable to induce the CPE effect (De Rycke *et al*., 1997; Nougayrede *et al*., 1999). To determine whether the presence of *cif* homologous genes in the strains was correlated with their ability to induce the CPE, we tested these strains in the CPE assay. Among the 12 *cif*-positive strains, only five strains induced the CPE. All the strains were FAS positive, showing that they were able to interact with the cells and that the TTSS system was functional in the conditions used for the assay (data not shown).

We hypothesized that the CPE-negative phenotype could result from a mutation of the *cif* gene. To determine the sequence of *cif* in various EPEC and EHEC, the locus was amplified by PCR using three sets of primers (Table 2 and Fig. 2). Analysis of the sequences showed that the five CPE-positive strains had a *cif* gene 100% identical at the amino acid level, whereas the seven CPE-negative strains had mutated *cif* genes (Fig. 4A). All mutations were in the 3′ end of the gene leading to a truncated Cif protein (Fig. 4A). Four types of mutation were identified: deletion, substitution or addition of a nucleotide or insertion of a partial IS*2* (Fig. 4C). Altogether, these results suggest that the CPE-negative phenotype of EPEC and EHEC strains resulted from the absence or mutation of *cif* and that the last 10 amino acids of the protein were essential for the effect.

The cloned cif gene restores CPE in the cif *mutant and in CPE-negative EHEC and EPEC strains*

To test the ability of Cif to restore or induce both the

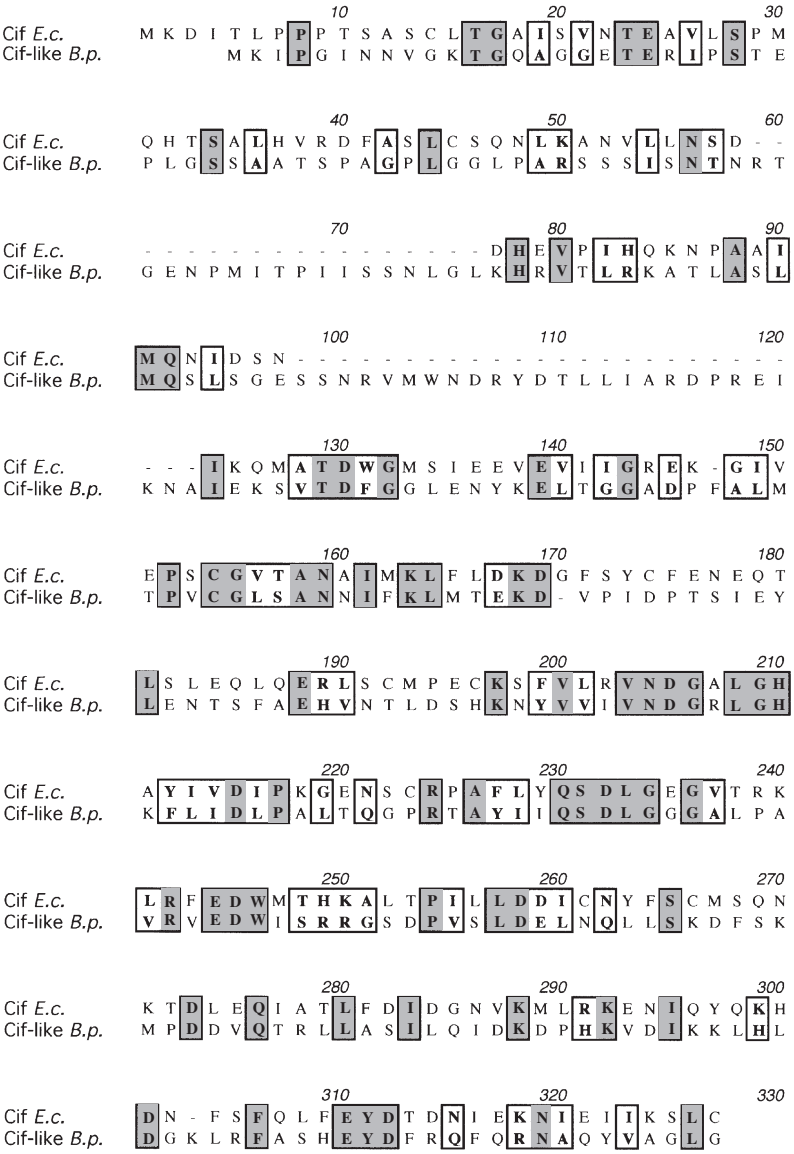

Fig. 3. Comparison of *E. coli* Cif with a putative Cif-like protein in *Burkholderia pseudomallei*. Fully conserved residues are indicated by a shaded dark grey background, and residues similar in properties are indicated by a shaded light grey background. The predicted amino acid sequence of the Cif-like protein was deduced from an ORF in contig 856 from the unfinished genome sequence of *B. pseudomallei* strain K96243. These sequence data were produced by the *B. pseudomallei* sequencing group at the Sanger Institute and can be obtained from ftp://ftp.sanger.ac.uk/pub/pathogens/bps.

cytoskeletal modifications and the inhibition of mitosis on HeLa cells, a low-copy plasmid expressing the full-length Cif from E22 was constructed and used to complement the *cif* mutant of E22. This plasmid, pCif, was also transformed in EPEC E2348/69, EHEC Sakai and PMK5 and the laboratory K-12 strain MG1655, and transformants were tested in the CPE assay. We stained nuclei and focal adhesions of HeLa cells 72 h after interaction with the bacteria transformed or not transformed with pCif. The cells in contact with EPEC or EHEC hosting pCif showed the accumulation of focal adhesions with nuclei and cell enlargement similar to cells exposed to wild-type EPEC E22 (Fig. 5A, 2, 4 and 6). Cells exposed to non-transformed bacteria or to the K-12 strain MG1655 expressing Cif behaved like control cells (Fig. 5A, 1, 3 and 5; not shown). Analysis of DNA by flow cytometry showed that cells exposed to E22*cif*, E2348/69 and Sakai transformed with pCif accumulated in the G_2/M phase at a distribution of 84.7%, 78.1%, and 71.6%, respectively, whereas upon interaction with parental strains, the percentages of cells in G_2/M were 20.4%, 23.3% and 16.4%, respectively (Fig. 5B), and the majority of cells were in the G_1 phase. Similar results were obtained when plasmid pHa-Cif-His expressing the tagged Cif was used to transform E22*cif*, E2348/69, Sakai and PMK5 (data not shown). These results showed that Cif alone was able to confer the CPE phenotype in EHEC and EPEC strains with a functional LEE-encoded TTSS.

Cif is a TTSS effector molecule

To confirm that Cif is a substrate of the TTSS, the EPEC

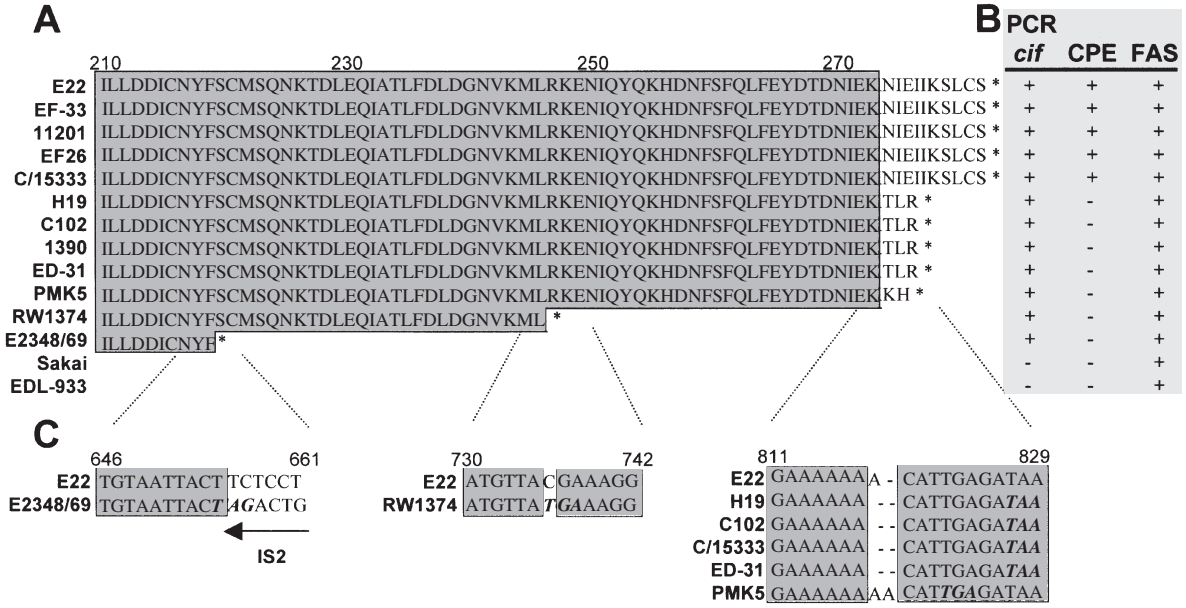

Fig. 4. The CPE is correlated with the presence of a *cif* full gene.
A. Alignment of the deduced last 72 C-terminal amino acids of Cif proteins from strains E22 rabbit EPEC O103:H2, EF-33 human EPEC O111, 11201 human EPEC O125:H6, EF-26 human EPEC O125:H6, C/15333 bovine EPEC O26, H19 human EHEC O26:H11, C102 rabbit EPEC O26:H11, 1390 pig EPEC O45, ED-31 human EHEC O111, PMK5 human EHEC O103:H2, RW1374 bovine STEC O103:H2 and E2348/69 human EPEC O127:H6. Identical residues are highlighted in grey. Asterisks indicate a stop codon. No homologous *cif* gene is present in the Sakai human EHEC O157:H7 and EDL-933 human EHEC O157:H7 strains.
B. Presence of the *cif* homologous gene tested by PCR, and CPE and FAS response of the challenged strains upon interaction with HeLa cells. In each CPE-negative strain, the *cif* gene is mutated in the C-terminal sequence or is absent from the genome.
C. Nature of mutation in the *cif* nucleotide sequences. Identical residues are highlighted in grey. Stop codons are represented in bold italics. Numbers above the nucleotide sequence indicate the position in the 846 bp *cif* gene. The *cif* gene in E2348/69 is interrupted at nucleotide 654 by a sequence identical to the right inverted terminal repeat of the transposable DNA element IS*2* (arrow).

E22 strain mutated in *cif*, *espB* (translocation deficient), or in *escN* (secretion deficient) were transformed with pHa-Cif-His and grown in DMEM buffered with Hepes to activate the TTSS. Pellets and supernatants were analysed by Western blotting with anti-HA antibodies. As a control, we used the *cif* mutant transformed with a plasmid expressing a non-secreted Ha-LacZ-His fusion. Analysis of the pelleted fractions showed that the Ha-Cif-His and the control Ha-LacZ-His fusions were expressed at a similar level in each strain (Fig. 6A). Ha-Cif-His was detected in the supernatants of the *cif* and *espB* mutants hosting pHa-Cif-His, but not in the supernatant of the *escN* mutant hosting pHa-Cif-His (Fig. 6A). Similar results were observed when pellets and supernatants were analysed by Western blotting with anti-His antibodies (data not shown), confirming that Cif secretion required a functional TTSS.

To investigate whether Cif was translocated into the host cells, HeLa cells were infected with the E22 strains mutated in *cif*, *espB* or both *eae* and *tir* and transformed with pHa-Cif-His or pHa-LacZ-His. Infected cells were fractionated into a cytoplasm- and membrane-soluble fraction and into an insoluble fraction containing adherent bacteria, host nuclei and the cytoskeleton component. Analysis by Western blotting revealed that Cif was only detected in the cytoplasm- and membrane-soluble fraction of cells infected with mutants with a functional TTSS (Fig. 6B). In contrast, Ha-Cif-His and Ha-LacZ-His were detected in the insoluble fraction of all the strains (Fig. 6B). Altogether, these results showed that Cif was translocated inside the host cells and that this translocation also required a functional TTSS.

To confirm this result, we constructed a fusion between the full-length Cif and the catalytic domain of CyaA from *Bordetella pertussis*. The resulting plasmid pCif-Cya was transformed into the wild-type strain E22, as well as mutants for *cif*, *espB*, *escN* or *eae/tir*, and then the transformants were used to infect HeLa cells. Expression of the fusion protein was confirmed by Western blotting with anti-Cya antiserum (data not shown). After infection, the cells were lysed, and the level of intracellular cAMP was determined. The wild-type strain and mutants for *cif* or *eae/tir* showed an efficient translocation of the fusion protein Cif-Cya associated with a high level of intracellular cAMP, whereas the strains defective for the translocation apparatus (*espB* mutant) or defective for the secretion apparatus (*escN* mutant) did not translocate Cif-Cya as they did not increase the level of intracellular cAMP (Fig. 6C).

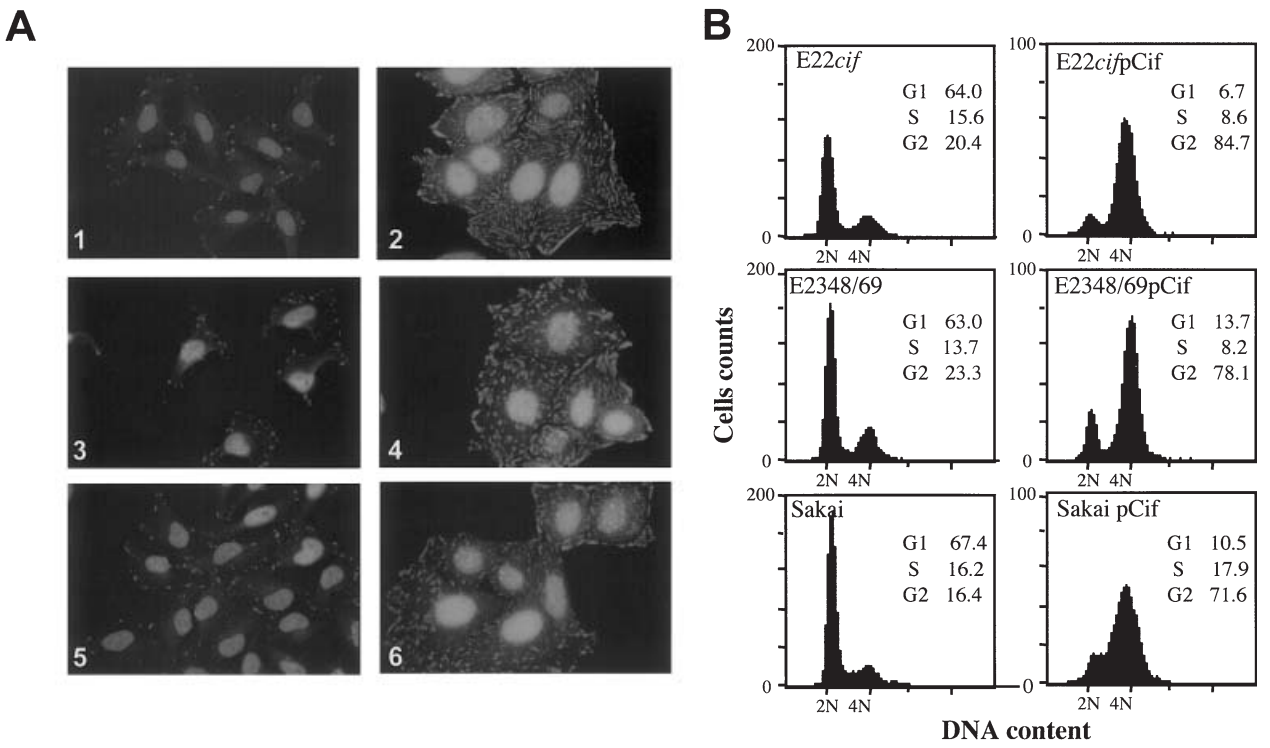

Fig. 5. Expression of the native E22 Cif protein in EPEC and EHEC strains mutated or defective for *cif*.
A. Effect of Cif expression on focal adhesions and nuclei. HeLa cells were exposed for 4 h to E22 *cif* (1) and E22 *cif* complemented with pCif (2), for 3.5 h with E2348/69 (3) and E2348/69 transformed with pCif (4), and for 6 h with Sakai (5) and Sakai transformed with pCif (6). Cells were cultivated 72 h after exposure to bacteria, then focal adhesions and nuclei were stained with anti-vinculin antibodies and DAPI respectively.
B. Effect of Cif expression on the cell cycle. HeLa cells were synchronized at the G_1/S transition by a double-thymidine block and were infected as before. Cells were harvested 22 h after G_1/S release and analysed for cell cycle distribution by flow cytometry.

These results confirm that Cif is a type III effector molecule translocated into host cells.

Cif inhibition of mitosis is associated with the inhibition of Cdk1 activation

Mitosis is regulated by the phosphorylation status of the M phase-promoting factor (MPF) formed by the complex between the regulatory cyclin B1 and the cyclin-dependent kinase Cdk1. The MPF is activated at the G_2/M transition by dephosphorylation of Cdk1 on Thr-14 and Tyr-15 residues. To check whether the inhibition of mitosis mediated by Cif was associated with inactive Cdk1, we exposed G_1/S synchronized HeLa cells to different EPEC O103 strains expressing Cif or not. Then, 26 h after the G_1/S release (22 h after interaction), the DNA content of the infected HeLa cells was determined by flow cytometry (Fig. 7A), and the corresponding level of Cdk1 phosphorylation was determined by Western blot analysis (Fig. 7B and C). As a control for a G_2/M cell cycle arrest and the accumulation of the slow-migrating hyperphosphorylated Cdk1 isoform, we used HeLa cells treated with CDT-I, a toxin that prevents Cdk1 dephosphorylation (Comayras *et al.*, 1997).

HeLa cells infected with E22 or E22*cif* pCif behaved like cells exposed to CDT-I and accumulated in G_2/M (Fig. 7A). In contrast, non-infected cells and cells infected with the *cif* mutant or with the mutant for the translocation apparatus (*espB*) were not arrested, although cycle progression was slowed down compared with uninfected control cells (Fig. 7A).

The MPF was purified from cell lysates using $p13^{suc1}$ affinity, and the phosphorylation of Cdk1 was analysed by Western blotting with an anti-Cdk1-Tyr-15 antibody. Infection of the cells with the wild-type EPEC strain or with the complemented *cif* mutant led to the accumulation of the inactive hyperphosphorylated isoform of Cdk1 in contrast to cells exposed to *cif* or *espB* mutant strains (Fig. 7B). To assess the different levels of the Cdk1 isoforms, we stripped the membrane and probed it with an antibody allowing the detection of all the isoforms of Cdk1. The fastest migrating band, which is Cdk1 that has been dephosphorylated at both Tyr-15 and Thr-14, is the predominant band in the cells infected by the *cif* and *espB* mutants or non-infected cells, whereas the slowest migrating band, which is the fully phosphorylated inactive Cdk1, is clearly predominant in cells infected by E22 or E22*cif* complemented with pCif (Fig. 7C). Similar results were

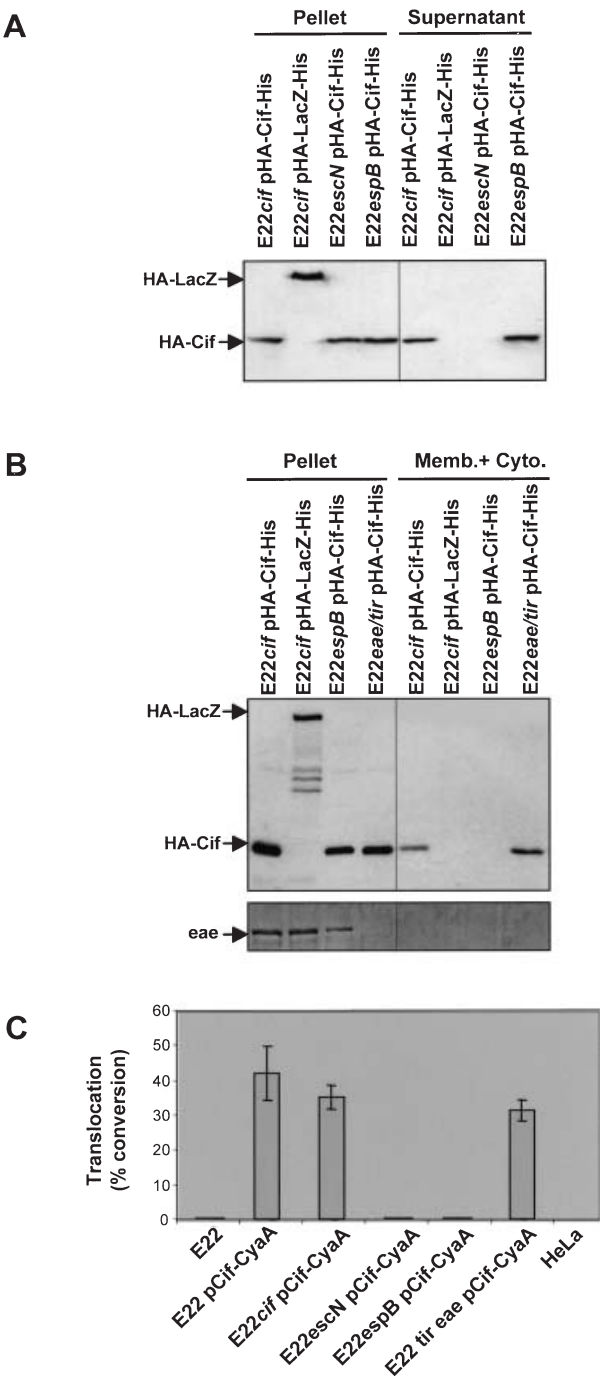

Fig. 6. Cif is a TTSS effector molecule.
A. Cif is a type III secreted protein. Bacterial pellets and TCA-precipitated supernatant proteins were resolved by 12% SDS-PAGE, blotted and probed with anti-HA antibodies. Positions of the Ha-Cif-His and the Ha-LacZ-His proteins are shown by arrows.
B. Cif is translocated into the host cell in an *espB*-dependent process. Infected HeLa cells were fractionated into adherent bacteria and host nuclei/cytoskeletal fractions (Pellet) and 'Triton X100-soluble membrane' and cytoplasmic fractions (Memb. + Cyto.). The samples were resolved by 10% SDS-PAGE, blotted and probed with anti-HA antibody. The membrane was stripped and probed with a specific intimin antiserum to confirm the absence of contaminating bacteria in the Memb. + Cyto. fractions.
C. Translocation of Cif–CyaA is TTSS dependent. The capability of the strains to mediate translocation of Cif–CyaA into infected cells was tested by the conversion assay as described in *Experimental procedures*. Each assay was performed at least three times. The vertical bars indicate the standard error.

obtained when the HeLa cell lysates were analysed directly by Western blotting with anti-Cdk1 antiserum (data not shown). These results indicate that Cif inhibition of the G_2/M transition results from some action that leads to a failure of dephosphorylating Cdk1.

Discussion

In EPEC and EHEC, five type III translocated effectors have been characterized so far: Tir, EspF, Map, EspG and EspH. The genes coding for these effector molecules as well as those coding for the TTSS are located on the LEE pathogenicity island. A recent study by Tu *et al*. (2003), using CyaA fusions, suggested that all type III secreted effectors coded by the LEE have now been identified. In the present study, we report the identification of a novel type III effector, called Cif for cycle inhibiting factor, which is the first *E. coli* TTSS translocated effector not coded by the LEE. EPEC and EHEC strains producing a full-length Cif trigger in HeLa cells an irreversible cytopathic effect (CPE). This CPE is characterized by the progressive recruitment of focal adhesions, assembly of stress fibres and the arrest of cell proliferation without entering mitosis. The effect of Cif was linked to the maintenance of the cyclin-dependent kinase Cdk1, a key effector driving entry into mitosis, in a premitotic, tyrosine-phosphorylated state.

The localization of the *cif* gene on a λ-like prophage demonstrates that the repertoire of the TTSS virulence factor is not restricted to the LEE in EPEC and EHEC. The increasing availability of bacterial genome sequence data highlights the unexpected importance of bacteriophages in the genetic diversity of bacteria as an important mechanism for evolution and adaptability to new hosts or ecological niches by the direct horizontal transfer of new genes (Wagner and Waldor, 2002). The recently published genomes of EDL933 and Sakai O157:H7 EHEC strains have shown that the genome of pathogenic *E. coli* contains a large number of bacteriophages carrying ORFs coding for putative proteins of unknown function (Ohnishi *et al*., 2001). We can assume that the number of TTSS effectors present in EHEC or EPEC will increase rapidly in the near future.

Localization of type III effectors on prophages has already been described in *Salmonella* spp. strains. Several effective and putative substrates for SPI-1 and SPI-2 TTSS are encoded on λ-like Gifsy prophages present in *Salmonella* (Miao and Miller, 2000, Ho *et al*., 2002). The well-characterized effector SopE, secreted by the SPI-1 TTSS and involved in efficient entry of *Salmonella* into

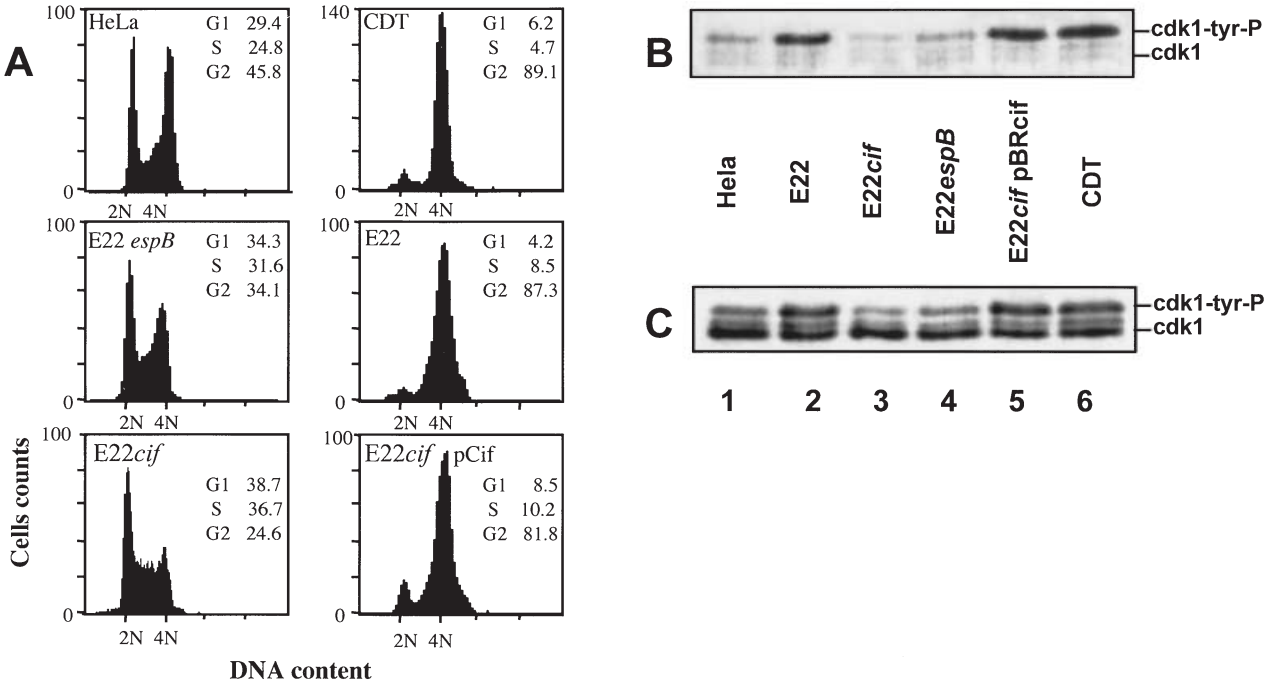

Fig. 7. Cif-mediated G_2/M cell cycle arrest leads to the accumulation of Cdk1 in an inactive hyperphosphorylated state. HeLa cells synchronized at the G_1/S border by a double-thymidine block were infected for 4 h with the bacteria or treated for 1 h with CDT-I and harvested 26 h after the G_1/S release.
A. Cell cycle distribution was analysed by flow cytometry. The percentage of cells in each cell cycle phase is indicated.
B. Tyr-15-cdk1 phosphorylation status was assessed by immunoblotting with anti-Cdk1-Tyr-15 antibody.
C. The blot was stripped and probed with anti-Cdk1 antibody to visualize each isoform of Cdk1. Non-infected HeLa cells and cells treated with CDT were used as controls for normal and Cdk1 hyperphosphorylation states respectively.

host cells, is encoded on a temperate P2-like phage called SopE Φ. This phage is not widely distributed in *Salmonella* bacteria but is present in the *S. typhimurium* isolates that have caused major outbreaks in the UK and Germany in the 1970s and 1980s. This suggests that SopE Φ contributed to the enhanced adaptability or pathogenicity of these strains (Mirold *et al*., 1999). In EPEC and EHEC, acquisition of other virulence factors such as Cif that can use the pre-existing TTSS to gain access directly to the target cell cytoplasm could also confer to these strains specific advantages in their pathogenic process. Whether the λ-like prophage encoding Cif is functional and able to disseminate to other strains by lysogenic conversion remains to be checked, but we have preliminary data showing that antibiotic treatments can induce the phage carrying *cif* in the rabbit EPEC strain E22.

There is a striking homology between the phenotypes of cells infected with EPEC and EHEC translocating Cif and cells treated with the cytolethal distending toxins (CDTs) produced by several Gram-negative bacteria species. Indeed, cultured cells exposed to CDT exhibit a number of similar effects including cell cycle arrest, enlargement, Cdk1 phosphorylation (Comayras *et al*., 1997; Pérès *et al*., 1997; Whitehouse *et al*., 1998; Cortes-Bratti *et al*., 1999) and actin stress fibre formation (Aragon *et al*., 1997; Cortes-Bratti *et al*., 1999). CDT is a secreted holotoxin encoded by a cluster of three adjacent genes (*cdtA*, *cdtB*, *cdtC*). CDT-B is the active subunit exerting its effect as a nuclease that damages the host DNA and triggers cell cycle arrest. CDT-B shows the conservation of functionally important residues involved in the catalytic and metal ion binding of several diphosphodiesterases such as the human DNase I (Elwell and Dreyfus, 2000; Lara-Tejero and Galan, 2000). As with other DNA-damaging agents, CDTs block proliferation of mammalian cells by activating DNA damage-induced checkpoint responses in an ATM-dependent manner in both proliferating and non-proliferating cells (Li *et al*., 2002). However, Cif has no homology with DNase I, and we have as yet no evidence that Cif can induce DNA damage. Therefore, it is possible that Cif hijacks the mitotic checkpoint pathway(s) without inducing DNA damage. For example, Cif might act directly on Cdk1 or on proteins that interact directly with Cdk1, such as the CDC25 phosphatase that carries out the reaction which activates Cdk1 for entry into mitosis. Our ongoing work is directed at defining the biochemical mechanisms underlying Cif-mediated inactivation of Cdk1 kinase.

We have shown in a small collection of EPEC and EHEC strains that *cif* was widely distributed in these

pathogens. However, a significant percentage of the *cif* genes is mutated among strains of different serotypes. Epidemiological studies are now in progress on a larger collection of pathogenic *E. coli* to evaluate the incidence of strains producing a functional Cif. The severity or recurrence of the disease associated with the identified strains will help us to appreciate the relevance of Cif in *E. coli* pathogenesis. We have shown that Cif is not required to induce the A/E lesion in the rabbit ileal loop model. This lesion is central in EPEC and EHEC pathogenesis. However, TTSS-dependent but intimin-independent effects triggered by certain EPEC strains could be important *in vivo* and account, for instance, for the persistent diarrhoea (Marchès *et al*., 2000). It is likely that Cif exerts its effect on cells that normally undergo continuous replication, such as the intestinal epithelium, which undergoes perpetual regeneration, fuelled by a population of multipotent stem cells located at the base of the crypts of Lieberkuhn or those associated with the immune system. By inducing cell cycle arrest, Cif could influence this renewal and developmental process to facilitate bacterial intestinal colonization by increasing the number of cells that could be permissive for their attachment, as has been suggested for CDT in a recent review (Lara-Tejero and Galan, 2002). Likewise, Cif could target intestinal T cells, which are crucial in initiating and regulating innate and adaptive mucosal immune responses.

Besides the role of Cif as a 'classical' EPEC and EHEC virulence factor, we should also consider that the carriage of bacteria-injecting proteins able to interfere with the host cell cycle represents a long-term risk of carcinogenesis. Indeed, the possible link between bacterial infection/carriage and tumorigenesis is an emerging theme in bacterial pathogenesis. *Helicobacter pylori* is now recognized as an important aetiological cofactor of gastric cancers, and several other bacteria, *Salmonella enterica* serovar *typhi*, *Citrobacter rodentium* and *Bartonella* spp., are suspected to be implicated in carcinogenesis (Lax and Thomas, 2002). The direct or indirect activation of RhoA by other bacterial toxins such as *E. coli* cytotoxic necrotizing factor (CNF), dermonecrotic toxin (DNT) of *Bordetella* spp. or *Pasteurella multocida* toxin (PMT) is suspected to confer a tumorigenic potential to these strains. PMT is a mitogenic protein mediating the activation of focal adhesion kinase (FAK) via RhoA transduction signalling, and an increased level of FAK activation promotes cell transformation, which is associated with many cancers (Lax and Thomas, 2002). If Cif is not found to be relevant in direct pathogenesis, its expression by persistent and chronically infecting bacteria promoting both Rho signalling and cell cycle perturbations could represent a more dangerous long-term risk for transformation and tumour development in infected tissues.

In conclusion, Cif is a new member of the expanding family of bacterial cyclomodulins that target the eukaryotic cell cycle. Certain cyclomodulins (such as CDT and now Cif) could have the ability to inhibit clonal proliferation of lymphocytes and could therefore represent a powerful strategy for immune evasion. These cyclomodulins could also impair intestinal barrier integrity and allow the entry of pathogenic bacteria into the body or could prolong the local existence of pathogenic bacteria by blocking the shedding of the epithelium. In contrast, other cyclomodulins (such as PMT) promote cellular proliferation and represent novel virulence mechanisms acting by interfering with the normal pathways of cell differentiation and development. The role of these cyclomodulins in bacterial virulence and also in carcinogenesis awaits further study and will delineate new perspectives in basic research and applications for the therapy of animal and human infections.

Experimental procedures

Bacterial strains, plasmids and cell lines

Bacterial strains and plasmids used in this study are listed in Table 1. Bacterial strains were cultured in Luria–Bertani (LB) broth or in Dulbecco's modified Eagle medium (DMEM) buffered with 25 mM Hepes (pH 7.4). None of these strains produce a cytolethal distending toxin (CDT) as tested by Southern blotting, PCR and lysate cytotoxic assays (data not shown). Antibiotics were used at the following final concentrations: carbenicillin 50 $\mu g\ ml^{-1}$, kanamycin 50 $\mu g\ ml^{-1}$ and chloramphenicol 25 $\mu g\ ml^{-1}$. Human epithelial HeLa cells (ATCC CCL-2) were cultivated in Eagle minimum essential medium (MEM) supplemented with 10% fetal calf serum (FCS), L-glutamine (200 mM) and gentamicin (80 $\mu g\ ml^{-1}$). Rabbit epithelial RK13 cells (ATCC CCL-37) were cultivated in DMEM with 10% FCS, L-glutamine (200 mM) and gentamicin (80 $\mu g\ ml^{-1}$). Both cell lines were incubated at 37°C in a 5% CO_2 atmosphere. Synchronization at the G_1/S border was performed on non-confluent HeLa cells by the double thymidine block method as described previously (Comayras *et al*., 1997).

Interaction between epithelial cells and bacteria

Twenty-four hours before the interaction, cells were trypsinized and seeded in 96-well tissue culture plates (Falcon) to give 7.5×10^3 cells per well in a 150 µl volume of MEM without antibiotic. In other instances, interaction assays were carried out in tissue culture Petri dishes (100 × 20 mm; Falcon) seeded with 8×10^5 cells, in a 16 ml volume of medium, or in eight-well Laboratory-Tek chamber slides (Nunc) seeded with 1.5×10^4 cells per well, in a volume of 300 µl. The day before the interaction, bacteria were cultivated statically overnight in LB except for Sakai and EDL-933 strains, which were cultivated in DMEM with 25 mM Hepes. Interactions were carried out in MEM buffered with 25 mM Hepes supplemented with 5% FCS and 1% mannose, with a starting inoculum of 10^2 bacteria per cell. After a 4 h interaction period, cells were washed with Earle's balanced saline solution and fixed for the FAS assay or were incubated further

Table 1. *E. coli* strains and plasmids used in this study.

Strains or plasmids	Genotype or description	Reference, company or colleague who gave the strain or plasmid
E22	Prototype rabbit EPEC of serotype O103:H2	Nougayrede *et al*. (1999)
E22 *espB*	E22 *espB::aphT*, EspB-	Nougayrede *et al*. (1999)
E22 *escN*	E22 *escN::aphT*, EscN-	This study
E22 *eae/tir*	E22 *eae::aphT tir::cat*, Eae-, Tir-	This study
E22*cif*	E22 *cif::km* Cif-	This study
1F12	E22 *cif::TZN*, Cif-	This study
20B8	E22 *cif::TZN*, Cif-	This study
4F6	E22 *cif::TZN*, Cif-	This study
E2348/69	Prototype human EPEC of serotype O127:H6	Jerse *et al*. (1990)
Sakai	Prototype human EHEC of serotype O157:H7	Hayashi *et al*. (2001)
EDL-933	Human EHEC of serotype O157:H7	Perna *et al*. (2001)
H19	Human EHEC of serotype O26:H11	Oswald *et al*. (2000)
11201	Human EPEC of serotype O125:H6	De Rycke *et al*. (1997)
PMK5	Human EHEC of serotype O103:H2	Oswald *et al*. (2000)
C102	Rabbit EPEC of serotype O26:H11	De Rycke *et al*. (1997)
RW1374	Bovine STEC of serotype O103:H2	Dr Lothar Wieler
1390	Porcine EPEC of serogroup O45	Dr Josée Harel
C/15333	Bovine EPEC of serogroup O26	Dr Hywell Ball
EF-33	Human EPEC of serogroup O111	Dr Stefano Morabito
EF-26	Human EPEC of serotype O125:H6	Dr Stefano Morabito
ED-31	Human EHEC of serogroup O111	Dr Stefano Morabito
MG1655	Prototype K-12	Blattner *et al*. (1997)
BL21CodonPlus(DE3)-RIL	*E. coli* host designed for high-level protein expression	Stratagene
XL1-Blue	*E. coli* host for cloning	Stratagene
Plasmids		
pCif	pBRSK vector expressing Cif	This study
pHa-Cif-His	pHB6 vector expressing a Cif tagged with HA at N-terminus and His_6 at C-terminus	This study
pCif-CyaA	pSIF003-RI vector expressing a Cif–CyaA fusion	This study
pHa-LacZ-His	pHB6 vector expressing LacZ tagged with HA at N-terminus and His_6 at C-terminus	This study
pFX1	pBK-CMV plus 3.9 kbp *Sau*3AI insert from E22 genomic DNA	This study
pFX13	pBK-CMV plus 3.8 kbp *Sau*3AI insert from E22 genomic DNA	This study
pHB6	Vector for HA N-terminal and His_6 C-terminal fusion	Roche
pGEM-T Easy	Cloning vector	Promega
pBluescript KS	High-copy cloning vector	Stratagene
pBK-CMV	Excised vector from λZap	Stratagene
pBRSK	Low-copy-number vector derived from pBR328 and used for complementation	Marchès *et al*. (2000)
pSIF003-RI	Vector for formation of *cyaA* translational fusions	Tu *et al*. (2003)
pUC4K	Supply kanamycin cassette	Pharmacia
pACYC184	Template for PCR amplification of chloramphenicol cassette	New England BioLabs
pKD46	Helper plasmid	Datsenko and Wanner (2000)
pKD4	Template for PCR amplification of kanamycin cassette for red recombinase-mediated recombination	Datsenko and Wanner (2000)
pKNG101	Suicide vector	Kaniga *et al*. (1991)

for the CPE assay at 37°C in a 5% CO_2 atmosphere in MEM with 10% FCS and 80 μg ml^{-1} gentamicin. Cellular alterations resulting from the interaction with bacteria were analysed after 1, 2 or 3 days.

Bacterial secretion profile

Overnight bacterial cultures were diluted 1:100 in DMEM buffered with 25 mM Hepes and incubated at 37°C in a 5% CO_2 atmosphere to an optical density of 0.5 at 600 nm. One hour after IPTG induction, samples were centrifuged (8000 *g* for 10 min), and supernatants were filtered (0.22 μm; Millipore) and concentrated with 10% (v/v) trichloroacetic acid (TCA) and a 1 h incubation at 4°C. Then, samples were centrifuged (8000 *g* for 20 min at 4°C), and the pellets were washed twice for 5 min at 4°C with cold acetone. The dried pellets were resuspended in Laemmli sample buffer and boiled before analysis by Western blotting.

Fractionation of HeLa cells infected with EPEC

Infected cells were washed three times with ice-cold PBS and scraped into 1 ml of buffer containing 10 mM imidazole (pH 7.4), 250 mM sucrose, 10 mM $MgCl_2$, 0.5 mM NaF, 1 mM Na_3VO_4 and complete protease inhibitor cocktail (Roche). The cells were then lysed by passages through a 22-gauge needle and centrifuged at 3000 *g* for 15 min to pellet bacteria, unbroken HeLa cells, host nuclei and the cytoskeleton (pellet fraction). The pellet fractions were resuspended in Laemmli sample buffer. The supernatants were treated by addition of

Triton X-100 (0.1% final concentration) to release membrane-associated proteins present in this fraction. After 5 min of incubation at 4°C, the supernatants were filtered (0.22 μm; Millipore) and precipitated with TCA. The pellets containing host cytoplasmic and internal membrane-associated proteins (Memb. + Cyto.) were resuspended in Laemmli sample buffer. The different fractions were then analysed by Western blotting.

Western immunoblot analysis

Protein samples were resolved by SDS-7% PAGE and blotted onto nitrocellulose membrane (Schleicher and Schuell). Primary and secondary antibodies were used at the following dilutions: mouse anti-HA 1:50 000 (Roche), rabbit anti-intimin 1:1000 (a gift from Dr Gadi Frankel), rabbit anti-cdc2 p34 1:100 (Santa Cruz Biotechnology), mouse anti-cyclin B1 1:100 (Santa Cruz Biotechnology), rabbit anti-phospho cdc2 1:1000 (Cell Signaling), goat anti-rabbit horseradish peroxidase (HRP) conjugated 1:2000 (Cell Signaling) and goat anti-mouse HRP conjugated 1:5000 (Sigma). Blots were developed with the Lumiglo chemiluminescence detection system (Cell Signaling).

Immunofluorescence microscopy

Cell monolayers were fixed with 3% formaldehyde and permeabilized for 5 min in PBS with 0.25% Triton. Monolayers were blocked with PBS supplemented with 3% bovine serum albumin (BSA) and incubated with mouse anti-vinculin antibody (clone Vin-11-5; Sigma) or rat anti-α-tubulin (clone YL1/2; Sera Laboratory) diluted in PBS with 3% BSA. Primary antibodies were detected with a secondary goat anti-mouse antibody or goat anti-rat antibody coupled to fluorescein isothiocyanate (FITC; Immunotech). Polymerized actin was stained with rhodamine-phalloidin (Molecular Probes). Samples were mounted with Vectashield (Vector) supplemented with DAPI to allow the labelling of DNA. Coverslips were observed with a Leica microscope.

Construction and screening of insertion transposon mutants library

E22 was mutagenized using the EZ::TN Kan-2 insertion kit (Epicentre). Transposition mutants were screened for their ability to induce the CPE on HeLa cells using 96-well tissue culture plates. Monolayers were stained with Giemsa and examined by light microscopy for mutants not able to induce large mononucleated cells. Genomic DNA of three selected mutants was digested with *Eco*RI and analysed by Southern blotting with a probe specific for the kanamycin resistance gene of the transposon. Fragments between 2 kb and 2.5 kb were cloned into pBluescript KS (Stratagene) and selected on agar plates with kanamycin. Insertion points were identified by sequencing with the primers provided with the EZ::TN kit (Epicentre).

Construction and screening of genomic DNA library

A genomic library was generated by partial digestion of E22 genomic DNA with *Sau*3AI and ligation of the resulting fragments the into the precut *Bam*HI λ Zap Express arms according to the manufacturer's instructions (Stratagene). Plates containing ≈40 000 pfu were hybridized with a probe obtained by PCR with cif-int-s and cif-int-as primers. Two positive phage clones were isolated and excised into the pBK-CMV phagemid. These plasmids, pFX1 and pFX13, were analysed by restriction digestion and sequenced.

Construction of pCif, pHa-Cif-His and pCif-CyaA plasmids

The *cif* gene was amplified using primers cif-rbs-s and cif-as and cloned into pGEM-T-Easy. The resulting plasmid was digested with *Not*I, and the fragment carrying *cif* was cloned into pBRSK giving plasmid pCif, which expresses Cif under the control of the *lac* promoter. To generate a HA and 6×His-tagged Cif protein, *cif* was amplified with primers Cif-HAs and Cif-HAas and cloned into pGEM-T-easy. The *Hin*dIII and *Eco*RI fragment containing *cif* was cloned into the expression vector pHB6. The resulting pHa-Cif-His plasmid allows the expression of a Cif protein fused to an N-terminal haemagglutinin (HA) and to a C-terminal six-histidine (His) epitope. For the Cif–Cya fusion, *cif* was amplified with primers cif-cya-sens and cif-cya-antisens. The PCR product was cloned into the *Bam*HI site of pSIF003-RI allowing the in frame fusion of cloned *cif* with the *cyaA* gene. The three resulting plasmids, pCif, pHa-Cif-His and pCya-Cif, were verified by sequencing.

Construction of E22 mutant strains for Eae/Tir, EscN and Cif

To generate the double *eae* and *tir* mutant of strain E22, the *cat* cassette obtained by PCR from pACYC184 was ligated into *Bam*HI-cut pKSTir2.1 (Marchès *et al*., 2000). The *Apa*I–*Spe*I fragment of the resulting plasmid carrying *tir* interrupted by *cat* was subcloned into the pKNG101 suicide plasmid and electroporated into E22*eae::aphT* (Nougayrede *et al*., 1999). Strain E22*eae::aphT tir::cat* was selected as described previously (Kaniga *et al*., 1991). The correct mutation of *tir* was checked by PCR and Southern blotting. To generate the *escN* mutant of strain E22, the flanking regions of *escN* were amplified by PCR with two primer pairs, escN-rbs-s and escN-Bam-as, escN-Bam-s and escN-C-term, containing the needed restriction sites (Table 2). The products were cloned into pGEM-T-easy and used to construct a suicide vector containing the two segments separated by the *kan* cassette taken from pUC4K. The fragments were cloned sequentially into the suicide vector pKNG101 and electroporated into E22. To generate a *cif* deletion mutant of strain E22, the *cif* gene was replaced by a gene encoding kanamycin resistance using the lambda red recombinase system (Datsenko and Wanner, 2000). The kanamycin resistance gene was amplified from pKD4 by PCR with primers cif-FRT-sens and cif-FRT-antisens. The product was treated with *Dpn*I and introduced into E22 carrying pKD46. Colonies containing the *cif::kan* knock-out were then obtained as described previously (Datsenko and Wanner, 2000).

Cell cycle analysis

The cell cycle distribution of HeLa or RK13 cells exposed to

Table 2. List of primers.

Name	Sequence (from 5′ to 3′)
cif-int-s	AACAGATGGCAACAGACTGG
cif-int-as	AGTCAATGCTTTATGCGTCAT
cif-rbs-s	CGTGAAGGAGTGAGATATGAAAGACATTACC
cif-as	CTGAATCATTTTACCGTATGG
cif-HAs	GAAGGAGTGAGATATGAAAGACATAAGCTTTCC
cif-HAas	CAAAAGCTAAGAATTCAGTGATTTTATTATCTC
cif-cya-sens	ATGGATCCTAAAGAGGAGAAATTAACTATGAAAGACATTACCCTTCCCC
cif-cya-antisens	CGGGATCCAACTACATAGTGATTTTATTATCTCAAT
escN-rbs-s	AGGAACGGTAAATGATTTTAGAGCA
escN-Bam-as	GGATCCAAGCGTGGATTGAGGTAAAAGC
escN-Bam-s	GGATCCTCCCGCGCTGGAAAGAATGAA
escN-C-term as	TAATCGCTGCTTTTGCTCATCA
cif-FRT-sens	CATAGAGTGAATGAATGAGATATGAAAGACATTACCCTTCCTGTGTAGGCTGGAGCTGCTTCG
cif-FRT-antisens	AAGTTCAGCCTAGGCTATGCTGAACCGGTTTTCCTGAATCACATATGAATATCCTCCTTAG
phage-s	TCGATACAACAGAGACAAATG
ybhB-sens	ATAATATTTCACCGCATCTGG
cat-1	CCCGGGATCCGTCGTAAGAGGTTC
cat-2	CCAATAGGATCCTTAAAAAAATTAC

EPEC or EHEC strains was determined by flow cytometric analysis. Cells were trypsinized, resuspended in phosphate-buffered saline (PBS) and fixed in 75% ethanol for 1 h at 4°C. They were then washed and resuspended in PBS containing propidium iodide (10 μg ml^{-1}; Sigma) and RNase A (250 μg ml^{-1}; Sigma). Flow cytometric analysis was performed with a FACScalibur flow cytometer (Becton Dickinson) using the red (PI) emission (630 nm) for DNA quantification. The data from 10^4 cells were collected and analysed using CELLQUEST software (Becton Dickinson).

Phosphorylation status of Cdk1 in HeLa cells exposed to EPEC or EHEC strains

HeLa cells (5×10^5 cells) were lysed as described previously (Comayras *et al.*, 1997) in a 50 mM Tris-HCl buffer (pH 7.4) containing 5 mM EDTA, 10 mM $MgCl_2$, 200 mM NaCl, 50 mM glycerophosphate, 25 mM NaF, 1 mM Na_3VO_4, 1 mM dithiothreitol (DTT), protease complete inhibitor cocktail (Roche) and 0.1% Triton X-100. Then, the lysates were incubated overnight at 4°C with $p13^{suc1}$ agarose beads allowing affinity purification of Cdk1 (cdc2) and associated proteins. Purified complexes were resolved by 12% SDS-PAGE and transferred to nitrocellulose membranes. The level of the inactive tyrosine-phosphorylated isoform of Cdk1 was revealed with rabbit anti-Cdk1-Tyr-15 antibodies (1:1000; Cell Signaling) followed by HRP-conjugated goat anti-rabbit antibodies (1:2000; Cell Signaling). The blots were then stripped, and the three isoforms of Cdk1 were revealed using rabbit anti-Cdk1 p34 antibodies followed by HRP-conjugated goat anti-rabbit antibodies.

Determination of Cif–CyaA translocation

We used the conversion assay to measure the conversion of intracellular [^{3}H]-ATP into [^{3}H]-cAMP in infected HeLa cells (Salomon, 1991). Briefly, HeLa cells (4×10^6 cells) were incubated with 5 ml of DMEM containing [^{3}H]-adenine (3 μCi ml^{-1}; Amersham). After 3 h incubation at 37°C in a 5% CO_2 atmosphere, the cells were washed with PBS and infected with the different EPEC strains. Half an hour later, IPTG was added, and the incubation was allowed to proceed for an additional hour. The infected HeLa cells were lysed in 1 ml of cold PCA lysis solution (2.5% perchloric acid, 0.1 mM cAMP). The amount of [^{3}H] cAMP formed and the total ^{3}H uptake were measured in duplicate for each assay as described previously (Tu *et al.*, 2003). These values were used to calculate the percentage conversion, which reflects the translocation efficiency.

Rabbit ligated intestinal loop assay

The rabbit ligated ileal loop assay was performed as described previously (Marchès *et al.*, 2000). Briefly, 1 ml of an overnight LB bacterial culture (10^9 cfu) was injected into ligated intestinal loops of 3-month-old New Zealand rabbits. On the following day, rabbits were euthanized with sodium pentobarbital. Samples were taken and prepared for transmission electron microscopy.

Nucleotide sequence accession number

The nucleotide sequences of several Cif loci have been sequenced and submitted to the GenBank database. The accession numbers are AF497476, AY128535, AY128536, AY128537, AY128538, AY128539, AY128540, AY128541, AY128542, AY128543, AY128544 and AF536197.

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