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Cycle Inhibiting Factors (CIFs) Are a Growing Family of Functional Cyclomodulins Present in Invertebrate and Mammal Bacterial Pathogens

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Abstract

The cycle inhibiting factor (Cif) produced by enteropathogenic and enterohemorrhagic Escherichia coli was the first cyclomodulin to be identified that is injected into host cells via the type III secretion machinery. Cif provokes cytopathic effects characterized by G2 and G3 cell cycle arrests, accumulation of the cyclin-dependent kinase inhibitors (CKIs) p21waf1/cip1 and p27kip1 and formation of actin stress fibres. The X-ray crystal structure of Cif revealed it to be a divergent member of a superfamily of enzymes including cysteine proteases and acetyltransferases that share a conserved catalytic triad. Here we report the discovery and characterization of four Cif homologs encoded by different pathogenic or symbiotic bacteria isolated from vertebrates or invertebrates. Cif homologs from the enterobacteria Yersinia pseudotuberculosis, Photorhabdus luminescens, Photorhabdus asymbiotica and the β-proteobacterium Burkholderia pseudomallei all induce cytopathic effects identical to those observed with Cif from pathogenic E. coli. Although these Cif homologs are remarkably divergent in primary sequence, the catalytic triad is strictly conserved and was shown to be crucial for cell cycle arrest, cytoskeleton reorganization and CKIs accumulation. These results reveal that Cif proteins form a growing family of cyclomodulins in bacteria that interact with very distinct hosts including insects, nematodes and humans.

Introduction

Pathogenic bacteria have developed sophisticated arsenals of virulence factors that hijack eukaryotic host functions to their own benefit. One of the pathways targeted by several bacterial effectors is the eukaryotic cell cycle. These toxins, termed cyclomodulins, can promote cell proliferation or, conversely, inhibit cell growth and modulate differentiation by blocking cell cycle progression [1,2]. The Cycle Inhibiting Factor (Cif) is a cyclomodulin injected into eukaryotic cells by the type III secretion system (T3SS) of enteropathogenic and enterohemorrhagic Escherichia coli (EPEC and EHEC). Cif from pathogenic E. coli triggers an irreversible cytopathic effect characterized by cell cycle arrests at the G2/M and G1/S phase transitions and, at least in HeLa cells, reorganization of the actin network [3–6]. In contrast to other cyclomodulins such as the cytolethal distending toxin [7] or colibactin [8], Cif is not a genotoxin nor an activator of DNA-damage checkpoint pathways that lead to phosphorylation of cyclin-dependent kinase 1 and consequent G2- arrest [5]. Both G1 and G2 arrests induced by Cif are correlated with the accumulation of the cyclin-dependant kinase inhibitors (CKIs) p21waf1/cip1 and p27kip1 (hereafter referred as p21 and p27), which actively participate in the control of cell cycle progression. These accumulations result from inhibition of their proteasome-mediated degradation [6].

Cif is composed of a C-terminal active domain (residues 21–282) and an exchangeable N-terminal translocation signal encoded by the first ~20 amino acids [9]. The crystal structure of a truncated form of EPEC Cif (lacking the first 99 amino acids) was recently determined. The presence of a conserved catalytic triad comprising Cys109, His165 and Gln185, revealed that Cif is a divergent member of a superfamily of enzymes that includes cysteine proteases, acetyltransferases and transglutaminases [10]. The three amino acids that comprise the triad are essential for Cif’s ability to induce cytopathic effects in eukaryotic cells as mutation of these residues leads to loss of function [10].

In EPEC and EHEC, Cif is not encoded within the locus of enterocyte effacement (LEE), which includes T3SS machinery genes and other effectors, but by a temperate lambdoid phage [11]. The cif gene has been widely disseminated by phage conversion within the natural population of E. coli, but positively selected within LEE-encoding strains [11]. Since Cif targets the...
cell cycle, a fundamental process conserved in all eukaryotic cells, it is reasonable to speculate that Cif homologs contribute to the pathogenicity of other bacterial species.

In the present study, four homologs of Cif have been identified and characterized in pathogenic or symbiotic bacteria: *Burkholderia pseudomallei*, *Yersinia pseudotuberculosis*, *Photorhabdus luminescens* and *Photorhabdus asymbiotica*. The four Cif homologs are functional and induce cell cycle arrest, p21 and p27 accumulation and actin cytoskeleton rearrangement in HeLa cells in an identical manner to EPEC Cif. The catalytic triad identified in the EPEC Cif crystal structure is strictly conserved in the homologs (at the sequence level) and is involved in their cytopathic activity since mutation of the critical cysteine residue leads to loss of function. Therefore, Cif proteins form a conserved family of cyclomodulins present in both symbionts and pathogens of vertebrate and invertebrate hosts.

**Results**

Genes encoding Cif-like proteins are present in the genomes of *Yersinia, Burkholderia* and *Photobacterium* species

The cyclomodulin Cif was initially identified and characterized in pathogenic *E. coli* (CifEc) [3]. Sequence database searches using BLAST [12] revealed that CifEc shares similarity with hypothetical proteins encoded by the genome of four other bacterial species (Table 1). CifEc exhibited a high degree of similarity with Ypk1971 (56% identity), a protein encoded by the human pathogen *Yersinia pseudotuberculosis* strain YPIII [13]. *Y. pseudotuberculosis* infection in humans causes gastroenteritis characterized by a self-limited mesenteric lymphadenitis that mimics appendicitis. CifEc was also similar to a protein encoded by the open reading frame bpss1385 from *B. pseudomallei* strain K96243 (26% identity). *B. pseudomallei* is the causative agent of melioidosis, an important cause of sepsis in east Asia and northern Australia [14]. Putative Cif homologs were also detected in two *Photobacterium* species: *P. luminescens*, a symbiotic bacterium for the soil nematode *Heterorhabditis* and a pathogen for a broad range of insects [15] and *P. asymbiotica*, an emerging human pathogen [16]. The proteins encoded by pha4011 (*P. luminescens*) and pha4011 (*P. asymbiotica*) share 23 and 26% of identity with CifEc, respectively. Interestingly, these four bacterial species in which cifEc-like genes were found all possess at least one T3SS. Proteins Ypk1971, Bpss1385, Pha2515 and Pha4011 are hereafter referred to as CifYp, CifBp, CifPl and CifPa, respectively. Finally, it should also be noted that a truncated putative protein (GOS5485515) obtained from the translation of a DNA fragment isolated from surface water marine samples [17,18] also shows sequence similarity to CifEc.

The degree of conservation and the phylogenetic relationship between Cif homologs were analysed by constructing a multiple sequence alignment and a phylogenetic tree using the Neighbour-Joining method (Fig. 1). CifPl and CifPa clustered together and were separated from a second group consisting of CifEc and CifYp. CifYp was the most divergent protein, located to a branch between the two groups. This phylogenetic tree matches the accepted bacterial taxonomy since *B. pseudomallei* belongs to the β-proteobacteria class whereas all others are enterobacteriaceae belonging to the γ-proteobacteria class.

**Genes encoding Cif-like proteins are found in highly rearranged DNA regions**

In *E. coli*, the cif gene is located on an inducible lambdoid prophage spread widely amongst EPEC and EHEC strains (Fig. 2)
In *Photorhabdus* strains, *cifPl* and *cifPa* are located downstream of a region displaying a high degree of similarity to a prophage described in *Serratia entomophila* (Fig. 2) [19]. This prophage is integrated 5 to 6 times in the genome of both *Photorhabdus* species [20] and encodes genes for several putative virulence factors, notably a putative T3SS effector protein homologous to *YopT* from *Yersinia*. This phage has no homology with the lambdoid prophage found in *E. coli* isolates but displays some similarity to bacteriocins and R-type pyocins [21]. In *B. pseudomallei* strain K96243 (Fig. 2), *cifBp* is located between two vestigial transposase genes on chromosome II near the *hop* cluster, which codes for one of the three T3SS present in *B. pseudomallei* [22]. Comparison of sequenced genomes from different *B. pseudomallei* strains reveals that the organization of this locus is highly variable. *B. pseudomallei* strains S13 and 9 contain additional genes, encoding putative transposases, which are inserted near *cifBp* (Fig. 2). In *B. pseudomallei* strain 1106a, this region is deleted and *cifBp* is absent. These data suggest that DNA transposition events could have lead to the heterogeneous distribution of the *cifBp*-like gene in *B. pseudomallei* strains. Among the sequenced strains of *Y. pseudotuberculosis*, only the strain YPIII possesses a gene with similarity to *cifBp*. Comparison of the genetic environment between YPIII and other *Y. pseudotuberculosis* strains revealed that *cifBp* is positioned within a chromosomal locus previously described as the insertion site of *ypm*, a gene coding for a superantigenic toxin in strain AH [23]. Both *ypm* and *cifBp* are located downstream of a 26-bp sequence called *yrs* which is homologous to *dif*, a site-specific recombination target used by filamentous bacteriophages for host chromosome integration. Deletions in the *yrs* locus occur at a higher frequency compared to others regions within the chromosome [23]. Genetic instability at this locus could explain the heterogeneous distribution of both *cifBp* and *ypm* genes in the *Y. pseudotuberculosis* population.

In conclusion, each *cifEc*-like gene is associated either with mobile genetic elements, such as phages, or is located in region of

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**Figure 2. Genetic organization of the *cif*-like genes loci from *E. coli* strain B171, *P. luminescens* strain TT01, *P. asymbiotica* strain ATCC43949, *B. pseudomallei* strains K96243, S13, 9 and 1106a and *Y. pseudotuberculosis* strains YPIII, AH and 9314/74.** Open reading frames (ORFs) are represented by horizontal arrows and designation of first and last ORF from each schematic are indicated. Vertical arrows indicate position of the *yrs* sequence (*Yersinia* recombination site). doi:10.1371/journal.pone.0004855.g002
proteins are functional homologs of CifEc, the E22 strain was previously described [3]. To test whether the Cif-like proteins derived from the marine metagenome is not available, this phenotype characterization of cells infected with EPEC producing CifBp, CifPl and CifPa were all readily overexpressed and purified in a soluble form (see Materials and Methods). However, despite many efforts, it was not possible to obtain a purified soluble form of CifYp, at levels necessary for activity assays using the BioPORTER delivery system. As previously reported [5], treatment of HeLa cells with BioPORTER mixed with purified CifEc leads to cell enlargement and formation of actin stress fibres (Fig. 5), identical to the phenotype observed with the infection model. However, as protein delivery with BioPORTER is not as efficient as bacterial infection [5], only ~50% of the treated cells exhibit morphological alterations (not shown). Studies of cell cycle patterns were therefore realized using G1/S synchronized cells to improve visualization of G2 arrest. In contrast to cells incubated with the lipid delivery agent mixed with PBS alone, cells treated with BioPORTER+CifEc accumulated in G2 phase (38% of Cif Ec-treated cells contained 4N DNA-content against 10% for PBS-treated cells). Lipofection of purified CifEc into HeLa cells led to actin stress fibres and cell accumulation in G2 phase (27%) (Fig. 5), confirming the functionality of CifEc observed with the infection assays. Introduction of purified CifPl or CifPa into HeLa cells with BioPORTER also led to cell enlargement, cytoskeleton alteration and accumulation of cells with 4N DNA content (40 and 25% for PBS- and BioPORTER-treated cells, respectively) versus 10% for PBS treated cells, see Fig. 5). Therefore, CifPl and CifPa are functional homologs of CifEc. As these phenotypes are observed with purified proteins, the results demonstrate that the proteins alone are sufficient to induce the Cif-associated cytopathic effects.

As it was not possible to introduce CifYp into cells using either the infection or BioPORTER treatments, the function of this protein to induce cytopathic phenotypes on HeLa cells was analysed using the infection model. In contrast to cells infected with E22Δcif carrying an empty vector, cells infected with E22Δcif producing CifBp developed cell distension and actin stress fibres indistinguishable from those induced by a CifEc-expressing strain (Fig. 4B). CifBp also blocked cell cycle progression, as demonstrated by the accumulation of G2 arrested cells containing 4N DNA content (Fig. 4B). These phenotypes were not induced when CifBp was expressed in an escN mutant (T3SS ATPase defective mutant), β-lactamase activity was no longer detected in infected cells, confirming that translocation of CifBp-TEM by E22 strain is T3SS-dependent (data not shown).

Since CifBp can be injected by the T3SS of E22, the capacity of the protein to induce cytopathic phenotypes on HeLa cells was analysed using the infection model. However, as protein delivery with BioPORTER is not as efficient as bacterial infection [5], only ~50% of the treated cells exhibit morphological alterations (not shown). Studies of cell cycle patterns were therefore realized using G1/S synchronized cells to improve visualization of G2 arrest. In contrast to cells incubated with the lipid delivery agent mixed with PBS alone, cells treated with BioPORTER+CifEc accumulated in G2 phase (38% of Cif Ec-treated cells contained 4N DNA-content against 10% for PBS-treated cells). Lipofection of purified CifEc into HeLa cells led to actin stress fibres and cell accumulation in G2 phase (27%) (Fig. 5), confirming the functionality of CifEc observed with the infection assays. Introduction of purified CifPl or CifPa into HeLa cells with BioPORTER also led to cell enlargement, cytoskeleton alteration and accumulation of cells with 4N DNA content (40 and 25% for CifPl and CifPa, respectively) versus 10% for PBS treated cells, see Fig. 5). Therefore, CifPl and CifPa are functional homologs of CifEc. As these phenotypes are observed with purified proteins, the results demonstrate that the proteins alone are sufficient to induce the Cif-associated cytopathic effects.

As expected, the CifEc-TEM fusion protein was properly translocated, as demonstrated by detection of intracellular β-lactamase activity (Fig. 4A). TEM activity was also detected in cells infected with E22Δcif producing CifEc-TEM, but levels of β-lactamase activity for CifEc-TEM, CifPl-TEM and CifPa-TEM were similar to the basal level detected with the negative control (TEM alone, Fig. 4A). Since TEM fusion proteins were produced to similar levels in the bacteria (Fig. 4A), absence of intracellular TEM activity likely results from inefficient recognition and/or injection of CifEc-TEM, CifPl-TEM and CifPa-TEM by the T3SS of EPEC. The lower translocation level of CifBp-TEM compared to CifEc-TEM probably also reflects a poor recognition of the secretion/translocation signal (STS) of CifBp, by the T3SS from EPEC. Indeed, when this fusion protein was expressed in an escN mutant (T3SS ATPase defective mutant), β-lactamase activity was no longer detected in infected cells, confirming that translocation of CifBp-TEM by E22 strain is T3SS-dependent (data not shown).

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CifBp, CifPl and CifPa are functional homologs of CifEc

As the EPEC T3SS was not able to translocate CifEc, CifEc and CifYp into infected cells, the cytopathic activity of these CifEc-like proteins was investigated using purified recombinant samples combined with a lipid mediated delivery system (BioPORTER) as previously described [5]. The effects of CifEc delivered with this system were also investigated. CifEc, CifPl and CifPa all showed the same activity and are therefore a suitable method to test the active function of Cif-associated cytopathic effects. An EPEC strain deleted for its chromosomal cifEc gene (E22Δcif) has previously been described [3]. To test whether the Cif-like proteins are functional homologs of CifEc, the E22Δcif strain was complemented with a plasmid encoding each of the cifEc-like genes, and these bacteria were used to infect cultured HeLa cells. Since the whole amino acid sequence of the putative protein derived from the marine metagenome is not available, this truncated protein was not included in these assays. Before phenotypic characterization of cells infected with EPEC producing the Cif homologs, the translocation efficiency of the proteins by the EPEC T3SS was monitored using the TEM/CCF2 assay [9].
Figure 3. The three residues of the ClfEc catalytic triad are conserved among members of the Cif protein family. (A) ClustalW alignment between ClfEc, ClfYp, ClfBp, ClfPl, ClfPa and GOS_548515. Fully conserved residues are indicated by a red background and amino acids conserved more than 60 or 80% are indicated by a yellow or an orange background respectively. The cysteine, histidine and glutamine residues that form the catalytic triad of ClfEc are indicated with blue stars.

(B) Position of the fully conserved residues in the three-dimensional structure of ClfEc. Side chain carbon atoms of residues comprising the catalytic triad are colored cyan. The remaining fully conserved residues cluster in three regions, as described in the text. Residues colored yellow, including glycine positions indicated by spheres, are P107, G110, A113, N159, L163-G164, S186-G189, G191, D200-W201; in green are D170, D172, E264-D266; in purple are K118-L119 and N273.

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Figure 4. Cif\textsubscript{Ec} is injected by the EPEC T3SS and induces cell cycle arrest and stress fibre formation in HeLa cells. (A) Translocation of Cif\textsubscript{Ec}-TEM, Cif\textsubscript{Bp}-TEM, Cif\textsubscript{Pl}-TEM, Cif\textsubscript{Pa}-TEM and Cif\textsubscript{Yp}-TEM fusions by the T3SS of EPEC strain E22. HeLa cells were loaded with CCF2/AM substrate and were infected for 2 and a half h with E22.<sub>Dcif</sub> hosting plasmids expressing TEM alone or the different Cif-TEM fusions. Upper panel: intracellular β-lactamase activity detected by measuring cleavage of the CCF2/AM, as described in Material and Methods. This ratio represents the relative translocation efficiency [9]. Experiments were performed in triplicate and error bars represent standard errors of the mean. Lower panel: synthesis of TEM fusions proteins were quantified in bacteria just before the translocation assays by western blot with anti-TEM antibodies. (B) G1/S synchronized HeLa cells were exposed for 90 min to E22.<sub>Dcif</sub> hosting either empty vector or the plasmids expressing Cif\textsubscript{Ec} or Cif\textsubscript{Bp}, washed and incubated with antibiotic for 20 or 72 h. Upper panels: F-actin was labelled with phalloidin-rhodamine (red) and DNA with DAPI (blue) 72 h post-infection. Bars represent 20 μm. Lower panels: cell cycle distribution was analysed by flow cytometry 20 h post-infection. 2N and 4N populations are indicated. doi:10.1371/journal.pone.0004855.g004
protein was analysed directly by expressing cifYp in HeLa cells. CifYp and CifEc, used as a positive control, were expressed as a translational fusion with the fluorescent reporter protein GFP, allowing quantification of GFP-Cif expression in transfected cells. GFP alone was also transfected as a negative control. Among the GFP positive population, 96% of cells expressing GFP-CifEc had a 2N DNA content whereas the 2N population of cells expressing GFP alone was only 82% (Fig. 6). Consistent with previous studies demonstrating that Cif could also induce G1/S arrest [6], this result demonstrates that the cell cycle of transfected cells expressing GFP-CifEc was blocked in G1 (2N DNA content). As expected, the cell cycle arrest was not observed when the critical cysteine residue from the catalytic triad of CifEc was substituted (Fig. 6). Expression of GFP-CifYp in HeLa cells also led to accumulation of GFP-positive cells with 2N DNA content (96% against 82% for cells expressing GFP alone), demonstrating that CifYp induced a cell cycle arrest in G1 phase similarly to CifEc (Fig. 6). This result indicates that Cif from Y. pseudotuberculosis is a functional homolog of CifEc.

The conserved catalytic triad is critical for the activity of Cif homologs

Most of the conserved residues in Cif proteins are clustered in discrete regions (Fig. 3). The cysteine, histidine and glutamine residues forming the catalytic triad in CifEc were shown to be critical for activity [10]. To determine whether an equivalent functional catalytic site exists in the Cif homologs, the conserved cysteines in CifBp, CifPl and CifPa (C90, C128 and C123 respectively) were substituted with a serine residue, and the corresponding proteins were purified prior to delivery into HeLa cells using the BioPORTER system. In contrast to the wild-type proteins, the cysteine variants did not induce cell enlargement and stress fibre formation (Fig. 7A). Further, analysis of DNA content revealed that accumulation of G2-arrested cells did not occur when cells were treated with the cysteine variants (Fig. 7B). Expression of the cysteine variant from CifYp by transfection in HeLa cells also revealed that the cell cycle was not arrested in contrast to cells producing the wild-type protein (Fig. 6). These results demonstrate that the conserved cysteine residue is critical for Cif activity. Also, as the histidine and glutamine residues that complete the triad are also conserved in the sequences of the Cif homologs, this suggests that catalytic triads also exist in CifBp, CifPl, CifPa and CifYp.

Cif homologs induce p21 and p27 accumulation in cells

It has recently been shown that the cytopathic activity of CifEc is correlated to the accumulation of CKIs p21 and p27, two important regulators of cell cycle progression [6]. Since all Cif homologs appear to share the same catalytic triad and induce identical cytopathic phenotypes in HeLa cells, we wonder if they could hijack the same signaling pathways, despite the fact that two of these proteins are produced by bacteria colonizing insects and nematodes. Western-blot analysis of HeLa cells treated with purified Cif homologs indicated that levels of p21 and p27 increase in the presence of wild-type CifBp, CifPl and CifPa (Fig. 8). An intact catalytic triad is integral to this accumulation as CKIs levels were not affected when cells were treated with the cysteine variants (Fig. 8). This accumulation of p21 and p27 suggests that the molecular mechanisms involved in Cif cytotoxicity on HeLa cells are identical for CifEc and the Cif homologs.

Discussion

CifEc proteins belong to a family of cyclomodulins that inhibit host cell proliferation by inducing G1/S and G2/M phase transition blocks [3,6]. In this study, functional homologs of Cif
from pathogenic E. coli have been identified in Y. pseudotuberculosis, B. pseudomallei, P. luminescens and P. asymbiotica. These homologs possess the same capacity as Cif Ec to induce cell cycle arrest, actin stress fibre formation and p21 and p27 CKIs accumulation when introduced into HeLa cells, suggesting they target the same substrates. Each of the Cif homologs possesses a predicted catalytic triad as identified in the crystal structure of Cif Ec. This triad is involved in the cytotoxic activity of each Cif homolog as substitution of the conserved cysteine residue in any of the proteins leads to inactivation.

In pathogenic E. coli, cif is located on an inducible lambda prophage that has spread widely within the natural population of E. coli [11]. Analysis of the genetic locus containing cif in other bacteria reveals that cif genes from Photorhabdus species are also located downstream of a prophage, while cif from B. pseudomallei and Y. pseudotuberculosis are inserted in highly rearranged DNA regions leading to heterogeneous distribution within bacterial populations. In addition, GC content of cif genes shows substantial deviation from the general pattern within their respective genome. In light of these data, cif genes are proposed to have been acquired by horizontal gene transfer and could be defined as xenologs according to the nomenclature proposed by Koonin et al. [25]. The phylogenetic relationship between the different xenologs is in agreement with the bacterial taxonomy since Cif from B. pseudomallei, the only β-proteobacteria, is the most divergent protein. This indicates that protein sequence variation is, to some extent, a consequence of speciation events and suggests that cif genes were probably acquired early during bacterial evolution. All Cif-producing bacteria encode at least one T3SS that could inject the effector into host cells during infection. It is interesting to speculate that tight association between horizontally acquired effectors and the T3SS machinery in bacteria is a consequence of selective pressure since advantages conferred by effector acquisition will occur only if the recipient organism produces the secretion/translocation machinery. Such an association has already been described in E. coli and Salmonella enterica serovar Typhimurium where phage-encoded T3SS effectors were associated with T3SS producing isolates [11,26].

Bacteria harboring the cif gene spend part of their life cycle in association with eukaryotic organisms. While E. coli, B. pseudomallei, Y. pseudotuberculosis and P. asymbiotica are mammalian pathogens [13,14,16,27], both Photorhabdus species are pathogenic for insects and symbiotic to nematodes [15,28,29]. Like the Cif proteins, other families of T3SS effectors are produced by bacterial pathogens that target distinct hosts. For example, a number of proteins belonging to the YopT cysteine protease family have been described in mammalian, insect and plant pathogens [30]. Although the overall sequence identity at the amino acid level is not extensive, every member of the YopT family shows several invariant residues including a cysteine, a histidine and an aspartate that form a putative catalytic triad. Representatives from the YopT-like family interfere with diverse host immune responses and display protease activity dependent on an intact catalytic triad. Representatives from the YopT-like family interfere with diverse host immune responses and display protease activity dependent on an intact catalytic triad. Representatives from the YopT-like family interfere with diverse host immune responses and display protease activity dependent on an intact catalytic triad. Representatives from the YopT-like family interfere with diverse host immune responses and display protease activity dependent on an intact catalytic triad.
structural homology to AvrPphB. Although the residues that form
the catalytic triad in each protein are different (C/H/D for
AvrPphB and C/H/Q for Cif), the overall folds and residues
comprising the catalytic triads superimpose well [10].

YopJ-like proteins form a second family of T3SS effectors
produced by different animal and plant pathogens that also possess
conserved residues forming a predicted catalytic triad, which is
required for protease activity [34]. YopJ, the archetypal member
of this family, is an essential virulence factor produced by
Yersinia which blocks MAPK and NFκB pathways resulting in inhibition of
host immune responses [35,36]. In contrast to members of the Cif
protein family, that induce similar phenotypes in HeLa cells,
proteins belonging to the YopT or the YopJ family appear to
generate different responses in eukaryotic cells. For example,
AvrA, a Salmonella YopJ-like T3SS effector (56% identity with
YopJ), does not induce the same host responses observed for YopJ
[37]. Further studies are required to determine whether the
conserved cytopathic effects induced by Cif proteins in HeLa cells,
notably cell cycle arrest, also occur in their respective host cells (gut
enterocytes for intestinal pathogens, insect cells for Photorhabdus
species, etc).

Interestingly, the plant symbiont Rhizobium sp. strain NGR234
produces NopJ, a YopJ-like protein and NopT, a T3SS effector
belonging to YopT-like family [38–40]. Both cysteine proteases
were shown to be involved in the host-specific nodulation response
of legumes [38,40]. Symbiotic bacteria deploy somewhat similar
strategies for colonizing host cells as those used by mammalian pathogens. The T3SS is, for example, required for host cell invasion by a variety of symbiotic bacteria [41–43]. As the Cif-producing Photorhabdus species are not only insect pathogens but also nematode symbionts, it is tempting to speculate that Cif may also contribute to the symbiotic process. Further, CdtB, the active monomer of the cyclomodulin CDT, is expressed in Hamiltonella defensa, a symbiont of pea aphids [44]. It maybe that symbionts use cyclomodulins like CDT and Cif to modulate, rather than globally deregulate, host signaling pathways resulting in initiation of symbiosis. Future studies will rely on further molecular (in vitro) analysis and in vivo models to achieve a full understanding of the roles of Cif in microbial pathogenesis, commensalism and symbiosis.

Materials and Methods

Cell line, bacterial strains and plasmids

HeLa cells (ATCC CCL-2) were cultured in Dulbecco’s modified Eagle medium (DMEM; Invitrogen) supplemented with 10% foetal calf serum (FCS; Eurobio) and 80 μg/mL gentamicin at 37°C in a 5% CO₂ atmosphere. For synchronization in G1/S phase, HeLa cells were treated with 2 mM thymidine (Sigma) for 18 h, washed three times with Hank’s balanced salt solution (HBSS; Invitrogen), incubated in normal medium for 9 h and treated again with 2 mM thymidine for 16 h. Bacterial strains and plasmids used in this study are listed in Table 2. Bacteria were cultured in Luria-Bertani (LB) broth or in interaction medium (DMEM with 25 mM Heps and 5% FCS). Antibiotics were used at the following concentrations: chloramphenicol 20 μg mL⁻¹ and kanamycin 25 μg mL⁻¹.

Construction of plasmids expressing Cif and Cif-Tem proteins

To construct plasmids suitable for expressing Cif homologs from B. pseudomallei, Y. pseudotuberculosis, P. luminescens and P. asymbiotica in EPEC, cifBP, cifPl, cifPa and cifEc genes were amplified from respective genomic DNA with primers adding a XhoI restriction site at the start codon and a BamHI (or XhoI for cifBP) restriction site after the stop codon. PCR products were digested and ligated into the corresponding sites of pBR1MCS-2 [46]. Plasmids encoding translational fusion between the different Cif proteins and the β-lactamase TEM-1 were obtained by cloning cif genes into the pKTEM vector. Briefly, cifBP, cifPl, cifPa, and cifEc genes were amplified from pEL1, pEL2, pEL3, pEL4 and pEL5 respectively using primers with XbaI-XhoI restriction sites (or XhoI-HindIII for pEL4), digested and cloned into the corresponding sites of pKTEM. The resulting plasmids pEL19, pEL20, pEL26, pEL21 and pEL03 encode Cif-TEM, CifBP-TEM, CifPa-TEM, CifPl-TEM and CifEc-TEM fusion proteins respectively. All the constructs were verified by DNA sequencing (Cogenics, France).

Purification of CifBP, CifPl, CifPa and CifEc proteins

For production of recombinant protein, the genes encoding cifBP, cifPl, cifPa and cifEc were cloned into the pET28a vector (Novagen). The resulting constructs encoded proteins with an N-terminal 6xHis tag. Plasmids were named pMB1, pPC1, pCC3 and pC008 respectively. The plasmid for expression of 6xHis-CifEc has been described elsewhere [5]. Mutations of the conserved cysteine residues were obtained by inverse PCR using pET28a based constructs as a template and oligonucleotides containing specific base changes. All the constructs were verified by DNA sequencing (Cogenics, France). After transformation into the E. coli BL21-CodonPlus®(DE3)-RIPL strain (Stratagene), bacteria were grown in LB to an OD₆₀₀,ₘ₉₉₉ of ~0.6 then induced with 0.5 mM IPTG for 3 h at 37°C. Purification of native proteins was achieved by Ni-NTA chromatography as recommended by the manufacturer (Qiagen) and, if necessary, gel filtration. Samples were then dialysed against PBS, aliquoted and stored at ~80°C.

Construction of plasmids expressing GFP-Cif fusion proteins and transfection assays

Plasmids encoding translational fusions between the fluorescent reporter protein GFP and CifBP, or CifPl, or CifPa, or CifEc, were obtained by cloning cif genes (encoding the wild-type or the cysteine variant forms) into the pTagGFP-C vector (Evrogen). The resulting plasmids were verified by DNA sequencing (Cogenics, France). After transformation into the E. coli BL21 (DE3)-RIPL strain (Stratagene), bacteria were grown in LB to an OD₆₀₀,ₘ₉₉₉ of ~0.6 then induced with 0.5 mM IPTG for 3 h at 37°C. Purification of native proteins was achieved by Ni-NTA chromatography as recommended by the manufacturer (Qiagen) and, if necessary, gel filtration. Samples were then dialysed against PBS, aliquoted and stored at ~80°C.

Infection, translocation and BioPORTER assays

For infection experiments, bacterial strains were cultured overnight in LB broth then diluted 1:100 in interaction medium for 3 h at 37°C in a 5% CO₂ atmosphere. HeLa cells were washed with HBSS and infected for the indicated time in interaction medium with a multiplicity of infection (MOI) of 100 bacteria per cell (except as otherwise noted). After the infection, cells were washed with HBSS then cultivated for the indicated times in DMEM medium supplemented with 10% FCS and 200 μg mL⁻¹ gentamicin.

Translocation levels of Cif-TEM fusion proteins were determined using CCF2/AM (Invitrogen) as a substrate for intracellular TEM enzyme as described previously [9]. Briefly, HeLa cells seeded in black 96-well plates were loaded for 1 h at 37°C with 1.7 μM CCF2/AM diluted in DMEM with 2 mM probenecid and then infected for

![Figure 8. Cif homologs induce p21 and p27 accumulation in cells.](image)

HeLa cells were treated with PBS or purified Cif proteins (wild-type (WT) or cysteine variants (C/S) as indicated), in combination with BioPORTER. Cell extracts were probed with anti-p21, anti-p27 and anti-actin antibodies 24 h post-treatment.

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2 and a half h with bacteria expressing TEM fusion proteins. Fluorescence was quantified in a microplate reader (TECAN Infinite M200) with excitation set at 410 nm (9 nm bandwidth) and emission at 450 nm for blue fluorescence and 520 nm for green fluorescence (20 nm bandwidth). Translocation was expressed as the emission ratio at 450/520 nm. To determine the expression level of TEM fusion proteins in bacteria, bacterial cultures with identical OD600 nm were pelleted, resuspended in SDS-PAGE sample buffer, boiled for 5 min and subjected to western blot analysis with anti-TEM-1 antibodies (QED Biosciences).

For BioPORTER assays, 80 ml of purified proteins (250 mg ml⁻¹) (or PBS as a negative control) were added to one BioPORTER tube (Genlanits) and resuspended in 420 or 920 ml of DMEM. The samples were added to the cells grown in BD Falcon culture slides or in 6-well plates and incubated for 4 h. BioPORTER mixes were replaced by fresh complete medium and the cells were incubated for 16–72 h.

Actin stress fibre and cell cycle analyses

For cell morphology and actin cytoskeleton visualization, cells were fixed for 15 min in PBS supplemented with 4% formaldedehyde, permeabilized with 0.1% Triton X-100 and stained with rhodamine-phalloidin (Molecular Probes) and DAPI (Sigma). Images were acquired with a DMIREB fluorescence microscope equipped with a DFC300FX digital camera (Leica). Cell cycle distribution analyses were performed as previously described [47]. Briefly, cells were grown on 6-well plates, synchronized in G1/S phase and infected or treated with BioPORTER. The cells were exposed to trypsin, washed, fixed with ethanol, stained with propidium iodide and analyzed by flow cytometry. Percentages of G2 populations were calculated using the Dean-Jett-Fox model from the FlowJo software (Tree Star).

Western Blot analyses

For Western blot analyses, 6 × 10⁵ cells were lysed in 80 ml of SDS-PAGE sample buffer, sonicated for 2 s to shear DNA and then boiled for 5 min. Protein samples were resolved on 4–12% NuPage gradient gels (Invitrogen) and blotted on PVDF membranes. Membranes were blocked in TBST (10 mM Tris pH 7.8, 150 mM NaCl, 0.1% Tween20) 5% non-fat dry milk, then probed with primary antibody (0.5 mg ml⁻¹) in TBST 5% non-fat dry milk. Primary antibodies were: anti-actin (ICN), anti-
p21 and anti-p21 (Santa Cruz Biotechnology). Bound antibodies were visualized with horseradish peroxidase-conjugated secondary antibody. Acquisitions were performed with a Molecular Imager ChemiDoc XRS system (Bio-Rad). Protein levels were quantified with Quantity One Software (Bio-Rad) and normalized with actin level.

Bioinformatic analyses

The search for proteins sharing similarity with CifE was performed using BLAST on the NCBI server and MaGe system on the Genoscope server for private access to the genome of P. aeruginosa (Sanger Institute). Genetic organization of the cif-like genes loci were determined using Artemis software from the Sanger Institute and MaGe system. Multiple alignments of Cif sequences were generated with ClustalW and edited using GeneDoc software. Based on this alignment, the unrooted phylogenetic tree was obtained using Phylip's Draw software.

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Author Contributions

Conceived and designed the experiments: GJ FT ASL RN JME EO. Performed the experiments: GJ CVC FT MJR ASL RN. Analyzed the data: GJ FT ASL RN JME EO. Contributed reagents/materials/analysis tools: GJ EO. Wrote the paper: GJ CVC FT MJR ASL RN RZ AG JME EO.

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