Protein expression, characterization, crystallization and preliminary X-ray crystallographic analysis of a Fic protein from *Clostridium difficile*

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Fic domains in proteins are found in abundance in nature from the simplest prokaryotes to animals. Interestingly, Fic domains found in two virulence factors of Gram-negative bacteria have recently been demonstrated to catalyse the transfer of the AMP moiety from ATP to small host GTPases. This post-translational modification has attracted considerable interest and a role for adenylation in pathology and physiology is emerging. This work was aimed at the structural characterization of a newly identified Fic protein of the Gram-positive bacterium *Clostridium difficile*. A constitutively active inhibitory helix mutant of *C. difficile* Fic was overexpressed in *Escherichia coli*, purified and crystallized by the vapour-diffusion technique. Preliminary X-ray crystallographic analysis shows that the crystals diffract to at least 1.68 Å resolution at a synchrotron X-ray source. The crystals belonged to the orthorhombic space group $P2_12_12_1$, with unit-cell parameters $a = 45.6$, $b = 80.8$, $c = 144.7$ Å, $\alpha = \beta = \gamma = 90^\circ$. Two molecules per asymmetric unit corresponds to a Matthews coefficient of $2.37 \text{ Å}^3 \text{ Da}^{-1}$ and a solvent content of 48%.

1. Introduction

Pathogens rely on virulence factors to facilitate infection and colonization in plants, animals and humans. Infections by toxigenic *Clostridium difficile* strains are the leading cause of nosocomial antibiotic-associated diarrhoea (AAD) in industrialized countries, and frequently affect people who have previously received antibiotic treatment (Hunt & Ballard, 2013; Miller *et al.*, 2011).

The most well characterized virulence factors in *C. difficile* are the large clostridial toxins (LCTs) TcdA and TcdB, which both inactivate the Rho GTPases in host cells by glycosylation of a threonine residue in the switch I region (Just, Selzer *et al.*, 1995; Just, Wilm *et al.*, 1995). Intoxication of cells by LCTs leads to disruption of the actin cytoskeleton, resulting in cell rounding, detachment and cell death. However, a study performed on clinical isolates from an American hospital revealed that 8.5% of the isolates did not contain the pathogenicity locus encoding TcdA and TcdB (Geric *et al.*, 2004). Therefore, it is possible that not all the virulence factors from this pathogen are known, and hence identifying and understanding the basic function of its proteins in mediating infection tropism and pathogenesis is an important future research goal.

Recent new findings have unravelled a new type of modification by bacterial Fic (filamentation induced by cAMP) proteins which also affects residues of the switch I region of Rho GTPases. The injected virulence factor VopS of *Vibrio parahaemolyticus* has been shown to catalyse the covalent attachment of AMP from ATP to threonine 37 of RhoA (Yarborough *et al.*, 2009). As for LCTs, this adenylation triggers an inactivation of the Rho proteins and the ensuing destruction of the actin cytoskeleton. The activity depends on a conserved histidine residue, which is placed in a highly conserved active-site motif present in all Fic sequences: HPF_x(D/E)GN(G/K)-R_xR. Moreover, the bacterial virulence factor IbpA of the respiratory pathogen *Histophilus somni* also disrupts the actin cytoskeleton (Worby *et al.*, 2009). IbpA bears two Fic domains, of which one domain has recently been shown to catalyse the adenylation of RhoA, Rac1 and Cdc42. Here, however, AMP is cross-linked to a conserved tyrosine of the switch I region. Structural

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Table 1

Macromolecule-production information.

Source organism	<i>C. difficile</i> R20291 (NC_013316)
DNA source	Genomic DNA
Forward primer	5'-GGGGCATATGAACAATTCCTTTTATAGATAAATG-3'
Reverse primer	5'-GGGGCTCGAGTTTCCATAACATTTTATATACTTC-3'
Cloning vector	pET-21b
Expression vector	pET-21b
Expression host	<i>E. coli</i> BL21
Complete amino-acid sequence of the construct produced	MNNSFLDKLIETKELKNSLYNVLKHNFYHANKIAGSTFTTE- ALALLLDKNVVTGRHTLDDVQETVNSSYVFDTVIDSLKEK- ITHNFRNLHSSLIFNTTLHSGRMAGIYKTIPTNMILGTDV- SIAQPFVEPEPKLDELIEWYYSQSEVSIKIVIAEFHYRFELI- HPFQDGNIGRFRVMLKQMLENNLPKIVSWSEDLRYRNS- LNSCSLGNVPLIEYLSLEDFREVYKMLWKLEHHHHHH

studies of Fic proteins from Gram-negative bacteria reveal a common core consisting of eight α -helices (Das *et al.*, 2009; Xiao *et al.*, 2010; Luong *et al.*, 2010). Furthermore, the activity of Fic proteins is shown to be controlled by an ATP-binding-site obstruction mechanism mediated by an inhibitory α -helix (Engel *et al.*, 2012).

However, much remains to be learnt about this newly discovered enzymatic reaction, which may be shared by eukaryotes. The primary sequences of the two IbpA Fic domains are homologous to the Fic domain of human HypE (huntingtin yeast-interacting protein E; Faber *et al.*, 1998; Kinch *et al.*, 2009). HypE has also been shown to adenylate RhoA, Rac and Cdc42 (Worby *et al.*, 2009). Nevertheless, the expression of HypE in cells does not trigger a collapse of the actin cytoskeleton, which might be owing to tight regulation of its activity in cells.

The bacterial Fic proteins characterized thus far originate from the group of Gram-negative bacteria. Recently, we discovered that the genomes of various virulent *C. difficile* strains contain at least one Fic sequence (CDR20291_0569) with the potential of functioning as a virulence factor similarly to VopS and IbpA. The activity and potential host cell target of this *C. difficile* Fic protein (CdFic) are still unknown.

Here, we report the cloning, expression, purification and crystallization of a presumed constitutively active inhibitory helix mutant of CdFic as well as initial crystallographic analysis of diffraction data collected to 1.68 Å resolution. Furthermore, to validate the nature and structural integrity of the purified protein, we chose to characterize the protein by mass spectrometry and circular-dichroism (CD) spectroscopy. The results provide a solid foundation for determining the crystal structure of CdFic and thus understanding both the principle of activation as well as the reaction mechanism.

2. Materials and methods

2.1. Cloning

The *cdr20291_0569* gene from the NCBI Gene Database was amplified from genomic DNA of *C. difficile* strain R20291 (accession No. YP_003217073) using the primers indicated in Table 1. The resulting PCR product was digested with *Nde*I and *Xho*I restriction nucleases and ligated into the pET-21b expression plasmid. Codons 31 (TCT) and 35 (GAG) encoding serine and glutamate, respectively, were substituted by alanine codons (GCT and GCG) using QuikChange site-directed mutagenesis according to the manufacturer's instructions (Agilent Technologies). This plasmid expresses C-terminally polyhistidine-tagged CdFic^{SE/AA} upon induction by isopropyl β -D-1-thiogalactopyranoside (IPTG).

Table 2

Crystallization.

Method	Sitting-drop vapour diffusion
Plate type	Cryschem plate (Hampton Research)
Temperature (°C)	21
Protein concentration (mg ml ⁻¹)	8–9
Buffer composition of protein solution	20 mM Tris pH 7.4, 5% (v/v) glycerol, 50 mM NaCl, 5 mM β -mercaptoethanol
Composition of reservoir solution	0.2 M MgCl ₂ ·6H ₂ O, 25% (w/v) PEG 3350, 0.1 M HEPES pH 7.5
Volume and ratio of drop	4 μ l, 3:1 (protein:reservoir)
Volume of reservoir (ml)	0.5

2.2. Macromolecule production and characterization

Both the wild-type CdFic and the mutant CdFic^{SE/AA} constructs were transformed into chemically competent *Escherichia coli* (BL21) cells by heat shock. The bacteria were grown in Luria broth medium containing 100 μ g ml⁻¹ ampicillin at 37°C in an incubator shaking at 210 rev min⁻¹ until the OD_{600 nm} reached 0.4. Subsequently, protein expression was induced with 1 mM IPTG for 5 h at 37°C. The cells were harvested by centrifugation at 6000 rev min⁻¹ for 15 min and then resuspended in lysis buffer (50 mM Tris pH 7.4, 300 mM NaCl, 0.1% Nonidet NP-40, 5 mM β -mercaptoethanol). The cells were lysed by sonication and the lysate was cleared by centrifugation at 15 000 rev min⁻¹ at 4°C for 40 min. The resulting supernatant was filtered with a 0.45 μ m syringe filter before application onto a pre-packed 1 ml HisTrap FF Crude column (GE Healthcare) charged with Ni²⁺ and washed with 20 column volumes of 50 mM Tris pH 7.4, 300 mM NaCl, 5 mM β -mercaptoethanol, 5% (v/v) glycerol, 20 mM imidazole. The protein was finally eluted with an imidazole gradient from 20 to 250 mM using the same buffer. As a final step before performing the circular-dichroism measurements, a monodisperse protein sample was obtained using a Superdex 200 HiLoad 16/600 size-exclusion column (GE Healthcare) running in 20 mM Tris-HCl pH 7.4, 5% (v/v) glycerol, 5 mM β -mercaptoethanol. For molecular-weight (MW) estimation, the protein was loaded onto a pre-calibrated size-exclusion Superdex 75 HR 10/30 column (GE Healthcare) and compared with the elution volumes of standard proteins (Gel Filtration Calibration LMW, GE Healthcare). The protein sample was concentrated to 8–9 mg ml⁻¹ and buffer-exchanged to storage buffer (Table 2) using a 20 ml 5K MWCO Vivaspinn concentrator (Sartorius VS2012) and stored at –20°C for later use.

2.3. Crystallization

Initial screening was carried out in 24-well Cryschem sitting-drop vapour-diffusion plates (Hampton Research) using the Index screen (Hampton Research; Table 2) by mixing 2 μ l protein solution with 2 μ l reservoir solution. Optimization of the crystallization conditions was also performed in Cryschem plates, varying the PEG type and content, the salt concentration and the drop composition. A drop composition of 3 μ l protein solution and 1 μ l reservoir solution yielded large crystals with high diffraction power. Drops were streak-seeded with a freshly prepared seed stock using a Seed Bead (Hampton Research). All chemicals for optimization were reagent grade from Sigma-Aldrich or Merck.

2.4. X-ray data collection and processing

Single crystals were transferred into a drop of reservoir solution containing 22% (v/v) glycerol using a mounted CryoLoop (Hampton Research) and were then flash-cooled in liquid nitrogen. Data were collected at –173°C on beamline ID23-2 (wavelength 0.99987 Å)

at the ESRF (Grenoble, France) and were processed with *XDS* (Kabsch, 2010) (Table 3). Matthews parameters were calculated using *MATTHEWS_COEF* as implemented in *CCP4* (Matthews, 1968; Winn *et al.*, 2011).

3. Results and discussion

The CdFic^{SE/AA} mutant was readily expressed and purified with yields of approximately 40 mg pure protein from 1 l expression culture.

Table 3

Data collection and processing.

Values in parentheses are for the outer shell.

Diffraction source	ID23-1, ESRF
Wavelength (Å)	0.99987
Temperature (°C)	−173
Detector	Pilatus 6M-F
Crystal-to-detector distance (mm)	313.8
Rotation range per image (°)	0.15
Total rotation range (°)	104.1
Exposure time per image (s)	0.037
Space group	<i>P2₁2₁2₁</i>
<i>a</i> , <i>b</i> , <i>c</i> (Å)	45.55, 80.80, 144.73
α , β , γ (°)	90.00, 90.00, 90.00
Mosaicity (°)	0.073
Resolution range (Å)	50.0–1.68 (1.73–1.68)
Total No. of reflections	235572 (19675)
No. of unique reflections	61488 (5071)
Completeness (%)	99.4 (99.6)
Multiplicity	3.81 (3.86)
$\langle I/\sigma(I) \rangle$	16.58 (2.07)
$R_{\text{meas}}^{\dagger}$ (%)	4.5 (64.0)
$CC_{1/2}^{\ddagger}$	99.8 (86.4)
Overall <i>B</i> factor from Wilson plot (Å ²)	32.6

$\dagger$ R_{meas} is the redundancy-independent *R* factor calculated on intensities (Diederichs & Karplus, 1997): $R_{\text{meas}} = \frac{\sum_{hkl} \{N(hkl)/[N(hkl) - 1]\}^{1/2} \sum_i |I_i(hkl) - \langle I(hkl) \rangle|}{\sum_{hkl} \sum_i I_i(hkl)}$. $\ddagger$ Percentage of correlation between intensities from random half-data sets (Karplus & Diederichs, 2012).

Figure 1

Purification and oligomeric state of CdFic^{SE/AA}. (a) Coomassie-stained 4–15% gradient SDS-PAGE of purified CdFic^{SE/AA}. Lane *M*, molecular-weight marker (labelled in kDa). Lane 1, elution from the HisTrap affinity column, 1.8 µg protein. Lane 2, as lane 1 but with 5.4 µg protein. In addition to the dense band corresponding to CdFic^{SE/AA} next to the 25 kDa band of the marker, there are two bands (bands 1 and 2) just above the 10 kDa band of the marker. These bands correspond to a degradation product of CdFic^{SE/AA} as determined by mass-spectrometric analysis. (b) Size-exclusion chromatography analysis of the oligomeric state of isolated CdFic^{SE/AA} using a standard Superdex 75 HR 10/30 column. The arrows at the top indicate the elution volumes of proteins of known mass applied to the same column (indicated in kDa; V_0 , void volume). The elution volume of CdFic^{SE/AA} (9.95 ml) suggests that the molecule is present as a dimer in solution. Comparison with elution volumes of standard proteins suggests a molecular weight of ~57.5 kDa, while the theoretical molecular weight of the dimer is 56.2 kDa.

Figure 2

Crystals of CdFic^{SE/AA}. (a) Crystals grown by streak-seeding with a 1:1 drop ratio of protein:reservoir solution. Crystals of similar size diffracted synchrotron X-ray radiation to 3.1 Å resolution (data not shown). (b) Optimization of the drop ratio to 3:1 protein:reservoir solution resulted in significantly larger crystals growing up to 1 mm in size. These larger crystals showed significantly improved diffraction and a 1.68 Å resolution data set was collected as shown in Fig. 3 and Table 3.

Almost pure CdFic^{SE/AA} was obtained by His-tag immobilized metal-ion affinity chromatography (Fig. 1a). The protein eluted at approximately 9.95 ml from a standard Superdex 75 HR 10/30 column as a monodisperse protein sample corresponding to a molecular weight of about 57.5 kDa (Fig. 1b). This suggests that the free protein (theoretical molecular weight 28.1 kDa) exists as a dimer in solution. Two contaminants of approximately 12–13 kDa proved very difficult to remove (Fig. 1a). In an attempt to remove these bands we also tested binding to a 1 ml HiTrap Q HP anion-exchange column. However, although CdFic^{SE/AA} binds efficiently to the column in a buffer consisting of 50 mM Tris-HCl pH 7.4, 5 mM NaCl, 5 mM β-mercaptoethanol the contaminating band was still present even after extensive washing and a gradient elution (results not shown). Mass spectroscopy indicates that the contaminants originate from degradation of CdFic^{SE/AA} (Supplementary Fig. S1¹). In order to corroborate the sequence-based assumption that CdFic^{SE/AA} adopts a Fic fold and that the fold of the mutant is comparable to wild-type CdFic, we performed CD spectroscopy (Supporting Information §S2, Supplementary Fig. S3 and Supplementary Table S1). Modelling of secondary-structure fractions obtained from the CD spectra showed that both wild-type CdFic and CdFic^{SE/AA} consist on average of 37%

¹ Supporting information has been deposited in the IUCr electronic archive (Reference: PG5042).

α -helix, 19–20% β -strand, 16–17% turn and 24–25% unordered structure. This is consistent with the predominantly helical nature of Fic proteins in the PDB (for example, PDB entries 3let, P. H. Luong, L. N. Kinch, C. A. Brautigam, N. V. Grishin, D. R. Tomchick & K. Orth, unpublished work; 3n3u, Xiao *et al.*, 2010; 2g03, Midwest Center for Structural Genomics, unpublished work; 3eqx, Das *et al.*, 2009; 2f6s, Midwest Center for Structural Genomics, unpublished work; and 3cuc, Joint Center for Structural Genomics, unpublished work), although they have on average as much as 58% α -helix and only 3% β -sheet.

Initial crystallization screening yielded a crystallization hit using Index condition No. 84 (Hampton Research) consisting of 0.2 M $\text{MgCl}_2 \cdot 6\text{H}_2\text{O}$, 0.1 M HEPES pH 7.5, 25% (w/v) PEG 3350. Numerous small crystals of $\text{CdFic}^{\text{SE/AA}}$ appeared within two weeks and continued to grow slightly larger during the following week. Upon streak-seeding new drops in similar conditions using crystals from the initial hit, new similarly sized crystals appeared within 24 h (Fig. 2a). The nature of the crystals was subsequently confirmed by SDS-PAGE, showing a protein band of the correct molecular weight, and initial data were collected to 3.1 Å resolution (data not shown) on beamline ID23-2 at the ESRF, Grenoble (Gabadinho *et al.*, 2010). Significantly larger sized crystals were subsequently obtained by setting up 4 μl drops with a 1:3 reservoir:protein solution ratio (Fig. 2b) which appeared 24 h after seeding and grew to maximum size within a week (0.8 $\times$ 0.5 $\times$ 0.1 mm). The flash-cooled $\text{CdFic}^{\text{SE/AA}}$ crystals diffracted to at least 1.68 Å resolution on ESRF beamline

ID23-1 (Fig. 3) and a full data set was collected and processed by XDS (Kabsch, 2010). A summary of the data-processing statistics is shown in Table 3. The diffraction images showed visible spots to approximately 2 Å resolution and hence the crystal-to-detector distance was set to a maximum resolution of 1.68 Å at the edge. However, the high $\text{CC}_{1/2}$ value in the outer resolution shell from the XDS processing suggests that valuable diffraction extends beyond this resolution. The crystals belonged to the orthorhombic space group $P2_12_12_1$, with unit-cell parameters $a = 45.6$, $b = 80.8$, $c = 144.7$ Å, $\alpha = \beta = \gamma = 90^\circ$. *phenix.xtriage* analysis (Zwart *et al.*, 2005) indicated no pathologies. The Matthews coefficient was estimated using CCP4 (Winn *et al.*, 2011) as $2.37 \text{ Å}^3 \text{ Da}^{-1}$, with two molecules in the asymmetric unit and a solvent content of 48% (Matthews, 1968). Together with the gel-filtration elution profile, this could suggest that a biological $\text{CdFic}^{\text{SE/AA}}$ dimer could form the crystallographic asymmetric unit.

In summary, the cloning, purification, crystallization and preliminary crystallographic analysis of the *C. difficile* $\text{Fic}^{\text{SE/AA}}$ mutant reported here form the foundations for determining the structure of Fic in its active conformation. The structure will provide a better understanding of the catalytic mechanism of Fic as well as the exact function of the inhibitory helix. Furthermore, the structure of wild-type CdFic as well as co-crystallization with ATP and the protein target substrate will further elucidate the mechanism behind this class of enzymes.

4. Related literature

The following references are cited in the Supporting Information for this article: Compton & Johnson (1986), Gobom *et al.* (1999), Jensen & Wilm (2002), Manavalan & Johnson (1987), Perkins *et al.* (1999), Shevchenko *et al.* (2006), Sreerama & Woody (2000) and Whitmore & Wallace (2008).

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References

- Compton, L. A. & Johnson, W. C. Jr (1986). *Anal. Biochem.* **155**, 155–167.
- Das, D. *et al.* (2009). *Proteins*, **75**, 264–271.
- Diederichs, K. & Karplus, P. A. (1997). *Nature Struct. Biol.* **4**, 269–275.
- Engel, P., Goepfert, A., Stanger, F. V., Harms, A., Schmidt, A., Schirmer, T. & Dehio, C. (2012). *Nature (London)*, **482**, 107–110.
- Faber, P. W., Barnes, G. T., Srinidhi, J., Chen, J., Gusella, J. F. & MacDonald, M. E. (1998). *Hum. Mol. Genet.* **7**, 1463–1474.
- Gabadinho, J. *et al.* (2010). *J. Synchrotron Rad.* **17**, 700–707.
- Geric, B., Rupnik, M., Gerding, D. N., Grabnar, M. & Johnson, S. (2004). *J. Med. Microbiol.* **53**, 887–894.
- Gobom, J., Nordhoff, E., Mirgorodskaya, E., Ekman, R. & Roepstorff, P. (1999). *J. Mass Spectrom.* **34**, 105–116.
- Hunt, J. J. & Ballard, J. D. (2013). *Microbiol. Mol. Biol. Rev.* **77**, 567–581.
- Jensen, O. N. & Wilm, M. (2002). *The Protein Protocols Handbook*, edited by J. Walker, pp. 693–710. Totowa: Humana Press.
- Just, I., Selzer, J., Wilm, M., von Eichel-Streiber, C., Mann, M. & Aktories, K. (1995). *Nature (London)*, **375**, 500–503.
- Just, I., Wilm, M., Selzer, J., Rex, G., von Eichel-Streiber, C., Mann, M. & Aktories, K. (1995). *J. Biol. Chem.* **270**, 13932–13936.
- Kabsch, W. (2010). *Acta Cryst.* **D66**, 125–132.
- Karplus, P. A. & Diederichs, K. (2012). *Science*, **336**, 1030–1033.
- Kinch, L. N., Yarbrough, M. L., Orth, K. & Grishin, N. V. (2009). *PLoS One*, **4**, e5818.

Figure 3
Synchrotron X-ray diffraction pattern collected from a single cryocooled $\text{CdFic}^{\text{SE/AA}}$ mutant crystal corresponding to that shown in Fig. 2(b). Clear diffraction was visible to approximately 2 Å resolution. The inset shows a close-up view of the reflections.

- Luong, P., Kinch, L. N., Brautigam, C. A., Grishin, N. V., Tomchick, D. R. & Orth, K. (2010). *J. Biol. Chem.* **285**, 20155–20163.
- Manavalan, P. & Johnson, W. C. Jr (1987). *Anal. Biochem.* **167**, 76–85.
- Matthews, B. W. (1968). *J. Mol. Biol.* **33**, 491–497.
- Miller, B. A., Chen, L. F., Sexton, D. J. & Anderson, D. J. (2011). *Infect. Control Hosp. Epidemiol.* **32**, 387–390.
- Perkins, D. N., Pappin, D. J., Creasy, D. M. & Cottrell, J. S. (1999). *Electrophoresis*, **20**, 3551–3567.
- Shevchenko, A., Tomas, H., Havlis, J., Olsen, J. V. & Mann, M. (2006). *Nature Protoc.* **1**, 2856–2860.
- Sreerama, N. & Woody, R. W. (2000). *Anal. Biochem.* **287**, 252–260.
- Whitmore, L. & Wallace, B. A. (2008). *Biopolymers*, **89**, 392–400.
- Winn, M. D. *et al.* (2011). *Acta Cryst.* **D67**, 235–242.
- Worby, C. A., Mattoo, S., Kruger, R. P., Corbeil, L. B., Koller, A., Mendez, J. C., Zekarias, B., Lazar, C. & Dixon, J. E. (2009). *Mol. Cell*, **34**, 93–103.
- Xiao, J., Worby, C. A., Mattoo, S., Sankaran, B. & Dixon, J. E. (2010). *Nature Struct. Mol. Biol.* **17**, 1004–1010.
- Yarbrough, M. L., Li, Y., Kinch, L. N., Grishin, N. V., Ball, H. L. & Orth, K. (2009). *Science*, **323**, 269–272.
- Zwart, P. H., Grosse-Kunstleve, R. W. & Adams, P. D. (2005). *CCP4 Newsl. Protein Crystallogr.* **43**, contribution 7.