Expression, purification, crystallization and crystallographic analysis of the N-terminal domain of translocated intimin receptor

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Translocated intimin receptor (Tir) is an *Escherichia coli*-encoded protein that is transported into the host cell through a sophisticated bacterial type III secretion system (T3SS). Tir anchors the infected cell membrane twice using both its N- and C-termini from inside the host cytoplasm for signalling. It plays a key role in enterohemorrhagic *Escherichia coli* (EHEC) infection, attaching and effacing (A/E) lesions and intracellular signal transduction. Here, the overexpression, purification and crystallization of its N-terminal intracellular domain are reported. The crystal belonged to the orthorhombic space group $I4_122$, with unit-cell parameters $a = b = 59.79$, $c = 183.11$ Å. The asymmetric unit contained one molecule, with a solvent content of 51% and a V_M of 2.55 Å³ Da⁻¹.

1. Introduction

Enterohaemorrhagic *Escherichia coli* (EHEC) is a subset of pathogenic *E. coli* that can cause a wide spectrum of diseases, including diarrhoea and haemorrhagic colitis, in humans. Haemorrhagic colitis occasionally progresses to haemolytic uraemic syndrome (HUS), a disease characterized by acute and chronic renal failure and haemolytic anaemia, as well as thrombocytopenia (Tilden *et al.*, 1996; Karch *et al.*, 2005). In the elderly, the case fatality rate for HUS can be as high as 50%. *E. coli* O157:H7 (EHEC O157:H7) has been recognized as a cause of this syndrome (Gasser *et al.*, 1955; Karch *et al.*, 2005). The infectious dose is very low (100 colony-forming units), and humans can easily acquire EHEC O157:H7 by direct contact with animal carriers or *via* the ingestion of undercooked ground beef or other animal products or of contaminated vegetables and fruit. The impact of EHEC infection on public health is therefore quite large, considering the ease with which the infection is transmitted and the severity of the disease caused.

EHEC causes diseases in humans and animals mainly by intimate attachment of bacteria to host cells. The adhesion of pathogenic *E. coli* to the host-cell surface is mediated through the interaction of intimin on the pathogen surface with its translocated receptor, translocated intimin receptor (Tir), on the host cells (DeVinney *et al.*, 1999). Tir is a bacterial protein which is injected into the host cell through the type III secretion system (T3SS; Tobe *et al.*, 2006), acting as a cellular receptor for intimin and anchoring infected cell membrane

Table 1

Macromolecule-production information.

Source organism	Enterohaemorrhagic <i>E. coli</i> (EHEC)
DNA source	EHEC O157:H7
Forward primer	5'-ATCGCATATGCGTGCCTATTT-3'
Reverse primer	5'-ATCGCTCGAGCTCCAGTATCCT-3'
Cloning vector	pET-21a (Novagen)
Expression vector	pET-21a (Novagen)
Expression host	<i>E. coli</i>
Complete amino-acid sequence of the construct produced	MALFTPVNRNSMADSGDNRASDVPLPVPNPMRLAA-SEITLNDGFEVLHDHGPLDLNRQIGSSVFRV-ETQEDGKHIAGVQRNGVETSVVLSQDEYARLQ-SIDPEGKDKFVFTGGRGGAGHAMVTVASDITE-ARQRILEHHHHH

twice with both its N- and C-termini inside the host cytoplasm for signalling (Kenny *et al.*, 1997; Hartland *et al.*, 1999).

The interaction between intimin and Tir is essential for the attachment of EHEC to the host cell, as well as subsequent infection, and the structure of the complex between intimin and the extracellular domain of Tir has been determined (Fairman *et al.*, 2012). The intracellular N- and C-terminal domains of Tir have been shown to play an important role in A/E lesion formation (Kenny *et al.*, 1996) and to be involved in actin binding and intracellular signal transduction for initiating EHEC infection, respectively.

Despite the important physiological functions of the intracellular N-terminal and C-terminal domains of Tir, no structural information on either the N-terminal or the C-terminal domains of Tir has been reported to date. Here, we report the cloning, expression, purification and crystallization of the N-terminal domain of Tir (Tir-N) as well as preliminary crystallographic analysis of X-ray diffraction data collected to 2.6 Å resolution. We believe that comprehensive structural studies of Tir-N should provide insight into its specific functions and the mechanism of infection of host cells by EHEC.

2. Materials and methods

2.1. Macromolecule production

The gene encoding the intracellular N-terminal domain of Tir (Tir-N; residues 46–181) was PCR-amplified using the chromosomal DNA of EHEC H7:O157 as the template. The PCR product was cloned into the pET-21a vector (Novagen) using NdeI/XhoI restriction sites, resulting in the overexpressed protein containing an N-terminal 6×His tag. The sequence of the insert gene was verified by DNA sequencing.

The plasmid containing the Tir-N gene was transformed into *E. coli* BL21 (DE3) cells (Novagen). The cells were grown at 37°C after inoculation from an overnight culture until they reached an optical density (OD₆₀₀) of 0.7 in LB medium supplemented with 25 mg ml⁻¹ kanamycin. The protein was overexpressed after adding 1 mM isopropyl β-D-1-thiogalactopyranoside (IPTG) to the culture at 20°C. 16 h after induction with IPTG, the cells were harvested by centrifugation at 3000g (30 min, 4°C) and disrupted by sonication in lysis buffer (10 mM Tris-HCl, 25 mM NaCl, 5% glycerol pH 8.0) on ice. The crude lysate was centrifuged at 12 000g for 30 min

at 4°C. The supernatant containing the soluble protein was loaded onto a nickel-nitrilotriacetic acid (Ni-NTA) column (Qiagen) and was washed with five column volumes of wash buffer (10 mM Tris-HCl, 25 mM NaCl, 5% glycerol, 50 mM imidazole pH 8.0). The protein was eluted with elution buffer (10 mM Tris-HCl, 25 mM NaCl, 5% glycerol, 100 mM imidazole pH 8.0). The eluted protein was buffer-exchanged into 10 mM Tris-HCl, 25 mM NaCl, 5% glycerol pH 8.0 by dialysis and treated with bovine thrombin (Invitrogen) to remove the 6×His tag (16 h, 4°C). Subsequently, the protein was applied onto an Ni-NTA column and the nonbinding fractions were concentrated for gel-filtration chromatography using a Superdex 200 HR 26/60 column (GE Healthcare, USA) to remove the noncleaved form. The column had previously been equilibrated with gel-filtration buffer (20 mM Tris-HCl, 10 mM Na₂HPO₄, 300 mM NaCl, 5 mM DTT pH 8.0). The eluted fractions were concentrated to 10, 15 and 20 mg ml⁻¹.

Figure 1
Mass-spectrometric (a) and SDS-PAGE (b) analysis of Tir-N. The purified and monomeric fraction of Tir-N from the size-exclusion column was used for crystallization trials. Lane M, molecular-mass marker (labelled in kDa).

Table 2
Crystallization.

Method	Sitting-drop vapour diffusion
Plate type	24-well VDX plates
Temperature (K)	293
Protein concentration (mg ml ⁻¹)	10
Buffer composition of protein solution	20 mM Tris-HCl, 10 mM Na ₂ HPO ₄ , 300 mM NaCl, 5 mM DTT pH 8.0
Composition of reservoir solution	2.0 M NaCl, 0.1 M sodium acetate pH 4.5
Volume and ratio of drop	1 µl, 1:1
Volume of reservoir (µl)	400

1 µl pure protein (10 mg ml⁻¹) was added to 9 µl pure H₂O and investigated by matrix-assisted laser desorption/ionization-time-of-flight (MALDI-TOF) analysis (ultrafleXtreme, Bruker, Vienna, Austria). The purity of the protein was checked by 15% SDS-PAGE and the protein homogeneity was determined to be >95% (Fig. 1). The recombinant protein contains additional amino-acid residues at the N-terminus originating from the plasmid, giving a total of 145 residues, and the final yield of the purified protein was approximately 3 mg per litre of cell culture. Protein-production information is summarized in Table 1.

2.2. Crystallization

In the initial crystallization trials, we screened conditions for obtaining protein crystals using commercial screening kits and the sitting-drop vapour-diffusion method with a 1:1 ratio of protein and reservoir solution in 24-well VDX plates (Hampton Research, USA) at 20°C (Jancarik & Kim, 1991; Cudney *et al.*, 1994). Each hanging drop was equilibrated over 400 µl reservoir solution. Initial microcrystals were obtained after 7 d from three conditions: (i) 3.5 M NH₄Cl, 0.1 M sodium acetate pH 4.6, (ii) 2.2 M NaCl, 0.1 M sodium acetate pH 4.6, and (iii) 4.0 M NaNO₃, 0.1 M sodium acetate pH 4.6. The crystallization conditions were further optimized to improve the crystal quality. Crystals of maximum size were obtained using a solution consisting of 2.0 M NaCl, 0.1 M sodium acetate pH 4.5 (Fig. 2 and Table 2).

Figure 2
Crystals of Tir-N obtained by the hanging-drop method at 293 K in 2.0 M NaCl, 0.1 M sodium acetate pH 4.5. The scale bar is 0.1 mm in length.

Table 3
Data collection and processing.

Values in parentheses are for the outer shell.

Diffraction source	BL17A, KEK Photon Factory
Wavelength (Å)	0.9790
Temperature (K)	100
Detector	ADSC Quantum 210
Crystal-to-detector distance (mm)	327
Rotation range per image (°)	1
Total rotation range (°)	360
Exposure time per image (s)	1
Space group	<i>I</i> ₄ 22
Unit-cell parameters (Å, °)	<i>a</i> = 59.79, <i>b</i> = 59.79, <i>c</i> = 183.11, $\alpha = \beta = \gamma = 90$
Mosaicity (°)	0.4
Resolution range (Å)	56.84–2.60 (2.74–2.60)
Total No. of reflections	151674
No. of unique reflections	5490
Completeness (%)	100 (100)
Multiplicity	27.6 (28.7)
$\langle I/\sigma(I) \rangle$	24.6 (4.8)
<i>R</i> _{r.i.m.}	0.09 (0.764)
Overall <i>B</i> factor from Wilson plot (Å ²)	61.4

2.3. Data collection and processing

The crystals were flash-cooled in liquid nitrogen after soaking in cryoprotectant (20% glycerol added to the crystallization buffer).

X-ray diffraction data were collected at 100 K on beamline BL17A at the KEK Photon Factory, Japan. A total rotation range of 360° was covered using 1.0° oscillation and 1 s exposure per frame. The wavelength of the synchrotron X-ray beam was 0.9790 Å and the crystal-to-detector distance was set to 327 mm (Garman & Schneider, 1997). X-ray diffraction data were collected to 2.6 Å resolution (Fig. 3). Data were integrated, scaled and merged using *MOSFLM* and *SCALA*.

Figure 3
X-ray diffraction image of Tir-N.

from the CCP4 software package (Winn *et al.*, 2011). Data-collection and processing statistics are summarized in Table 3.

3. Results and discussion

The N-terminal domain of Tir (Tir-N) plays an important role in A/E lesion formation because it is involved in the mechanism of the adhesion of pathogenic *E. coli* to the host-cell surface. Tir-N was cloned, expressed, purified and crystallized. The purified protein was examined using SDS-PAGE (Fig. 1*b*) and mass spectrometry (Fig. 1*a*), indicating that the molecular weight of Tir-N was 15.8 kDa, as predicted by analyzing its sequence, and that the purity of the protein was greater than 95%. The protein was crystallized in three conditions as described above. We further improved the crystal quality and obtained crystals of maximum size using a solution consisting of 0.1 M sodium acetate, 2.0 M NaCl pH 4.5 (Fig. 2). The crystal diffracted to 2.6 Å resolution (Fig. 3). Systematic extinctions clearly revealed that the crystal belonged to space group $I4_122$, with unit-cell parameters $a = 59.79$, $b = 59.79$, $c = 183.11$ Å. Solvent-content analysis indicated that there was one molecule in the asymmetric unit, with a V_M value (Matthews, 1968; Chruszcz *et al.*, 2008) of $2.55 \text{ Å}^3 \text{ Da}^{-1}$ and a solvent content of 51%. Experimental phasing using a selenomethionine derivative and structure determination are currently under way.

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