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Received 2 July 2010
Accepted 11 September 2010

Crystallization and preliminary X-ray studies of native and mutant intimin from enterohaemorrhagic *Escherichia coli*

Enterohaemorrhagic *Escherichia coli* (EHEC) O157:H7 is a primarily food-borne bacterial pathogen that is capable of causing life-threatening human infections and poses a serious challenge to public health worldwide. The bacterial outer-membrane protein intimin plays a key role in the initiation process of EHEC infection. In this study, intimin from EHEC O157:H7 (Int188) and its N916Y mutant (IntN916Y) were purified and crystals of both were obtained using the hanging-drop vapour-diffusion method at 291 K. Data were collected from Int188 and IntN916Y crystals to 2.8 and 2.6 Å resolution, respectively. The crystal of Int188 belonged to the orthorhombic space group C2, with unit-cell parameters $a = 235.16$, $b = 44.81$, $c = 129.12$ Å, $\alpha = \gamma = 90$, $\beta = 97.53^\circ$. The crystal of IntN916Y belonged to space group $P2_12_12_1$, with unit-cell parameters $a = 43.78$, $b = 92.49$, $c = 100.05$ Å, $\alpha = \beta = \gamma = 90^\circ$.

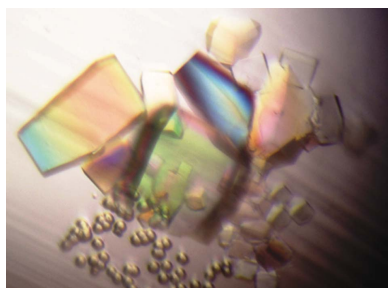
1. Introduction

Escherichia coli is a facultative anaerobe which can be divided into two major groups: pathogenic *E. coli* and virulent *E. coli* (Welinder-Olsson & Kaijser, 2005; Bell, 2002). Enterohaemorrhagic *E. coli* (EHEC) O157:H7 has been recognized as one of the most notorious pathogens among the pathogenic *E. coli* strains, featuring an extremely virulent serotype, and has been responsible for a series of severe gastrointestinal illnesses with life-threatening consequences in North America, Europe and China (Mead *et al.*, 1999; Henderson & Jablensky, 2010; Watanabe *et al.*, 1999; Wang *et al.*, 2004).

Like most mucosal pathogens, infection by EHEC O157:H7 follows a common cycle: colonization at the mucosal sites, evasion of the host defence, multiplication and host damage (Spears *et al.*, 2006). Many virulence factors [*e.g.* Shiga toxin (O'Brien *et al.*, 1992) and intimin (Donnenberg *et al.*, 1993)] or pathogenicity islands (PAIs) are involved in the general virulence of EHEC (Perna *et al.*, 1998). It is noteworthy that intimin has been demonstrated to be responsible for generation of the attaching and effacing (A/E) lesion (Donnenberg *et al.*, 1993).

Currently, intimin is classified into a large family of adhesion proteins that are capable of evoking A/E lesions. These adhesion proteins are generally divided into five types (α , β , γ , δ and ϵ) on the basis of their divergent C-terminal domains (Adu-Bobie *et al.*, 1998; Oswald *et al.*, 2000). The intimin of EHEC O157:H7 is classified into the γ -division (designated intimin- γ), while that of enteropathogenic *E. coli* (EPEC) is of the α -type. In addition, it has been found that the Tir proteins are specific intimin receptors and are quite different between EHEC O157:H7 and EPEC, especially in their pattern of phosphorylation after the infiltration of host cells. This implies that the function of intimin may vary to some extent between EHEC and EPEC (DeVinney *et al.*, 1999).

Here, we report the purification, crystallization and preliminary crystallographic analysis of recombinant native intimin Tir-binding domain (Int188) and its IntN916Y mutant.



2. Materials and methods

2.1. Cloning

The DNA fragment encoding intimin D2D3 (residues 747–934) of EHEC was amplified from bacterial genomic DNA isolated from EHEC O157:H7 strain EDL933. PCR was carried out with *Pfu* polymerase and utilized the primers Int-F (5'-GAA TTC CAT ATG GCG ACT GAG GTC ACT-3') and Int-R (5'-CCG CTC GAG TTA TTC TAC ACA AAC-3'). The PCR product was digested with *NdeI* and *XhoI* and ligated into pET21a vector (Novagen). A mutant, pET21a-IntN916Y, was inadvertently obtained in this experiment. The recombinant plasmids were verified by DNA sequencing.

2.2. Protein expression and purification

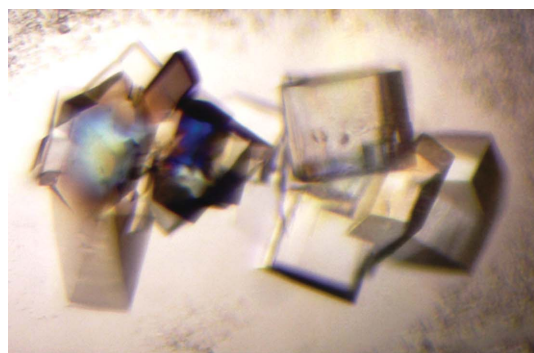
The two recombinant plasmids (pET21a-Int188 and pET21a-IntN916Y) were transformed into *E. coli* strain BL21 (DE3) and the cells were cultured at 310 K in LB medium containing 50 mg l⁻¹ ampicillin (Sigma, USA). When the optical density at 600 nm (OD₆₀₀) reached 0.8, the cultures were induced with 1.0 mM isopropyl β-D-1-thiogalactopyranoside (IPTG; Sigma, USA) and maintained at 310 K for ~3.5 h. The cells were then harvested by centrifugation at 5000 rev min⁻¹ (12 min, 277 K). The cell pellets were resuspended in cold PBS (140 mM NaCl, 2.7 mM KCl, 10 mM Na₂HPO₄ and 1.8 mM KH₂PO₄). After being lysed by sonication and centrifuged at 16 000g, the pellet was washed three times with a solution consisting of 20 mM Tris-HCl, 100 mM NaCl, 1 mM EDTA, 1 mM DTT and 0.5% Triton X-100. Int188 and IntN916Y in the form of inclusion bodies were washed and then dissolved in guanidine buffer (20 mM Tris pH 8.0, 1 mM EDTA, 6 M guanidine).

Refolding of purified inclusion bodies was carried out as described by Garboczi *et al.* (1992) with minor modifications (Zhou *et al.*, 2004). The inclusion bodies were dissolved in a solution consisting of 10 mM

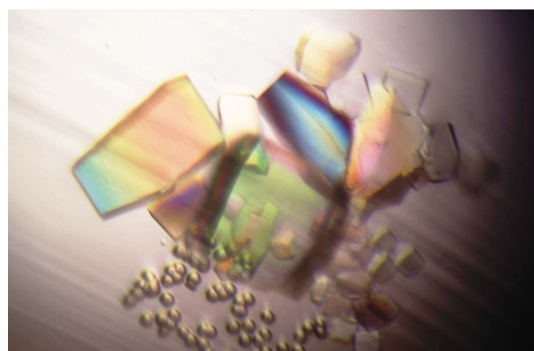
Tris-HCl pH 8.0 and 8 M urea. After 24 h at 277 K, the soluble portion was concentrated and purified using Resource Q anion-exchange chromatography followed by Superdex 75 size-exclusion chromatography in 20 mM Tris pH 8.0, 50 mM NaCl.

2.3. Crystallization

The purified Int188 (20.7 kDa) and IntN916Y (20.7 kDa) proteins were dialyzed against crystallization buffer (20 mM Tris-HCl pH 8.0, 50 mM NaCl). Crystals of Int188 and IntN916Y were obtained by the hanging-drop method at 291 K. Initial screening was performed using

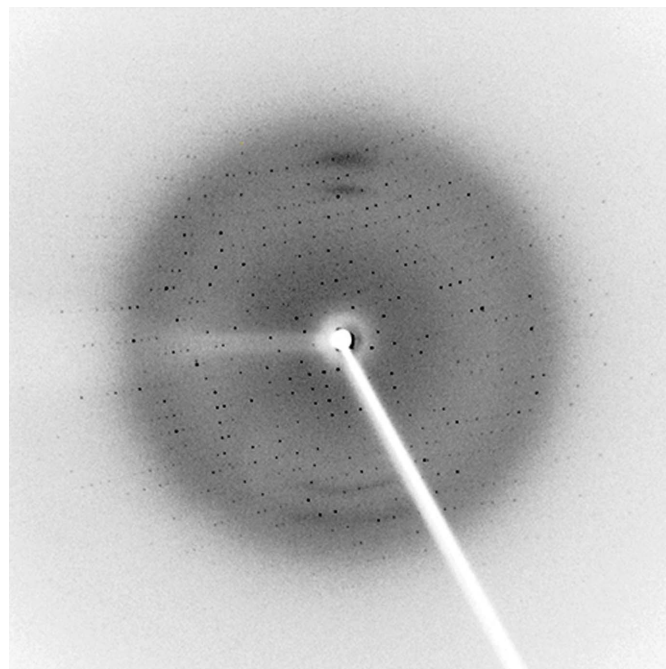


(a)

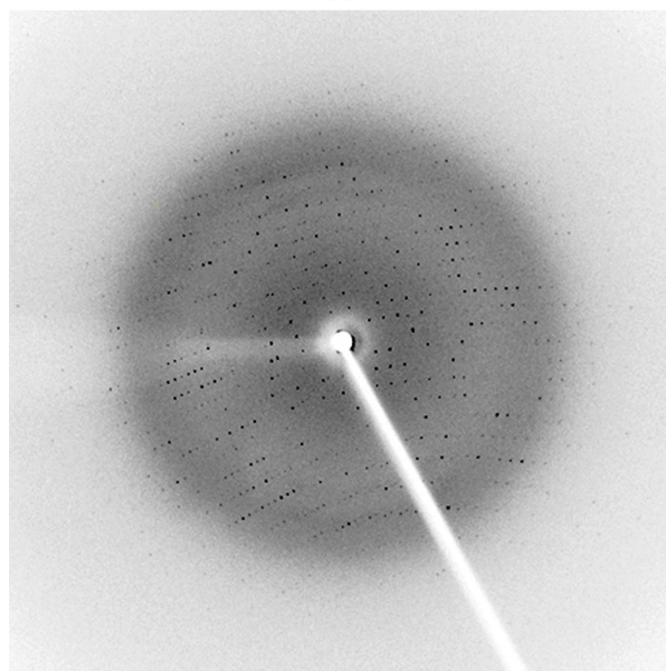


(b)

Figure 1
Typical crystals of native Int188 (a) and IntN916Y (b).



(a)



(b)

Figure 2
X-ray diffraction images from native Int188 (a) and IntN916Y (b) crystals.

Table 1

X-ray diffraction data-collection and refinement statistics for intimin crystals.

Values in parentheses are for the highest resolution shell.

	Int188	IntN916Y
Space group	C2	P2 ₁ 2 ₁ 2 ₁
Resolution (Å)	46.21–2.80 (2.90–2.80)	41.98–2.60 (2.69–2.60)
Unit-cell parameters (Å, °)	$a = 235.16, b = 44.81, c = 129.12,$ $\alpha = \gamma = 90.00, \beta = 97.53$	$a = 43.78, b = 92.49, c = 100.05,$ $\alpha = \beta = \gamma = 90.00$
Total reflections	109738 (36217)	90399 (12932)
Unique reflections	32396 (10691)	13058 (1932)
Completeness (%)	96.3 (92.7)	99.7 (100)
$R_{\text{merge}}^{\dagger}$	0.230 (0.432)	0.132 (0.356)
$\langle I/\sigma(I) \rangle$	4.1 (2.4)	9.6 (4.5)

$\dagger R_{\text{merge}} = \sum_{hkl} \sum_i |I_i(hkl) - \langle I(hkl) \rangle| / \sum_{hkl} \sum_i I_i(hkl)$, where $I_i(hkl)$ is the intensity of unique reflection hkl and $\langle I(hkl) \rangle$ is the average over symmetry-related observations of unique reflection hkl .

Crystal Screen and Crystal Screen 2 (Hampton Research). 1 μ l droplets of protein solution (at 5, 10 and 15 mg ml⁻¹) mixed with an equal amount of reservoir solution were equilibrated against 200 μ l reservoir solution. Crystals were first observed in 4 d using condition No. 30 of Crystal Screen (30% PEG 8000, 0.2 M ammonium sulfate). After several rounds of optimization, a more promising crystallization condition was obtained (25% PEG 8000, 0.18 M ammonium sulfate) at 277 K; the concentrations of Int188 and IntN916Y used were 10 and 15 mg ml⁻¹, respectively (Fig. 1).

2.4. X-ray diffraction, data collection and processing

Data collection was performed in-house using a Rigaku MicroMax-007 rotating-anode X-ray generator operated at 40 kV and 20 mA (Cu K α ; $\lambda = 1.5418$ Å) and equipped with an R-Axis VII⁺⁺ image-plate detector. The crystals were flash-frozen in liquid nitrogen after addition of 15% (v/v) glycerol to the mother liquor, mounted in nylon loops and flash-cooled in a cold nitrogen-gas stream at 100 K using an Oxford Cryosystem. The crystals of EHEC Int188 and IntN916Y diffracted to 2.8 and 2.6 Å resolution, respectively (Fig. 2). Data were processed and scaled with *CrystalClear* (Pflugrath, 1999).

3. Results and discussion

The open reading frame of Int188 consists of 564 bp coding for 188 amino-acid residues. The mutant (IntN916Y) obtained in this experiment has only one residue (N916Y) that differs from Int188. In this study, the fragments of intimin (Int188) and its mutant (IntN916Y) were cloned into pET21a(+) plasmid with *Nde*I and *Xho*I restriction sites. The two recombinant proteins were expressed as inclusion bodies in *E. coli*. The refolded proteins were purified using Resource Q anion-exchange chromatography followed by Superdex 75 size-exclusion chromatography. Crystallization was carried out by the hanging-drop method at 291 K. Crystals of Int188 and the

IntN916Y mutant were successfully obtained (using 25% PEG 8000, 0.18 M ammonium sulfate) at 277 K after optimization (Fig. 1). Native (Int188) and mutant (IntN916Y) data sets were collected to 2.8 and 2.6 Å resolution, respectively, using a Rigaku MicroMax-007 generator. The crystal of Int188 belonged to the orthorhombic space group C2, with unit-cell parameters $a = 235.16, b = 44.81, c = 129.12$ Å, $\alpha = \gamma = 90, \beta = 97.53^\circ$. The crystal of IntN916Y belonged to space group P2₁2₁2₁, with unit-cell parameters $a = 43.78, b = 92.49, c = 100.05$ Å, $\alpha = \beta = \gamma = 90^\circ$. Selected data statistics are shown in Table 1. Int188 has six molecules in the asymmetric unit; the calculated V_M (Matthews, 1968) and solvent content are 2.72 Å³ Da⁻¹ and 54.82%, respectively. IntN916Y has two molecules in the asymmetric unit; the calculated V_M and solvent content are 2.45 Å³ Da⁻¹ and 49.85%, respectively.

We would like to express our gratitude to Jun Liu and Dr Christopher Vavricka for their revisions of the manuscript. This work was completed in the laboratory of Professor George F. Gao at the Institute of Microbiology, Chinese Academy of Sciences (IMCAS) and was supported by grants from the National Natural Science Foundation of China (30670167 and 30770514).

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