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Crystallization and preliminary X-ray diffraction analysis of Wza outer-membrane lipoprotein from *Escherichia coli* serotype O9a:K30

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A novel integral membrane lipoprotein, Wza, from *Escherichia coli* serotype O9a:K30 has been purified and crystallized. Wza is required for the surface expression of the serotype K30 group 1 capsular polysaccharide of *E. coli*; closely related homologues are found in other bacteria that produce extracellular polysaccharides. The Wza crystals belong to the orthorhombic space group $P2_12_12_1$, with unit-cell parameters a = 94.6, b = 215.5, c = 218.5 Å. A data set to 3.0 Å with 99.8% completeness and an $R_{\rm merge}$ of 10.5% has been collected from a single crystal.

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1. Introduction

Escherichia coli produces more than 80 structurally and immunochemically distinct capsular polysaccharides termed K antigens (Jann & Jann, 1997). These polymers, varying in composition, linkage specificity and substitution, are required for cell viability and interactions between a bacterium and its host (Whitfield *et al.*, 1994). The capsular K antigens of *E. coli* are classified into four groups based on their surface organization, the mechanism involved in their assembly, the structure of their biosynthetic gene loci and the regulation of their expression (Whitfield & Roberts, 1999). Of these four groups, groups 1 and 2 predominate. K antigens are cell surface-associated and are synthesized at the inner membrane before being translocated to the cell surface. However, in Gram-negative bacteria, the outer membrane serves as a selectively permeable protective barrier and presents a significant obstacle to macromolecular translocation.

The group 1 K30 antigen from *E. coli* (O9a:K30) is present on the cell surface and the genes involved in synthesis and transport are encoded at a locus called *cps* (Drummelsmith & Whitfield, 1999). The group 1 capsule clusters are characterized by the presence of four genes: *orfx*, *wza*, *wzb* and *wzc* (Rahn *et al.*, 1999). Initial biochemical characterization by Drummelsmith & Whitfield (2000) reported that the *wza* gene encodes an outer integral membrane lipoprotein termed $\rm Wza_{K30}$. The apparent molecular weight of the Wza protein in solution is 40 232 Da, compared with the value of 39 553 Da computed from the amino-acid sequence. This is because the N-terminal cysteine is modified by acylation by palmitic acid and diacylglycerol (Drummelsmith & Whitfield, 2000; Nesper *et al.*, 2004). This protein has been identified as being required for the export of polysaccharides to the cell surface. Electron microscopy of the purified protein showed the presence of ring-like structures with a large pore in the middle (Drummelsmith & Whitfield, 2000). The rings were clearly made up of multiple subunits, although the precise number was unclear. Recent two-dimensional crystal electron microscopy (Nesper *et al.*, 2004) coupled with image reconstruction by Fourier filtering suggests that the structure contains eight electron-dense areas, corresponding to eight subunits. The electron-microscopy images showed the presence of a pore with a diameter of 1.6 nm and a protein radius of 7.1 nm. These structures are reminiscent of the multimeric secretins used by many bacteria to translocate folded or partially folded proteins across bacterial outer membranes (Thanassi, 2002), suggesting that there may be a common principle in the export of several large macromolecules across the outer membrane.

As yet, there is no structure of any protein that shares homology with this putative polysaccharide exporter, nor is there any detailed structure or molecular characterization of any polysaccharide exporter. Understanding the basis of polysaccharide export is not only important in the study of bacteria, but also seems likely to shed light on protein-export systems. Towards the goal of obtaining detailed structural information, we have recently succeeded in purifying and crystallizing Wza from *E. coli* with its modified N-terminal cysteine. In this paper, we report our preliminary crystallographic characterization of the protein.

2. Materials and methods

2.1. Expression and purification

We based our method closely on the protocol reported in the biochemical characterization of $\rm Wza_{K30}$; some of the steps were

changed to improve the crystallizability of the protein. Wza was overexpressed from plasmid pWQ126 in *E. coli* LE392. The bacterial cells harvested from a 10 l fermentor batch culture in Luria broth were resuspended in 120 ml 20 m*M* sodium phosphate pH 7. Bacterial cells were disrupted using a French press (110 MPa). The crude extract was fractionated by ultracentrifugation. The supernatant solution was discarded and the pellet containing cell envelopes was resuspended in 160 ml 20 m*M* sodium phosphate pH 7 and 2% *N*-lauroylsarcosine (Sigma, St Louis, MO, USA) to solubilize the inner membrane (Filip *et al.*, 1973). The pellet was solubilized at room temperature with rolling for 1 h. The extract was centrifuged again for 1 h at 289 K. The supernatant solution was then discarded and the pellet, now enriched in outer membranes, was resuspended in 160 ml 20 m*M* sodium phosphate, 50 m*M* NaCl and 0.5% *N*-tetradecyl-*N*,*N*-dimethyl-3-ammonio-1-propanesulfonate (SB3-14; Sigma). The membranes were solubilized overnight at room temperature with rolling. The insoluble materials were then removed by centrifugation at 85 000*g* for 1 h at 289 K.

Purification of Wza was performed using anion-exchange chromatography. A Biorad Q (Bio-Rad, Canada) column was used and equilibrated with 20 m*M* sodium phosphate, 50 m*M* NaCl and 0.5% SB3-14. The protein was eluted at 12% using a solution consisting of 20 m*M* sodium phosphate, 1 *M* NaCl and 0.5% SB3-14. The fractions containing Wza were pooled and dialysed overnight against 20 m*M* sodium phosphate, 50 m*M* NaCl. The Biorad Q column was also used to exchange the detergent to *n*-dodecyl-β-D-maltoside (DDM; Anatrace, USA). The protein was washed with 100 ml 20 m*M* Tris pH 8.5, 50 m*M* NaCl and 0.008% DDM and eluted with 20 m*M* Tris–HCl pH 8.5, 1 *M* NaCl and 0.008% DDM. Minor contaminants were removed by hydroxyapatite chromatography (Bio-Rad, Canada). The sample was dialysed overnight in 20 m*M* Tris base pH 8, 50 m*M* NaCl and 0.008% DDM. Purity was checked at all stages with SDS–PAGE using Coomassie blue staining for revelation. The yield was 30 mg of pure protein per 10 l of culture.

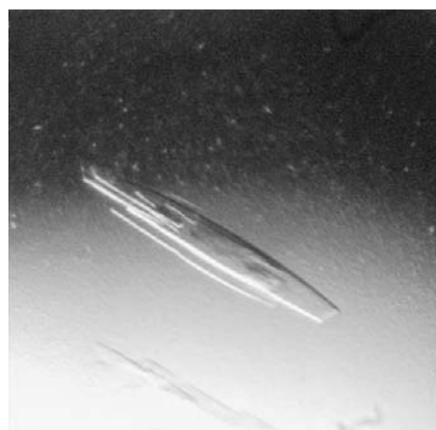

Figure 1
Crystal clusters of Wza_{K30} from *E. coli*. The crystal has dimensions of 1.0 × 0.1 × 0.1 mm.

2.2. Crystallization

Crystallization trials were performed using the hanging-drop vapour-diffusion system at 293 K. The protein solution contained 20 m*M* Tris base pH 8, 50 m*M* NaCl, 0.008% DDM and 8.5 mg ml^{-1} protein. Each drop was prepared by mixing 0.5 µl protein solution with the same volume of reservoir solution. The drops were suspended over 0.5 ml reservoir solution. Intitial crystallization conditions were screened using the hanging-drop method and a screen developed by Professor So Iwata (Iwata, 2003). Small crystals appeared in 24 different conditions in less than 5 min. Crystallization quality (judged by eye) was improved by ultracentrifugation of the protein-precipitate mix to remove aggregates prior to addition to the crystallization tray (Horsefield *et al.*, 2003). After this step, the crystallization conditions (PEG, NaCl, $MgSO_4$ and protein concentration) were systematically varied to identify the optimal point. The largest single crystals appeared after 24 h using 6 mg ml^{-1} protein solution containing 0.008% DDM, 100 m*M* NaCl in 8% PEG 4000, 100 m*M* sodium citrate, 50 m*M* NaCl and 50 m*M* $MgSO_4$. The crystals grew to final dimensions of 1.0 × 0.1 × 0.1 mm after 2 d (Fig. 1).

2.3. Data collection

The quality of Wza crystals, as judged by their diffraction pattern, varies in an unpredictable manner from batch to batch of protein and within the same batch, even in the same well. In our experience of this protein, poor-looking crystals give low-resolution diffraction, whilst apparently good crystals (by visual inspection) can give poor data. Prior to data collection, the crystals were flash-frozen in a cold nitrogen stream. Crystals were cryoprotected using an increasing concentration of glycerol in mother liquor (2–20%), which always contained 0.008% DDM. The preliminary characterization of the Wza crystals was performed using a Rigaku RU-HR3 rotating-anode generator operated at 40 kV and 100 mA. The crystals diffracted to 6 Å with this X-ray source.

Higher resolution data (3.0 Å) were collected from a single crystal at ESRF beamline ID14EH1 using an ADSC Quantum 4 CCD detector (Fig. 2). To reduce the overlap of the reflections, exposures were collected as 0.25° oscillations. The exposure time was 10 s for each 0.25° image. The crystal-to-detector distance was 300 mm and the X-rays had a wavelength of 0.933 Å.

3. Results and discussion

Indexing of the data indicated the cell was primitive orthorhombic, with unit-cell parameters a = 94.6, b = 215.5, c = 218.5 Å, $\alpha = \beta = \gamma = 90°$. Data were processed using the program *MOSFLM* (Leslie, 1992) and merged and scaled using the program *SCALA* from the *CCP*4 program suite (Evans, 1997). Data were merged satisfactorily in Laue group *P*222. Sufficient data were collected along the axis (at least 60

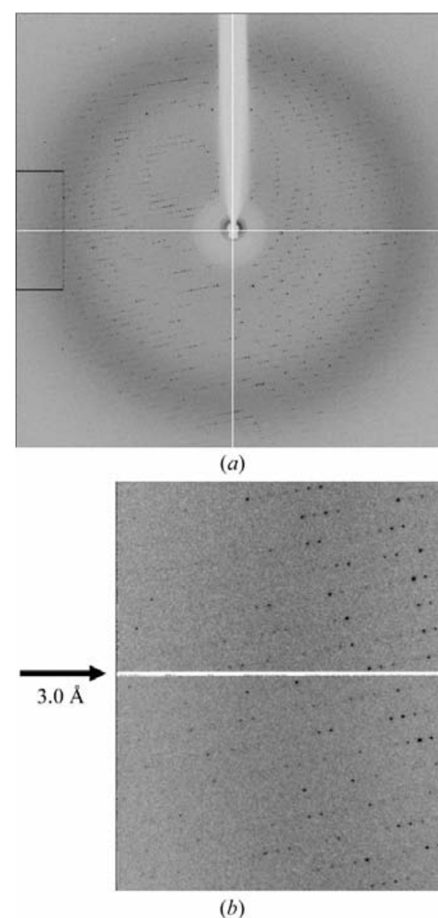

Figure 2
(*a*) X-ray diffraction pattern from the Wza_{K30} crystal collected at ID14EH1, ESRF. The crystal-to-detector distance was 300 mm and the oscillation angle was 0.25°. (*b*) Close-up of the above image; diffraction spots extend to 3.0 Å.

Table 1
Summary of data-collection statistics.

Values in parentheses are for the highest resolution shell (3.16–3.0 Å).

Wavelength (Å)	0.933
Resolution (Å)	87.7–3.0
Space group	$P2_12_12_1$
Unit-cell parameters (Å, °)	a = 94.6, b = 215.5, c = 218.5, $\alpha = \beta = \gamma = 90$
No. measured reflections	1867695
No. unique reflections	90374
Multiplicity	8.1 (8.1)
$I/\sigma(I)$	6.6 (2.2)
Completeness (%)	99.8 (99.8)
$R_{\rm merge}$† (%)	10.5 (34.4)

† $R_{\rm merge} = \sum_{hkl}\sum_i |I_i - \langle I\rangle| / \sum_{hkl}\sum_i \langle I\rangle$, where I_i is the intensity of the ith measurement of a reflection with indices hkl and $\langle I\rangle$ is the weighted mean of the reflection intensity.

measurements for each axis) for us to confidently identify twofold systematic absences and the space group was thus determined to be $P2_12_12_1$. A summary of the data collection is presented in Table 1. Taking into account the existence of eight subunits as shown in the two-dimensional crystal electron-microscopy data (Nesper *et al.*, 2004), calculation of the Matthews coefficient suggests the presence of one octamer in the asymmetric unit with a value of 3.4 $Å^3$ Da^{-1} and a water content of 63.5% based on a molecular weight of 40 232 Da per monomer. High $V_{\rm M}$ values and solvent contents are common in crystals of membrane-protein complexes owing to the detergent micelle surrounding the proteins (Iwata *et al.*, 1998; Ostermeier *et al.*, 1995). Structure determination attempts using mercury acetate and thallium bromide co-crystals are in progress.

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