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Purification, crystallization and preliminary diffraction studies of the *Pseudomonas aeruginosa* strain K122-4 monomeric pilin

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The monomeric pilin from *Pseudomonas aeruginosa* strain K122-4 has been crystallized and preliminary X-ray diffraction data have been collected. Pilin is the monomeric subunit of the type IV pilus, the dominant adhesin of the opportunistic pathogen *P. aeruginosa*. The K122-4 pilin crystallizes as a dimer in space group *P*1, with unit-cell parameters $a = 40.19$, $b = 38.93$, $c = 37.22$ Å, $\alpha = 66.38$, $\beta = 111.12$, $\gamma = 93.74^\circ$. Diffraction data were collected using a synchrotron-radiation source and were processed to 1.54 Å *d*-spacing.

1. Introduction

The opportunistic pathogen *Pseudomonas aeruginosa* is of significant concern to cystic fibrosis patients (Tummler *et al.*, 1997), burn victims (Bang *et al.*, 1998; Bang *et al.*, 2002) and immunosupressed or immunocompromized individuals (Rivera & Nicorta, 1982; Todd *et al.*, 1989). The dominant factor employed in the initiation of *P. aeruginosa* infections are the type IV pili, which are long fibre-like structures assembled from ~15 kDa pilin monomers (Irvin *et al.*, 1990; Irvin, 1993). It has been shown that although each pilin monomer contains a functional receptor-binding site, binding occurs only at the tip of the pilus structure (Lee *et al.*, 1994). Although the pilins from different *P. aeruginosa* strains exhibit significant sequence variability, it has been shown that the pilins bind to the same receptor, the glycosphingolipids asialo-GM_1 and asialo-GM_2 (Lee *et al.*, 1994; Yu *et al.*, 1994), and display a conserved antigenic epitope (Sheth *et al.*, 1995). Therefore, the receptor-binding site of the pilin is a likely target for anti-infective agents. Indeed, Sheth *et al.* (1994) have demonstrated that the disaccharide β-D-GalNAc(1–4)β-D-Gal, present in both asialo-GM_1 and asialo-GM_2, is sufficient to inhibit pilin binding. Further investigation into the structural basis of receptor activity by pili has been undertaken (Schweizer *et al.*, 1998); however, the exact nature of the pilin–carbohydrate interaction remains unclear (Audette *et al.*, 2003).

Several studies have shown that the receptor-binding site of *P. aeruginosa* pilins resides in a C-terminal disulfide-bonded loop (Irvin *et al.*, 1989; Wong *et al.*, 1995). We have previously shown that in *P. aeruginosa* strain K (PAK) pilin this loop contains two consecutive β-turns that define a shallow solvent-exposed pocket dominated by main-chain atoms. We have proposed that this pocket represents the receptor-binding site and that it is conserved in spite of high sequence divergence through retention of the main-chain structure (Hazes *et al.*, 2000). Structures of two or more highly divergent *P. aeruginosa* pilins should reveal whether this retention of function indeed arises from the structural conservation of this pocket or arises by some other molecular mechanism. K122-4 pilin, which shares approximately 40% sequence identity with PAK pilin in the receptor-binding loop, has been selected for this purpose.

Structures of full-length type IV pilins are available for *Neisseria gonorrhoeae* strain MS11 pilin (Parge *et al.*, 1995) and PAK pilin (Craig *et al.*, 2003), but their low solubility makes them hard to handle. In order to facilitate crystallization, we have deleted the first 28 residues, which form a fully exposed and extremely hydrophobic α-helix (Hazes *et al.*, 2000). These recombinant pilins are highly soluble and retain biological function; comparison between truncated and full-length PAK pilins shows no differences in the receptor-binding loop structure (Craig *et al.*, 2003). Binding assays also confirm retention of function for truncated K122-4 pilin (Keizer *et al.*, 2001) and PAK pilin (R. T. Irvin, unpublished results). Crystal structures of truncated type IV pilins are now available for PAK pilin (Hazes *et al.*, 2000) and *Vibrio cholerae* pilin (Craig *et al.*, 2003). An NMR structure of truncated K122-4 pilin has also been reported (Keizer *et al.*, 2001); unfortunately, the region of greatest interest, the receptor-binding loop, could not be unambiguously assigned owing to spectral overlap.

Since the *N. gonorrhoeae* and *V. cholerae* pilins do not share the receptor specificity of the *P. aeruginosa* pilins and as the K122-4 pilin solution structure does not fully define the receptor-binding site, our K122-4 crystals will give us the first opportunity to determine the molecular mechanism of conserved receptor

specificity in *P. aeruginosa* pilins. Here, we report the purification, crystallization and preliminary X-ray diffraction studies of the truncated K122-4 pilin monomer. These and future studies should shed light on the nature of carbohydrate binding by the pilins and lead to the development of more effective anti-pilin therapeutics.

2. Methods and results

2.1. Cloning, expression and purification

The K122-4 pilin gene was excised from a pRLD plasmid (Tripet *et al.*, 1996) containing truncated K122-4 [*pilA*(Δ1–28); Keizer *et al.*, 2001] by cleavage with *Eco*RI and *Hin*dIII. The DNA was isolated from a 1.2% agarose gel using standard procedures and ligated in-frame into the pMAL-p2 expression vector (NEB) for periplasmic expression of the monomeric pilin as a maltose-binding protein (MBP)–*pilA*(Δ1–28) fusion protein. *Escherichia coli* strain ER2507 (NEB) was transformed with the expression vector and transformed cells were cultured at 310 K with shaking in LB broth containing carbenicillin (50 μg ml^{-1}) to an absorbance of A_{600} = 0.5–0.7. Protein expression was induced by the addition of isopropyl β-D-thiogalactopyranoside to a final concentration of 1 m*M* and allowed to proceed for 3 h prior to harvesting. The MBP-K122-4 fusion protein was released from the periplasmic space using an osmotic shock protocol (Neu & Heppel, 1965) and the periplasmic fraction was passed through a 0.45 μm filter to remove cellular debris. The filtered periplasmic solution was then loaded onto an amylose resin column (NEB) equilibrated with column buffer (10 m*M* Tris pH 7.4, 50 m*M* sodium chloride, 1 m*M* EDTA) on an Akta Purifier (Amersham Biosciences) at 277 K. The column was then washed with two column volumes of column buffer, after which the MBP-K122-4 fusion protein was eluted from the column with an elution buffer consisting of column buffer and 5 m*M* maltose. The collected MBP-K122-4 fusion protein was trypsinized for 15 min on ice to release the K122-4 pilin monomer from the MBP; the ratio of trypsin to protein was 1:500 and cleavage was halted by the addition of a tenfold excess of phenylmethylsulfonyl fluoride to the solution. The monomeric K122-4 pilin was then purified by cation-exchange chromatography on Resource 15S resin (Amersham Biosciences) using a linear gradient of 0–1 *M* sodium chloride at 295 K. The purity and identity of the monomeric pilin was assessed by SDS–PAGE, immunoblotting with a rabbit polyclonal anti-pilus antibody (Doig *et al.*, 1988; Schweizer *et al.*, 1998), amino-acid analysis, N-terminal protein sequencing and MALDI–TOF mass-spectrometric analysis. The purified K122-4 truncated pilin exhibited a single band on SDS–PAGE analysis which reacted with the anti-pilus antibody and exhibited a single mass-spectral peak at 12 837.57 Da; the theoretical molecular weight of the K122-4 pilin is 12 822.37 Da. N-terminal sequencing revealed the first seven residues as ISEFARA, corresponding to four residues from the cloning construct (ISEF) and the first three residues of the truncated K122-4 Δ(1–28) pilin.

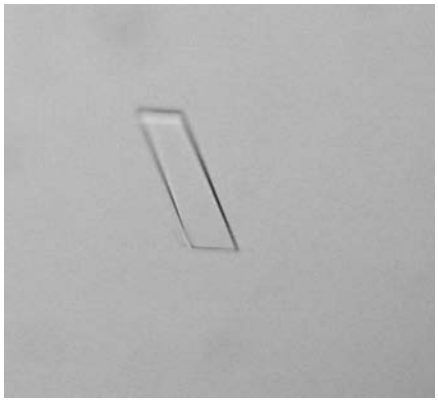

Figure 1
A crystal of the monomeric K122-4 pilin. Typical dimensions are 0.25 × 0.1 × 0.05 mm.

2.2. Crystallization

All crystallization experiments were performed using the hanging-drop vapour-diffusion method at 295 K. Initial crystals of the K122-4 pilin were grown in 1–2 d from 2 μl drops containing equal volumes of protein (20 mg ml^{-1} in 10 m*M* Tris pH 7.4, 100 m*M* sodium chloride) and reservoir solution [35%(*w*/*v*) PEG 4000, 100 m*M* sodium cacodylate pH 6.0, 100 m*M* monobasic potassium phosphate]. These initial crystals were used as a microseed stock for further crystallization experiments. Crystals suitable for X-ray diffraction analysis (typical dimensions of 0.25 × 0.1 × 0.05 mm; Fig. 1) were grown from 3 μl drops containing equal volumes of protein, reservoir [30%(*w*/*v*) PEG 4000, 100 m*M* sodium cacodylate pH 6.0, 100 m*M* monobasic potassium phosphate] and microseed solution. Crystals were mounted in cryoloops (Hampton Research) and flash-cooled by direct immersion into liquid nitrogen prior to X-ray diffraction analysis.

Table 1
Summary of data-collection statistics.

Values in parentheses are for the highest resolution shell.

Resolution (Å)	37–1.54 (1.55–1.54)
No. of observations	62889
No. of unique reflections	26457
Completeness (%)	93.1 (88.5)
R_{sym}†	0.059 (0.096)
$\langle I/\sigma(I)\rangle$	16.5 (6.6)
B factor from Wilson plot (Å^2)	14.0

† $R_{sym} = \sum_j(|I_j - \langle I_j\rangle|)/\sum I_j$, where j represents a set of observations of equivalent reflections and $\langle I\rangle$ is the average intensity of the reflection.

2.3. Data collection and reduction

X-ray diffraction data were collected on beamline 8.3.1 at the Advanced Light Source. Data were collected in 180 images on a ADSC Quantum 315 CCD detector with a crystal-to-detector distance of 175 mm. Intensity data were collected at 100 K using a wavelength of 1.1 Å with a 1° oscillation per image. Diffraction data were processed using *MOSFLM* (Leslie, 1992) and *SCALA* from the *CCP*4 suite of programs (Collaborative Computational Project, Number 4, 1994). The K122-4 pilin crystallizes in space group *P*1, with unit-cell parameters a = 40.19, b = 38.93, c = 37.22 Å, α = 66.38, β = 111.12, γ = 93.74°. Analysis of the self-rotation function revealed a single peak 13σ above the background (κ = 180°), indicating that there is a K122-4 dimer in the unit cell. With two molecules in the unit cell, V_M is 1.93 Å^3 Da^{-1} (Matthews, 1968) and the solvent content is 33.8%. Data-collection statistics are summarized in Table 1. Structure solution of the K122-4 pilin *via* molecular replacement is currently being undertaken and co-crystallization of suitable pilin–carbohydrate complexes is being investigated.

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