

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 *P1*, 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.

Received 28 May 2003
 Accepted 14 July 2003

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 immunosuppressed 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-GM₁ and asialo-GM₂ (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-GM₁ and asialo-GM₂, 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*(Δ 1–28); Keizer *et al.*, 2001] by cleavage with *EcoRI* and *HindIII*. 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*(Δ 1–28) 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 ($50 \mu\text{g ml}^{-1}$) 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 mM 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 $0.45 \mu\text{m}$ filter to remove cellular debris. The filtered periplasmic solution was then loaded onto an amylose resin column (NEB) equilibrated with column buffer (10 mM Tris pH 7.4, 50 mM sodium chloride, 1 mM 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 mM 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.

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 mM Tris pH 7.4, 100 mM sodium chloride) and reservoir solution [35%(w/v) PEG 4000, 100 mM sodium cacodylate pH 6.0, 100 mM 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 $0.25 \times 0.1 \times 0.05 \text{ mm}$; Fig. 1) were grown from 3 μl drops containing equal volumes of protein, reservoir [30%(w/v) PEG 4000, 100 mM sodium cacodylate pH 6.0, 100 mM 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 ($\text{\AA}$)	37–1.54 (1.55–1.54)
No. of observations	62889
No. of unique reflections	26457
Completeness (%)	93.1 (88.5)
$R_{\text{sym}}^{\dagger}$	0.059 (0.096)
$\langle I/\sigma(I) \rangle$	16.5 (6.6)
B factor from Wilson plot ($\text{\AA}^2$)	14.0

$\dagger R_{\text{sym}} = \sum_j (|I_j - \langle I_j \rangle|) / \sum_j I_j$, 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 $\text{\AA}$ with a 1° oscillation per image. Diffraction data were processed using *MOSFLM* (Leslie, 1992) and *SCALA* from the *CCP4* suite of programs (Collaborative Computational Project, Number 4, 1994). The K122-4 pilin crystallizes in space group *P1*, with unit-cell parameters $a = 40.19$, $b = 38.93$, $c = 37.22 \text{ \AA}$, $\alpha = 66.38$, $\beta = 111.12$, $\gamma = 93.74^\circ$. Analysis of the self-rotation function revealed a single peak 13σ above the background ($\kappa = 180^\circ$), indicating that there is a K122-4 dimer in the unit cell. With two molecules in the unit cell, V_M is $1.93 \text{ \AA}^3 \text{ Da}^{-1}$ (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.

We thank A. M. Brigley and L. Price for technical assistance. This work was supported by operating grants from the Canadian Institutes for Health Research to RTI (MOP-38004) and BH (MOP-42448). X-ray diffraction data were collected at beamline 8.3.1 of the Advanced Light Source (ALS) at Lawrence Berkeley Laboratory under an agreement with the Alberta Synchrotron Institute (ASI). The ALS is operated by the Department of Energy and supported by the National Institutes of Health. Beamline 8.3.1 was funded by the National Science Foundation, the University of California and Henry Wheeler. The ASI synchrotron-access program is supported by grants from the Alberta Science and Research Authority

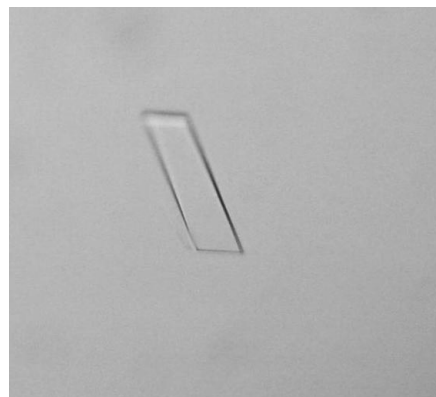


Figure 1

A crystal of the monomeric K122-4 pilin. Typical dimensions are $0.25 \times 0.1 \times 0.05 \text{ mm}$.

(ASRA) and the Alberta Heritage Foundation for Medical Research (AHFMR). GFA acknowledges salary support from the AHFMR; BH is an AFHMR scholar.

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