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Expression, purification and preliminary crystallographic analysis of *Pseudomonas aeruginosa* RocR protein

Masayo Kotaka,[a,b]* Sujit Dutta,[a] Hooi Chen Lee,[a] Mitchell J. M. Lim,[a] Yeehwa Wong,[a] Feng Rao,[a] Edward P. Mitchell,[c] Zhao-Xun Liang[a] and Julien Lescar[a,d]*

[a]School of Biological Sciences, Nanyang Technological University, Singapore 637551, Singapore, [b]Department of Physiology, University of Hong Kong, Hong Kong, [c]European Synchrotron Radiation Facility, Grenoble BP 220, France, and [d]AFMB, CNRS UMR6098 Marseille, France

Correspondence e-mail: masayo@hku.hk, julien@ntu.edu.sg

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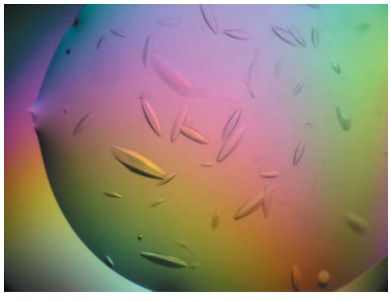

Pseudomonas aeruginosa RocR, an EAL-domain protein which regulates the expression of virulence genes and biofilm formation, has been cloned and expressed in *Escherichia coli* and purified. Here, the crystallization and preliminary diffraction analysis of RocR are reported. The X-ray diffraction data were processed to a resolution of 2.50 Å. The crystals belonged to space group $P6_122$ or $P6_522$, with unit-cell parameters $a = 118.8$, $b = 118.8$, $c = 495.1$ Å, $\alpha = \beta = 90$, $\gamma = 120^\circ$.

1. Introduction

Pseudomonas aeruginosa is a common bacterium which acts as an opportunistic pathogen, causing infection in humans with compromised host defences (Costerton *et al.*, 1999; Lyczak *et al.*, 2000). Chronic and acute infections caused by *P. aeruginosa* are most commonly found in severe burns victims, cystic fibrosis patients and users of contact lenses for extended periods of time (Lyczak *et al.*, 2000). These chronic infections are notoriously difficult to treat owing to the formation of antibiotic-resistant biofilms (Hall-Stoodley *et al.*, 2004; Mikkelsen *et al.*, 2007).

The RocSAR system (also known as the SadARS system) is a two-component regulatory signalling system in *P. aeruginosa* that regulates biofilm maturation (Kulasekara *et al.*, 2005, 2006; Kuchma *et al.*, 2005). This system controls bacterial biofilm maturation and virulence gene expression through regulation of the transcription of *cup* fimbrial gene clusters and genes of the type III secretion system (Kuchma *et al.*, 2005; Kulasekara *et al.*, 2005). This regulatory system is composed of a sensor histidine kinase, RocS1, and two response regulators, RocA1 and RocR.

RocR is the negative response regulator in the RocSAR system and regulates *cup* gene expression by antagonizing the activity of RocA1 (Kulasekara *et al.*, 2005). It contains an N-terminal CHEY-like domain and a C-terminal EAL domain with phosphodiesterase activity. The EAL domain of RocR hydrolyses cyclic di-GMP using a general base-catalyzed mechanism in the presence of Mg^{2+} ion (Rao *et al.*, 2008, 2009). Cyclic di-GMP is a ubiquitous second messenger in bacteria which regulates various virulence-related factors such as virulence gene transcription, biofilm formation, motility and adhesion (Galperin, 2004; Jenal & Malone, 2006). The intracellular concentration of cyclic di-GMP is controlled by the opposing activities of diguanylate cyclases and phosphodiesterases: di-GMP is synthesized by cyclic diguanylate cyclases, whereas it is hydrolysed by phosphodiesterases (Ross *et al.*, 1991).

Here, we report the protein expression, purification, crystallization and preliminary diffraction analysis of RocR.

2. Experimental procedures

2.1. Cloning, expression and purification

The cloning, expression and purification of RocR were performed as previously described (Rao *et al.*, 2008), with slight modification. Briefly, the open reading frame encoding RocR was amplified from the genomic DNA of *P. aeruginosa* PAO-1 (ATCC) and cloned into pET26b (Novagen) *via* the *Nde*I and *Not*I sites. The resulting plasmid

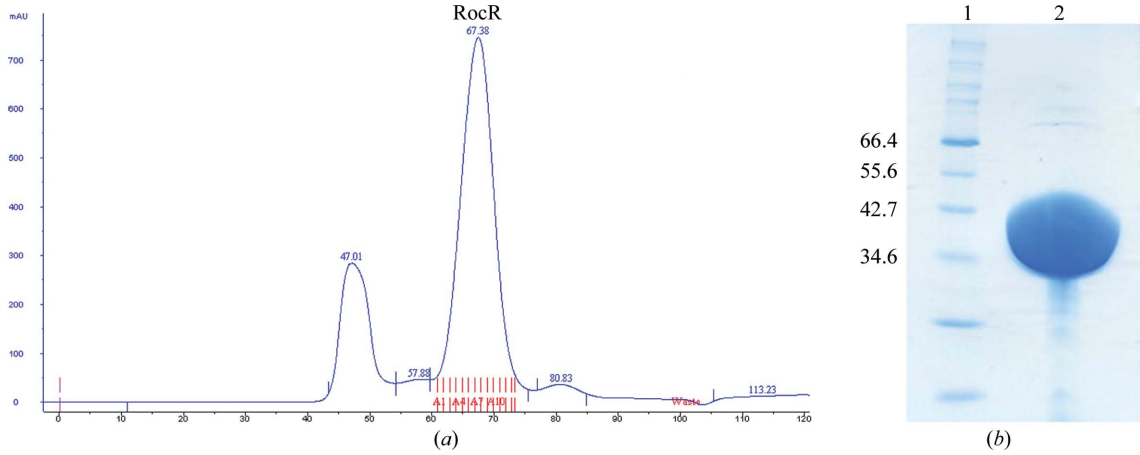

Figure 1
(*a*) Gel-filtration profile of RocR on a Superdex200 16/60 column (GE Healthcare). RocR elutes at a position corresponding to approximately 160 kDa. (*b*) 12% SDS–PAGE analysis of purified RocR. Lane 1, molecular-weight markers (kDa). Lane 2, concentrated RocR after gel filtration.

coding for C-terminally 6×His-tagged RocR, with a molecular weight of 43 901.6 Da, was verified by DNA sequencing and transformed into *Escherichia coli* expression strain BL21 (DE3) (Novagen). Cells were grown at 310 K in Luria–Bertani (LB) medium supplemented with 30 µg ml^{-1} kanamycin. Upon reaching an OD_{600} of 0.8, the culture was cooled to 301 K and induced by addition of isopropyl β-D-1-thiogalactopyranoside (IPTG) to a final concentration of 0.1 m*M*. After an incubation period of 16 h, the cells were harvested at 4000*g* for 15 min. The bacterial pellet was resuspended in lysis buffer (50 m*M* Tris–HCl pH 7.5, 250 m*M* NaCl, 10 m*M* imidazole, 5% glycerol, 0.5 m*M* DTT) supplemented with Complete EDTA-free protease inhibitor (Roche) and subjected to sonication. The lysate was cleared by centrifugation at 20 000*g* for 1 h. The supernatant was loaded onto a HiTrap HisTrap column (GE Healthcare) pre-equilibrated with lysis buffer and the protein was eluted with a 10–250 m*M* imidazole gradient in 50 m*M* Tris–HCl pH 7.5, 250 m*M* NaCl, 5% glycerol and 0.5 m*M* DTT. The eluant was concentrated using Amicon Ultra centrifugal concentrators (30 kDa cutoff, Millipore) and subjected to size-exclusion chromatography using a Superdex200 16/60 column (GE Healthcare) in 20 m*M* Tris–HCl pH 7.5, 40 m*M* KCl, 2 m*M* DTT and 5% glycerol, with RocR eluting at a position corresponding to a tetramer (Fig. 1*a*). At each step in the purification procedure, fractions were analysed by SDS–PAGE and appropriate fractions were pooled. After size-exclusion chromatography, RocR was concentrated to approximately 10 mg ml^{-1} using Amicon Ultra centrifugal concentrators (30 kDa cutoff, Millipore; Fig. 1*b*).

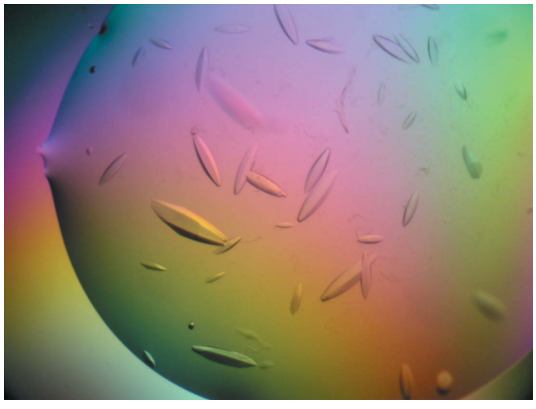

Figure 2
Crystals of RocR. Crystals typically grew to maximum dimensions of 0.3 × 0.1 × 0.1 mm.

2.2. Crystallization

An automated initial crystallization screen was performed using a CyBio crystal-creator robot (Jena Biosciences) with RocR at a concentration of 10 mg ml^{-1}. 200 nl protein solution was added to an equal volume of the crystallization solution using the sitting-drop vapour-diffusion method. Crystals were obtained at 291 K from PEG/Ion Screen (Hampton Research) condition No. 36 (0.2 *M* sodium tartrate, 20% PEG 3350). Optimization of the condition gave crystals from 0.2 *M* sodium tartrate, 0.1 *M* Na HEPES pH 7.0–7.2 and 16–22% PEG 3350 (Fig. 2).

2.3. X-ray diffraction analysis

Before data collection, crystals were transferred to a cryoprotectant containing 0.2 *M* sodium tartrate, 0.1 *M* Na HEPES pH 7.0–7.2, 16–22% PEG 3350 and 30% glycerol for 5–10 s and cooled to 100 K in a gaseous nitrogen stream using an Oxford cryosystem. A full data set was collected using a Quantum CCD image plate on beamline 13B1 at the National Synchrotron Radiation Research Centre (NSRRC, Taiwan) using a single crystal. The distance between the crystal and the image plate was set to 450 mm and the images were recorded with 0.5° oscillation per image and an exposure time of 10 s per frame (Fig. 3). Diffraction intensities were integrated and scaled to 2.50 Å resolution with *DENZO* and *SCALEPACK* (Otwinowski & Minor, 1997).

3. Results and discussion

The DNA segment encoding *P. aeruginosa* RocR was cloned into pET26b expression vector, resulting in a C-terminal 6×His tag. Recombinant RocR was expressed in *E. coli* BL21 (DE3) cells and purified (Figs. 1*a* and 1*b*). Crystals of RocR first appeared after one week of incubation in the initial crystallization screen at 291 K. Optimization resulted in crystals of dimensions 0.1 × 0.1 × 0.3 mm (Fig. 2).

Data were collected from a single RocR crystal and processed to 2.50 Å resolution at the corners of the image plate (Fig. 3). The space group was determined to be $P6_122$ or $P6_522$, with unit-cell parameters

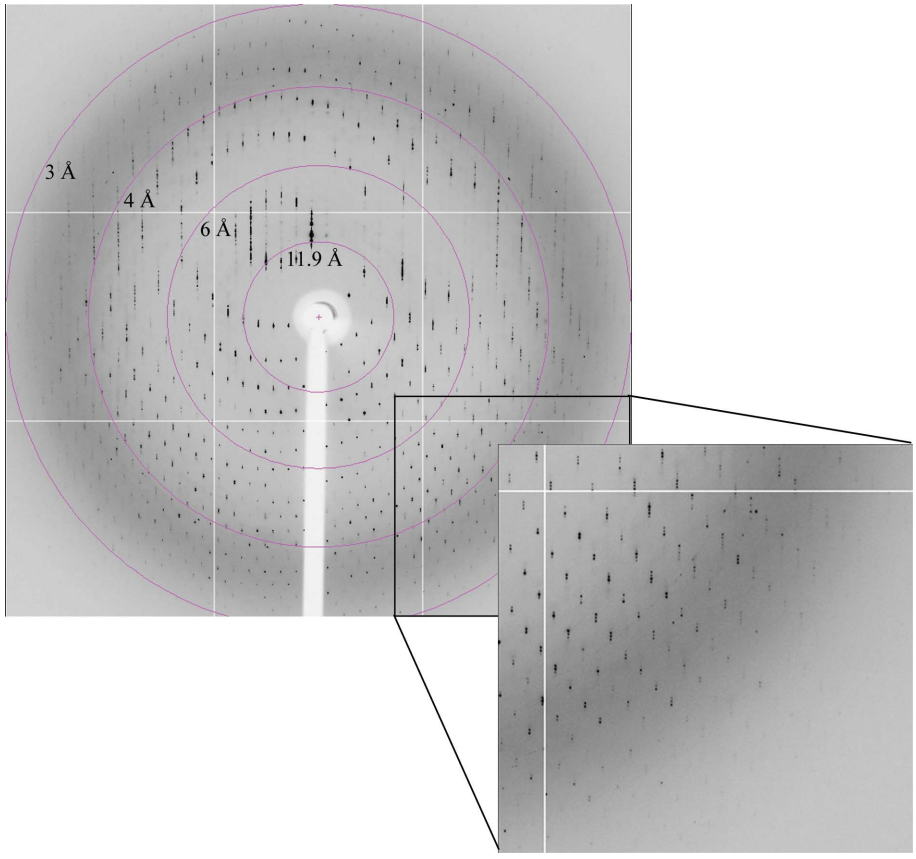

Figure 3
Diffraction image of a RocR crystal collected at NSRRC, Taiwan. The resolution limits of the X-ray diffraction are shown. The insert shows that diffraction extends beyond 3 Å and thus the data were processed to 2.5 Å resolution.

Table 1
Data-collection statistics for RocR.

Values in parentheses are for the highest resolution shell.

X-ray source	13B1, NSRRC
Wavelength (Å)	1.00
Space group	$P6_122$ or $P6_522$
Unit-cell parameters (Å, °)	$a = b = 118.8$, $c = 495.1$, $\alpha = \beta = 90$, $\gamma = 120$
Molecules per ASU	4
Resolution (Å)	30.0–2.50 (2.59–2.50)
Completeness (%)	83.2 (40.9)
No. of reflections	721820
No. of unique reflections	60479
Redundancy	11.9 (8.8)
Average $I/\sigma(I)$	61.9 (2.9)
$R_{\rm merge}$† (%)	4.6 (49.5)

† $R_{\rm merge} = \sum_{hkl}\sum_i |I_i(hkl) - \langle I(hkl)\rangle| / \sum_{hkl}\sum_i I_i(hkl)$.

$a = 118.8$, $b = 118.8$, $c = 495.1$ Å, $\alpha = \beta = 90$, $\gamma = 120°$. The asymmetric unit was estimated to contain four RocR molecules and the Matthews coefficient was determined to be 2.86 Å^3 Da^{-1}, corresponding to a solvent content of 57% (Matthews, 1968), which is consistent with the gel-filtration data (Fig. 1*a*). No clear peak was present in either the $\kappa = 180°$ section or the $\kappa = 90°$ section of the self-rotation function, despite the likelihood that RocR forms a tetramer with 222 symmetry. Details of the data-collection statistics are shown in Table 1. Unfortunately, as there are currently no structural homologues with reasonable homology (over 30%) in the PDB to serve as a search probe, molecular replacement for phase determination proved to be difficult. Attempts to solve the structure using the putative diguanylate cyclase phosphodiesterase from *Thiobacillus denitrificans* (PDB code 2r6o), which shares 32% homology to residues 149–385 of RocR, and CHEY domains (*e.g.* PDB codes 1p6u, 2ayx and 3eqz), which share 25–29% homology to the 120 N-terminal residues of RocR, as search probes for molecular replacement were unsuccessful. Both heavy-atom derivatization and selenomethionine incorporation of the protein for MIR and MAD/SAD phasing, respectively, are being actively pursued in order to solve the structure of RocR.

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