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Crystallization and preliminary X-ray analysis of the TetR-like efflux pump regulator SimR

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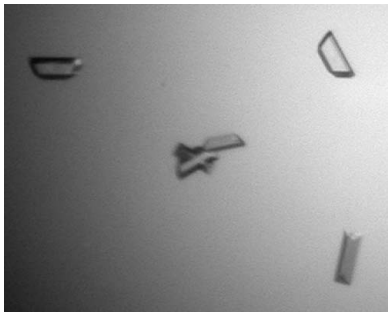
Crystals of SimR were grown by vapour diffusion. The protein crystallized with trigonal symmetry and X-ray data were recorded to a resolution of 2.3 Å from a single crystal at the synchrotron. SimR belongs to the TetR family of bacterial transcriptional regulators. In the absence of the antibiotic simocyclinone, SimR represses the transcription of a divergently transcribed gene encoding the simocyclinone efflux pump SimX in *Streptomyces antibioticus* by binding to operators in the *simR–simX* intergenic region. Simocyclinone binding causes SimR to dissociate from its operators, leading to expression of the SimX efflux pump. Thus, SimR represents an intimate link between the biosynthesis of simocyclinone and its export, which may also provide the mechanism of self-resistance to the antibiotic in the producer strain.

1. Introduction

Most antibiotics are potentially lethal to the producing organism and therefore there must be mechanisms to ensure that the machinery responsible for the export of the mature antibiotic is in place at the time of biosynthesis. Simocyclinone (Schimana *et al.*, 2000) is a potent DNA gyrase inhibitor that is produced by *Streptomyces antibioticus* Tü 6040 (Edwards *et al.*, 2009; Flatman *et al.*, 2005; Oppegard *et al.*, 2009). Within the simocyclinone-biosynthetic cluster are two divergently transcribed genes, *simR* and *simX*, that encode proteins that resemble the TetR/TetA repressor–efflux pump pair that causes widespread resistance to clinically important tetracyclines (Chopra & Roberts, 2001). Transcription of *simX* is controlled by SimR, which directly represses the *simX* and *simR* promoters by binding to two operator sites in the *simX–simR* intergenic region (Le *et al.*, 2009). Simocyclinone abolishes DNA binding by SimR, inducing expression of the SimX efflux pump and thus providing a mechanism that couples the biosynthesis of simocyclinone to its export. TetR-family transcriptional regulators are widespread in the bacterial kingdom (Ramos *et al.*, 2005) and there are representative structures of over 100 of these in the Protein Data Bank (Yu *et al.*, 2010). However, the mode of action has been fully elucidated at the molecular level for relatively few of these proteins (Orth *et al.*, 2000; Schumacher *et al.*, 2001; Itou *et al.*, 2010; Miller *et al.*, 2010; Reichheld *et al.*, 2009; Yu *et al.*, 2010). Here, we report the crystallization and preliminary X-ray analysis of SimR as the first step towards determining the molecular basis of its action.



2. Materials and methods

2.1. Protein expression and purification and crystallization

The *simR* gene of *S. antibioticus* Tü 6040, encoding a protein of 259 amino acids, was chemically synthesized and codon-optimized (GenScript) for expression in *Escherichia coli* and then ligated into the pETM11-*Nde*I plasmid to give the expression vector pIJ10495. The SimR protein produced from this vector has an N-terminal TEV protease-cleavable His tag. This adds a further 26 amino acids to the native protein (with sequence MKHHHHHHPMSDYDIPTTENLYFQGA), giving a total deduced molecular mass of 32 222 Da. The

expression vector pIJ10495 was transformed into *E. coli* strain BL21 (DE3) pLysS and a 7 ml overnight culture of the cells was used to inoculate a 500 ml culture of Luria–Bertani medium containing 50 µg ml^{-1} kanamycin and 30 µg ml^{-1} chloramphenicol. The cells were grown at 310 K to an $OD_{600\,nm}$ of around 0.4. The culture was then cooled to 293 K and dimethylsulfoxide (DMSO) was added to a final concentration of 0.15%(*v*/*v*) before induction of protein expression by the addition of isopropyl β-D-thiogalactopyranoside to a final concentration of 0.3 m*M*. The addition of DMSO was essential for a high yield of soluble SimR, probably because it induces the expression of chaperones that assist in the folding of SimR *in vivo*. The culture was left shaking for 3 h at 303 K. Harvested cells were resuspended in 50 m*M* Tris–HCl pH 8.4, 300 m*M* NaCl, 5%(*v*/*v*) glycerol containing Complete EDTA-free protease-inhibitor cocktail (Roche) and lysed by sonication (three cycles of 20 s with 40 s resting on ice in between each cycle). The cell debris was removed by centrifugation at 84 000*g* for 30 min and the supernatant was filtered through a 0.45 µm membrane. It was then applied onto a 1 ml Ni-loaded Hi-Trap Chelating HP column (GE Healthcare) connected to an ÄKTA FPLC system (Pharmacia) that had been equilibrated with buffer *A* (50 m*M* Tris–HCl pH 8.4, 300 m*M* NaCl, 50 m*M* imidazole). Protein was eluted from the column using an increasing (50–500 m*M*) imidazole gradient in the same buffer. SimR fractions were identified using SDS–PAGE, pooled together and concentrated to approximately 5–10 mg ml^{-1} using a Vivaspin 6 10 kDa cutoff concentrator (Vivascience). His-tagged SimR is prone to precipitation and further purification by gel-filtration chromatography resulted in very poor yields (data not shown). Although attempts to cleave the affinity tag with TEV protease were successful (as judged by SDS–PAGE), the resultant sample still adhered to the Ni-affinity resin when reapplied onto the Hi-Trap Chelating HP column and could not be eluted even with buffer containing 1.0 *M* imidazole (data not shown). Therefore, for all subsequent preparations a single-column purification procedure was used and the His tag was not cleaved. Directly after SDS–PAGE analysis the pooled fractions were exchanged into crystallization buffer (25 m*M* Tris–HCl pH 8.4, 300 m*M* NaCl) using a Zeba desalting micro column (Thermo Scientific).

Dynamic light scattering (DLS) was used to monitor the solution properties of the purified sample. For this purpose, approximately 30 µl protein solution was centrifuged through a 0.1 µm Ultrafree filter (Millipore) to remove particulate material before introduction into a 12 µl microsampling cell. The cell was inserted into a DynaPro Titan molecular-sizing instrument at 293 K (Wyatt Technology). A minimum of ten scattering measurements were obtained and the resulting data were analysed using the *DYNAMICS* software package (Wyatt Technology).

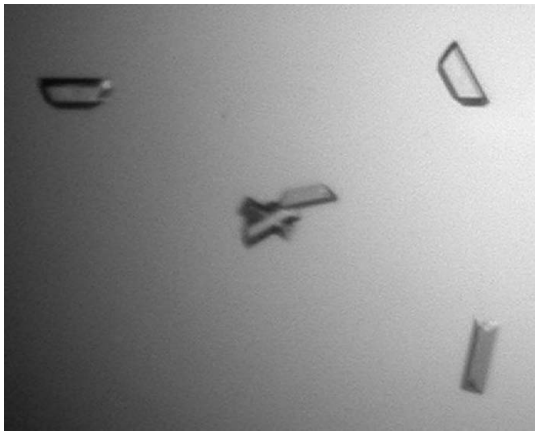


Figure 1
Single crystals of SimR, with approximate dimensions of 200 × 100 × 100 µm.

Crystallization trials of His-tagged SimR were set up using an OryxNano robot (Douglas Instruments Ltd) in sitting-drop vapour-diffusion format with 96-well MRC plates (Molecular Dimensions) using a variety of commercially available screens (Molecular Dimensions and Qiagen) at a constant temperature of 293 K. Drops consisted of 0.3 µl protein solution mixed with 0.3 µl precipitant solution and the reservoir volume was 50 µl; the protein concentration was approximately 5 mg ml^{-1}. Improved crystals were subsequently obtained by refining the successful conditions in a hanging-drop format using 24-well VDX plates (Molecular Dimensions) over a reservoir volume of 1 ml.

2.2. X-ray data collection

Crystals were mounted for X-ray data collection using LithoLoops (Molecular Dimensions) and were flash-cooled by plunging into liquid nitrogen and stored in Uni-puck cassettes prior to transport to the synchrotron. Crystals were subsequently transferred robotically to the goniostat on station I03 of the Diamond Light Source (Oxfordshire, England) and maintained at 100 K with a Cryojet cryocooler (Oxford Instruments). Diffraction data were recorded using an ADSC Quantum 315 CCD detector with the wavelength set to 0.9763 Å and were then processed using *MOSFLM* (Leslie, 2006) and *SCALA* (Evans, 2006).

3. Results and discussion

N-terminally His-tagged SimR was overexpressed and purified with an approximate yield of 40 mg protein from 500 ml culture and was judged to be greater than 95% pure by SDS–PAGE analysis. DLS analysis gave a peak accounting for 99.9% of the mass of the sample with a polydispersity of 16.5%. From these results the molecular size was estimated at 70 kDa, being close to the value expected for a SimR homodimer (64 kDa).

Preliminary crystals of SimR grew within two weeks of setup with 17%(*w*/*v*) PEG 10 000, 0.2 *M* ammonium acetate in 0.1 *M* bis-tris pH 5.5 as the precipitant. Improved crystals were subsequently obtained overnight from 2%(*w*/*v*) PEG 10 000, 0.2 *M* ammonium acetate in 0.1 *M* bis-Tris pH 5.5, with maximum dimensions of approximately 200 × 100 × 100 µm (Fig. 1). We found that subjecting SimR stock solutions to heat treatment prior to setting up crystallizations resulted in better quality crystals. SimR (10 mg ml^{-1}) was incubated at 310 K for 2 min and then cooled in running water at ∼283 K for 1 min and the solution was cleared of precipitated protein by centrifugation through a Millipore 0.1 µm Ultrafree filter before crystallization. SimR crystals were cryoprotected by a three-step transfer (25, 50 and 100% cryosolution) to the final cryosolution [crystallization solution with the addition of 30%(*w*/*v*) PEG 1500 in place of an equivalent volume of water].

Native X-ray data were collected from a single SimR crystal: a total of 200 × 0.45° oscillation images were recorded in a continuous sweep to a maximum resolution of 2.3 Å. Indexing was consistent with a rhombohedral lattice, with unit-cell parameters $a = b = 116.62$, $c = 110.58$ Å. Analysis using the program *POINTLESS* (Evans, 2006) indicated that the space group was *H*32. Data-collection and processing statistics are summarized in Table 1. Estimation of the content of the asymmetric unit indicated that a single His-tagged SimR subunit was most likely, giving a solvent content of 45% and a crystal-packing parameter (V_M) of 2.25 Å^3 Da^{-1} (Matthews, 1968).

Table 1
Summary of X-ray data for SimR.

Values in parentheses are for the outer resolution shell.

No. of crystals	1
Beamline	I03, Diamond Light Source, England
Wavelength (Å)	0.9763
Detector	ADSC Quantum 315 CCD
Crystal-to-detector distance (mm)	368.6
Rotation range per image (°)	0.45
Total rotation range (°)	90.0
Resolution range (Å)	48.50–2.30 (2.42–2.30)
Space group	*H*32
Unit-cell parameters (Å)	$a = b = 116.62$, $c = 110.58$
Mosaicity (°)	0.79
Total no. of measured intensities	63659 (6804)
Unique reflections	12693 (1786)
Multiplicity	5.0 (3.8)
Mean $I/\sigma(I)$	10.2 (2.3)
Completeness (%)	98.0 (95.2)
$R_{\rm merge}$†	0.093 (0.523)
$R_{\rm meas}$‡	0.104 (0.604)
Wilson *B* value (Å^2)	57.9

† $R_{\rm merge} = \sum_{hkl}\sum_i |I_i(hkl) - \langle I(hkl)\rangle| / \sum_{hkl}\sum_i I_i(hkl)$. ‡ $R_{\rm meas} = \sum_{hkl}[N/(N-1)]^{1/2} \times \sum_i |I_i(hkl) - \langle I(hkl)\rangle| / \sum_{hkl}\sum_i I_i(hkl)$, where $I_i(hkl)$ is the *i*th observation of reflection *hkl*, $\langle I(hkl)\rangle$ is the weighted average intensity for all observations *i* of reflection *hkl* and *N* is the number of observations of reflection *hkl*.

A partial molecular-replacement solution for SimR was obtained with the *BALBES* pipeline (Long *et al.*, 2008; with an MR score of 2.63) using the structure of an *S. coelicolor* putative transcriptional regulator (PDB code 2hxi; 25.4% sequence identity to SimR; K. Tan, X. Xu, H. Zheng, A. Savchenko, A. Edwards & A. Joachimiak, unpublished work) as the search model. This template was also the top hit that was found by the *FUGUE* fold-prediction server (http://tardis.nibio.go.jp/fugue/prfsearch.html; Shi *et al.*, 2001) based on the amino-acid sequence of SimR, with a *Z* score of 31.2. In addition, a 3.4 Å resolution single-wavelength anomalous dispersion data set was collected from a crystal of selenomethionine-substituted protein (not shown). The combination of these two sources of phase information enabled us to solve the SimR structure. Full details of the structure-determination process and refinement of the resultant model will be reported elsewhere.

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