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Crystallization and preliminary X-ray diffraction characterization of RpfF, a key DSF synthase from *Stenotrophomonas maltophilia*

Xian-Ya Lin,[a] Shu-Ju Liao,[a] Ko-Hsin Chin,[b] Hui-Ling Shih,[c] Gwan-Han Shen,[d] Andrew H.-J. Wang[c] and Shan-Ho Chou[a,b]*

[a]Institute of Biochemistry, National Chung-Hsing University, Taichung 40227, Taiwan, [b]National Chung-Hsing University Biotechnology Center, National Chung-Hsing University, Taichung 40227, Taiwan, [c]Core Facility for Protein Crystallography, Academia Sinica, Nankang, Taipei, Taiwan, and [d]Department of Internal Medicine, Taichung Veterans General Hospital, Taichung, Taiwan

Correspondence e-mail: shchou@nchu.edu.tw

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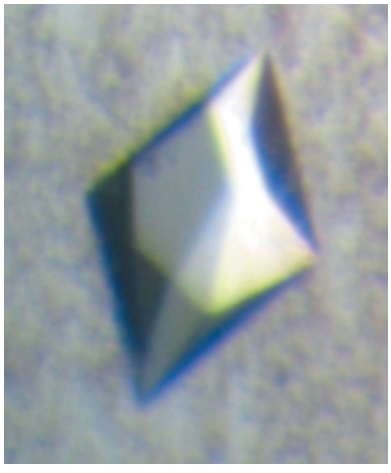
Stenotrophomonas maltophilia has emerged as a critical nosocomial opportunistic pathogen in the last few years. It is resistant to many clinically useful antibiotics; hence, new ways of combatting this bacterium are essential. Diffusible signal factor (DSF) dependent quorum sensing is a major mechanism of virulence induction in *S. maltophilia*, with RpfF playing a key role in DSF biosynthesis. Inhibiting *S. maltophilia* RpfF (*Sm*RpfF) function *via* small-molecule interference may constitute a new way of treating *S. maltophilia* infection. *Sm*RpfF was therefore overexpressed in *Escherichia coli*, purified and crystallized using the hanging-drop vapour-diffusion method. The crystals belonged to the tetragonal space group $P4_12_12$ or $P4_32_12$, with unit-cell parameters $a = b = 148.51$, $c = 122.82$ Å, and diffracted to a resolution of 2.25 Å.



1. Introduction

Antibiotic resistance has developed against virtually every antibiotic employed (Clatworthy *et al.*, 2007). Recently, a new paradigm of targetting bacterial virulence factors rather than bacterial viability has been proposed (Zhang, 2003; Zhang & Dong, 2004; Raffa *et al.*, 2005; Rasmussen & Givskov, 2006*a*,*b*; Bjarnsholt & Givskov, 2007; Clatworthy *et al.*, 2007). Since many opportunistic pathogenic bacteria rely on quorum sensing (QS) to regulate expression of their virulence factors, proteins involved in the QS signal network may serve as new targets for developing the next generation of drugs.

Stenotrophomonas maltophilia is a newly emerging bacterium that deserves much attention (Johnson & Duckworth, 2008; Looney *et al.*, 2009). The sequencing of its genome has recently been completed (Crossman *et al.*, 2008), revealing an organism with a remarkable capacity for drug and heavy-metal resistance. Similar to *Xanthomonas campestris* (Barber *et al.*, 1997; Slater *et al.*, 2000; Zhang & Dong, 2004; Ryan *et al.*, 2006; Dong *et al.*, 2007; He & Zhang, 2008), *S. maltophilia* contains an *rpf* gene cluster (for **r**egulator of **p**athogenicity **f**actors) that is believed to be the major determinant for quorum sensing and for control of pathogenicity gene expression (Fouhy *et al.*, 2007). The *rpf* cluster comprises a number of *rpf* genes involved in regulating the synthesis of extracellular enzymes and polysaccharides. Of these *rpf* gene products, RpfF is the key protein responsible for diffusible signal factor (DSF) synthesis (Tang *et al.*, 1991). Importantly, mutation of the *rpf*F gene was found to significantly reduce the tolerance of *S. maltophilia* to many antimicrobial compounds, indicating that *S. maltophilia* RpfF (*Sm*RpfF) is a potential target for the discovery of drugs based on this novel principle (Fouhy *et al.*, 2007).

However, in order to target RpfF it is necessary to obtain a more thorough understanding of its tertiary structure. Although RpfF has been found to bear some relatedness to enoyl-CoA hydratase, its structure is still unknown and remains to be determined. In this paper, we report the crystallization and preliminary X-ray diffraction characterization of *Sm*RpfF.

2. Materials and methods

2.1. Cloning, expression and purification

The *rpf*F gene fragment was PCR-amplified directly from a local *S. maltophilia* genome with a forward 5′-*TACTTCCAATCCAATG-CT*ATGTCTGCAGTACGCCCCATCA primer and a reverse 5′-*TT ATCCACTTCCAATG*TCAGGCCGGGTCGCCATT primer (the linker sequences are italicized). A ligation-independent cloning (LIC) approach (Aslanidis & de Jong, 1990) was carried out to obtain the desired construct according to a previously published protocol (Wu *et al.*, 2005). The final construct codes for an N-terminal His_6 tag, a 17-amino-acid linker and the *Sm*RpfF target protein (289 amino acids) under the control of a T7 promoter. The vector was transformed into *Escherichia coli* BL21 (DE3) C41 host cells, which were grown in LB medium at 310 K until an OD_{600} of 0.8 was attained. Overexpression of the His_6-tagged target protein was induced by the addition of 0.5 m*M* IPTG at 293 K for 20 h. The cells were harvested, resuspended in equilibration buffer (20 m*M* Tris–HCl pH 8.0, 500 m*M* NaCl and 5 m*M* β-mercaptoethanol) and lysed using a microfluidizer (Microfluidics). Most tagged target proteins were present in the soluble fraction (Fig. 1). After centrifugation, the target protein was purified by immobilized metal-affinity chromatography (IMAC) on a nickel column (Sigma) and eluted with a 50–300 m*M* imidazole gradient in 20 m*M* Tris–HCl pH 8.0, 500 m*M* NaCl and 5 m*M* β-mercaptoethanol. The fractions containing *Sm*RpfF were monitored by SDS–PAGE, recombined and dialyzed repeatedly against 20 m*M* Tris–HCl pH 8.0, 500 m*M* NaCl and 5 m*M* β-mercaptoethanol. After buffer exchange, the His_6 tag and linker were cleaved from *Sm*RpfF by TEV (tobacco etch virus) protease at 295 K for 16 h and removed by immobilized metal-affinity chromatography (IMAC) on a nickel column (Sigma). For crystallization, *Sm*RpfF was further purified by FPLC (ÄKTA, Pharmacia Inc.) on an anion-exchange column (DEAE Sepharose FF). The fractions eluted with 20 m*M* Tris pH 8.0, 1 *M* NaCl were combined and dialyzed against 20 m*M* Tris pH 8.0, 500 m*M* NaCl and 5 m*M* β-mercaptoethanol. The final protein (292 amino acids) had greater than 95% purity and contained a non-native tripeptide (Ser-Asn-Ala) followed by the target protein sequence of 289 amino acids (the GenBank accession No. for *Sm*RpfF is DQ_4156711 and the protein ID is ABD594531). SeMet-substituted *Sm*RpfF was expressed in a similar way except that the cells were induced in SeMet-containing M9 minimal medium when an OD_{600} of 0.8 was reached. The overexpression and purification of SeMet-substituted *Sm*RpfF were monitored by SDS–PAGE as shown in Fig. 1.

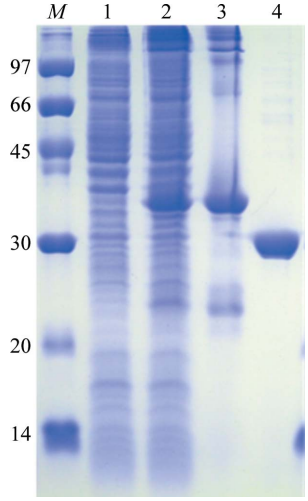
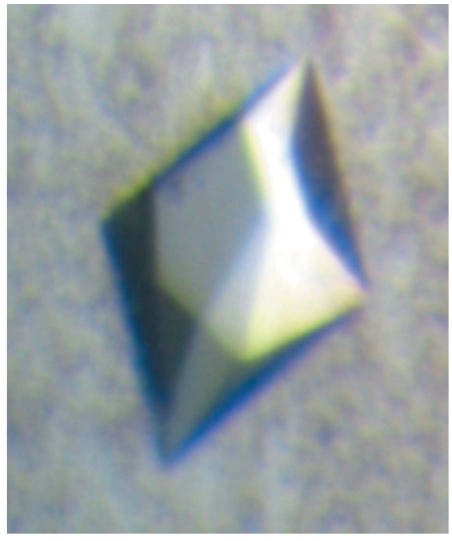


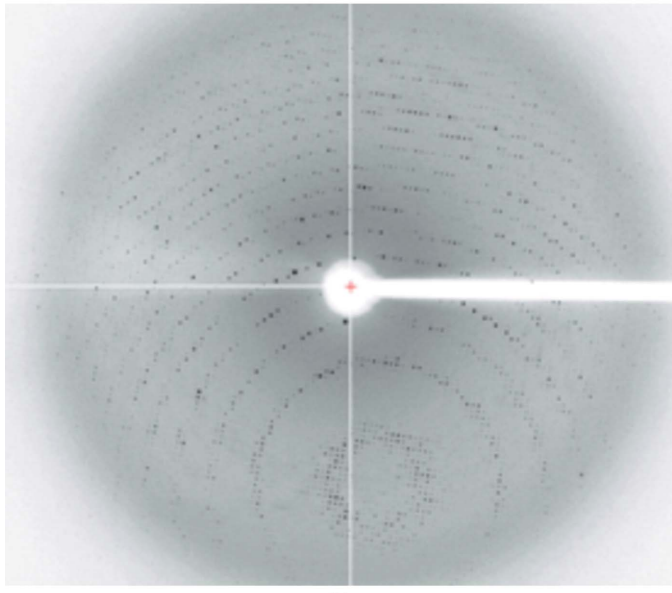
Figure 1
SDS–PAGE monitoring of the overexpression and purification of *Sm*RpfF. Lane *M*, molecular-weight marker standards (kDa); lane 1, whole cell lysate before IPTG induction; lane 2, whole cell lysate after IPTG induction; lane 3, purified *Sm*RpfF before TEV cleavage; lane 4, purified *Sm*RpfF after TEV cleavage.

2.2. Crystallization

For crystallization, the protein was concentrated to 4 mg ml^{-1} in 20 m*M* Tris–HCl pH 8.0, 500 m*M* NaCl and 5 m*M* β-mercaptoethanol using an Amicon Ultra-10 (Millipore). Screening for crystallization conditions of *Sm*RpfF was performed using the sitting-drop vapour-diffusion method in 96-well plates (Hampton Research) at 298 K. 1 µl



(*a*)



(*b*)

Figure 2
Crystal and diffraction pattern of *Sm*RpfF. (*a*) Crystal of *Sm*RpfF obtained by the hanging-drop vapour-diffusion method. The final optimized crystallization condition was 0.1 *M* sodium cacodylate buffer pH 6.5, 0.1 *M* NaH_2PO_4 and 1 *M* sodium acetate. The dimensions of these diamond-shaped crystals were approximately 0.12 × 0.07 × 0.03 mm. (*b*) Picture of the diffraction pattern of *Sm*RpfF collected at the Taiwan NSRRC synchrotron facility (beamline 13B1) from a flash-frozen crystal in 20%(*v*/*v*) glycerol cryoprotectant. The exposure time was 10 s and the oscillation range was 0.5° per frame, with a crystal-to-detector distance of 220 mm.

Table 1
Summary of native and Se-MAD crystallographic data for *Sm*RpfF.

Values in parentheses are for the outermost shell.

	Inflection	High remote	Native
Beamline	NSRRC BL13B1		
Wavelength (Å)	0.97913	0.96391	0.97622
Space group	$P4_12_12$ or $P4_32_12$	$P4_12_12$ or $P4_32_12$	$P4_12_12$ or $P4_32_12$
Unit-cell parameters (Å)	$a = b = 147.79$, $c = 122.04$	$a = b = 147.78$, $c = 122.04$	$a = b = 148.51$, $c = 122.82$
Resolution range (Å)	30–3.46 (3.58–3.46)	30–3.46 (3.58–3.46)	30–2.25 (2.33–2.25)
Unique observations	18195 (1739)	18153 (1720)	64872 (6401)
Redundancy	9.3 (8.8)	9.3 (8.9)	7.0 (4.9)
Completeness (%)	99.7 (97.9)	99.6 (97.6)	98.8 (98.7)
$R_{\rm merge}$† (%)	17.7 (62.8)	14.3 (63.5)	6.9 (51.9)
$\langle I/\sigma(I)\rangle$	13.1 (3.1)	13.8 (3.1)	22.4 (3.1)

† $R_{\rm merge} = \sum_{hkl}\sum_i |I_i(hkl) - \langle I(hkl)\rangle| / \sum_{hkl}\sum_i I_i(hkl)$, where $I_i(hkl)$ is the intensity of the ith observation of reflection hkl and $\langle I(hkl)\rangle$ is the average intensity of redundant measurements of reflection hkl.

drops comprising equal volumes of protein solution and reservoir solution were equilibrated against 50 µl reservoir solution. Initial screens, including Hampton Research sparse-matrix Crystal Screens 1 and 2, a systematic PEG–pH screen and the PEG/Ion Screen, were performed using a Gilson C240 crystallization workstation. Diamond-shaped crystals appeared in 3 d from a reservoir solution comprising 0.1 *M* sodium cacodylate buffer pH 6.5, 0.1 *M* NaH_2PO_4 and 1 *M* sodium acetate. Crystals suitable for diffraction experiments were grown by mixing 1.5 µl protein solution with 1.5 µl reagent solution and equilibrating against 500 µl reservoir solution. The crystals reached maximum dimensions of 0.12 × 0.07 × 0.03 mm after one week (Fig. 2*a*).

2.3. Data collection

The *Sm*RpfF crystals were soaked in a cryoprotectant solution comprising reservoir solution plus 20%(*v*/*v*) glycerol. X-ray diffraction data were collected on National Synchrotron Radiation Research Center (NSRRC) beamline 13B1 in Taiwan. A native data set was collected to 2.25 Å resolution and a two-wavelength multiple anomalous dispersion (MAD) data set was collected to a resolution of 3.46 Å. The data were indexed and integrated using the *HKL*-2000 processing software (Otwinowski & Minor, 1997), yielding three data sets, each 99% complete, with $R_{\rm merge}$ values in the range 6.9–17.7%. The *Sm*RpfF crystals belonged to the tetragonal space group $P4_12_12$ or $P4_32_12$, which cannot be discriminated until the final structure has been solved. The data-collection statistics are summarized in Table 1 and an X-ray diffraction image is shown in Fig. 2(*b*).

3. Results and discussion

In this manuscript, we report the successful cloning, expression and crystallization of RpfF from *S. maltophilia*. We have also obtained high-resolution X-ray diffraction data from native crystals and moderate-resolution data from SeMet-substituted crystals of *Sm*RpfF (Table 1 and Fig. 2). Analyses of the diffraction intensities indicated that the space group was either $P4_12_12$ or $P4_32_12$, with unit-cell parameters $a = b = 147.79$, $c = 122.04$ Å. The Matthews coefficient $V_{\rm M}$ (Matthews, 1968) suggested the presence of three, four or five *Sm*RpfF protein molecules per asymmetric unit, with solvent contents of 63.64, 51.52 or 39.40%, respectively. There are a total of 12 methionine residues in *Sm*RpfF. Their substitution by SeMet should provide sufficient phasing power to solve the *Sm*RpfF structure. In fact, we have already obtained the preliminary Se substructure (data not shown). We are confident that we have the data required for structure solution and work towards solution is currently ongoing.

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