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Citation for published version:

Quin, M, Newman, J, Firbank, S, Lewis, RJ & Marles-Wright, J 2008, 'Crystallization and preliminary X-ray analysis of RsbS from *Moorella thermoacetica* at 2.5 Å resolution', *Acta Crystallographica Section F Structural Biology and Crystallization Communications*, vol. 64, no. Pt 3, pp. 196-199.
<https://doi.org/10.1107/S1744309108003849>

Digital Object Identifier (DOI):

[10.1107/S1744309108003849](https://doi.org/10.1107/S1744309108003849)

Link:

[Link to publication record in Edinburgh Research Explorer](#)

Document Version:

Publisher's PDF, also known as Version of record

Published In:

Acta Crystallographica Section F Structural Biology and Crystallization Communications

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Received 16 January 2008
 Accepted 5 February 2008

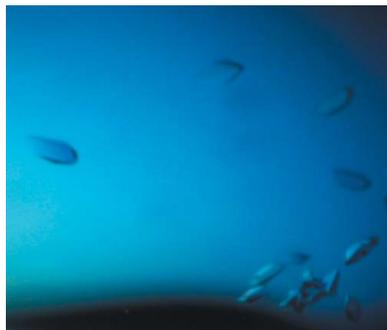
Crystallization and preliminary X-ray analysis of RsbS from *Moorella thermoacetica* at 2.5 Å resolution

The thermophilic bacterium *Moorella thermoacetica* possesses an *rsb* operon that is related to the genetic locus common to many Gram-positive bacteria that regulates the activity of the stress-responsive sigma factor σ^B . One of the gene products of this operon is RsbS, a single STAS-domain protein that is a component of higher order assemblies in *Bacillus subtilis* known as 'stressosomes'. It is expected that similar complexes are found in *M. thermoacetica*, but in this instance regulating the biosynthesis of cyclic di-GMP, a ubiquitous secondary messenger. Selenomethionine-labelled *MtRsbS* protein was crystallized at room temperature using the hanging-drop vapour-diffusion method. Crystals belonging to space group $P2_12_12_1$, with unit-cell parameters $a = 51.07$, $b = 60.52$, $c = 89.28$ Å, diffracted to 2.5 Å resolution on beamline I04 of the Diamond Light Source. The selenium substructure was solved using *SHELX* and it is believed that this represents the first reported *ab initio* crystal structure to be solved using diffraction data collected at DLS.

1. Introduction

Moorella thermoacetica, previously known as *Clostridium thermoaceticum*, is a rod-shaped Gram-positive thermophilic acetogen that was originally isolated from horse manure (Fontaine *et al.*, 1942). The normal habitat for *M. thermoacetica* is soil and it grows at an optimum temperature of 328–333 K; however, other strains have been isolated from spoiled canned foods, possibly owing to the microorganism's ability to produce heat-resistant spores that can withstand temperatures as high as 394 K (Byrer *et al.*, 2000). *M. thermoacetica* converts glucose to acetate via the acetyl-CoA pathway, using CO₂, nitrate and nitrite or thiosulfate as terminal electron acceptors (Drake & Daniel, 2004; Carlier & Bedora-Faure, 2006).

Because this species is a spore-forming Gram-positive bacterium, it would seem reasonable to expect that *M. thermoacetica* might employ a similar system to deal with environmental stress to that of *Bacillus subtilis*, the Gram-positive endospore-forming paradigm. The response of *B. subtilis* to environmental stress is organized by σ^B , a subunit of RNA polymerase (Hecker & Volker, 2001). The availability of σ^B to form the holo-form of RNA polymerase is controlled predominantly by the gene products of the *rsb* operon (Kang *et al.*, 1996; Wise & Price, 1995). At the very top of this signalling cascade stands the stressosome, a >1.5 MDa signalling complex comprising in the minimal sense RsbR, RsbS and RsbT (Delumeau *et al.*, 2006; Chen *et al.*, 2003). However, unlike in *B. subtilis*, the RsbRST module in *M. thermoacetica* (*MtRsbRST*) does not regulate stress resistance (Pane-Farre *et al.*, 2005). Instead, the presence of a GGDEF domain downstream of the *rsbRST* operon indicates that *M. thermoacetica* utilizes the RsbRST module to coordinate biosynthesis of the nucleotide cyclic diguanosine monophosphate (cyclic di-GMP; Romling & Amikam, 2006). Cyclic di-GMP was recognized as a signalling molecule during the 1980s by Benziman and coworkers, regulating cellulose synthesis in *Gluconacetobacter xylinum* (Ross *et al.*, 1990; Amikam & Benziman, 1989), but its ubiquity indicates its generic importance to signal transduction.



Stressosomes in *B. subtilis* comprise in the minimal sense multiple copies of RsbS, a single STAS (sulfate transporter and anti-antisigma factor) domain protein that associates with several molecules of RsbR to form >1.5 MDa complexes that sequester RsbT (Pane-Farre *et al.*, 2005; Delumeau *et al.*, 2006; Chen *et al.*, 2003; Kim *et al.*, 2004). RsbT is released during the response to stress by phosphorylating conserved serine and threonines in the STAS domains of RsbS, RsbR (and paralogues) and in doing so transduces the stress signal to σ^B . Because of the high degree of sequence conservation, it is anticipated that the *MtRsbRST* module forms similar structures in regulating secondary messenger biosynthesis. In this paper, we report the expression, purification, crystallization and preliminary X-ray analysis of the 123-amino-acid 13 566 Da *MtRsbS* protein, the crystal structure of which will provide a structural basis for the interpretation of the interactions in the core of the stressosome and of the binding and release of RsbT (Chen *et al.*, 2003; Pane-Farre *et al.*, 2005).

2. Overexpression and purification of *MtRsbS*

A DNA fragment encompassing the *rsbS* gene from *M. thermoacetica* ATC39073 (NCBI gene ID 3832355) was amplified from *M. thermoacetica* chromosomal DNA using the Pfu ultrahigh-fidelity polymerase (Roche). The oligonucleotides RsbSFwd, 5'-GGAATT-CATATGAGTAGCCGGGTGCCCATCCTG-3', and RsbSRev, 5'-CGGGATCCTTATCGCTGCTCCATTCCGGGC-3', were used as PCR primers (the *Nde*I and *Bam*HI restriction sites, respectively, are shown in bold). The 372-base-pair product was cloned into the pET24a vector and the resultant plasmid was named S41.

Escherichia coli B834 cells were transformed with the resulting plasmid and used to produce protein as follows. A 100 ml overnight culture was inoculated into 1 l minimal M9 medium supplemented with amino acids (with selenomethionine replacing methionine), vitamins and 50 mg ml⁻¹ kanamycin, which was then incubated at 308 K. When the culture reached mid-log phase (OD₆₀₀ = 0.4), expression of the protein was induced by adding IPTG to a final concentration of 1 mM. The cells were grown for an additional 16 h at 291 K and harvested by centrifugation.

The harvested cells were resuspended in buffer A (20 mM Tris-HCl pH 7.5, 1 mM DTT) and disrupted by sonication. The crude cell lysate was centrifuged at 15 000g for 30 min at 277 K and the supernatant was further clarified by passing it through a 0.45 µm syringe-filter device (Millipore). The protein was loaded onto a 25 ml

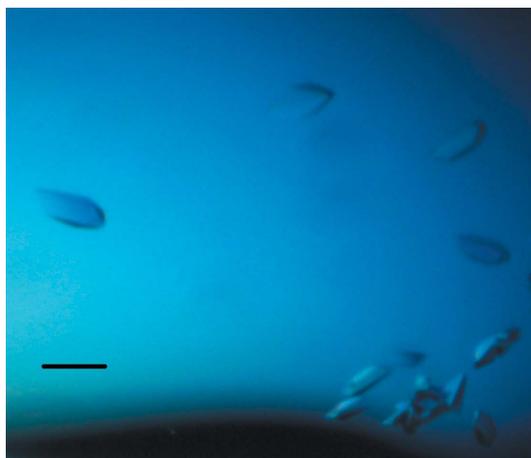


Figure 1
Crystals of *MtRsbS*. The black scale bar represents 100 µm.

Table 1

Data-collection and processing statistics for *MtRsbS*.

Values in parentheses are for the highest resolution shell.

Data collection	
X-ray source	DLS (I04)
Wavelength (Å)	0.9699
Detector	ADSC CCD
Crystal-to-detector distance (mm)	250
Space group	<i>P</i> 2 ₁ 2 ₁ 2 ₁
Unit-cell parameters	
<i>a</i> (Å)	51.07
<i>b</i> (Å)	60.52
<i>c</i> (Å)	89.28
Resolution	2.5 (2.64–2.5)
<i>V</i> _M (Å ³ Da ⁻¹)	2.54
Solvent content (%)	51.62
Monomers in ASU	2
Data processing	
No. of observed reflections	145887
No. of unique reflections	10052
Completeness	99.8 (100)
Anomalous completeness	99.9 (100)
Multiplicity	14.5 (15.0)
Anomalous multiplicity	7.8 (7.9)
Mean <i>I</i> /σ(<i>I</i>)	22.4 (6.2)
<i>R</i> _{merge} [†]	0.114 (0.478)

[†] $R_{\text{merge}} = \frac{\sum_{hkl} \sum_i |I_i(hkl) - \langle I(hkl) \rangle|}{\sum_{hkl} \sum_i I_i(hkl)}$, where $I_i(hkl)$ is the *i*th observation of reflection *h* and $\langle I(hkl) \rangle$ is the weighted average intensity for all observations *i* of reflection *h*.

Q-Sepharose fast-flow anion-exchange column (Amersham Biosciences) pre-equilibrated with buffer A and bound proteins were eluted over a 250 ml linear gradient to buffer A with 1 M NaCl. Fractions containing *MtRsbS* as identified by SDS-PAGE were further purified by gel filtration using an S75 16/60 size-exclusion column pre-equilibrated with buffer B (20 mM Tris-HCl pH 7.5, 200 mM NaCl, 1 mM DTT). Purified protein was subsequently concentrated to 6 mg ml⁻¹ for crystallization experiments.

3. Crystallization of RsbS

Crystallization of purified protein in 20 mM Tris-HCl pH 7.5, 200 mM NaCl, 1 mM DTT buffer was initially performed with commercially available screens from Qiagen using the sitting-drop vapour-diffusion technique. Each experiment was set up using a Mosquito (TTP Labtech) nanolitre pipetting robot and MRC crystallization plates containing 100 µl each of 96 conditions from the PACT screen. 100 nl protein solution was pipetted into the sample well of each of the 96 wells of the crystallization plate followed by 100 nl of each crystallant. Plates were sealed with clear sealing films and allowed to equilibrate at 294 K. *MtRsbS* crystals were observed in a number of conditions (PACT B10, B11, D10, D11, F4 and F9). An optimization screen for each of these conditions was set up in 24-well trays using the hanging-drop vapour-diffusion technique with 1 ml of crystallant in the reservoir and 1 µl protein solution mixed with 1 µl crystallant. Crystals appeared in 2 d from drops containing 0.2 M potassium thiocyanate, 0.1 M bis-tris propane pH 6.5, 20% (w/v) PEG 3350 (Fig. 1).

4. X-ray analysis

4.1. Data collection and processing

The crystals were transferred to a cryoprotectant solution containing 0.2 M potassium thiocyanate, 0.1 M bis-tris propane pH 6.5, 20% (w/v) PEG 3350 and 20% (v/v) PEG 300, mounted in a

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