Crystallographic analysis of RsmA, a ribosomal RNA small subunit methyltransferase A from *Staphylococcus aureus*

Yang Liu,^{a,b} Yuwei Zhu,^{a,b} Maikun Teng^{a,b*} and Xu Li^{a,b*}

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^aHefei National Laboratory for Physical Sciences at Microscale, Innovation Center for Cell Signaling Network, School of Life Science, University of Science and Technology of China, Hefei, Anhui 230026, People's Republic of China, and ^bKey Laboratory of Structural Biology, Hefei Science Center of CAS, Chinese Academy of Science, Hefei, Anhui 230026, People's Republic of China. *Correspondence e-mail: mkteng@ustc.edu.cn, sachem@ustc.edu.cn

RsmA, a ribosomal RNA small subunit methyltransferase from *Staphylococcus aureus*, catalyzes the *N*⁶ methylation of adenine in 16S rRNA. In this study, RsmA from *Staphylococcus aureus* was cloned, expressed, purified and crystallized. The crystal belonged to space group C2, with unit-cell parameters $a = 84.38$, $b = 157.76$, $c = 96.50$ Å, $\beta = 95.04^\circ$. X-ray diffraction data were collected to a resolution of 3.2 Å. The self-rotation function and the Matthews coefficient suggested the presence of two molecules in the asymmetric unit.

1. Introduction

The ribosomal RNAs (rRNAs) from all living organisms contain a number of both base- and 2'-*O*-methylated nucleotides (Bachelier & Cavallé, 1997). The conservation of these methylated residues is not strict or pervasive, but the great majority of the methylation sites are present in the same region of rRNA: the peptidyl-transferase centre in large ribosomal subunit rRNA and the decoding centre in small ribosomal subunit rRNA (Decatur & Fournier, 2002). On the basis of this observation and of numerous functional studies, it was suggested that rRNA methylations play a role in the efficiency and fidelity of mRNA decoding by the ribosome.

Detailed studies of rRNA methylation have mainly focused on several model prokaryotic and eukaryotic organisms such as *Escherichia coli*, *Thermus thermophilus*, *Saccharomyces cerevisiae*, *Haloarcula marismortui*, *Arabidopsis thaliana* and *Homo sapiens*. The studies of rRNAs from other species are still limited. To date, almost all rRNA methylation sites in the 70S ribosome of *E. coli* are known. In *E. coli*, analysis of the methylation sites in the 30S ribosomal subunit revealed two main functions, subunit-assembly control and translation-initiation control, while the main function of 50S subunit methylation may be stabilization of the RNA structure (Sergeeva *et al.*, 2014).

Bacterial rRNA methylation can cause specific resistance to some antibiotics, most frequently to aminoglycosides, macrolides and lincosamides (Chow *et al.*, 2007). On the other hand, there are also examples of antibiotic sensitivity caused by rRNA methylation. Methylation of the G527 nucleotide of the 16S rRNA leads to streptomycin sensitivity (Brulé *et al.*, 1998). Methylation of the A1518 and A1519 nucleotides of the 16S rRNA causes kasugamycin sensitivity (Kellner *et al.*, 2010), while methylation of ribose at nucleotides 1409 in the 16S rRNA and 1920 in the 23S rRNA is essential for the antimicrobial activity of capreomycin (Horie *et al.*, 1985).

Table 1
Cloning details.

Source organism	<i>S. aureus</i> strain NCTC 8325
DNA source	3920324
Forward primer†	5′-CGCGGATCCATGTTGGATAATAAGATATTG- CAACAC-3′
Reverse primer‡	5′-CCGCTCGAGTTAATTTCTAATTGAGGGAAT- TTTTTC-3′
Cloning vector	p28 (Novagen, a modification of pET-28a) with a hexahistidine tag (MGHHHHHH) at the N-terminus
Expression vector	p28 (Novagen, a modification of pET-28a) with a hexahistidine tag (MGHHHHHH) at the N-terminus
Expression host	<i>E. coli</i> Rosetta (DE3) cells (Novagen)
Complete amino-acid sequence of the construct produced	MGHHHHHMGSLDNKDIAITPSRTRALLDKYGFN- FKKSLGQNFLIDVNIINNIIDASDIDAQTGVI- EIGPGMGSLTEQLARHAKRVLAFEIDQRLIPV- LNDTLSPYDNTVTINEDILKANIKEAVENHLQ- DCEKIMVVANLPYYITPILLNLMQQDIPIDG- YVMMQKEVGERLNAEVGSKAYGSLSIIVVQYY- TETSKVLTVPKSVFMPPNVDISIVVKLMQRTE- PLVTVDNEEAFFKLAKAAFAQRRTKINNYYQN- YFKDQKQHKVEVLQWLEQAGIDPRRRGETLSI- QDFAKLYEEKKKFPQLEN

† The BamHI site is underlined. ‡ The XhoI site is underlined.

Staphylococcus aureus, a Gram-positive microorganism, is an important human pathogen which can cause diseases that range in severity from mild to life-threatening. *S. aureus* is notorious for its ability to become resistant to any antibiotic. Some severe syndromes or pathologies, including pneumonia, sepsis and endocarditis, have been compounded by the emergence of methicillin-resistant and vancomycin-resistant *S. aureus* (Miller *et al.*, 2005). Recently, a ribosomal RNA small subunit methyltransferase A (RsmA) has been identified in *S. aureus* and is conserved throughout evolution: as KsgA in *E. coli* (Desai & Rife, 2006), Dim1 in *S. cerevisiae* (Lafontaine *et al.*, 1995), mitochondrial mtTFB in *H. sapiens* (Seidel-Rogol *et al.*, 2003) and KsgA in *Coxiella burnetii* (PDB entry 3tqs; Franklin *et al.*, 2015). Previous studies have revealed that members of the KsgA/Dim1 methyltransferase family undertake the highly conserved dimethylation of two adjacent adenosine bases in the loop of helix 45, near the 30 end of the small subunit rRNA, to produce *N*⁶,*N*⁶-dimethyladenosine (O’Farrell *et al.*, 2004). The KsgA/Dim1 methyltransferase family plays an important role in the assembly of the small ribosomal subunit. In the absence of KsgA, 30S subunit biogenesis is slowed; in particular, rRNA processing is retarded when compared with 30S subunit biogenesis in the presence of KsgA (Connolly *et al.*, 2008). The structures of the KsgA/Dim1 methyltransferase family members are very similar to each other. They share a two-domain architecture, with a larger N-terminal domain consisting of a Rossmann-like fold characteristic of *S*-adenosylmethionine-utilizing MTases (known as the AdoMet-dependent MTase fold) and a smaller C-terminal domain consisting of 4–5 α -helices (O’Farrell *et al.*, 2004). On the basis of the functional and structural conservation in the KsgA/Dim1 methyltransferase family, *S. aureus* RsmA might also play an indispensable role in the assembly of the small ribosomal subunit, and might be a possible antimicrobial drug target in *S. aureus*. To investigate the structural

characteristics of *S. aureus* RsmA in rRNA methylation and the assembly of the small ribosomal subunit, a crystallization and preliminary X-ray crystallographic study was carried out and is reported here.

2. Materials and methods

2.1. Cloning, expression and purification

The *rsmA* gene was amplified from *S. aureus* strain NCTC 8325 by polymerase chain reaction (Table 1). The PCR fragment was digested by the BamHI and XhoI restriction endonucleases and then inserted into expression vector p28 (Novagen, a modification of pET-28a) to give a hexahistidine tag (MGHHHHHH) at the N-terminus of the recombinant RsmA. After sequencing, the plasmid was transformed into *E. coli* Rosetta (DE3) cells (Novagen). The transformant was grown in 1 l Luria–Bertani (LB) medium containing 50 μ g ml^{−1} kanamycin at 310 K. When an OD₆₀₀ of 0.6–0.8 was reached, 1 mM isopropyl β -D-1-thiogalactopyranoside (IPTG)

Figure 1
(a) SDS-PAGE analysis of RsmA. (b) Size-exclusion chromatography of RsmA. The elution volume of RsmA is 15.46 ml, which is consistent with a monomeric state (black curve); the red curve indicates the elution positions of standard molecular-weight protein markers (labelled in kDa).

Table 2
Crystallization.

Method	Vapour diffusion
Plate type	Hanging-drop plate
Temperature (K)	289
Protein concentration (mg ml ⁻¹)	25
Buffer composition of protein solution	25 mM Tris-HCl pH 7.5, 500 mM NaCl
Composition of reservoir solution	1.6 M lithium chloride, 0.1 M Tris-HCl pH 8.0
Volume and ratio of drop	2 µl, 1:1
Volume of reservoir (µl)	200

was added for induction. After 22 h of induction at 289 K, the cells were harvested by centrifugation at 8000g for 7 min.

The harvested cells were suspended in buffer A (25 mM Tris-HCl pH 7.5, 500 mM NaCl) and lysed by sonication on ice. The soluble portion was obtained after centrifugation at 12 000g for 35 min and was applied onto a Ni-NTA column (Qiagen) pre-equilibrated with buffer A. The bound protein was eluted with buffer A containing 200 mM imidazole. The collected fractions were concentrated to 2 ml by centrifugal ultrafiltration (Millipore, 10 kDa cutoff) and the target protein was purified using a HiLoad 10/300 Superdex 200 gel-filtration chromatography column (GE Healthcare) previously equilibrated with buffer A. All purification steps were performed at 288 K. The purity of the target protein was estimated by SDS-PAGE (Fig. 1*a*). Macromolecule-production information is summarized in Table 1.

2.2. Crystallization

The recombinant RsmA was concentrated to 25 mg ml⁻¹ in buffer A (calculated from the A_{280} using a molar absorption coefficient of 21 890 M⁻¹ cm⁻¹; Eppendorf BioPhotometer plus) by centrifugal ultrafiltration (Millipore; 10 kDa cutoff) prior to crystallization trials. Initial crystallization trials were performed using the hanging-drop vapour-diffusion method by mixing 1 µl protein solution and 1 µl reservoir solution and equilibrating against 200 µl reservoir solution using the Crystal Screen, Crystal Screen 2, Index, PEG/Ion and PEG/Ion 2 reagent kits (Hampton Research) at 289 K. Crystals

Figure 2
Crystals of recombinant RsmA grown under the condition 1.6 M lithium chloride, 0.1 M Tris-HCl pH 8.0.

Table 3
Data-collection and refinement statistics.

Values in parentheses are for the outer shell.

Diffraction source	BL-17U, SSRF
Wavelength (Å)	1.28211
Temperature (K)	100
Detector	Jupiter CCD
Crystal-to-detector distance (mm)	250
Rotation range per image (°)	1
Total rotation range (°)	180
Exposure time per image (s)	1
Space group	C2
Unit-cell parameters (Å, °)	$a = 84.38$, $b = 157.76$, $c = 96.50$, $\beta = 95.04$
Mosaicity (°)	0.868
Resolution range (Å)	30.00–3.20 (3.31–3.20)
Total No. of reflections	78114
No. of unique reflections	20444
Completeness (%)	98.9 (98.4)
Multiplicity	3.8 (3.8)
$\langle I/\sigma(I) \rangle$	14.6 (2.8)
$R_{\text{r.i.m.}}^{\dagger}$ (%)	7.5 (39.5)
Overall B factor from Wilson plot (Å ²)	56.7

$\dagger R_{\text{r.i.m.}} = \sum_{hkl} \{ [N(hkl)/[N(hkl) - 1]]^{1/2} \sum_i |I_i(hkl) - \langle I(hkl) \rangle| / \sum_{hkl} \sum_i I_i(hkl) \}$ is the redundancy-independent merging R factor, where $N(hkl)$ is the data multiplicity.

were observed in several conditions after 2 d. Single crystals suitable for X-ray diffraction measurement grew in drops consisting of 1.6 M lithium chloride, 0.1 M Tris-HCl pH 8.0 (Fig. 2). Crystallization information is summarized in Table 2.

2.3. Data collection and processing

For data collection, crystals were first flash-cooled in liquid nitrogen with a cryoprotectant solution consisting of 1.6 M lithium chloride, 0.1 M Tris-HCl pH 8.0, 20%(v/v) glycerol and were then transferred into a nitrogen-gas stream at 100 K.

Figure 3
A representative diffraction image of an *S. aureus* RsmA crystal.

X-ray diffraction data were collected on beamline 17U1 at the Shanghai Synchrotron Radiation Facility (SSRF) using a Jupiter CCD detector (Fig. 3). All frames were collected at 100 K using a 1° oscillation angle. The crystal-to-detector distance was set to 250 mm. The complete diffraction data set was subsequently processed using *HKL-2000* (Otwinowski & Minor, 1997). The detailed data-processing statistics are shown in Table 3.

3. Results and discussion

Full-length recombinant RsmA from *S. aureus* was cloned, overexpressed and purified as a monomer in solution (as calculated by gel-filtration chromatography; Fig. 1*b*), and the final yield of the recombinant protein was as high as 15 mg per litre of culture. Prismatic crystals grew in 2 d. SDS-PAGE of the washed crystals and the purified RsmA revealed that no proteolysis took place during the crystallization process (Fig. 1*a*). A total of 180 diffraction images were recorded from a single crystal. The diffraction data were processed to 3.2 Å resolution. The RsmA crystals belonged to space group *C*2, with unit-cell parameters $a = 84.38$, $b = 157.76$, $c = 96.50$ Å, $\beta = 95.04^\circ$. The Matthews coefficient calculated using the *CCP4* suite (Winn *et al.*, 2011) suggested that the presence of two to five monomers in the asymmetric unit would give a reasonable V_M ($1.83\text{--}4.57$ Å³ Da⁻¹; Matthews, 1968). The self-rotation function only showed clear peaks in the $\kappa = 180^\circ$ section, indicating the presence of an NCS dimer. Therefore, two molecules may be present in the asymmetric unit, with a solvent content of 73.10% (Winn *et al.*, 2011).

Structure determination of recombinant *S. aureus* RsmA has been attempted by the molecular-replacement method using the solution structure of the dimethyladenosine transferase (KsgA) from *C. burnetii* as a search model (PDB entry 3tqs; 32% amino-acid sequence identity; Franklin *et al.*, 2015). However, a molecular-replacement solution could not be found and experiments to obtain crystals of selenomethionine-substituted protein are currently being performed. Experiments to obtain *S. aureus* RsmA crystals that diffract to higher resolution and attempts to obtain crystals of RsmA–SAM or RsmA–RNA (with a 22-base RNA fragment from 16S rRNA; AGCCGUAUCGGAAGGUGCGGCU; Tu *et al.*, 2009) complexes are also under way.

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