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Expression, purification and crystallization of adenosine 1408 aminoglycoside-resistance rRNA methyltransferases for structural studies

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Abstract

High-level resistance to a broad spectrum of aminoglycoside antibiotics can arise through either N7-methyl guanosine 1405 (m⁷G1405) or N1-methyl adenosine 1408 (m¹A1408) modifications at the drug binding site in the bacterial 30S ribosomal subunit decoding center. Two distinct families of 16S ribosomal RNA (rRNA) methyltransferases that incorporate these modifications were first identified in aminoglycoside-producing bacteria but were more recently identified in both human and animal pathogens. These resistance determinants thus pose a new threat to the usefulness of aminoglycosides as antibiotics, demanding urgent characterization of their structures and activities. Here, we describe approaches to cloning, heterologous expression in *E. coli*, and purification of two A1408 rRNA methyltransferases: KamB from the aminoglycoside-producer *Streptoalloteichus tenebrarius* and NpmA identified in a clinical isolate of pathogenic *E. coli* ARS3. Antibiotic minimum inhibitory concentration (MIC) assays and *in vitro* analysis of KamB and NpmA using circular dichroism (CD) spectroscopy, *S*-adenosyl-L-methionine (SAM) binding by isothermal titration calorimetry and 30S subunit methylation assays showed both enzymes were soluble, folded and active. Finally, crystals of each enzyme complexed with SAM were obtained, including selenomethionine-derived KamB, that will facilitate high-resolution X-ray crystallographic analyses of these important bacterial antibiotic-resistance determinants.

INTRODUCTION

Detailed molecular structure-function studies of antibiotic resistance determinants in bacterial populations are urgently needed to support development of new strategies to counter their increasing threat to the clinical use of antibiotics. Among the mechanisms of resistance to the

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ribosome-targeting aminoglycoside antibiotics are the expression of enzymes that chemically modify either the drug or its binding site within the 16S ribosomal RNA (rRNA) [1]. Among the latter group, two families of aminoglycoside-resistance rRNA methyltransferase enzymes have been identified that confer overlapping but distinct resistance phenotypes against different structural classes of aminoglycosides [2]. These enzymes methylate either N1 of A1408 or N7 of G1405 to produce m¹A1408 and m⁷G1405 respectively. The aminoglycoside-resistance 16S rRNA methyltransferases were first identified in drug-producing bacteria [3] but are now increasingly being identified as a cause of aminoglycoside resistance in both human and animal pathogens [4,5]. Recently, major advances were made with the first high resolution crystal structure determination of two examples of the G1405 methyltransferase family, Sgm [6] and RmtB [7], from aminoglycoside-producing and pathogenic bacteria respectively. However, to date, equivalent structures for the A1408 methyltransferases are lacking. Thus methods for expression and purification of these proteins to facilitate their detailed structural and functional characterization are urgently needed.

Here, we describe the expression, purification and characterization of the A1408 rRNA methyltransferases KamB from the nebramycin producer *Streptoalloteichus tenebrarius* [8,9] and its plasmid-borne ortholog NpmA from human pathogenic *Escherichia coli* ARS3 [10]. The recombinant enzymes expressed from synthetic genes exhibit characteristic resistance phenotypes in *E. coli*, are active *in vitro* and form protein crystals suitable for high-resolution structural analyses. Furthermore, we have found the approaches described here to be generally applicable to other rRNA methyltransferases of both the Class I and IV (SPOUT) RNA methyltransferase superfamilies [12].

MATERIALS AND METHODS

Construction of plasmids encoding functional A1408 rRNA methyltransferases

Genes encoding methyltransferases KamB (NCBI CAF33037) and NpmA (NCBI BAF80809) were synthesized with codon optimization for expression in *E. coli* (GeneArt). The *kamB* gene included DNA sequence encoding an additional 60 amino acids at the protein N-terminus identified as critical for expression of an active methyltransferase enzyme [2,11]. Both were cloned into the vector pET44a (Novagen) as *NdeI*/*HindIII* fragments using restriction enzyme sites incorporated during gene synthesis to produce expression plasmids pET44a-KamB and pET44a-NpmA. The expressed proteins contained a short N-terminal peptide sequence (MGS) from translation of sequence corresponding to an additional restriction enzyme site (*Bam*HI) 3' of the *NdeI* cloning site. No other additional amino acids or purification tags were added to the authentic KamB or NpmA protein sequences.

Antibiotic minimum inhibitory concentrations (MIC) were measured in liquid media for *E. coli* BL21 (DE3) transformed with empty pET44a, pET44a-KamB or pET44a-NpmA plasmid. Initial cultures for each experiment were grown to saturation in Luria-Bertani (LB: 5 g yeast extract, 10 g peptone from casein, and 10 g sodium chloride in 1 L deionised water) medium supplemented with 100 µg/ mL ampicillin. 10 ml fresh LB medium containing 0.05 mM isopropyl-β-D-thio-galactoside (IPTG) and various concentrations (0 – 1200 µg/ mL) of kanamycin, gentamicin, neomycin or apramycin was inoculated at 1:100 dilution using each starter culture and incubated at 37 °C for 16 hours with vigorous shaking. Assays were performed at least in triplicate and the MIC was defined as the lowest concentration of antibiotic for which no growth could be detected ($A_{600} < 0.05$).

Protein expression and purification

Chemically competent *E. coli* BL21 (DE3) were transformed with pET44a-KamB or pET44a-NpmA, a single colony of each used to inoculate 10 ml of LB medium with 100 µg/ mL

ampicillin and cultures grown to saturation at 37 °C. These cultures were used to inoculate (1:100 dilution) autoinduction media [13] containing 100 µg/ ml ampicillin and protein expression was induced by incubation to culture saturation at 37 °C with vigorous shaking for ~20 hours.

Cell pellets were resuspended in 20 mM Tris buffer (pH 8.0 for KamB and 7.0 for NpmA) containing 150 mM NaCl, 2 mM β-mercaptoethanol and 100 µM PMSF, and lysed by sonication for 2 minutes (cycling 1 s on, 2 s off, level 5.5; Misonix S3000). Insoluble cell debris was removed by centrifugation at 21,000 rcf for 40 minutes and the supernatant filtered sequentially through 5 µm and 0.45 µm filters (Millipore). Samples were then loaded onto a 20 ml HiPrep Heparin 16/10 FF column attached to an ÄKTApurifier 10 (GE Healthcare). Unbound proteins were washed from the column with 100 ml (5 CV) of Buffer A (20 mM Tris buffer, pH 8.0 or pH 7.0, 150 mM NaCl and 2 mM β-mercaptoethanol) and eluted using a 200 ml (10 CV) 0–100% linear gradient of Buffer B (20 mM Tris pH 8.0 or pH 7.0, 1 M NaCl and 2 mM β-mercaptoethanol). Eluted proteins were analyzed by SDS-PAGE and fractions containing the target protein pooled and concentrated using Amicon Ultra centrifugal concentration devices (10,000 MWCO; Millipore). Concentrated proteins were further purified on a HiLoad™ 26/20 Superdex 200 gel filtration column (GE Healthcare), eluting over 1.5 CV of Buffer A. Target protein content at each stage of purification and the final sample quality was assessed by SDS-PAGE.

Isothermal Titration Calorimetry (ITC)

Purified proteins (1.4 – 1.8 mg/ml) were dialyzed exhaustively against 20 mM Tris buffer (pH 8.0 for KamB and 7.0 for NpmA) containing 150 mM NaCl. The final dialysis buffer was used to prepare *S*-adenosyl-L-methionine (SAM) solution (2 or 2.5 mM). A VP-ITC microcalorimeter (Microcal) was used to perform 35×10 µl injections of SAM into protein or buffer (to measure heats of dilution). ITC experiments were performed in triplicate and analyzed using Origin 7 software.

In vitro methyltransferase Assays

E. coli (MRE600) 30S and 50S subunits were isolated essentially as described previously [14]. Methylation reactions contained 32 pmol of ribosomal subunits, 32 pmol of purified KamB or NpmA, 0.7 µCi (13.2 µCi/ mmol) or 1.0 µCi (10.0 µCi/ mmol) *S*-adenosyl-L-[methyl-³H]-methionine ([³H]-SAM; GE Healthcare) and methylation buffer (50 mM Hepes-KOH pH 7.5, 10 mM MgCl₂, 50 mM NH₄Cl, and 5 mM β-mercaptoethanol) adjusted to 40 µL final volume. Before addition to the reaction mixture, ribosomal subunits were preheated at 42 °C. Each reaction was sampled at time 0, 5, 10, 20, 30, 40 and 60 minutes, and 5 µl of the reaction mixture added directly to 50 µL of ice-cold 5% trichloroacetic acid. Methyltransferase activity of total proteins from purification steps was measured in the same way except that reactions were sampled at only 0 and 10 minute time points. Quenched samples were filtered under vacuum through GF/C filter discs (Whatman) and washed with 3 × 100 µL of ice-cold 5% trichloroacetic acid and 2 × 100 µL of ethyl alcohol. Liquid scintillation counting was performed on a Beckman LS 5000TD liquid scintillation counter in Ultima Gold™ F (Perkin Elmer).

Circular Dichroism (CD) spectroscopy

Purified proteins were dialyzed exhaustively against 20 mM NaHPO₄ buffer (pH 8.0 for KamB and 7.0 for NpmA) containing 150 mM NaF. KamB (0.4 mg/mL) and NpmA (0.3 mg/mL) CD spectra from 260 to 190 nm with 0.2 nm step size were collected on a Jasco J-810 spectropolarimeter using a 1.0 mm path length cell thermostated at 20 °C. Five individual spectra for each protein were recorded, averaged and background corrected using the instrument software.

Analysis of protein secondary structure was performed using Dichroweb [15]. Each available deconvolution algorithm was tested and CDSSTR [16] found to give the best fit to the experimental data. The values for KamB and NpmA secondary structure content are the averaged output from CDSSTR using each of the three possible reference spectra data sets for the wavelength range used (260–190 nm).

Protein crystallization and X-ray diffraction studies

Crystallization conditions were identified from commercial screens of 96 conditions (Molecular Dimensions or Hampton Research) using a Phoenix nanoliter dispensing robot (Art Robbins Instruments). Crystallization was performed by sitting drop vapor diffusion at 20 °C using KamB or NpmA (10 mg/ml), with or without 1 mM SAM or 1 mM S-adenosylhomocysteine (SAH), in Tris buffer (pH 8.0 and 7.0, respectively) and 150 mM NaCl. Crystals of *apo*-KamB, KamB-SAH, KamB-SAM and SeMet-KamB-SAM were grown from essentially identical conditions. Two different NpmA-SAM and one NpmA-SAH crystal forms were grown under two different solution conditions. Cryoprotection for low temperature X-ray data collection was achieved by adding or increasing the concentration of PEG 400, MPD or polyethyleneglycol, or by drawing the looped crystal through PFPE PFO-X175/08 (Hampton Research). Diffraction experiments were performed at the Southeast Region Collaborative Access Team (SER-CAT) beamline at the Advanced Photon Source (APS). Complete data sets were collected and processed for each crystal.

RESULTS

Expression and purification of A1408 rRNA methyltransferases

Synthetic KamB and NpmA genes optimized for expression in *E. coli* were used to create recombinant protein expression plasmids based on the pET44a vector. Protein expression in *E. coli* BL21(DE3) was accomplished using the autoinduction method of Studier [13] and monitored by SDS-PAGE (Figure 1). Following cell lysis by sonication and centrifugation to remove insoluble cell debris, supernatants contained 260 – 400 mg total soluble protein per liter of culture (6 – 9 g cells) including an estimated 90 – 160 mg of target protein (details shown for KamB in Table 1). KamB and NpmA were purified from the supernatant using the same two-step chromatographic procedure. Filtered supernatant was first applied to a heparin sepharose affinity column (see Materials and Methods), unbound proteins washed out and the target protein eluted over a linear NaCl gradient (150 – 1000 mM). SDS-PAGE was used to identify KamB or NpmA containing fractions and these were pooled and concentrated for further purification using a Superdex 200 gel filtration column. Comparison to protein standards (10–600 kDa; data not shown) run on the same column indicated that each protein eluted at a volume corresponding to a molecular weight of 20–25 kDa, in good agreement with calculated values for KamB and NpmA of 23.7 and 25.1 kDa, respectively.

Recombinant KamB and NpmA proteins are active in bacteria and in vitro

Before testing the activity of purified KamB and NpmA proteins *in vitro*, we first measured the ability of these recombinant proteins to confer resistance to bacteria in minimal inhibitory concentration (MIC) assays. When overexpressed from the pET constructs, both enzymes conferred characteristic resistance spectra [2], *i.e.* high level resistance to kanamycin, neomycin and apramycin but lower resistance to gentamicin (Table 2).

The A1408 rRNA methyltransferases are SAM-dependent enzymes, proposed from sequence analyses [11] to possess a Class I methyltransferase SAM-binding core fold. The binding of SAM to KamB and NpmA was measured using isothermal titration calorimetry (ITC) and a dissociation constant (K_d) of ~80 μ M obtained for both enzymes (Figure 2). Binding of SAM by these A1408 16S rRNA methyltransferases is thus ~4 fold weaker than for Sgm (K_d ~18

μM), the only G1405 aminoglycoside-resistance methyltransferase for which such measurements have been made to date [6,17], and ~17 fold weaker than for RsmC (K_d 4.8 μM), another 16S rRNA targeting methyltransferase [18].

To complete the characterization of the recombinant KamB and NpmA proteins we performed *in vitro* ribosome 30S subunit methylation assays using [³H]-SAM. Methylation assays were performed using equivalent amounts of total protein before or after heparin-affinity purification and with fully purified KamB and NpmA (Figure 3). Differences in 30S methylation were only observed for the crude or partially purified total protein samples at the early time sampling points (shown for 10 minutes in Figure 3A), presumably due to the high expression levels of each protein and thus significant recombinant enzyme activity present in each. These data demonstrate that the purification procedure enriches enzyme activity, as expected from the increased homogeneity (Figure 1), and that these recombinant enzymes robustly methylate 30S subunits. As expected, the A1408 16S rRNA methyltransferases have no activity against 50S subunits or naked 16S rRNA (data not shown). Thus our approaches to expression and purification produce large quantities of highly homogeneous and active protein, suitable for structural analyses.

KamB and NpmA form protein crystals that diffract to high resolution

To begin structural characterization of the A1408 rRNA methyltransferases we collected circular dichroism (CD) spectra for KamB and NpmA (Figure 4). These data indicate that the proteins are folded, soluble and possess a mixed α/β structure as expected for Class I methyltransferases [12].

Approximately 500 – 1000 crystallization conditions were screened at 20 °C for KamB and NpmA as the *apo*-enzyme or complex with SAM or SAH. Crystals were obtained for *apo*-KamB, KamB-SAM, NpmA-SAM and NpmA-SAH (Table 3), and the initial conditions for each optimized if required by systematic variation of each component. Complete data sets for KamB-SAM and three different crystal forms of NpmA-SAM were collected at the SER-CAT beamline at APS. All have been processed with good statistics and one complex of each protein (both $P2_1$ forms) proved to be suitable for high resolution structure determination. To facilitate phasing and structure determination, selenomethionine-derived KamB protein was expressed, purified and crystallized using essentially identical procedures, and a complete data set to 2.0 Å resolution collected at the selenium peak at the SER-CAT beamline.

DISCUSSION

The design and synthesis of *E. coli* codon optimized gene constructs for the aminoglycoside-resistance A1408 16S rRNA methyltransferases has allowed the generation of new protein expression constructs that routinely yield in excess of 100 mg purified target protein per liter of bacterial culture. Both KamB and NpmA methyltransferases were expressed without addition of sequences, such as 6 × His, that enable purification but may potentially interfere with protein function or crystallization. Instead, we relied on a two-step procedure combining heparin affinity and gel filtration chromatographies to produce highly homogeneous protein. The success of this purification strategy is, at least part, likely attributable to the very high expression levels of the target proteins, KamB and NpmA. However, we have successfully used the same purification strategy with other enzymes, including several Class I G1405 16S rRNA methyltransferases and the Class IV (SPOUT) A1067 23S rRNA methyltransferase Tsr where expression was more modest (5–20 mg/L culture; data not shown). In the latter example, where Tsr contained an N-terminal 6 × His, heparin affinity chromatography was found to be as effective for producing homogeneous and crystallizable protein as the published procedure using Ni²⁺ affinity [19]. Thus, where protein expression is robust, this strategy appears to be

a generally applicable method for purification of RNA methyltransferases suitable for structural studies.

The data presented here provide the first comparative *in vitro* functional characterization of A1408 methyltransferases from aminoglycoside-producer (KamB) and human pathogen (NpmA) bacteria. Our data suggest that these enzymes employ similar mechanisms of SAM binding and, despite relatively low sequence identity (~30%), are likely to adopt very similar three dimensional structures. Our success in identifying crystallization conditions for both KamB and NpmA will underpin future detailed structural and functional characterization of these important bacterial antibiotic resistance determinants.

Abbreviations

APS	Advanced Photon Source
CD	circular dichroism
CV	column volumes
ITC	isothermal titration calorimetry
KamB	kanamycin-apramycin-resistance (rRNA) methyltransferase
m¹A	N1-methyl adenosine
m⁷G	N7-methyl guanosine
MIC	minimum inhibitory concentration
MME	monomethylether
MPD	2-methyl-2,4-pentanediol
MWCO	molecular weight cut-off
NpmA	'novel plasmid-mediated methyltransferase A'
PEG	polyethylene glycol
PFPE	perfluoropolyether
rcf	relative centrifugal force ($\times g$)
rRNA	ribosomal RNA
SAH	S-adenosylhomocysteine
SAM	S-adenosyl-L-methionine
SER-CAT	SouthEast Region Collaborative Access Team

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REFERENCES

1. Jana S, Deb JK. Molecular understanding of aminoglycoside action and resistance. Appl. Microbiol. Biotechnol 2006;70:140–150. [PubMed: 16391922]
2. Savic M, Lovric J, Tomic TI, Vasiljevic B, Conn GL. Determination of the target nucleosides for members of two families of 16S rRNA methyltransferases that confer resistance to partially

- overlapping groups of aminoglycoside antibiotics. *Nucleic Acids Res* 2009;37:5420–5431. [PubMed: 19589804]
3. Cundliffe E. How antibiotic-producing organisms avoid suicide. *Annu. Rev. Microbiol* 1989;43:207–233. [PubMed: 2679354]
 4. Long, KS.; Vester, B. Resistance to antibiotics in bacteria through modification of nucleosides in 23S ribosomal RNA. In: Grosjean, H., editor. *DNA and RNA Modification Enzymes: Comparative Structure, Mechanism, Functions, Cellular Interactions and Evolution*. Austin, TX: Landes Bioscience; 2009.
 5. Conn, GL.; Savic, M.; Macmaster, R. Resistance to antibiotics in bacteria through modification of nucleosides in 16S ribosomal RNA. In: Grosjean, H., editor. *DNA and RNA Modification Enzymes: Comparative Structure, Mechanism, Functions, Cellular Interactions and Evolution*. Austin, TX: Landes Bioscience; 2009.
 6. Husain N, Tkaczuk KL, Tulsidas SR, Kaminska KH, Cubrilo S, Maravic-Vlahovicek G, Bujnicki JM, Sivaraman J. Structural basis for the methylation of G1405 in 16S rRNA by aminoglycoside resistance methyltransferase Sgm from an antibiotic producer: a diversity of active sites in m7G methyltransferases. *Nucl. Acids Res*. 2010 doi:10.1093/nar/gkq1122.
 7. Schmitt E, Galimand M, Panvert M, Courvalin P, Mechulam Y. Structural bases for 16 S rRNA methylation catalyzed by ArmA and RmtB methyltransferases. *J. Mol. Biol* 2009;388:570–582. [PubMed: 19303884]
 8. Holmes DJ, Drocourt D, Tiraby G, Cundliffe E. Cloning of an aminoglycoside-resistance-encoding gene, kamC, from *Saccharopolyspora hirsuta*: comparison with kamB from *Streptomyces tenebrarius*. *Gene* 1991;102:19–26. [PubMed: 1840536]
 9. Tamura T, Ishida Y, Otoguro M, Hatano K, Suzuki K. Classification of '*Streptomyces tenebrarius*' Higgins and Kastner as *Streptoalloteichus tenebrarius* nom. rev., comb. nov., and emended description of the genus *Streptoalloteichus*. *Int J Syst Evol Microbiol* 2008;58:688–691. [PubMed: 18319479]
 10. Wachino J, Shibayama K, Kurokawa H, Kimura K, Yamane K, Suzuki S, Shibata N, Ike Y, Arakawa Y. Novel plasmid-mediated 16S rRNA m1A1408 methyltransferase, NpmA, found in a clinically isolated *Escherichia coli* strain resistant to structurally diverse aminoglycosides. *Antimicrob. Agents Chemother* 2007;51:4401–4409. [PubMed: 17875999]
 11. Kosciński L, Feder M, Bujnicki JM. Identification of a missing sequence and functionally important residues of 16S rRNA: m(1)A1408 methyltransferase KamB that causes bacterial resistance to aminoglycoside antibiotics. *Cell Cycle* 2007;6:1268–1271. [PubMed: 17495534]
 12. Schubert HL, Blumenthal RM, Cheng XD. Many paths to methyltransfer: a chronicle of convergence. *Trends Biochem. Sci* 2003;28:329–335. [PubMed: 12826405]
 13. Studier FW. Protein production by auto-induction in high-density shaking cultures. *Protein Expression Purif* 2005;41:207–234.
 14. Moazed D, Stern S, Noller HF. Rapid chemical probing of conformation in 16 S ribosomal RNA and 30 S ribosomal subunits using primer extension. *J Mol Biol* 1986;187:399–416. [PubMed: 2422386]
 15. Whitmore L, Wallace BA. Protein secondary structure analyses from circular dichroism spectroscopy: Methods and reference databases. *Biopolymers* 2008;89:392–400. [PubMed: 17896349]
 16. Sreerama N, Woody RW. Estimation of protein secondary structure from CD spectra: Comparison of CONTIN, SELCON and CDSSTR methods with an expanded reference set. *Anal. Biochem* 2000;287:252–260. [PubMed: 11112271]
 17. Savic M, Ilic-Tomic T, Macmaster R, Vasiljevic B, Conn GL. Critical residues for cofactor binding and catalytic activity in the aminoglycoside resistance methyltransferase Sgm. *J. Bacteriol* 2008;190:5855–5861. [PubMed: 18586937]
 18. Sunita S, Purta E, Durawa M, Tkaczuk KL, Swaathi J, Bujnicki JM, Sivaraman J. Functional specialization of domains tandemly duplicated within 16S rRNA methyltransferase RsmC. *Nucleic Acids Res* 2007;35:4264–4274. [PubMed: 17576679]
 19. Dunstan MS, Hang PC, Zelinskaya NV, Honek JF, Conn GL. Structure of the thiostrepton resistance methyltransferase-S-adenosyl-L-methionine complex and its interaction with ribosomal RNA. *J. Biol. Chem* 2009;284:17013–17020. [PubMed: 19369248]

FIGURE 1. SDS-PAGE analysis of KamB protein expression and purification

For induction of protein expression ('Cells'), samples shown correspond to 'uninduced' (*lane U*) and 'induced' (*lane I*) BL21 (DE3) cells grown in LB and autoinduction medium, respectively (sample loading was matched for cell density measured by absorbance at 600 nm). Purification from soluble supernatant (*lane S*) is indicated by analysis of samples following Heparin affinity (*lane 1*) and subsequent gel filtration (*lane 2*) chromatographies. *Lane M* is protein molecular weight marker (sizes indicated on right).

FIGURE 2. Measurement of A1408 16S rRNA methyltransferase-SAM binding by isothermal titration calorimetry (ITC)
Representative titrations and the derived dissociation constants (K_d) for (A) KamB-SAM and (B) NpmA-SAM interaction. K_d values shown are the average of three independent experiments.

FIGURE 3. Analysis of A1408 16S rRNA methyltransferase activity using an *in vitro* 30S subunit methylation assay

(A) Enrichment of methyltransferase activity during protein purification monitored by incorporation of ^3H into 30S subunits from [^3H]-SAM after 10 minutes. (B) Time course assays using final purified KamB and NpmA methyltransferases.

FIGURE 4. Circular dichroism (CD) spectroscopic analysis of KamB and NpmA
Protein CD spectra were deconvoluted using the CDSSTR algorithm via Dichroweb to produce the indicated protein secondary structure contents (see Materials and Methods).

TABLE 1

Summary of KamB protein purification.

Purification Step	KamB	
	Total protein/ gram cells (mg)	Estimated purity (%)
Cleared lysate	43.4	35 – 40
Heparin	21.0	95
Gel filtration	14.5	99

TABLE 2

Analysis of A1408 rRNA methyltransferase activity in bacterial culture (MIC assay).

Plasmid	Minimum Inhibitory Concentration (MIC; $\mu\text{g/ml}$)			
	Kanamycin	Gentamicin	Neomycin	Apramycin
None	10	<5	10	20
pET44a	10	<5	10	20
pET44a-KamB	>1200	100	800	>1200
pET44a-NpmA	>1200	400	>1200	>1200

TABLE 3

Crystallization and preliminary analysis of KamB and NpmA protein crystals.

Protein	KamB-SAM	NpmA-SAM	NpmA-SAH	NpmA-SAM
Crystallization Conditions	200–300 mM Tris-HCl (pH 8.0), KSCN, 10–25% PEG 2000 MME.	100 mM Hepes (pH 7.5), 13–18% PEG 6000, 3–6% MPD.	100 mM Hepes (pH 7.5), 13–18% PEG 6000, 3–6% MPD.	100 mM Bicine pH 9.0, 7–12% PEG 20000, 1–2.5% Dioxane.
Crystal
Cryoprotection	Added 20–25% PEG400	Increased MPD to 25%	Drawn through PFPE	Added 25% ethylene glycol
Space group	$P2_1$	$P2_1$	$P2_12_12_1$	$P3_2$
Maximum Resolution (Å)	1.7	1.8	3.0	2.8
$R_{merge}^{a,b}$	0.05 (0.285)	0.067 (0.395)	0.088 (0.585)	0.064 (0.449)
$I / \sigma I$	16.8 (3.4)	20 (3.4)	18.5 (2.5)	18.1 (3.1)
Completeness (%)	99.1 (92.0)	99.9 (99.7)	96.5 (97.9)	99.8 (99.9)
Redundancy	3.8 (3.6)	4.6 (4.3)	3.1 (3.1)	4.0 (4.0)

^aValues in parenthesis are for the highest resolution shell.^b $R_{merge} = \sum_{hkl} \sum_i |I_i(hkl) - \langle I(hkl) \rangle| / \sum_{hkl} \sum_i I_i(hkl)$.