A Novel $N,N$-8-Amino-8-demethyl-$d$-riboflavin Dimethyltransferase (RosA) Catalyzing the Two Terminal Steps of Roseoflavin Biosynthesis in *Streptomyces davawensis* 

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Background: The biosynthetic pathway for the unique antibiotic roseoflavin from *Streptomyces davawensis* is unknown. A novel $N,N$-8-amino-8-demethyl-$d$-riboflavin dimethyltransferase (RosA) was purified from *S. davawensis*. RosA catalyzes the two terminal steps of roseoflavin biosynthesis.

Significance: RosA is the first enzyme of roseoflavin biosynthesis to be identified. The Gram-positive bacterium *Streptomyces davawensis* was first isolated from a Philippine soil sample in a screening program for antibiotic-producing organisms. *S. davawensis* synthesizes the red riboflavin (vitamin $B_{12}$) analog roseoflavin (RoF) (Scheme 1; compound 12) (1, 2). RoF is the only known natural riboflavin analog with antibiotic activity. RoF is toxic to Gram-positive but also to Gram-negative bacteria provided that the compound is able to enter the cell (3, 4). The molecular mechanism of action of RoF and its biosynthesis as well as the resistance mechanism of the producer strain are currently not understood (5). The synthesis of presumably inactive flavin cofactors, *i.e.* roseoflavin-5’-monophosphate and roseoflavin adenine dinucleotide, may at least in part explain its antibiotic properties (6). In addition, roseoflavin-5’-monophosphate may block FMN-riboswitches, rendering cells riboflavin auxotrophic (7–9). The genome of *S. davawensis* has been sequenced in our laboratory to near completion (99%), and publication of these data is planned for early 2012.

It was postulated that RoF is synthesized from riboflavin via 8-amino-7-deoxy-8-methyl-8-demethyl-D-riboflavin (MAF) (10, 11). The major lines of evidence were 1) incorporation of $^{14}$C of [2- and U-$^{13}$C]guanine and [2-$^{14}$C]riboflavin into RoF, 2) no incorporation of $^{14}$C of [8-$^{14}$C]guanine into RoF, and 3) formation of [2-$^{14}$C]roseoflavin upon the addition of [2-$^{14}$C]AF or [2-$^{14}$C]MAF (11). Accordingly, *S. davawensis* is able to convert a vitamin into an antibiotic vitamin analog. This strategy for the synthesis of an antimicrobial compound seems to be very economical as the precursors for antibiotic synthesis are readily available. Furthermore, many microorganisms have efficient vitamin transporters that catalyze the uptake of vita-

*The abbreviations used are: RoF, roseoflavin; AF, 8-amino-8-demethyl-$d$-riboflavin; MAF, 8-methylamino-8-demethyl-$d$-riboflavin; SAM, $S$-adenosyl methionine.*
RosA from S. davawensis

**SCHEME 1.** The enzymatic conversion of AF and MAF into RoF. The SAM-dependent reaction releases S-adenosyl-L-homocysteine (AHC).

### TABLE 1

| Strains or plasmid | Relevant characteristics | Source or reference |
|--------------------|--------------------------|---------------------|
| S. davawensis       | Wild-type strain         | Japan Society for   |
| S. albus            | Wild-type strain         | Culture Collections,|
| S. lividans 66 TK24 | str-6, SLP2’, SLP3’     | Streptomyces strain |
| E. coli Rosetta 2 (DE3) | Expression strain      | DSMZ strain no.     |
| E. coli ET12567(pUB307) | Expression vector      | 41208               |
| pET24a(+)          | pET24a(+):rosA          | This work           |
| pFJ01              | pET24a(+):rosA-His6     | This work           |
| pFJ02              | acc(3)JV(Apr), (cos)3’, IncPα oriT, colE1 origin, intE<sup>21</sup> | This work           |
| pFJ436             | pOJ436 carrying a 38,277-bp insert containing the putative ros gene cluster | Ref. 20             |
| pSET152            | acc(3)JV(Apr), oriT(RK2), pUC18 origin, int<sup>21</sup> | Ref. 20             |
| pFJ04              | pSET152 carrying a 10,321-bp insert with the putative ros gene cluster derived from pFJ03 | This work           |
| pUC18              | Cloning vector          | Ref. 16             |
| pIJ773             | Source of the apramycin resistance gene aac(3)IV and oriT | 18                   |
| pFJ05              | pUC18 cloning vector containing the apramycin resistance gene aac(3)IV and oriT from pIJ773 | This work           |
| pFJ06              | pFJ05 containing a 509-bp fragment internal to rosA; used for inactivation of rosA | This work           |

EXPERIMENTAL PROCEDURES

**Bacterial Strains and Growth Conditions**—The plasmids and strains used in this work are shown in Table 1. *S. davawensis* (Streptomyces strain 768) was aerobically grown at 37 °C and pH 7.2 in a nutrient broth (YS) containing yeast extract (10 g/liter) and soluble potato starch (10 g/liter). *Escherichia coli* was cultivated on LB. For growth of precultures or small scale cultures (up to 200 ml), baffled Erlenmeyer flasks were used for good aeration and cell dispersion. The cultures were agitated at 220 rpm in an orbital shaker. For growth on solid media agar-agar (16 g/liter) was added. To produce spore suspensions, a maltose soybean growth medium was prepared containing maltose (20 g/liter), soybean meal (20 g/liter), and agar-agar (20 g/liter). At a larger scale (for recombinant protein production) *E. coli* was grown in a 15-liter bioreactor (Bioengineering, Wald, Switzerland) at 1000 rpm, 37 °C, and an aeration of 1.0 vvm in a total volume of 10 liters. *S. davawensis* was also grown in a 15-liter bioreactor. A total of 5 × 10<sup>8</sup> *S. davawensis* spores were used to inoculate 10 liter of YS medium. Growth conditions were kept constant at 700 rpm, 37 °C, and an aeration of 0.3 vvm in a total volume of 10 liter until stationary phase, where production of RoF occurred.

**Isolation of Total DNA and Other Molecular Biology/Cloning Techniques**—For the isolation of total DNA from *S. davawensis*, a modified protocol of the Kirby Mix Procedure was used (15). Other molecular biology techniques were carried out according to standard protocols (16).

**N,N-8-Amino-8-demethyl-D-riboflavin Dimethyltransferase Assay and HPLC Analysis of Flavins**—*N,N-8-Amino-8-demethyl-D-riboflavin* dimethyltransferase (RosA) activity was
measured in a final volume of 1 ml of 50 mM Tris-HCl (pH 8.0) containing 200 μM AF and 2 mM S-adenosyl methionine (SAM). The mixture was preincubated at 37 °C for 5 min, and the reaction was started by the addition of SAM. After appropriate time intervals, an aliquot was removed and applied directly to an HPLC column (REPROSIL-PUR C_{18}-AQ, 5-μm particle size, 250 × 4 mm; Dr. A. Maisch HPLC-GmbH, Ammerbuch-Entringen, Germany). The following solvent system was used at a flow rate of 0.8 ml/min: 40% (v/v) methanol, 100 mM formic acid, 500 mM ammonium formate (REPROSIL-PUR C_{18}-AQ, 5-μm particle size, 250 × 2 mm; Dr. A. Maisch HPLC-GmbH) with the Agilent 1260 Infinity system and a 6130 Quadrupole ESI-MS (Agilent Technologies, Santa Clara, CA). RosA activity is expressed as μmol of RoF formed/min from AF and SAM. Assays were carried out by varying the concentration of the substrate AF (ranging from 16 to 326 μM) to determine the $K_m$ and $k_{cat}$ of the first methylation step under saturating conditions of SAM (2 mM). To determine the $K_m$ and $k_{cat}$ for SAM, AF was kept constant at saturating conditions (200 μM), and the concentration of SAM was varied (ranging from 25 to 500 μM). The initial reaction rates were plotted against the substrate concentrations, and the kinetic constants $K_m$ and $V_{max}$ were evaluated from the best fit to the Michaelis-Menten equation using nonlinear regression in the SigmaPlot software. The turnover numbers, $k_{cat}$, were calculated with the subunit molecular mass of 38 kDa for RosA. The pH/temperature dependence was determined using the rate of change of absorption at 505 nm in a spectrophotometer (Ultraspec 3100 pro, GE Healthcare).

**Purification of Native N,N-8-Amino-8-demethyl-d-riboflavin Dimethyltransferase from S. davawensis**—To wet-packed stationary phase cells of *S. davawensis* suspended in 50 mM Tris-HCl (pH 8.0), EDTA, free Complete Protease Inhibitor Mixture (Roche Applied Science) was added according to the recommendations of the manufacturer. Cells were then disintegrated in a French press system at 2000 bar and 10 °C. Cell debris was removed by centrifugation at 10,000 × g for 30 min and at 100,000 × g for 1 h. Solid (powdered) ammonium sulfate was added to the cell-free extract to achieve 30% saturation. After centrifugation at 10,000 × g, the supernatant was brought to 60% ammonium sulfate saturation and centrifuged as described above. The protein pellet was dissolved in loading buffer (1 M ammonium sulfate, 50 mM Tris-HCl, 5 mM DTT, 1 mM EDTA (pH 8.0)) for hydrophobic interaction chromatography with phenyl-Sepharose™ High Performance (GE Healthcare). All the following chromatographic steps were performed using the ÄKTApurifier™ system (GE Healthcare). After equilibration of the column and application of the sample, dimethyltransferase eluted within a step gradient of 50% elution buffer (50 mM Tris-HCl, 5 mM DTT, 1 mM EDTA, 10% glycerol (pH 8.0)). Active fractions were pooled, and the buffer was changed to anion exchange chromatography loading buffer (50 mM Tris-HCl, 2 mM DTT, 1 mM EDTA, pH 8.0, 10% glycerol) with Hiprep Desalting columns (GE Healthcare). The sample was loaded on a Mono Q 5/50 GL column (GE Healthcare) previously equilibrated with AEX (GE Healthcare) loading buffer and eluted by a linear gradient of 0–0.125 M ammonium sulfate in 50 mM Tris-HCl, 2 mM DTT, 1 mM EDTA, pH 8.0, and 10% glycerol. Active fractions were pooled and concentrated with Vivaspin concentrators (Sartorius Stedim Biotech, Goettingen, Germany) with a molecular weight cutoff of 10,000 at 4,000 × g. The last purification step was size exclusion chromatography on a HiLoad 16/60 Superdex 200 Prep Grade column (GE Healthcare) with 50 mM Tris-HCl, 2 mM DTT, 1 mM EDTA (pH 8.0) and 10% glycerol. Finally active fractions were concentrated with Vivaspin concentrators as described above and subjected to sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and stained with Coomassie Brilliant Blue R-250. The purest fraction was used for subsequent protein identification by MS/MS.

**De Novo Peptide Sequencing by MS/MS**—The Protein band was excised from SDS-PAGE and destained using ammonium bicarbonate and acetonitrile. In-gel digestion was performed overnight at 37 °C using trypsin after saturation of the gel pieces. Peptides were extracted from the gel, and peptide sequences were determined using Ultraflex Maldi TOF/TOF (Bruker, Ettlingen, Germany). Peptide sequences were compared with protein sequence data deduced from the genomic sequence of *S. davawensis*.

**Heterologous Overexpression of RosA from *S. davawensis***—To construct the expression vectors pET24a(+)-rosA (pFJ01) and pET24a(+)-rosA-His₆ (pFJ02) introducing an additional C-terminal His_tag tag, Ndel/Xhol-treated pET24a(+) (Merck KGaA, Darmstadt, Germany) and the modifying oligonucleotides rosfAv (5′-ACT TAC ATA TGC GGC CGG AAC CGA CC-3′) and rosArev (5′-ATA ACT CGA GTC AGC CGG CCG TGC C-3′) were used. *E. coli* Rosetta 2 (DE3) (Merck KGaA) was transformed with pET24a(+)-rosA (pFJ01) and pET24a(+)-rosA-His₆ (pFJ02). For rosA and rosA-His₆ expression, the resulting strains were grown at 37 °C on LB medium. Expression was induced by adding 0.1 mM isopropylthiogalactoside after the culture had reached an absorbance of 0.6. After 5 h of further aerobic incubation, cells were harvested by centrifugation.

**Purification of Recombinant His-tagged RosA from *E. coli***—Chromatographic steps were performed using the AKTApurifier™ system (GE Healthcare). Frozen cell paste of *E. coli* Rosetta 2 (DE3) (6 g) overproducing RosA was resuspended in 30 ml of Histrap (GE-Healthcare) binding buffer (50 mM Na₂HPO₄ (pH 7.4), 500 mM NaCl, 20 mM imidazole). Cells were passed twice through a French press at 1500 bar. All subsequent centrifugation steps were performed at 10,000 × g and 4 °C. Centrifugation for 45 min removed cell debris and unbroken cells. The clear lysate was filtered (0.2 μm) and applied to a 5-ml HisTrap column after equilibration with loading buffer. When the UV signal returned to base line, elution of His₆-tagged protein was induced with 50% elution buffer (50 mM Na₂HPO₄ (pH 7.4), 500 mM NaCl, 500 mM imidazole). Aliquots of the fractions were analyzed by SDS-PAGE and staining with Coomassie Brilliant Blue R-250. The apparently homogenous fractions were tested directly for N,N-8-amino-8-demethyl-d-riboflavin dimethyl-
RosA from S. davawensis

transferase activity and stored at −20 °C. The enzyme was stable for at least five months under these conditions.

Ligand Binding Titrations—Titrations were performed in 100 mm Tris-HCl, pH 7.4, by systematically varying the RosA concentration. The spectra were recorded between 300 and 650 nm with a Uvikon 933 spectrophotometer (BioTek Kontron Instruments AG). The dissociation constant (K_D) for AF was estimated by determining the concentration of free and bound AF by means of membrane centrifugation in Microcon filter devices (Millipore). Flavins were analyzed by HPLC.

Isolation of Total RNA from S. davawensis and RT-PCR Analysis—S. davawensis was grown in YS to mid-exponential growth phase for 15 h (no RoF was present in the culture medium) and to the late stationary phase for 24 h (until RoF was present in the culture medium). Total RNA was isolated using a modified method of Chomczynski and Sacchi (17). The mycelium was harvested by centrifugation at 4 °C, immediately frozen in liquid nitrogen, and broken by shearing in a mortar. About 400 mg of the lysate was suspended in 4 ml of buffer A (4 M guanidinium thiocyanate, 25 mM sodium citrate (pH 7), 0.5% N-lauroyl sarcosine, 100 mM β-mercaptoethanol). Subsequently, 400 μl of 2 M sodium acetate (pH 4), 4 ml of phenol (pH 4), and 800 μl of chloroform/isoamyl alcohol (49:1; v/v) were added, and the mixture was incubated on ice for 10 min. After centrifugation (30 min, 10,000 × g, 4 °C) the aqueous phase was mixed with an equal volume of isopropyl alcohol and incubated at −20 °C for 1 h. After centrifugation (20 min, 10,000 × g, 4 °C) the precipitate was washed with 70% ethanol, air-dried, and resuspended in 100 μl of RNase free water. Dissolved RNA was purified with the RNeasy RNA isolation kit (Qiagen, Hilden, Germany), and on-column DNase I digest was performed twice according to the protocol of the manufacturer to eliminate chromosomal DNA contamination. All aqueous solutions were prepared using diethylpyrocarbonate-treated water. RNA was used as the template for RT-PCR to probe for the presence of rosA (and gltA, see below) mRNA. Oligonucleotides rosA401bpfw (5′-TCG TTC GAC TTC GGG CGC TTC A-3′) and rosA401bprv (5′-ACC AGC ACC AGC ATG TCG AGG T-3′) were used for detection of the rosA transcript, and the oligonucleotides gltA408bpfw (5′-ACA CCG CCG CCT ATA AAT CCG C-3′) and gltA408bprv (5′-ACG GTG GCC CGA TGG ACT TCT T-3′) were used to probe for the transcript of the S. davawensis citrate synthase gene gltA (control).

Gene Disruption of rosA—A 1284-bp DNA fragment containing an apramycin resistance cassette and Streptomyces oriT was generated from pIJ773 (18) by XbaI treatment. The latter fragment was bluntly Klenow treated and ligated to PscI-digested (and subsequently blunted) pUC18 (16) to generate pFJ05. A 509-bp fragment internal to rosA was amplified by PCR using the modifying oligonucleotides rosKnOu01fw (5′-TAT CAA GCT TGA TGG GCG ACC TCG AGG T-3′) and rosKnOu01rv (5′-TAT GGA TCC CCC AGT CCT CCA GCA C-3′) and was ligated to HindIII/BamHI-digested pFJ05 to give pFJ06. The resulting plasmid was conjugated to S. davawensis using the non-methylating E. coli strain ET12567 and the helper plasmid pUB307 (19). Exconjugants were selected with 50 μg/ml apramycin and 20 μg/ml nalidixic acid on MS-agar. Spores of the rosA disruption mutant and the wild-type strain were used to inoculate 25 ml of TSB-medium. After 48 h of growth, 10 ml of the precultures were used to inoculate a total volume of 100 ml of YS medium (roseoflavin production medium). The cultures were grown for 72 h to the stationary phase, and the production of flavins was monitored using HPLC/MS. The site-specific integration of the plasmid was verified by PCR using oligonucleotides SEQSDknou01fw (5′-GAC CGG GGT GCT GGT GGA CG-3′), SEQSDknou01rv (5′-CGA TGC CGC TCG CCA GTC GA-3′), SEQSDknou2fw (5′-GGG AAT AAG GGC GAC ACG GAA ATG-3′), and SEQSDknou2rv (5′-ATC GCC GGC TGG TCG AATC TCG TCG-3′), and genomic DNA was derived from S. davawensis wild type or the rosA disruption strain. The corresponding PCR products covered the plasmid sequence and rosA at both junctions.

Construction of a Genome Library—Total DNA (108 μg) of S. davawensis was partially digested with MboI to fragments with approximate sizes of 40 kb. The 40-kb fragments were dephosphorylated with FastAP (Fermentas, St. Leon-Rot, Germany) and extracted once using phenol:chloroform:isoamyl
alcohol (25:24:1) followed by chloroform extraction and ethanol precipitation. The shuttle cosmid vector pOJ436 (20) was digested with KspAI(HpaI), dephosphorylated, and subsequently digested with BamHI. The resulting two fragments were extracted once using phenol:chloroform:isoamyl alcohol (25:24:1) followed by chloroform extraction and ethanol precipitation and ligated to the prepared genomic fragments. The ligation reaction was used for in vitro packaging with MaxPlax™ Lambda Packaging Extracts. E. coli ET12567(pUB307) (19). The latter sequences) and pSET152 (New England Biolabs, Frankfurt, Germany). The corresponding plasmid pFJ04 could be isolated from a transformant strain. The plasmid pFJ04 was used to transform E. coli NEB10β cells (New England Biolabs, Frankfurt, Germany). The corresponding plasmid pFJ04 was used to transform E. coli ET12567(pUB307) (19). The latter strain subsequently was conjugated to S. albus according to standard protocols (15) to ensure plasmid transfer. Transformants and exconjugants were grown to stationary phase and analyzed with respect to RoF synthesis using HPLC. Both, culture supernatants and cell-free extracts were analyzed.

**Protein Concentration Determination**—Protein concentration was estimated by the method of Bradford (21).

**Nucleotide Sequence Accession Numbers**—The sequence reported here has been deposited in the GenBank™ data base under the accession number FR750395.

**RESULTS**

*N,N-8-Amino-8-demethyl-α-riboflavin Dimethyltransferase Activity Is Present in a Cell-free Extract from S. davawensis in the RoF Production Phase**—Under laboratory conditions using a starch and yeast extract containing culture broth, RoF synthesis in *S. davawensis* was observed in the stationary phase of growth. It was postulated by Juri et al. (10) that two subsequent methylation reactions convert AF to RoF via MAF (Scheme 1). Indeed, by using a cell-free extract from RoF producing *S. davawensis* cells, we could measure the SAM-dependent synthesis of both MAF and RoF from AF employing a novel enzyme assay.
containing SAM and AF. The specific activity for the formation of RoF was 27 microunits mg\(^{-1}\) of total protein. Under these reaction conditions, both products, MAF and RoF, could be detected and isolated by HPLC with subsequent identification by mass spectrometry (Fig. 1). Using a cell-free extract from exponentially growing \(S. dava"wensis\) cells lacking RoF production, formation of MAF and RoF was not observed.

**RosA from S. dava"wensis**

![FIGURE 4. A](image)

**Purification of N,N-8-Amino-8-demethyl-d-riboflavin Dimethyltransferase (AF Dimethyltransferase) from S. dava"wensis and Identification of the Corresponding Gene**—An AF dimethyltransferase activity was enriched from a cell-free \(S. dava"wensis\) extract (stationary phase culture) by a three-step chromatography procedure. The final active fractions still contained at least six different proteins (as judged by Coomassie Brilliant Blue staining) and at least six different proteins (as judged by Coomassie Brilliant Blue staining).

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Blue R-250 staining) (Fig. 2A). From one of the major proteins present in one of the final active fractions with an estimated molecular mass of about 40 kDa, a peptide sequence of GVLED-WADADAVR was determined by mass spectrometry. This sequence matched a predicted peptide fragment of a hypothetical protein deduced from a yet uncharacterized open reading frame (sda77720; GenBank™ FR750395) located in the middle of a (hypothetical) ros gene cluster within the S. davawensis genome (Fig. 3). The deduced primary structure of sda77720 was compared with structures of other proteins in the public databases (BLASTp) and was found to be weakly similar to other dimethyltransferases (Fig. 4). In particular, the putative dimethyltransferase did show weak sequence similarity to TylM1 and DesVI, two well characterized N,N-dimethyltransferases from Streptomyces fradiae and Streptomyces venezuelae (23), respectively. The latter enzymes have an aminohexose sugar substrate and are active in the biosynthesis of mycinamino and desosamine, found in several macrolide antibiotics. TylM1 and DesVI each possess all three of the consensus sequence motifs (I-III) (Fig. 4) typical for methyltransferases that use SAM as co-substrate (24). Motif I of TylM1 and DesVI is thought to be a variant of the canonical methyltransferase motif GXGXXG, which is found in glycine N-methyltransferases and forms part of the SAM binding pocket (25). Motifs I-III are not present in the deduced primary structure of sda77720. Still, this comparison on a sequential level suggested that ORF sda77720 may encode for a methyltransferase activity necessary to convert AF into RoF. To confirm this, ORF sda77720 was expressed in E. coli using pET-24a(+) (pFJ01). Because the G+C content (70.64%) of our target gene is very high and contains rare codons that may hamper efficient expression, an E. coli strain providing the necessary rare tRNAs was employed. A cell-free extract of a pFJ01-containing strain was tested for AF dimethyltransferase activity and compared with controls harboring the empty expression vector pET-24a(+). A cell-free extract of the strain containing pFJ01 had a specific activity for the formation of RoF of 2.0 milliunits mg\(^{-1}\) of total protein. The control strain did not produce AF dimethyltransferase activity. We concluded from these experiments that ORF sda77720 very likely codes for an enzyme, which is active in the final two steps of the RoF biosynthetic pathway. Thus, ORF sda77720 tentatively was named rosA being the first gene of the RoF pathway to be identified.

**Overproduction, Purification, and Characterization of Recombinant S. davawensis RosA from E. coli**—Both the native form (using pFJ01) and a C-terminal His\(_{5,6}\)-tagged version of RosA (using pFJ02) were produced in E. coli. Cell-free extracts of the corresponding recombinant E. coli strains (both produced similar amounts of RosA as judged by SDS-PAGE) were compared with respect to their specific RosA activities (2.0 milliunits mg\(^{-1}\) of total protein using pFJ01; 1.9 milliunits mg\(^{-1}\) of total protein using pFJ02). Thus, no significant difference in specific activity was detected for the two enzyme versions, indicating that the C-terminal His\(_{5,6}\) tag did not negatively affect the activity of RosA. His\(_{5,6}\)-tagged RosA was purified by immobilized metal affinity chromatography to apparent homogeneity using a standard protocol (Fig. 2B). His\(_{5,6}\)-tagged RosA was employed for the subsequent biochemical characterization of this novel enzyme. The identity of recombinant RosA was confirmed by N-terminal peptide sequencing and de novo peptide sequencing using MS/MS. The apparent subunit molecular mass of about 40 kDa, as revealed by SDS-PAGE (Fig. 2B, lane 3), correlated well with the predicted value of 37,935 Da based on the translated peptide sequence. Gel filtration with molecular weight

**FIGURE 5.** Time course of RosA-catalyzed N-methyl transfer reactions (A) and determination of steady-state kinetic parameters (B and C) for the first methylation of A) catalyzed by RosA. A, , AF; B, MAF; C, roseoflavin. B, rates of individual reactions were obtained by incubating varying amounts of AF (16–326 \(\mu\)M) with 2.3 \(\mu\)M RosA and 2.0 mM SAM. The initial rates were plotted against the AF concentrations. To determine the kinetic constants \(K_m\) and \(V_{max}\) the data were fit to the Michaelis-Menten equation using SigmaPlot. C, as above, rates of individual reactions were obtained by incubating varying amounts of SAM (25–500 \(\mu\)M) with 2.3 \(\mu\)M RosA and 200 \(\mu\)M AF. The initial rates were plotted against the SAM concentrations. To determine the kinetic constants \(K_m\) and \(V_{max}\) the data were fit to the Michaelis-Menten equation using SigmaPlot.
RosA from *S. davawensis*

![Graph showing absorption spectra](image)

**FIGURE 6. Titration of RosA with AF.** Different amounts of RosA (0–70.5 μM) were incubated with AF (200 μM), and the absorbance spectra were recorded between 300 and 650 nm. The arrow indicates the absorbance maximum (476 nm). The inset shows the change in absorbance at 476 nm due to the formation of the RosA-AF complex as a function of the concentration of protein. AU, absorbance units.

**FIGURE 7. Change in expression of the gene rosA in the time course of growth of a *S. davawensis* culture.** The level of rosA transcript was assessed by RT-PCR. Lane 1, the template RNA was isolated during the RoF production phase, and *rosA* specific primers were used. A band corresponding to a fragment of the *rosA* gene was detected (arrow). Lane 2, the template RNA was isolated during the RoF production phase, and *gltA*-specific primers were used (positive control, *gltA* is a constitutively expressed gene coding for citrate synthase). Lane 3, the template RNA was isolated during exponential growth phase (no RoF synthesis) and *rosA*-specific primers were used. Lane 4, the template RNA was isolated during exponential growth phase, and *gltA*-specific primer were used. Lanes 5 and 6 show the RT-PCR control reactions not containing reverse transcriptase and *rosA* specific primers. The template RNA was isolated during exponential growth phase and roseoflavin production phase, respectively. Lanes M, 1-kb markers.

standards suggested that His₆-tagged RosA exists as a monomer. The apparent pH optimum of RosA was 8.8, and the apparent temperature optimum of RosA was 52 °C. *N,N*-8-Amino-8-demethyl-D-riboflavin dimethyltransferase from *S. davawensis* is a colorless enzyme with no additional absorbance between 400 nm and the protein peak at 280 nm.

**Kinetic Characterization of His₆-tagged RosA**—The purified His₆-tagged RosA protein of *S. davawensis* was kinetically characterized. First of all, the time course of RosA-catalyzed mono- and dimethylation reactions of AF were studied to determine the rate constants of each step. The progress of the reaction was monitored over a period of 90 min by taking aliquots from the incubation at appropriate time intervals followed by HPLC analysis. The percent conversion of substrate to monomethylated intermediate and dimethylated product was estimated on the basis of the integration of the corresponding HPLC peaks. The incubation was conducted under saturating concentrations of SAM. Monotonic consumption of the substrate AF and the concomitant formation of the dimethylation product RoF were observed (Fig. 5A). The concentration of the monomethylated intermediate increased to 50% of overall conversion when the reaction was initiated, and the reaction rate of the first methylation step decreased slightly, whereas the reaction rate of the second methylation step increased slowly. When the data were fit to the rate law of irreversible unimolecular consecutive reactions, rate constants of 0.07 min⁻¹ and 0.02 min⁻¹ for the respective mono- and dimethylation steps of the RosA reaction were deduced.

The kinetic parameters for the monomethylation step catalyzed by RosA were determined by following the rate of substrate (AF) consumption. The values of $K_m$ and $V_{max}$ were calculated from the best fit to the Michaelis-Menten equation using nonlinear regression in the SigmaPlot software. In these experiments the concentration of one substrate (AF or SAM) was kept fixed, whereas that of the other varied. When the concentration of SAM was maintained at 2.0 mM (saturation), a $K_m$ of 57.7 ± 9.2 μM and a $k_{cat}$ of 22.0 ± 1.1 min⁻¹ (Fig. 5B) ($V_{max}$ of 0.58 ± 0.03 μmol min⁻¹ mg protein⁻¹) were determined for AF. Similarly, when the concentration of AF was maintained at 200 μM (saturation), the $K_m$ for SAM was determined to be 28.6 ± 4.2 μM, and the $k_{cat}$ (assuming a RosA monomer of 38 kDa) was estimated to 14.8 ± 0.5 min⁻¹ (Fig. 5C). MAF was not available to our laboratory, and therefore, the kinetic parameters for the second half of methylation by following the rate of formation of the dimethylated product RoF could not be performed. Riboflavin and 8-hydroxy-8-demethyl-D-riboflavin (in this compound the 8-amino group of AF is replaced by a hydroxyl group) were available and tested as substrates. Using the latter substrates, no activity was found.
**Ligand Binding Titrations**—His$_{6}$-tagged RosA was purified from a recombinant *E. coli* strain and used for the subsequent titration experiment. A constant amount of the substrate AF was incubated with varying amounts of RosA (Fig. 6). A dissociation constant ($K_{d}$) for AF of 10 μM was estimated by determining the concentration of free and bound AF by means of membrane centrifugation in Microcon filter devices (Millipore).

**Transcriptional Analysis of rosA in *S. davawensis***—Total RNA was prepared from two different *S. davawensis* cultures in the standard yeast extract/starch medium. One culture was grown to the stationary phase and produced RoF. The other culture was harvested in the exponential growth phase where no RoF was present in the cultures. Total RNA from both cultures was analyzed by reverse transcription PCR using specific *rosA* primers. A signal was detected in total RNA derived from RoF-producing cells only (Fig. 7, lane 1). This finding suggests that *rosA* is an active gene and that it is associated with RoF biosynthesis. Sequence analysis upstream of ORF sda77160 revealed a single sequence similar to the described *Streptomyces* (vegetative) consensus promotor TTGACR (−35) and TASSR (−10) (Fig. 3) (26).

**Inactivation of ORF sda77220 Leads to a *S. davawensis* Strain That Synthesizes AF but Not MAF or RoF**—The gene *rosA* (ORF sda77220) was inactivated by insertion of a cassette conferring resistance to the antibiotic apramycin. The corresponding *rosA*-deficient strain was grown to the stationary phase in standard YS-broth. The culture supernatant and a cell-free extract were analyzed with respect to flavin synthesis by HPLC/MS. Neither MAF nor RoF was detected (Fig. 8). Instead, accumulation of AF was observed (Fig. 8) showing that inactivation of *rosA* did not affect synthesis of the remaining enzymes of the pathway. Site-specific integration of the cassette was checked by PCR. BLASTn analysis did not reveal any other likely integration site in the *S. davawensis* genome other than *rosA*.

**The rosA Gene Cluster from *S. davawensis***—**Does Not Support RoF Production in *S. albus* and *S. lividans***—To study the function of the putative RoF gene cluster in *S. davawensis* expression, experiments employing the closely related species *S. albus* and *S. lividans*, which are both unable to produce RoF, were carried out. To obtain a subgenomic fragment containing *S. davawensis* ORFs sda77160, sda77170, sda77180, sda77190, sda77200, sda77210, roSA, sda77230, sda77240, and sda77250, a cosmids library was prepared using the integrative *Streptomyces* vector pOJ436. About 4000 colonies of the library were screened by colony lifts using a digoxigenin-labeled *rosA* probe. One of the lysed colonies produced a strong hybridization signal. The cosmids from the corresponding strain (pFJ03) was isolated and characterized using restriction endonucleases. The restriction pattern of the subgenomic DNA insert perfectly matched the expected restriction pattern, suggesting that an intact subgenomic fragment has been cloned. DNA sequencing revealed that the cosmid pFJ03 contained an insert of 38,277 bp. A DNA fragment containing the putative *ros* gene cluster (sda77160, sda77170, sda77180, sda77190, sda77200, sda77210, roSA, sda77230, sda77240, and sda77250) and the natural putative promotor (see Fig. 3) was excised from plasmid pFJ03 using restriction endonucleases and ligated to integrative *Streptomyces* plasmid pSET152 carrying no additional promoter sequence in front of the multiple cloning site (FJ04) (27). Expression experiments produced negative results, *i.e.* the corresponding recombinant *S. albus* and *S. lividans* strains (transformed with FJ04) were unable to synthesize RoF. In the latter strains, RosA activity (0.5 microunits mg protein$^{-1}$) was detected showing that expression of the heterologous gene cluster indeed occurred. These results suggest that the gene cluster sda77160, sda77170, sda77180, sda77190, sda77200, sda77210, roSA, sda77230, sda77240, and sda77250 does not
RosA from *S. davawensis*

TABLE 2

| ORF       | Amino acids (no.) | Similarity                                                                 |
|-----------|-------------------|---------------------------------------------------------------------------|
| sda77160  | 435               | 35% identity (aa 134–379) to “radical SAM domain-containing protein from Methanosoaeta thermophila PT” (GenBank™ accession no. YP_842640) |
| sda77170  | 508               | 26% identity (aa 115–441) to “radical SAM domain protein from Desulfovibrio thermosulfidooxidans AS03-1” (GenBank™ accession no. EF134877) |
| sda77180  | 553               | 36% identity (aa 204–560) to “AMP-dependent synthetase and liggase from Rhodofex ferrireducens T118” (GenBank™ accession no. YP_523400) |
| sda77190  | 91                | 29% identity (aa 21–72) to “acyl carrier protein from Acholeplasma laidlawii PG-8A” (GenBank™ accession no. ABX80867) |
| sda77200  | 406               | 37% identity (aa 157–406) to β-ketoacyl-acyl carrier protein synthase II from Thermodesulfovibrio yellowstonii DSM 11347” (GenBank™ accession no. ACL20203) |
| sda77210  | 167               | 34% identity (aa 41–119) to “cholesterase superfamily protein Rhodopseudomonas palustris DX-1” (GenBank™ accession no. EFCZ7112) |
| sda77220  | 347               | RosA                                                                 |
| sda77230  | 222               | 30% identity (aa 62–202) to “fructose-2,6-bisphosphatase from Pelotomaculum thermopropionicum SI” (accession no. BAF59496) |
| sda77240  | 261               | 53% identity (aa 135–253) to “phytanyl-CoA dioxygenase from Streptomyces thermochromogenensis BCW-1” (GenBank™ accession no. AD09555) |
| sda77250  | 157               | 76% identity (aa 115–151) to “hypothetical protein from Streptomyces scabies” (GenBank™ accession no. YP_003494084) |

contain all genes necessary for RoF biosynthesis. The functions of the remaining ORFs are unclear; analysis of their primary structures revealed best hits, which are shown in Table 2.

**DISCUSSION**

It was suggested earlier by Juri et al. (10) that AF was an intermediate in the biosynthesis of the riboflavin analog RoF in *S. davawensis*. The results reported here now show that this indeed is the case. We were able to partially purify a novel N,N-8-amino-8-demethyl-d-riboflavin dimethyltransferase from *S. davawensis*, which converts AF in two steps to RoF. Both methylation reactions depend on the methyl group donor SAM. The corresponding gene identified in the genome of *S. davawensis* was named *rosA* and was found to be located in a cluster comprising a total of 10 genes. The inactivation of *rosA* led to a MAF/RoF-deficient strain accumulating AF, unequivocally showing that *rosA* is responsible for the terminal two steps in RoF biosynthesis. The primary structure of RosA is similar (up to 35% at the amino acid level) to several SAM-dependent N-methyl- and O-methyl-transferases (Fig. 4). RosA apparently is a new member of a small family of enzymes that is capable of catalyzing a *N,N*-dimethylation reaction. RosA shares low similarities to several characterized *N,N*-dimethyltransferases, such as PEM2 (28), ErmSF (29), TylM1, and DesVI (23).

RosA activity and *rosA* transcripts were only detectable in the RoF production phase. AF apparently is a good substrate for the enzyme (*K_μ_ = 55.7 ± 9.2 μM; *K_D_ = 10.0 μM) and thus most likely also is the natural substrate in *S. davawensis*. It was reported earlier (23) that reactions catalyzed by dimethyltransferases are generally slow, with *k_cat* values in the range of 1 s⁻¹. The *k_cat* = 0.37 ± 0.02 s⁻¹ of RosA is within this range.

The functions of the other putative genes (Table 2) of the *ros* gene cluster (Fig. 3) are unclear. No obvious candidate genes responsible for reactions of the riboflavin biosynthetic pathway are present in the cluster.

Heterologous expression of the putative riboflavin gene cluster in *S. albus* and *S. lividans* did not lead to RoF production, although RosA activity was observed (0.5 microunits mg protein⁻¹), which indicates that heterologous gene expression was successful. However, the latter activity is relatively weak as compared with the RosA activity in *S. davawensis* (27 microunits mg protein⁻¹) and shows that the expression level of the cluster in the heterologous hosts was low. Possibly, this low expression level was not enough to support RoF synthesis. Our conclusion, however, is that not all the genes required for RoF biosynthesis are located within the *ros* gene cluster. In many cases the genes involved in the synthesis of secondary metabolites were found to be clustered. Known exceptions to this are, e.g. the genes involved in the synthesis of monoenomyein (31), ansamitocin (32), and pristinamycin (33).

*S. davawensis* (in contrast to *S. lividans* and *S. albus*) is RoF-resistant. *S. lividans* and *S. albus*, both, were transformed with the putative *ros* gene cluster (using the plasmids described above); however, the resulting recombinant strains were not RoF-resistant. This indicates that the gene(s) for RoF resistance is not present within this first *ros* gene cluster.

In summary, we have reported the identification of a novel biosynthetic gene cluster and the identification and characterization of the first enzyme of the RoF biosynthetic pathway in *S. davawensis*. Our discoveries also have practical implications for research in the field of streptomycetes. For example, the probably unique enzyme RosA could be used to determine promoter activity in bacteria of the genus *Streptomyces*, as other reporter systems, e.g. lacZ-based assays, did not perform well in these bacteria. Because both RoF and especially AF have antibacterial (9, 30) and also antimalarial activity, the identification of the corresponding pathway is also important with respect to the construction of a AF/RoF-overproducing strain and the possibility to generate chemically altered compounds with improved properties. Furthermore, it is now possible to synthesize ¹³C or ¹⁴C-labeled MAF and RoF. The latter antibiotic flavin derivatives may be important in identifying flavoenzymes inhibited by these compounds.

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