Flavoproteins Are Potential Targets for the Antibiotic Roseoflavin in *Escherichia coli*

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The riboflavin analog roseoflavin is an antibiotic produced by *Streptomyces davawensis*. Riboflavin transporters are responsible for roseoflavin uptake by target cells. Roseoflavin is converted to the flavin mononucleotide (FMN) analog roseoflavin mononucleotide (RoFMN) by flavokinase and to the flavin adenine dinucleotide (FAD) analog roseoflavin adenine dinucleotide (RoFAD) by FAD synthetase. In order to study the effect of RoFMN and RoFAD in the cytoplasm of target cells, *Escherichia coli* was used as a model. *E. coli* is predicted to contain 38 different FMN- or FAD-dependent proteins (flavoproteins). These proteins were overproduced in recombinant *E. coli* strains grown in the presence of sublethal amounts of roseoflavin. The flavoproteins were purified and analyzed with regard to their cofactor contents. It was found that 37 out of 38 flavoproteins contained either RoFMN or RoFAD. These cofactors have different physicochemical properties than FMN and FAD and were reported to reduce or completely abolish flavoprotein function.

The Gram-positive bacterium *S. davawensis* JCM 4913 (1, 2) synthesizes the antibiotic roseoflavin, a structural riboflavin (vitamin B₂) analog (2, 3). For *Bacillus subtilis*, *S. davawensis*, and *Corynebacterium glutamicum*, it was shown that roseoflavin is taken up via riboflavin transporters (4–8). Moreover, it was found that roseoflavin is converted to the flavin cofactor analogs roseoflavin mononucleotide (RoFMN) and roseoflavin adenine dinucleotide (RoFAD) by flavokinases (EC 2.7.1.26) and FAD synthetases (EC 2.7.7.2) *in vitro* (9, 10) (Fig. 1A).

RoFMN was reported to reduce expression of genes involved in riboflavin biosynthesis and/or transport in *B. subtilis*, *Streptomyces coelicolor*, and the human pathogen *Listeria monocytogenes* (10–13). These genes are all controlled by FMN riboswitches (14), regulatory elements which are negatively affected by RoFMN. This reduction of gene expression at least in part explains why roseoflavin acts as an antibiotic. For example, reduced expression of the FMN riboswitch-controlled riboflavin-biosynthetic genes *ribEMAH* in *S. coelicolor* (caused by RoFMN) led to a significantly decreased level of riboflavin synthase (RibE) activity (10) and consequently to a reduced supply of riboflavin. In another study, the addition of roseoflavin to riboflavin-auxotrophic *L. monocytogenes* led to reduced expression of the FMN riboswitch-controlled riboflavin transporter gene *lmo1945* and to a reduced supply of riboflavin as well (11). In this study, an *L. monocytogenes* strain was described that contained a mutant FMN riboswitch which was not blocked by RoFMN. This strain constitutively transcribed *lmo1945* and showed a significant decrease in roseoflavin sensitivity; however, it was still roseoflavin sensitive. We therefore concluded that additional targets for roseoflavin must be present in *L. monocytogenes* and very likely in other bacteria as well.

Approximately 1 to 3% of all bacterial proteins depend on the riboflavin-derived cofactors FMN or FAD (15) and thus are plausible targets for the flavin cofactor analogs RoFMN and RoFAD. Indeed, some FMN- or FAD-dependent enzymes (flavoenzymes) were found to be less active or completely inactive in combination with RoFMN or RoFAD (9, 16, 17). Notably, these flavoenzymes were loaded with the cofactor analogs *in vitro* (following apoenzyme purification) and not *in vivo* (as in our study). A very recent

report describes the analysis of the FMN-dependent homodimeric azobenzene reductase (AzoR) (EC 1.7.1.6) from *Escherichia coli* in complex with RoFMN (18). The work shows that RoFMN binds to AzoR apoenzyme with an even higher affinity than FMN. Structural analysis revealed that RoFMN binding did not affect the overall topology of the enzyme and also did not interfere with dimerization of AzoR. Most importantly, AzoR-RoFMN holoenzyme was found to be less active (7 to 30% of AzoR-FMN activity, depending on the substrate) in a standard assay. The redox potential of AzoR-bound FMN was –145 mV, and the redox potential of AzoR-bound RoFMN was –223 mV. These different redox properties were reported to be responsible for the reduced activity of AzoR-RoFMN compared to that of AzoR-FMN (18).

With regard to riboflavin metabolism, more is known in *B. subtilis* than is known for any other microorganism, and thus a variety of control experiments were carried out in this study employing this bacterium. *B. subtilis* (in contrast to *E. coli*) is naturally roseoflavin sensitive due to the presence of a riboflavin transporter (RibU) which also is able to catalyze roseoflavin uptake (6) (see above). In *B. subtilis* and *E. coli* riboflavin is synthesized in a series of enzymatic reactions starting from GTP and ribulose 5-phosphate (19). The following nomenclature (1) with regard to riboflavin-biosynthetic enzymes was used throughout this text (Fig. 1): RibA, GTP cyclohydrolase II (EC 3.5.4.25) function; RibB, 3,4-dihydroxy-2-butanone-4-phosphate synthase (EC 4.1.99.12) function; RibD, riboflavin-specific deaminase function (EC 3.5.4.26); RibG, riboflavin-specific reductase function (EC

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FIG 1 (A) The conversion of riboflavin (top) into FMN/FAD and of roseoflavin (bottom) into roseoflavin-5'-phosphate (roseoflavin mononucleotide; RoFMN) and roseoflavin adenine dinucleotide (RoFAD). In many bacteria the flavokinase and the FAD synthetase reaction are catalyzed by a single (bifunctional) enzyme. (B) A schematic view of the *Escherichia coli* strains CpXFMN and CpXFAD employed for the *in vivo* generation of flavoproteins loaded with the FMN/FAD cofactor analogs RoFMN and RoFAD. The genes of 40 different recombinant flavoproteins (FP; gray ovals) are expressed using the expression plasmid pCA24N (20). Upon induction of protein synthesis, riboflavin (RF) or roseoflavin (RoF) is added to the growth medium. Both flavins are taken up via RibM (the corresponding gene *ribM* from *Corynebacterium glutamicum* replaces *manX* in the *E. coli* chromosome). *E. coli* naturally does not produce a flavin transporter. The His₆-tagged recombinant flavoproteins were combined with FMN, FAD, RoFMN, or RoFAD, purified using affinity chromatography, and analyzed with regard to their cofactor content employing HPLC/MS. The genes *ribA*, *ribDG*, *ribH*, and *ribB* encoding riboflavin-biosynthetic enzymes are shown (see introduction). The gene *ribB* is controlled by the FMN riboswitch *sroG*. (C) Schematic view of a *Bacillus subtilis* wild-type strain which naturally contains the gene *ribU* encoding a flavin transporter. This gene is controlled by an FMN riboswitch. The riboflavin-biosynthetic genes *ribDG*, *ribE*, *ribAB*, *ribH*, and *ribT* are organized in a single transcription unit and are controlled by an FMN riboswitch. The gene for the bifunctional flavokinase/FAD synthetase (*ribCF*) is located elsewhere in the chromosome. The flavoproteins are not overproduced in *B. subtilis* and bind to RoFMN or RoFAD when cells are treated with riboflavin or roseoflavin.

1.1.1.193); RibH, lumazine synthase function (EC 2.5.1.78); and RibE, riboflavin synthase function (EC 2.5.1.9). Moreover, flavokinase was renamed RibF (EC 2.7.1.26), and FAD synthetase was renamed RibC (EC 2.7.7.2).

In order to identify additional flavoprotein targets for roseoflavin-derived cofactors, the present study was initiated. *E. coli* was chosen as a model since a complete set of open reading frame (ORF) clones, containing all predicted *E. coli* flavoprotein genes,

was available for systematic functional analyses through the ASKA (*a* complete set of *E. coli* K-12 ORF archive) library (20). Our results show that under our experimental conditions, 37 out of 38 *E. coli* flavoproteins bind either RoFMN or RoFAD. Very likely, the activities of these flavoproteins are reduced by RoFMN or RoFAD, as found previously for AzoR (18).

MATERIALS AND METHODS

Chemicals and materials. Roseoflavin was obtained from MP Biomedicals and was dissolved in dimethyl sulfoxide (DMSO) if not otherwise stated. Roseoflavin mononucleotide (RoFMN) was enzymatically prepared using recombinant human flavokinase as described previously (21). All other chemicals were from Sigma-Aldrich. Restriction endonucleases and other cloning reagents were purchased from Fermentas.

Bacterial strains and growth conditions. *E. coli* was cultivated at 37°C on lysogeny broth (LB) containing the appropriate antibiotics. For growth of precultures or small-scale cultures (up to 200 ml), baffled Erlenmeyer flasks were used for good aeration and cell dispersion. *B. subtilis* wild type (Marburg 168) (22) was aerobically cultivated on LB at 37°C. The MIC for roseoflavin was determined as described previously (23).

Homologous expression of *ribCF*. The gene for *E. coli* RibCF was amplified by PCR using genomic DNA as a template and the modifying primers 5'-TTTGGACATATGAAGCTGATACGCGG-3' and 5'-ATCTC GAGTTAAGCCGGTTTGTAGCC-3'. The restriction endonuclease sites NdeI and XhoI are underlined. The NdeI/XhoI-treated PCR product was ligated to the NdeI/XhoI-digested expression vector pET28a(+) (Invitrogen) to give pET28a(+)-N-ribCF. The latter plasmid produced an N-terminally His₆-tagged version of RibCF (RibCF-NHis6).

Synthesis and purification of recombinant RibCF. *E. coli* BL21 (DE3) harboring pET28a(+)-N-ribCF was grown in LB (containing 1 μM riboflavin) to an optical density at 600 nm (OD₆₀₀) of 0.5. Synthesis of recombinant RibCF-NHis6 was stimulated by adding 1 mM isopropyl thiogalactopyranoside (IPTG) to the cultures, which were grown for another 12 h. Cells were harvested by centrifugation (3,500 × *g*) and stored at -20°C. Frozen cell paste (6 g) was resuspended in 30 ml of binding buffer (50 mM Na₂HPO₄, pH 7.4, 500 mM NaCl, 10 mM imidazole). Cells were passed twice through a French press at 2.0 × 10⁸ Pa. Centrifugation (10,000 × *g* and 4°C) for 20 min removed cell debris and unbroken cells. The lysate was cleared by ultracentrifugation (106,000 × *g* for 30 min and 4°C) and applied to a 5-ml HisTrap column after equilibration with loading buffer. Chromatographic steps were performed using an ÄKTA purifier system (GE Healthcare). When the UV signal returned to baseline, elution of the His₆-tagged protein was induced by stepwise increases in the concentration of the 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 G-250. Protein concentration was determined by the method of Bradford using bovine serum albumin (BSA) as a standard.

Flavokinase/FAD synthetase assay. Flavokinase activity was measured in a final volume of 1 ml of 50 mM potassium phosphate (pH 7.5) containing, e.g., 50 μM riboflavin, 1 mM ATP, 12 mM NaF, 6 mM MgCl₂, and 24 mM Na₂SO₃. The mixture was preincubated at 37°C for 5 min, and the reaction was started by addition of the enzyme. After appropriate time intervals, an aliquot was removed and applied directly to a high-performance liquid chromatography (HPLC) column. Flavokinase activity is expressed as nanomoles of FMN formed from riboflavin and ATP. The reaction velocity, *v*, was determined separately for each substrate concentration by linear regression using multiple data points. The substrate concentrations were tested in triplicate, and the data were found to be highly reproducible. The kinetic constants *K_m* and *V_{max}* were evaluated with the Michaelis-Menten equation using SigmaPlot (Erkrath, Germany). The turnover numbers, *k_{cat}*, were calculated with the subunit molecular mass of 35 kDa for *E. coli* RibCF. FAD synthetase was measured accordingly using, e.g., 50 μM FMN or RoFMN as a substrate.

Preparation of cell extracts. Cell extracts of *B. subtilis* and *E. coli* strains CpXFMN and CpXFAD were prepared by passing the cells twice through a French press at 2.0 × 10⁸ Pa. Centrifugation (10,000 × *g* and 4°C) for 45 min removed cell debris and unbroken cells. The lysates were cleared by ultracentrifugation (106,000 × *g* for 30 min at 4°C). The proteins in the supernatant were precipitated (5-min incubation at room temperature) by the addition to 1% (vol/vol) of an aqueous solution of trichloroacetic acid (50%, wt/vol). The samples were centrifuged (10,000 × *g* and 4°C) and filtered (cellulose acetate membrane; 0.2-μm pore size), and the flavins in the supernatant were analyzed by HPLC-mass spectrometry (HPLC/MS). In order to measure the protein-bound flavins only, the cleared lysates were subjected to size exclusion chromatography on a HiTrap desalting column (GE Healthcare) in 50 mM ammonium acetate, pH 7.0. The protein fraction was concentrated with Vivaspin concentrators (GE Healthcare) and fully denatured by treatment with 1% trichloroacetic acid (see above). The samples were centrifuged (10,000 × *g*), filtered, and analyzed by HPLC/MS with regard to their flavin cofactor contents. Control experiments (data not shown) revealed that FMN, FAD, RoFMN, and RoFAD, were stable under these conditions (see also reference 24).

HPLC analysis of flavins. Flavins were analyzed by HPLC/MS essentially as described previously (25). A Poroshell 120 EC-C₁₈ column (2.7-μm particle size, 50 mm by 3 mm; Agilent, Santa Clara, CA) was employed. The following solvent system was used at a flow rate of 5 ml/min: 18% (vol/vol) methanol–20 mM formic acid–20 mM ammonium formate (pH 3.7). Detection of riboflavin, FMN, and FAD was carried out photometrically at 445 nm, and detection of roseoflavin, RoFMN, and RoFAD was carried out photometrically at 503 nm.

Overproduction of predicted flavoproteins in *E. coli*. Expression plasmids containing a subset of known or predicted *E. coli* flavoprotein genes were available through the ASKA library (20). This library is based upon the genomic sequence data of *E. coli* K-12. The strains containing predicted flavoprotein genes were ordered from the ASKA library. The corresponding plasmids were purified, and the inserts were proof digested. The plasmids subsequently were used to transform either *E. coli* CpXFMN or CpXFAD, depending on whether the gene products were predicted to contain FMN or FAD as a cofactor. The flavoproteins are listed in Table S1 in the supplemental material. Western blotting was carried out using standard procedures and anti-pentahistidine antibodies (Qiagen).

RESULTS

***In vivo* generation of recombinant flavoproteins using specialized strains of *E. coli*.** The *E. coli* strains CpXFMN and CpXFAD (Fig. 1B) were constructed in order to produce recombinant flavoproteins loaded with RoFMN or RoFAD (instead of FMN and FAD) *in vivo*. *E. coli* does not contain an uptake system for flavins and thus is naturally resistant to the antibiotic roseoflavin (MIC of >50 μg/ml) (2). The introduction of heterologous riboflavin transporters, however, generates *E. coli* strains which are able to efficiently import flavins and consequently are roseoflavin sensitive (4–6, 26). *E. coli* CpXFMN and CpXFAD (Fig. 1B) are derivatives of *E. coli* CmpX131 (26). This strain overproduces the flavin transporter RibM (PnuX) from *C. glutamicum* (6) and is riboflavin auxotrophic (*rib* mutant) due to the chromosomal deletion of the gene *ribE* coding for riboflavin synthase (EC 2.5.1.9). RibM in *E. coli* CmpX131 allows the uptake of essential riboflavin. *E. coli* CpXFMN is different from CmpX131 in that it carries the gene *FMN1* (replacing *ribE* which is under the control of the σ⁷⁰-dependent promoter *ribEp5* [27]) coding for the monofunctional flavokinase from *Schizosaccharomyces pombe* (28). This enzyme produces FMN from riboflavin and ATP and RoFMN from roseoflavin and ATP. *FMN1* was inserted into the chromosome in order

FIG 2 Growth of *Escherichia coli* CpXFMN (A) and CpXFAD (B) on lysogeny broth in the presence of different concentrations of the antibiotic roseoflavin (RoF). Both strains are riboflavin auxotrophic. Both strains overproduce the riboflavin transporter PnuX from *Corynebacterium glutamicum* and thus are able to grow on LB, which contains about 1 μM riboflavin. Both strains show reduced growth in the presence of roseoflavin. The addition of riboflavin (RF) enhances growth of the riboflavin-auxotrophic strains.

to enhance intracellular synthesis of FMN analogs. The objective was to generate high levels of RoFMN within the cytoplasm of CpXFMN upon the addition of roseoflavin to the growth medium. In the following experiments, CpXFMN was used as a host for the overproduction of a series of *E. coli* FMN-dependent flavoproteins to be tested *in vivo* for RoFMN binding. Analogously, *E. coli* CpXFAD was used for the analysis of FAD-dependent flavoproteins. CpXFAD contains an additional copy of *E. coli* *ribCF* (replacing *ribE*) (see above) encoding the endogenous bifunctional flavokinase/FAD synthetase which produces both FMN (from riboflavin and ATP) and FAD (from FMN and ATP) (see below). This gene was introduced in order to enhance intracellular synthesis of RoFAD. Notably, *E. coli* CpXFMN as well as *E. coli* CpXFAD contains *ribCF* under the control of its natural promoter

and at the original site in the chromosome. CpXFMN and *E. coli* CpXFAD are riboflavin auxotrophic due to the deletion of the *ribE* gene (replaced by *FMN1* or *ribCF*) (see above). Riboflavin auxotrophy was important for this initial experiment to allow regulation of cofactor loading of the recombinant flavoproteins. *E. coli* CpXFMN (roseoflavin MIC₅₀ of 2 μg/ml) and *E. coli* CpXFAD (roseoflavin MIC₅₀ of 2 μg/ml) showed reduced growth in the presence of different amounts of roseoflavin (Fig. 2; see also Fig. S1 in the supplemental material).

RoFMN (but not RoFAD) is present in the cytoplasm of roseoflavin-treated bacteria. The following experiment was carried out in order to characterize the *E. coli* strains CpXFMN and CpXFAD (and *B. subtilis* [see below]) with regard to the synthesis of RoFMN and RoFAD upon treatment with roseoflavin (Fig. 1A). CpXFMN and CpXFAD were grown to an OD₆₀₀ of 0.5 and treated with riboflavin or roseoflavin (50 μM each). The cells were cultivated for another 14 h, thoroughly washed, and disrupted. The corresponding cell extracts were treated with trichloroacetic acid in order to fully denature the proteins (and in order to release all present flavins), and the samples were analyzed by HPLC/MS with regard to their cofactor contents (Table 1). The data show that in riboflavin-treated CpXFMN as well as in riboflavin-treated CpXFAD, FMN and FAD were present in very similar concentrations (113 to 119 nmol FMN/g of total soluble protein; 98 to 99 nmol FAD/g of total soluble protein). As a control, a similar experiment was carried out with a wild-type *B. subtilis* strain (Marburg 168). As stated in the introduction, this organism is naturally roseoflavin sensitive due to the presence of a riboflavin transporter, RibU (6). Moreover, this strain contains the bifunctional flavokinase/FAD synthetase RibCF (formerly named RibC) which was found to produce *in vitro* RoFMN and RoFAD (9) from roseoflavin and ATP (Fig. 1C). In riboflavin-treated *B. subtilis*, higher levels of FMN and FAD were found, which, however, were within a similar range (226 nmol FMN/g of total soluble protein; 319 nmol FAD/g of total soluble protein) as levels in *E. coli*. The analysis of cell extracts of roseoflavin-treated cells (Table 1) revealed that in *E. coli* as well as in *B. subtilis* FMN was present at significantly lower concentrations (5 to 17 times lower) than RoFMN. In roseoflavin-treated *E. coli* cells, FAD was present at concentrations very similar to those in riboflavin-treated *E. coli* cells. In *B. subtilis*, however, three times less FAD was found in roseoflavin-treated cells than in riboflavin-treated cells. RoFAD

TABLE 1 FMN, FAD, and RoFMN, but not RoFAD, are present in cell extracts of *E. coli* strains CpXFMN and CpXFAD and *B. subtilis* treated with roseoflavin

Treatment and strain ^a	Amt of cofactor (nmol/g of total soluble cellular protein) ^b			
	FMN	FAD	RoFMN	RoFAD
RF				
CpXFMN	113 ± 5	99 ± 2	0	0
CpXFAD	119 ± 4	98 ± 5	0	0
<i>B. subtilis</i>	226 ± 13	319 ± 7	0	0
RoF				
CpXFMN	9 ± 13	122 ± 18	171 ± 2	0
CpXFAD	24 ± 17	118 ± 5	137 ± 8	0
<i>B. subtilis</i>	29 ± 1	111 ± 4	140 ± 6	0

^a RF, riboflavin; RoF, roseoflavin.

^b The controls were treated with riboflavin.

FIG 3 *Escherichia coli* RibCF is a bifunctional flavokinase/FAD synthetase and also produces roseoflavin mononucleotide (RoFMN) and roseoflavin adenine dinucleotide (RoFAD) *in vitro*. Assay mixtures containing 50 μ M riboflavin or roseoflavin, 1 mM ATP, 12 mM NaF, 6 mM $MgCl_2$, and 24 mM Na_2SO_3 were incubated at 37°C for 5 min. Purified RibCF (His₆ tagged; 0.08 mg/ml) from *Escherichia coli* was added, and the mixtures were incubated for 0 min (A and D), 10 min (B and E), or 60 min (C and F). An aliquot was removed from the assay mixtures, and flavins were analyzed by HPLC/MS. Peak intensity is given in arbitrary absorbance units (AU). The chromatograms in panels A to C show three resolved peaks of riboflavin and/or FMN and/or FAD. Similar reaction results employing roseoflavin instead of riboflavin are shown in panels D to F where RoFMN and/or RoFAD was synthesized.

was not found in either *E. coli* or *B. subtilis*, tentatively suggesting that this cofactor analog was not available for flavo-apoprotein binding. RoFMN was present at relatively high concentrations (137 to 171 nmol FMN/g of total soluble protein) in *E. coli* and *B. subtilis* and thus apparently was available for flavo-apoprotein binding.

***E. coli* RibCF does not produce RoFAD in the presence of FMN.** The following experiment was carried out in order to find an explanation for the fact that RoFAD was not found in roseoflavin-treated *E. coli* cells. In *S. davawensis* and *S. coelicolor*, a bifunctional flavokinase/FAD synthetase RibC was reported to synthesize RoFMN and RoFAD *in vivo* (9, 10). A similar protein (35% identity on the amino acid level) is present in *E. coli* (27). The corresponding gene *ribCF* was reported to be essential (29). In order to confirm the function of *ribCF*, gene expression experiments were carried out. RibCF was overproduced in *E. coli* as a

His₆-tagged recombinant protein and purified (see Fig. S2 in the supplemental material). Biochemical analysis revealed that RibCF is a bifunctional flavokinase/FAD synthetase converting riboflavin and ATP to FMN and FAD *in vitro* (Fig. 3A to C). Moreover, RibCF synthesized RoFMN and RoFAD from roseoflavin and ATP (Fig. 3D to F). The kinetic parameters for the synthesis of FMN/FAD and RoFMN/RoFAD by RibCF were determined by following the rate of substrate 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 (see Fig. S3 in the supplemental material). In these experiments the concentration of the substrate ATP was kept fixed while the concentrations of riboflavin/FMN and roseoflavin/RoFMN varied. Table 2 summarizes the kinetic data for RibCF. The data suggest that roseoflavin is a better substrate for the flavokinase domain of RibCF than the “natural” substrate riboflavin. In contrast, FMN is a better substrate for the FAD synthetase function of RibCF than RoFMN. When riboflavin and roseoflavin were present in equal amounts (50 μ M) as substrates for the flavokinase reaction, FMN and RoFMN were produced by RibCF in roughly equal amounts (Fig. 4A). When FMN and RoFMN were present in equal amounts (50 μ M) as substrates for the FAD synthetase reaction, RoFAD was not produced (Fig. 4B). Even when FMN (2.5 μ M) and RoFMN (100 μ M) were present in very different amounts as substrates for the FAD synthetase reaction, RoFAD was not produced. The latter findings explain why RoFAD was not detected in the cytoplasm of *E. coli* (Table 1).

The soluble proteomes of *E. coli* and *B. subtilis* are targets for roseoflavin. The following experiment was carried out in order to test whether *E. coli* and *B. subtilis* proteins bind roseoflavin-derived cofactors. The roseoflavin-sensitive strains *E. coli* CpXFMN and CpXFAD and roseoflavin-sensitive wild-type *B. subtilis* cells were grown to an OD₆₀₀ of 0.5 in LB and treated with either riboflavin (50 μ M; control) or roseoflavin (50 μ M). The cells were further incubated until they entered the stationary growth phase. The final cell densities of the roseoflavin-treated cultures were significantly lower than those of the riboflavin-treated cells. *E. coli* CpXFMN grew to an OD₆₀₀ of 4.3 (riboflavin-treated cells) and to an OD₆₀₀ of 1.6 (roseoflavin-treated cells). *E. coli* CpXFAD grew to an OD₆₀₀ of 4.1 (riboflavin-treated cells) and to an OD₆₀₀ of 1.5 (roseoflavin-treated cells). *B. subtilis* grew to an OD₆₀₀ of 3.6 (riboflavin-treated cells) and to an OD₆₀₀ of 1.8 (roseoflavin-treated cells). From all cultures cell extracts were prepared. In contrast to the experiments described above (Table 1), where the total concentration of flavins in cell extracts of *E. coli* was determined, the extracts now were applied to a gel filtration column in order to remove free, non-protein-bound flavins. The protein fraction was

TABLE 2 Kinetic constants for the bifunctional flavokinase/FAD synthetase reactions of RibCF (35 kDa) from *Escherichia coli* with different flavin substrates

Substrate	RibCF kinetic data			
	K_m (μ M)	V_{max} (nmol min ⁻¹ mg protein ⁻¹)	k_{cat} (s ⁻¹)	k_{cat}/K_m (μ M ⁻¹ s ⁻¹)
Riboflavin (FK)	2	660	0.39	0.20
Roseoflavin (FK)	1	726	0.42	0.42
Flavin mononucleotide (FS)	4	110	0.06	1.5 10^{-2}
Roseoflavin mononucleotide (FS)	6	42	0.02	4 10^{-3}

^a FK, flavokinase; FS, FAD synthetase.

FIG 4 The bifunctional flavokinase/FAD synthetase of *Escherichia coli* (RibCF) produces roseoflavin adenine dinucleotide (RoFAD) in the absence of FMN only. (A) Time course for the synthesis of FMN and roseoflavin mononucleotide (RoFMN) (the assay was carried out as described in the legend of Fig. 3). (B) Time course for the synthesis of FAD and roseoflavin adenine dinucleotide (RoFAD).

collected, concentrated, and fully denatured by treatment with trichloroacetic acid. The samples subsequently were neutralized, filtered, and analyzed by HPLC/MS with regard to their flavin cofactor contents. In Table 3 the data for *E. coli* and *B. subtilis* are summarized. Total soluble protein derived from roseoflavin-treated *E. coli* cells contained RoFMN in addition to FMN and FAD but no RoFAD. The standard deviations of the data were high, which was probably due to the strong dilution of the samples upon gel filtration, and it is therefore difficult to interpret the data. Still, it is obvious that RoFMN can be extracted in significant amounts from the soluble proteomes of *E. coli* and from *B. subtilis*. We hypothesized that it was the flavoprotein portion (flavoproteome) of the soluble *E. coli/B. subtilis* proteome from which RoFMN had been released.

Analysis of *E. coli* flavoproteins with regard to binding of flavin cofactors or cofactor analogs. In order to test whether it is the flavoproteome which binds RoFMN or RoFAD, the following experiments were carried out. A list of known and predicted fla-

voproteins of the bacterium *E. coli* was published recently (15). Expression plasmids (based on pCA24N) containing a subset of the corresponding genes were available through the ASKA library (20). These expression plasmids were used to transform either *E. coli* CpXFMN or CpXFAD, depending on whether the flavoprotein gene products were predicted to contain FMN or FAD as a cofactor (15, 27). The 40 analyzed proteins and their ASKA accession numbers (20) are listed in Table S1 in the supplemental material. The newly generated strains were employed in order to generate His₆-tagged flavoproteins *in vivo*. The strains were grown on LB to an OD₆₀₀ of 0.4 in the presence of limiting amounts of riboflavin. Subsequently, 0.1 mM IPTG was added to the cultures, which induced the production of the different recombinant proteins from pCA24N (Fig. 1B). At the same time riboflavin was added in order to stimulate the synthesis of FMN and FAD within the cytoplasm and in order to stimulate the loading of the recombinant flavoproteins with FMN and/or FAD. The cultures were grown to the stationary phase. The recombinant proteins were purified from these cells by affinity chromatography and analyzed with regard to their flavin content (μ M cofactor per μ M protein) using HPLC/MS. The data of this experiment are summarized in Table S1 in the supplemental material. The concentrations of the protein preparations were different and ranged from 50 μ M to 400 μ M. Two recombinant proteins, IspH (30) and UxaC (31), were reported to not depend on flavin cofactors and, indeed, were found to not bind FMN or FAD under the conditions of our experiment.

None of the 40 recombinant proteins was fully loaded with cofactor (100%), indicating that FMN and FAD were present in limiting amounts for apoprotein binding. Some flavoprotein preparations were deep yellow (CysJ, FadH, Fpr, Gor, Lpd, MetF, MurB, NfnB, NorW, PoxB, PutA, TrxB, WrbA, and YieF) and were loaded to about 40 to 90% with cofactor. Other recombinant flavoproteins, however, were loaded to about 2% with cofactor only. Notably, it was found that upon *in vivo* loading with cofactors, most flavoproteins contained both FMN and also FAD (see Fig. S4 in the supplemental material). For example, the purified flavoprotein chorismate synthase (EC 4.6.1.4) (AroC; 145 μ M) contained 0.5 μ M FMN and 2.2 μ M FAD (see Table S1 in the supplemental material). AroC was annotated as an FMN-dependent protein (27). However, inspection of the literature revealed that AroC synthesizes chorismate when supplied with FMNH₂

TABLE 3 FMN, FAD, and RoFMN, but not RoFAD, can be extracted from the soluble proteome of *E. coli* strains and *B. subtilis* (wild type) treated with roseoflavin

Treatment and strain ^a	Amt of cofactor (nmol/g of total soluble cellular protein) ^b			
	FMN	FAD	RoFMN	RoFAD
RF				
CpXFMN	65 ± 32	108 ± 36	0	0
CpXFAD	76 ± 18	108 ± 38	0	0
<i>B. subtilis</i>	138 ± 66	93 ± 36	0	0
RoF				
CpXFMN	27 ± 40	68 ± 12	84 ± 41	0
CpXFAD	5 ± 3	70 ± 13	81 ± 2	0
<i>B. subtilis</i>	69 ± 11	40 ± 13	50 ± 15	0

^a RF, riboflavin; RoF, roseoflavin.

^b The controls were treated with riboflavin.

FIG 5 RoFMN and/or RoFAD is a ligand for flavoproteins of *Escherichia coli*. Different *E. coli* CpXFMN and CpXFAD strains overproducing 40 different (putative) *E. coli* (flavo)proteins were grown to an OD₆₀₀ of 0.4 in the presence of limiting amounts of riboflavin. IPTG was added to the cultures in order to induce oversynthesis of the different recombinant His₆-tagged *E. coli* flavoproteins. At the same time, roseoflavin was added, and the strains were grown to the stationary phase. The recombinant proteins were purified and analyzed with regard to their flavin cofactor content using HPLC/MS. The flavin content (percent) shows how much RoFMN/RoFAD (black columns) and/or FMN/FAD (gray columns) was found in flavoproteins purified from roseoflavin-treated strains.

and also when supplied with FADH₂ although a preference for FMNH₂ (the reduced form of FMN) over FADH₂ was found (32). This is in line with our results using the *in vivo* loading approach. Also, CysJ (33) and FadH (34) were reported to bind both cofactors. Since the concentration of FMN is more than twice as high in riboflavin-treated CpXFMN or CpXFAD than in the untreated cells, we cannot exclude the possibility that our conditions led to an abnormal cofactor profile in the recombinant proteins. The fact that other flavoproteins (DadA, Gor, NorW, TrxB, Lpd, and PutA) contained the expected cofactor to 100%, however, suggests that our *in vivo* approach is close to wild-type conditions. Notably, NfnB previously was described to contain FAD but later was reclassified as an FMN-containing protein (35). This is in line with our *in vivo* approach, where NfnB was purified predominantly in its FMN form (97% FMN).

In a set of experiments similar to those described above, CpXFMN or CpXFAD cells were treated with roseoflavin instead of riboflavin. The recombinant proteins were again purified by affinity chromatography and analyzed with regard to their flavin content. The data of this experiment are summarized in Fig. 5 and Table S1 in the supplemental material. *E. coli* does not grow in the absence of essential riboflavin, FMN, or FAD, which explains why FMN and/or FAD (in addition to RoFMN and/or RoFAD) was found in the recombinant flavoproteins purified from roseoflavin-treated cells. As expected, IspH and UxaC (see above) were found to not bind FMN, FAD, RoFMN, or RoFAD under the conditions of our experiment. For the flavoprotein lipoamide dehydrogenase (Lpd) (36), RoFAD or RoFMN binding was not observed. Lpd apparently was the only flavoprotein which did not bind a roseoflavin-derived cofactor using our *in vivo* approach. Notably, Lpd isolated from riboflavin-grown cells was almost completely loaded with FAD (90%). The remaining 37 enzymes were all found to bind either RoFMN or RoFAD. The ratio of the percentage of RoFMN to RoFAD (Fig. 5) shows how much RoFMN/RoFAD was found in flavoproteins purified from roseoflavin-treated strains. Please note also that in these expression experiments, none of the recombinant proteins was fully loaded

with cofactor and that the percent values merely represent the proportion of the proteins which were found to contain any of the flavins. For example, MurB (42 μM) contained FAD (3.9 μM) and RoFMN (0.6 μM) and thus significantly more FAD than RoFMN even in roseoflavin-treated cells. In contrast, AzoR, Dfp, Dld, Fpr, NorV, NorW, PdxH, PyrD, Wrba, and YieF contained significantly more RoFMN or RoFAD than FMN/FAD and appear to be the main targets for roseoflavin. For example, AzoR (40 μM) contained RoFMN (8.9 μM) and FMN (0.4 μM) and thus significantly more RoFMN than FMN. Surprisingly, RoFAD was found in some flavoproteins although our previous experiments suggested that RoFAD was not present in the *E. coli* host for flavoprotein binding.

DISCUSSION

We hypothesized that FMN- or FAD-dependent proteins would constitute targets for the roseoflavin-derived cofactor analogs RoFMN and RoFAD and therefore initiated the present study. The soluble proteome of roseoflavin-treated *E. coli* and *B. subtilis* cells, indeed, contained RoFMN and provided initial support for this hypothesis (Table 3). Notably, in these experiments (flavo)proteins were not overproduced but were present at normal levels. The cofactor analog RoFAD and roseoflavin, however, were not found in the soluble proteome of roseoflavin-treated *E. coli* and *B. subtilis* cells. Notably, in *Streptomyces* RoFAD was detected in the cytoplasm, albeit in 7 to 12 times lower concentrations than RoFMN (10). Our *in vitro* data suggested that the *E. coli* flavokinase/FAD synthetase RibCF produced RoFAD only when the concentration of FMN was below 1%. This could explain why RoFAD was not found in cell extracts of *E. coli* (where FMN is present in higher concentrations). However, in some of the flavoproteins purified from the different recombinant *E. coli* strains, small amounts of RoFAD were detected. Possibly, the purification of the His₆-tagged flavoproteins led to an enrichment of RoFAD, which then was present above the detection limit of our HPLC/MS method.

The fact that 37 out of 38 overproduced *E. coli* flavoproteins

contained either RoFMN or RoFAD when purified from roseoflavin-treated recombinant *E. coli* cells further supported our idea that roseoflavin targets flavoproteins in addition to FMN riboswitches. It is important to keep in mind that in these experiments, *E. coli* flavoproteins were overproduced, which of course does not reflect the wild-type situation. It is possible that the overproduced flavoproteins bind unusual levels of roseoflavin-derived cofactors. Moreover, the *E. coli* strains used for overproduction of the different flavoproteins were riboflavin auxotrophic and contained additional flavokinase/FAD synthetase genes. Thus, the recombinant cells contained unusual amounts of cofactors and cofactor analogs, which could have caused an unusual binding of roseoflavin-derived cofactors. Lpd, however, when purified from roseoflavin treated cells, did not contain any RoFMN although this cofactor analog was present in high concentrations. Also, the data in Table 1 suggest that even in wild-type cells (*B. subtilis*), a substantial amount of RoFMN is present in the cytoplasm and is available for flavo-apoprotein binding. All in all, we therefore think that our *E. coli* system may very well be close to the wild-type situation and thus is useful for the identification of target flavoproteins for roseoflavin also from other organisms. Notably, the *E. coli* flavoproteins AzoR, Dfp, Dld, Fpr, NorV, NorW, PdxH, PyrD, WrbA, and YieF contained large amounts of RoFMN and probably are the main targets for roseoflavin in this organism.

The cofactor analogs RoFMN/RoFAD have different physicochemical properties and thus may disturb the overall structure of flavoproteins, affect multimerization, or be inactive cofactors due to an altered reactivity. Moreover, the covalent attachment via the C-8 α of the flavin to the apoenzyme may be disturbed. Consequently, RoFMN/RoFAD binding may lead to partial or complete inactivation of flavoproteins (37–39). Flavoproteins carry out a wide variety of different biochemical reactions (40, 41), and it is thus plausible that inactive flavoproteins lead to reduced cell growth. Moreover, since flavoproteins seem to be present in all organisms, it is very likely that at least one target protein for roseoflavin is present in most (if not all) organisms. In our experiments a 50 μ M concentration of the antibiotic roseoflavin was used, a concentration which will not be present in a natural setting. However, even if the activity of a few essential enzymes would only be slightly reduced in the presence of RoFMN/RoFAD, this may constitute a disadvantage for competing cells in a natural habitat. A few reports deal with the *in vitro* reconstitution of apo-flavoenzymes with RoFMN or RoFAD. The corresponding holoenzymes [L-lactate oxidase from *Aerococcus viridans* (17), NAD(P)H:flavin oxidoreductase from *Beneckea harveyi* (39), rabbit liver pyridoxamine 5'-phosphate oxidase (42), and pig kidney D-amino acid oxidase (9)] were all less active or completely inactive, which tentatively explains why roseoflavin is an antibiotic.

Vitamin analogs in principle have multiple cellular targets since many vitamins (as precursors of enzyme cofactors) are active at more than one site in the cell. As a result, the frequency of developing resistance to antimicrobials based on vitamin analogs is expected to be significantly lower (43). This is not true in the case for roseoflavin in, e.g., *B. subtilis*, since a single mutation either in the *ribG* FMN riboswitch or in the flavokinase/FAD synthetase gene *ribC* may lead to riboflavin oversynthesis and consequently to a roseoflavin-resistant phenotype (44–46). It may be true, however, for bacteria which do not control their riboflavin metabolism employing FMN riboswitches (47) and which are not deregulated by point mutations. If these organisms contain

RoFMN/RoFAD-sensitive flavoproteins (which is likely), more mutation events are necessary in order to generate roseoflavin-resistant cells.

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