UNIVERSIDADE ESTADUAL PAULISTA
"JÚLIO DE MESQUITA FILHO"
INSTITUTO DE BIOCIÊNCIAS – RIO CLARO

PROGRAMA DE PÓS-GRADUAÇÃO EM CIÊNCIAS BIOLÓGICAS
(MICROBIOLOGIA APLICADA)

Riboflavin analogs from *Streptomyces davawensis* as antiinfectives: mode of action, mechanism of resistance of the producer and metabolization by humans

DANIELLE BISCARO PEDROLLI

Tese apresentada ao Instituto de Biociências do Campus de Rio Claro, Universidade Estadual Paulista, como parte dos requisitos para obtenção do título de Doutor em Ciências Biológicas (Microbiologia Aplicada).

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Orientadora: Profa. Dra. Eleonora Cano Carmona

Co-orientador: Prof. Dr. Matthias Mack

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RESUMO

Nas bactérias Gram-positivas *Streptomyces davawensis* e *Streptomyces coelicolor* a transcrição dos genes responsáveis pela biossíntese de riboflavina (RF; vitamin B_2) *ribBMAH* é iniciada a partir do promoter da riboflavina (P_{rib}). Nas duas espécies um *ribB* FMN *riboswitch* está presente imediatamente à montante em relação a P_{rib}, regulando a expressão gênica ao nível traducional. *S. davawensis* produz o atibiótico roseoflavina (RoF), um análogo de riboflavina, o qual é convertido a roseoflavina 5´-mononucleotídeo (RoFMN) no citoplasma das células alvo. *S. davawensis* é resistente a RoF, enquanto que *S. coelicolor* é sensível a RoF. Através de experimentos de transcrição/tradução *in vitro* foi demonstrado que o *ribB* FMN *riboswitch* de *S. coelicolor* é desligado na presença de RoFMN. Em contraste, o *ribB* FMN *riboswitch* de *S. davawensis* mostrou-se mais ativo na presença de RoFMN. Compatível com esse resultado, a atividade enzimática correspondente ao produto do primeiro gene (*ribB*) do *cluster ribBMAH* foi fortemente reduzida em *S. coelicolor*, mas não em *S. davawensis*, quando ambas foram cultivadas em meio acrescido de RoF. Através de mutagênese sítio-dirigida foi identificado um único nucleotideo (A61) na estrutura do *ribB* FMN *riboswitch* de *S. davawensis* como sendo responsável pela resposta diferencial a RoFMN. A introdução do *ribB* FMN *riboswitch* de *S. davawensis* em *S. coelicolor* gerou uma linhagem resistente a RoF.

O presente trabalho apresenta evidências de que o *ribB* FMN *riboswitch* de *S. coelicolor* é controlado termodinamicamente, enquanto que o *ribB* FMN *riboswitch* de *S. davawensis* é controlado cineticamente. Os últimos resultados muito provavelmente explicam as diferentes respostas com relação a RoFMN observada para os dois *ribB* FMN *riboswitches* quase idênticos. Em resumo, os experimentos desse trabalho mostram que um FMN *riboswitch* altamente especializado é responsável por conferir à *S. davawensis* resistência ao antibiótico RoF e, ao mesmo tempo, mostram que FMN *riboswitches* de fato são alvos para análogos de flavina.

Análogos de flavina possuem reconhecido potencial para serviren de estruturas básicas para o desenvolvimento de novos antibióticos. Notavelmente, um segundo análogo de flavina com atividade antibiótica, 8-demethyl-8-amino-riboflavina (AF), foi identificado em *S. davawensis*. AF é o precursor direto de RoF. Como um primeiro passo em direção ao entendimento do metabolismo dos análogos de RF em mamíferos/humanos, as enzimas

flavoquinase (EC 2.7.1.26) e FAD sintetase (EC 2.7.7.2) foram estudadas. A flavoquinase humana converteu eficientemente roseoflavina (RoF) e 8-demethyl-8-amino-riboflavina (AF) em roseoflavina mononucleotideo (RoFMN) e 8-demethyl-8-amino-riboflavin mononucleotideo (AFMN), respectivamente. A FAD sintetase humana foi capaz de aceitar RoFMN como substrato, mas não AFMN. Consequentemente, roseoflavina adenina dinucleotideo (RoFAD) foi sintetizada pela última enzima, mas 8-demethyl-8-amino-riboflavina adenina dinucleotideo (AFAD) não. Os cofatores RoFMN, AFMN e RoFAD possuem diferentes propriedades físico-químicas quando comparados com FMN e FAD. Além disso, os cofatores análogos tem o potencial de tornar flavoenzimas inativas, o que pode afetar negativamente o metabolismo humano. Em testes de inibição, apenas RoF foi capaz de inibir a flavoquinase humana. Em resumo, os últimos resultados sugerem que AF possui um menor potencial tóxico e pode ser o mais adequado para uso como estrutura base no desenvolvimento de compostos antimicrobianos.

Palavras chave: *Streptomyces davawensis*. *Streptomyces coelicolor*. Biosíntese de riboflavina. FMN *riboswitches*. Roseoflavina. Análogos de flavina. Flavoquinase. FAD sintetase.

ABSTRACT

In the Gram-positive soil bacterium *Streptomyces davawensis* and in the closely related *Streptomyces coelicolor* transcription of the riboflavin (RF; vitamin B_2) biosynthetic genes *ribBMAH* originates from the riboflavin promoter (P_{rib}). In both species a *ribB* (ribo)flavin mononucleotide (FMN) binding riboswitch is present immediately downstream of P_{rib} regulating gene expression at the translational level. *S. davawensis* produces the antibiotic roseoflavin (RoF), a RF analog, which is converted to roseoflavin mononucleotide (RoFMN) within the cytoplasm of target cells. *S. davawensis* is RoF resistant, whereas *S. coelicolor* is RoF sensitive. *In vitro* transcription/translation experiments revealed that the *S. coelicolor ribB* FMN riboswitch was turned off in the presence of RoFMN. In contrast, the *S. davawensis ribB* FMN riboswitch was found to be more active in the presence of RoFMN. In line with this finding was that the activity of the product of the first gene (*ribB*) of the *ribBMAH* cluster was strongly reduced in *S. coelicolor*, but not in *S. davawensis*, when cells were grown in the presence of RoF. Sequence alignments and site directed mutagenesis experiments identified a single nucleotide (A61) within the *S. davawensis ribB* FMN riboswitch which is responsible for its specific response to RoFMN. Introduction of the *S. davawensis ribB* FMN riboswitch to *S. coelicolor* produced a RoF resistant *S. coelicolor* strain.

The present work gives evidence that the *ribB* FMN riboswitch of *S. coelicolor* is thermodynamically controlled whereas the *S. davawensis ribB* FMN riboswitch is kinetically controlled. The latter finding most likely explains the different response of the two almost identical *ribB* FMN riboswitches with respect to RoFMN. Altogether, the present results show that a highly specialized FMN riboswitch confers RoF resistance to *S. davawensis* and conversely shows that FMN riboswitches indeed are targets for flavin analogs.

Flavin analogs have the outstanding potential to serve as basic structures for the development of novel antiinfectives. Notably, a second flavin analog with antibiotic activity, 8-demethyl-8-amino-riboflavin (AF), has been identified in *S. davawensis*. AF is the direct precursor to RoF. As a first step towards understanding the metabolism of RF analogs in mammals/humans, the key enzymes flavokinase (EC 2.7.1.26) and FAD synthetase (EC 2.7.7.2) were investigated. Human flavokinase efficiently converted RoF and AF to RoFMN and 8-demethyl-8-amino-riboflavin mononucleotide (AFMN), respectively. Human FAD-synthetase accepted RoFMN but not AFMN as a substrate. Consequently, roseoflavin adenine

dinucleotide (RoFAD) was synthesized by the latter enzyme but not 8-demethyl-8-amino-riboflavin adenine dinucleotide (AFAD). The cofactor analogs RoFMN, AFMN and RoFAD have different physicochemical properties as compared to FMN and FAD. RoFMN, AFMN and RoFAD have the potential to produce inactive flavoenzymes, and thus may negatively interfere with human metabolism. In inhibition tests, only RoF was found to inhibit human flavokinase. In summary, the results suggest that AF has a lower toxic potential and may be better suited as a lead structure to develop antimicrobial compounds.

Keywords: *Streptomyces davawensis*. *Streptomyces coelicolor*. Riboflavin biosynthesis. FMN riboswitches. Roseoflavin. Flavin analogs. Flavokinases. FAD synthetases.

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Contents

1. Introduction

1.1 Flavins and flavoproteins

Riboflavin (RF), also called vitamin B_2 (Fig. 1), is a yellow pigment synthesized by all plants and many microorganisms. Animals and some prokaryotic and eukaryotic microorganisms (e.g. *Corynebacterium pyogenes, Streptococcus pyogenes, Listeria monocytogenes*, some lactic acid bacteria, mycoplasmas, spirochetes, rickettsiae and protists) cannot synthesize riboflavin *de novo* and thus, have to obtain it from dietary sources. Many Gram-positive bacteria seem to be able to import riboflavin from the environment into the cytoplasm, whereas most Gram-negative bacteria depend on the endogenous synthesis of this vitamin (FISCHER & BACHER, 2005; ABBAS 2011).

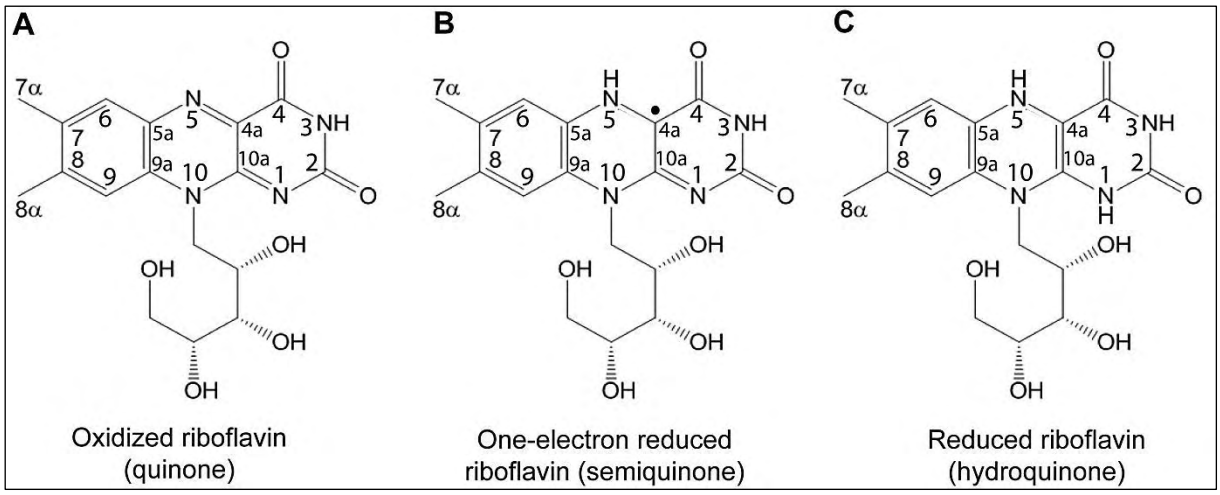

Figure 1. The structure of 7,8-dimethyl-10-(1′-D-ribityl)isoalloxazine or riboflavin (RF), also known as vitamin B_2. (A) Oxidized riboflavin or quinone state; (B) one-electron reduced riboflavin or semiquinone (radical) state. The solid circle represents an electron at C4a; (C) two-electron reduced riboflavin (fully reduced) or hydroquinone state.

In all organisms, riboflavin serves as the direct precursor for the flavoenzyme cofactors flavin mononucleotide (FMN) and flavin adenine dinucleotide (FAD). Both flavins are essential for all organisms (GERDES et al., 2002). Whereas free riboflavin apparently does not directly play any biological role, the flavin cofactors FMN and FAD are the active groups of a large number of enzymes generally called flavoenzymes (FRAAIJE & MATTEVI, 2000).

Flavins are amphipathic molecules allowing a large variety of different interactions with the flavoenzyme and also with the substrate. The diversity of the biological activity of flavins is supported by its isoalloxazine moiety. It exists in three redox states: oxidized or quinone state, one-electron reduced or semiquinone (radical) state, and two-electron reduced (fully reduced) or hydroquinone state (ABBAS, 2011) (Fig. 1). The oxidized isoalloxazine ring system is planar in solution and in most protein structures. In a few proteins, however, it is distorted, influencing its ability to accept one or two electrons. The radical state is thermodynamically unstable in aqueous solution. It represents approximately 5% of equimolar mixtures of fully oxidized and fully reduced flavins. The ring system of fully reduced flavins is bent along the N5–N10 axis, and in free flavins, the bent conformation places the N10 substituent in a pseudoaxial position above the plane of the isoalloxazine ring system (HASFORD & RIZZO, 1998).

Most flavin cofactors are noncovalently bound to flavoenzymes (90%). Covalent attachment of FMN occurs at either the 8-α (methyl) or the C6-position of the isoalloxazine ring (Fig. 1), and sometimes at both sites. A novel attachment site of FMN at the ribitylphosphate side chain was recently discovered. Apparently, covalent attachment of FAD is more common as compared to FMN and always occurs *via* the 8-α (methyl) position. In rare cases FAD is bicovalently attached at both 8-α (methyl) and the 6-position of the isoalloxazine ring (MACHEROUX et al., 2011). The biochemical utility of FMN and FAD is based on the redox-active isoalloxazine ring system, which is capable of one-electron and two-electron transfer reactions and, most importantly, of dioxygen activation (MASSEY, 1994). By database screening, Marcheroux et al. (2011) analyzed 374 flavin-dependent proteins with regard to their function, structure and distribution. Twenty two archaeal, eubacterial, protozoan and eukaryotic genomes were considered. Among the 276 fully classified flavoenzymes found, 91% were oxidoreductases, 4.3% were transferases, 2.9% were lyases, 1.4% were isomerases and 0.4% belonged to the ligase group. The majority of flavoenzymes bind FAD (75%) rather than FMN (25%). The complete list also includes some flavoproteins without a demonstrated enzymatic activity (MACHEROUX et al., 2011).

1.2 Riboflavin transport and biosynthesis

Flavin importers (uptake systems) have been identified and characterized in *Bacillus subtilis* and some other bacteria (VOGL et al., 2007; GRILL et al., 2007). Two main kinds of bacterial importers have been already described. *B. subtilis* YpaA is a proton-riboflavin symporter with high affinity for its substrate (K_M = 5 - 20 nM) and is related to RibU riboflavin transporter from *Lactococcus lactis*. Whereas the *Corynebacterium glutamicum* riboflavin transporter PnuX is an energy-independent facilitator for riboflavin with poorer affinity (K_M = 11 μM) (VOGL et al., 2007). PnuX from *C. glutamicum* is similar (40% at the amino acid level) to RibM (23.7 kDa) from *Streptomyces davawensis*. The gene for the latter protein is present in the *S. davawensis* riboflavin biosynthetic gene cluster *ribBMAH* which is controlled by an FMN riboswitch directly upstream of *ribB* (GRILL et al., 2007; LEE et al., 2009). Besides riboflavin, RibM from *S. davawensis* also accepts the riboflavin analog roseoflavin as a substrate (HEMBERGER et al., 2011). A third bacterial importer class ImpX has been identified in *Fusobacterium nucleatum*, but it has not been yet functionally characterized (SERGANOV et al., 2009). YpaA, ImpX, and PnuX do not share similar

primary structures, a fact that is also reflected in the predicted protein topologies that suggest 5 transmembrane domains (TMDs) for YpaA and orthologs, 6 to 7 TMDs for PnuX and orthologs, and 8 to 10 TMDs for most members of the ImpX family. *Escherichia coli* and other Gram-negative bacteria do not have flavin importers and thus are dependent on endogenous flavin synthesis (VOGL et al., 2007).

In *Saccharomyces cerevisiae* riboflavin uptake is mediated by an energy-independent facilitator encoded by *Mch5p*. The transporter is localized in the plasma membrane and is apparently regulated by the cellular flavin content (REIHL & STOLZ, 2005)

Mammals are not able to synthesize riboflavin and therefore are dependent on absorption from dietary sources. In humans riboflavin is absorbed in the small intestine and colon from dietary and bacterial sources. After absorption, riboflavin is delivered by the blood to tissues. Three human flavin transporters have been described, hRFT1, hRFT2 and hRFT3. The riboflavin transporter RFT1 was also found in rat kidney cells. None of them exhibits similarity to the bacterial transporters RibU and ImpX, or to the yeast transporter Mch5p. The RFT transporter family is predicted to have 10 putative membrane-spanning domains. Human RFT transporters seem to specifically recognize the flavin isoalloxazine ring as a substrate. The riboflavin transporters hRFT1 and hRFT2 probably play important roles in intestinal riboflavin absorption, whereas hRFT3 is strongly produced in the brain (YONEZAWA et al., 2008; YAO et al., 2010).

The pathways of riboflavin biosynthesis are similar in bacteria, fungi and plants, being surprisingly identical in eubacteria and plants. Riboflavin biosynthesis starts from one molecule of guanosine 5'-phosphate (GTP) and two molecules of ribulose-5'-phosphate (Fig. 2). The first reaction is catalyzed by GTP cyclohydrolase II (EC 3.5.4.25) which removes C-8 from GTP producing formate, and also releases pyrophosphate. Yeasts and some bacteria (e.g. *E. coli*) contain separate genes coding for GTP cyclohydrolase II, whereas plants and other bacteria (e.g. *B. subtilis*) contain a fused gene coding for a bifunctional enzyme GTP cyclohydrolase II/ 3,4-dihydroxy-2-butanone-4-phosphate synthase (EC 4.1.99.12). Archaea and some eubacteria also contain a GTP cyclohydrolase III, which hydrolyzes the imidazole ring of GTP but does not remove the resulting formyl group from the formamide, as does GTP cyclohydrolase II. Thus, the GTP cyclohydrolase III activity must be complemented by a formamide hydrolase. In eubacteria (e.g. *E. coli* and *B. subtilis*) the second and third reactions are catalyzed by one deaminase/reductase bifunctional enzyme. The hydrolytic deamination is catalyzed by the diaminohydroxyphosphoribosylaminopyrimidine deaminase (EC 3.5.4.26)

domain, and the reduction of the ribosyl side chain is catalyzed by the 5-amino-6-(5-phosphoribosylamino)uracil reductase (EC 1.1.1.193) enzyme domain, which uses NADPH for reduction of the product generated in the second reaction. The forth reaction is the dephosphorylation of 5-amino-6-ribitylamino-2,4(*1*H,*3*H) pyrimidinedione 5'-phosphate, which mechanism has not been elucidated yet. The pyrimidine precursor of riboflavin is converted to 3,4-dihydroxy-2-butanone 4-phosphate in the fifth reaction. The enzyme responsible for the latter reaction (3,4-dihydroxy-2-butanone-4-phosphate synthase; EC 4.1.99.12) is a homodimer in *E. coli* but in *B. subtilis* is part of a bifunctional enzyme that also contains the GTP cyclohydrolase II activity. The lumazine synthase (EC 2.5.1.78) catalyzes the condensation of 5-amino-6-ribitylamino-2,4(*1*H,*3*H) pyrimidinedione with 3,4-dihydroxy-2-butanone-4-phosphate in the sixth reaction. The final step in riboflavin biosynthesis, the dismutation of two molecules of 6,7-dimethyl-8-ribityllumazine into riboflavin and 5-amino-6-ribitylamino-2,4(*1*H,*3*H) pyrimidinedione, is catalyzed by RF synthase (EC 2.5.1.9). The reaction produces riboflavin and recycles the substrate of the lumazine synthase reaction. Notably, in Bacillaceae, lumazine synthase probably forms a complex comprising an icosahedral capsid of 60 lumazine synthase subunits and a core of three RF synthase subunits. The latter finding suggests that substrate channeling occurs (FISCHER & BACHER, 2005; ABBAS 2011).

In the Gram-positive model bacterium *B. subtilis* the riboflavin biosynthetic genes *ribGBAHT* are transcribed from the *rib*-promoter (P_{rib}). The corresponding gene products RibG, RibB, RibA and RibH synthesize riboflavin from GTP and ribulose-5'-phosphate (SONENSHEIN et al., 2001). The function of *ribT* remains elusive. FMN controls riboflavin biosynthesis in *B. subtilis* by binding the aptamer portion of an FMN riboswitch (hereafter called *ribG* FMN riboswitch) present in the 5'-untranslated (UTR) region of *ribGBAHT* causing transcription termination (MIRONOV et al., 2002; WINKLER et al., 2002).

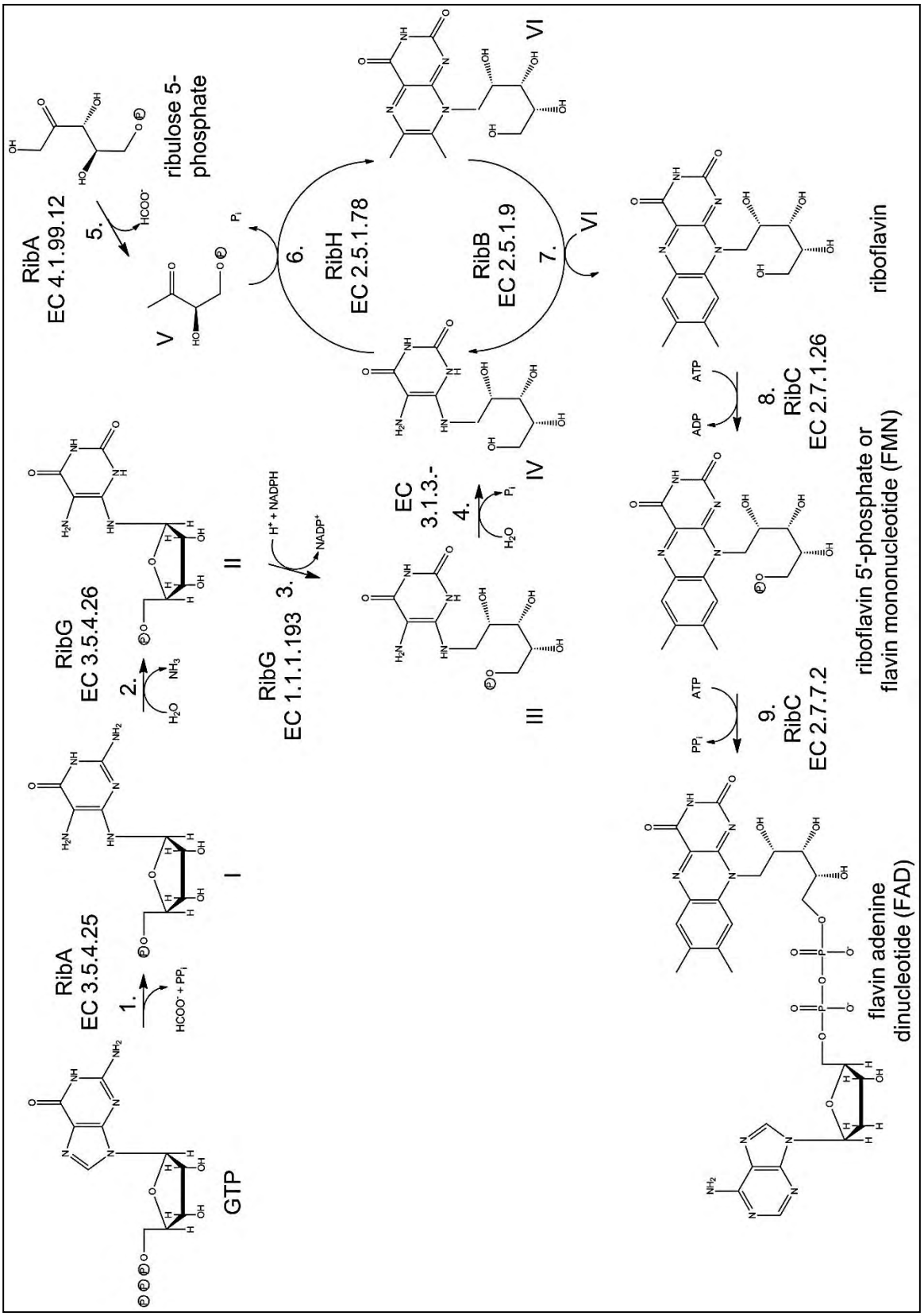

Figure 2. The biosynthetic pathway for riboflavin, FMN and FAD in bacteria according to Fischer & Bacher (2005). The enzymatic steps (1–9) responsible for the conversion of the precursors GTP and ribulose 5-phosphate into riboflavin, FMN and FAD are shown. EC

numbers of the corresponding enzymes are shown. The names of the respective gene products are shown for the Gram-positive bacterium *Bacillus subtilis* above the arrows, whereby RibA and RibG are bifunctional enzymes. Enzyme and gene names are different depending on the organism. I, 2,5-diamino-6-ribosylamino-4(*3*H)- pyrimidinone 5'-phosphate; II, 5-amino-6-ribosylamino-2,4(*1*H,*3*H)- pyrimidinedione 5'-phosphate; III, 5-amino-6-ribitylamino-2,4(*1*H,*3*H)-pyrimidinedione 5'-phosphate; IV, 5-amino-6-ribitylamino- 2,4(*1*H,3H)-pyrimidinedione; V, (3S)-3,4-dihydroxy-2-butanone 4-phosphate; VI, 6,7-dimethyl-8-D-ribityllumazine. Two molecules of VI dismutate (step 7) to give IV and riboflavin.

In the Gram-positive bacteria *Streptomyces davawensis*, *Streptomyces coelicolor* and *Streptomyces avermitilis* some of the genes involved in riboflavin biosynthesis form transcription units also regulated by riboswitches. In Streptomycetes two riboflavin gene clusters (*ribBMAH* and *ribG/Y*) are present (GRILL et al., 2007). The corresponding gene products RibBMAH and RibG are similar to the *B. subtilis* enzymes RibGBAH and are responsible for riboflavin biosynthesis. In *S. davawensis* the gene cluster formed by *ribB* (riboflavin synthase), *ribM* (riboflavin and roseoflavin permease), *ribA* (bifunctional GTP cyclohydrolase II/3,4-dihydroxy-2-butanone-4-phosphate synthase) and *ribH* (lumazine synthase) are controlled by an FMN riboswitch, hereafter called *ribB* FMN riboswitch (LEE et al., 2009). At another *locus*, a *ribG* gene coding for pyrimidine deaminase/reductase of the riboflavin biosynthetic pathway and *ribY* (or *ribA*) coding for a second (monofunctional) GTP cyclohydrolase II apparently are not under control of a riboswitch element (GRILL et al., 2007).

1.3 FMN and FAD biosynthesis

Riboflavin is quickly and almost quantitatively converted to FMN and FAD within the cell cytoplasm. FMN is synthesized from riboflavin and ATP by flavokinase (RFK, EC 2.7.1.26) and FAD is synthesized from FMN and ATP by FAD synthetase (FADS, EC 2.7.7.2) (DEMAIN, 1972; FISCHER & BACHER, 2005) (Fig. 3). ATP has been described as the phosphoryl donor for flavokinases from a large number of organisms, but that seems not to be the case for archaeal kinases. The RFK from *Methanocaldococcus jannaschii* was described as a CTP-dependent enzyme (AMMELBURG et al., 2007; MASHHADI et al., 2008), but its FAD synthetase displays a broader substrate specificity accepting ATP, CTP and also GTP as donor nucleotide (MASHHADI et al., 2010).

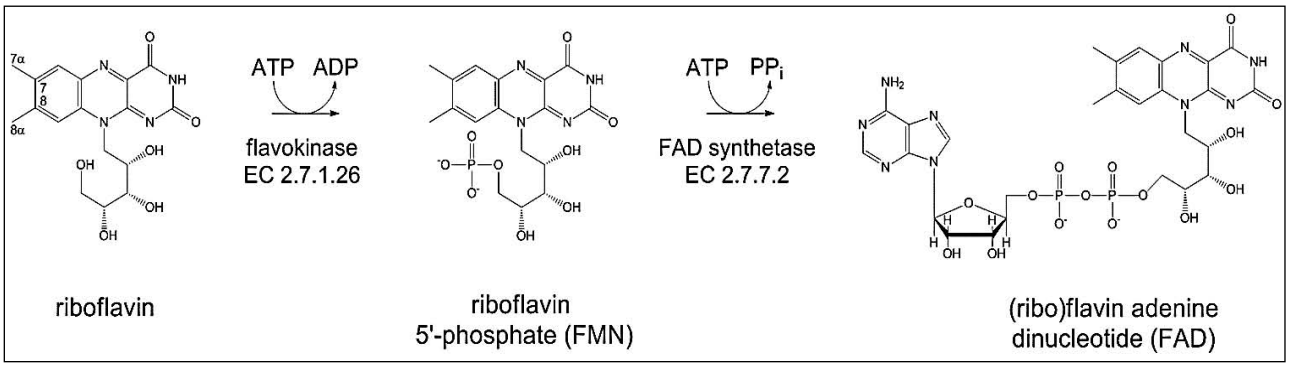

Figure 3. The enzymatic conversion of riboflavin into FMN by flavokinase and of FMN into FAD by FAD synthetase.

In bacteria, both functions of RFK and FADS are usually present in a single bifunctional flavokinase/FAD synthetase, the gene product of *ribF* (*E. coli*) or *ribC* (*B. subtilis*) (MACK et al., 1998; GERDES et al., 2002). In eukaryotes (e.g. *S. cerevisiae* and humans) and in the archeon *Methanocaldococcus jannaschii*, RFK and FADS exist as separate monofunctional enzymes (KEARNEY, 1952; KARTHIKEYAN et al., 2003; BRIZIO et al., 2006; AMMELBURG et al., 2007; MASHHADI et al., 2010). In *B. subtilis* and a few closely related *bacilli*, a monofunctional RFK encoded by the gene *ribR* is present in addition to bifunctional RibC. RibR probably plays a regulatory or supplementary role in flavin cofactor biosynthesis (SOLOVIEVA et al., 1999). The flavokinase reaction is irreversible, but the adenylylation of FMN to FAD is usually reversible in bifunctional enzymes.

Flavokinase and FAD synthetase are essential enzymes in *S. cerevisiae*, *E. coli* and *B. subtilis*, since knock out of corresponding genes proved to be lethal (WU et al., 1995; MACK et al., 1998; SANTOS et al., 2000; GERDES et al., 2002).

Human flavokinase (Fmn1p; encoded by the gene *RFK*) is an 18.5 kDa protein, whose tridimensional structure has been determined (KARTHIKEYAN et al., 2003) (Fig. 4). The RFK-riboflavin ternary complex structure shows that the isoalloxazine ring of the substrate is bound in a deep pocket between parts of the outer surface of a β-barrel. The hydrophobic dimethylbenzene edge sits at the bottom of this pocket and is surrounded by the hydrophobic residues I53, V69, F116, L122 and I126. The 2,4-pyrimidindione (uracil) moiety of the isoalloxazine ring points toward the opening of the pocket. All the amino acid residues that interact with riboflavin are highly conserved throughout the RFK family (KARTHIKEYAN et al., 2003).

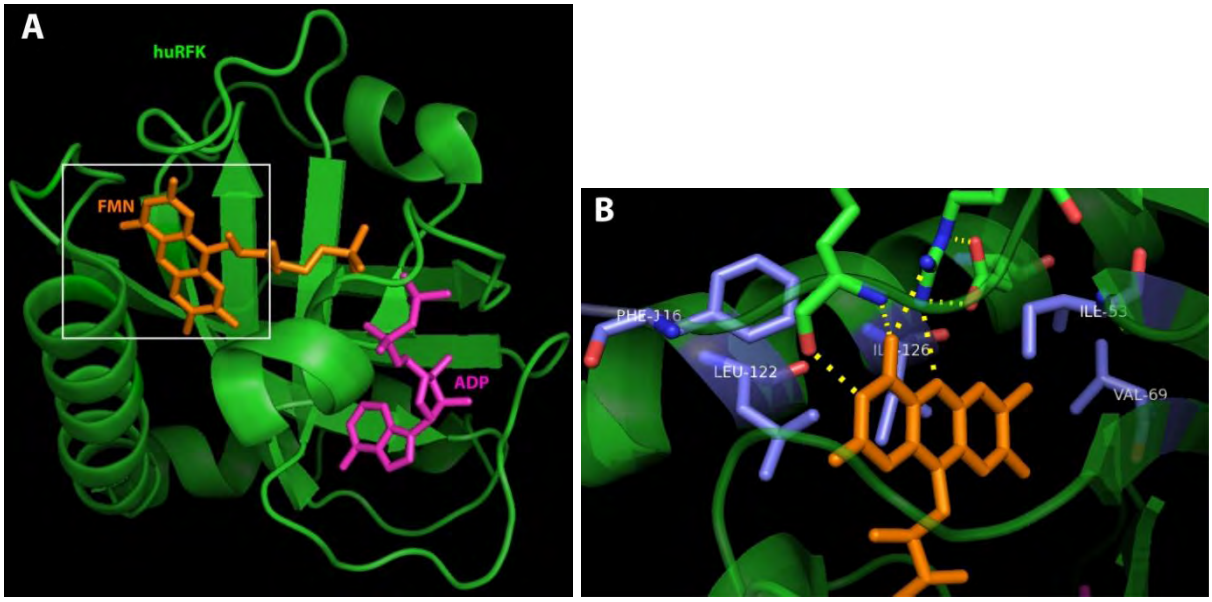

Figure 4. Tridimensional structure of human riboflavin kinase (huRFK) structure. (A) huRFK bound to flavin mononucleotide (FMN) and to adenosine 5'-diphosphate (ADP); the white square marks the binding pocked portion showed in detail in B. (B) huRFK binding pocket: blue chains show the hydrophobic environment created by the residues I53, V69, F116, L122 and I126; yellow discontinuous lines represent hydrogen bounds (Protein Data Bank code: 1NBO; KARTHIKEYAN et al., 2003).

At least two isoforms of human FAD synthetase exist, which are the gene products of *FLAD1* transcript variant 1 (65 kDa) and transcript variant 2 (54 kDa). Isoform 1 probably represents the mitochondrial enzyme whereas isoform 2 is a cytosolic enzyme. Both enzymes have already been overproduced in *E. coli*. Isoform 1 could easily be recovered from the cell-free extract but isoform 2 was mainly produced as insoluble protein in the heterologous host *E. coli* (BRIZIO et al., 2006; GALLUCCIO et al., 2007; TORCHETTI et al., 2010).

1.4 Regulation of cellular flavin content

In general, the catalytic efficiencies and reaction rates of the enzymes involved in riboflavin biosynthesis are very low, indicating a relatively small cellular demand for riboflavin and flavin coenzymes (FISCHER & BACHER, 2005; ABBAS, 2011). The amount of flavins in the cytoplasm in principle can be regulated at the enzyme level or at the level of gene expression.

Very little is known about the regulation of flavin synthesis at the level of enzyme activity. In *B. subtilis* the reactions catalyzed by bifunctional RibA (GTP cyclohydrolase II/

3,4-dihydroxy-2-butanone 4-phosphate synthase) are rate-limiting in riboflavin synthesis. The integration of one additional *ribA* copy into the chromosome improved riboflavin production by an industrial *B. subtilis* strain. However, expression of additional copies of GTP cyclohydrolase II (only the C-terminal part of RibA) reduced cell growth, suggesting that an excess of GTP cyclohydrolase II activity exerts a negative effect on cell viability (HÜMBELIN et al., 1999). A feedback regulatory mechanism was described for 6,7-dimethyl-8-ribityllumazine synthase of *Pichia guilliermondii*, which is inhibited by riboflavin but not FAD (ABBAS & SIBIRNY, 2011).

Another regulatory mechanism of cellular flavin content is the adenylylation of FMN by FAD synthetase. The reaction rate is lower and the affinity of the FAD synthetase for the substrate is poorer as compared to the flavokinase. Thus, in the cytoplasm riboflavin is readily converted to FMN, but the conversion of FMN slower allowing FMN accumulation (KEARNEY et al., 1979; MACK et al., 1998; GRILL et al., 2008).

More is known with respect to the regulation of flavin biosynthesis at the level of gene expression. The so called FMN riboswitches are probably the most important control modules of flavin concentration within bacterial cells. FMN riboswitches are found in the 5'-untranslated region (UTR) of bacterial mRNA coding for riboflavin biosynthetic and/or transporter genes. They are able to directly sense the FMN concentration within the cytoplasm and control gene expression without the need for protein assistance. In *B. subtilis* two FMN riboswitches have been described, one controls the riboflavin biosynthetic cluster *ribGBAH(T)* and another controls the gene *ypaA* coding for a flavin importer (VITRESCHAK et al., 2002; LEE et al., 2009). A similar control system is present in *F. nucleatum*, where one FMN riboswitch controls expression of the *ribHDE(B/A)* cluster and another controls the transporter gene *impX* (SERGANOV et al., 2009). In *E. coli* only the *ribB* gene (coding for 3,4-dihydroxy-2-butanone-4-phosphate synthase) is predicted to be under control of an FMN riboswitch (VITRESCHAK et al., 2002), and in the pathogenic Gram-positive bacterium *Listeria monocytogenes*, unable to synthesize riboflavin *de novo*, one FMN riboswitch has been annotated upstream of the putative riboflavin transporter gene *lmo1945* (MANSJÖ & JOHANSSON, 2011).

Regulation of expression of riboflavin biosynthetic genes can also be triggered by other factors besides flavins, e.g. superoxide-generating agents and iron deficiency (ABBAS & SIBIRNY, 2011).

1.5 Riboswitches

Experimental proofs of the existence of non-coding RNAs carrying metabolite-sensing domains able to control gene expression were first published in 2002 (MIRONOV et al., 2002; WINKLER et al., 2002). Since then new riboswitches have been described and classified into 17 classes based on a conservative approach, wherein representatives are grouped either by similar global architecture or by similar binding site architecture. The most widespread riboswitches classes are the coenzyme-sensing (TPP, adenosylcobalamin and FMN), the amino acid-sensing (lysine and glycine) and the nucleotide-sensing (guanine and adenine) riboswitches (BREAKER, 2010).

Riboswitches are RNA sequences almost exclusively located in the 5'-untranslated regions (5'-UTRs) of the mRNA whose expression they control. Genes under control of riboswitches are usually involved in the synthesis and/or transport of their cognate ligand. The tertiary structure formed by the riboswitch sequence is able to directly bind a specific metabolite and control gene expression without need for protein factor. The complete riboswitch sequence carries two functional domains: an aptamer and an expression platform. The aptamer is a highly conserved sequence that has evolved to bind to a specific metabolite. The ability of the riboswitch to recognize and bind a particular molecule relies on its tertiary structure. Riboswitches of a given class tend to fold into the similar tertiary structure to sense their cognate ligand. The expression platform is located downstream of the aptamer, where it interprets the ligand binding status of the aptamer and regulates gene expression accordingly. Although competing alternative folding structures adjacent to the aptamer could possibly disturb riboswitch function, expression platforms sequences tend to be far less conserved as compared to aptamer domains (BLOUNT & BREAKER, 2006; BREAKER, 2010; BREAKER, 2011).

All riboswitch classes characterized to date have only been found in eubacteria. The exception is the thiamine pyrophosphate (TPP)-sensing riboswitch, which is also present in archaea and eukaryotes (WACHTER, 2010).

Aptamers can be structurally preorganized in the absence of ligand, but ligand binding induces at least some structural reorganization or stabilization of aptamer substructures that consequently influences the folding and function of the downstream expression platform. This metabolite-dependent interplay between aptamer and expression platform is exploited by the riboswitch to modulate gene expression through various mechanisms (BREAKER, 2010).

Among the bacterial riboswitches described until now, two mechanisms are commonly employed to control gene expression involving either transcription termination or translation initiation. As an example, the well characterized FMN riboswitches are shown in the figure 5. If the cognate metabolite is not present when the 5′-UTR is transcribed (“gene ON”), riboswitches controlled by transcription termination usually fold into a structure called anti-terminator that does not interfere with the expression of the adjacent open reading frame (ORF) (Fig 5A). When present in a sufficiently high concentration, the metabolite binds to the riboswitch aptamer, which induces the formation of a stable stem followed by a run of uridine residues constituting an intrinsic transcription terminator (Rho independent terminator) (BREAKER, 2010). The formation of the terminator stem causes RNA polymerase to halt transcription and to eventually release the DNA template and the nascent RNA product (“gene OFF”) (Fig. 5B). For an effective control, metabolite binding must occur during the short time frame between aptamer formation and synthesis of the terminator sequence by RNA polymerase. The well characterized *ribG* FMN riboswitch of *B. subtilis* and the *impX* FMN riboswitch of *F. nucleatum* are examples of riboswitches that control gene expression by transcription termination (VITRESCHAK et al., 2002; WICKISER et al., 2005b; SERGANOV et al., 2009; BREAKER, 2010).

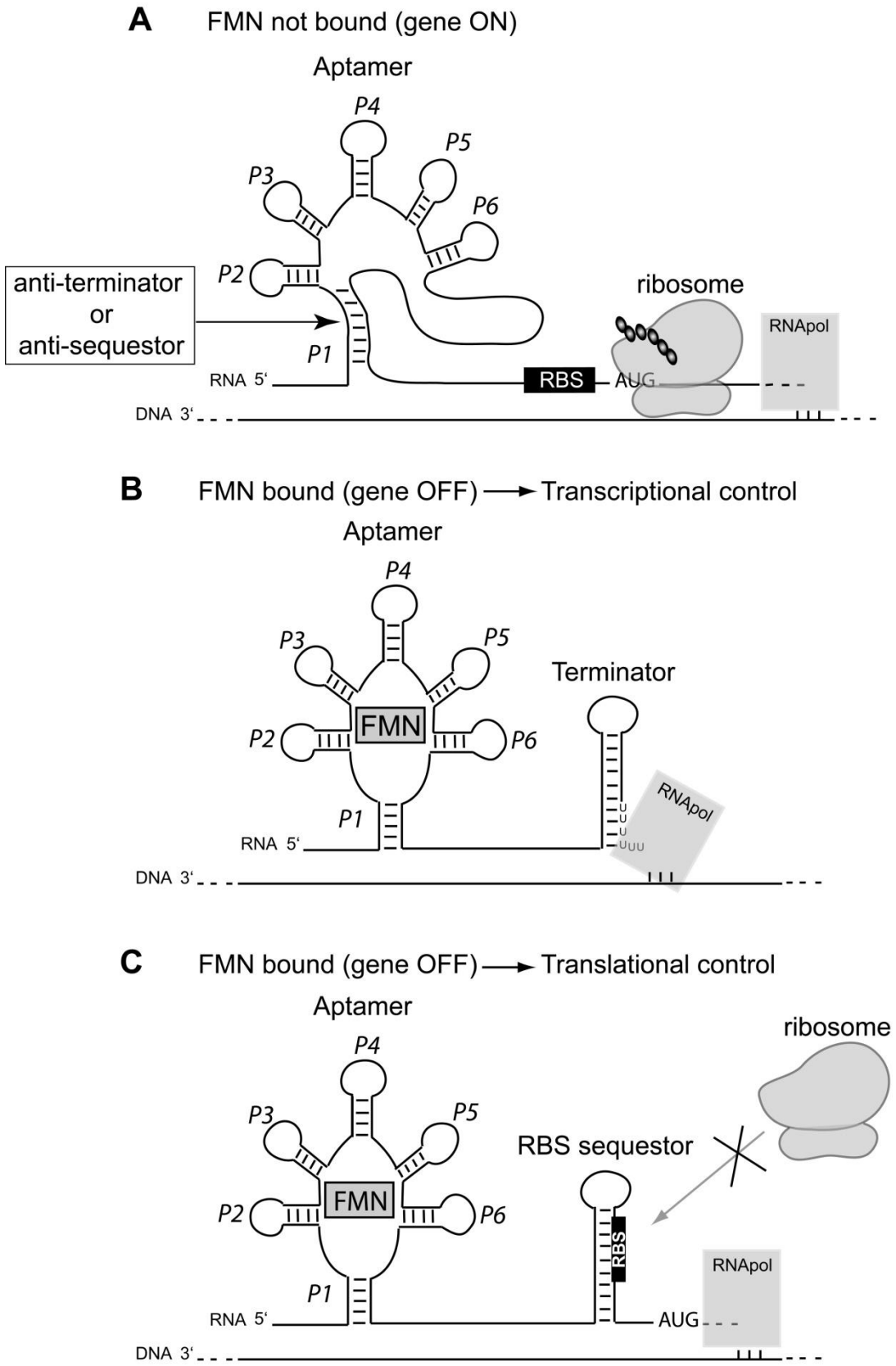

Figure 5. Common mechanisms employed by already described riboswitches to regulate gene expression. The FMN riboswitch, which can control gene expression either by transcription termination or translation prevention, is exemplarily shown. (A) at low FMN concentration the aptamer does not bind the metabolite and folds into the anti-termination or anti-sequestor

stem, thus transcription proceeds, translation initiates and protein expression is achieved; (B) at high FMN concentration the aptamer binds the metabolite and folds to a specific structure (butterfly-like scaffold) which allows the terminator stem to form causing transcription termination; (C) alternatively at high FMN concentrations the aptamer binds the metabolite and folds to a specific structure (butterfly-like scaffold) allowing the RBS sequestor stem to form. Consequently, transcription proceeds but ribosome access to the ribosomal binding site (RBS) is prevented and translation cannot initiate.

Similar to the transcriptional control, mutually-exclusive base-paired structures are exploited by riboswitches to control ribosome access to the ribosomal binding site (RBS) or Shine-Dalgarno (SD) sequence *via* an RBS-sequestor stem formation (Fig 5C), thereby regulating translation initiation. Riboswitches controlling translation initiation can either deactivate or activate gene expression. The prevention of translation initiation, the most common mechanism among the already described riboswitches, exploits a sequestor that, upon ligand binding, overlaps only the RBS or both RBS and start codon of the adjacent gene. Thus, RNA polymerase can still proceed with transcription but ribosome access to the RBS is prevented and translation cannot initiate. The *ypaA* FMN riboswitch responsible for regulation of a flavin transporter in *B. subtilis* is an example of a riboswitch that modulates gene expression by preventing translation initiation (VITRESCHAK et al., 2002; BREAKER, 2010). This mechanism could be used by cells to regulate translation from full-length mRNAs. However, it is also possible that the transcription terminator protein Rho recognizes nascent mRNAs that are not being actively translated therefore causing premature transcription termination (CIAMPI, 2006). Moreover, translation initiation control by riboswitches can also function to activate translation by exposing the RBS or RBS and start codon upon metabolite binding. In this case, in the absence of the cognate metabolite, RBS is trapped in the riboswitch secondary structure. Upon metabolite binding to the aptamer, RBS is released from base pairing and exposed to the ribosome (WANG et al., 2008).

Control of splicing reactions is the only regulatory mechanism described for eukaryotic riboswitches investigated up to now. In some fungal and plant TPP riboswitches, a 5’ splice site is blocked by base pairing with nucleotides of the aptamer. Consequently, the spliceosome cannot access it. At high TPP concentrations, the riboswitch aptamer binds this metabolite and stabilizes an RNA secondary structure that does not involve base pairing with the 5’ splice site nucleotides. Once the 5’ splice site is available, the spliceosome can act on it (WACHTER, 2010; BREAKER, 2011). In the green alga *Chlamydomonas reinhardtii*, it has been demonstrated that *THIC* (gene involved in the TPP biosynthesis) pre-mRNAs are

alternatively spliced in the riboswitch-containing intron with the ratio of splice products being dependent on cellular TPP levels. The *THIC* pre-mRNA contains an aptamer in a cassette exon, which is spliced out under conditions of low cellular TPP levels leading to an mRNA that encodes the full-length protein (Fig. 6A). Elevated TPP levels result in retention of an exon, which introduces a premature termination codon and thereby prevents expression of the functional protein (Fig. 6B) (WACHTER, 2010).

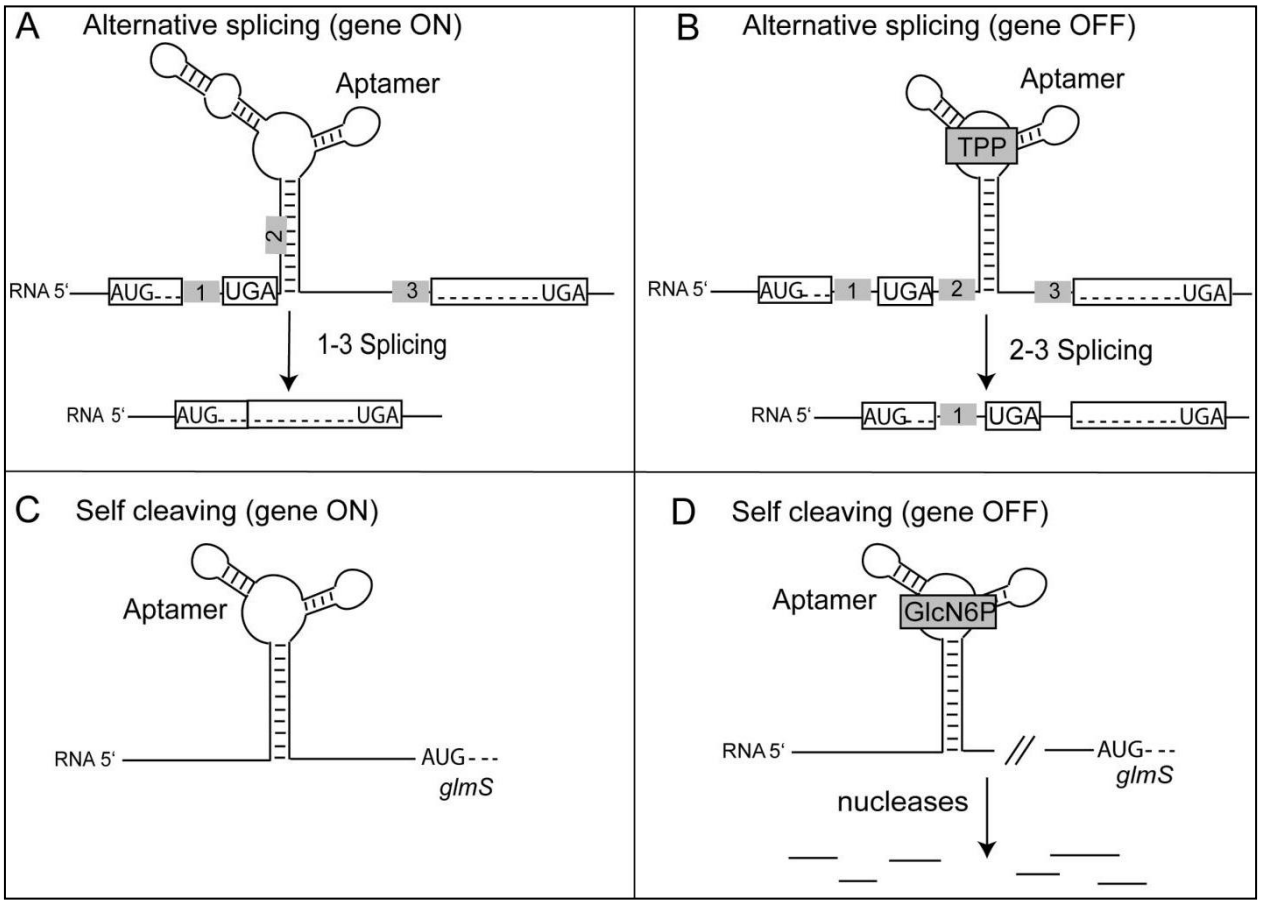

Figure 6. Rare mechanisms of riboswitch-mediated gene regulation among the already described riboswitches. The *THIC* TPP riboswitch from *Chlamydomonas reinhardtii* (A and B) and the *glmS* ribozyme riboswitch (C and D) are exemplarily shown. (A) At low TPP concentration the secondary structure assumed by the aptamer overlaps the splicing site 2 directing the spliceosome to the sites 1 and 3. Splicing at sites 1 and 3 generates a mature mRNA encoding full-length protein. (B) At high TPP concentrations the aptamer binds the metabolite and folds into a specific structure that exposes the splicing site 2. Splicing at sites 2 and 3 generates a mature mRNA containing an intron fragment which introduces a premature termination codon preventing expression of functional THIC protein. (C) At low glucose-6-phosphate (GlcN6P) concentration the ligand-dependent self-cleaving ribozyme is not active, thus the mRNA is not modified and the GlcN6P synthase is translated. (D) At high GlcN6P concentration the aptamer binds the metabolite and activates the ribozyme activity

causing self-cleavage of the mRNA; cleaved mRNA is readily digested by nucleases. The structures represented in this figure are simple illustrations; they do not intend to reproduce the secondary structures of the described riboswitch aptamers.

Another mechanism of riboswitch action is present in some Gram-positive bacteria, which mediates gene control by modulation of ribozyme activity. The *glmS* riboswitch stimulates a self-cleaving activity upon glucosamine-6-phosphate binding (COPPINS et al., 2007; BREAKER, 2010). At high GlcN6P concentration the aptamer binds the metabolite and activates the ribozyme activity causing self-cleavage of the mRNA. The cleaved mRNA is readily digested by nucleases (Fig. 6D). It has been shown that the *glmS* ribozyme is fully folded in solution prior to binding and that the GlcN6P ligand acts as a coenzyme and participates in the cleavage reaction without inducing a conformational change (TINSLEY et al., 2007).

Additionally, riboswitches do not necessarily function to exclusively control the expression of adjacent ORFs. In *Listeria monocytogenes*, two *S*-adenosylmethionine (SAM) riboswitches (SreA and SreB) can also function in *trans* and act as noncoding RNAs, thus controlling expression of the virulence regulator PrfA by binding to the 5'-untranslated region of its mRNA (LOH et al., 2009).

1.6 FMN riboswitches

Up to now, the tridimensional structure of only one FMN riboswitch (RFN element) has been determined. SERGANOV et al. (2009) solved the crystal structures of the *Fusobacterium nucleatum impX* FMN riboswitch bound to FMN, FAD, riboflavin and roseoflavin. The structure does not fold by collinear stacking of adjacent helices, unlike other large RNAs. Instead, it adopts a unique butterfly-like scaffold, stapled together by two nearly identical domains formed by the stem-loops P2–P6 and P3–P5 (see Fig. 5) that are connected by the FMN-bound junction. FMN is positioned within the junctional site and is specifically bound to RNA through interactions with the isoalloxazine ring and with the phosphate moiety mediated by Mg^{2+}. The interactions between the FMN and riboswitch aptamer critically depend on the physiological concentration of Mg^{2+} and can be enhanced further by addition of K^{+}. FAD, riboflavin, roseoflavin and lumiflavin have much lower affinity for the riboswitch than FMN. The binding of riboflavin and roseoflavin requires several nucleotides to be slightly re-positioned in relation to the FMN-bound structure. Moreover, additional spatial

adjustments are necessary to accommodate the dimethylamino group of roseoflavin. According to that study the intrinsic plasticity of the FMN-binding pocket and the availability of large openings make the riboswitch an attractive target for structure-based design of FMN-like antimicrobial compounds.

Recent studies have demonstrated slow kinetics of association and dissociation for the FMN riboswitch complex, suggesting a kinetically driven riboswitch mechanism. It was observed that the *ribG* FMN riboswitch of *B. subtilis* does not achieve thermodynamic equilibrium with the FMN ligand by the time the RNA polymerase reaches the decision point between transcription elongation or termination. Thus, the riboswitch control relies on the metabolite association rate. This explains why the concentration required to trigger efficient transcription termination (T_{50}) is more than 10-fold higher than the K_D value measured for the minimal aptamer domain. Additional factors such as transcriptional pause sites were also observed to provide more time for the ligand to bind before the genetic decision is made (WICKISER et al., 2005b).

The kinetic characteristics of FMN riboswitches are consistent with the recognition principles identified in the three dimensional structure of the *F. nucleatum* riboswitch, which as the *ribG* FMN riboswitch of *B. subtilis* controls gene expression by transcription termination. Riboswitch folding requires conformational adjustments upon FMN binding, which may account for the slow association rate. Subsequent FMN release is likely to be slowed down due to envelopment of the ligand by the RNA (SERGANOV et al., 2009; SERGANOV, 2009).

Recent works showed that some antibacterial compounds function by targeting riboswitches (BLOUNT & BREAKER, 2006). It was found that roseoflavin *in vitro* binds with high affinity to the *B. subtilis* FMN riboswitch aptamer and *in vivo* reduces expression of a P_{lysC} driven *B. subtilis* FMN riboswitch-*lacZ* reporter gene (LEE et al., 2009). Notably, in this study the relatively strong promoter P_{lysC} was used instead of the natural promoter P_{rib}. Similar *in vivo* studies using the natural (relatively weak) promoter P_{rib} of *B. subtilis* were also performed, however, the repressing effect of roseoflavin could only be shown in strains overproducing a flavin importer protein (*S. davawensis* RibM) (OTT et al., 2009). Importantly, the study mentioned above (LEE et al., 2009) also showed, that the genes *ribGBAHT* controlled by the FMN riboswitch are required for optimal growth of *B. subtilis*. Accordingly, a compound which blocks FMN riboswitches should induce riboflavin auxotrophy and consequently reduce cell growth.

1.7 Streptomycetes

Streptomycetes are Gram-positive bacteria which have a genome with a relatively high G+C content. The members of the genus *Streptomyces* are soil bacteria widely spread in nature. Their ability to colonize the soil is greatly facilitated by growth as a vegetative hyphal mass which can differentiate into spores that are responsible for spread and persistence. Streptomycetes have been intensively studied for decades due to their ability to produce secondary metabolites with biological activities including antibacterial, antifungal, antiparasitic and antitumor drugs. The ecological role of antibiotic production by Streptomycetes is probably the defense of the colony during aerial mycelium development and it probably does not provide ability to the producer to invade a substrate with an established population. Antibiotic production is usually growth phase dependent. Under laboratory conditions it begins in the stationary phase for liquid cultures and coincides with the beginning of morphological differentiation in cultures on solid surfaces (agar-agar containing culture plates). Most antibiotics are synthesized through complex biosynthetic pathways. The genes involved in these pathways are usually clustered and contain pathway-specific regulatory genes which act as transcriptional activators and are themselves subject to control. In addition to these positive effectors, antibiotic synthesis may also be subject to metabolite repression and/or inhibition by readily utilized sources of nitrogen, phosphate and glucose (KIESER, 2000).

Most genetic studies of antibiotic regulation have used *S. coelicolor* A3(2) that is the wild type without plasmids. Like many other Streptomycetes, *S. coelicolor* has a single linear chromosome (8.6 Mbp) with a centrally located origin of replication (*ori*C) and terminal inverted repeats (TIRs) carrying covalently bound proteins on the free 5' ends. Essential genes, like those related to cell division, DNA replication, transcription, translation and amino acid biosynthesis, are located in the core region of the chromosome. In contrast, non-essential genes, such as those coding for secondary metabolites and hydrolytic exoenzymes, are present in the chromosome arms. Most Streptomycetes have self-transmissible plasmids that can be either circular or linear and, like the chromosome, carry TIRs and proteins at their 5' ends. *S. coelicolor* has two plasmids, SCP1 (365 Kbp linear plasmid) and SCP2 (31 Kbp circular plasmid) (BENTLEY, 2002).

Streptomyces phages, both lytic and temperate, are abundant. Among temperate phages, some lysogenize by integrating site-specifically into the host chromosome (e.g. at the bacteriophage ΦC31 attachment site). The ΦC31 integrase acts on an *attB* site and an *attP* site to form *attL* and *attR* without requirement of an accessory factor for integration (KIESER, 2000).

In *S. davawensis*, which also has a linear chromosome (8.9 Mbp), riboflavin biosynthetic genes have been identified at two locations. The open reading frames of the riboflavin gene cluster *ribBMAH* and *ribGY* (or *ribGA*) show a high degree of similarity on the amino acid and also on the nucleotide level to riboflavin biosynthetic genes within the framework of the genome projects of *Streptomyces coelicolor* (BENTLEY et al., 2002) and *Streptomyces avermitilis* (IKEDA et al., 2003). By comparative genomics techniques it was predicted that the *ribBMAH* genes of *S. coelicolor* are regulated by an FMN riboswitch element which most probably controls gene expression by preventing ribosome access to the ribosomal binding site (RBS) (VITRESCHAK et al., 2002). *S. davawensis* also has an FMN riboswitch in front of *ribB*, and given the high level of sequence similarity with the *S. coelicolor* FMN riboswitch it is reasonable to assume that it also regulates gene expression at the translational level.

1.8 Flavin analogs

Only few natural riboflavin analogs are known. Among them, cofactor F_{420} and the molybdopterin are found in different group of organisms. Roseoflavin, which is produced by *Streptomyces davawensis*, calls special attention because it is the only known natural flavin analog with antibiotic activity. Moreover, *S. davawensis* is the only organism known to produce roseoflavin.

Cofactor F_{420} (5-deaza-7,8-didemethyl-8-hydroxyriboflavin) and its precursor F_0 (so called 5-deazaflavins) (Fig. 7A) are two-electron carriers found in archaea and mycobacteria (DI MARCO et al., 1990; BASHIRI et al., 2010). It has been shown that F_{420} has a direct and important role in archaeal energy metabolism whereas its role in mycobacterial metabolism has not been completely elucidated yet. F_{420} is used by *Streptomyces* for lincomycin and tetracycline biosynthesis, and it has been found to serve as a second chromophore in DNA photolyases of Archaea and cyanobacteria. In Mycobacterium F_{420} is used by F_{420}-dependent glucose-6-phosphate dehydrogenase (MACK & GRILL, 2006; PURWANTINI &

MUKHOPADHYAY, 2009). The isoalloxazine chromophore of F_{420} is structurally very similar to that of the flavins (FMN and FAD), although it is functionally similar to $NAD(P)^+$ (DI MARCO et al., 1990; BASHIRI et al., 2010).

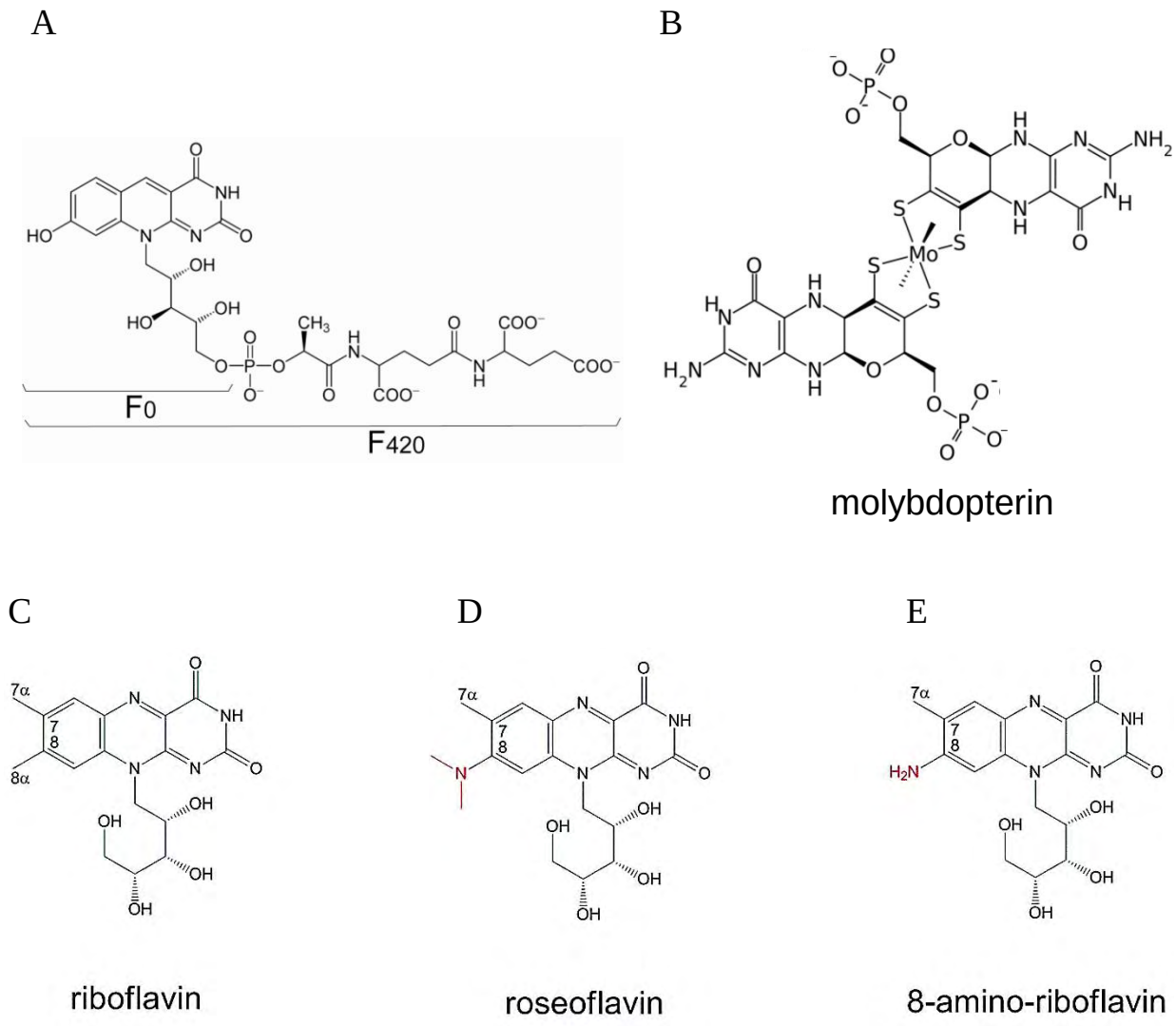

Figure 7. The structure of (A) cofactor F_{420} (5-deaza-7,8-didemethyl-8-hydroxyriboflavin) and its precursor F_0 (so called 5-deazaflavins); (B) molybdopterin; (C) 7,8-dimethyl-10-(D-1′-ribityl)isoalloxazine or riboflavin (RF), also known as vitamin B_2; (D) 8-dimethyl-amino-8-demethyl-D-riboflavin or roseoflavin (RoF); (E) 8-demethyl-8-amino-D-riboflavin (AF). The C8- substituent groups of RoF and AF (in relation to RF) are red marked.

Molybdopterin (Fig. 7B) is a molybdenum-carrying cofactor involved in several enzymatic redox reactions. Molybdopterin-containing enzymes catalyze the transfer of an oxygen atom in a two-electron redox reaction. It is found in bacteria, plants, and animals. A

relatively large number of gene products are involved in its biosynthesis and the pyrimidine ring of molybdopterin is derived from that of a guanine precursor, probably GTP. Like the riboflavin derived cofactors (FMN and FAD), the molybdopterin cofactor may be found either as the mononucleotide or the dinucleotidic form (ROMÃO et al., 1997; MACK & GRILL, 2006).

Roseoflavin (8-dimethyl-amino-8-demethyl-D-riboflavin; RoF) and its precursor 8-demethyl-8-amino-riboflavin (AF) (OTANI et al., 1974; JURI et al., 1987; JANKOWITSCH et al., 2011) are produced by the Gram-positive bacterium *Streptomyces davawensis* and are the only known natural flavin analogs with antibiotic activity. Roseoflavin is found in the *S. davawensis* culture medium and easily identified by the characteristic pink color. Although no specific RoF exporter has been found yet, the secretion seems to be very efficient. As an intermediate compound in roseoflavin biosynthesis, 8-amino riboflavin is not secreted, it accumulates in the cells. In an *S. davawensis rosA* deficient strain, 8-amino riboflavin was found in the culture medium. Apparently, in the absence of RosA 8-amino riboflavin accumulates and is secreted by *S. davawensis* (JANKOWITSCH et al., 2011).

Roseoflavin and 8-amino riboflavin differ from riboflavin at position C8 in the isoalloxazine ring. Riboflavin carries a methyl group at C8, whereas roseoflavin carries a dimethyl-amino group and 8-amino riboflavin has an amino substituent at C8 (Fig. 7D and E). Both compounds show antimicrobial activity against Gram-positive bacteria such as *Bacillus subtilis* but also against Gram-negative bacteria if uptake systems for flavins/flavin analogs are present (OTANI et al., 1974; GRILL et al., 2007). The minimal inhibitory concentration (MIC) of roseoflavin was found to be 1.56 µg ml^{-1} for *B. subtilis*, and varied from 0.25 to 6.25 µg ml^{-1} for *Staphylococcus aureus* (depending on the dilution method used) (OTANI et al., 1974).

In *Streptomyces davawensis* roseoflavin is synthesized during the stationary growth phase most likely from GTP and ribulose-5-phosphate through riboflavin, 8-demethyl-8-amino-D-riboflavin (AF) and 8-methylamino-8-demethyl-D-riboflavin (MAF) (JURI et al., 1987). Recently, the gene and enzyme responsible for the conversion of AF to MAF and finally to RoF in *S. davawensis* has been identified (JANKOWITSCH et al., 2011). The complete biosynthetic pathway and the genes and enzymes involved on it are still unknown.

Like riboflavin, roseoflavin and 8-amino riboflavin most likely are activated by flavokinase and FAD synthetase within the cytoplasm (Fig. 8). In roseoflavin sensitive *B. subtilis*, roseoflavin is converted to the FMN/FAD-analogs roseoflavin mononucleotide

(RoFMN) and roseoflavin adenine dinucleotide (RoFAD) by bifunctional RibC (GRILL et al., 2008). This suggests that RoFMN rather than roseoflavin is the active (toxic) compound in the cytoplasm of target cells. It was initially hypothesized that a specialized enzyme RibC not synthesizing RoFMN was present in *S. davawensis* conferring roseoflavin resistance to the producer organism. However, RibC was biochemically characterized and found to produce RoFMN and RoFAD with a similar efficiency as RibC from *B. subtilis* (GRILL et al., 2008). Similar bifunctional enzymes are present in the roseoflavin sensitive bacteria *S. albus*, *S. avermitilis*, *S. lividans* and *S. coelicolor* and it is very likely that imported flavin analogs are as well phosphorylated and adenylylated in these organisms. It was shown, that for D-amino acid oxidase (DAAO) from *Sus scrofa* RoFAD is an inactive cofactor (GRILL et al., 2008). *E. coli* and other members of the Enterobacteriaceae seem not to be affected by roseoflavin. As these organisms are devoid of a transport system for riboflavin, their resistance is probably due to a very limited uptake of roseoflavin (MACK & GRILL, 2006; GRILL et al., 2007; HEMBERGER et al., 2011).

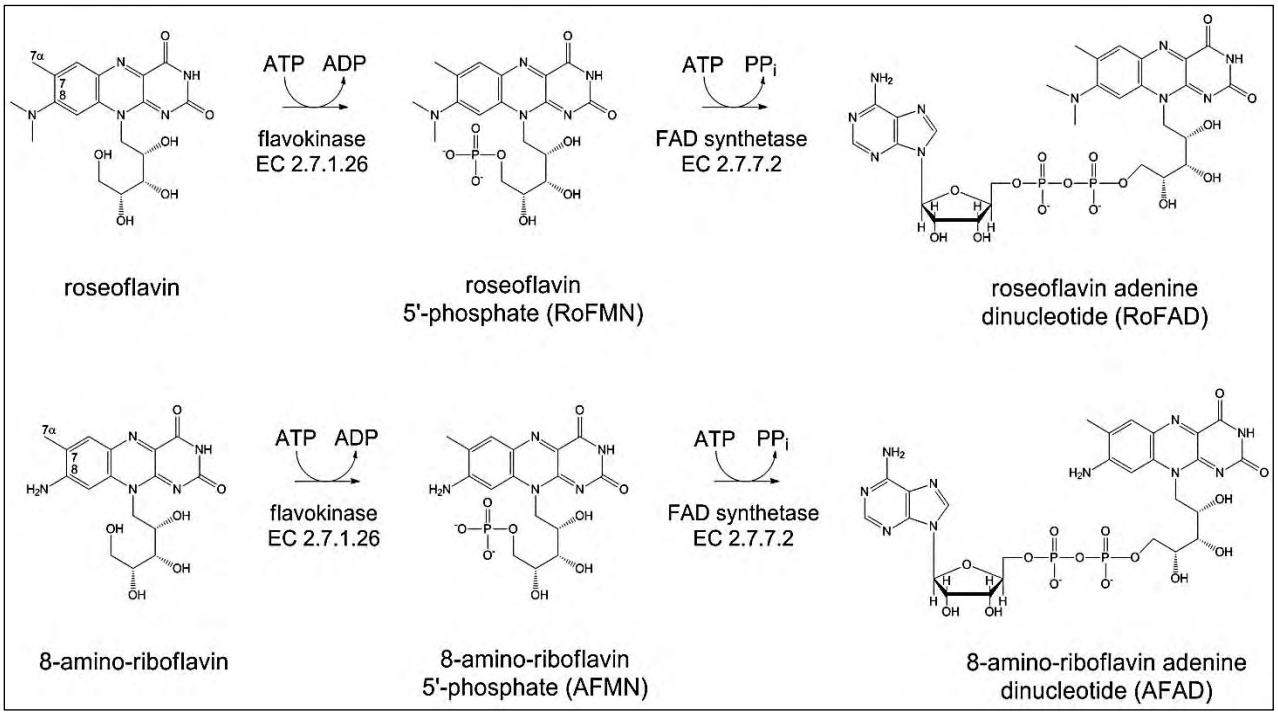

Figure 8. The enzymatic conversion of roseoflavin (top) into roseoflavin mononucleotide (RoFMN) and roseoflavin adenine dinucleotide (RoFAD) and of (8-demethyl)-8-amino-riboflavin (bottom) into (8-demethyl)-8-amino-riboflavin mononucleotide (AFMN) and (8-demethyl)-8-amino-riboflavin adenine dinucleotide (AFAD).

Chemical synthesis has already produced several riboflavin analogs, some of them with significant antibacterial or antiprotist activity. Synthetic analogs of riboflavin differing with respect to the substituent at C_7, C_8 or C_{10} were examined for their toxicity to *Tetrahymena pyriformis*. All analogs with altered substituents at C_7 or C_8 (7-methyl-8-ethyl, 7,8-diethyl, 7-chloro-8-methy, 7-methyl-8-chloro 7-methyl-8-bromoflavin) were able to inhibit growth to some extent. The analogs had an inhibitory effect even when administrated in association with small amounts of riboflavin (0.24 µM). A 7-ethyl analog with a bis(2-hydroxyethyl)aminoethyl side chain was the most potent antagonist (WALLACE & HOLMLUND, 1980). In another study a riboflavin-dependent mutant of *Pichia guilliermondii* was used as a test organism. The antagonistic properties were distinctly displayed only by D-ribityl derivatives of riboflavin with a substitution of CF_3, Cl, H, NH_2 and $N(CH_3)_2$ at C7 or C8. The analogs which were phosphorylated by flavokinase from *P. guilliermondii* were shown to be effective antivitamins. The inhibition of growth by the antivitamins on yeast was competitively eliminated by adding riboflavin to the growth medium (KASHCHENKO et al., 1982). Kasai et al. (1979) reported the anti-riboflavin activity of 8-O-alkyl derivatives of riboflavin in some Gram-positive bacteria, and another report describes the anti-riboflavin activity of 8-N-alkyl analogs of roseoflavin in some Gram-positive bacteria (KASAI et al., 1978). The latter report claims that the anti-riboflavin activity of the analogs could not be completely explained by the difference of the redox potential as compared to riboflavin.

It was reported that flavin analogs may be good steric replacements for riboflavin but not catalytic substitutes (HASFORD & RIZZO, 1998). Accordingly, these analogs may combine with FMN- or FAD-dependent flavoenzymes, reduce their activity and negatively affect the cell metabolism. For D-amino acid oxidase (EC 1.4.3.3) from *Sus scrofa* it was shown that RoFAD is an inactive cofactor (GRILL et al., 2008). Riboflavin analogs with electron-donating substituents at position 8 were described to be inert to several biological reductants and consequently were thought not to function as redox-active components (roseoflavin E_0` = –222 mV; riboflavin $E_0$` = –208 mV). Furthermore, it was reported that protonation of flavin analogs which carry an amino group at C8 have a strongly altered redox potential, e.g. for protonated roseoflavin an E_0` of +190 mV was published (HASFORD & RIZZO, 1998).

1.9 Riboflavin analogs as potential novel anti-infective drugs

Antibiotic-resistant organisms have emerged in the past two decades and now represent a threat to human health. Examples include methicillin-resistant staphylococci, pneumococci resistant to both penicillin and macrolides, vancomycin-resistant enterococci, and multidrug-resistant strains of *Mycobacterium tuberculosis*. Resistance has also become a major problem in the treatment of malaria (NORRBY et al., 2005). The commercially available anti-infective drugs have a very narrow spectrum of cellular targets. Therefore, new antibiotics targeting different cellular processes are urgently required to overcome drug resistance (BLOUNT & BREAKER, 2006).

Vitamin analogs are interesting antimicrobials for two reasons. First, many microorganisms have efficient vitamin transporters, which catalyze the rapid uptake of vitamins and also vitamin analogs (VOGL et al., 2007). Consequently, the delivery of the vitamin analog to the target molecules is very efficient. Second, many vitamin analogs have multiple cellular targets and thus the chance of developing resistance is much less likely.

In a very recent study it was investigated how roseoflavin affected FMN-riboswitch mediated gene-expression, growth and infectivity of the human bacterial pathogen *Listeria monocytogenes* (MANSJÖ & JOHANSSON, 2011). *L. monocytogenes* is unable to synthesize riboflavin *de novo* and therefore it is dependent on riboflavin uptake from environment. A putative flavin transporter (similar to YpaA of *B. subtilis*) is present in *L. monocytogenes* genome and is regulated by an FMN riboswitch, the only one found in the bacterium up to now. The results showed that roseoflavin strongly affects the growth of *L. monocytogenes*, which was inhibited at concentrations as low as 1 µM. Riboswitches form structured receptors that are among the most selective of any RNA drug target. This highly conserved riboswitch structure has been widely perceived as a promising specific therapeutic target, since riboswitch-mediated gene regulation has not been described in humans until now (BLOUNT & BREAKER, 2006).

An ongoing research at the Australian National University (Canberra, Australia) was already able to show that roseoflavin strongly inhibits *in vitro* proliferation of *Plasmodium falciparum* the causative agent of malaria in humans. Increasing the concentration of riboflavin in the culture medium reduced the antiplasmodial activity of roseoflavin, suggesting a mechanism of action related to the transport and/or utilization of riboflavin by the parasite. Consistent with this, roseoflavin reduced riboflavin accumulation by infected

erythrocytes and isolated parasites but not by uninfected erythrocytes (Kylie Easton, personal communication).

1.10 Objectives of the work

In the present work, the riboflavin analogs roseoflavin and 8-amino riboflavin, and their respective phosphorylated and adenylylated forms, were evaluated in regards to the molecular activity using two different approaches:

1. It was hypothesized earlier that some antibacterial compounds may function by targeting riboswitches (BLOUNT & BREAKER, 2006). Roseoflavin, the only known natural riboflavin analog with antibiotic activity, was found to bind to FMN riboswitches and affect gene expression (LEE et al., 2009; OTT et al., 2009). In order to understand the mechanism of roseoflavin resistance of the producer organism *Streptomyces davawensis* and shed light on the mechanism of action of roseoflavin, the FMN riboswitches from *Bacillus subtilis*, *Streptomyces coelicolor* and *S. davawensis* were compared *in vitro* and *in vivo* with respect to their ability to control gene expression upon treatment with flavins and flavin analogs (Chapter I).

2. Riboflavin analogs have the outstanding potential to serve as basic structures for the development of novel antiinfectives (MACK & GRILL, 2006) and it is possible that in the future they will be able to meet the urgent need for new molecules to fight multiresistant microorganisms (GERDES et al., 2002). In parallel to developing flavin analogs as antiinfectives it is important to study their metabolism in humans. Therefore, the activity of human flavokinase and human FAD synthetase on roseoflavin and 8-amino riboflavin was tested (Chapter II).

2. Materials & Methods

2.1 Chemicals

All chemicals were purchased from Sigma-Aldrich (Munich, Germany), if not otherwise specified. Restriction endonucleases and other cloning reagents were purchased from Fermentas (St Leon Rot, Germany). Bacto peptone and bacto yeast extract were from Difco (Becton Dickinson, Heidelberg, Germany).

Riboflavin (RF), flavin mononucleotide (FMN) and flavin adenine dinucleotide (FAD) were purchased from Sigma (Munich, Germany). roseoflavin was obtained from Chemos (Regenstauf, Germany). The riboflavin analog 8-demethyl-8-amino riboflavin (AF) was prepared synthetically and was a gift from Peter Macheroux (Dept. of Biochemistry, Graz University of Technology, Austria). Roseoflavin mononucleotide (RoFMN), roseoflavin adenine dinucleotide (RoFAD) and 8-demethyl-8-amino riboflavin mononucleotide (AFMN) were enzymatically synthesized from ATP and the corresponding flavin (see section 2.6.4).

2.2 Media and medium additives

Lysogeny Broth (LB) for *E. coli* (SAMBROOK & RUSSELL, 2001)

Component	Final concentration
Tryptone	10 g L^{-1}
Yeast extract	5 g L^{-1}
NaCl	10 g L^{-1}

pH adjusted to 7.0-7.2 with 1.0 M NaOH.

Low salt LB medium for *E. coli*

Component	Final concentration
Tryptone	10 g L^{-1}
Yeast extract	5 g L^{-1}
NaCl	5 g L^{-1}

pH adjusted to 7.5 with 1.0 M NaOH.

SOC medium for *E. coli* (SAMBROOK & RUSSELL, 2001)

Component	Final concentration
Tryptone	20.0 g L^{-1}
Yeast extract	5.0 g L^{-1}
NaCl	0.5 g L^{-1}
KCl*	2.5 mM
$MgCl_2$*	10.0 mM
Glucose**	0.04%

pH adjusted to 7.0 with 1.0 M NaOH.

*KCl and $MgCl_2$ were sterilized by filtration (0.2 µm cellulose acetate syringe filter) and added after autoclaving.

**20% glucose stock solution was separately autoclaved.

GTY medium for *E. coli*

Component	Final concentration
Tryptone	2.5 g L^{-1}
Yeast extract	12.5 g L^{-1}
Glycerol*	10%

pH adjusted to 7.0-7.2 with 1.0 M NaOH.

*50% glycerol stock solution was separately autoclaved.

YPD medium for *Pichia pastoris* (1% yeast extract, 2% peptone, 2% glucose)

Component	Final concentration
Tryptone	20 g L^{-1}
Yeast extract	10 g L^{-1}
Glucose*	20 g L^{-1}

pH adjusted to 7.5 with 1.0 M NaOH.

*200 g L^{-1} glucose stock solution was separately autoclaved.

Nutrient broth for Streptomycetes (YS) (KIESER, 2000)

Component	Final concentration
Yeast extract	2 g L^{-1}
Soluble potato starch	10 g L^{-1}

pH adjusted to 7.0-7.2 with 1.0 M NaOH.

Sporulation broth for Streptomycetes (MS) (KIESER, 2000)

Component	Final concentration
Mannitol	20 g L^{-1}
Soybean meal	20 g L^{-1}
Agar-agar	20 g L^{-1}

pH adjusted to 7.0-7.2 with 1.0 M NaOH.

The soybean meal was first added to tap water and dissolved by incubation at 100°C.

To the conjugation plates 10 mM $MgCl_2$ (sterilized with 0.2 µm cellulose acetate syringe filter) was added after autoclaving.

2xYT broth for conjugation (KIESER, 2000)

Component	Final concentration
Tryptone	16 g L^{-1}
Yeast extract	5 g L^{-1}
NaCl	5 g L^{-1}

pH adjusted to 7.0-7.2 with 1.0 M NaOH.

Minimal medium (MM) for Streptomycetes

Component	Final concentration
KH_2PO_4	1.5 g L^{-1}
K_2HPO_4	2.0 g L^{-1}
$(NH_4)_2SO_4$	1.4 g L^{-1}
$CaCl_2$	0.1 g L^{-1}
$MgSO_4$	0.1 g L^{-1}
Glucose*	10.0 g L^{-1}
Trace elements**	1x

pH adjusted to 7.0-7.2 with 1.0 M NaOH.

*200 g L^{-1} glucose stock solution was separately autoclaved.

** 500x trace elements solution was added after autoclaving.

Trace elements solution 500x

Component	Final concentration (mg L^{-1})
$ZnCl_2$	40 mg L^{-1}
$FeCl_3$ x $6H_2O$	200 mg L^{-1}
$CuCl_2$ x $2H_2O$	10 mg L^{-1}
$MnCl_2$ x $4H_2O$	10 mg L^{-1}
$Na_2B_4O_7$ x $10H_2O$	10 mg L^{-1}
$(NH_4)_6Mo_7O_{24}$ x $4H_2O$	10 mg L^{-1}

Sterilized by filtration (0.2 µm cellulose acetate syringe filter).

Medium additives (stock solutions)

Substance	Concentration
Ampicillin	100 mg mL^{-1} in water
Apramycin	50 mg mL^{-1} in water
Chloramphenicol	25 mg mL^{-1} in ethanol 100%
Kanamycin	25 mg mL^{-1} in water
Nalidixic acid	25 mg mL^{-1} in NaOH 0.15 M
Zeocin	100 mg mL^{-1} in water
Riboflavin	20 mM in DMSO*
Roseoflavin	20 mM in DMSO*
IPTG**	1 M in water

*DMSO: dimethyl sulfoxide

**IPTG: Isopropyl β-D-1-thiogalactopyranoside

2.3 Microbiological methods

2.3.1 Bacterial strains and growth conditions

Streptomyces davawensis (*Streptomyces strain* 768; a wild-type strain) was aerobically grown at 37°C and pH 7.2 in a nutrient broth (YS). *Streptomyces coelicolor* (DSM 40233; a wild-type strain) was aerobically grown at 30°C in YS. When required 50 µg mL^{-1} apramycin and/or 25 µg mL^{-1} nalidixic acid were added to the growth media.

For riboflavin synthase and growth curve experiments, *S. davawensis* and *S. coelicolor* were aerobically grown at 30°C on minimal medium (MM).

For the production of spores the Streptomycetes were grown for five days at 37°C (*S. davawensis*) or 30°C (*S. coelicolor*, *S.avermitilis* and *S. albus*) on mannitol soybean broth (MS) until the aerial mycelium was visible. The spores were harvested by adding 1 mL 0.1% Tween 20 solution to the plates (9 cm diameter) and gentle agitation of the plates. The spores within the resulting spore solution were separated from the mycelium by filtration through sterile cotton plugs. Finally, the spores in the filtrate were resuspended in 0.1% Tween 20.

E. coli Top10 was used as a host for plasmid cloning and was aerobically grown at 37°C on Lysogeny Broth (LB). When required 100 µg ml^{-1} ampicillin or 25 µg ml^{-1} zeocin were added to the growth media.

E. coli BL21 and *E. coli* Rosetta (DE3) were used as hosts for gene expression experiments and were aerobically grown at 37°C on LB. When required, 34 µg ml^{-1} chloramphenicol and/or 100 µg ml^{-1} ampicillin were added to the growth media. Oversynthesis of recombinant proteins was stimulated by adding 1 mM IPTG.

E. coli GM2163 (*dam-*, *dcm-*) carrying the self-transmissible plasmid pUB307 was used as donor strain for conjugation and was aerobically grown at 37°C on Lysogeny Broth (LB). When required 25 µg ml^{-1} chloramphenicol, 25 µg ml^{-1} kanamycin and 25 µg ml^{-1} apramycin were added to the growth media.

Pichia pastoris X33 (Invitrogen) was used as a host for the heterologous expression of the gene for human FAD synthetase (transcript variant 2). Recombinant *P. pastoris* strains were cultured on YPD supplemented with 100 µg ml^{-1} of the antibiotic zeocin. *P. pastoris* cultures were grown at 30°C for 4 days for the constitutive expression of human FAD synthetase.

Precultures or small scale cultures (up to 200 mL) of all organisms were performed in baffled Erlenmeyer flasks 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 L^{-1}) was added to YS, LB or YPD.

2.3.2 Preparation of competent cells for dsDNA uptake and cell transformation

Preparation of chemically competent *E. coli* cells (SAMBROOK & RUSSELL, 2001)

An overnight *E. coli* culture (OD_{600} 4.0) was used to inoculate 100 ml of LB in 1-liter flasks with to an OD_{600} of 0.05 and incubated at 37°C with agitation (220 rpm). When the OD_{600} of the cultures reached 0.4, the cell suspension was transferred to ice-cold falcon tubes and cells were harvested by centrifugation at 2,500 x g for 10 minutes at 4°C. The supernatant was decanted, the cell pellet was resuspended in ice-cold 0.1 M $CaCl_2$ and incubated on ice for 20 min. The cell suspension was again centrifuged and the pellet resuspended in 2 mL ice-cold 0.1 M $CaCl_2$/10% glycerol solution. Aliquots of the cell suspensions were stored at -80°C. For transformation, about 25 ng dsDNA were added to a 50 µL aliquot of the competent cells and incubated on ice for 30 min. The mixture was subsequently incubated at 42°C for 30 sec and placed on ice for 2 min. SOC medium was added (250 µL) and the cells were incubated for 1h at 37°C. The cell culture was spread on LB-agar containing the appropriate antibiotic(s) and incubated over-night at 37°C.

Preparation of electrocompent *E. coli* cells (SAMBROOK & RUSSELL, 2001)

An overnight *E. coli* culture (OD_{600} 4.0) was used to inoculate 100 ml of LB in 1-liter flasks with to an OD_{600} of 0.05 and incubated at 37°C with agitation (220 rpm). When the OD_{600} of the cultures reached 0.4, the cell suspension was transferred to ice-cold falcon tubes and cells were harvested by centrifugation at 2,500 x g for 20 minutes at 4°C. Cells were subsequently harvested by centrifugation at 2,500 x g for 20 minutes at 4°C. The supernatant was decanted and the cell pellet was washed one time with 100 ml of ice-cold pure H_2O, another time with 50 ml of ice-cold 10% glycerol, another time with 5 ml of ice-cold 10% glycerol, and finally resuspended in 1 ml of ice-cold GYT medium. The cell suspension was diluted to a concentration of 2 x 10^{10} cells ml^{-1} (OD_{600} = 1 is equivalent to ~ 2 x 10^8 cells ml^{-1}) with ice-cold GYT. Aliquots of the cell suspensions were stored at -80°C. For electroporation, 40 µl of the electrocompetent cells solution and about 25 ng of dsDNA (volume of 1-2 µl) were pipetted into ice-cold electroporation cuvette. The cuvette was incubated on ice for 5 min. The electroporation apparatus was set to deliver an electrical pulse of 25 µF capacitance, 2.5 kV, and 200 ohm resistance (low range 200, high range 500). The cuvette was electrically pulsed at a time constant of 4-5 milliseconds. Immediately after the pulse 1 ml of SOC was added to the cuvette and the resulting cell suspension was incubated for 1h at 37°C and 220 rpm. The cell culture was then spread on LB-agar containing the appropriate antibiotic (or low salt LB-agar containing zeocin) and incubated over-night at 37°C.

Preparation of electrocompetent *P. pastoris* cells

An overnight *P. pastoris* culture (OD_{600} 9.0) was used to inoculate 100 ml of YPD in 1-liter flasks with to an OD_{600} of 0.1 and incubated at 30°C with agitation (250 rpm). When the OD_{600} of the cultures reached 1.5, the cell suspension was transferred to ice-cold falcon tubes and cells were harvested by centrifugation at 2,000 x g for 10 minutes at 4°C. The supernatant was decanted and the cell pellet was resuspended in 100 mL YPD/ 20 mL 1 M HEPES pH 8.0 (sterilized by filtration).To the cell suspension 2.5 mL 1 M DTT was added and it was incubated for 15 min at 30°C. Subsequently, 400 mL of ice-cold water was added and the resulting suspension was centrifuged at 2,000 x g for 10 minutes at 4°C. The cell pellet was washed one time with 250 mL ice-cold and another time with 50 mL. A final wash with 20 mL 1 M sorbitol was done and the cell suspension was again centrifuged. Finally the resulting pellet was resuspended in 400 µL 1 M sorbitol and aliquots were stocked at -80°C. For electroporation, an 80 µl aliquot of the competent cells was mixed with 2 µl (about 5 µg) of

linearized dsDNA (digested with *Bgl*II) and transferred to an ice-cold electroporation cuvette. The cuvette was incubated on ice for 5 min. The electroporation apparatus was set to deliver an electrical pulse of 25 µF capacitance, 1.5 kV, and 200 ohm resistance (low range 200, high range 500). The cuvette was pulsed at a time constant of 4-5 milliseconds. Immediately after the pulse 1 ml of ice-cold 1 M sorbitol was added to the cuvette and the resulting cell suspension was incubated for 2h at 30°C and 250 rpm. The cell culture was spread on YPD-agar containing 1 M sorbitol and 100 µg mL^{-1} zeocin and incubated for 3 days at 30°C.

2.3.3 Selection and analysis of spontaneous roseoflavin resistant *Streptomyces coelicolor* mutants

S. coelicolor spores (10^8) were spread on MS agar containing 200 µM roseoflavin and the inoculated plates were incubated at 30°C for four days. The largest (apparently RoF resistant) colonies were isolated and used as an inoculum for small scale (5 ml) cultures in YS broth. The isolated strains were aerobically grown for 16 h at 30°C and cells were harvested by centrifugation. Genomic DNA was extracted from the different isolates using the "Genomic DNA Extraction Kit" from Fermentas (Heidelberg, Germany) and was used as a template for the subsequent PCR reactions. The FMN riboswitch region including the *rib* promoter was amplified by PCR using the primers scFMNforward 5'-ACA GTC GGT TCC TCT CCA CG-3' and scFMNreverse 5'-CTT CGA CGA TTC CGG TGA A-3'. The resulting PCR products were subjected to DNA-sequencing.

2.4. Molecular biology methods

2.4.1 Isolation of total DNA and other molecular biology/cloning techniques

For the isolation of total DNA from Streptomycetes a modified protocol of the "Kirby Mix Procedure" was used (KIESER, 2000). Site directed mutagenesis was carried out using mutagenic primers and the QuikChange II XL kit (Invitrogen, Darmstadt, Germany). Nucleotide exchange was also carried out using linkers prepared mixing two complementary oligonucleotides carrying *Psy*I and *Sex*AI compatible ends. The oligonucleotides used in this work are listed in Table 1.

Table 1. Modifying oligonucleotides (restriction sites are underlined and exchanged nucleotides are in bold).

Oligonucleotide Name	Oligonucleotide Sequence	Used for
RFNdav.HindIII_fw	5'-GAT AGT ACA AGC TTT GAC AGC AAG TCG GTT CCT C-3'	*S. davawensis ribB* FMN riboswitch amplification (translational fusion)
RFNdav.coe.BamHI_rv	5'-CGC AGG ATC CCT TCG ACG ATT CCG GTG AA-3'	*S. davawensis/S. coelicolor ribB* FMN riboswitch amplification (translational fusion)
RFNcoe.HindIII_fw	5'-GAT AGT ACA AGC TTA CAG TCG GTT CCT CTC CAC G-3'	*S. coelicolor ribB* FMN riboswitch amplification (translational fusion)
RFNdav.coe.NotI_rv	5'-CGC AGC GGC CGC CTT CGA CGA TTC CGG TGA A-3'	*S. davawensis/S. coelicolor ribB* FMN riboswitch amplification (transcriptional fusion)
RFNbs.HindII_fw	5'-GAT AGT ACA AGC TTT AAG GAC AAA TGA ATA AAG ATT GTA TC-3'	*B. subtilis ribG* FMN riboswitch amplification (translational fusion)
RFNbsBamHI_rv	5'-CGC AGG ATC CCC AGC TTC ATA TAA TAC TCT TC-3'	*B. subtilis ribG* FMN riboswitch amplification (translational fusion)
RFNbs.NotI_rv	5'-CGC AGC GGC CGC CCA GCT TCA TAT AAT ACT CTT C-3'	*B. subtilis ribG* FMN riboswitch amplification (transcriptional fusion)
RFNdavII.HindII_fw	5'-GAT AGT ACA AGC TTT CG ACC ATC CGA CCG TAC G -3'	*S. davawensis ribA* FMN riboswitch amplification (translational fusion)
RFNdavII.BamHI_rv	5'-CGC AGG ATC CCG ACG CTT TCG AGT GGG-3'	*S. davawensis ribA* FMN riboswitch amplification (translational fusion)
RFNdavII.NotI_rv	5'-CGC AGC GGC CGC CGA CGC TTT CGA GTG GG-3'	*S. davawensis ribA* FMN riboswitch amplification (transcriptional fusion)
Ribdav.XbaI_fw	5´-GAT AGTA CTC TAG ACC CGC GAG CAG GGA GGT TCT C-3´	*S. davawensis ribBMAH* amplification
Ribdav.EcoRV_rv	5´-TAC GCA GAT ATCG TGG TGC CCA TGC TCA GCT G-3´	*S. davawensis ribBMAH* amplification
scFMNforward	5'-ACA GTC GGT TCC TCT CCA CG-3'	*S. coelicolor ribB* FMN riboswitch amplification for mutant screening
scFMNreverse	5'-CTT CGA CGA TTC CGG TGA A-3'	*S. coelicolor ribB* FMN riboswitch amplification for mutant screening
pT7luc(ATG51GCA)_fw	5'-G AGG GCC CGG ATC CAA **GCA** GAA GAC GCC AAA AAC-3'	site-directed mutagenesis of *luc* start codon (translational fusions)
pT7luc(ATG51GCA)_rv	5'-GTT TTT GGC GTC TTC **TGC** TTG GAT CCG GGC CCT C-3'	site-directed mutagenesis of *luc* start codon (translational fusions)
RFNdav(A61G)_fw	5'-GTC CGC GAC CCG **G**CC GCT TCC AGC-3'	site-directed mutagenesis of *S. davawensis ribB* FMN riboswitch (translational fusion)

RFNdav(A61G)_rv	5'-GCT GGA AGC GGC **C**GG GTC GCG GAC-3'	site-directed mutagenesis of *S. davawensis ribB* FMN riboswitch (translational fusion)
RFNdav(C62T)_fw	5' -CGA CCC GA**T** CGC TTC CAG CGG C- 3'	site-directed mutagenesis of *S. davawensis ribB* FMN riboswitch (translational fusion)
RFNdav(C62T)_rv	5' -GCC GCT GGA AGC GA**T** CGG GTC G- 3'	site-directed mutagenesis of *S. davawensis ribB* FMN riboswitch (translational fusion)
RFNdav(T66C)_fw	5' -GAC CCG ACC GC**C** TCC AGC- 3'	site-directed mutagenesis of *S. davawensis ribB* FMN riboswitch (translational fusion)
RFNdav(T66C)_rv	5' -GCT GGA **G**GC GGT CGG GTC- 3'	site-directed mutagenesis of *S. davawensis ribB* FMN riboswitch (translational fusion)
RFNdav(G74A)_fw	5'-CGC TTC CAG CGA CCG GTT GAC CAG-3'	site-directed mutagenesis of *S. davawensis ribB* FMN riboswitch (translational fusion)
RFNdav(G74A)_rv	5'-CTG GTC AAC CGG **T**CG CTG GAA GCG-3'	site-directed mutagenesis of *S. davawensis ribB* FMN riboswitch (translational fusion)
RFNdav(A61G+C62T)_fw	5'-GTC CGC GAC CCG G**T**C GCT TCC AGC GG-3'	site-directed mutagenesis of *S. davawensis ribB* FMN riboswitch (translational fusion)
RFNdav(A61G+C62T)_rv	5'-CCG CTG GAA GCG **A**CC GGG TCG CGG AC-3'	site-directed mutagenesis of *S. davawensis ribB* FMN riboswitch (translational fusion)
RFNcoe(G61A)_fw	5'-CCG CGA CCC GA**T** CGC CTC CAG-3'	site-directed mutagenesis of *S. coelicolor ribB* FMN riboswitch (translational fusion)
RFNcoe(G61A)_rv	5'-CTG GAG GCG A**T**C GGG TCG CGG-3'	site-directed mutagenesis of *S. coelicolor ribB* FMN riboswitch (translational fusion)
Linker.RFNdav(A61G+C62T+T66C+G74A)_fw	5´-CG**G** **T**CG C**C**T CCA GCG **A**CC GGT TGA-3´	linker for nucleotide exchange of *S. davawensis ribB* FMN riboswitch (translational fusion)
Linker.RFNdav(A61G+C62T+T66C+G74A)_rv	5´-CCT GGT CAA CCG G**T**C GCT GGA **G**GC G**A**C CG-3´	linker for nucleotide exchange of *S. davawensis ribB* FMN riboswitch (translational fusion)
hFADS2.EcoRI_fw	5´-AGC CGA ATT CGC CAT GAC TTC TAG GGC CTC TGA ACT TTC TC-3`	human FAD synthetase (isoform 2) amplification
hFADS2. XhoI_rv	5´-GAC CCT CGA GCC TGT GCG GGA GTT CCG-3`	human FAD synthetase (isoform 2) amplification

2.4.2 Preparation of plasmids used for *in vitro* transcription/translation

The plasmid constructs for generating translational fusions contained a T7 promoter (T7), a downstream FMN riboswitch, the corresponding endogenous ribosomal binding site (RBS) and the first six codons (including the start codon) of the gene downstream of the riboswitch in translational fusion with the firefly luciferase reporter gene (Fig. 9). Two different FMN riboswitches were PCR amplified using *S. davawensis* genomic DNA as a template: The *ribB* FMN riboswitch contained twenty two base pairs of *ribB* (including the start codon GTG) and 487 bp upstream of the *ribB* start codon. The *ribA* FMN riboswitch contained twenty two base pairs of *ribA* (including the start codon ATG) and 443 bp upstream of the *ribA* start codon. Genomic DNA from *S. coelicolor* was used to amplify the *ribB* FMN riboswitch (RFNBcoe) from the latter bacterium containing twenty two base pairs of *ribB* (including the start codon GTG) and 485 bp upstream of the *ribB* start codon. The *B. subtilis ribG* FMN riboswitch DNA fragment contained twenty two bp of *ribG* (including the initial ATG codon) and 292 bp upstream of the *ribG* start codon. The four DNA fragments described above were treated with *Hin*dIII/*Bam*HI and ligated to *Hin*dIII/*Bam*HI digested pT7luc^{mod} (Promega, Mannheim, Germany) (Fig. 9). The latter plasmid was generated from pT7luc by changing the start codon ATG of the luciferase gene *luc* to GCA by site directed mutagenesis. In addition, the ribosomal binding site originally present in pT7luc upstream of the reporter gene *luc* was removed by treatment with *Hin*dIII and *Bam*HI. The newly constructed plasmids were named pT7luc_RFNBdav, pT7luc_RFNAdav, pT7luc_RFNBcoe and pT7luc_RFNGsub. The plasmid constructs for generating transcriptional fusions were produced as follows. The DNA fragments RFNBcoe, RFNBdav, RFNAdav, RFNGsub were treated with *Hin*dIII/*Not*I and ligated to *Hin*dIII/*Not*I digested pT7luc. As a result, all the DNA fragments were placed downstream of the T7 promoter and upstream of the ribosomal binding site already present in pT7luc. The vectors generated were named pT7luc(RBS)_RFNBdav, pT7luc(RBS)_RFNAdav, pT7luc(RBS)_RFNBcoe and pT7luc(RBS)_RFNGsub. All the pT7luc constructs were transformed into *E. coli* Top10 cells (Invitrogen). DNA vectors were further isolated from *E. coli* and double purified using DNA purifying columns (Fermentas) and eluted with nuclease-free water.

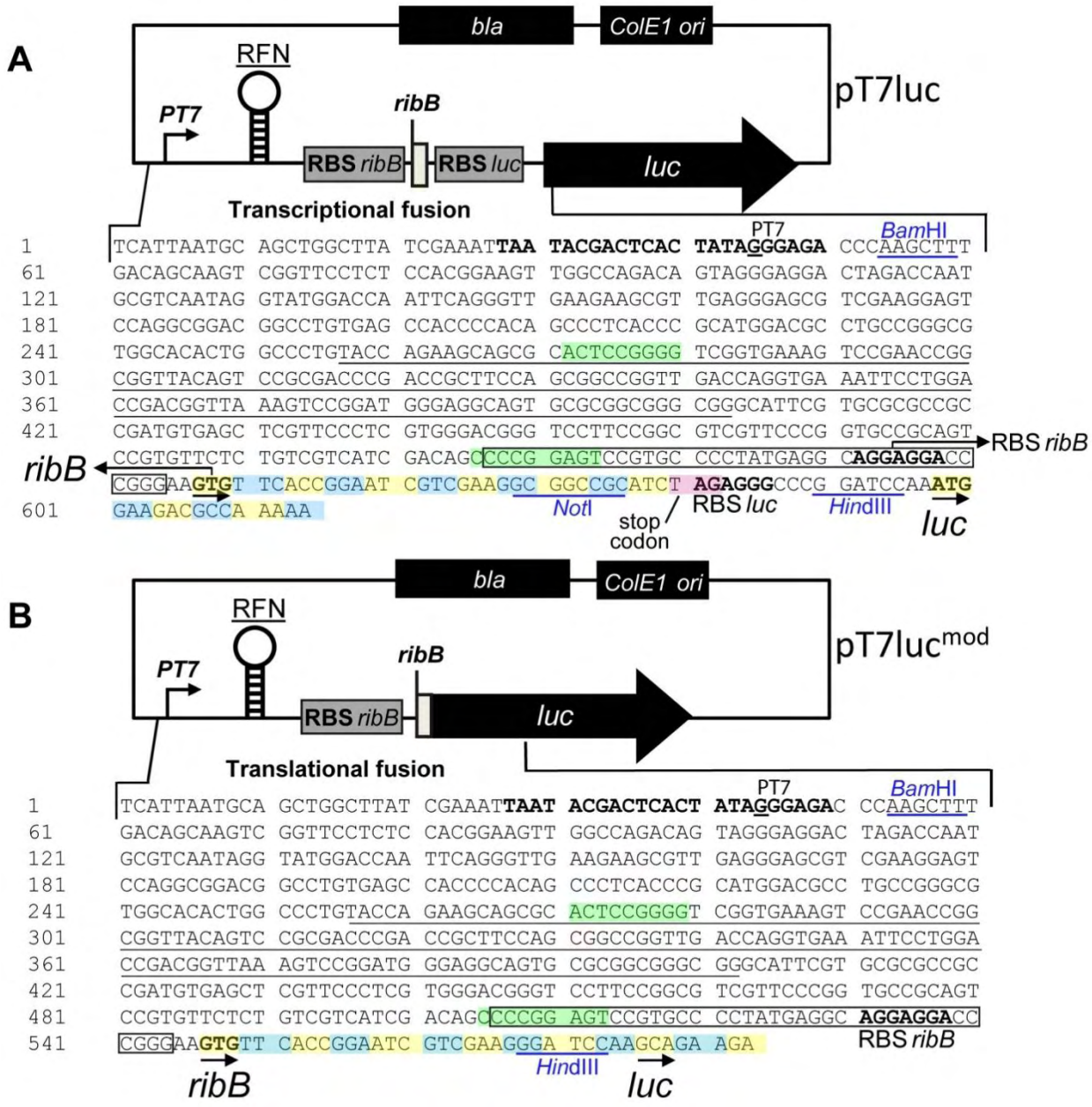

Figure 9. Scheme of the reporter plasmid used for testing FMN riboswitches from *Bacillus subtilis*, *Streptomyces coelicolor* and *Streptomyces davawensis* using *in vitro* transcription/translation. (A) Plasmid (pT7luc) used for the generation of transcriptional fusions. The DNA sequence exemplarily is shown for the transcriptional fusion construct used for testing the *ribB* FMN riboswitch of *S. davawensis*. (B) Plasmid (pT7luc^{mod}) used for the generation of translational fusions. The DNA sequence exemplarily is shown for the translational fusion construct used for testing the *ribB* FMN riboswitch of *S. davawensis*. The sequence in bold (top) is the promoter from bacteriophage T7, transcription starts with G (underlined). The underlined sequence (middle) is the FMN riboswitch aptamer; the boxed sequence forms the hairpin responsible for RBS sequestration; the green sequences are complementary to each other and form the anti-RBS sequestor. The ribosomal binding sites RBS are in bold: RBS *ribB* is part of the FMN riboswitch and RBS *luc* was originally present

in the plasmid. The translated sequences are yellow/blue marked and the red highlighted nucleotides in A mark a stop codon.

2.4.3 Coupled *in vitro* transcription/translation assays

The coupled TK/TL assay was performed using the *E. coli* T7 S30 Extract System for Circular DNA kit (Promega, Mannheim, Germany). The TK/TL mix was freshly prepared mixing the "T7 extract solution", the "S30 premix" and the "amino acid mix" in the proportion 3:4:1 (v/v/v) (according to the manufacturer's directions) and combined with 1 µL of a plasmid solution (the plasmid concentration was adjusted to produce a similar luciferase activity in all control assays) and with 1 µL of a flavin solution or 1 µL of nuclease-free water (see table below). The final mixture was incubated at 30°C for 5 min in a water bath. The reaction was stopped by adding 90 µL of a bovine serum albumin containing solution (1 mg mL^{-1} BSA, 2 mM DTT, 25 mM Tris-phosphate, pH 7.8). The resulting solution was used to determine the luciferase activity in a microtiter plate reader (Tecan Genios Pro microplate reader, Tecan, Mainz, Germany). T_{50} values were estimated by fitting the plot of the luciferase activity percentage versus the flavin concentration with a first-order exponential decay equation, using SigmaPlot 9 software (Systat Software).

In vitro transcription/translation reaction mixtures were prepared as follows:

Component	Control	Treatment	Concentration
TK/TL mix	8.0 µL	8.0 µL	–
Flavin solution	–	1.0 µL	10 to 180 µM
Nuclease-free water	1.0 µL	–	–
Vector solution	1.0 µL	1.0 µL	0.2 to 10 ng μL^{-1}

2.4.4 *In vitro* transcription assays

FMN riboswitch RNA molecules (containing both the aptamer and the expression platform) *in vitro* were synthesised by T7 RNA polymerase (Promega, Mannheim) using the plasmids generating translational fusion as templates. The DNA templates were linearized by digestion with *Eco*RV. Two different experiments were performed:

1. mRNA molecules were synthesised in the absence of flavins according to the table below (FM1). The reaction mixture was incubated at 30°C for 4h in water bath. The

resulting RNA solution was used for fluorescence quenching assays (see section 2.5.5 for fluorescence measurements).

2. Fluorescence quenching measurements were performed during the *in vitro* transcription reaction (see section 2.5.5 for fluorescence measurements). The reaction mixture was prepared according to the table below (FM2) and directly incubated in the luminometer at 30°C for 30 min.

In vitro transcription reaction mixtures were prepared as follows:

Component	FM1*	FM2**
Transcriptional 5x buffer	10 µL	10 µL
DTT 100 mM	5 µL	5 µL
Ribonuclease inhibitor (recombinant RNasin)	1.25 µL (50 units)	1.25 µL (50 units)
rNTP mix (2.5 mM each)	10 µL	10 µL
Linearized DNA (in nuclease-free water)	10 µL (1 µg)	1 µL (1.5 nM)
Flavin 10 µM (FMN or RoFMN)	–	5 µL
T7 RNA polymerase	1 µL (20 units)	1 µL (20 units)
Nuclease-free water	Up to 50 µL	Up to 50 µL

*reaction mixture used to produce large amounts of mRNA.

**reaction mixture used for simultaneous *in vitro* transcription and fluorescence measurements.

2.4.5 Cloning of the riboflavin gene cluster from *Streptomyces davawensis*

A genomic DNA fragment containing the *ribBMAH* gene cluster including the promoter and the *ribB* FMN riboswitch and also the genes *hisGE* immediately downstream of *ribH* was amplified by PCR using the modifying oligonucleotides forward 5´-GAT AGT ACT CTA GAC CCG CGA GCA GGG AGG TTC TC-3´ and reverse 5´-TAC GCA GAT ATC GTG GTG CCC ATG CTC AGC TG -3´ (restriction endonuclease sites are underlined). The PCR product was treated with *Xba*I/*Eco*RV and ligated to the *Xba*I/*Eco*RV digested integrative *S. coelicolor*/*E. coli* shuttle vector pSET152 (conferring apramycin resistance to Streptomycetes) (BIERMAN et al., 1992). The resulting plasmid was named pSET_Ribdav. The latter plasmid was used to transform wild-type *S. coelicolor* (see below) in order to generate the recombinant strain *S. coelicolor*::P_{rib}RFN^{dav}*ribBMAH*^{dav}. A variant of the latter strain, *S. coelicolor*::P_{rib}RFN^{davA61G}*ribBMAH*^{dav}, was generated by transforming wild-type *S.*

coelicolor using pSET_RibdavA61G. The latter plasmid was generated from pSET_Ribdav and contained the single nucleotide exchange A61A within the S. *davawensis ribB* FMN riboswitch. In order to introduce this point mutation pSET_Ribdav was digested with *Xba*I/*Sex*AI in order to release a 320 bp DNA-fragment. An almost identical synthetic 320 bp DNA-fragment containing the nucleotide exchange A61G and the restriction sites *Xba*I (5'-end) and *Sex*AI (3'-end) was produced by overlapping oligonucleotides and PCR (GeneArt, Regensburg, Germany) and was ligated to *Xba*I/*Sex*AI digested pSET_Ribdav.

2.4.6 Transformation of Streptomycetes by conjugation

A culture (50 mL) of the *E. coli* GM2163 (pUB307) donor strain containing the plasmids pSET152, pSET_ $P_{rib}RFN^{dav}$*ribBMAH*^{dav} or pSET_ $P_{rib}RFN^{davA61G}$*ribBMAH*^{dav} was grown to an OD_{600} of 0.4. The *E. coli* cells were harvested by centrifugation (2,500 x g at 4°C), washed twice in LB and resuspended in 1.0 mL LB. *Streptomyces coelicolor* (DSM 40233) spores were used as recipients. 50 µL of a spore suspension (10^{10} spores per mL) were added to 500 µL of 2xYT broth and incubated at 50°C for 10 min to promote germination. *E. coli* donor cells (500µL) were added to the prepared spores and the mixture was spread on MS containing 10 mM $MgCl_2$. The conjugation plates were incubated for 16-20 h at 30°C. Subsequently, the surface of each plate was overlaid with 1 ml of water containing 500 µg nalidixic acid and 1 mg apramycin. The plates were incubated for 5 days at 30°C. The exconjugant colonies were isolated and grown on MS plates containing 25 µg mL^{-1} nalidixic acid and 50 µg mL^{-1} apramycin (KIESER, 2000). The correct integration of the plasmids was verified by PCR.

2.4.7 Construction of plasmids for recombinant gene expression

The plasmid used for the synthesis of His_6-tagged recombinant human flavokinase in *E. coli* strain BL21 was constructed earlier (KARTHIKEYAN et al., 2003). The gene for human FAD synthetase (transcript variant 2) was amplified by PCR from pH6EX3-$_{UTR}$h-FADS2 (BRIZIO et al., 2006) employing the forward and reverse modifying primers 5`-AGC CGA ATT CGC CAT GAC TTC TAG GGC CTC TGA ACT TTC TC- 3` and 5`-GAC CCT CGA GCC TGT GCG GGA GTT CCG-3`. The restriction endonuclease sites *Eco*RI and *Xho*I are underlined. The *Eco*RI/*Xho*I treated PCR product was ligated into the *Eco*RI/*Xho*I digested expression vector pGAPZA (zeocin resistance) (Invitrogen, Darmstadt, Germany). The resulting plasmid was used to transform *P. pastoris* X33. A corresponding transformant

strain overproduced human FAD synthetase (isoform 2) carrying the additional C-terminal amino acids GSSRGGRQLGPEQKLISEEDLNSAVD and the additional C-terminal amino acids HHHHHH (His_6-tag).

Gene insertion events at the *GAP* promoter locus arise from a single crossover event between the locus and the P*GAP* region on the pGAPZ vector. This results in the insertion of one or more copies of the vector upstream or downstream of the *GAP* locus. Genes under control of the GAP promoter are constitutively expressed in *P. pastoris*.

2.4.8 Agarose gel electrophoresis

Plasmids and other DNA sequences were separated on 1% agarose gels and stained with ethidium bromide according to standard protocols (SAMBROOK & RUSSELL, 2001).

2.5 Biochemical methods

2.5.1 Liquid chromatography-mass spectrometry (LC-MS)

All the LC-MS analyses were performed in an Agilent 1220 Infinity LC, using a reverse-phase column (ReproSil-Pur C18 AQ 5µm; 2.0 mm x 250 mm; Dr. Maisch GmbH, Ammerbich-Entringen, Germany). The following solvent system was used at a flow rate of 0.3 ml min^{-1}: 30% (v/v) methanol, 20 mM formic acid and 20 mM ammonium formate (pH 3.7). Detection of flavins was carried out with a diode array detector and a 6130 Single Quadrupole MS (Agilent Technologies, Waldbronn, Germany).

2.5.2 Protein concentration determination

Protein concentration was estimated by the method of Bradford (BRADFORD, 1976) using BSA as a standard.

2.5.3 SDS-PAGE

Proteins were separated by SDS-PAGE on 7-20% polyacrylamide gels performed according to Laemmli (1970).

2.5.4 Purification of recombinant proteins

Both His_6-tagged (RFK and FADS) proteins were purified from cell free extracts by column chromatography using Ni^{2+}-nitrilotriacetic acid-agarose (GE Healthcare, Munich,

Germany). Samples were applied to the column in binding buffer (20 mM sodium phosphate, 0.5 M NaCl, 20 mM imidazole, pH 7.4) at a flow rate of 1 mL min^{-1}. Elution was carried out by applying increasing a gradient of elution buffer (20 mM sodium phosphate, 0.5 M NaCl, 500 mM imidazole, pH 7.4). Fractions containing the purified enzyme were pooled and desalted using a Sephadex G-25 column (GE Healthcare) equilibrated with 50 mM potassium phosphate pH 7.4.

2.5.5 Fluorescence measurements

The intensity of fluorescence emission was determined at a wavelength of 535 nm with an excitation at 485 nm. Assays were performed on the basis of the intrinsic fluorescence of FMN and RoFMN, which is quenched upon the specific interaction of the flavins with the riboswitch aptamers. RoFMN fluorescence was about 20 times weaker as compared to FMN. Each experiment was performed three times at 25°C using a Tecan Genios Pro microplate reader and 96-well black plates (FluoroNunc, Nunc A/S, Kamstrupvej, Denmark). FMN riboswitch RNA molecules (containing both the aptamer and the expression platform) were *in vitro* synthesised by T7 RNA polymerase (Promega, Mannheim) using the plasmids generating translational fusion as templates. As a negative control plasmid pT7luc containing the T7 promoter and the luciferase gene only was used. RNA (1 µM) was mixed with FMN (1 µM) or RoFMN (1 µM) in 50 mM potassium phosphate pH 7.4 containing 2 mM $MgCl_2$ and 2 mM KCl. The measurement started immediately after addition of the RNA solution and fluorescence emission was followed during 10 min.

FMN (1 µM) and RoFMN (1 µM) induced fluorescence quenching was also measured during the time course of *in vitro* transcription reactions which were performed using linearized plasmid (1.5 nM) as a template. The reaction was incubated directly in the plate reader at 30°C and fluorescence emission was followed during 30 min.

2.5.6 In-line probing assay

In-line probing assays were essentially performed as described by Regulski & Breaker (2008). 50 pM (FMN riboswitch aptamer of *S. davawensis* or *S. coelicolor*) of each 5' radiolabeled RNA was incubated at 23°C for 40-48 hours in 20 mM $MgCl_2$, 100 mM KCl and 50 mM Tris-HCl pH 8.3 with FMN or RoFMN. Denaturing 10% PAGE was used to separate RNA cleavage products. The radiolabeled cleavage products were visualized using a Phosphorimager (GE Healthcare). ImageQuant software (GE Healthcare, Boston, USA) was

used to quantify the product bands. K_D values were estimated by plotting the fraction modulated (F) by the ligand versus the logarithm of the ligand concentration [L] at modulating sites and by fitting the data to the equation F = [L]/([L]+ *K*D) using SigmaPlot 9(Systat Software). It was assumed that there was no modulation in the absence of the ligand and complete modulation in the presence of the highest ligand concentration tested.

2.6 Enzyme assays

2.6.1 Firefly luciferase activity assay

In a 96-well microplate (OptiPlate-96, white opaque 96-well microplate, PerkinElmer, Rodgau, Germany) 10 µL of TK/TL reaction mixture were mixed with 50 µL of Luciferase Assay Reagent (Promega, Mannheim). The resulting mixture was incubated for 10 sec at 26°C with agitation in a Tecan Genios Pro microplate reader. The luminometer was programmed to perform a 2-second measurement delay followed by a 10-second measurement read for luciferase activity. Measurements were done each 30 sec until the light emission became constant.

2.6.2 *N,N*-8-amino-8-demethyl-D-riboflavin dimethyltransferase (RosA) assay

RosA activity was determined by measuring the roseoflavin formation from 8-demethyl-8-amino riboflavin and *S*-adenosyl methionine (SAM). The reaction was performed in 50 mM Tris-HCl buffer pH 8.0 containing 200 µM 8-amino riboflavin and 2 mM SAM. The mixture was preincubated at 52°C for 5 min and the reaction was started by addition of *S. davawensis* cell-free extract. After appropriate time intervals, an aliquot was removed and analyzed by LC-MS (JANKOWITSCH et al., 2011).

2.6.3 Measurement riboflavin synthase (RibB) in Streptomycetes

Spore suspensions (10^8 spores) from *S. davawensis* and *S. coelicolor* were used to inoculate 1 L flasks containing 100 mL YS broth and cultures were grown overnight (14 h). The mycelia were harvested by centrifugation (4,000 x g), washed three times and suspended in 100 mL minimal medium (1.5 g KH_2PO_4 L^{-1}, 2.0 g L^{-1} K_2HPO_4, 1.4 g L^{-1} $(NH_4)_2SO_4$, 0.1 g L^{-1} $CaCl_2$, 0.1 g L^{-1} $MgSO_4$, 0.2 ml trace elements solution, pH 7.2) containing 50 µM riboflavin (in DMSO), 50 µM roseoflavin (in DMSO) or DMSO (control). The trace elements solution (500x) contained $ZnCl_2$ (40 mg L^{-1}), $FeCl_3$ x $6H_2O$ (200 mg L^{-1}), $CuCl_2$ x $2H_2O$ (10

mg L^{-1}), $MnCl_2$ x $4H_2O$ (10 mg L^{-1}), $Na_2B_4O_7$ x $10H_2O$ (10 mg L^{-1}) and $(NH_4)_6Mo_7O_{24}$ x $4H_2O$ (10 mg L^{-1}). Following the medium change the cells were grown for another 20, 30 or 40 h. Again, the cells were harvested by centrifugation. The mycelia were washed five times with 100 mM potassium phosphate pH 7.4, suspended in the same buffer and disrupted using a French press (2,000 bar, 10 °C). The resulting cell extract was tested for riboflavin synthase activity: Assay mixtures containing 100 mM potassium phosphate (pH 7.4), 10 mM EDTA, 7.5 mM 2-mercaptoethanol and 0.6 mM 6,7-dimethyl-8-ribityllumazine were pre-warmed (5 min, 37°C) and the reaction was started by the addition of cell extracts. Riboflavin formation was monitored by LC-MS.

2.6.4 Flavokinase and FAD synthetase enzyme assays

Flavokinase activity was measured in a final volume of 2 ml of 50 mM potassium phosphate (pH 7.5) containing 25-350 µM flavin, 1 mM ATP, 6 mM NaF, 12 mM $MgCl_2$ and 24 mM sodium dithionite ($Na_2S_2O_4$). The mixture was preincubated at 37°C for 5 min and the reaction was started by addition of the enzyme. After appropriate time intervals aliquots were taken and trichloroacetic acid (TCA) was added to a final concentration of 1% to precipitate proteins. The aliquots were centrifuged (13,000 x g for 2 min) and applied to LC-MS. FAD synthetase activity was measured as described for the flavokinase but using 25-180 µM of 5´-monophosphorylated flavins as a substrate. Flavokinase and FAD synthetase activities are expressed as micromoles of phosphorylated or adenylylated flavins formed from the corresponding flavin and ATP. All apparent K_M and V_{max} values were determined from Lineweaver-Burk plots.

2.6.5 Preparation of RoFMN, RoFAD and AFMN

Roseoflavin mononucleotide (RoFMN), roseoflavin adenine dinucleotide (RoFAD) and 8-demethyl-8-amino-riboflavin mononucleotide (AFMN) were not commercially available and were enzymatically synthesized. RoFMN and AFMN were produced by the human flavokinase using roseoflavin and 8-amino-riboflavin, respectively, and ATP as substrates. RoFAD was produced by the human FAD synthetase using RoFMN and ATP as substrates. The compounds were purified by preparative HPLC and the identities of the compounds were verified by LC-MS (Fig. 10).

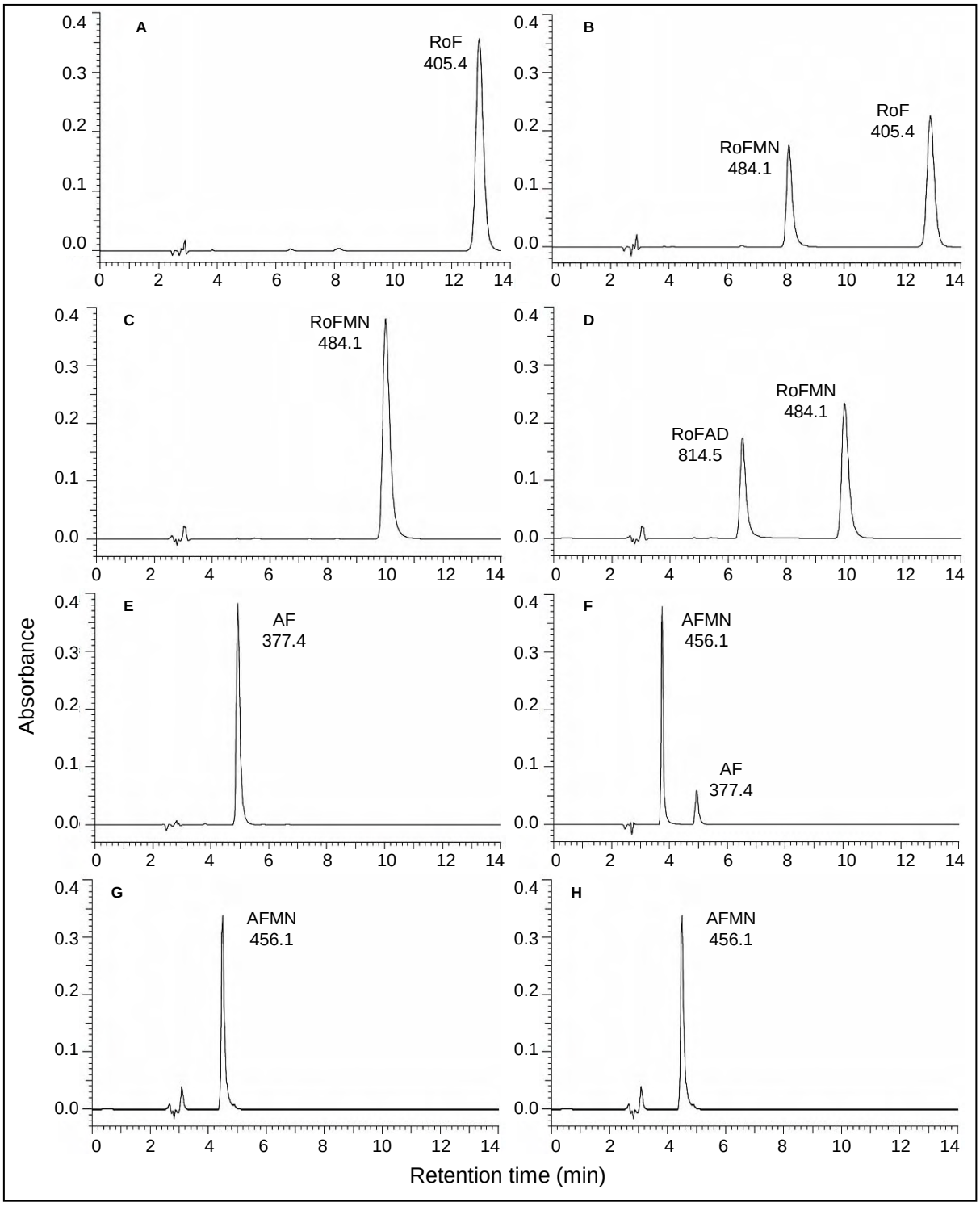

Figure 10. Enzymatic synthesis of phosphorylated/adenylylated flavins. Reaction mixtures containing different flavin substrates and human flavokinase (huRFK) or human FAD synthetase (hFADS2) were incubated at 37ºC; samples were taken and analyzed by HPLC/mass spectrometry. A (0 min) and B (10 min) Roseoflavin mononucleotide (RoFMN) formed from RoF and ATP upon addition of huRFK. C (0 min) and D (10 min) RoFAD formed from RoFMN and ATP upon addition of hFADS. E (0 min) and F (10 min) AFMN

formed from AF and ATP upon addition of huRFK. G (0 min) and H (120 min) reaction mixture containing AFMN, ATP and hFADS. No formation of AFAD from AFMN and ATP upon addition of hFADS was observed.

2.6.6 Measurement of flavokinase/FAD synthetase activity in human hepatocytes

Human hepatocytes (HepG2 cells) were cultured in RPMI 1640 medium (Sigma Aldrich) with L-glutamine (2 mM) supplemented with 10% fetal bovine serum (PAA Laboratories), at 37 °C in a 5% CO_2 environment. The cells were lysed in lysis buffer (50 mM Tris–HCl, pH 7.5; 150 mM NaCl; 0.5% Nonidet P-40; 50 mM NaF; 1 mM phenylmethylsulfonyl fluoride) at 4 °C for 30 min. Cell-free extracts were used directly in the flavokinase/FAD synthetase assay or for LC-MS analysis.

CHAPTER I. The mechanism of roseoflavin resistance in *Streptomyces davawensis*

3. Results

3.1 Roseoflavin inhibits growth of *Streptomyces coelicolor* and other Streptomycetes but not of *Streptomyces davawensis*

It was reported earlier that roseoflavin inhibits growth of the Gram-positive bacteria *Staphylococcus aureus*, *Bacillus subtilis*, *Bacillus cereus and Micrococcus luteus (Sarcina lutea)* (OTANI et al., 1974). It was not reported, however, whether *S. davawensis* is resistant towards its own antibiotic. Therefore, in this work, *S. davawensis* and some closely related species, *Streptomyces lividans*, *Streptomyces coelicolor*, *Streptomyces albus* and *Streptomyces avermitilis* were tested with respect to roseoflavin sensitivity. Spores of *S. lividans*, *S. coelicolor*, *S. albus* and *S. avermitilis* were not able to produce colonies on a solid growth medium containing 200 µM roseoflavin, evidencing that these bacteria are RoF sensitive (Fig. 11). The three latter species all contain a gene homologous to *S. davawensis ribM* which was shown to be responsible for riboflavin and roseoflavin uptake (GRILL et al., 2007;

HEMBERGER et al., 2011). The *S. lividans* genomic data have not been published yet, and thus, the presence of a *ribM* homologous gene could not be verified. However, since according to the present results *S. lividans* is roseoflavin sensitive, it is very likely that a *ribM* homologous gene is present in this species as well. In contrast to the other *Streptomyces* species tested, spores of *S. davawensis* were very well able to germinate and produce colonies in the presence of 200 µM roseoflavin showing that *S. davawensis* is RoF resistant (Fig. 11).

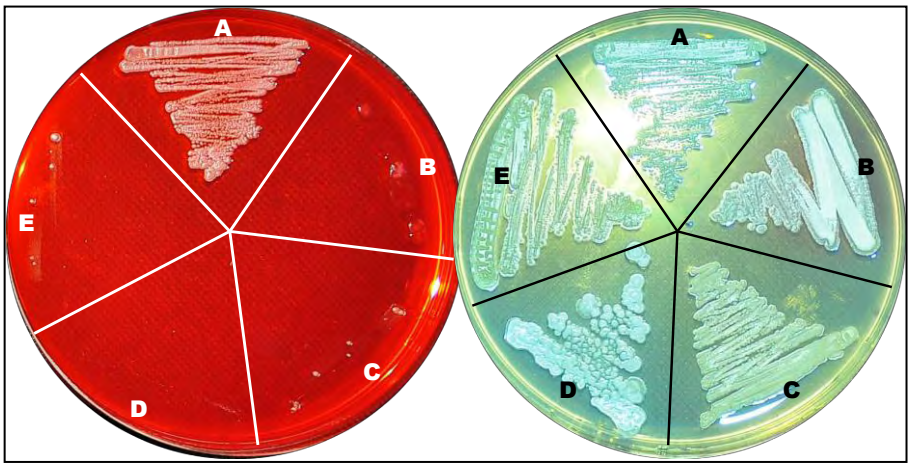

Figure 11. *S. davawensis* (A) and the closely related species *Streptomyces lividans* (B), *Streptomyces coelicolor* (C), *Streptomyces albus* (D) and *Streptomyces avermitilis* (E) were tested with respect to RoF sensitivity. Streptomycetes spores were applied to sectors of YS plate (right) and were incubated aerobically for 48 h at 30°C. The spores of all species germinated and produced colonies. The same amount of spores of each bacterium was applied to sectors of a YS plate (left) containing the antibiotic RoF (200 µM) and the plate was as well incubated aerobically for 48 h at 30°C.

3.2 RoFMN and RoFAD were present in the cytoplasm of *Streptomyces coelicolor* and *Streptomyces davawensis* when cells were grown in the presence of roseoflavin

S. coelicolor was cultured in the presence of sub-lethal amounts of roseoflavin (50 µM). Exponentially growing cells were harvested, thoroughly washed and disrupted. Subsequently, the corresponding cell-free extracts were analysed by LC-MS for their flavin content which was normalized to the total protein content of the cells. Analysis of *S. coelicolor* cell-free extracts revealed the presence of both RoFMN (1.2 µM ± 0.3 µM) and RoFAD (0.1 µM ± 0.05 µM). The same experiment was carried out with *S. davawensis* and the concentration of RoFMN/RoFAD was found to be very similar (RoFMN: 1.4 µM ± 0.2

µM; RoFAD: 0.2 µM ± 0.1 µM). *S. davawensis* produces roseoflavin in the stationary growth phase and therefore a stationary culture also was analysed without adding RoF. Cell free-extracts from these cells were found to contain 0.9 µM RoFMN (± 0.1 µM) and 0.1 µM RoFAD (± 0.03 µM). Free flavins (RF and RoF) were detected in both bacteria in trace amounts only (<0.01 µM). Thus, *S. davawensis* is roseoflavin resistant but is not a riboflavin overproducer.

3.3 The addition of roseoflavin reduces riboflavin synthase activity in *Streptomyces coelicolor* but not in *Streptomyces davawensis*

Expression of the *ribBMAH* genes was monitored in *S. coelicolor* and in *S. davawensis* cultures by measuring the activity of riboflavin synthase (RibB), the product of the first gene of the cluster. RibB catalyses the last step in riboflavin biosynthesis, the dismutation of 6,7-dimethyl-8-ribityllumazine to riboflavin and 5-amino-6-ribitylamino-2,4(1*H*,3*H*)-pyrimidinedione. *S. coelicolor* and *S. davawensis* were grown on YS medium overnight, then the mycelia were harvested by centrifugation, washed and inoculated on minimal medium containing 50 µM riboflavin, 50 µM roseoflavin or no flavin (control). RibB activity was measured at different time points (20 h, 30 h and 40 h) following the change of the culture medium and the addition of flavins (Table 2). It was found that upon addition of riboflavin to cultures of *S. coelicolor*, RibB activity decreased up to 48% (± 7.1%; 40 h value). When roseoflavin was added to the cultures, a stronger decrease of RibB activity was detected (20 h: to 20.7 % ± 3.0%; 30 h: to 20.8 % ± 7.5%). After 40 h of growth in the presence of roseoflavin no RibB activity was detected (0%) in cells of *S. coelicolor*. In *S. davawensis* the addition of riboflavin reduced RibB activity up to 23.5% (± 8.3%; 40 h value). The addition of roseoflavin, however, had no such effect (RibB activity remained at >90%) indicating that expression of the *ribBMAH* genes in *S. davawensis* was not affected by this antibiotic.

The data (see Table 2; control levels) also revealed that the specific RibB activity in *S. davawensis* (20 h: 0.485 µM mg^{-1} h^{-1} ± 0.040) was quite similar to what was found in *S. coelicolor* at that time point (20 h: 0.319 µM mg^{-1} h^{-1} ± 0.015). However, in *S. davawensis* RibB activity strongly increased at 30 h of growth and at 40 h was almost 60 times higher as compared to *S. coelicolor*. This, however, still did not lead to an increased riboflavin level in the cytoplasm of *S. davawensis*.

Table 2. Monitoring of expression of the *ribBMAH* genes by measuring the activity of riboflavin synthase (RibB). The activities are presented per mg of total protein (A) or normalized to 100% (control level) (B).

A		**MM 20h** ($\mu M\ mg^{-1}\ h^{-1}$)	**MM 30h** ($\mu M\ mg^{-1}\ h^{-1}$)	**MM 40h** ($\mu M\ mg^{-1}\ h^{-1}$)	**YS 40h** ($\mu M\ mg^{-1}\ h^{-1}$)
S. davawensis	control	0.485 ± 0.040	1.862 ± 0.151	5.140 ± 0.502	0.451 ± 0.050
	RF 50µM	0.225 ± 0.030	0.614 ± 0.060	1.208 ± 0.100	0.226 ± 0.018
	RoF 50µM	0.474 ± 0.050	1.781 ± 0.070	4.575 ± 0.313	1.168 ± 0.235
S. coelicolor	control	0.319 ± 0.015	0.254 ± 0.040	0.088 ± 0.005	–
	RF 50µM	0.176 ± 0.014	0.129 ± 0.011	0.042 ± 0.003	–
	RoF50µM	0.066 ± 0.002	0.053 ± 0.004	0	–

B		**MM 20h (%)**	**MM 30h (%)**	**MM 40h (%)**	**YS 40h (%)**
S. davawensis	control	100 ± 8.2	100 ± 8.1	100 ± 9.7	100 ± 11.0
	RF 50µM	46.4 ± 13.3	33.0 ± 9.7	23.5 ± 8.3	50.1 ± 8.0
	RoF 50µM	97.7 ± 10.5	95.6 ± 3.9	90.0 ± 6.8	259.8 ± 20.1
S. coelicolor	control	100 ± 4.7	100 ± 15.7	100 ± 5.7	–
	RF 50µM	55.1 ± 7.9	50.8 ± 8.5	47.7 ± 7.1	–
	RoF 50µM	20.7 ± 3.0	20.8 ± 7.5	0	–

When grown on a minimal medium *S. davawensis* produced comparably small amounts of roseoflavin (1 µM). However, when grown in a yeast extract/starch (YS) culture medium containing small amounts (4 µM) of the precursor riboflavin considerably more roseoflavin (19 µM) was produced by *S. davawensis*. Therefore similar experiments, as described above, were performed with *S. davawensis* growing on YS. In cells of these cultures a strongly increased RibB activity (259.8%, ± 20.1%) was detected upon addition of roseoflavin to the cultures. In contrast, the addition of riboflavin reduced RibB activity to 50.1% (± 8%). An increase of RibB activity in *S. davawensis* upon stimulation with

roseoflavin is perfectly in-line with the fact that riboflavin is the precursor of roseoflavin (MATSUI, et al., 1979) and thus is needed in larger amounts when roseoflavin synthesis is triggered in the stationary phase of growth. RibB activity of *S. coelicolor* could not be detected in cells grown on YS medium, probably because the flavin content in YS (4µM) repressed the *ribBMAH* genes expression.

3.4 The *ribB* FMN riboswitches of *Streptomyces davawensis* and *Streptomyces coelicolor* regulate at the translational level

Four steps in riboswitch formation are critical for efficient gene expression modulation: appropriate aptamer folding, speed of transcription process, association rate between cognate metabolite and riboswitch aptamer and folding of the expression platform. The speed of transcription process depends on intrinsic cell characteristics like RNA polymerase speed and presence of transcriptional pause sites in the DNA sequence, and also depends on cell nutrition parameters like nucleobases availability (WINKLER et al., 2002; BREAKER, 2010). The metabolite binding rate depends on the ligand structure itself and also on the extension of rearrangements in the riboswitch aptamer necessary to accommodate the cognate metabolite (SERGANOV, 2009). Aptamer and expression platform folding probably depends on salt concentration (especially Mg^{2+} and K^{+}) and on the mRNA primary structure, since alternative folding structures can compete with the aptamer or the terminator/sequestor for the same nucleotides (SERGANOV, 2009; BREAKER, 2010). In this case, it might be important that the aptamer has folded before a long stretch of downstream RNA has been synthesized. The same principle is valid for the ribosomal binding site sequestor. All the conditions involved in these four steps point to the importance of the analysis of riboswitch function in the context of an active transcription process. Following this direction, a suitable *in vitro* transcription/translation assay was established based on the plasmid pT7luc (Promega, Mannheim, Germany) in order to be able to quantify the activity of FMN riboswitches from different bacteria.

Between the bacteriophage T7 promoter and the luciferase reporter gene, FMN riboswitches from *B. subtilis*, *S. coelicolor* and *S. davawensis* were placed. Two different plasmid constructs were generated for each FMN riboswitch producing transcriptional (Fig. 9A) as well as translational fusions (Fig. 9B). The plasmids subsequently were used as templates for a T7 RNA polymerase based prokaryotic *in vitro* transcription/translation assay. Before testing the yet uncharacterized *Streptomyces* riboswitches the test system was

validated using the well described *ribG* FMN-riboswitch from *B. subtilis* (MIRONOV et al., 2002; WINKLER et al., 2002). The presence of FMN in the *in vitro* transcription/translation reaction reduced luciferase gene expression regardless of whether the plasmid generating a transcriptional fusion (reduction to 40%; ± 2%) or the plasmid generating a translational reporter gene fusion (reduction to 42%; ± 3%) was employed. Apparently, the FMN riboswitch from *B. subtilis* acts at the transcriptional level as was reported earlier (MIRONOV et al., 2002; WINKLER et al., 2002). In contrast, the FMN riboswitches from *S. coelicolor* and *S. davawensis* did not respond to FMN when the plasmids generating transcriptional fusions were used as templates for *in vitro* transcription/translation. However, FMN reduced luciferase gene expression using the plasmids for translational fusions: A very similar reduction to 40% (± 2%) was observed for the *S. coelicolor* as well as for the *S. davawensis* FMN riboswitch. The latter result shows that the FMN riboswitches of *S. davawensis* and *S. coelicolor* indeed regulate at the translational level as was suggested earlier (VITRESCHAK et al., 2002).

3.5 RoFMN triggers FMN riboswitches to repress gene expression

It was previously reported that roseoflavin *in vivo* is able to reduce expression of an FMN riboswitch-*lacZ* reporter gene in the RoF sensitive bacterium *B. subtilis* (LEE et al., 2009; OTT et al., 2009) and additionally, it was shown using in-line probing experiments that roseoflavin directly can bind to FMN riboswitch aptamers *in vitro* (LEE et al., 2009). Another work showed that the bifunctional flavokinase/FAD synthetase of *B. subtilis* (RibC) accepts roseoflavin as a substrate and synthesizes RoFMN and RoFAD (GRILL et al., 2008) suggesting that in *B. subtilis* roseoflavin following uptake by RibU (VOGL et al., 2007) is converted intracellularly to RoFMN and RoFAD. Hence it appeared feasible that RoFMN and not RoF was the true effector of FMN riboswitches. Indeed, the addition of increasing amounts of RoFMN (0-180 µM) reduced the activity of the luciferase reporter-gene of the *B. subtilis* FMN riboswitch *in vitro* by almost 60% (Fig. 12). Notably, a very similar effect (reduction by 60%) was found when FMN was added. The addition of riboflavin and roseoflavin had no such an effect (Fig. 12).

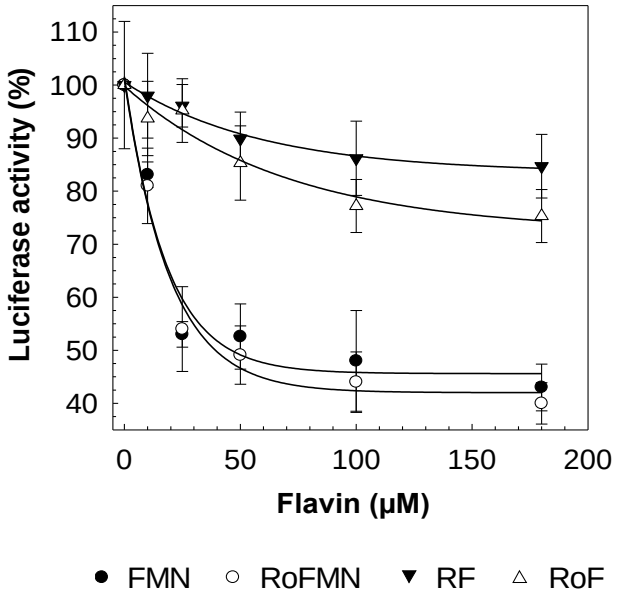

Figure 12. RoFMN triggers FMN riboswitches to repress gene expression. A reporter plasmid producing a transcriptional fusion of the *ribG* FMN riboswitch of *Bacillus subtilis* and the reporter gene *luc* (coding for firefly luciferase) was used as a DNA template in an *in vitro* transcription/translation assay driven by RNA polymerase of bacteriophage T7. The addition of FMN (solid circles) resulted in less activity of Luc indicating that less *luc* transcript was produced. Roseoflavin mononucleotide (RoFMN) (open circles) had a very similar effect. The addition of riboflavin (solid triangles) or RoF (open triangles) also resulted in reduced luciferase activity, however, the effect was much smaller.

3.6 The *Streptomyces coelicolor* and *Streptomyces davawensis ribB* FMN riboswitches respond differently to the flavin analogs RoFMN and AFMN

Sequence analysis revealed that, as in *S. davawensis*, expression of the *ribBMAH* riboflavin biosynthetic gene cluster present in the chromosome of the model Streptomycete *S. coelicolor* may be regulated by an FMN riboswitch (VITRESCHAK et al., 2002), hereafter called *ribB* FMN riboswitch.

According to the experiments up to this point, the results suggested that formation of RoFMN targeting FMN riboswitches is responsible for the antibiotic effect of roseoflavin. Since *S. davawensis*, in contrast to *S. coelicolor*, was found to be RoF resistant it was hypothesized that the *ribB* FMN riboswitches of the two organisms react differently with respect to treatment with RoFMN. In order to test this, the two *ribB* FMN riboswitches were characterized using *in vitro* transcription/translation assays (Fig. 13A and B) employing plasmid constructs for translational fusions. For the *S. coelicolor ribB* FMN riboswitch the

data revealed that both FMN and RoFMN significantly reduced the amount of luciferase activity *in vitro*. In the case of FMN a reduction by 62% (± 7%) was observed, in the case of RoFMN the reduction was 75%, (± 6%). Similar experiments (Fig. 13B) were carried out with translational fusions to the *S. davawensis ribB* FMN riboswitch and it was found that luciferase activity was clearly reduced upon addition of FMN (reduction by 63%, ± 7%) to the assay mixture. The extent of reduction (by 62%, ± 7%) was similar to what was found for the *S. coelicolor* riboswitch. Most strikingly however, the addition of RoFMN to the assay containing the *S. davawensis* FMN riboswitch resulted in an increase of luciferase activity by 28% (± 9%).

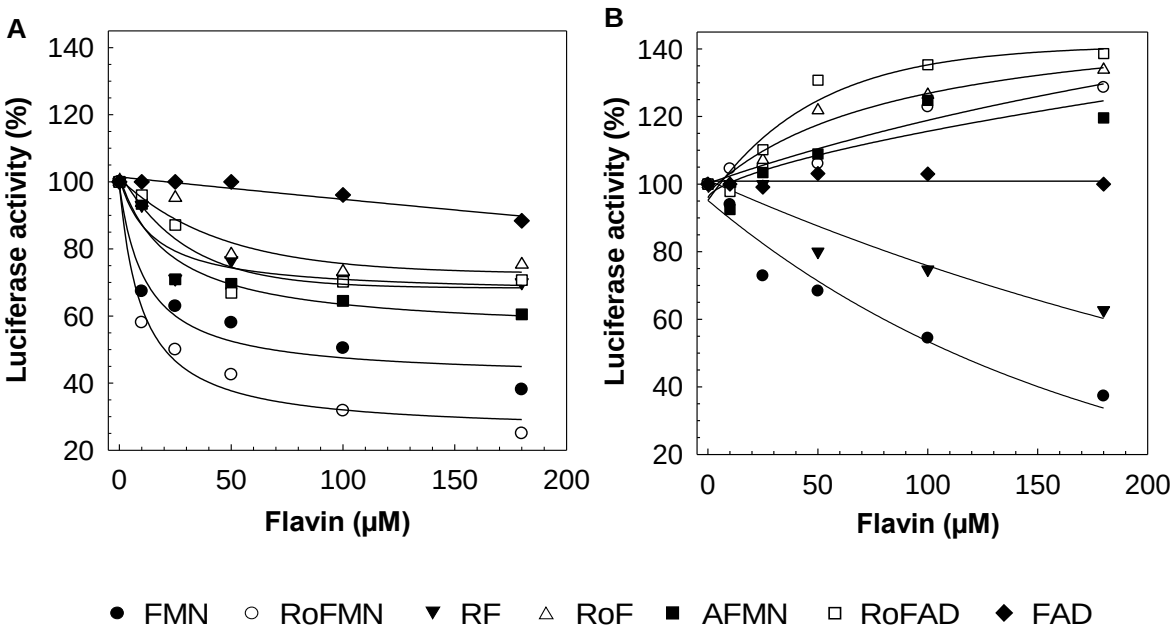

Figure 13. The *ribB* FMN riboswitches of *Streptomyces coelicolor* and *Streptomyces davawensis* are affected differently by different flavin analogs. Reporter plasmids producing translational fusions of the *ribB* FMN riboswitches of *S. coelicolor* (A) and *S. davawensis* (B) and the reporter gene *luc* were tested with respect to their activity in the presence of different flavins using *in vitro* transcription/translation assays.

The amount of flavin needed for a 50% reduction (T_{50}) of luciferase activity in the transcription/translation assays is a measure for the apparent ligand affinity of the FMN riboswitch aptamers. The *B. subtilis ribG* aptamer apparently did not discriminate against FMN and RoFMN showing similar T_{50} for both, 46 µM (± 6 µM) for FMN and 40 µM (± 4 µM) for RoFMN. The *S. coelicolor ribB* aptamer appeared to be more sensitive to RoFMN

showing a T_{50} of 20 µM (± 5 µM) whereas for FMN a concentration of 50 µM (± 8 µM) was necessary to trigger 50% of reduction in gene expression. T_{50} of the *S. davawensis ribB* aptamer showed the poorest apparent affinity for FMN trigging 50% of luciferase activity reduction at 102 µM (± 6 µM). For RoFMN activation was observed and thus T_{50} was not calculated.

The FMN riboswitches from *S. coelicolor* and *S. davawensis* were also tested with respect to modulation by other flavins. Notably, another flavin analog produced by *S. davawensis* was tested, the flavin 8-demethyl-8-amino-riboflavin (AF). AF (the 5'-phosphorylated form is AFMN) is the direct precursor of roseoflavin (JANKOWITSCH et al., 2011) and was found to exhibit antibiotic activity as well (M. Mack, unpublished results). The *S. coelicolor* FMN riboswitch was found to be affected by all flavins tested (RoF, RF, RoFMN, FMN, RoFAD, FAD, and AFMN) except for FAD (Fig. 13A). The affinity of the *S. coelicolor* riboswitch for these flavins appeared to be much poorer (T_{50} > 180 µM) than for FMN or RoFMN. Except for FMN, only riboflavin was able to decrease the luciferase activity level when testing the *S. davawensis* FMN riboswitch (T_{50} > 180 µM) (Fig. 13B). RoFAD, RoFMN, RoF and AFMN stimulated luciferase expression by up to 38% (180µM RoFAD). FAD did not cause any effect.

3.7 Analysis of a second (*ribA*) FMN riboswitch present in the genome of *Streptomyces davawensis*

A genome wide screen in *S. davawensis* identified a second sequence element ("*ribA* FMN riboswitch") matching the consensus for FMN riboswitch aptamers (Fig. 15). Other candidate FMN riboswitches were not found in the 8.9 Mbp *S. davawensis* genome. The *ribA* FMN riboswitch is present in the 5'-UTR of a gene cluster with unknown function (comprising seven genes). Interestingly, the product of the first putative gene SDA76970 within this cluster is similar (up to 54% on the amino acid level) to 3,4-dihydroxy-2-butanone 4-phosphate synthases from other microorganisms. The latter enzymes catalyse an initial step in riboflavin biosynthesis, the formation of 3,4-dihydroxy-2-butanone 4-phosphate (FISCHER & BACHER, 2005).

The *ribA* FMN riboswitch was analysed using *in vitro* TK/TL, and, in contrast to the *S. davawensis ribB* FMN riboswitch, was found to be less active in the presence of RoFMN (T_{50} = 25 µM ± 4 µM) and FMN (T_{50} = 94 µM ± 5 µM) (Fig. 14). Besides RoFAD, all the other flavins tested negatively affected expression of the luciferase gene under control of this FMN

riboswitch. Furthermore, only the translational fusion of this second FMN riboswitch produced a decrease in luciferase activity upon FMN addition strongly suggesting that the *ribA* FMN riboswitch also operates at the translational level.

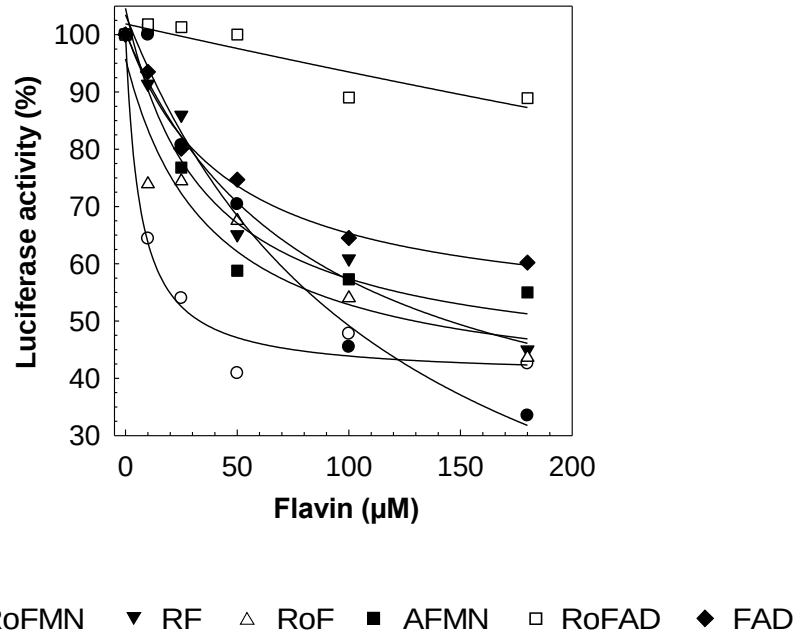

Figure 14. The *ribA* FMN riboswitch present in the genome of *Streptomyces davawensis*. The activity of the *ribA* FMN riboswitch was tested using the *in vitro* TK/TL luciferase based system in the presence of different flavins. The FMN *ribA* riboswitch (in contrast to the FMN *ribB* riboswitch) was found to be sensitive with respect to the addition of RoFMN (open circles). Besides RoFAD (closed circles), all the other flavins tested negatively affected expression of the luciferase gene under control of this FMN riboswitch.

The putative *ros* gene cluster responsible for synthesizing roseoflavin from riboflavin is located 20 kb downstream of SDA76970 (similar up to 54% on the amino acid level to 3,4-dihydroxy-2-butanone 4-phosphate synthases, *ribA*, from other microorganisms), whereas additional putative riboflavin biosynthetic genes are present 25 kb upstream of SDA76970. The following experiment was performed in order to find out whether the *ribA* FMN riboswitch contributes to regulation of roseoflavin biosynthesis. *S. davawensis* was cultivated in YS supplemented with riboflavin or roseoflavin and the activity of RosA in a cell-free extract was determined. RosA is responsible for the last step in roseoflavin biosynthesis, converting 8-demethyl-8-amino riboflavin into roseoflavin (JANKOWITSCH et al., 2011). RosA activity was not affected by the addition of roseoflavin or riboflavin to the culture,

suggesting (in light of our *in vitro* experiments) that the *ribA* FMN riboswitch is not directly involved in the regulation of *rosA* expression. In summary, the physiological role of the *ribA* FMN riboswitch remains elusive.

3.8 Predicted secondary structures of FMN riboswitches from *Bacillus subtilis*, *Streptomyces davawensis* and *Streptomyces coelicolor*

Based on predicted and experimentally determined riboswitches structures previously published (LEE et al., 2009; SERGANOV et al., 2009) and using the software R2R (WEINBERG & BREAKER, 2010), the aptamer structures of the FMN riboswitches used in this study were drawn (Fig 15). Despite of sequence divergences, all four FMN riboswitches (RFNGsub, RFNBcoe, RFNBdav and RFNAdav) seem to assume a similar secondary structure based on six stem-loops *P1* to *P6*. In all the predicted structures the anti-terminator/ anti-sequestor stem is formed through base-pairing of a specific stretch in the expression platform and 8 or 9 nucleotides located in the *P1* stem and in the *P1*/*P2* inter-loop sequences within the aptamer. A sequence of four guanines (Fig. 15, sequence between *P1* and *P2*) seems to be characteristic for FMN riboswitches anti-terminator/ anti-sequestor stems (VITRESCHAK et al., 2002).

Figure 16A shows the predicted secondary structure of the *S. davawensis ribB* FMN riboswitch including the aptamer and the expression platform. The RBS sequestor stem forms 114 bp downstream of the aptamer and overlaps the ribosomal binding site (RBS). Aptamer and RBS sequestor sequences are highly conserved among Streptomycetes FMN riboswitches (Fig 16B and C), but the connecting stretch between them (without any apparent function) is highly variable. Among the riboswitches sequences analysed, the *S. davawensis ribB* riboswitch is unique: no other riboswitch sequence carries an adenine at position 61 or the nucleotides cytosine and thiamine at positions 261 and 262 (or at corresponding positions in relation to the RFNBdav).

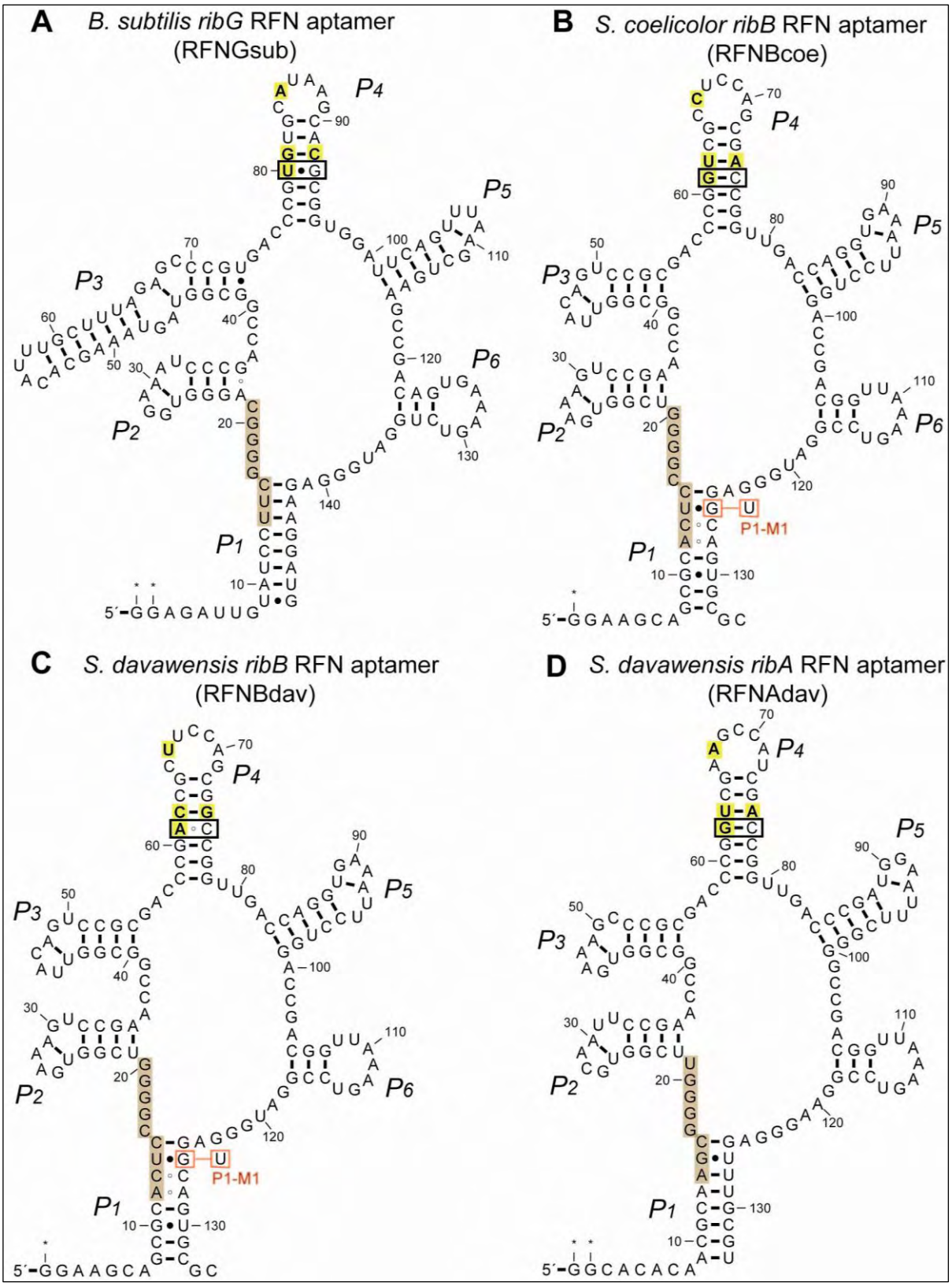

Figure 15. The aptamer portions (5' to 3') of FMN riboswitches (RFN) from *Bacillus subtilis* (controlling the riboflavin biosynthetic genes *rib<u>G</u>BAHT*), *Streptomyces coelicolor* (controlling the riboflavin biosynthetic genes *rib<u>B</u>MAH*) and *Streptomyces davawensis* (controlling the riboflavin biosynthetic genes *rib<u>B</u>MAH*). In *S. davawensis* a second FMN

riboswitch controlling the putative riboflavin biosynthetic gene *ribA* has been identified. The *ribB* FMN riboswitch aptamers of *S. davawensis* and *S. coelicolor* differ at 4 nucleotide positions only (yellow). The nucleotide A61/G61 responsible for RoF resistance/sensitivity (and the corresponding nucleotide in the *B. subtilis* aptamer) is boxed in black. The *ribA* FMN riboswitch aptamer of *S. davawensis* and the *ribG* FMN riboswitch aptamer of *B. subtilis* are similar in the *P1* (stem)-loop. The nucleotides marked brown are responsible for anti-termination/anti-sequestration. Single point mutations (boxed red) were placed in the P1 stem of the aptamers in order to weaken ligand affinities in the in-line probing assays (see results section). The G residues (asterisks) at the 5' end of the aptamers were added to increase the yield of transcription by T7 RNA polymerase in the in-line probing assays.

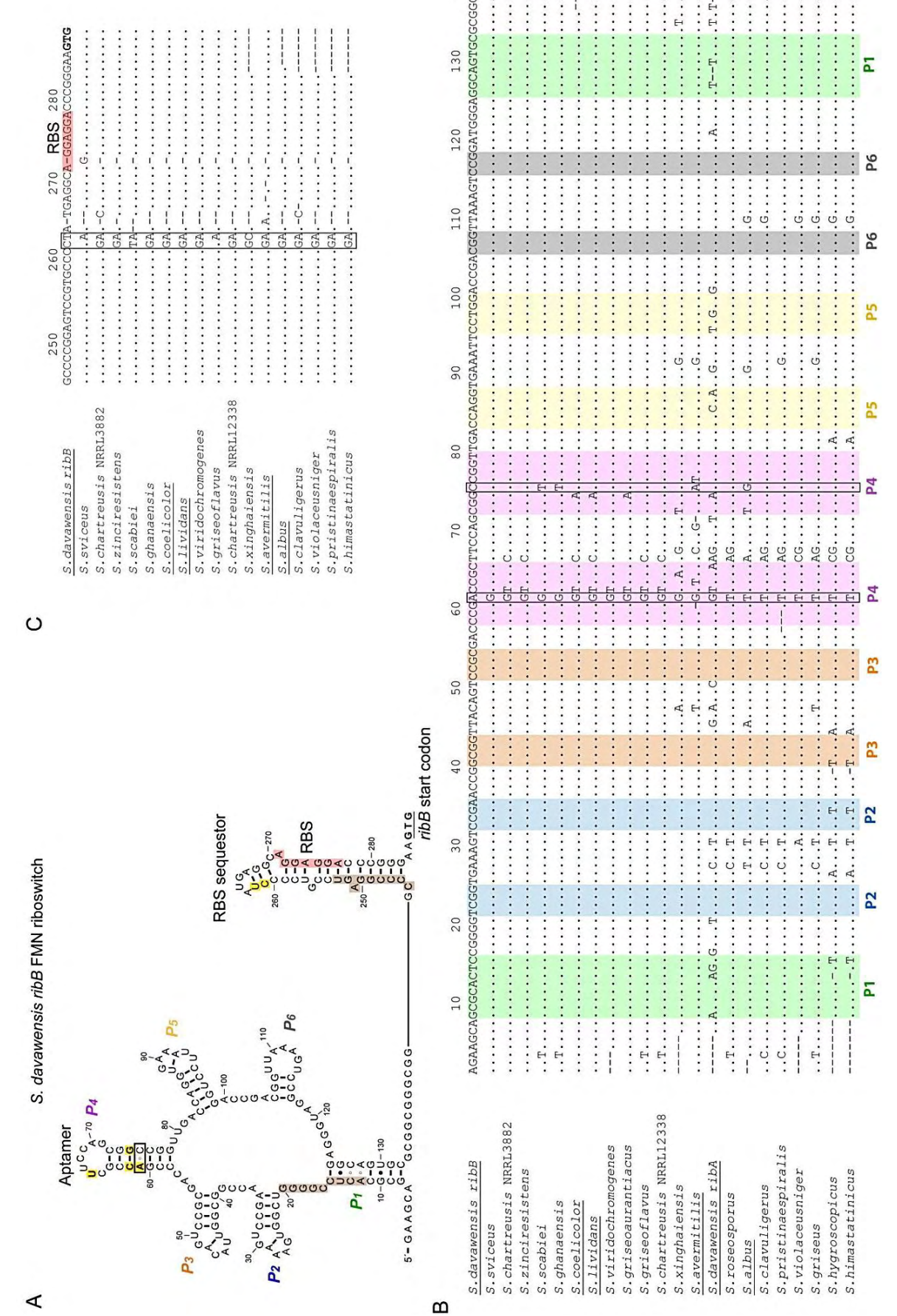
A
S. davawensis ribB FMN riboswitch
Aptamer
P1
P2
P3
P4
P5
P6
RBS sequestor
RBS
ribB start codon
B
C
S.davawensis ribB
S.sviceus
S.chartreusis NRRL3882
S.zinciresistens
S.scabiei
S.ghanaensis
S.coelicolor
S.lividans
S.viridochromogenes
S.griseoaurantiacus
S.griseoflavus
S.chartreusis NRRL12338
S.xinghaiensis
S.avermitilis
S.davawensis ribA
S.roseosporus
S.albus
S.clavuligerus
S.pristinaespiralis
S.violaceusniger
S.griseus
S.hygroscopicus
S.himastatinicus
RBS

Figure 16. Sequence alignment of *Streptomyces* FMN-riboswitches. (A) *S. davawensis ribB* FMN riboswitch (aptamer and expression platform): *P1 – P6* identify the stem-loops in the aptamer structure; yellow marks highlight sequence differences in the aptamer and RBS sequestor between the *S. davawensis* and *S. coelicolor* riboswitches; the important nucleotide 61 is black boxed; solid line represents a highly variable stretch with no apparent function; nucleotides marked brown are complementary to each other and responsible for anti-RBS sequestration; ribosomal binding site (RBS) sequence is red marked. (B) Aptamer nucleotide sequences of FMN riboswitches from different Streptomycetes. The riboswitches are highly similar and only the nucleotides differing from the *S. davawensis ribB* FMN riboswitch (top sequence) are shown. The *S. davawensis* FMN riboswitch sequence is the only sequence containing an A at position 61 (boxed nucleotide). The other *Streptomyces* species show either a G or a T (U) at this position. Sequences marked with the same color combine to form one of the six stem-loops shown in A. (C) RBS-sequestor sequences of FMN riboswitches from different Streptomycetes: ribosomal binding site (RBS) sequence is red marked and important nucleotides are black boxed.

3.9 The nucleotide A61 of the *Streptomyces davawensis* riboswitch confers resistance to roseoflavin

The *ribB* FMN riboswitch aptamers of *S. coelicolor* and *S. davawensis* differ at 4 nucleotide positions only (nucleotides 61, 62, 66 and 74; Fig. 15B and C). Sequence analysis of *ribB* FMN riboswitches of RoF-sensitive *Streptomyces* species tested in this work (*S. coelicolor, S. lividans, S. albus* and *S. avermitilis*) revealed that RoF-resistant *S. davawensis* is the only species to contain an A at position 61 (A61) (Fig. 16B). *S. coelicolor* (G61), *S. lividans* (G61), *S. albus* (T61) and *S. avermitilis* (G61) have either a G or a T at that position. Interestingly, as the *S. coelicolor ribB* FMN riboswitch, the RoFMN-sensitive *ribA* FMN riboswitch has a guanine nucleotide at the position corresponding to 61A in the *ribB* FMN riboswitch of *S. davawensis* (Fig. 15D). Position 62 appeared not to be a key residue with respect to RoF/RoFMN resistance since *S. avermitilis* and *S. albus*, like *S. davawensis*, contain a C at that position. The same was true for nucleotides 66 and 74, where *S. albus* and *S. avermitilis ribB* FMN riboswitches contain T66 and G74, respectively, as found in *S. davawensis*. Notably, none of the 23 *Streptomyces* FMN riboswitches listed in the RFAM database (GARDNER et al., 2010) revealed the nucleotide pair 61AC75 (Fig. 16A and B). Even the *ribG* FMN riboswitch aptamer of *B. subtilis* do not have the nucleotide pair AC at the corresponding position, instead it has the base pair UG.

Variants of the riboswitch aptamers of *S. coelicolor* and *S. davawensis* were generated by site-directed mutagenesis and tested using *in vitro* transcription/translation. The single

nucleotide exchange A61G produced a mutant *S. davawensis* FMN riboswitch (RFNdavA61G) which, in contrast to the wild-type riboswitch, apparently was negatively affected by both FMN and RoFMN: The luciferase activity produced in the transcription/translation assays decreased upon addition of FMN and also upon addition of RoFMN (Fig. 17B). In contrast the wild-type riboswitch was negatively affected by FMN but found to be more active in the presence of RoFMN (Fig. 17A, see also Fig. 13B). The single nucleotide exchanges C62T (Fig. 17C) and G74A (Fig. 17E) produced mutant *S. davawensis* FMN riboswitches which were less sensitive with respect to both FMN and RoFMN indicating that destabilization of the P4 stem (Fig. 16A) affects aptamer/riboswitch function. The exchange T66C responded like the wild type to FMN and RoFMN (Fig. 17D). Activity of the mutant FMN riboswitches A61G/C62T/G74A (Fig. 17F) and A61G/C62T/G74A/T66C (Fig. 17G; corresponds to the *S. coelicolor* riboswitch aptamer) was reduced in the presence of RoFMN, strongly suggesting that A61 indeed is critical with respect to RoF resistance. Most interestingly, the single mutation G61A within the *S. coelicolor* FMN riboswitch (removing Watson-Crick DNA base pairing at nucleotide 61G-75C) produced a RoFMN-insensitive FMN riboswitch responding like the *S. davawensis* FMN riboswitch with respect to the addition of RoFMN (Fig. 17H).

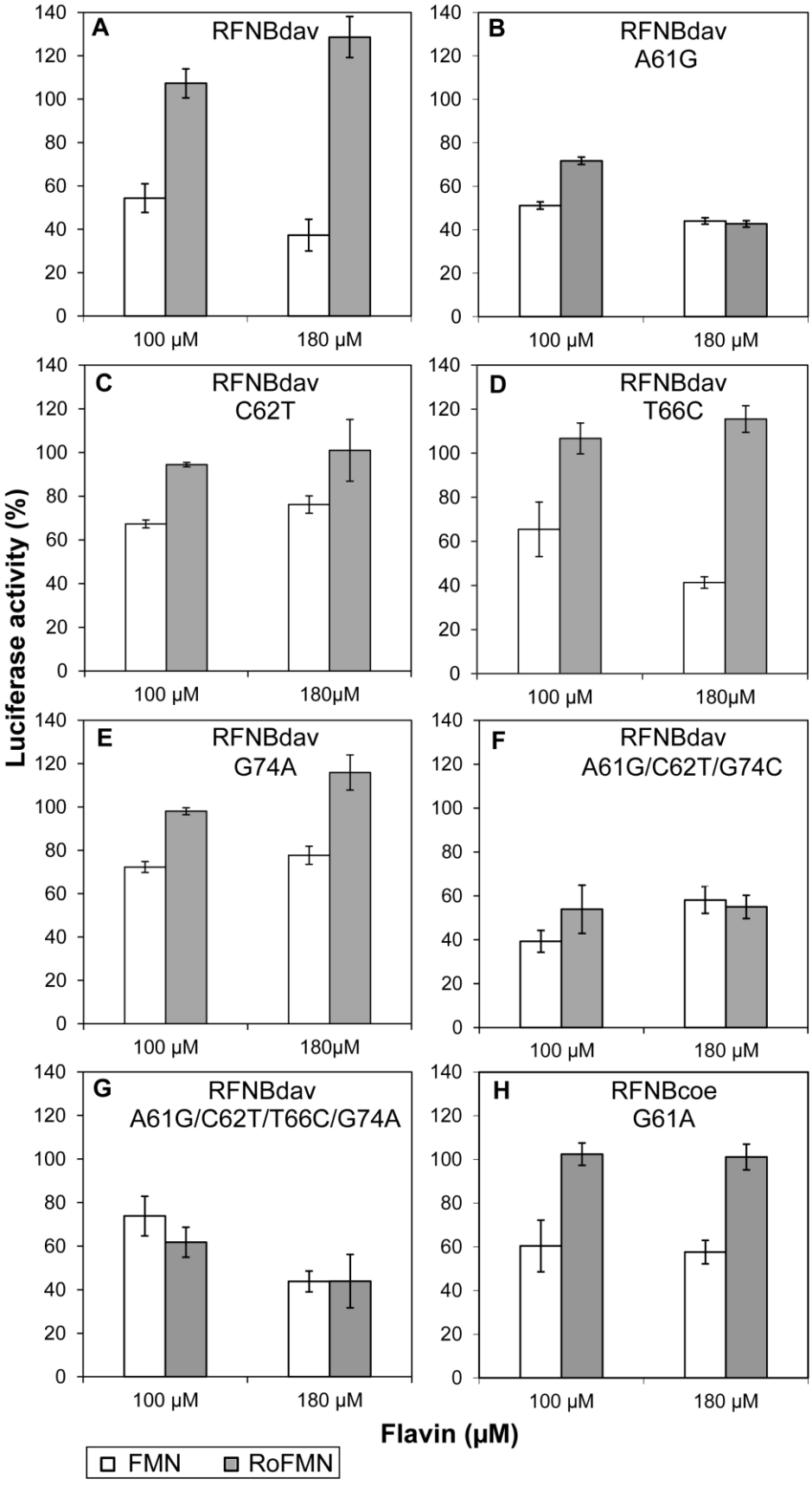

Figure 17. Nucleotide A61 of the *Streptomyces davawensis* riboswitch is responsible for the discrimination between FMN and RoFMN. Reporter plasmids producing translational luc-

fusions of *ribB* FMN riboswitches from *S. davawensis* and *S. coelicolor* were employed as templates for in vitro transcription/translation assays (see Fig. 2). Wild-type (A) and mutant FMN riboswitches of *S. davawensis* (B, C, D, E, F and G) and a mutant FMN riboswitch of *S. coelicolor* (H) were tested in the presence of FMN or RoFMN. The single nucleotide exchange A61G (B) produced an *S. davawensis* FMN riboswitch which, in contrast to the wild-type riboswitch (A), was negatively affected by both FMN and RoFMN. The single nucleotide exchanges C62T (C), T66C (D) and G74A (E) produced similar results as in (A). Most interestingly, the single mutation G61A within the *S. coelicolor* FMN riboswitch aptamer (removing Watson-Crick DNA base pairing at nucleotide 61G-75C) produced a RoFMN-insensitive FMN riboswitch (H). Mean values of three independent experiments are shown.

3.10 The *Streptomyces davawensis* FMN riboswitch confers RoF resistance to *Streptomyces coelicolor*

The following experiment was initiated in order to *in vivo* validate the *in vitro* results suggesting that a single nucleotide change conferred resistance to *S. davawensis*. The *S. davawensis ribBMAH*-genes including the RoFMN insensitive (wild-type) *ribB* FMN riboswitch and the corresponding promoter P_{rib} were stably integrated into the chromosome of *S. coelicolor* by homologous recombination at the Φ31 site using pSET152. The resulting strain *S. coelicolor*::P_{rib}RFN^{dav}*ribBMAH*^{dav} was able to grow on YS medium containing 200 µM roseoflavin and thereby found to be RoF resistant (Fig. 18A). It has long been known that oversynthesis of RF in the cytoplasm results in RoF resistance (MATSUI et al., 1982). Therefore, it was important to exclude the possibility that increased activities of the products of the chromosomally integrated *S. davawensis ribBMAH* genes (possibly leading to increased RF levels) were responsible for RoF resistance. In order to exclude this possibility, the *S. davawensis ribBMAH*-genes including the RoF sensitive A61G mutant FMN riboswitch were chromosomally integrated in *S. coelicolor*. The corresponding recombinant strain *S. coelicolor*::P_{rib}RFN^{davA61G}*ribBMAH*^{dav} was grown on a solid medium in the presence of roseoflavin and was found to grow only poorly (Fig. 18A). Similarly, when grown in liquid YS, only little growth was observed (Fig. 18B). The result of this experiment unequivocally showed that a single nucleotide exchange was responsible for RoF resistance and that the *S. davawensis ribB* FMN riboswitch was not negatively affected by RoFMN.

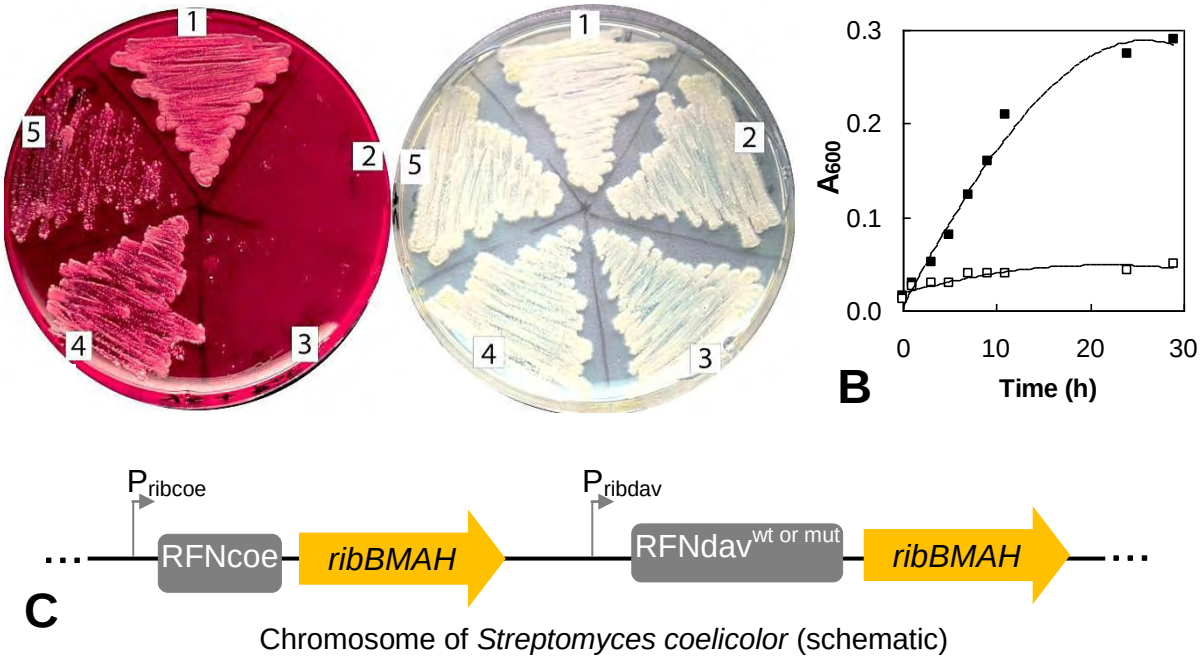

Figure 18. The *S. davawensis* FMN riboswitch confers RoF resistance to *S. coelicolor*. To each of the sectors of the YS plates shown about $5x10^4$ spores of different *Streptomyces* strains (1. wild-type *S. davawensis*; 2. wild-type *S. coelicolor*; 3. *S. coelicolor*::pSET152; 4. *S. coelicolor*::P_{rib}RFN^{dav}*ribBMAH*^{dav}; 5. *S. coelicolor*::P_{rib}RFN^{davA61G}*ribBMAH*^{dav}) were applied and the plates were incubated at 37°C for 60 h. The strain in sector 3 is a control containing pSET152 only (conferring apramycin resistance) at Φ31. (A) The plate to the left contained 200 µM RoF, the plate to the right did not contain any antibiotic. (B) Growth of the strains *S. coelicolor*::P_{rib}RFN^{dav}*ribBMAH*^{dav} (solid squares) and *S. coelicolor*::P_{rib}RFN^{davA61G}*ribBMAH*^{dav} (open squares) was also monitored in a liquid culture (minimal medium). (C) Schematic drawing showing the chromosome of recombinant *S. coelicolor* strains containing two different gene clusters controlled by two different promoters and two different FMN *ribB* riboswitches (RFNBcoe, from *S. coelicolor*, and RFNBdav from *S. davawensis*).

To test whether the integrated *ribBMAH*-genes were indeed expressed under control of P_{rib} and the *S. davawensis* FMN riboswitch, riboflavin synthase (RibB) activity was determined for both recombinant *S. coelicolor* strains (Table 3). Upon addition of RF to the culture medium of the RoF resistant strain *S. coelicolor*::P_{rib}RFN^{dav}*ribBMAH*^{dav} RibB activity decreased to 42% (± 11%), while roseoflavin addition only caused a small reduction in activity (91%; ± 18%), similar to *S. davawensis* wt RibB behaviour. In case of the RoF sensitive *S. coelicolor*::P_{rib}RFN^{davA61G}*ribBMAH*^{dav} strain RibB activity decreased to 58% (± 7%) upon addition of riboflavin to the culture medium. An even stronger decrease of RibB activity was found when roseoflavin was added to the cultures (47%, ± 8%), showing that the

mutation A61G in the riboswitch leads to a roseoflavin response similar to what was found for *S. coelicolor* wild-type. Notably, the total specific RibB activities were very similar in all *S. coelicolor* strains irrespective whether the *S. davawensis ribBMAH* genes (including the *rib*-promoter and the FMN-riboswitches) were present or not, suggesting that the *S. coelicolor* cells still responded to the same flavin concentration regardless the presence of two copies of *ribBMAH* genes with two FMN *ribB* riboswitches. Furthermore, the *rib*-promoters from *S. coelicolor* and *S. davawensis in vivo* had an almost identical activity (data not shown). In summary, the data show that nucleotide A61 was responsible for RoF resistance of the recombinant strain *S. coelicolor*::P_{rib}RFN^{dav}*ribBMAH*^{dav}.

Table 3. Riboflavin synthase (RibB) activity in different recombinant *Streptomyces coelicolor* strains. The activities were normalized to 100% (control level).

Substance	**Sc::P_{rib}RFN^{dav} *ribBMAH*^{dav} (%)**	**Sc::P_{rib}RFN^{davA61G} *ribBMAH*^{dav} (%)**
control	100 ± 12	100 ± 10
RF 50µM	42 ± 11	58 ± 7
RoF 50µM	91 ± 18	47 ± 8

3.11 A spontaneous roseoflavin resistant mutant of *Streptomyces coelicolor* carries the point mutation A61G within the FMN riboswitch

Spores of *S. coelicolor* were plated on a growth medium supplemented with roseoflavin (250 µM) and spontaneous RoF resistant cells (producing colonies) were detected with a frequency of 1 x 10^{-8}. Only the large colonies which resembled in size to colonies of *S. coelicolor* growing on plates without roseoflavin were analysed further. The regions upstream of the *ribBMAH* gene clusters (comprising the promoters P_{rib} and the FMN riboswitches) of twenty independent RoF resistant strains were amplified by PCR (500 bp fragments) and analysed by DNA-sequencing. One out of twenty isolated resistant strains showed the mutation A61G in the FMN riboswitch. Other mutations were not found upstream of the *ribBMAH* genes in any of the RoF resistant strains which were analysed.

3.12 *In vitro* binding of flavins to the riboswitch aptamers of *Streptomyces coelicolor* and *Streptomyces davawensis*

An in line-probing assay was used in order to *in vitro* test *ribB* FMN riboswitch aptamers from *S. coelicolor* and *S. davawensis* for RoFMN or FMN binding. Internucleotide linkages in regions of structured RNA tend to spontaneously cleave with a lower rate constant than linkages present in unstructured regions. Hence, in-line probing data generated at various ligand concentrations allow the determination of dissociation constant (K_D) values for RNA-ligand interactions (REGULSKI & BREAKER, 2008). We found that the affinities of both aptamers were exceedingly high for FMN and also for RoFMN ($K_D << 5$ nM). Indeed, the K_D values were so small that they could not reliably be measured with in-line probing. To overcome this, single point mutations were introduced in the aptamers that were expected to weaken the affinities, but not alter the ligand specificities. Accordingly, a single mutation was placed in the *P1* stem, since it was known that similar mutations in other riboswitches weaken affinities (WICKISER et al., 2005a) (Fig. 15). The K_D estimates using in-line probing are summarized in Table 4. It was found that all RNA aptamers clearly favoured FMN over RoFMN by at least a factor of 2. This result was surprising given the expectation that the *S. davawensis* aptamer might be clearly less affected by RoFMN i.e. would show a much higher K_D value as compared to FMN. In contrast, the values revealed that the *S. davawensis* aptamer does not selectively discriminate against RoFMN under equilibrium conditions.

The control of gene expression mediated by metabolite-sensing riboswitches is predicted to be either thermodynamically or kinetically driven (WICKISER et al., 2005a; WINKLER & BREAKER, 2005). For example, it has been shown that the *B. subtilis ribG* FMN riboswitch does not reach thermodynamic equilibrium with FMN in a timeframe that is relevant for gene control. Rather, the riboswitch is kinetically driven, and therefore the rate constant for ligand association is more meaningful than the K_D value when evaluating the concentration of ligand needed to trigger gene control. The working hypothesis at this point was that the *S. davawensis ribB* riboswitch functions as a kinetically driven riboswitch as well, and thus differently responds to FMN/RoFMN on a seconds or minutes timescale. Since in-line probing assays extended for 40 hours, this kinetic discrimination effect in the case of the *S. davawensis ribB* FMN riboswitch (i.e. a slower rate of association of RoFMN) may have been masked by a slower off rate of RoFMN as compared to FMN.

Table 4. Ligand binding by FMN riboswitches. ImageQuant software (Molecular Dynamics, Sunnyvale, USA) was used to quantify the product bands generated by in-line probing experiments and the data were used to estimate the dissociation constants (*K*D) of ligands.

RNA	Ligand	K_d (nM)
RFNBcoe	FMN	<<5
RFNBcoe	RoFMN	<<5
RFNBdav	FMN	<<5
RFNBdav	RoFMN	<<5
RFNAdav	FMN	≈ 5
RFNAdav	RoFMN	≈ 5
RFNBcoe_P1-M1	FMN	109
RFNBcoe_P1-M1	RoFMN	250
RFNBdav_P1-M1	FMN	62
RFNBdav_P1-M1	RoFMN	125

3.13 The *ribB* FMN riboswitches of *Streptomyces davawensis* and *Streptomyces coelicolor* operate differently

Fluorescence measurement assays were conducted in order to study the basis of the ligand-riboswitch interaction for the *ribB* FMN riboswitches from *S. coelicolor* and *S. davawensis*, whereby the kinetically driven FMN *ribG* riboswitch from *B. subtilis* was used as a control (WICKISER et al., 2005b) (Fig. 19). Riboswitch RNAs (aptamer portion in combination with the expression platform) *in vitro* were synthesized by T7 RNA polymerase and were mixed with FMN (1 µM) or RoFMN (1 µM). As expected (WICKISER et al., 2005b), the *B. subtilis* riboswitch did not induce fluorescence quenching due to ligand (FMN or RoFMN) binding. Very similar results were found for the *S. davawensis* FMN riboswitch, which apparently also formed an RNA unable to bind either FMN or RoFMN when it was complete and, therefore, the *S. davawensis ribB* FMN riboswitch behaviour is compatible with a kinetically controlled element. The situation appeared to be substantially different in *S.*

coelicolor. The presence of the *S. coelicolor* FMN riboswitch RNA led to significant fluorescence quenching of FMN and RoFMN (Fig. 19A and B), which indicated strong binding of the ligand. The latter result suggested that the *S. coelicolor* riboswitch (in contrast to the *S. davawensis* riboswitch) may remain receptive for a considerably longer time indicating that it could be triggered thermodynamically.

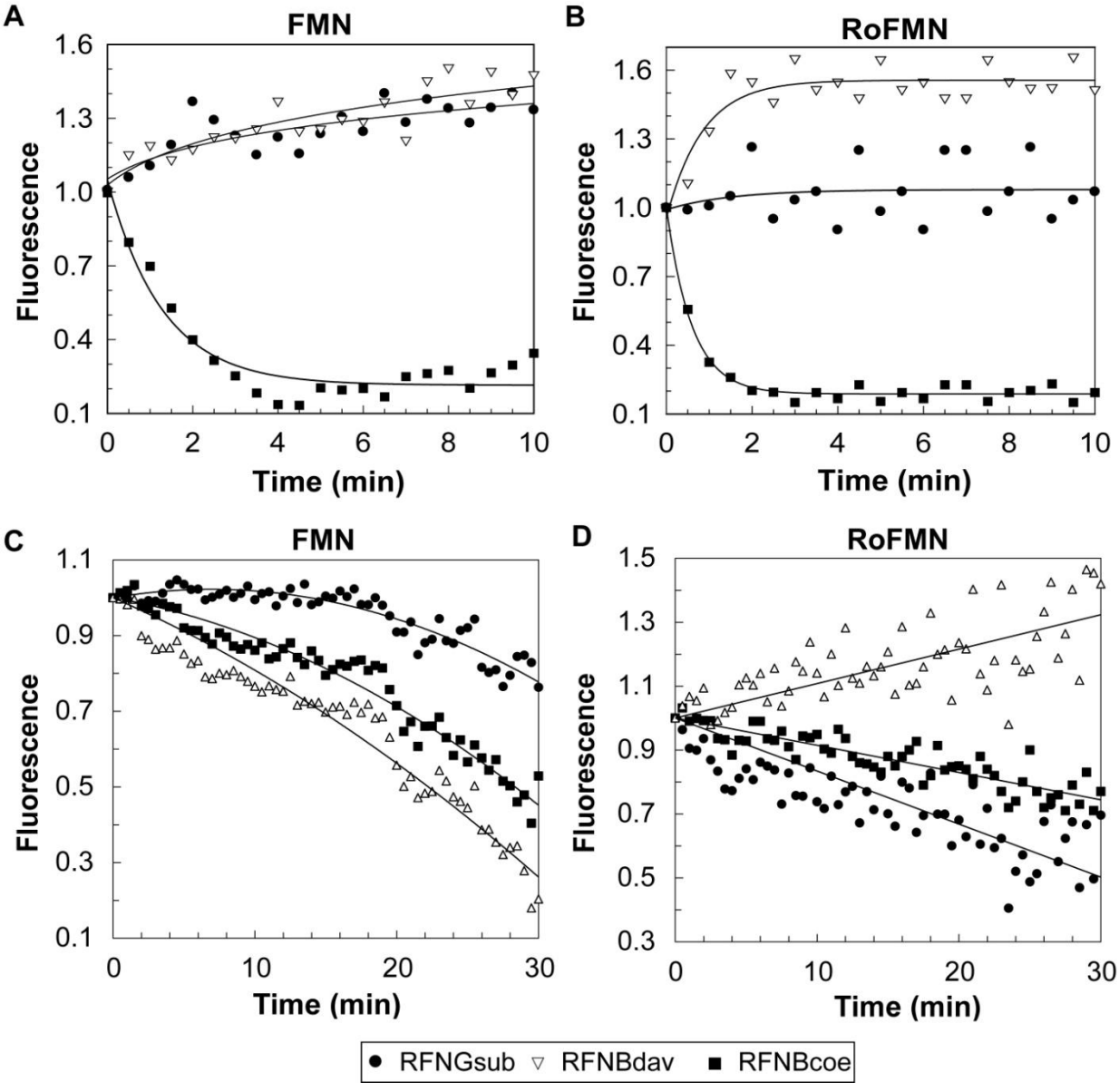

Figure 19. Monitoring the *ribB* FMN riboswitch-FMN or RoFMN complex formation by real-time fluorometry. Each image shows the change of fluorescence with time. The measurements were carried out in order to study the mechanism of the FMN/RoFMN-riboswitch interaction for the FMN riboswitches from *Streptomyces coelicolor* (RFNBcoe) and *Streptomyces davawensis* (RFNBdav). The kinetically driven *ribG* FMN riboswitch from *Bacillus subtilis* (RFNGsub) was used as a control. Riboswitch RNAs (aptamer portion in

combination with the expression platform) *in vitro* were synthesized by T7 RNA polymerase and were mixed with 1 µM FMN or 1 µM RoFMN. Fluorescence quenching due to FMN- (A) or RoFMN (B) -binding was determined. The change of fluorescence was also measured during the *in vitro* transcription process in the presence of 1µM FMN (C) or 1µM RoFMN (D). The observed fluorescence for each experiment was corrected to yield a plot where the initial signal was 1 (corresponding to the fluorescence of the free flavin).

In the subsequent experiment fluorescence quenching induced by FMN or RoFMN was monitored during the formation of the riboswitch RNAs in the *in vitro* transcription process. FMN (1 µM) or RoFMN (1 µM) were added to the *in vitro* transcription assay and fluorescence was measured for 30 min (Fig. 19C and D). The nascent riboswitches of *B. subtilis* and the *S. coelicolor* induced fluorescence quenching in the presence of FMN and RoFMN, indicating that both ligands are able to bind to these RNA molecules. However, in contrast to the completed RNA, the *S. davawensis* riboswitch as the nascent transcript was able to bind FMN but was unable to bind RoFMN. This finding was perfectly in line with the results generated by the *in vitro* transcription/translation assays which revealed that RoFMN did not negatively affect *S. davawensis* riboswitch function.

4. Discussion

The antibiotic roseoflavin from *S. davawensis* only affects organisms which have an uptake system for riboflavin whereby RF transporters apparently also catalyze the import of roseoflavin (VOGL et al., 2007; ABBAS & SIBIRNY, 2011; HEMBERGER et al., 2011). Following uptake, cytoplasmic roseoflavin is converted to RoFMN and RoFAD by bifunctional flavokinases/FAD synthetases (RibC or RibF) (GRILL et al., 2008). The latter cofactors combine with flavoenzymes and there is good evidence that enzymes containing RoFMN or RoFAD as cofactors in general are less active or completely inactive (GRILL et al., 2008). However, additional targets for roseoflavin (RoFMN) seem to be present in bacteria. *In vivo* studies using *lacZ* fusion experiments recently suggested that binding of flavin analogs to the aptamer portions of FMN riboswitches leads to reduced gene expression (LEE et al., 2009; OTT et al., 2009). Most importantly, one of the studies (LEE et al., 2009) unequivocally showed that expression of the *rib* genes controlled by the FMN riboswitch is indispensable for *B. subtilis* cell growth. Consequently, turning FMN riboswitches off leads to

riboflavin auxotrophy and, in an environment devoid of riboflavin, cells will not be able to grow.

An independent proof that roseoflavin and other flavin analogs indeed target FMN riboswitches now are presented here through *in vitro* and *in vivo* experiments with respect to RoF resistance of the producer strain *S. davawensis* in direct comparison to the RoF sensitive model actinomycete *S. coelicolor*. It was found that *S. davawensis* is RoF resistant to concentrations of roseoflavin (200 µM), exceeding the level naturally synthesized by *S. davawensis* (about 20 µM). In addition, it could be shown that RoFMN is indeed present in the cytoplasm of *S. davawensis* and also of *S. coelicolor* cells treated with roseoflavin supporting the previous finding that flavokinases are responsible for the activation of flavin analogs (GRILL et al., 2008).

The *in vitro* transcription/translation data strongly suggest that in *S. davawensis* and in *S. coelicolor* (as in other FMN riboswitch containing bacteria) expression of the *rib* genes is controlled by the amount of FMN present in the cytoplasm. Furthermore, it was clearly demonstrated that the FMN riboswitches of *S. davawensis* and *S. coelicolor*, in contrast to the *ribG* FMN riboswitch of *B. subtilis* (MIRONOV et al., 2002; VITRESCHAK et al., 2002; WINKLER et al., 2002), function at the translational level.

The *ribB* FMN riboswitches of *S. davawensis* and *S. coelicolor* were found to respond very differently with respect to the addition of RoFMN to *in vitro* transcription/translation assays. The *S. coelicolor ribB* FMN riboswitch was turned off by RoFMN, i.e. reporter gene expression was repressed in the presence of this ligand. In contrast, the *S. davawensis ribB* FMN riboswitch was turned on in the presence of RoFMN, i.e. reporter gene expression was stimulated in the presence of RoFMN. Both riboswitches, however, apparently were repressed by FMN. The *in vitro* transcription/translation results were strongly supported by different levels of riboflavin synthase (RibB) activity, which were detected in the two Streptomycetes upon treatment with riboflavin and roseoflavin, respectively. RibB is encoded by the first gene of the *ribBMAH* cluster and thus was an ideal reporter to monitor the activity of the translationally controlled FMN riboswitch. In cells of *S. coelicolor* that were grown in the presence of 50 µM riboflavin RibB activity was reduced up to 48% (40 h value) as compared to the untreated control. This reduction nicely corresponds to the *in vitro* transcription/translation data where (at 50 µM FMN) a reduction of the riboswitch activity to about 58% was observed. In cells of *S. coelicolor* that were grown in sublethal amounts of roseoflavin (50 µM) RibB activity was only 21% (20 h and 30 h values) as compared to the control, and was even reduced to 0% (40 h value). This reduction was even stronger than

observed in *in vitro* transcription/translation experiments where the riboswitch caused a reduction of Luc levels to about 40% at 50 µM RoFMN, indicating that *in vivo* the blocking effect of RoFMN can really cause riboflavin auxotrophy. When *S. davawensis* was grown in a minimal medium containing riboflavin (50 µM), RibB activity was also reduced (up to 24%), similar to what was observed for *S. coelicolor*. However, in contrast to *S. coelicolor*, RibB remained at 96% in RoF grown *S. davawensis* cells and even increased to 273% when a medium was used which supported roseoflavin production. Notably, the *ribA* FMN riboswitch present in *S. davawensis* was turned off by both, FMN and RoFMN, a finding that served as a valuable control judging the *in vitro* transcription/translation data.

The critical residue responsible for the different response of *S. davawensis* and of *S. coelicolor* to RoFMN is nucleotide 61 of the aptamer portion of FMN riboswitches (Fig. 15), as shown by *in vitro* transcription/translation experiments (Fig. 17) and by *in vivo* experiments using a recombinant *S. coelicolor* strain containing the *S. davawensis* FMN riboswitch (Fig. 18). An A at nucleotide 61 (*S. davawensis*) accounts for RoF resistance, a G at that position (*S. coelicolor* and other *Streptomyces* species) allows a Watson/Crick-Base pair and results in RoF sensitivity. Notably, the RoF sensitive *ribA* FMN riboswitch of *S. davawensis* also contains a GC pair at this critical position. Moreover, the isolation of a spontaneous roseoflavin resistant *S. coelicolor* mutant carrying the point mutation G61A within the *ribB* FMN riboswitch further supported the above findings. The surprising finding that a single nucleotide exchange within a 150 nucleotide ssRNA-molecule completely changed the response to RoFMN demonstrates that RNA molecules display a high degree of specificity and versatility.

A considerable number of RoF resistant strains were found to be deregulated with respect to RF biosynthesis and overproduce riboflavin (MATSUI et al., 1982). These strains carry mutations in either *ribC* (coding for a bifunctional flavokinase/FAD synthetase) or within the FMN riboswitch. The riboflavin overproducing phenotype in RibC deficient strains can be explained by reduced synthesis of FMN (MACK et al., 1998) whereas FMN riboswitch mutations lead to an FMN riboswitch which is not affected by FMN and consequently does not terminate transcription (MIRONOV et al., 2002; WINKLER et al., 2002). Some RoF resistant strains, however, interestingly do not oversynthesise riboflavin. These strains probably carry mutant FMN riboswitches that are regulated by FMN but not by RoFMN as it is the case for the *S. davawensis ribB* FMN riboswitch.

Most importantly, however, the data discussed above also shed new light on the molecular mechanism of action of roseoflavin. *In vitro*, RoFMN seems to block the *S.*

coelicolor FMN riboswitch more efficiently as compared to FMN, which could explain why roseoflavin is able to inhibit growth and act as an antibiotic. The latter is strongly supported by the *in vivo* data which showed that RibB activity was strongly reduced in *S. coelicolor* upon treatment with roseoflavin. Even if the supply with essential RF/FMN/FAD would only be slightly reduced in the presence of roseoflavin, this may constitute a major disadvantage for competing cells in a natural habitat.

Fluorescence measurements using complete riboswitches (including the aptamer and expression platform portions) were carried out and we found that the *S. davawensis* FMN riboswitch was unable to bind FMN or RoFMN although the aptamer alone has an excellent ability to bind both flavins with high affinities (see in-line probing data). The same was true for the *B. subtilis* FMN riboswitch. The *S. coelicolor* FMN riboswitch, however, was able to bind FMN and also RoFMN in this assay which suggested that the two *Streptomyces* riboswitches, although structurally very similar, are controlled differently. The latter was supported by the fact that fluorescence quenching was caused by nascent *S. davawensis* FMN riboswitch RNA in the presence of FMN suggesting that the *nascent* RNA was very well able to bind FMN (Fig. 19C). Similar results were generated using the *S. coelicolor* and the *B. subtilis* FMN riboswitches. The addition of RoFMN to this assay, however, produced a very different result for the *S. davawensis* FMN riboswitch. In contrast to the other FMN riboswitches the nascent *S. davawensis* riboswitch RNAs were not able to bind RoFMN (Fig. 19D). The *S. coelicolor* riboswitch RNA remains receptive to the modulator for a relatively long time (thermodynamic control) and thus can negatively be affected by RoFMN and FMN. The *S. davawensis* riboswitch, however, only remains receptive for modulator binding in the relatively short time frame between aptamer formation and synthesis of the anti-ribosomal binding site stem by RNA polymerase (kinetic control). Possibly, RoFMN binds slower to the *S. davawensis ribB* FMN riboswitch RNA molecules as compared to FMN due to the A61 nucleotide. In this case, RoFMN is not able to bind the aptamer and to generate the sequestering RNA structure preventing the translation initiation in the right time frame. In contrast, FMN binding is considerably faster, which explains why the *S. davawensis* riboswitch is still regulated by FMN. This work presents evidences that nucleotide A61 within the *S. davawensis* riboswitch is responsible for the slower binding of RoFMN. Possibly, the downstream expression platform of the *S. davawensis* riboswitch as well plays an important role with respect to the discrimination between RoFMN/FMN. This could explain why the *S. coelicolor* G61A mutant riboswitch does not lead to an *increase* of reporter activity as was found for the *S. davawensis* riboswitch. Thus, although nucleotide A61 in the aptamer of the

latter mutant slows down RoFMN binding, the downstream *S. coelicolor* expression platform remains receptive for RoFMM keeping translation at the 100% level. Moreover, the phenotype of *S. coelicolor*::P_{rib}RFN^{davA61G}*ribBMAH*^{dav} (poor growth) can be explained as follows: The latter strain contains a mutant *S. davawensis* riboswitch aptamer (A61G) which allows RoFMN to bind faster. In fact, it was expected this strain to be as sensitive towards roseoflavin as is wild-type *S. coelicolor*, which naturally contains G61 in its aptamer. However, *S. coelicolor*::P_{rib}RFN^{davA61G}*ribBMAH*^{dav} in addition to its mutant A61G aptamer contains the downstream *S. davawensis* expression platform, which helps to reduce RoFMN sensitivity and consequently allows sparse growth.

It is more difficult to find an explanation for the significantly increased activity of the *S. davawensis ribB* FMN riboswitch in the presence of RoFMN. The fluorescence measurements argue against RoFMN binding to the complete riboswitch. In contrast, in-line probing data suggest strong binding of RoFMN to the aptamer portion under equilibrium conditions. Structural experiments will have to be carried out in order to resolve this apparent contradiction and possibly will reveal that RoFMN leads to a conformational change promoting access of the ribosomes to their binding site within the 5'-UTR of the *ribBMAH* mRNA.

CHAPTER II. Metabolization of flavin analogs by humans

5. Results

5.1 Overproduction and purification of recombinant human flavokinase and human FAD synthetase in *E. coli* and *P. pastoris*

Human flavokinase and FAD synthetase (transcript variant 2) were separately overproduced as His_6-tagged recombinant enzymes in *Escherichia coli*. Human flavokinase (19.4 kDa, including His_6-tag) could easily be purified (>90%) as an active soluble enzyme by affinity chromatography from a cell-free extract of a corresponding recombinant *E. coli* strain (Fig. 20, lane 2). The purification of active His_6-tagged human FAD synthetase from *E. coli*, however, was more challenging. The latter enzyme (overproduced in *E. coli*) apparently was not soluble under the applied conditions and purification of sufficient amounts of active enzyme was not possible (data not shown). Therefore, His_6-tagged human FAD synthetase was overproduced employing an alternative expression system, the methylotrophic yeast *P. pastoris*. Purification of His_6-tagged human FAD synthetase (57.6 kDa, , including His_6-tag)

from a recombinant overproducing *P. pastoris* strain was successful and yielded soluble and pure (>90%) enzyme (Fig. 20, lane 3). It was already reported previously that overproduction/purification of soluble human FAD synthetase was difficult (BRIZIO et al., 2006; GALLUCCIO et al., 2007). *P. pastoris* proved to be an efficient alternative strategy to overproduce this enzyme. Slower growth of *P. pastoris* (as compared to *E. coli*) may have promoted correct folding in turn leading to enhanced solubility of the recombinant protein. Potential glycosylation sites (N-X-S/T) are absent in human FAD synthetase. Furthermore, a band-shift of the enzyme upon treatment with endoglycosidase H was not observed (data not shown) and therefore it was concluded that glycosylation by the host *P. pastoris* apparently was not responsible for enhanced solubility.

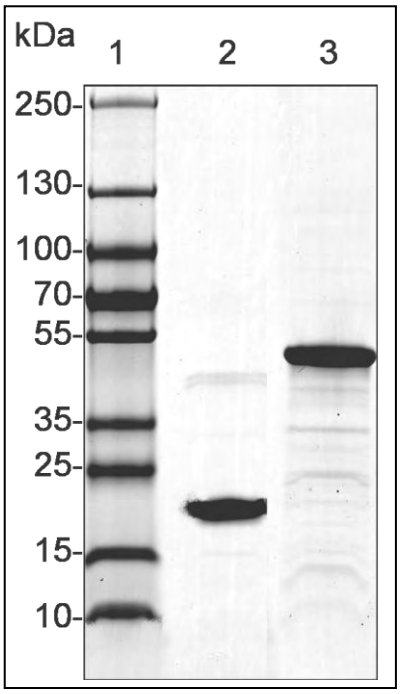

Figure 20. His_6-tagged human flavokinase (lane 2) was purified to near homogeneity from a cell-free extract of a recombinant *Escherichia coli* strain using immobilized metal affinity chromatography. Accordingly, His_6-tagged (soluble) human FAD synthetase (lane 3) was purified from a cell-free extract of a recombinant *Pichia pastoris* strain. Protein samples were analyzed by SDS-PAGE/Coomassie Brilliant Blue R-250 staining. Lane 1, molecular mass marker (in kDa).

5.2 The effect of sodium dithionite on the activity of human flavokinase and human FAD synthetase and kinetic constants for the natural substrates riboflavin and FMN

Some bacterial bifunctional flavokinases/FAD synthetases previously were found to be more active in the presence of reducing agents (GRILL et al., 2008; KEARNEY et al., 1979; SPENCER et al., 1976). In fact, the *B. subtilis* bifunctional flavokinase/FAD synthetase RibC was reported to be specific for reduced flavins (dihydroriboflavin/$FMNH_2$), although, it could not completely be ruled out that the tertiary structure of the enzyme itself was affected by the reducing agent (KEARNEY et al., 1979). For human flavokinase we found that the addition of sodium dithionite (24 mM) enhanced the activity (V_{max}) by a factor of 1.8 (Table 5 and Fig. 21A and B). The apparent K_M value for riboflavin was 117 µM in the absence, and 36 µM in the presence of sodium dithionite (V_{max} 1,667 U mg^{-1} total protein). The k_{cat}/K_M ratio was 2.6 x 10^{-3} in the absence, and 1.4 x 10^{-2} in the presence of sodium dithionite. These data indicate that the reduced form of riboflavin (dihydroriboflavin), which upon reduction assumes a folded configuration, may have a higher affinity towards the active site of flavokinase, as compared to planar oxidized riboflavin.

Table 5. Kinetic constants for human flavokinase with natural flavin substrate.

Substrate	K_M (µM)	V_{max}* (U mg^{-1})	k_{cat} (s^{-1})	k_{cat}/K_M (μM^{-1} s^{-1})
Riboflavin (without $Na_2S_2O_4$)	117	909	0.3	2.6 10^{-3}
Riboflavin (24 mM $Na_2S_2O_4$)	36	1667	0.5	1.4 10^{-2}

* Specific activities (U mg^{-1}) are in µmoles min^{-1} mg^{-1} protein.

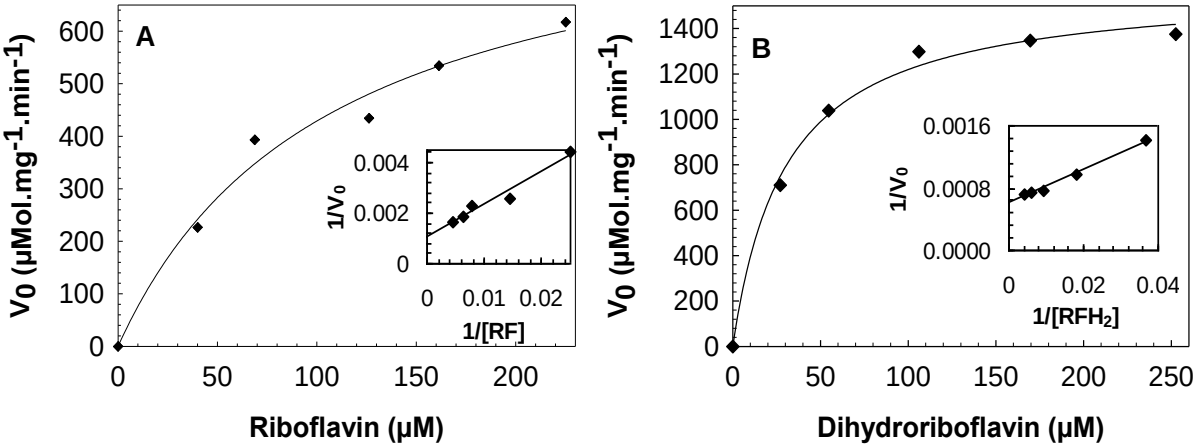

Figure 21. Determination of steady-state kinetic parameters for the phosphorylation of riboflavin (RF) by recombinant human flavokinase in the absence (A) and presence (B) of the reducing agent sodium dithionite. Rates of individual reactions were obtained by incubating varying amounts of riboflavin (0 to 250 µM) with 2.3 µM human flavokinase and 1.0 mM ATP as described in the materials and methods section. The initial rates (V_0) were plotted against the riboflavin concentrations to obtain the data. The insert graphs show the Lineweaver-Burk plots of the data.

Similar results (with respect to the addition of a reducing agent) were obtained for human FAD synthetase (Fig. 22), which apparently was more active (factor of 4.9) in the presence of sodium dithionite (Table 6). Notably, using the latter assay conditions, it was determined an activity V_{max} of 88 U mg^{-1} for this enzyme which was much higher than what has been reported previously (6.8 mU mg^{-1}) (BRIZIO et al., 2006). The apparent K_M value for the adenylylation reaction was 109 mM FMN in the absence, and 68 mM FMN in the presence of sodium dithionite. The latter values were also different to what has previously been reported (K_M for FMN of 1.5 µM) (BRIZIO et al., 2006). The presence or absence of sodium dithionite cannot be responsible for these large discrepancies. Earlier, *E. coli* was used as a host for overproduction of largely insoluble human FAD synthetase (BRIZIO et al., 2006) and the conflicting data can be attributed to this. In comparison with FAD synthetase human flavokinase *in vitro* apparently is more active (about 19-fold) and thus is able to provide sufficient amounts of FMN for the following FAD synthetase reaction. Notably, FAD contributes the majority (about 90%) of total flavins in most tissues (KARTHIKEYAN et al., 2003).

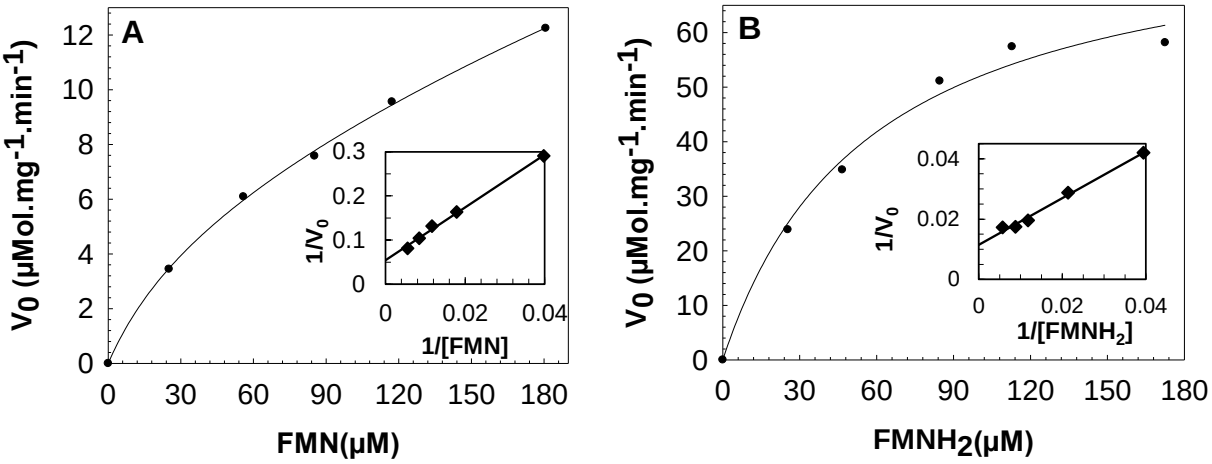

Figure 22. Determination of steady-state kinetic parameters for the adenylylation of flavin mononucleotide (FMN) by recombinant human FAD synthetase in the absence (A) and presence (B) of the reducing agent sodium dithionite. Rates of individual reactions were obtained by incubating varying amounts of riboflavin (0 to 180 µM) with 0.9 mM human FAD synthetase and 1.0 mM ATP as described in the materials and methods section. The initial rates (V_0) were plotted against the FMN concentrations to obtain the data. The insert graphs show the Lineweaver-Burk plots of the data.

Table 6. Kinetic constants for human FAD synthetase with natural flavin substrate

Substrate	K_M	V_{max}^*	k_{cat}	k_{cat}/K_M
	µM	U mg^{-1}	s^{-1}	µM^{-1} s^{-1}
FMN (without $Na_2S_2O_4$)	109	18	0.02	8.3 10^{-4}
FMN (24 mM $Na_2S_2O_4$)	68	88	0.08	1.2 10^{-3}

* Specific activities (U mg^{-1}) are in µmoles min^{-1} mg^{-1} protein.

5.3 Human flavokinase accepts roseoflavin and 8-amino riboflavin as substrates

As a first step towards analyzing the metabolism of flavin analogs in humans, roseoflavin (Fig. 23A) and 8-amino riboflavin (Fig. 23B) were tested as substrates for human flavokinase (see also Fig. 10A and B; 10E and F). Both flavin analogs were good substrates with turnover numbers (k_{cat}) being three times as high when compared to k_{cat} values of (dihydro)riboflavin. The K_M –values for the phosphorylation of the flavin analogs thereby were significantly higher (RoF, 160 mM; AF, 885 µM) when compared to the K_M value for the reaction with (dihydro)riboflavin (36 mM) (Table 7).

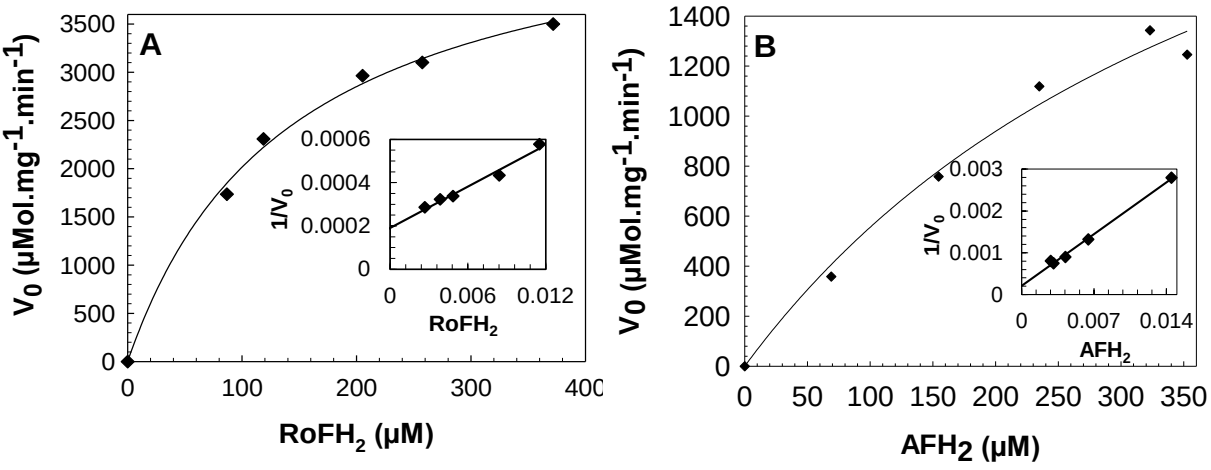

Figure 23. Determination of steady-state kinetic parameters for the phosphorylation of roseoflavin (A) and 8-Aminoriboflavin (B) by recombinant human flavokinase in the presence of the reducing agent sodium dithionite. Rates of individual reactions were obtained by incubating varying amounts of reduced roseoflavin ($RoFH_2$) or reduced 8-amino riboflavin (AFH_2) (0 to 370 µM) with 2.3 µM human flavokinase and 1.0 mM ATP as described in the materials and methods section. The initial rates (V_0) were plotted against the riboflavin concentrations to obtain the data. The insert graphs show the Lineweaver-Burk plots of the data.

Table 7. Kinetic constants for human flavokinase with flavin analogs as substrates.

Substrate	K_M (µM)	V_{max}* (U mg^{-1})	k_{cat} (s^{-1})	k_{cat}/K_M ($\mu M^{-1}\ s^{-1}$)
Roseoflavin (24 mM $Na_2S_2O_4$)	160	5000	1.6	$1.0\ 10^{-2}$
8-Amino riboflavin (24 mM $Na_2S_2O_4$)	885	5000	1.6	$1.8\ 10^{-3}$

* Specific activities (U mg^{-1}) are in µmoles min^{-1} mg^{-1} protein.

First, it can be concluded from the kinetic data that there is apparently enough space in the active site of the enzyme to accommodate the dimethylamino group of roseoflavin. Inspection of the three-dimensional structure of human flavokinase (KARTHIKEYAN et al., 2003) indeed suggests that roseoflavin could fit into the active site without strongly disturbing the overall structure of the enzyme (Fig. 24A). The same should in principle be true for 8-amino riboflavin (Fig. 24B). However, for the latter substrate a significantly higher K_M value was found suggesting that substrate binding is less efficient. AF contains a hydrophilic amino

group at C8 and a hydrophobic methyl group at C7 (C7a) (Fig. 7). In contrast, the isoalloxazine ring of the natural substrate riboflavin contains two hydrophobic methyl groups (C8a and C7a) (‘‘dimethylbenzene portion’’). The structure of human flavokinase revealed that this hydrophobic dimethylbenzene portion of riboflavin resides at the bottom of a pocket surrounded by the hydrophobic residues I53, V69, F116, L122 and I126 (KARTHIKEYAN et al., 2003). The hydrophilic C8 amino group of AF does not allow hydrophobic interactions (Fig. 24B) and thus could be the reason for the higher K_M value of 8-amino riboflavin. Roseoflavin (K_M = 160 µM) clearly is a better substrate as compared to 8-amino riboflavin (K_M = 885 µM). An explanation for this may be that the more hydrophobic dimethylamino group at C8 of roseoflavin allows interaction with the hydrophobic environment I53, V69, F116, L122 and I126 of human flavokinase (Fig. 24A). In contrast to riboflavin, the flavin analogs roseoflavin and 8-amino riboflavin may become protonated at the C8 amino group during the enzymatic reaction. Protonation in turn may cause an electrostatic repulsion enhancing release of the reaction products. The latter could explain why the apparent k_{cat} values of roseoflavin and 8-amino riboflavin are significantly higher as compared to the k_{cat} value of the natural substrate riboflavin.

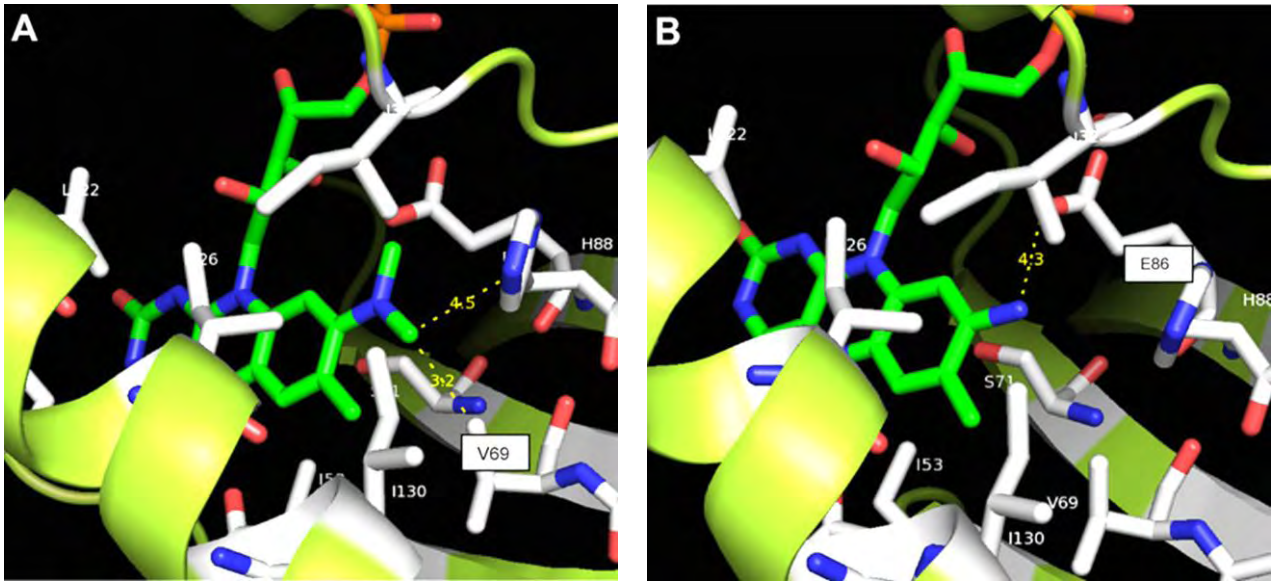

Figure 24. (A) The FMN-binding site of human flavokinase is shown whereby FMN was *in silico* replaced by roseoflavin-5’-phosphate (RoFMN). RoFMN was fitted using PyMol, the distances are in Å. One of the methyl groups of RoFMN apparently is close to the hydrophobic amino acid residue V69. (B) The FMN-binding site of human flavokinase is shown whereby FMN was in silico replaced by (8-demethyl)-8-amino-riboflavin 5’-phosphate (AFMN). AFMN was fitted using PyMol, the distances are in Å. The hydrophilic amino

group at C8 of AFMN may not be able to interact with the hydrophobic pocket of the enzyme (I53, V69, F116, L122 and I126) which naturally accommodates the dimethylbenzene portion of FMN. The hydrophilic amino group at C8 of AFMN also may be repulsed by E86. It is important to note that the crystal which originally was used for solving the structure contained FMN (not RoFMN or AFMN) (KARTHIKEYAN et al., 2003).

If flavin analogs in the future should be used to treat infectious diseases they will be present in the cell in addition to the natural flavins RF, FMN and FAD. In order to study the influence of different flavin species on the activity of human flavokinase the enzyme was tested in the presence of both riboflavin and roseoflavin at the same time. The addition of roseoflavin apparently reduced the activity of human flavokinase, an inhibition constant of 109 µM (± 29) was determined (Fig. 25A). The observed inhibition effect was probably caused by competition between the two substrates for the enzyme active site since roseoflavin was also phosphorylated in the reaction. Interestingly, 8-amino riboflavin did not affect human flavokinase activity even at a maximum concentration of AF (200 µM) just below its solubility limit (Fig. 25B). 8-amino riboflavin was never converted to AFMN in the presence of riboflavin, but after all riboflavin had been converted to FMN, AFMN could then be detected. The human flavokinase has affinity one order of magnitude poorer for 8-amino riboflavin than for riboflavin (both in the reduced form), what possibly explains the clear preference for riboflavin over 8-amino riboflavin in the mixture.

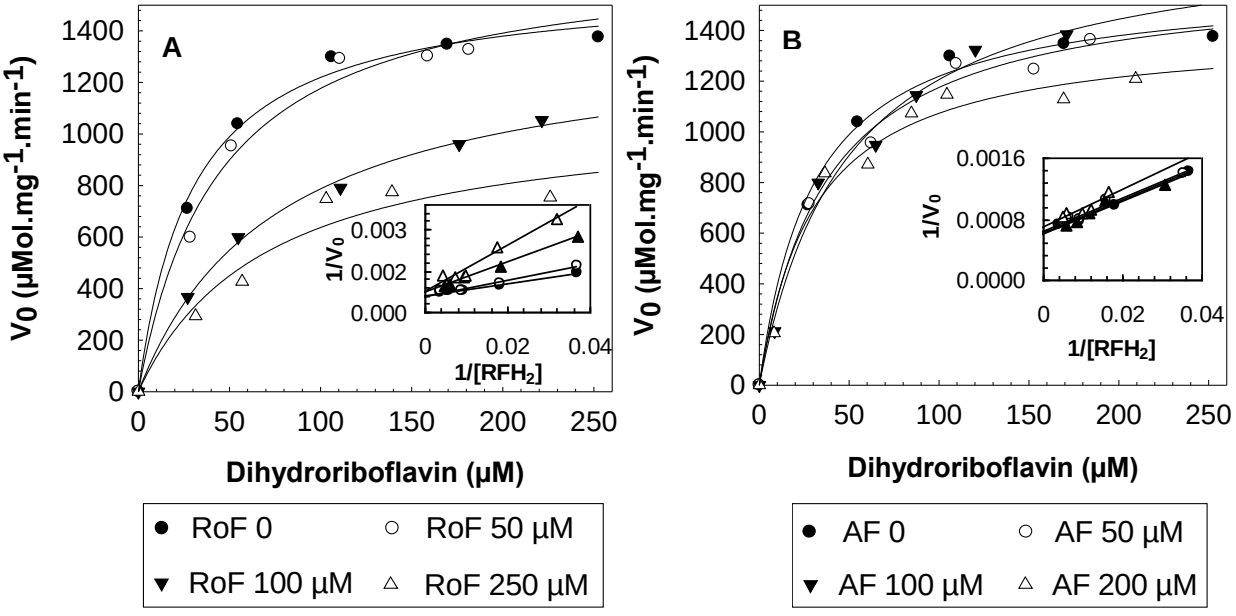

Figure 25. Inhibition of human flavokinase by the toxic riboflavin analogs roseoflavin (A) and 8-demethyl-8-aminoriboflavin (B). (A) The upper curve (solid circles) shows the

saturation of human flavokinase with increasing concentrations of riboflavin. Upon addition of 50 µM roseoflavin the initial activity of the enzyme was slightly reduced (open circles). Addition of 100 µM (solid triangles) and 200 µM roseoflavin (open triangles) strongly reduced enzyme activity. The insert graphs show the Lineweaver-Burk plot of the data for determining the inhibition constant K_i. (B) Human flavokinase was assayed as in (A) in the presence of 8-demethyl-8-aminoriboflavin, however, no inhibition of the enzyme was observed.

5.4 Human FAD synthetase accepts RoFMN but not AFMN as a substrate

The following experiments were carried out in order to study the metabolism of RoFMN and AFMN, which, according to the results reported above, were synthesized *in vitro* by human flavokinase. The latter enzyme was used to produce the cofactor analogs RoFMN and AFMN, which were not commercially available (Fig. 10A and B; 10E and F). RoFMN was a good substrate (K_M 116 µM; V_{max} 77 U mg^{-1}, k_{cat} 0.08) for human FAD synthetase when compared to the natural substrate FMN (K_M 68 µM; V_{max} 88 U mg^{-1}, k_{cat} 0.07) (Fig. 26; Table 8). The structure of human FAD synthetase is not yet available, however, the structure of the corresponding enzyme from yeast has been solved (LEULLIOT et al., 2010). This work revealed that the dimethylbenzene portion of FMN fills a hydrophobic pocket upon binding to the enzyme. Assuming a similar structure for the FMN binding site in the human enzyme, and, in light of our kinetic data, we conclude that RoFMN with its relatively hydrophobic dimethylamino group at C8 effectively binds to the protein. This seems not to be the case for AFMN (for reasons described for apparent reduced binding of 8-amino riboflavin to human flavokinase) since no activity was measured employing this substrate under the standard assay conditions (Fig. 10G and H).

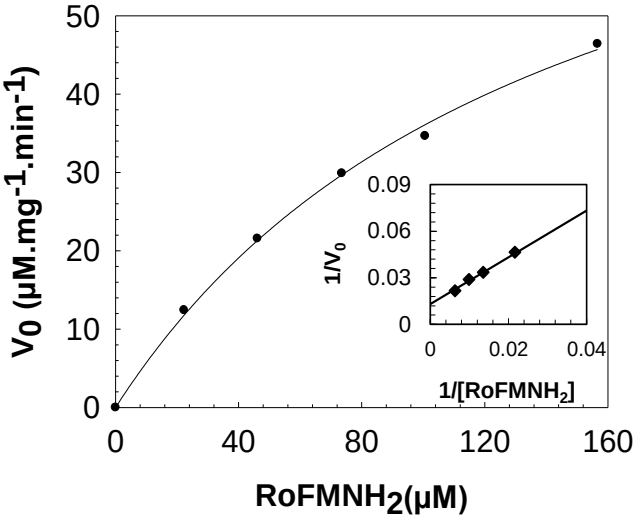

Figure 26. Determination of steady-state kinetic parameters for the adenylylation of Roseoflavin mononucleotide in the presence of the reducing agent sodium dithionite ($RoFMNH_2$) by recombinant human FAD synthetase. Rates of individual reactions were obtained by incubating varying amounts of riboflavin (0 to 180 μM) with 0.9 mM enzyme and 1.0 mM ATP as described in the materials and methods section. The initial rates (V_0) were plotted against the RoFMN concentrations to obtain the data. The insert graphs show the Lineweaver-Burk plots of the data.

Table 8. Kinetic constants for human FAD synthetase with flavin analogs as substrate

Substrate	K_M	V_{max}*	k_{cat}	k_{cat}/K_M
	μM	U mg^{-1}	s^{-1}	$\mu M^{-1}\ s^{-1}$
RoFMN (24 mM $Na_2S_2O_4$)	116	77	0.07	$6.0\ 10^{-4}$
AFMN (24 mM $Na_2S_2O_4$)	–	0	–	–

* Specific activities (U mg^{-1}) are in μmoles min^{-1} mg^{-1} protein.

Human FAD synthetase was also tested in the presence of both flavin substrates, FMN and RoFMN (Fig. 27). The presence of RoFMN, however, did not negatively affect the adenylylation of FMN carried out by human FAD synthetase. As described in the case of the flavokinase preference for riboflavin over 8-amino riboflavin, the human FAD synthetase also do not convert RoFMN into RoFAD until all the FMN added to the reaction is completely converted to FAD. Additionally, the later enzyme has affinity one order of magnitude poorer for RoFMN than for FMN (both in the reduced form). Thus, RoFMN would possibly not

affect the human FADS activity *in vivo* if the individual is continuously supplied with riboflavin. The same could be true for the human flavokinase in case of treatment with 8-amino riboflavin.

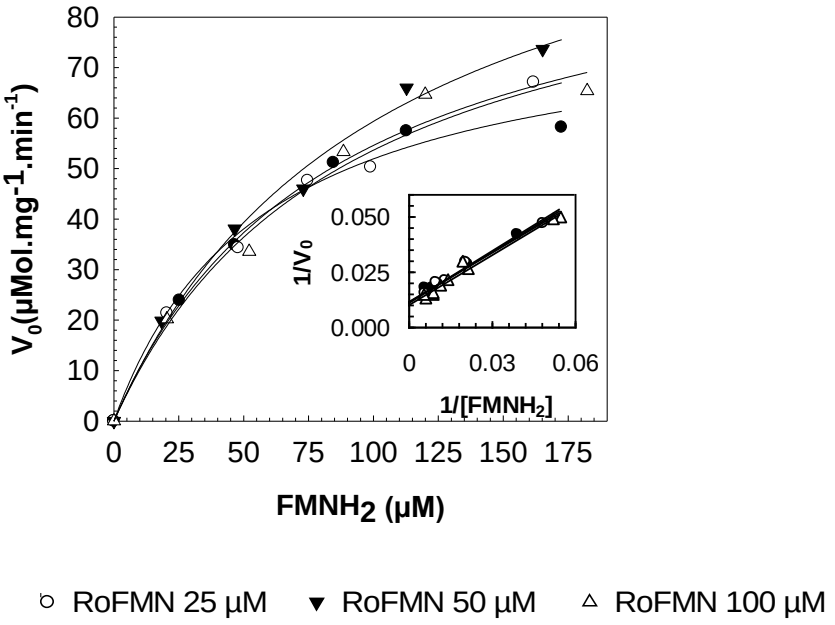

Figure 27. Inhibition of human FAD synthetase by the phosphorylated form of roseoflavin, roseoflavin-5'-phosphate (RoFMN). The curves show the saturation of FAD synthetase with increasing concentrations of flavin mononucleotide, FMN. Upon addition of RoFMN (25 µM, open circles; 50 µM, solid triangles; 100 µM, open triangles) the initial activity of the enzyme did not change, and, apparently, no inhibition of the enzyme occurred.

5.5 A hepatocyte cell-free extract catalyzes the formation of FMN, RoFMN and AFMN

Cell-free extracts from freshly grown human hepatocytes were tested with respect to flavokinase and FAD synthetase activity using the substrates riboflavin, 8-amino riboflavin and roseoflavin and the corresponding 5´-phosphates FMN, RoFMN and AFMN (100 µM each), respectively. The flavokinase reaction, i.e. the 5´-phosphorylation of riboflavin to FMN (0.4 µMol min^{-1} mg^{-1} total protein), of roseoflavin to RoFMN (0.7 µMol min^{-1} mg^{-1} total protein) and of 8-amino riboflavin to AFMN (1.1 µMol min^{-1} mg^{-1} total protein) could be measured. The latter data suggest that synthesis of the flavin cofactor analogs RoFMN and AFMN also occurs *in vivo*. Furthermore, roseoflavin and 8-amino riboflavin seem to even be better substrates as compared to riboflavin, a finding, which supports our data generated using

recombinant human flavokinase. FAD synthetase activity was not detected in hepatocyte cell-free extracts. This was not surprising since the data using recombinant enzymes revealed that FAD synthetase activity was much lower (at least 19-fold) as compared to flavokinase activity.

Human hepatocytes were also grown in a medium supplemented with riboflavin, roseoflavin or 8-amino riboflavin (70 µM each). From each culture, 2 x 10^7 cells were collected and cell-free extracts were prepared and analyzed by LC-MS. FAD (1.7 nMol), FMN (2.5 nMol) and trace amounts of riboflavin were found in cells treated with RF. Also RoFAD (1.5 nMol), RoFMN (0.5 nMol) and trace amounts of roseoflavin were found in cells treated with roseoflavin. No flavin analog could be detected in cytoplasm extracts after treatment with 8-amino riboflavin. The results show that human hepatocytes are able to import riboflavin and also roseoflavin. Moreover, the human flavokinase and the human FAD synthetase apparently are able to *in vivo* activate both flavins. Notably, human hepatocytes seem to be unable to import 8-amino riboflavin.

6. Discussion

In mammals, dietary riboflavin is actively absorbed by intestinal cells *via* specific plasma membrane transporter(s) (YAO et al., 2010; YONEZAWA et al., 2008). For bacterial riboflavin transporters, roseoflavin was found to be a good substrate (VOGL et al., 2007), apparently human riboflavin transporters accept roseoflavin as substrate as well. According to the present *in vitro* studies using human flavokinase and FAD synthetase the flavin analogs roseoflavin and 8-amino riboflavin are efficiently converted to the cofactor analogs RoFMN, AFMN and RoFAD. The relatively high K_M value for the phosphorylation of 8-amino riboflavin (885 mM) and the fact that AFMN was not adenylylated at all suggest that AF has a lower toxic potential as compared to roseoflavin. Since most flavoenzymes within the cell use FAD as a cofactor, 8-amino riboflavin, having a good antibacterial potential, is probably a better lead structure for the development of novel antiinfectives based on flavin analogs. It cannot be ruled out, however, that flavin analogs and/or their degradation products negatively interfere with human metabolism.

The synthesis of RoFMN and AFMN by human hepatocyte cell-free extracts supports the data which were generated *in vitro* using recombinant enzymes and strongly suggests that cofactor analogs are also generated *in vivo*. It can only be speculated on the molecular activity of the potentially toxic flavin cofactor analogs RoFMN, AFMN and RoFAD in human cells. It was reported that flavin analogs may be good steric replacements for riboflavin but not catalytic substitutes (HASFORD & RIZZO, 1998). Accordingly, RoFMN, AFMN and RoFAD may combine with FMN- or FAD-dependent flavoenzymes, reduce their activity and negatively affect human metabolism (GRILL et al., 2008). Furthermore, it was reported that protonation of flavin analogs which carry an amino group at C8 have a strongly altered redox potential, e.g. for protonated roseoflavin an E_0` of +190 mV was published (HASFORD & RIZZO, 1998). It remains to be elucidated whether the different physicochemical properties of RoFMN, AFMN and RoFAD are indeed relevant for human physiology.

7. Conclusion

The absence of similar structures in mammals turns riboswitches interesting targets for new antiinfective drugs. Besides, FMN riboswitches are even more interesting targets since in bacteria they control the biosynthesis and/or uptake of the essential vitamin riboflavin.

For 38 years (since its first description OTANI et al., 1974) the roseoflavin mechanism of action remained obscure. Since roseoflavin also reduces growth of protozoa and animals (OTANI et al., 1997) not employing FMN riboswitches, the observed activity of RoF/RoFMN apparently is not the only explanation for its toxicity. A few tested flavoenzymes were found to be less active when loaded with roseoflavin cofactors. RoFMN and RoFAD very likely are inactive cofactors and lead to decreased activity of flavoenzymes.

The number of genes encoding flavin-dependent proteins varies greatly in the genomes analyzed so far, and covers a range from approximately 0.1% to 3.5% of the predicted genes. For *S. coelicolor* 2.7% of all enzymes appear to be flavoenzymes (MACHEROUX et al., 2011). A similar amount is expected in *S. davawensis*, which of course brings up the question as to why *S. davawensis* can actively grow in the presence of its

own antibiotic. Now, the data described in chapter I clearly show that the main cellular target for RoFMN are FMN riboswitches. At the same time, the results also show that roseoflavin resistance in *S. davawensis* is mediated by a specialized FMN riboswitch which does not turn off gene expression upon RoFMN binding. Rather, the *ribB* FMN riboswitch from *S. davawensis* increases the expression rate in the presence of RoFMN. Most probably by increasing the expression of riboflavin biosynthetic genes, *S. davawensis* overcomes the problem with inactive cofactors by competition of FMN with RoFMN and FAD with RoFAD for the flavoenzymes, and, at the same time, meets its need for riboflavin, the direct precursor for roseoflavin synthesis (JANKOWITSCH et al., 2011).

Notably, roseoflavin sensitive cells contain multiple targets for roseoflavin, FMN riboswitches and also flavoenzymes (MACHEROUX et al., 2011), which makes flavin analogs even more attractive as basic structures for developing novel antibacterial compounds. The current knowledge with respect to roseoflavin activity and resistance is summarized in the figure 28.

The compound 8-amino riboflavin seems to be an interesting lead structure for the development of novel antiinfectives, i.e. it is potentially less toxic to human cells (as compared to roseoflavin) and its phosphorylated form (AFMN) targets FMN riboswitches as well as does RoFMN.

Despite of its apparent ancient origin, riboswitches were only described 20 years ago, and have been intensively studied in the last ten years. Still, its mechanism of action is not completely understood. It was predicted by Breaker (2010) that a kinetically driven riboswitch could be tuned to respond to a different concentration of metabolite by accruing mutations in the aptamer that would change the rate constant for ligand association. Now, the present work shows that kinetically driven riboswitches can also be tuned to respond to different metabolite analogs.

This work on roseoflavin underscores the potential generality of targeting riboswitches with new antibacterial drugs. Roseoflavin is a unique chemical being the only known natural compound negatively affecting riboswitches. Since riboswitches are widespread in bacteria one can expect that a large number of highly interesting natural antiinfectives synthesized by yet unknown organisms remain to be discovered.

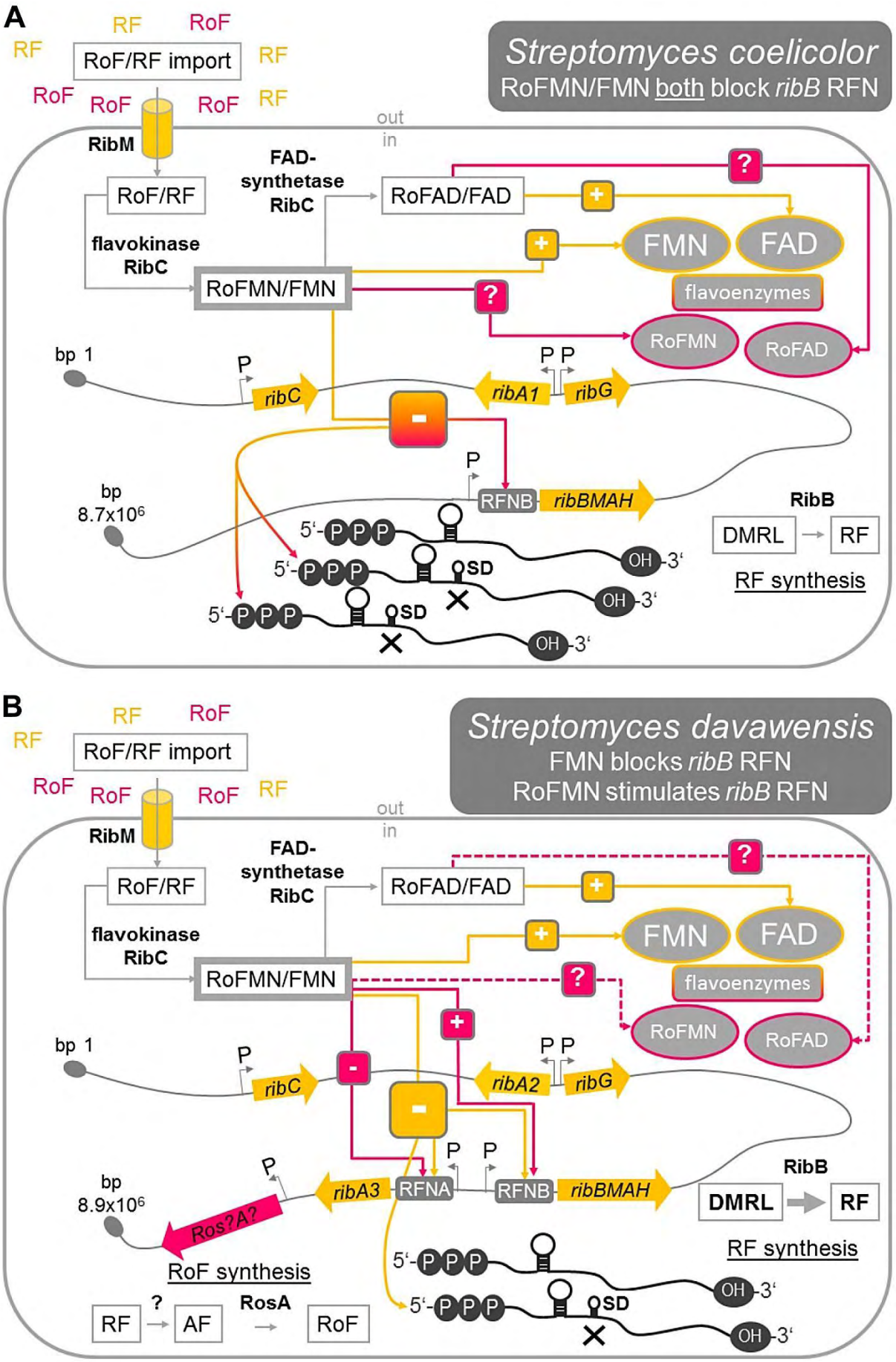
A
Streptomyces coelicolor
RoFMN/FMN both block ribB RFN
RF
RoF
RoF/RF import
RibM
out
in
RoF/RF
FAD-synthetase RibC
RoFAD/FAD
flavokinase RibC
RoFMN/FMN
FMN
FAD
flavoenzymes
RoFMN
RoFAD
bp 1
P
ribC
ribA1
ribG
RFNB
ribBMAH
bp 8.7x10⁶
5'-PPP
OH-3'
SD
RibB
DMRL
RF
RF synthesis
B
Streptomyces davawensis
FMN blocks ribB RFN
RoFMN stimulates ribB RFN
ribA2
ribA3
RFNA
Ros?A?
RoF synthesis
RosA
AF
bp 8.9x10⁶

Figure 28. Molecular responses of *Streptomyces coelicolor* (A) and *Streptomyces davawensis* (B) to riboflavin (RF) and roseoflavin (RoF). The scheme shows the probable mode of action of the riboflavin (RF) analog roseoflavin (RoF), which is naturally synthesized by *Streptomyces davawensis*. (A) Schematic drawing of a RoF-sensitive *Streptomyces coelicolor* cell. Uptake of flavins (RF and RoF) is catalyzed by RibM encoded by *ribM* of the *ribBMAH* cluster. Expression of *ribBMAH* is controlled by the *rib*-promoter P (see arrow) in combination with a *ribB* FMN riboswitch (RFNB) (translational control *via* sequestration of the ribosomal binding site, SD). RF and RoF are both substrates for RibC, a bifunctional flavokinase/FAD-synthetase (encoded by *ribC*), which produces the cofactors FMN/FAD and the cofactor analogs RoFMN/RoFAD within the cytoplasm. The latter flavin derivatives combine with flavoenzymes. RoFMN and RoFAD are less active as cofactors and their incorporation probably produces flavoenzymes with reduced activity (pink box). Possibly, *S. davawensis* contains flavoenzymes which are active in the presence of RoFMN/RoFAD. RFNB is a target for FMN and also RoFMN (yellow/pink box). Upon binding of the FMN/RoFMN ligand the 5'-untranslated region of the corresponding mRNA is not accessible for the ribosomes (SD, ribosomal binding site). This leads to reduced synthesis of the Rib enzymes. Exemplarily, riboflavin synthase encoded by *ribB* of the *ribBMAH* cluster is shown which is responsible for the last step in RF (bio)synthesis, the conversion of dimethylribityllumazine (DMRL) to RF. The genes *ribA* and *ribH* encode the bifunctional GTP-cyclohydrolase II/3,4 dihydroxybutanone-4-phosphate synthase and the ribityllumazine synthase, respectively. The missing genes for the bifunctional deaminase/reductase (RibG) of the riboflavin pathway is located at a different site within the 8.7 Mbp *S. coelicolor* linear chromosome (the telomeric ends are depicted as black circles; the *S. coelicolor* plasmid SCP is not shown) and are not controlled by FMN riboswitches. An additional gene (*ribA1*) encoding a second GTP-cyclohydrolase II is present. (B) Schematic drawing of a RoF-resistant *Streptomyces davawensis* cell. As in (A), however, the genome of *S. davawensis* contains a second FMN riboswitch (RFNA) controlling the synthesis of an additional 3,4 dihydroxybutanone-4-phosphate synthase (RibA3) with unknown function. RFNA is sensitive to RoFMN and FMN (yellow box, -; pink box, -), RFNB is only sensitive to FMN. Interestingly, RFNB is stimulated by RoFMN (pink box, +). The putative RoF biosynthetic gene cluster *ros?A?* is shown. Only the function of RosA has been confirmed, it catalyzes the methylation of 8-demethyl-8-amino-riboflavin to RoF. The *ros* genes are not controlled by a riboswitch. *S. davawensis* also contains a plasmid (SDP, not shown).

Scientific publications

The present study has generated two research articles:

Pedrolli, D. B.; Nakanishi, S.; Barile, M.; Mansurova, M.; Carmona, E. C.; Lux, A.; Gärtner, W.; Mack, M. The antibiotics roseoflavin and 8-demethyl-8-amino-riboflavin from *Streptomyces davawensis* are metabolized by human flavokinase and human FAD synthetase. *Biochemical Pharmacology*, v. 82, n.12, p.1853–1859, 2011.

Pedrolli, D.B.; Matern, A.; Wang, J.; Ester, M.; Siedler, K.; Breaker, R.R.; Mack M. A highly specialized FMN riboswitch responds very differently to similar ligands and confers roseoflavin resistance to *Streptomyces davawensis*. 2012.

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