APPENDIX

Uptake, metabolism and mode of action of the antibiotics roseoflavin and 8-demethyl-8-aminoriboflavin in *Listeria monocytogenes*

Running title: Flavin analogs as antibiotics

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References of the appendix

1. **Mansjo M, Johansson J.** 2011. The riboflavin analog roseoflavin targets an FMN-riboswitch and blocks *Listeria monocytogenes* growth, but also stimulates virulence gene-expression and infection. RNA Biol **8:**674-680.
2. **Serrano A, Frago S, Velazquez-Campoy A, Medina M.** 2012. Role of key residues at the flavin mononucleotide (FMN):adenylyltransferase catalytic site of the bifunctional riboflavin kinase/flavin adenine dinucleotide (FAD) Synthetase from *Corynebacterium ammoniagenes*. Int J Mol Sci **13:**14492-14517.
3. **Serrano A, Frago S, Herguedas B, Martinez-Julvez M, Velazquez-Campoy A, Medina M.** 2013. Key residues at the riboflavin kinase catalytic site of the bifunctional riboflavin kinase/FMN adenylyltransferase from *Corynebacterium ammoniagenes*. Cell Biochem Biophys **65:**57-68.
4. **Frago S, Martinez-Julvez M, Serrano A, Medina M.** 2008. Structural analysis of FAD synthetase from *Corynebacterium ammoniagenes*. BMC Microbiol **8:**160.

Figure legends of the appendix

Supplementary Fig. S1: Phosphorylation and adenylylation of riboflavin, roseoflavin and 8-demethyl-8-aminoriboflavin. The enzymatic conversion of riboflavin (top) to flavin mononucleotide (FMN) and flavin adenine dinucleotide (FAD), of roseoflavin (middle) to roseoflavin mononucleotide (RoFMN) and roseoflavin adenine dinucleotide (RoFAD) and of 8-demethyl-8-aminoriboflavin (bottom) to 8-demethyl-8-aminoriboflavin mononucleotide (AFMN) and 8-demethyl-8-aminoriboflavin adenine dinucleotide (AFAD). The reactions are catalyzed by flavokinases (2.7.1.26) and/or FAD synthetases (2.7.7.2.).

Supplementary Fig. S2: Sequence, proposed secondary structure i.e. expected transcriptional intermediate of the *Listeria monocytogenes* FMN riboswitch *Rli96* in the presence of FMN. *Rli96* controls synthesis of the *lmo1945* mRNA encoding the membrane-embedded riboflavin-binding subunit Lmo1945 (EcfS or RibU) of the energy-coupling factor (ECF) riboflavin uptake system. In the presence of FMN (FMN binds to the aptamer formed by P1-P6) a transcriptional terminator forms which prevents formation of the full-length *lmo1945* mRNA (1). The highlighted nucleotides (blue) are predicted to pair in the absence of FMN forming an anti-terminator loop, enabling transcription to proceed. The replacement of nucleotides G37/G38 by A37/A37 (highlighted in grey) generated an FMN riboswitch variant which did not cause transcription termination in the presence of FMN or RoFMN (1).

Supplementary Fig. S3: Lmo1945 facilitates riboflavin (RF) uptake. RF auxotrophic *Bacillus subtilis* Δ*ribU*::Kan^r Δ*ribB*::Erm^r cells expressing *lmo1945* from plasmid pHT01-*lmo1945* was grown in a minimal medium in the presence of indicated amounts of RF and IPTG (to stimulate expression of *lmo1945*) (left panel).

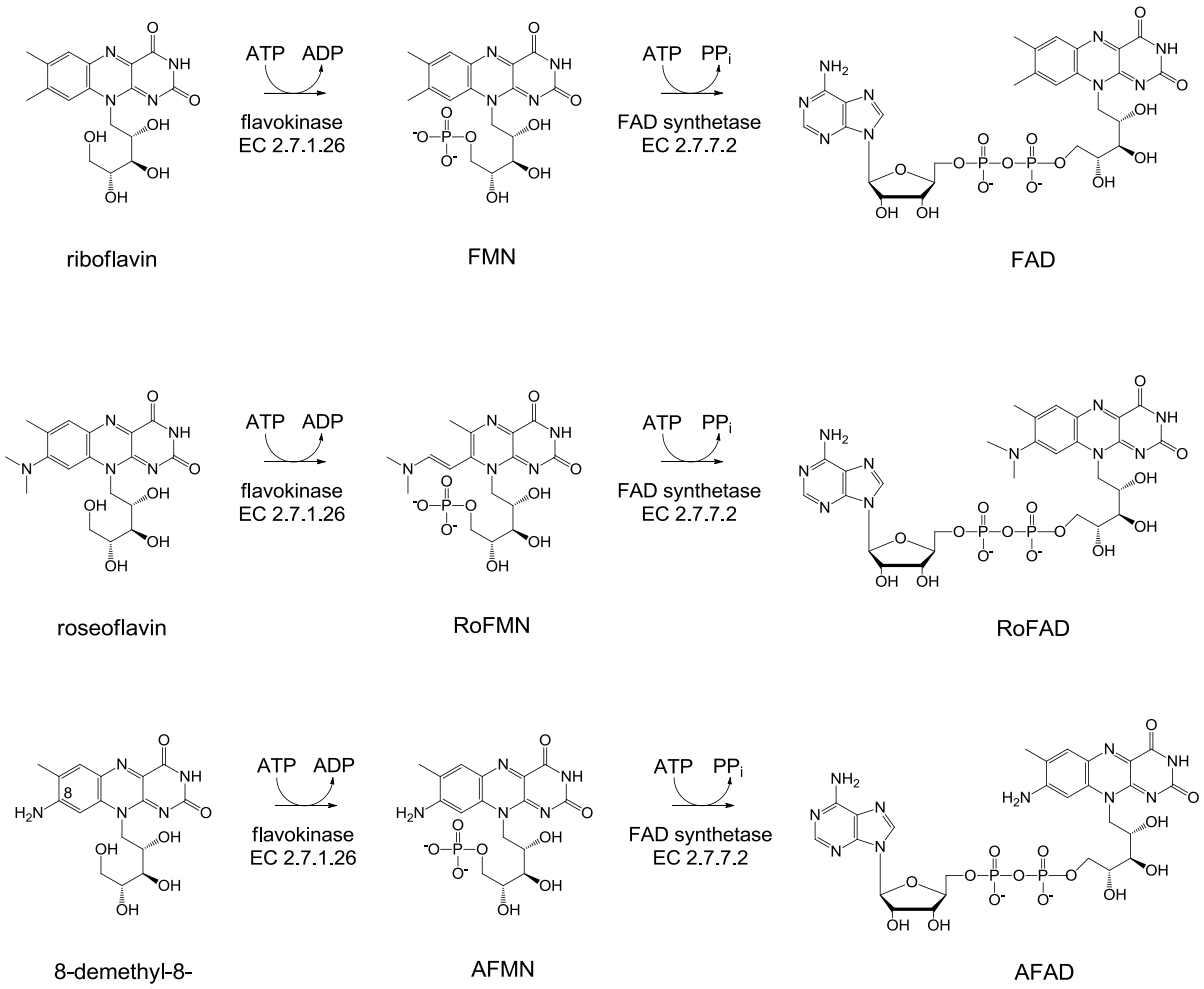
Growth was recorded at λ=600 nm. As a control, strains were transformed with the empty expression vector pHT01 (right panel). At 10 µM only the *lmo1945* expressing strain could grow. At 100 µM RF also the control strain could grow indicating that RF is able to cross the cytoplasmic membrane in the absence of a flavin transporter.

Supplementary Fig. S4: Lmo1945 facilitates roseoflavin (RoF) uptake. Riboflavin (RF) prototrophic *Bacillus subtilis* Δ*ribU*::Kan^r cells expressing *lmo1945* from plasmid pHT01-*lmo1945* were grown in a minimal medium in the presence of indicated amounts of RF and IPTG (to stimulate expression of *lmo1945*) (left panel). Growth was recorded at λ=600 nm. As a control, strains were transformed with the empty expression vector pHT01 (right panel). At 100 µM RoF growth of the *lmo1945* expressing strain was strongly reduced whereas the control strain grew to a higher cell density. At 50 µM RoF a similar effect was observed. Together these findings suggested that Lmo1945 was responsible for RoF uptake.

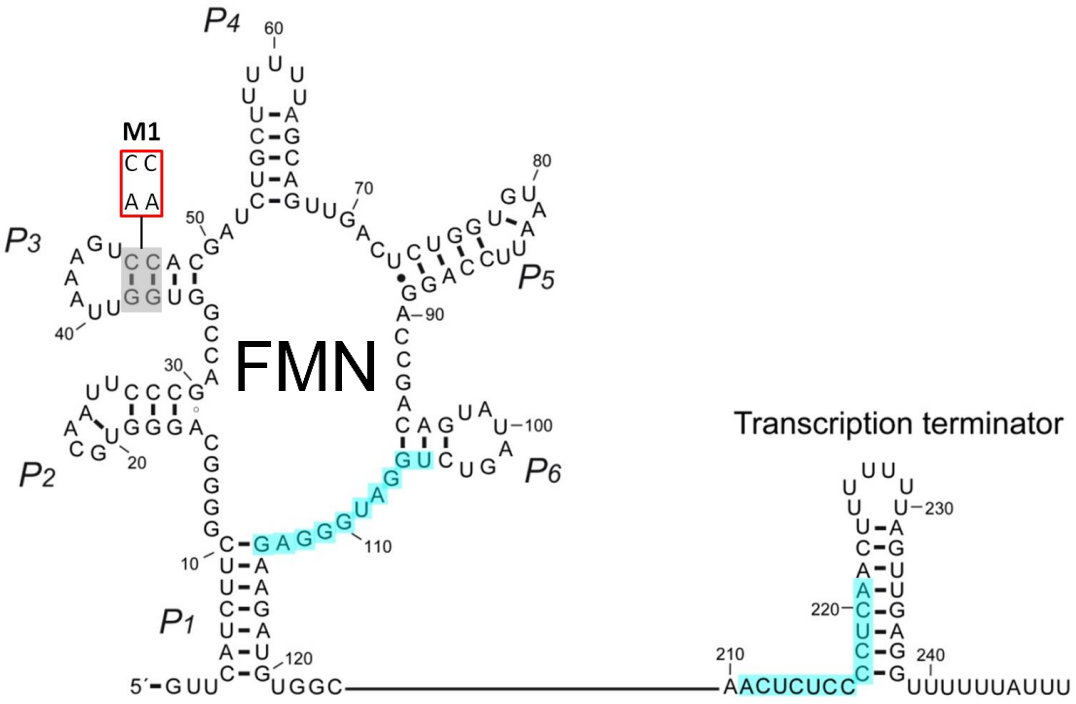
Supplementary Fig. S5: Multiple sequence alignment of representative primary structures of bifunctional bacterial flavokinases/FAD-synthetases. UniProtKB accession numbers for proteins are as follows: Cam_RibF, bifunctional flavokinase/FAD synthetase from *Cornyebacterium ammoniagenes*, Q59263; Eco_RibF, bifunctional flavokinase/FAD synthetase from *Escherichia coli*, P0AG40; Bsu_RibC, bifunctional flavokinase/FAD synthetase from *Bacillus subtilis*, P54575; Sco_RibC, bifunctional flavokinase/FAD synthetase from *Streptomyces coelicolor*, Q9Z530; Sdav_RibC1, bifunctional flavokinase/FAD synthetase from *Streptomyces davawensis*, K4R340; Lmo_RibC, bifunctional flavokinase/FAD synthetase from *Listeria monocytogenes*, Q8Y7F2; Lmo_0728, monofunctional FAD synthetase from *L. monocytogenes*, Q8Y914. The amino acids highlighted in blue and green were

reported to be relevant for the flavokinase function, whereas amino acids highlighted in red were reported to be relevant for the FAD synthetase function (2-4). (2-4). Conserved domains are boxed.

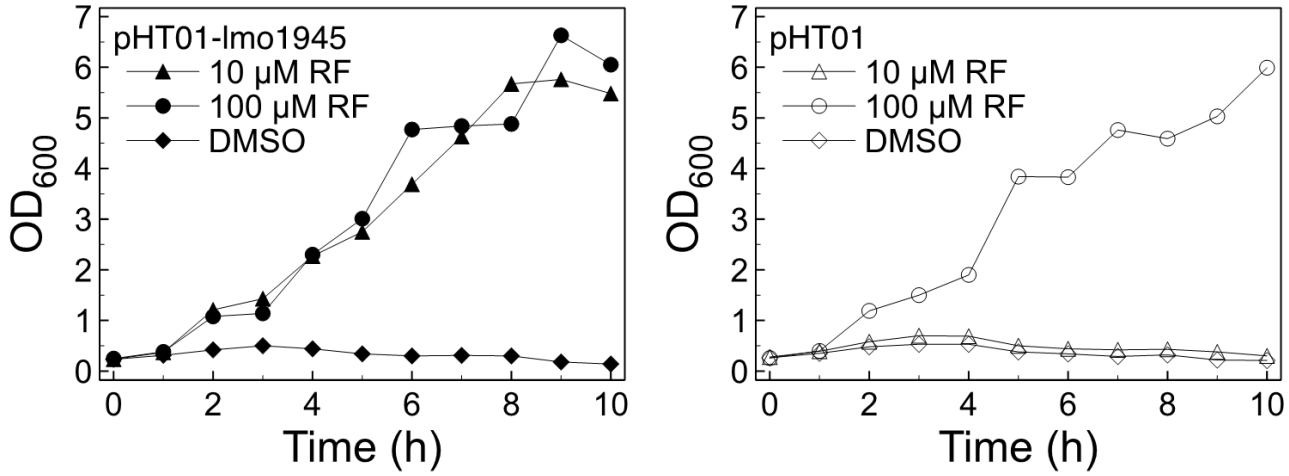
Supplementary Figure S1:



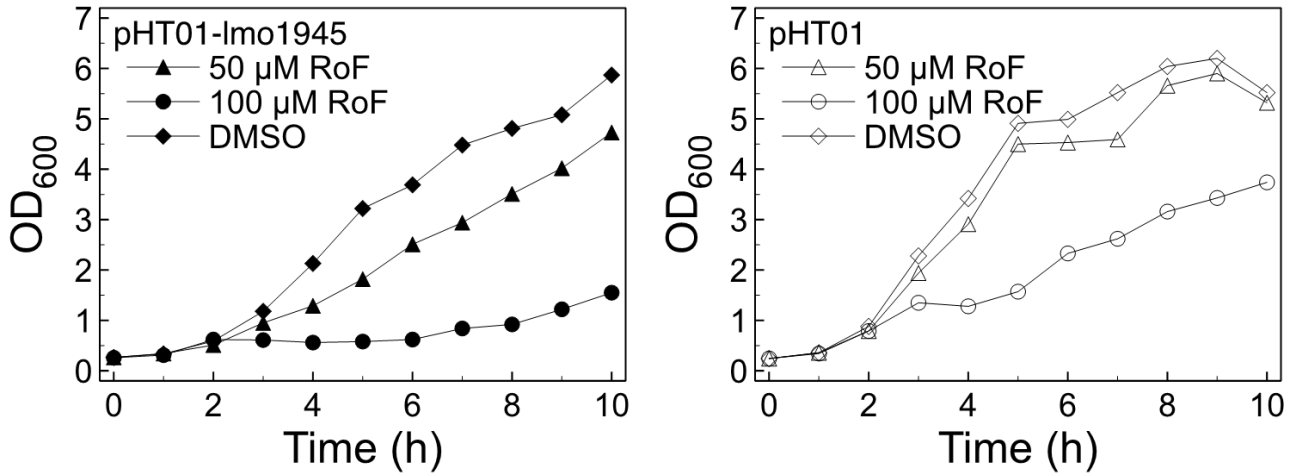
Supplementary Figure S2:



Supplementary Figure S3:



Supplementary Figure S4:



Supplementary Figure S5: