A dual control mechanism synchronizes riboflavin and sulphur metabolism in *Bacillus subtilis*

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Flavin mononucleotide (FMN) riboswitches are genetic elements, which in many bacteria control genes responsible for biosynthesis and/or transport of riboflavin (rib genes). Cytoplasmic riboflavin is rapidly and almost completely converted to FMN by flavokinases. When cytoplasmic levels of FMN are sufficient (“high levels”), FMN binding to FMN riboswitches leads to an increase of rib gene expression. We report here that the protein RibR counteracts the FMN-induced “turn-off” activities of both FMN riboswitches in *Bacillus subtilis*, allowing rib gene expression even in the presence of high levels of FMN. The reason for this secondary metabolic control by RibR is to couple sulfur metabolism with riboflavin metabolism.

RibR | FMN riboswitch | *Bacillus subtilis* | flavin mononucleotide | riboflavin

In the cytoplasm of *Bacillus subtilis* (as in other bacteria), riboflavin (vitamin B₂) is synthesized in a series of enzymatic reactions starting from GTP and ribulose-5-phosphate (1) (see pathway and enzymes in SI Appendix, Fig. S1). The bifunctional flavokinase/flavin adenine dinucleotide (FAD)-synthetase RibFC is responsible for the rapid and almost quantitative conversion of cytoplasmic riboflavin into its biologically active derivatives flavin mononucleotide (FMN) and FAD (2). FMN and FAD are cofactors of flavoenzymes, which carry out a large variety of different biochemical reactions (3). Notably, 1.4% of the *B. subtilis* proteins (58 out of 4,245) depend on either FMN or FAD (4), and it is the cytoplasmic FMN level that represents the measurand for the flavin gene regulatory system. The riboflavin biosynthetic genes ribDG, ribE, ribAB, ribH, and ribT of *B. subtilis* form a transcription unit (see SI Appendix, Fig. S2A). The 5’-untranslated region of the corresponding mRNA contains an FMN riboswitch (“ribDG FMN riboswitch”) that regulates expression of the gene cluster ribDG, ribE, ribAB, ribH, and ribT (5, 6). The ribDG FMN riboswitch consists of an FMN-responsive aptamer and an overlapping expression platform. FMN controls riboflavin biosynthesis in *B. subtilis* by binding to the aptamer portion of the ribDG FMN riboswitch. This leads to the formation of an intrinsic transcription terminator within the expression platform when FMN levels are high but to an alternative structure (allowing transcription of the downstream genes) when FMN levels are low. As a consequence of the latter, riboflavin is synthesized. A second FMN riboswitch (“ribU FMN riboswitch”) controls translation of the monocistronic ribU mRNA of the riboflavin importer RibU (see SI Appendix, Fig. S2B) (6, 7). The ribU FMN riboswitch operates by forming an intrinsic ribosomal binding site sequestrator when FMN levels are high but adopts an alternative structure when FMN levels are low (allowing translation of the ribU mRNA). As a consequence of the latter, riboflavin is transported into the cell.

*B. subtilis* RibR was described as a flavokinase with unknown cellular function (8). The gene ribR is part of a transcription unit comprising 12 genes (snaA, tcyJ, tcyK, tcyL, tcyM, tcyN, cmoO, cmoI, cmoJ, ribR, snaA, and ytmM) (Fig. L4) (9). The gene products of this operon (except for RibR) are involved in the uptake and degradation of sulfur compounds, and expression of these genes was found to strongly be enhanced in the presence of methionine or taurine (2-aminoethanesulfonic acid) (9–11). Using a yeast three-hybrid system, it was shown that RibR specifically interacts with the *B. subtilis* ribDG FMN riboswitch in vivo (12). This interaction could be located to the carboxy-terminus part of the protein, whereas the flavokinase activity of RibR tentatively was assigned to the N-terminal part of RibR (N-RibR). We now show that RibR regulates the activity of both *B. subtilis* FMN riboswitches and couples riboflavin synthesis and transport to sulfur metabolism.

**Results**

Synthesis of RibR Is Induced When Methionine and Taurine Are Present in the Growth Medium. The objective of this experiment was to verify the previous finding that synthesis of RibR is induced by methionine and taurine in *B. subtilis* (10). If true, this would allow us to selectively stimulate the production of RibR in a variety of experiments. To monitor RibR synthesis by Western blot analysis, a recombinant *B. subtilis* strain carrying ribR fused to nucleotides coding for a tandem affinity purification tag (“TAP-tag”) was generated. This strain was cultivated in a growth medium containing methionine and taurine as the sole sulfur sources or in a medium containing MgSO₄ as a sulfur source. As expected, the gene product RibR-TAP-tag was found to be synthesized only when methionine and taurine were present in the growth medium (see SI Appendix, Fig. S3).
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show that this indeed is the case and that there is a physiological need for such a secondary control.

RibR Induces Synthesis of the Riboflavin Transporter Gene ribU. The result of the next experiment suggested that RibR not only stimulates riboflavin biosynthesis but also is involved in regulation of riboflavin transport. The riboflavin transporter RibU of B. subtilis catalyzes uptake of riboflavin from the growth medium (13), and expression of the corresponding gene ribU is controlled by a second FMN riboswitch present in B. subtilis, the FMN-sensing ribU FMN riboswitch (6). To monitor synthesis of the membrane protein RibU by Western blot analysis, a recombinant B. subtilis strain (ariB:E::tet/ribU-his8) was used that produced a His<sub>6</sub>-tagged version of RibU (13). In addition, this strain was riboflavin auxotrophic (due to the deletion of ribE), which allowed us to control cytoplasmic riboflavin levels by adding different amounts of riboflavin to the culture medium. B. subtilis ΔribE::tet/ribU-his8 was cultivated in a medium containing MgSO<sub>4</sub> (RibR repression) or methionine and taurine (RibR induction). Riboflavin was added to the culture medium to allow growth of this riboflavins auxotrophic B. subtilis strain. Western blot analysis of membrane fractions revealed that RibU was produced upon induction of RibR at all levels, the FMN riboswitch was turned on and RibU was produced. (Lane 2) RibU was synthesized in the presence of methionine and taurine (RibR induction). In the wild-type strain, RibE activity was increased 10-fold upon induction of RibR. In contrast, RibE activity was not increased in the ribE::aph3 strain in the presence of methionine/taurine. The results of this experiment indicated that RibR is a regulator.

**Induction of RibR Leads to Enhanced Riboflavin Synthase Activity**

The result of the following experiment indicated that RibR is involved in the regulation of riboflavin biosynthesis. Riboflavin synthase (RibE) catalyzes the last step in riboflavin biosynthesis (see SI Appendix, Fig. S1) and was used as a “reporter enzyme” to monitor expression of the riboflavin biosynthesis genes ribDG, ribE, ribAB, ribH, and ribT. Expression of this transcription unit is controlled by the FMN-sensing ribDG FMN riboswitch (see SI Appendix, Fig. S2A). B. subtilis wild-type and a ribE deletion strain (Fig. 1A) were cultivated in the presence of either MgSO<sub>4</sub> (RibR repression) or methionine and taurine (RibR induction). In cell-free extracts of RibR-induced wild-type cells, RibE activity was increased 10-fold compared with the noninduced cells (Fig. 1B). The ribE deletion strain, however, did not show enhanced RibE activity upon treatment of the cells with methionine and taurine (Fig. 1B). Western blot analysis confirmed that the amount of RibE was higher in RibR-induced wild-type cells compared with the noninduced cells (see SI Appendix, Fig. S4), ruling out the possibility that a RibR-mediated modification of RibE was responsible for the observed increase in enzymatic activity. Induction of RibR in a recombinant B. subtilis strain using an isopropyl-β-D-thiogalactopyranoside (IPTG)-inducible expression plasmid (pHT01-ribR) validated the results obtained by RibR induction of a B. subtilis wild-type strain with methionine and taurine. In the recombinant strain, induction of RibR was achieved by adding IPTG instead of methionine/taurine. As expected, the addition of IPTG as well led to an increased level of RibE activity in cell-free extracts of the recombinant strain (52.0 ± 5.1 pmol min<sup>−1</sup> mg<sup>−1</sup> protein<sup>−1</sup>) compared with the control strain containing an empty expression vector (8.0 ± 0.9 pmol min<sup>−1</sup> mg<sup>−1</sup> protein<sup>−1</sup>).

Because RibR was reported to be an RNA binding protein (12) and riboflavin biosynthesis/transport (rib) genes are regulated by FMN riboswitches (6, 7), we hypothesized that RibR would interfere with FMN riboswitch function. The following experiments...
Affects Expression of a Reporter Gene Coupled to FMN riboswitches. (A) Different E. coli strains containing the expression plasmid pPrib-RiBDG-RFN-luc (Luc reporter gene luc transcriptionally fused to the B. subtilis ribDG FMN riboswitch) in addition to either pET28a-RibR (RibR), pET28a-RibU (RibU), or pET28a-RibRC-terminus (producing the N-RibR, amino acids 1–89) were cultured in the absence (control) or presence of 5 μM riboflavin. Transcription of the reporter gene initiated at the E. coli promoter in pPrib (1). The strains were grown to the exponential phase, and RibR synthesis was induced by IPTG. Luc activity was determined in cell-free extracts prepared from these cells. In the absence of IPTG-induced proteins (no protein), Luc activity was reduced upon addition of riboflavin to the cells. IPTG-induced coproduction of RibR and of C-RibR alleviated the regulating effect of the riboswitch and led to almost full Luc activity (control levels), although FMNH₂ (which was converted to the flavin) was present. Luc activity was not reduced even though FMN was present in the cells. Coproduction of N-RibR did not show this alleviating effect. (B) Similar expression experiments (using pPrib-RibU-RFN-luc) with similar results were produced using the B. subtilis ribU FMN riboswitch (“RibU-RFN”) transcriptionally coupled to luc.

**Fig. 3.** Coexpression of ribr affects expression of a reporter gene coupled to B. subtilis FMN riboswitches. (A) Different E. coli strains containing the expression plasmid pPrib-RiBDG-RFN-luc (Luc reporter gene luc transcriptionally fused to the B. subtilis ribDG FMN riboswitch) in addition to either pET28a-RibR (producing full-length RibR, RibR), pET28a-RibU (producing the C-RibR, amino acids 90–230), or pET28a-RibRC-terminus (producing the N-RibR, amino acids 1–89) were cultured in the absence (control) or presence of 5 μM riboflavin. Transcription of the reporter gene initiated at the E. coli promoter in pPrib (16). The strains were grown to the exponential phase, and RibR synthesis was induced by IPTG. Luc activity was determined in cell-free extracts prepared from these cells. In the absence of IPTG-induced proteins (no protein), Luc activity was reduced upon addition of riboflavin to the cells. IPTG-induced coproduction of RibR and of C-RibR alleviated the regulating effect of the riboswitch. Luc activity was not reduced even though FMN was present in the cells. Coproduction of N-RibR did not show this alleviating effect. (B) Similar expression experiments (using pPrib-RibU-RFN-luc) with similar results were produced using the B. subtilis ribU FMN riboswitch (“RibU-RFN”) transcriptionally coupled to luc.

Coexpression of ribr affects expression of a reporter gene coupled to B. subtilis FMN riboswitches. To study the presumed regulatory function of RibR in vivo, a specialized Escherichia coli strain CpxFMN was used (see SI Appendix, Fig. S5). This strain was transformed with a first plasmid that allowed monitoring of FMN riboswitch controlled luciferase gene (luc) expression. A second plasmid was introduced into the same strain allowing (co)production of different forms of RibR (see below in this section). Luciferase (Luc) activity was determined in the different strains, and the results of these experiments are summarized in Fig. 3A. In the absence of RibR, Luc activity was reduced upon treating the cells with riboflavin, suggesting that the ribDG FMN riboswitch of B. subtilis reduced expression of the reporter gene luc when exposed to the flavin. The coproduction of full-length RibR alleviated the regulating effect of the riboswitch and led to almost full Luc activity. This effect of RibR could be located to the C-terminal part of RibR (C-RibR, amino acids 90–230), as coproduction of N-RibR (amino acids 1–89) did not show this alleviating effect. Similar experiments with similar results were obtained by testing the ribU FMN riboswitch coupled to luc, and the corresponding data are summarized in Fig. 3B. The presence of the different versions of RibR (all His₆-tagged) in the test strain was confirmed by Western blot analysis using anti-penta-His antibodies. In all strains, similar amounts of RibR (or N-RibR or C-RibR) were found.

**Fig. 4.** The C-RibR counteracts the turn-off activities of FMN riboswitches in vitro. (A) The reporter plasmid pPrib-RiBDG-RFN-luc (see Fig. 3A) was used as a template for in vitro transcription/translation assays in the absence (control) or presence of FMNH₂ (the reduced form of FMN) and in the presence or absence of RibR (and variants thereof) (see SI Appendix, Fig. S6A). Purified RibR-His₆, rapidly precipitated, and thus these preparations could not be used in our subsequent in vitro studies. However, purified maltose binding protein (MBP) fusions of RibR (and variants thereof) (see SI Appendix, Fig. S6B) were soluble. The purification and biochemical characterization of RibR. To study B. subtilis RibR functions in vitro, we set out to purify His₆-tagged RibR from a recombinant E. coli strain (see SI Appendix, Fig. S6A). Purified RibR-His₆ rapidly precipitated, and thus these preparations could not be used in our subsequent in vitro studies. However, purified maltose binding protein (MBP) fusions of RibR (and variants thereof) (see SI Appendix, Fig. S6B) were soluble.
different fusion proteins (MBP–RibR, full-length RibR fused to MBP; MBP–N–RibR, RibR amino acids 1–89 fused to MBP; MBP–C–RibR, RibR amino acids 90–230 fused to MBP) were tested for flavokinase activity (see SI Appendix, Table S1). The data revealed that it was N-RibR that had flavokinase activity and that this enzymatic activity of RibR was about 21 times lower compared with the major flavokinase RibFC of B. subtilis (2). We concluded that RibR does not contribute significantly to the FMN pool within cells. Our biochemical studies also confirmed the previous finding (14) that RibR represents a flavokinase that is specific for reduced riboflavin (dihydroriboflavin or riboflavinH2) (SI Appendix, Table S1).

**Fig. 5.** RibR prevents transcription termination of the ribDG FMN riboswitch. A fragment of the reporter plasmid pHis–RibDG–RFN-luc (see Fig. 3A) was used as a template for an in vitro transcription assay in the absence (−) or presence (+) of FMNH2, as indicated and in the presence or absence of RibR (and variants thereof) purified as MBP fusions (see SI Appendix, Fig. S5B). Transcripts were generated using E. coli RNA polymerase. In the absence of FMNH2 (lane 1), transcription continued until RNA polymerase reached the end of the DNA template (see scheme; triangle represents the B. subtilis ribDG FMN riboswitch; arrow represents E. coli ribB promoter; luc2 represents the 5′ part of the luc gene), resulting in a full-length mRNA (FL). The transcripts were separated by denaturing agarose gel electrophoresis and detected using ethidium bromide staining. The intensity of the band indicated the amount of product mRNA synthesized. In the presence of FMNH2, transcription termination was mediated by the B. subtilis ribDG FMN riboswitch, and a shorter transcript of 304 nu was synthesized (T) (lane 2). This shorter transcript was also observed in the presence of FMNH2 and MBP (lane 3). The presence of full-length RibR (MBP–RibR) as well as the presence of the C-RibR (MBP–C-RibR) alleviated this terminating FMN riboswitch function and resulted in an increased amount of the full-length transcript (FL, 926 nu) (lanes 5 and 6). The addition of the N-RibR (MBP–N-RibR) did not have such an effect (lane 4).

**FMNH2 Is a Stronger Effector of the B. subtilis ribDG FMN Riboswitch Compared with FMN.** FMN is a planar compound, whereas the reduced form of FMN (FMNH2) has a roof-like shape (15). Moreover, RibR was shown to be specific for reduced flavins (see Purification and Biochemical Characterization of RibR). In light of this, we hypothesized that FMN riboswitches in general may have a different reactivity with regard to FMN and FMNH2 and tested the regulating activity of these structurally different effectors. A plasmid carrying the E. coli ribB promoter Prib (16) and the B. subtilis ribDG FMN riboswitch transcriptionally fused to the reporter gene luc was used as a template in an in vitro transcription/translation reaction (17). We measured a reduction of luc expression in the presence of FMN and also of FMNH2 (see SI Appendix, Fig. S7). The amount of flavin needed for a 50% reduction (T50) of Luc activity in the in vitro transcription/translation assay is a measure for the apparent ligand affinity of the FMN riboswitch aptamer domain and was estimated from the data in the Left panel (SI Appendix, Fig. S7). T50 for FMN was 12.4 μM and T50 for FMNH2 was 3.2 μM, indicating that FMNH2 was a more efficient effector. As a consequence, we used FMNH2 as an effector in the subsequent assays.

**C-RibR Counteracts the “Turn-Off” Activities of FMN Riboswitches.** To validate the results of the coexpression experiments suggesting a regulatory role for RibR, in vitro transcription/translation assays were carried out in the presence or absence of purified MBP–RibR or truncated versions thereof (MBP–N–RibR or MBP–C–RibR; see SI Appendix, Fig. S6B). We measured a reduction of reporter enzyme (Luc) activity upon addition of FMNH2 to the assay where only MBP was present (Fig. 4A). The addition of MBP–RibR and MBP–C–RibR alleviated the regulating effect of the ribDG FMN riboswitch; that is, almost full Luc activity was found even though the effector FMNH2 was present (Fig. 4A). This was not the case when MBP–N–RibR was added to the assay. Very similar results were obtained when the B. subtilis ribU FMN riboswitch was tested (Fig. 4B). In the case of the B. subtilis ribU FMN riboswitch, the alleviating effect of RibR appeared to be less pronounced but again could be allocated to the C-terminal part of the protein.

When the B. subtilis FMN riboswitches used in the in vitro transcription/translation assays were replaced by the well-characterized ribE FMN riboswitch of the bacterium Streptomyces davawensis (18), no alleviating effect of RibR was observed (Fig. 4C), showing that RibR activity was specific for the B. subtilis ribDG– and ribU FMN riboswitches. In another control experiment, two different previously described B. subtilis ribDG FMN riboswitch variants (5) were tested. The variant “Mut ON” (see SI Appendix, Fig. S8A) was reported to not be able to terminate transcription even in the presence of high levels of FMN (5). The variant “Mut OFF” (see SI Appendix, Fig. S8B) was reported to terminate transcription constitutively regardless of whether FMN was present or not (5). Both FMN riboswitch variants were tested in the presence of FMNH2 and were found to not be affected by MBP–RibR, MBP–C–RibR, or MBP–N–RibR.
RibR Prevents Transcription Termination of the ribDG FMN Riboswitch.

The following experiment was performed to show that RibR overrides the genetic decision of FMN riboswitches, which would explain why RibR leads to high levels of reporter enzyme activity even in the presence of FMNH$_2$ (which otherwise reduces luc gene expression; see C-RibR Counteracts the ‘Turn-Off’ Activities of FMN Riboswitches). A linear DNA that contained the E. coli ribB promoter Prib (16) and the B. subtilis ribDG FMN riboswitch transcriptionally fused to a truncated reporter gene luc was used as a template in an in vitro transcription reaction using E. coli RNA polymerase (Fig. 5). The DNA template was transcribed in the presence or absence of purified MBP (controls), MBP-RibR, or truncated versions thereof (MBP–N-RibR or MBP–C-RibR; see SI Appendix, Fig. S6D). As expected (6), we found a reduction of the full-length product mRNA upon addition of FMNH$_2$ to the assay reaction where only MBP was present and an increase of a shorter transcript representing the prematurely terminated mRNA (Fig. 5). The addition of MBP-RibR and MBP–C-RibR alleviated the regulating effect of the ribDG FMN riboswitch. An increased amount of the full-length transcript was detected even in the presence of FMNH$_2$, which in the absence of RibR led to transcription termination. The addition of MBP–N-RibR did not have such an effect, indicating that the C-RibR was responsible for the prevention of transcription termination (Fig. 5).

RibR in Vitro Binds to the Aptamer Domains of Both FMN Riboswitches in B. subtilis.

The following experiment was carried out to in vitro verify binding of RibR to FMN riboswitch RNAs, which previously has been shown to occur in vivo using a yeast three-hybrid system (12). An RNA molecule representing the aptamer domain of the ribDG FMN riboswitch was synthesized by in vitro transcription, radiolabeled, and incubated with purified preparations of either MBP–RibR, MBP–N-RibR, or MBP–C-RibR. Only the addition of MBP–RibR or MBP–C-RibR (representing the RNA binding part of RibR) produced a characteristic shift toward a higher molecular mass in an electrophoretic mobility shift assay. This indicated that a complex between the full-length aptamer RNA and RibR had formed (Fig. 6). The addition of MBP (control) or MBP–N-RibR (representing the flavokinase domain of RibR) did not result in such a shift. A shift was observed in the presence of FMN or FMNH$_2$ only. Similar results were obtained testing the aptamer domain of the B. subtilis ribU FMN riboswitch (Fig. 6).

RibR Is Not Directly Involved in Sulfur Metabolism.

The following experiment was done to investigate the physiological role of RibR. A B. subtilis wild-type strain and a ribR deletion strain (B. subtilis ribR::aph3) were cultivated to the exponential growth phase. Two different sulfur sources were used during cultivation: MgSO$_4$ (RibR repression) or methionine/taurine (RibR induction). Polar intracellular metabolites were extracted and analyzed using nontargeted flow injection time-of-flight mass spectrometry (19). In total, 11,161 ions were detected, of which 1,441 ions could be annotated as metabolites based on matching their accurate masses with the Kyoto Encyclopedia of Genes and Genomes (KEGG) B. subtilis compound database (20) (see Dataset S1). To determine the metabolic consequences of ribR deficiency, we computed fold changes and statistical significance of all detected metabolite ions between the ribR deletion strain and the wild-type in both growth media. The metabolomics data are consistent with ribR being expressed in the presence of methionine/taurine only, as we observed stronger metabolic changes in the methionine/taurine medium compared with the MgSO$_4$ medium (see SI Appendix, Fig. S9A). The data further strongly suggest that ribR is not directly involved in sulfur metabolism, as the replacement of ribR by a kanamycin resistance gene did not affect levels of metabolites of the major sulfur assimilation pathways (see SI Appendix, Fig. S9B). Induction of RibR did not result in increased levels of riboflavin/FMN/FAD or intermediates of their biosynthesis (see SI Appendix, Fig. S9C), although RibR induction enhanced RibE activity. An explanation for this may be that an increased level of FMN/FAD-dependent flavoproteins was present that reduced the total amount of soluble flavins. This prompted us to investigate whether levels of substrates and products of known B. subtilis flavoenzymes were affected by ribR inactivation. Indeed, we observed that several metabolites processed by flavoenzymes were present in higher amounts in the ribR-deficient strain compared with wild-type B. subtilis in the methionine/taurine medium (see SI Appendix, Fig. S9D).

Discussion

To the best of our knowledge, this is the first report showing that the activity of a metabolite-sensing riboswitch is modulated by a protein. Whereas it was obvious from our experiments that RibR affected FMN riboswitch-mediated control of gene expression, the physiological meaning of this regulator function was less clear. The B. subtilis gene cluster snaA, tcyJ, tcyK, tcyL, tcyM, tcyN, cmoO, cmoL, cmoA, ribR, snd4, and ytmN was tentatively explained by the fact that the flavoenzymes CmoL and CmoO required flavin cofactors for activity and that the flavokinase RibR would provide the cell with additional FMN. Our data follow a similar line but suggest that it is not the flavokinase activity of RibR that is of physiological relevance (RibR has a comparably low activity only) but rather its regulating function: RibR prevents transcription termination and consequently allows expression of the riboflavin biosynthesis genes ribDG, ribE, ribAB, ribH, and ribT at high levels of FMN. This biologically active end product of the riboflavin pathway otherwise would shut down gene expression. Accordingly, RibR prevents sequestration of the ribosomal binding site of the gene ribU, allowing synthesis of the riboflavin transporter RibU even at high levels of FMN. Both activities of RibR lead to an increased amount of riboflavin/FMN/FAD necessary to generate fully cofactor-loaded (active) flavoenzymes. In summary, RibR represents a superordinate regulator that is able to affect the activities of the mechanistically very different FMN riboswitches present in B. subtilis, allowing this bacterium to fine-tune riboflavin metabolism.

At present, it is unclear where exactly RibR binds the aptamer portions of the analyzed FMN riboswitch RNAs, how the RibR/RNA complexes look, and how FMN/FMN$_2$ affects complex formation of RibR. Also the function of the N-terminal flavokinase domain of RibR is unclear, as the C-terminal FMN riboswitch binding domain of RibR seems to be sufficient to carry out the regulator function. Structural studies are underway that hopefully will also shed light on the question of how RibR binding could prevent formation of the terminator or sequestator structures to promote the alternative antitermination/sequestator stem loops. Structural work on RibR could also lead to a better understanding of riboswitch function in general.

Materials and Methods

An extended version of the materials and methods is available in SI Appendix.

Chemicals.

All chemicals and oligonucleotides were from Sigma-Aldrich.
Bacterial Strains, Plasmids, and Growth Conditions. Information with regard to strain construction, plasmids, and growth conditions can be found in SI Appendix.

Purification of Recombinant Proteins. His₆-tagged RibR was purified from cell-free extracts by column chromatography using Ni²⁺-nitrilotriacetate-agarose (GE Healthcare). Fractions containing the purified enzyme were pooled and desalted using Vivaspin columns of the 10 kDa cutoff column (GE Healthcare). The different forms of RibR were produced as fusions to the well-soluble MBP of E. coli and purified according to standard protocols. Protein was determined according to Bradford (23).

Assay of RibR. RibE was assayed as described (18). Data are presented as mean ± SEM (n = 3).

Coexpression Experiments. These experiments were performed using E. coli CpxXFIMM transformed with either pPhb-RibbD-GRN-luc or pPhb-RibbRFN-luc generating two different recombinant strains. These two strains were transformed with PET28a either carrying ribR, ribR-C, or ribR-N. The strains were aerobically grown at 37 °C in LB to an OD₆₀₀ of 0.4 (in the absence or presence of 5 μM riboflavin). At this point, 100 μM IPTG was added to the cultures. The cells were grown for another 2 h. Cells were harvested by centrifugation (4,000 × g, 4 °C, 10 min) and suspended in 100 mM potassium phosphate pH 7.5. Cell-free extracts were prepared by passing the cells through a French press at 2,000 bar. Centrifugation (8,500 rpm, 4 °C, 20 min) removed cell debris and unbroken cells. Aliquots of 10 μL of a dilution of these cell-free extracts were mixed with 50 μL Luciferase Assay Reagent (Promega). Lucifer activity was determined using a microtiter plate reader (Tecan Genios Pro microplate reader) as described (17). Data are presented as mean ± SEM (n = 4).

In Vitro Transcription/Translation Assay. The transcription/translation assay was performed using the E. coli T7 S30 Extract System for Circular DNA Kit (Promega), which contains T7 RNA polymerase and, in addition, E. coli RNA polymerase (24). Lucifer activity was determined as described above. Reducing conditions were achieved by adding 100 μM Na₂S₂O₄. If not otherwise indicated, FMN or FMNH₂ was present at a concentration of 5 μM. Preparations of purified MBP-RibR, MBP-N-RibR, and MBP-C-RibR were added to a concentration of 1 μM. Data are presented as mean ± SEM (n = 4). Run-Off Transcription Assay. Plasmid pPhb-RibbDG–RFN-luc was treated with EcoRlSspI and the resulting fragment carrying Prib, the ribbDG FMN riboswitch, and a 578-bp fragment of luc was used as a template in a run-off transcription assay using E. coli RNA polymerase holoenzyme in a reaction containing 40 mM Tris·HCl pH 7.9, 6 mM MgCl₂, 2 mM spermidine, 10 mM NaCl, 10 mM DTT, 500 μM each nNTP, and 100 U Recombinant RNasin Ribonuclease Inhibitor (Promega). When indicated, 50 μM FMN in 100 mM sodium dithionite (FMNH₂) and 2 μM of protein were added. The transcripts were separated by using denaturing 2% (wt/vol) agarose gel electrophoresis and visualized by staining with ethidium bromide.

Electrophoretic Mobility Shift Assays. A 20 fmol sample of gel-purified in vitro-transcribed RNA was 5' labeled with [γ-³²P]ATP using T4 Polynucleotide Kinase. A 20 fmol sample of labeled RNA was mixed with 1 μg yeast tRNA and 1 μg heparin in a 15 μL reaction containing 100 mM Tris·HCl pH 7.0, 1.0 M KCl, 100 mM MgCl₂, and 50 μM FMN or FMNH₂. After 15 min of incubation at 37 °C, each sample was mixed with loading buffer (50%, vol/vol, glycerol; 0.2% bromophenol blue; 0.5x Tris-borate buffer) and loaded onto a running 6% native polyacrylamide gel, precooled to 4 °C, with 0.5x Tris-borate as a running buffer. The gel was dried for 30 min at 80 °C, and radioactivity was detected using a Fuji FLA-5000 phosphoimager.

Metabolome Analysis. The B. subtilis strains were cultivated in a minimal medium until reaching an OD₆₀₀ of 1.5. Methionine/taurine or MgSO₄ was added to the growth medium. Six replicates were performed for each culture and processed as described in SI Appendix.

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