SYNTHETIC RIBOSWITCHES FOR THE CONDITIONAL CONTROL OF GENE EXPRESSION IN STREPTOMYCES COELICOLOR

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We have demonstrated the portability of theophylline-dependent synthetic riboswitches for the conditional control of gene expression in Streptomyces coelicolor. The riboswitches mediate dose-dependent, up to 260-fold activation of reporter gene expression. Riboswitch regulation is a simple method requiring a sequence of only ~85 nt to be inserted between a transcriptional start site and the start codon; no additional auxiliary factors are necessary. The promoters galP2, ermEp1 and SF14 worked well in concert with the riboswitches. They allowed theophylline-dependent expression of not only the heterologous β-glucuronidase reporter gene but also dagA, an endogenous agarase gene. The successful combination of all tested promoters with the riboswitches underlines the orthogonality of riboswitch regulation. We anticipate that any additional natural or synthetic promoters can be combined with the riboswitch.

INTRODUCTION

Tools for the precise control of gene expression are indispensable for genetic studies. Furthermore, conditional gene expression systems are important for heterologous protein expression to control the timing and expression rate of a gene of interest. Attempts to overexpress recombinant proteins with strong constitutive promoters are often accompanied by negative side effects on bacterial cell growth and, in consequence, on the amount of the desired product. This is especially the case for toxic proteins. Model organisms possess a variety of such control devices, but robust and easy applicable systems are rare in other organisms.

Streptomyces coelicolor is a Gram-positive, filamentous GC-rich soil-dwelling bacterium. It undergoes complex morphological differentiation, involving the development of sporophyte and mycelia colonies and is characterized by a complex secondary metabolism. Bacteria of the genus Streptomyces are the major producers of industrially relevant compounds, such as antibiotics, enzymes, antiviral and even anticancer drugs (Demain & Sanchez, 2009). Their ability to express and secrete heterologous proteins in a properly folded and biologically active state makes them even more attractive for biotechnological applications (Anné et al., 2012).

Conditional gene expression systems available for Streptomyces are based on the control of transcription. The thiostrepton-inducible promoter pripA (Murakami et al., 1989) is currently the most commonly used regulation system in streptomycetes. However, as thioestrepton is an antibiotic, its application requires the heterologous expression of the resistance gene tsr in addition to an activator protein tipA (Holmes et al., 1993; Takano et al., 1995). Other conditional gene expression systems in streptomycetes include the tetracycline repressor/operator system (Rodríguez-Garcia et al., 2005) and the Rhodococcus rhodochrous P\textit{erm}R-NitR system, which is induced by \textit{y}-caprolactam (Herai et al., 2004). The T7 RNA polymerase was adapted for \textit{Streptomyces lividans} but its complexity and additional requirement for thioestrepton induction makes it less favourable (Fischer, 1996; Lussier et al., 2010). Common to all these systems; however, is the demand for the additional expression of regulatory proteins including their promoter and codon usage optimization.

The conditional control of gene expression by synthetic riboswitches circumvents these drawbacks because they do not rely on additional protein cofactors (Wittmann & Suess, 2012). Most natural riboswitches are located in the 5’UTR of an mRNA and are composed of a binding region (aptamer domain) and an expression platform that modulates gene expression (Breaker, 2012). The aptamer domain is capable of sensing very diverse metabolites with a high binding affinity and specificity. Upon ligand binding, the aptamer region undergoes a conformational change that is interpreted by the expression platform, resulting in altered gene expression by either premature transcription termination or the sequestration of the translation initiation site.

Based on these principles, various synthetic riboswitches have been engineered (Suess et al., 2008). Thereby, \textit{in vitro} selected aptamers have been employed as binding domains, which recognize non-toxic and cell permeable small
molecules that are not metabolized by the cell. One riboswitch designed for *Bacillus subtilis* makes use of a theophylline-binding aptamer (Jenison et al., 1994) linked to a communication module that performs a helix slipping. The riboswitch was integrated in close proximity to the Shine–Dalgarno (SD) sequence, thereby impairing ribosomal accessibility in the ligand free form. Binding of theophylline to the receptor domain leads to a local one-nucleotide shift within the helical communication module that frees the SD sequence, rendering translation possible (Suess et al., 2004).

In an alternative approach, a smart design of an expression platform resulted in a construct that prevents the accessibility of both the ribosome-binding site and the translational start codon in the absence of the ligand. Theophylline binding to the aptamer results in a structural rearrangement of the translation initiation region. It renders it accessible to the anti-SD sequence of the 16S rRNA (Fig. 1; Desai & Gallivan, 2004). By means of rational design and in silico structure predictions combined with cell-based screenings (Lynch et al., 2007; Lynch & Gallivan, 2009; Topp & Gallivan, 2008), a set of six riboswitch constructs was developed permitting inducible gene expression in a range of Gram-positive and Gram-negative bacteria (Topp et al., 2010).

Our results indicate that these theophylline-dependent riboswitches are applicable in *S. coelicolor*, too, and indeed represent a suitable alternative to the conditional gene expression systems currently available in these bacteria. We tested three well-studied constitutive promoters (*galP2*, *ermEp1* and *SF14*; Fornwald et al., 1987; Bibb et al., 1985; Labes et al., 1997) in combination with the riboswitches. We demonstrated that, especially with one variant (E*), a simple and highly reliable induction system was created. It is represented by a very low basal expression and a high activation ratio and does not employ accessory protein factors. The dose-dependence of the riboswitch reaction makes it possible – in combination with different promoters – to adjust the expression of a gene of interest to the desired level. We anticipate that these riboswitches will also be applicable to other *Streptomyces* species for the expression of both endogenous and heterologous proteins.

**METHODS**

**Streptomyces growth.** *S. coelicolor* M145 strains were cultivated at 28 °C on mannitol–soy flour (MS) agar, R2YE or MM (Kieser et al., 2000) for spore suspension preparation, conjugation or direct detection of β-glucoronidase (GusA) activity. Agar was supplemented with theophylline (Sigma) or caffeine (Fluka) directly before pouring. Submerged cultures were grown at 28 °C in tryptic soy broth (TSB) medium (Kieser et al., 2000) on a rotary shaker incubator at 150 r.p.m.

**Genetic manipulations.** *E. coli* DH5α was used for all plasmid manipulations. The methylation-defective *E. coli* strain ET15267/pU28002 was used for intergeneric conjugation to *S. coelicolor* M145 (Flett et al., 1997). All constructs were verified by DNA sequencing (Seqlab Sequence Laboratories). Purification of plasmids, PCR products and enzyme digests were performed with kits from Qiagen and Zypto Research. Synthetic oligonucleotides were purchased from Sigma (sequences are given in Table S1, available in *Microbiology* online). Phusion DNA polymerase and restriction enzymes were purchased from New England BioLabs.

The β-glucoronidase gene (*gusA*) was amplified from pSETGUS (Myronovskyi et al., 2011) in an overlap PCR using the oligonucleotides pGus1–4 (Table S1). Hereby, an AgeI restriction site was introduced directly behind the start codon and an XbaI site 3′ of *gusA*. After digestion by BglII and XbaI, the fragments were cloned into the corresponding sites of vector pLuci, a derivative of pAR933a (Rodríquez-Garcia et al., 2005) where the tetR gene had been removed by PCR. The resulting pGusA vector carried the reporter gene in the opposite direction relative to the remaining genes on the vector to prevent readthrough of the neighbouring genes. A synthetic transcriptional terminator (generated by annealing of the oligonucleotide terminator 1–4, Table S1) was inserted downstream of *gusA* via the XbaI restriction site to ensure proper transcription termination.

The oligonucleotides *galP2*-a/b, *ermE*-a/b and *SF14*-a/b were annealed to generate the three corresponding promoters that were cloned into pGusA via BglII and Acc65I, resulting in the plasmids pGusT-†*galP2*, pGusT-ermE and pGusT-SF14, respectively. Riboswitch constructs were PCR-amplified from pBAV1K-A/B/D/E/SF14 using the primer ribo-FWD-†*KpnI* and riboREV-Agel (Table S1). The forward primer ribo-C-FWD-†*KpnI* was used for riboswitch construct C with pBAV1K-C as template. All fragments A to E* were digested by *KpnI* and *AgeI* and cloned into the corresponding sites of the vectors pGusT-†*galP2/-ermE/-SF14*. The plasmids were transferred to *S. coelicolor* M145 by intergeneric conjugation (Flett et al., 1997).

**Fig. 1.** The theophylline-dependent riboswitch E* and proposed switching. Nucleotides of the theophylline aptamer important for ligand binding are shown in bold, the SD sequence and the start codon are shown in red.
dagA was amplified from the chromosome using the oligonucleotides dag_f/dag_r (omitting the start codon). The PCR product was cloned into pGusT-ermE and pGusT-ermE-E* using the restriction sites AgeI and XbaI giving pT_dagA and pTE_dagA, respectively. These plasmids where then used to complement the dag\(^{-}\) strain J801 by integeneric conjugation (Hodgson & Chater, 1981).

**Measurement of \(\beta\)-glucuronidase activity.** For direct detection of GusA activity in agar-grown colonies, 4–5 day plates were flooded with X-Gluc solution (X-Gluc DIRECT, 50 µg ml\(^{-1}\) in DMF), incubated at 28 °C for 2 h and then photographed.

For spectrophotometric measurement of GusA activity, cell lysates were prepared as follows: 10\(^8\) pre-germinated spores of *S. coelicolor* strains were inoculated in 50 ml TSB medium containing theophylline/caffeine as described. Mycelia were harvested after 90 h of cultivation, washed with distilled water and centrifuged again. The subsequent disruption of cells was done using a FastPrep-24 instrument (MP Biomedicals, 6 \(\times\) 30 s at 6.0 m s\(^{-1}\)). Mycelia were mixed with 200 µl glass beads (0.25–0.5 mm) in 50 mM NaHPO\(_4\) pH 7.0, 0.1 % Triton X-100, 5 mM DTT (Gus buffer). Mycelial debris was removed by centrifugation at 4 °C. Protein concentration of the supernatant was determined by Bradford assay in 1–25 µg of total protein in a total volume of 750 µl Gus buffer incubated at 28 °C for 15 min. The reaction was started by addition of 80 µl 200 mM p-nitrophenyl-\(\beta\)-D-glucuronide (Glycosynth) and stopped with 300 µl 15 mM Na\(_2\)CO\(_3\) upon appearance of a yellowish coloration. The absorption at 415 nm was measured and divided by the amount of protein used and the reaction time to calculate Gus units GU [A\(_{415}\) 5,5 µg \(\times\) min \(^{-1}\)]]. All assays were conducted in triplicate and repeated at least once. Data are presented as the mean ± SD.

**Gran’s test for agar decomposition.** A agarase activity measurements were performed as described previously (Vockenhuber & Suess, 2012). In detail, 10\(^8\) spores in a volume of 5 µl were dropped on a modified basal salts medium (MBSM) plate (Brawner et al., 1985) with 2 % agar as the only carbon source. The inoculated plates were incubated for 5 days at 28 °C, then stained for 1 h with Lugol solution (Roth) at 4 °C. For semiquantitative analysis of agarase expression, the ratio of colony- to halo-diameter was calculated as measured by ImageJ software (http://rsb.info.nih.gov/ij/). Each colony was measured in both horizontal and vertical directions to control for differences in colony shape.

### RESULTS

**Theophylline-dependent activation of gene expression in *S. coelicolor***

Six synthetic theophylline-dependent riboswitches (Topp et al., 2010; Table S2) were cloned in front of a \(\beta\)-glucuronidase reporter gene (*gusA*) driven by the constitutive promoter *ermE*\(_1\). A construct without a riboswitch but including the core SD motif 5'-GGAGG-3' nine nucleotides upstream of the *gusA* start codon was used as a control. A copy of the plasmids was integrated into the *attB* site of the *S. coelicolor* genome. The exconjugants were streaked out on agar plates supplemented with or without 2 mM theophylline and cultivated for three days. The GusA activity then was directly visualized by addition of the chromogenic substrate X-Gluc. The blue precipitate 5,5'-dibromo-4,4'-dichloro-indigo appeared at 2 h incubation, indicating GusA expression. The results are shown in Fig. 2a. Activation of reporter gene expression by theophylline was detectable for the riboswitches A, B, E and E* whereas C and D showed no GusA activity at all. The positive control showed constitutive GusA expression independent of theophylline supplementation.

The theophylline-dependent activation of *gusA* expression was quantified. The strains were cultivated in TSB medium with and without 4 mM theophylline for 90 h, and GusA activity (GU) was determined (Fig. 2b). Correlating with the agar plate experiments, the strains with the riboswitches A, B, E and E* showed an inducible GusA expression upon addition of theophylline, but to different extents (32-, 3-, 12- and 30-fold, respectively). The background expression was very low for both riboswitches A and E*; B and E showed leaky GusA expression in the absence of theophylline. Overall, riboswitch E* represents the best regulatory device because of its high dynamic range (30-fold) combined with a very low basal expression.

![Fig. 2](image-url) Analysis of different theophylline-dependent riboswitches in *S. coelicolor*. (a) X-Gluc staining of *S. coelicolor* strains growing on MS agar expressing *ermE*\(_1\)-driven *gusA* (wt) under control of the theophylline riboswitches A, B, C, D, E or E*. The medium was supplemented with 2 mM theophylline (theo) for induction. (b) Measurement of GusA activity of liquid cultures grown for 90 h in the absence (closed bars) and presence of 4 mM theophylline (open bars). Theophylline was present from the beginning. The maximal induction is given above the respective bars.
Different promoters can be combined with the theophylline riboswitch

In the next step, the activation of riboswitch E* was compared at different expression levels. Fig. 3 shows the E*-gusA construct in combination with promoters of different expression strength ranging from weak (galP2) to medium (ermE) to strong (SF14). The integration of the riboswitch almost completely inhibited gene expression in the absence of theophylline for all three expression levels, albeit to different degrees (Figs 3 and S1, available in Microbiology Online). Induction of gene expression was then achieved by addition of theophylline, with ratios of about 30-fold for galP2-E* and ermE-E*. Interestingly, the strikingly low basal expression of the SF14–E* construct led to a 260-fold activation. However, the expression level of the construct without a riboswitch could only be achieved upon induction for galP2-E* (Fig. S1). Comparable results were also obtained for riboswitches A–E (Fig. S2).

The closely related compound, caffeine, was used as a control. It is distinguished from theophylline by only one methyl group, but does not bind to the riboswitch. No activation was obtained with caffeine, underlining the ligand specificity of riboswitch regulation (Fig. 3a). The specific activation of gene expression also was independent of the growth medium (Fig. S3).

Taken together, all tested promoters were combinable with the riboswitches indicating the portability of artificial riboswitch regulation. Therefore, it is likely that any further natural or synthetic promoter can be combined with the riboswitch. The smart combination of a certain promoter with one of the riboswitches will allow for adjusting the needed regulatory window.

Dose-dependent regulation

The construct ermEp1-E* was used to assess the dose-dependence of the riboswitch regulation. The strain was cultivated in TSB medium in the presence of 0, 1, 2, 3 or 4 mM theophylline, respectively. GusA activity was determined after 90 h. The data depicted in Fig. 4a provide evidence of the riboswitch acting in a dose-dependent manner. Increasing theophylline concentrations entailed higher levels of reporter gene expression for the riboswitch-controlled constructs whereas no influence on the expression of the wild-type was observed (Fig. S4). At the same time, a decrease in cell dry weight per millilitre was observed (Fig. S5). Theophylline concentrations higher than 4 mM resulted in a reduction of cell growth, which has already been reported for other Gram-positive (Topp, 2009) and Gram-negative bacteria (Desai & Gallivan, 2004).

For this reason, a quantitative restoration of GusA expression obtained by the riboswitch-lacking control strain cannot be achieved by raising theophylline concentrations for the ermEp1-E* construct. As a consequence, stronger promoters rather than increasing inducer concentrations must be combined with the riboswitch to gain higher levels of gene expression.

Next, the kinetics of induction and repression of gene expression were determined (Fig. 4). To achieve this, cells were grown for 48 h in the absence of theophylline, harvested and transferred into theophylline-containing TSB medium. Increased GusA activity was detectable already by 4 h after induction, reaching maximum expression after 8 h (Fig. 4b).

The reversibility of theophylline induction was analysed in a similar manner. After 48 h of growth in theophylline-containing medium the cells were washed and transferred

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**Fig. 3.** Activation ratio of the riboswitch E* at different expression levels. (a) X-Gluc staining of *S. coelicolor* strains growing on MS agar expressing gusA driven by three different promoters under control of the riboswitch E*. The medium was supplemented with 2 mM theophylline (theo) or caffeine (caff), or without anything (w/o) (b) Measurement of GusA activity of liquid cultures grown for 90 h in the absence (closed) and presence of 4 mM theophylline (open bars). Theophylline was present from the beginning. The maximal induction is given above the respective bars.
into fresh medium without inducer (Fig. 4c). Here, 8 h after the removal of theophylline, GusA activity was reduced by ~50%, and after 24 h it approached the values observed in non-induced cultures. It has to be noted that the reversal of induction is severely dependent on mRNA and protein stability. The time needed to shut expression off again might vary dramatically for each tested gene.

Riboswitch control of dagA expression

As proof of principle we used the E* riboswitch to regulate the expression of the extracellular agarase gene dagA in S. coelicolor. The dag- strain J801 was complemented with a plasmid carrying the agarase dagA under control of the ermEp1-E* construct. As a control the same construct without the riboswitch was used. All strains were tested for agarase activity in the presence of 0, 0.5, 1 or 2 mM theophylline (Fig. 5). Rising amounts of theophylline led to a dose-dependent increase of agarase expression indicated by the size of the white halo. No agarase activity could be detected on plates without theophylline, whereas upon induction with 2 mM theophylline an expression level of ~76% compared to the construct without riboswitch was reached. Using the same setup with the gusA gene as a reporter we could only reach ~40% induction (Fig. 2b). This again demonstrates the tight repression of this system but on the other hand also shows that the desired induction level can be adjusted for each gene of interest.

DISCUSSION

Here, we demonstrate the portability of theophylline-dependent riboswitches for use as genetic control elements in the model actinomycete S. coelicolor.

In general, the use of riboswitches might offer a simple method to obtain conditional gene expression since no auxiliary factors and exogenous regulators are required. As for the riboswitch constructs tested in this work, a sequence of only ~85 nt has to be inserted between a transcriptional start site and a gene of choice. All promoters examined in this study have proven to work well in concert with the riboswitches. They allowed theophylline-dependent expression of a heterologous GusA reporter gene but – most importantly – also of the endogenous agarase gene, dagA.

The variations in riboswitch activities may be rationalized by differing SD sequences and their respective spacing to the translational start codon. Translational efficiency strongly depends on complementarity between the SD sequence and the anti-SD sequence located on the 3’ end of the 16S rRNA. S. coelicolor 30S ribosomal subunits carry the anti-SD sequence 3’-UCUUUCCUCCACUAG-5’ (Baylis & Bibb, 1987) and the corresponding conserved SD minimal motif is 5’-GGAGG-3’. In general, for Gram-positive bacteria a strong complementarity is reported to be a stricter prerequisite for efficient translation initiation than for Gram-negatives such as E. coli (Band & Henner, 1984;

Fig. 4. Dose-dependence of the E* riboswitch. (a) Measurement of GusA activity of liquid cultures grown for 90 h in the absence of (closed bars) and supplemented with increasing concentrations of theophylline (Theo, open bars) or 4 mM caffeine (Caff). Theophylline and caffeine, respectively, were present from the beginning. The maximal induction is given above the respective bars. Cells were grown in liquid culture in (b) the presence or (c) the absence of 4 mM of theophylline for 48 h. Theophylline was added to the culture (b, time 0) or removed by harvesting and resuspending the cells in new medium without theophylline (c, time 0). GusA activity was measured 0.5, 2, 4, 8 and 24 h after that time.
McLaughlin et al., 1981). This, however, might not be valid for all streptomycetes, because studies with S. lividans – a close relative of S. coelicolor – showed that weaker SD sequences can also be employed for effective translation (Strohl, 1992). Nevertheless, the SD sequences of riboswitches C and D only provide four complementary base pairings to the anti-SD sequence, which seems to be insufficient for successful translation initiation.

The weak SD sequence of riboswitch D was optimized for Gram-positive bacteria either by inserting an AGG codon (generating riboswitch E) or by replacing the UAA codon by Gram-positive bacteria either by inserting an AGG codon (generating riboswitch E*) (Topp et al., 2010). The former now possesses the sequence 5′-AAGGAGGU-3′ where eight bases perfectly match the anti-SD sequence of S. coelicolor ribosomes. The SD sequence in riboswitch E* is located three nucleotides closer to the initiation codon and is predicted to form only seven base pair interactions (5′-AAGGAGG-3′) with the anti-SD sequence. Compared to riboswitch E*, the stronger complementarity between the SD sequence of riboswitch E and the 3′ end of 16S rRNA may contribute to relatively high GusA expression observed in the absence of theophylline. It has been demonstrated that an increased SD–anti-SD complementarity can surmount the hindrance of translation initiation by intranadir base-pairing of the ribosome-binding site (de Smit & van Duin, 1994).

Riboswitch A achieved the highest activation ratio (32-fold, Fig. 2b). This was attributed to the basal GusA expression observed in the absence of theophylline. It is noteworthy that this riboswitch originally was detected in an in vivo screen for the Gram-negative bacterium Acinetobacter baylyi (Topp et al., 2010) and comprises a purine-rich SD sequence. Here, six to seven bases including one or two wobble base pairs are involved in the recognition by the S. coelicolor anti-SD sequence. Due to its very efficient repression in absence of the ligand – even in combination with the strongest tested promoter SF14 (Fig. S2) – we anticipate that this switch might be the best choice for use with multi-copy plasmids, or when tight repression is required over high induction.

The riboswitch E* displayed the highest increase in protein production coupled with the lowest background activity. This makes it the most interesting candidate for controlled gene expression in S. coelicolor. We propose that riboswitch E* can be used for applications where protein yield is crucial, whereas construct A would be rather eligible for expression of toxic or otherwise detrimental proteins.

Taken together, our work with S. coelicolor confirms the apparently unrestricted applicability of the synthetic theophylline-riboswitches that was already shown for a variety of other bacterial species (Reynoso et al., 2012; Seeliger et al., 2012; Topp et al., 2010). All tested promoters in our study could be combined with the riboswitches. Therefore, it is likely that the use of any appropriate natural or synthetic promoter can be regulated. The dose-dependence which was shown exemplarily for the ermEp1-E* construct – in combination with the exchange-ability of the promoters – provides the unparalleled possibility to adjust for almost any desired gene expression level. In addition, the induction/repression profile (Fig. 4b, c) indicates that the system can rapidly be induced and switched off in the exponential growth phase and is independent of the growth medium (Fig. S3).

Remarkably, the observed regulation is highly specific. In contrast to theophylline, the structurally very similar molecule caffeine was incapable of activating gene expression. Caffeine is methylated at position N-7, which impairs recognition by the aptamer. The binding affinity for theophylline is 10 000 times greater than for caffeine (Jenison et al., 1994), which makes the utilized riboswitches highly specific and makes interference by other metabolites or media components extremely unlikely.

**Fig. 5.** Regulation of dagA expression by the E* riboswitch. (a) Agarase activity assay (Lugol staining) of S. coelicolor strain J801 (J801::dagA) complemented with ermEp1-driven dagA growing on MBSM plates with agar and glycerol as only carbon sources supplemented with increasing concentrations of theophylline. The area of clearing around a colony shows where agar has been utilized. Addition of theophylline has no effect on agarase expression if no riboswitch is present (wt). Addition of theophylline can almost completely restore agarase activity when dagA is under control of the riboswitch E*. (b) Quantification of agarase induction. Given is the level of agarase expression at a given theophylline concentration divided by the expression level in the absence of theophylline.
We assume that this system may become widely used in S. coelicolor and should also be easy to transfer to other Streptomyces species that currently lack toolsets for inducible gene expression.

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