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A FACS-Based Approach to Engineering Artificial Riboswitches

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Nucleic acids have established themselves as valuable building blocks for the construction of synthetic elements with a wide range of uses in biological research.[1] Indeed, nucleic acid enzymes (ribozymes and deoxyribozymes) and receptors (aptamers) have emerged as major players in the era of synthetic biology, and can be applied in such disciplines as biosensing,[2,3] inity purification,[4,5] nanodevices[6,7] and therapeutic development.[8,9] The success in isolating these elements can be largely attributed to the methods used to select for molecules that carry out a specific function from extremely large, random sequence libraries.[10,11] While a great deal of effort and progress has been made toward advancing these protocols in vitro, synonymous methods for selections within a cellular environment are far less developed. This shortcoming is becoming more relevant as our understanding of the diverse array of functions that nucleic acids—particularly RNA—have within cells continues to grow at an astounding pace.[12,13] To have the ability to produce these interesting new RNA molecules with custom-designed characteristics would be invaluable to biological research.

One of the most intriguing classes of newly discovered RNAs is a group of genetic control elements called riboswitches.[14,15] They reside within the untranslated regions (UTRs) of mRNA where they sense the metabolic status of the cell through directly binding a relevant ligand.[14–22] The binding event results in structural changes in the RNA, which ultimately leads to modulation of the level of expression of neighbouring gene or genes. The bulk of natural riboswitches that have been uncovered have been found in bacteria where they regulate the expression of a remarkable number of genes in response to a growing number of different metabolic cues.[23] From a molecular engineering point of view, riboswitches represent a highly desirable design for artificial gene expression systems. They have a very simple composition that consists of an RNA receptor domain (aptamer domain) and a neighbouring expression platform with a simple stem–loop architecture. Riboswitches are relatively small, cis-acting elements, usually encoded by 300 bases or less. These factors simplify the design, optimisation and troubleshooting associated with their creation. Riboswitches have shown the potential to be extremely efficient as well, and demonstrate both remarkable specificity and impressive induction levels in response to their cognate ligand.[21,22]

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Artificial riboswitches could be designed for a wide range of applications. One such possibility is the engineering of programmable cells. This could include the production of cells that have an array of genes under the control of different inducer molecules; this would open the door to experiments for working out the interplay of numerous genes or pathways. Programmed bacteria also have great potential as tools for environmental cleanup and drug delivery. Topp and Gallivan recently made progress toward this goal by employing an artificial riboswitch to create a strain of *E. coli* cells that migrate specifically toward the small molecule theophylline.[24] We believe that riboswitches also hold great potential as intracellular sensors for the detection and quantification of small molecules. While these fields are still largely unexploited, the continuing development of methods to create and modify riboswitches should make such systems possible.[25,26]

The goal of this work was to develop a fast, efficient and general intracellular selection method for isolating riboswitches from a library of sequences. In order to sort through a large number of sequences quickly and easily, we opted to use fluorescent proteins and fluorescence activated cell sorting (FACS). FACS can be used to sort individual cells based on their fluorescence intensity at rates that exceed 10^4 cells per second. Previously FACS has been employed to identify proteins with desirable properties from libraries of random mutants.[27–30] Here, we apply similar thinking to identify riboswitch mediated gene regulation from a library "potential riboswitches". Figure 1 A demonstrates the principle of our selection scheme in which cells that are highly fluorescent only in response to the small-molecule inducer of our choice are isolated by using two steps of FACS.

As a pilot project to demonstrate our selection scheme we chose to seek riboswitches that respond to the small molecule theophylline. We built a library of "potential riboswitches" that consisted of an RNA aptamer that binds theophylline connected to an intrinsic transcriptional terminator by a partially random linker region (Figure 1 B). Transcriptional terminators are RNA elements that consist of a simple stem–loop followed by a string of uracil residues.[31,32] When RNA polymerase encounters such an element it is released from the template and sequences downstream are not transcribed. We sought linker regions that were able to transduce the binding of theophylline to a disruption of the neighbouring transcriptional terminator stem; this would result in activating transcription of the downstream green fluorescent protein (GFP) gene. This riboswitch mechanism (transcriptional termination) appears to be the most common, and has not been exploited by artificial riboswitches that have been created to date.

Before beginning the selection process we first chose each of the elements for the construction of our riboswitch library. The TCT8-4 theophylline aptamer,[33] which has previously been

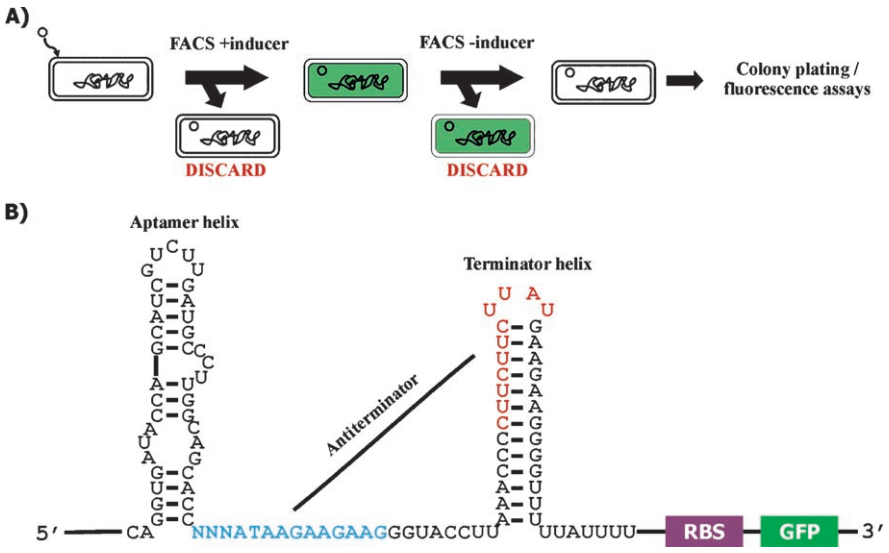

Figure 1. A) Selection scheme for the isolation of riboswitches. Cell sorting based on fluorescence intensity was used to isolate cells that were highly fluorescent in the presence of inducer, but less so in the absence of inducer. The small circle represents a plasmid-encoded library of riboswitch candidates within the 5′-UTR of GFP. B) Design of our library of potential theophylline-responsive riboswitches. The partially random linker region is shown in blue; N represents a completely random position and other positions are conserved 76% of the time with an 8% chance of being changed to one of the other three possible residues; RBS: ribosome binding site.

used for a wide range of proof-of-principle experiments, was chosen for a variety of reasons: it is very well characterized, it exhibits remarkable specificity for its ligand and it is functional in a cellular environment.[34–37] To choose a transcriptional terminator we cloned a panel of candidates upstream of GFP within our selection plasmid and carried out fluorescence assays. The terminator from the *B. subtilis* MetI riboswitch[22] was chosen as it appeared to be the most efficient within our system (data not shown). It should be noted that we only assayed terminators from riboswitches as it has been postulated that they contain pause signals that allow for efficient ligand binding and riboswitch function.[37,38] The final element of our library—the linker region—was biased to resemble the antiterminator from the MetI riboswitch to provide our library with an enhanced ability to disrupt the terminator (Figure 1B, see legend).

Since the goal of this project was to develop a selection method that could isolate rare active sequences from a library, we first cloned and sequenced eight random, unique library sequences to determine if they were theophylline responsive. These plasmids were assayed in cell-based fluorescence assays (see the Experimental Section) and none showed a significant theophylline response. We thus set out to test our selection method by cloning a large number of sequences into competent *E. coli* cells. By plating dilutions of this transformation it was determined that approximately 100000 positive clones were successfully transformed. Cells were grown in the presence of theophylline for approximately 6 h and sorted to keep only those with a relatively high fluorescence (Figure 2A). Choosing an induction time required striking a balance between allowing the induced GFP time to fold and minimizing growth time to avoid losing library diversity. We were particularly concerned about this because cells that express lower levels of GFP would presumably have a slight growth advantage.

The remaining cells, which had survived the first sort, were grown in the absence of theophylline, and those with a relatively low fluorescence were kept (Figure 2B). For our first round of selection we opted to use rather lenient gating in order to avoid losing cells that contained weakly active riboswitches. The astonishingly high proportion of cells with a low fluorescence in the second FACS sort indicated that our pool either contained a very large number of active sequences, or

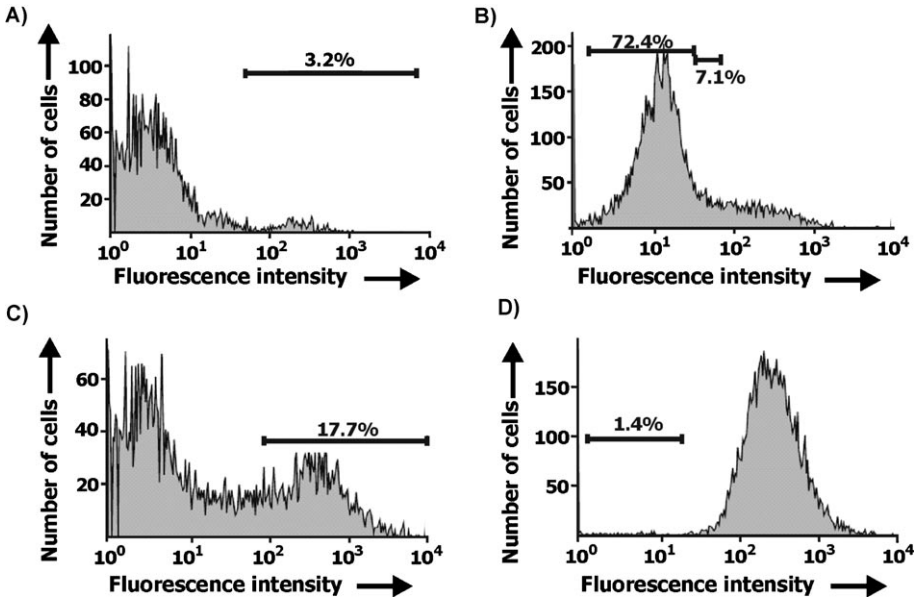

Figure 2. FACS results: A) selection round 1, with theophylline; B) selection round 1, without theophylline; C) selection round 2, with theophylline; D) selection round 2, without theophylline.

that the first round of FACS was too lenient and thus a large number of weakly fluorescent cells were able to pass through the first sort.

To examine the cells that had survived the selection, individual clones were isolated by being plated on agar plates. Single colonies were then grown in separate wells of 96-well plates and kept as frozen stocks. From these stocks duplicate plates were grown, one with theophylline and one without, and were assayed for fluorescence. The majority of the clones exhibited small changes in fluorescence in response to theophylline; this indicates that perhaps our FACS gating was too lenient. Several of the clones showed a very clear theophylline response and they were selected for further examination.

The clones examined showed a two- to fourfold increase in GFP expression in response to theophylline. Generally, there were no obvious trends observed that related the sequence of these constructs to their activity. We wanted to see if a second round of this selection process would yield a construct with a more significant theophylline response. The plasmid 6E12 exhibited the highest fold induction from round one and so it was chosen to be the basis of a new library for a second round of selection. At each of the 14 positions in the linker region, the new library had a 76% chance of retaining the 6E12 sequence, and an 8% chance of being mutated to each of the other three possible nucleotides.

The second selection was carried out in a similar manner to the first. The FACS spectrum for the first sort looked similar to that observed in the first round (Figure 2C), however a greater fraction of clones exhibited a high level of fluorescence. The spectrum for the second sort was much more consistent with what we predicted than in the first round (Figure 2D), and only a small percentage of cells passed our more stringent criteria. Follow-up 96-well plate fluorescence assays yielded a much higher proportion of theophylline-responsive clones than the first round; this indicates that the more stringent FACS criteria were beneficial. Also, several riboswitches with fold-induction values that exceeded the 6E12 riboswitch were uncovered; the most efficient of these was RS11A. The sequence of RS11A in the original random portion (blue nucleotides in Figure 1B) was ACTATATGAAGAGG and the remainder of the sequence (including the aptamer region, Figure 1B) did not incur any unexpected mutations.

Fluorescence assays both in the presence and absence of theophylline for 6E12, RS11A and RS11A with a point mutant that disrupted theophylline binding (C27A) are shown in Figure 3A. These data show that RS11A was nearly twice as efficient as its predecessor 6E12, and it owed its improved induction to a lower level of uninduced expression. The C27A mutant of RS11A showed no theophylline response; this indicates that aptamer binding was indeed responsible for the induction of GFP expression and implies a riboswitch-based mechanism of regulation. Figure 3B demonstrates the selectivity of RS11A for theophylline over caffeine—a close structural relative. It also demonstrates that the riboswitch was responsive to theophylline over a wide concentration range, up to 2 mM. Concentrations greater than 2 mM were not tested as they were detrimental to cell growth. It should be noted that

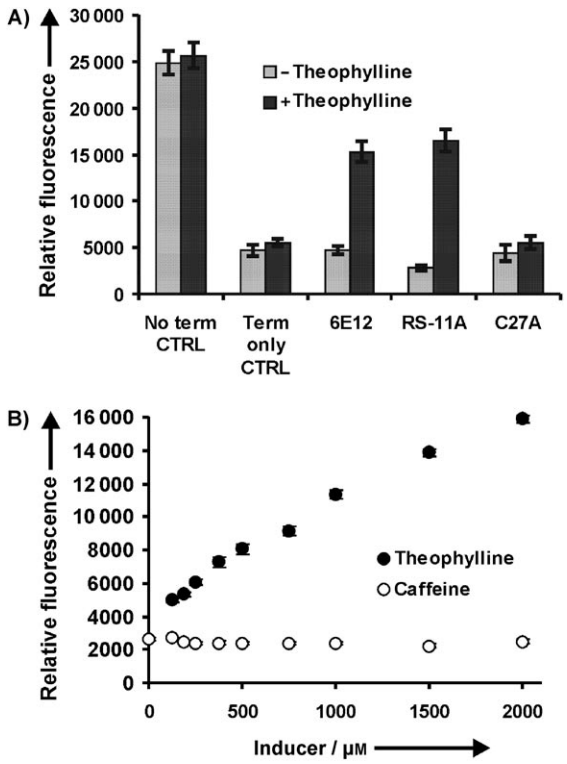

Figure 3. Performance of some selected riboswitches. A) Fluorescence assays of the top riboswitch candidates isolated from each round of selection. No term CTRL: construct that lacked the entire aptamer–linker–terminator region; Term only CTRL: construct with the MetI transcriptional terminator, but no aptamer and linker region. B) Fluorescence of RS11A in response to changes in the concentrations of theophylline and caffeine.

previous studies have shown that the intracellular concentration of theophylline in cultured *E. coli* is far lower than the extracellular concentration, presumably due to efflux.[39] This explains the high media concentration of inducer required to elicit a maximal response. The same phenomenon has been previously observed by other groups.[35,36]

We next sought to examine whether or not RS11A was acting at the level of transcription, as was intended by our library design. As a first step in this process we identified a potential antiterminator stem in RS11A that could compete with the formation of the terminator (Figure 4A). It is interesting to note that the putative antiterminator stem of RS11A was shifted slightly from the wild-type MetI antiterminator, upon which the original library was based. To determine whether RS11A used this terminator/antiterminator mechanism, we mutated the polyU tract that follows the terminator (Figure 4B). While these residues should be crucial for terminator function, they should not have a significant effect on RNA folding. As a control we mutated the polyU tract of the MetI terminator in a construct that lacked the aptamer and the linker region of RS11A. This mutation significantly disrupted terminator function. To our surprise, however, RS11A was completely unaffected by the polyU mutation. This implies that the antiterminator efficiently prevented the formation of the terminator both in the presence and absence of theophylline, and that RS11A

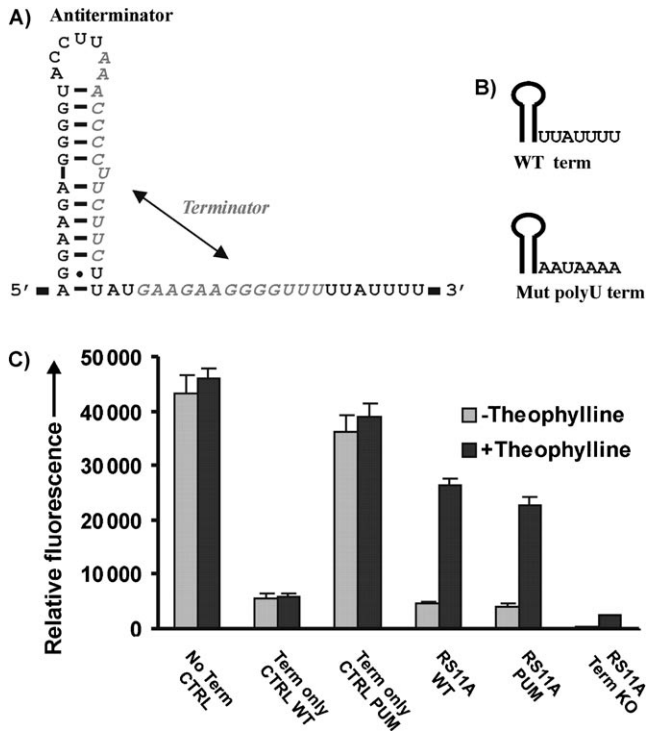

Figure 4. Probing the regulation mechanism of the RS11A riboswitch. A) Predicted antiterminator stem of RS11A. Residues shown in grey and italic letters represent the stem of the MetI terminator. B) Schematic representation of the wild-type and polyU mutant transcriptional terminators used to examine the RS11A mechanism. C) Fluorescence assay that compare various riboswitch constructs and controls and their response to theophylline; the controls are described in the legend of Figure 3. WT: construct with a wild-type MetI terminator; PUM: construct that had a MetI terminator with a mutated polyU tract; RS11A Term KO: an RS11A construct that completely lacked the MetI transcriptional terminator.

does not exert its effect through transcriptional termination (Figure 4C).

To further examine the role of the terminator, we made a construct that completely removed the terminator, but maintained the selected RS11A linker sequence. It was difficult to anticipate the effects of such a deletion, as it involved the removal a large chunk of sequence from the middle of the 5′-UTR. Interestingly, this construct retained its switching ability (sevenfold induction), but the level of expression (both induced and uninduced) fell dramatically. This implies that the removed sequence might not be involved in riboswitch activity, but that the distance of the riboswitch from translational signals is important for expression levels. The next obvious possibility for the RS11A mechanism was ribosome binding site sequestration/antisequestration[14]—the other predominant mechanism exploited in naturally occurring riboswitches. By examining the sequence manually and by using structure-prediction programs, a few possibilities for such structures were noted, but subsequent experiments dismissed these options.

To further probe the mechanism we performed in-line probing on the RS11A 5′-UTR, a method that probes for structural changes in RNA in response to ligand binding. Although some changes were noted in the aptamer and linker regions, the data did not provide a clear indication of mechanism (see the Supporting Information). Several groups have previously shown ligand-responsive expression in response to cloning an aptamer immediately upstream of a gene.[35,40–42] While the precise mechanism has not always been clear, it is evident that in the correct context the structural changes caused by aptamer–ligand binding can have a significant effect on neighbouring gene expression. For RS11A, we have selected for a sequence, and presumably an RNA structure, that places the aptamer in a suitable context to allow for theophylline-activated gene expression. This could perhaps occur through the ribosome's ability to access the different structures or at the level of RNA stability, although this is entirely speculative.

In summary, we have demonstrated a novel means of creating synthetic riboswitches that relies on FACS and fluorescence assays to isolate active riboswitches from a library. We believe that this general scheme offers some distinct advantages in engineering riboswitches in *E. coli*. Firstly, by selecting and screening for the best sequences, there is less reliance on clever engineering. Such designs are not usually generally applicable and can require a structural understanding of the aptamer that is not always available. FACS analysis allows for millions of individual cells to be sorted in less than an hour, which makes this strategy amenable to relatively high throughput selections. FACS also allows the user to choose gate settings that make it possible to fine-tune the stringency for each round of selection, and ultimately to target whatever expression levels they hope to achieve. It should be noted, however, that this method is limited with respect to the size of library that can be sorted. While high-efficiency cloning strategies can allow for much larger libraries to be produced than that used in this study, libraries that approach the size commonly used for in vitro experiments ($\sim 10^{15}$) are not obtainable and could not be sorted with this method. While sorting does allow for much more flexibility than systems that are strictly rationally designed, this method still requires a certain degree of engineering.

This proof-of-principle effort targeted a transcriptional-termination mechanism, although our data indicate that this is not what was obtained. We believe this to be a function of the library we used, as it is entirely possible that such a mechanism was not achievable within the confines of our design. The unexpected nature of what we isolated reinforces the advantages of selection over strict engineering for elements such as this and shows the power of the FACS method employed here. Application of this method to sort libraries that more efficiently target a given mechanism would presumably yield more efficient riboswitches. This could be achieved by more thoroughly designing a transcriptional-termination mechanism that includes carefully designed aptamer and terminator stems by using random elements to fine-tune the system. Alternatively, translational initiation is likely much simpler to target as only a few bases of sequence (the ribosome binding site) need to be structurally affected by ligand binding. Recently published work that uses such a library design demonstrated impressive induction levels and further supports this idea.[25] One advantage of riboswitches that work by transcriptional termination,

however, is that multiple copies of these elements could potentially be used to obtain very tightly regulated systems. The concept of multiple riboswitches that control the expression of a single gene has been exploited in natural systems.[21,43]

In addition to de novo creation of riboswitches, we feel that this method has the potential to be used for modifying those that have evolved naturally. Evolution has provided these elements with exquisite ligand-recognition characteristics and gene-regulation capabilities. This makes natural riboswitches a great starting point for the development of tools for synthetic biology. However, in many cases it is desirable to modify these riboswitches to exhibit different ligand specificity, to be responsive over a different ligand concentration range or to exhibit more or less tightly regulated expression. The speed and high-throughput capacity of the method demonstrated here make it an ideal choice for such endeavours.

Experimental Section

Growth media, strains, plasmids and molecular cloning: All cells were grown in Luria–Bertani (LB) broth supplemented with ampicillin (50 $\mu g\,mL^{-1}$). Library transformations were done by using *E. coli* DH5α max efficiency competent cells (Invitrogen); this strain was also used for FACS and 96-well fluorescence assays. All other experiments were carried out by using Nova blues (Novagen) *E. coli* cells. The pRB374 plasmid was used as the backbone for all constructs described. Sequence and detailed cloning information are provided as the Supporting Information.

All molecular cloning, including restriction digests, ligations, transformations, DNA phosphorylation and second-strand synthesis were carried out according to well established, standard protocols by following supplier instructions. Cloning of transcriptional terminators and library sequences was done by using chemically synthesized DNA that encoded the desired sequence (Mobix lab, McMaster University; Integrated DNA Technologies, Coralville, IA, USA). In some cases, two complementary DNA oligonucleotides with suitable overhangs for cloning into the appropriate restriction sites were annealed, phosphorylated by using T4 polynucleotide kinase (PNK) and ligated into the digested plasmid. For other constructs, DNA oligonucleotides (with the desired sequence flanked on either side by suitable restriction sites) were made into a double-stranded form by using a short primer and Klenow DNA polymerase, followed by restriction digestion and ligations. The sequences of the 6E12 and RS11A linker regions were 5′-ACTATATGAAGAGG-3′ and 5′-ATTGAAGGAAGAGG-3′, respectively.

Fluorescence activated cell sorting (FACS) and colony isolation: Prior to cell sorting, *E. coli* cells were washed several times with PBS and resuspended in PBS at a concentration of approximately $10^7\,cells\,mL^{-1}$. FACS was carried out by using a FACSVantage SE™ flow cytometry system (BD Biosciences). A 488 nm laser was used for excitation, and fluorescence was detected by using a $530\pm$ 15 nm band pass filter. Sorting rates that ranged from 800–12000 events per second were used, depending on the requirements of the particular experiment.

For the first round of selection, transformed cells were grown at 37 °C for ~6 h in media supplemented with theophylline (2 mM; Sigma) prior to being sorted. Positive cells were collected and stored (diluted in several volumes of growth media) at 4 °C for ~6 h. They were then grown at 37 °C in media that lacked theophylline for ~10 h prior to the second phase of FACS. The cells were diluted and plated on agar to yield ~100 cells per plate. Individual colonies were picked manually, grown, overnight, at 37 °C and stored as frozen stocks in LB supplemented with glycerol (15%). The second round of sorting was carried out in the same manner as described above, except that cells were grown at 37 °C for only 5 h (rather than 10 h) prior to the second phase of FACS. This was done to minimize selective growth advantages and other anomalies associated with long periods of bacterial cell growth.

Fluorescence assays: High-throughput cell assays were carried out in 96-well, clear polypropylene plates (Corning). Duplicate plates were prepared by pinning, and allowed to grow for approximately 24 h at 37 °C, one plate with theophylline and an identical plate without. Fluorescence for both sets of plates was measured by using a Tecan Safire fluorometer spectrophotometer with excitation/emission set at 488/517 nm. The values were normalized by subsequently measuring absorbance at 600 nm (OD_{600}) for both sets of plates. Colonies were chosen for examination based on the ratio of the corrected fluorescence values in the presence and absence of theophylline.

Low-throughput assays were carried out by picking individual colonies from plates of cells that harboured the plasmid of interest. These colonies were grown, overnight, at 37 °C, and diluted 1:1000 in fresh media supplemented with the indicated concentration of inducer. After 7 h of growth at 37 °C the OD_{600} was measured for each sample and approximately 3 mL of culture was centrifuged (the centrifuged volume was varied to correct for differences in OD_{600}) and the pellet was resuspended in PBS. The resuspension process led to the cultures being concentrated ~eightfold (from ~3 mL to 400 μL). This was done to optimize the signal strength to provide the most accurate possible data. A fraction of the resuspended cells (100 μL) was added to half-area, solid black 96-well plates (Corning) and measured for fluorescence as described above. Unless otherwise stated, the concentration of theophylline used for all experiments was 2 mM.

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[1] D. H. Bunka, P. G. Stockley, *Nat. Rev. Microbiol.* **2006**, *4*, 588–596.
[2] J. Liu, Y. Lu, *Methods Mol. Biol.* **2006**, *335*, 275–288.
[3] S. Hahn, S. Mergenthaler, B. Zimmermann, W. Holzgreve, *Bioelectrochemistry* **2005**, *67*, 151–154.
[4] D. Hutanu, V. T. Remcho, *Adv. Chromatogr.* **2007**, *45*, 173–196.
[5] S. Tombelli, M. Minunni, M. Mascini, *Biosens. Bioelectron.* **2005**, *20*, 2424–2434.
[6] U. Feldkamp, C. M. Niemeyer, *Angew. Chem.* **2006**, *118*, 1888–1910; *Angew. Chem. Int. Ed.* **2006**, *45*, 1856–1876.
[7] P. Guo, *J. Nanosci. Nanotechnol.* **2005**, *5*, 1964–1982.

[8] J. F. Lee, G. M. Stovall, A. D. Ellington, *Curr. Opin. Chem. Biol.* **2006**, *10*, 282–289.
[9] C. R. Ireson, L. R. Kelland, *Mol. Cancer. Ther.* **2006**, *5*, 2957–2962.
[10] A. D. Ellington, J. W. Szostak, *Nature* **1990**, *346*, 818–822.
[11] C. Tuerk, L. Gold, *Science* **1990**, *249*, 505–510.
[12] A. Serganov, D. J. Patel, *Nat. Rev. Genet.* **2007**, *8*, 776–790.
[13] J. S. Mattick, I. V. Makunin, *Hum. Mol. Genet.* **2006**, *15 Spec. No. 1*, R17–29.
[14] W. Winkler, A. Nahvi, R. R. Breaker, *Nature* **2002**, *419*, 952–956.
[15] A. Nahvi, N. Sudarsan, M. S. Ebert, X. Zou, K. L. Brown, R. R. Breaker, *Chem. Biol.* **2002**, *9*, 1043.
[16] N. Sudarsan, J. K. Wickiser, S. Nakamura, M. S. Ebert, R. R. Breaker, *Genes Dev.* **2003**, *17*, 2688–2697.
[17] W. C. Winkler, S. Cohen-Chalamish, R. R. Breaker, *Proc. Natl. Acad. Sci. USA* **2002**, *99*, 15908–15913.
[18] A. Roth, W. C. Winkler, E. E. Regulski, B. W. Lee, J. Lim, I. Jona, J. E. Barrick, A. Ritwik, J. N. Kim, R. Welz, D. Iwata-Reuyl, R. R. Breaker, *Nat. Struct. Mol. Biol.* **2007**, *14*, 308–317.
[19] C. E. Dann III, C. A. Wakeman, C. L. Sieling, S. C. Baker, I. Irnov, W. C. Winkler, *Cell* **2007**, *130*, 878–892.
[20] M. Mandal, R. R. Breaker, *Nat. Struct. Mol. Biol.* **2004**, *11*, 29–35.
[21] M. Mandal, M. Lee, J. E. Barrick, Z. Weinberg, G. M. Emilsson, W. L. Ruzzo, R. R. Breaker, *Science* **2004**, *306*, 275–279.
[22] W. C. Winkler, A. Nahvi, N. Sudarsan, J. E. Barrick, R. R. Breaker, *Nat. Struct. Biol.* **2003**, *10*, 701–707.
[23] B. J. Tucker, R. R. Breaker, *Curr. Opin. Struct. Biol.* **2005**, *15*, 342–348.
[24] S. Topp, J. P. Gallivan, *J. Am. Chem. Soc.* **2007**, *129*, 6807–6811.
[25] S. A. Lynch, S. K. Desai, H. K. Sajja, J. P. Gallivan, *Chem. Biol.* **2007**, *14*, 173–184.
[26] Y. Nomura, Y. Yokobayashi, *J. Am. Chem. Soc.* **2007**, *129*, 13814–13815.
[27] S. van den Berg, P. A. Löfdahl, T. Härd, H. Berglund, *J. Biotechnol.* **2006**, *121*, 291–298.
[28] B. P. Cormack, R. H. Valdivia, S. Falkow, *Gene* **1996**, *173*, 33–38.
[29] A. Aharoni, K. Thieme, C. P. Chiu, S. Buchini, L. L. Lairson, H. Chen, N. C. Strynadka, W. W. Wakarchuk, S. G. Withers, *Nat. Methods* **2006**, *3*, 609–614.
[30] S. W. Santoro, L. Wang, B. Herberich, D. S. King, P. G. Schultz, *Nat. Biotechnol.* **2002**, *20*, 1044–1048.
[31] P. Gollnick, P. Babitzke, *Biochim. Biophys. Acta Gene Struct. Expression* **2002**, *1577*, 240–250.
[32] E. Nudler, M. E. Gottesman, *Genes Cells* **2002**, *7*, 755–768.
[33] R. D. Jenison, S. C. Gill, A. Pardi, B. Polisky, *Science* **1994**, *263*, 1425–1429.
[34] G. R. Zimmermann, R. D. Jenison, C. L. Wick, J. P. Simorre, A. Pardi, *Nat. Struct. Biol.* **1997**, *4*, 644–649.
[35] S. K. Desai, J. P. Gallivan, *J. Am. Chem. Soc.* **2004**, *126*, 13247–13254.
[36] B. Suess, B. Fink, C. Berens, R. Stentz, W. Hillen, *Nucleic Acids Res.* **2004**, *32*, 1610–1614.
[37] J. K. Wickiser, M. T. Cheah, R. R. Breaker, D. M. Crothers, *Biochemistry* **2005**, *44*, 13404–13414.
[38] J. K. Wickiser, W. C. Winkler, R. R. Breaker, D. M. Crothers, *Mol. Cell* **2005**, *18*, 49–60.
[39] A. L. Koch, *J. Biol. Chem.* **1956**, *219*, 181–188.
[40] G. Werstuck, M. R. Green, *Science* **1998**, *282*, 296–298.
[41] I. Harvey, P. Garneau, J. Pelletier, *RNA* **2002**, *8*, 452–463.
[42] B. Suess, S. Hanson, C. Berens, B. Fink, R. Schroeder, W. Hillen, *Nucleic Acids Res.* **2003**, *31*, 1853–1858.
[43] N. Sudarsan, M. C. Hammond, K. F. Block, R. Welz, J. E. Barrick, A. Roth, R. R. Breaker, *Science* **2006**, *314*, 300–304.

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