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Genetic Screens and Selections for Small Molecules Based on a Synthetic Riboswitch That Activates Protein Translation

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Abstract: Genetic selection provides the most powerful method to assay large libraries of biomolecules for function. However, harnessing the power of genetic selection for the detection of specific, nonendogenous small-molecule targets in vivo remains a significant challenge. The ability to genetically select for small molecules would provide a reaction-independent mechanism to clone biosynthesis genes from large DNA libraries and greatly facilitate the exploration of large libraries of mutant enzymes for improved synthetic capabilities including altered substrate specificities and enhanced regio- or stereoselectivities. While remarkable progress has been made in developing genetic methods to detect small molecules in vivo, many of these methods rely on engineering small-molecule−protein interactions which remains a difficult problem, and the potential for some of these systems to assay large libraries is limited by the low transformation efficiency and long doubling time of yeast relative to bacteria. Herein, we demonstrate that synthetic riboswitches that activate protein translation in response to a specific small molecule can be used to perform sensitive genetic screens and selections for the presence of small molecules in *Escherichia coli*. We further demonstrate that the exquisite molecular discrimination properties of aptamers selected in vitro translate directly into an in vivo genetic selection system. Finally, we demonstrate that a cell harboring a synthetic riboswitch with a particular ligand specificity can be selectively amplified from a million-fold larger pool of cells containing mutant riboswitches that respond to a closely related ligand, suggesting that it is possible to use genetic selection in *E. coli* to discover synthetic riboswitches with new ligand specificities from libraries of mutant riboswitches.

Introduction

The remarkable diversity of small-molecule natural products is the result of billions of years of natural selection processes operating on biosynthesis genes. In recent years, great strides have been made in cloning the genes involved in natural product biosynthesis[1−3] and in applying the principles of Darwinian selection in the laboratory to evolve new biocatalysts that show altered substrate specificities, regioselectivities, and enhanced enantioselectivities.[4−8] Directed evolution experiments critically depend on the ability to generate molecular diversity and efficiently screen this diversity for the desired function.[9−11] While there are many effective methods for generating molecular diversity, including error-prone PCR,[12,13] DNA shuffling,[14−16] staggered extension,[17] incremental truncation,[18−20] as well as several others,[21−23] there are considerably fewer general methods of screening or selecting molecular diversity for desired biocatalytic function in cells.[9,11] The most common method for detecting biocatalytic function is high-throughput screening.[9,11] Typically, reactions are chosen such that either the product or the byproduct of a desired reaction produces a measurable change in absorbance[24] or fluorescence.[8,25] While screening methods are powerful, even the best high-throughput screening

(1) Ahlert, J.; Shepard, E.; Lomovskaya, N.; Zazopoulos, E.; Staffa, A.; Bachmann, B. O.; Huang, K.; Fonstein, L.; Czisny, A.; Whitwam, R. E.; Farnet, C. M.; Thorson, J. S. *Science* **2002**, *297*, 1173−6.
(2) Tang, L.; Shah, S.; Chung, L.; Carney, J.; Katz, L.; Khosla, C.; Julien, B. *Science* **2000**, *287*, 640−2.
(3) Cane, D. E.; Walsh, C. T.; Khosla, C. *Science* **1998**, *282*, 63−68.
(4) Arnold, F. H.; Volkov, A. A. *Curr. Opin. Chem. Biol.* **1999**, *3*, 54−9.
(5) Farinas, E. T.; Bulter, T.; Arnold, F. H. *Curr. Opin. Biotechnol.* **2001**, *12*, 545−51.
(6) Liebeton, K.; Zonta, A.; Schimossek, K.; Nardini, M.; Lang, D.; Dijkstra, B. W.; Reetz, M. T.; Jaeger, K. E. *Chem. Biol.* **2000**, *7*, 709−18.
(7) Reetz, M. T. *Angew. Chem., Int. Ed. Engl.* **2001**, *40*, 284−310.
(8) Joo, H.; Lin, Z.; Arnold, F. H. *Nature* **1999**, *399*, 670−3.
(9) Cohen, N.; Abramov, S.; Dror, Y.; Freeman, A. *Trends Biotechnol.* **2001**, *19*, 507−510.
(10) Lin, H.; Cornish, V. W. *Angew. Chem., Int. Ed. Engl.* **2002**, *41*, 4402−25.
(11) Olsen, M.; Iverson, B.; Georgiou, G. *Curr. Opin. Biotechnol.* **2000**, *11*, 331−7.
(12) Cadwell, R. C.; Joyce, G. F. *PCR Methods Appl.* **1994**, *3*, S136−40.
(13) Cadwell, R. C.; Joyce, G. F. *PCR Methods Appl.* **1992**, *2*, 28−33.
(14) Stemmer, W. P. C. *Proc. Natl. Acad. Sci. U.S.A.* **1994**, *91*, 10747−10751.
(15) Stemmer, W. P. C. *Nature* **1994**, *370*, 389−391.
(16) Crameri, A.; Raillard, S. A.; Bermudez, E.; Stemmer, W. P. C. *Nature* **1998**, *391*, 288−291.
(17) Zhao, H.; Giver, L.; Shao, Z.; Affholter, J. A.; Arnold, F. H. *Nat. Biotechnol.* **1998**, *16*, 258−61.
(18) Ostermeier, M.; Shim, J. H.; Benkovic, S. J. *Nat. Biotechnol.* **1999**, *17*, 1205−9.
(19) Lutz, S.; Ostermeier, M.; Moore, G. L.; Maranas, C. D.; Benkovic, S. J. *Proc. Natl. Acad. Sci. U.S.A.* **2001**, *98*, 11248−53.
(20) Ostermeier, M.; Nixon, A. E.; Shim, J. H.; Benkovic, S. J. *Proc. Natl. Acad. Sci. U.S.A.* **1999**, *96*, 3562−7.
(21) Coco, W. M.; Levinson, W. E.; Crist, M. J.; Hektor, H. J.; Darzins, A.; Pienkos, P. T.; Squires, C. H.; Monticello, D. J. *Nat. Biotechnol.* **2001**, *19*, 354−9.
(22) Hiraga, K.; Arnold, F. H. *J. Mol. Biol.* **2003**, *330*, 287−96.
(23) Volkov, A. A.; Shao, Z.; Arnold, F. H. *Nucleic Acids Res.* **1999**, *27*, e18.
(24) Moore, J. C.; Arnold, F. H. *Nat. Biotechnol.* **1996**, *14*, 458−467.
(25) Gutierrez, M. C.; Sleegers, A.; Simpson, H. D.; Alphand, V.; Furstoss, R. *Org. Biomol. Chem.* **2003**, *1*, 3500−6.

methods are limited by current instrumentation, and the library sizes that may be assayed in cells are typically limited to fewer than 10^6 members in a given experiment.[11]

The most powerful method for detecting function within large libraries of biomolecules in cells is genetic selection.[26,27] In a genetic selection experiment the survival of a cell is either directly or indirectly linked to the desired phenotype. As such, at the end of a genetic selection experiment, all of the surviving cells carry the desired trait. Because cells that do not display the desired trait do not survive, this type of stringent test allows extremely large libraries ($>10^8$ members) to be assayed for function.[26,27] The ability to select large libraries for function is particularly useful in the context of directed evolution experiments where the available sequence space is enormous and beneficial mutations are rare.[10] However, because genetic selections are typically limited to discovering enzymes that either complement a specific auxotrophy (such as a deficiency in amino acid biosynthesis)[28,29] or confer resistance to a toxin (such as antibiotic resistance),[16,30] their use in the discovery of biocatalysts through directed evolution has been limited.

To overcome these limitations we are interested in developing a genetic selection method that couples the life of a bacterium to the presence of a small molecule of our choosing. We envision that such a method will be useful in several contexts: for example, bacteria that depend on the presence of a small molecule for survival could potentially be used as selectable hosts in which to heterologously clone biosynthetic genes and pathways that are capable of producing the desired target molecule. Since these cells would depend only on the presence of the small-molecule product and not on the way in which it was produced, such a selection would be reaction independent and could, in principle, be used to discover a wide variety of biosynthesis enzymes, even if their reaction mechanisms were unknown. Furthermore, such bacteria could also be used as selectable hosts in directed evolution experiments in which the goal is to improve the selectivity or activity of a known biocatalyst. Since genetic selections are powerful and the transformation efficiencies of bacteria are high (in some cases, up to 10^{10} variants can be assayed simultaneously),[26] such a system could be useful for selecting rare catalytic function from large mutant libraries.

Fundamentally, using a genetic method to detect the presence of a small-molecule target requires a mechanism for sensing the target within a cell and a mechanism to transduce this sensing event into a cellular response, such as a change in gene expression. While remarkable progress has been made in developing genetic methods to sense small-molecule targets in cells,[31−34] many of these methods rely on engineering small-molecule−protein interactions, which remains a challenging problem. In a pioneering experiment, Werstuck and Green demonstrated that an RNA aptamer sequence originally selected to bind to a small molecule in vitro could subsequently be cloned in the 5′-untranslated region of a gene and used to regulate mRNA translation in a small-molecule-dependent fashion in eukaryotic cells.[35] This study and others[36−40] presaged more recent discoveries that bacterial mRNAs contain sequences that bind small-molecule metabolites to regulate gene expression at either the transcriptional or the translational level.[41−46] These "riboswitches" are reminiscent of artificial RNA aptamers that tightly and selectively bind to small molecules in vitro,[47,48] and their existence suggests that synthetic riboswitches based on in-vitro-generated aptamer sequences may be used to control gene expression in a small-molecule-dependent fashion. While others have demonstrated that artificial riboswitches can be used to control protein translation,[36−40] the potential of these systems to perform genetic selections for small molecules in cells remains unexplored.

We now report a synthetic riboswitch that activates protein translation in *Escherichia coli* upon binding the small-molecule theophylline, and we demonstrate that this switch can be applied in both genetic screens and selections to detect the presence of theophylline. We further demonstrate that the molecular recognition properties of an aptamer generated in vitro can be transferred into an in vivo genetic assay that couples the life of a bacterium to the presence of a specific, nonendogenous small molecule. Taken together, these results suggest a new strategy for detecting the small-molecule products of biocatalytic transformations in *E. coli*. In addition, we demonstrate the potential of in vivo genetic selection methods to enable the discovery of synthetic riboswitches that respond to different small-molecule targets in *E. coli*.

Experimental Section

General Considerations. All plasmid manipulations were performed according to standard cloning techniques,[49] and the sequences of all constructs have been verified by DNA sequencing at the NSF-supported Center for Fundamental and Applied Molecular Evolution at Emory University. Descriptions of the plasmids appear in the Supporting Information. Purification of plasmid DNA, PCR products, and enzyme digestions was performed using kits from Qiagen. Caffeine, theophylline, 3-methylxanthine, *o*-nitrophenyl-β-D-galactopyranoside, ampicillin, and chloramphenicol were purchased from Sigma. IPTG and X-gal were purchased from US Biological. Synthetic oligonucleotides were purchased from IDT. All experiments were performed in *E. coli* TOP10 cells (Invitrogen) cultured in media obtained from Difco.

(26) Woycechowsky, K. J.; Hilvert, D. *Eur. J. Biochem.* **2004**, *271*, 1630−7.
(27) Taylor, S. V.; Kast, P.; Hilvert, D. *Angew. Chem., Int. Ed. Engl.* **2001**, *40*, 3310−3335.
(28) Oue, S.; Okamoto, A.; Yano, T.; Kagamiyama, H. *J. Biol. Chem.* **1999**, *274*, 2344−2349.
(29) Yano, T.; Oue, S.; Kagamiyama, H. *Proc. Natl. Acad. Sci. U.S.A.* **1998**, *95*, 5511−5.
(30) Yano, T.; Kagamiyama, H. *Proc. Natl. Acad. Sci. U.S.A.* **2001**, *98*, 903−7.
(31) Azizi, B.; Chang, E. I.; Doyle, D. F. *Biochem. Biophys. Res. Commun.* **2003**, *306*, 774−80.
(32) Baker, K.; Bleczinski, C.; Lin, H.; Salazar-Jimenez, G.; Sengupta, D.; Krane, S.; Cornish, V. W. *Proc. Natl. Acad. Sci. U.S.A.* **2002**, *99*, 16537−42.
(33) Firestine, S. M.; Salinas, F.; Nixon, A. E.; Baker, S. J.; Benkovic, S. J. *Nat. Biotechnol.* **2000**, *18*, 544−7.
(34) Looger, L. L.; Dwyer, M. A.; Smith, J. J.; Hellinga, H. W. *Nature* **2003**, *423*, 185−90.
(35) Werstuck, G.; Green, M. R. *Science* **1998**, *282*, 296−8.
(36) Grate, D.; Wilson, C. *Bioorg. Med. Chem.* **2001**, *9*, 2565−70.
(37) Harvey, I.; Garneau, P.; Pelletier, J. *RNA* **2002**, *8*, 452−463.
(38) Hanson, S.; Berthelot, K.; Fink, B.; McCarthy, J. E.; Suess, B. *Mol. Microbiol.* **2003**, *49*, 1627−37.
(39) Suess, B.; Fink, B.; Berens, C.; Stentz, R.; Hillen, W. *Nucleic Acids Res.* **2004**, *32*, 1610−4.
(40) Suess, B.; Hanson, S.; Berens, C.; Fink, B.; Schroeder, R.; Hillen, W. *Nucleic Acids Res.* **2003**, *31*, 1853−8.
(41) Miranda-Rios, J.; Navarro, M.; Soberon, M. *Proc. Natl. Acad. Sci. U.S.A.* **2001**, *98*, 9736−9741.
(42) Nou, X.; Kadner, R. J. *Proc. Natl. Acad. Sci. U.S.A.* **2000**, *97*, 7190−7195.
(43) Nudler, E.; Mironov, A. S. *Trends Biochem. Sci.* **2004**, *29*, 11−7.
(44) Mandal, M.; Breaker, R. R. *Nat. Rev. Mol. Cell Biol.* **2004**, *5*, 451−63.
(45) Winkler, W. C.; Breaker, R. R. *ChemBioChem* **2003**, *4*, 1024−32.
(46) Winkler, W.; Nahvi, A.; Breaker, R. R. *Nature* **2002**, *419*, 952−6.
(47) Famulok, M. *Curr. Opin. Struct. Biol.* **1999**, *9*, 324−329.
(48) Wilson, D. S.; Szostak, J. W. *Annu. Rev. Biochem.* **1999**, *68*, 611−47.
(49) Sambrook, J.; Russell, D. W. *Molecular Cloning: A Laboratory Manual*, 3rd ed.; Cold Spring Harbor Laboratory Press: Cold Spring Harbor, NY, 2001.

Plate-Based Assays for β-Galactosidase Activity. LB/agar plates (100 mm diameter) containing ampicillin (50 μg/mL) and supplemented with either no small molecule, caffeine (500 μM), or theophylline (500 μM) were spread with 9 μL of a 20% IPTG solution and 50 μL of a 2% solution of X-gal in DMF and allowed to dry at 37 °C for 2 h. The wild-type synthetic riboswitch plasmid (pSKD177.2) was introduced into *E. coli* TOP10 cells by electroporation; the cells were allowed to recover in SOC media (500 μL) for 20 min at 37 °C. An aliquot (10 μL) was spread onto the above LB plates, and the cells were grown for 14 h at 37 °C, followed by incubation at 4 °C until blue colony color was readily visible on the theophylline-supplemented plate (48−96 h).

Quantitative Assays for β-Galactosidase Activity. Three separate colonies of *E. coli* TOP10 cells harboring either the no-aptamer (pLacZU1hpII), wild-type synthetic riboswitch (pSKD177.2), C27A synthetic riboswitch (pSKD185.1), or transcriptional fusion (pSKD323.1) plasmid were picked from an LB/agar plate containing ampicillin (50 μg/mL) and grown overnight at 37 °C in separate culture tubes containing LB-media (5 mL) supplemented with ampicillin (50 μg/mL). A 5 μL aliquot of the overnight cultures was used to inoculate 1.5 mL of LB-media supplemented with ampicillin (50 μg/mL) and, if necessary, appropriate concentrations of caffeine, theophylline, or 3-methylxanthine. Cells were grown at 37 °C with shaking to an OD_{600} of 0.3−0.5, and β-galactosidase activity was assayed spectrophotometrically by monitoring the hydrolysis of *o*-nitrophenyl-β-D-galactopyranoside following the protocol of Jain and Belasco.[50] All assays were conducted in triplicate, and data are presented as the mean ± s.e.m.

Plate-Based Chloramphenicol Resistance Assays. LB/agar plates containing ampicillin (50 μg/mL) and varying concentrations of chloramphenicol and either no small molecule, caffeine (500 μM), 3-methylxanthine (500 μM), or theophylline (500 μM) were streaked with *E. coli* TOP10 cells from a glycerol stock harboring the chloramphenicol-selectable wild-type synthetic riboswitch plasmid (pSKD314). Cells were grown for 18 h at 37 °C, and growth was assayed by visual inspection. Resistance assays were conducted in triplicate.

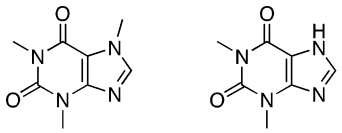
Solution-Based Chloramphenicol Resistance Assays. Three separate colonies of *E. coli* TOP10 cells harboring the chloramphenicol-selectable wild-type synthetic riboswitch plasmid (pSKD314) or the chloramphenicol-selectable C27A synthetic riboswitch plasmid (pSKD441.1) from an LB/agar plate containing ampicillin (50 μg/mL) were grown overnight at 37 °C with shaking in separate culture tubes containing LB media (5 mL) supplemented with ampicillin (50 μg/mL). Aliquots of 100 μL of a 1:1000 dilution of the overnight cultures in LB media were used to inoculate LB media to a final volume of 1 mL (10^5 cells per mL final concentration) supplemented with ampicillin (50 μg/mL) and varying concentrations of chloramphenicol and either no small molecule, caffeine (500 μM), 3-methylxanthine (500 μM), or theophylline (500 μM) in a 24-well plate (Costar). The culture plate was shaken at 215 rpm at 37 °C for 18 h, and OD_{600} readings were recorded for each well using a plate-reading spectrophotometer (BioTek). All growth studies were conducted in triplicate, and data are presented as the mean ± s.e.m.

Plate-Based Screening for a Functional Riboswitch, "Spiking Experiment". A 100 mm diameter LB/agar plate supplemented with ampicillin (50 μg/mL) and theophylline (1 mM) was spread with 20 μL of a 20% IPTG solution and 50 μL of a 2% solution of X-gal in DMF and allowed to dry at 37 °C for 2 h. The wild-type synthetic riboswitch plasmid (pSKD177.2) and C27A synthetic riboswitch plasmid (pSKD185.1) were electroporated as a 1 μL mixture into *E. coli* TOP10 cells. The ratios of the mixtures of the synthetic riboswitch plasmid (pSKD177.2) to the C27A synthetic riboswitch plasmid (pSKD185.1) were 1:1, 1:10, and 1:100 in a total volume of 1 μL, with the plasmid pSKD185.1 kept at a constant concentration of 100 ng/μL (~1.5×10^{10} plasmid molecules). The cells were allowed to recover in SOC (400 μL) for 15 min at 37 °C. An aliquot (1 μL) was diluted to 100 μL with SOC, 50 μL was spread on the above agar plates, and the cells were grown for 14 h at 37 °C, followed by incubation at 4 °C until blue colonies were readily visible (48−96 h). To determine the genotype of the blue and white colonies, individual colonies were cultured and their plasmids extracted and sequenced.

Plate-Based Selection for a Functional Riboswitch, "Spiking Experiment". The chloramphenicol-selectable wild-type synthetic riboswitch plasmid (pSKD314) and the chloramphenicol-selectable C27A synthetic riboswitch plasmid (pSKD441.1) were mixed, and 1 μL was used to electroporate 25 μL (~5×10^9 cells) *E. coli* TOP10 cells. The ratios of the plasmids pSKD314 to pSKD441.1 were 1:10^3, 1:10^4, 1:10^5, and 1:10^6 in a total volume of 1 μL with the plasmid pSKD441.1 remaining at a constant concentration of 100 ng/μL (~2.4×10^{10} plasmid molecules). The cells were allowed to recover in SOC (400 μL) for 1 h at 37 °C. A 50 μL aliquot of the recovery mixture was plated onto LB/agar plates supplemented with ampicillin (50 μg/mL). The cells were grown at 37 °C for 12 h, and then 1 mL of LB was spread onto the plates, and a sterile spreader was used to scrape the cells off the plate into the media. The LB−cell mixtures were mixed to 5 mL with LB supplemented with ampicillin (50 μg/mL) and grown at 37 °C until saturation (~10^9 cells/mL). To determine the total number of cells in the saturated culture, 1 μL of a 1:10^4 dilution of this culture was plated onto LB/agar containing ampicillin, the cells were grown at 37 °C for 12 h, and the colonies were counted. To select for the wild-type synthetic riboswitch, a 1−20 μL aliquot of the undiluted saturated culture was plated onto LB/agar plates supplemented with ampicillin (50 μg/mL), chloramphenicol (100 μg/mL), and theophylline (500 μM) such that 20−1000 colonies were found per plate after 18 h of growth at 37 °C. The ratios of the numbers of colonies on the chloramphenicol plates to those on the ampicillin-only plates were in all cases consistent with ratios of the original plasmids used in the electroporation. Plasmids were extracted from five colonies from each chloramphenicol selection plate and sequenced.

Results and Discussion

Creation of a Synthetic Riboswitch. We based our synthetic riboswitch on the previously described mTCT8-4 aptamer.[51] This aptamer binds theophylline tightly (K_d = 100 nM), discriminates against the structurally related caffeine by a factor of 10 000,[51] and is well characterized both biochemically[52] and structurally.[53] When cloned into the 5′-untranslated region (5′-UTR) of a reporter gene, this aptamer sequence has been shown to suppress protein translation in a theophylline-dependent fashion in eukaryotic translation extracts from wheat germ[37] or to activate expression of a reporter gene in a theophylline-dependent manner in the Gram-positive bacterium *Bacillus subtilis*.[39] However, there are no examples of synthetic riboswitches that operate in the Gram-negative bacterium *E. coli*. Since *E. coli* have high transformation efficiencies and well-understood genetics[49] and are the most commonly used host in directed evolution experiments,[4] we wished to test whether a synthetic riboswitch could be used for genetic control in *E. coli* in response to a specific small molecule.



caffeine ***theophylline***

(50) Jain, C.; Belasco, J. G. *Methods Enzymol.* **2000**, *318*, 309−332.

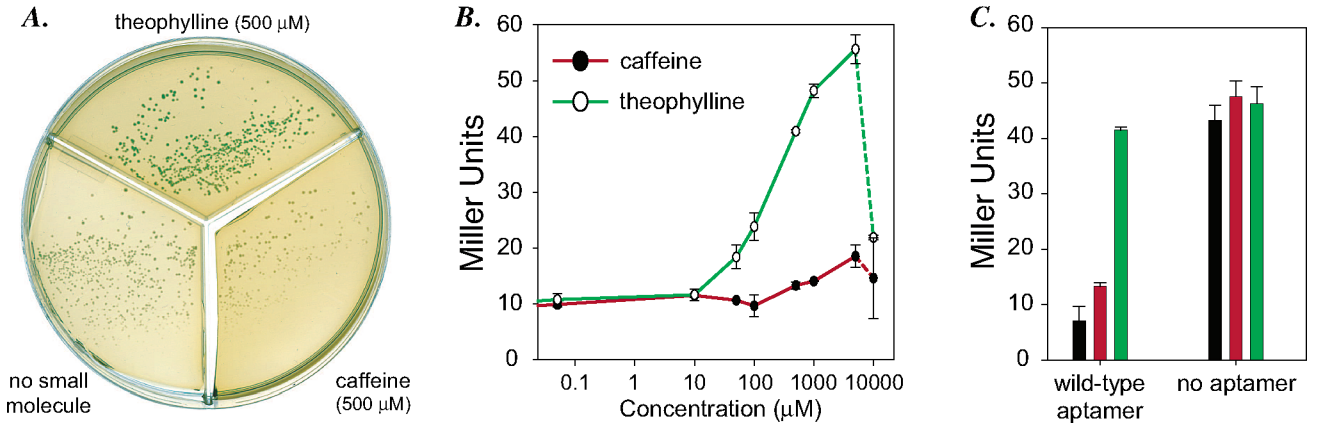


Figure 1. (A) Plate-based assay for β-galactosidase activity; color changes are clearly evident in response to theophylline but not caffeine. (B) Dose−response relationship for β-galactosidase activity (dashes indicate toxicity of the small molecules at very high concentrations). (C) β-Galactosidase activity in response to no small molecule (black), 500 μM caffeine (red), or 500 μM theophylline (green).

To create the synthetic riboswitch, we subcloned the mTCT8-4 aptamer sequence at a location five bp upstream of the ribosome binding site of the β-galactosidase reporter gene (IS10-*lac*Z) in the plasmid pLacZU1hpII.[50] This plasmid was originally designed to allow accurate measurement of small changes in β-galactosidase activity in vivo in response to proteins binding to sequences in the 5′-UTR, and it has many attractive features: Expression is tightly controlled at the transcriptional level by a weak IS10 promoter sequence, which is followed by translational terminators, a multiple cloning site in the 5′-UTR, a weak ribosome-binding site that limits translation, and a β-galactosidase gene fused to the C-terminus of a weakly translated IS10 transposase sequence.[50] These features allow accurate measurement of small changes in β-galactosidase activity in response to small-molecule binding in *E. coli*.[50]

Screening for Synthetic Riboswitch Activity. To test whether the synthetic riboswitch mediated gene expression in a theophylline-dependent manner, *E. coli* harboring this construct were grown on LB/agar supplemented with ampicillin, the chromogenic β-galactosidase substrate X-gal, IPTG [in these experiments, IPTG is not used to induce expression of the gene but rather as a weak inhibitor of β-galactosidase, which improves the signal-to-noise ratio in the detection of X-gal hydrolysis in experiments performed on solid media], and either no additional small molecule, 500 μM caffeine, or 500 μM theophylline. These cells were grown for 14 h at 37 °C to allow colonies to form and incubated at 4 °C to restrict growth and allow the blue color of the hydrolyzed X-gal to develop. After 48 h, cells grown in the presence of theophylline were distinctly blue whereas those grown in the presence of caffeine showed no visible color, suggesting that theophylline was activating the expression of β-galactosidase (Figure 1A).

To quantify the theophylline-dependent increase in β-galactosidase activity observed in the plate-based assay, we performed assays of cells harboring the synthetic riboswitch grown in liquid media using a modification of the method of Miller[50] (Figure 1B). Addition of theophylline produces a significant, dose-dependent increase in β-galactosidase activity, whereas addition of caffeine produces only small changes in the β-galactosidase activity over background. Interestingly, the level of β-galactosidase activity in the presence of theophylline (500 μM) is comparable to that of a control construct lacking the aptamer sequence, which suggests that the theophylline-bound aptamer sequence does not interfere with translation (Figure 1C).

The extracellular concentrations of theophylline needed to activate protein translation in *E. coli* are considerably higher than the K_d for theophylline binding to the aptamer measured in vitro.[51] Although the toxicity of theophylline at high concentrations (>5 mM, Supporting Information) prevents saturation of the response, we estimate that half-maximal response occurs at an extracellular theophylline concentration of approximately 250 μM (Figure 1B). Previous studies of theophylline uptake in *E. coli* have shown that at an external concentration of 10 μM, the intracellular concentration of theophylline is only 7 nM.[54] This suggests that either theophylline uptake in *E. coli* is slow, and thus equilibrium is not reached, or there is an active mechanism for theophylline efflux that maintains this concentration differential. In either case, increasing the extracellular concentration of theophylline should lead to a proportional increase in the intracellular concentration; however, in the case of an active efflux mechanism, this relationship would break down as the pump becomes saturated. The theophylline dose−response relationship suggests the possibility of an active efflux mechanism that becomes saturated at theophylline concentrations greater than 5 mM, leading to cell death. At concentrations below 5 mM, however, the extracellular and intracellular concentrations would be expected to vary linearly, and at an extracellular theophylline concentration of 250 μM, the expected intracellular concentration would be approximately 175 nM, which closely approximates the K_d for theophylline binding to the aptamer in vitro.[51] [Theophylline uptake could be measured using ^{14}C-labeled theophylline, but the large amounts needed to make accurate measurements over a wide range of concentrations rendered these experiments cost prohibitive. Attempts to permeabilize the cells to increase theophylline uptake using DMSO at concentrations of up to 5% produced only modest (<15%) increases in β-galactosidase activity.]

To confirm that the increase in β-galactosidase activity was the result of theophylline interacting specifically with the

(51) Jenison, R. D.; Gill, S. C.; Pardi, A.; Polisky, B. *Science* **1994**, *263*, 1425−1429.
(52) Zimmermann, G. R.; Shields, T. P.; Jenison, R. D.; Wick, C. L.; Pardi, A. *Biochemistry* **1998**, *37*, 9186−92.
(53) Zimmermann, G. R.; Jenison, R. D.; Wick, C. L.; Simorre, J. P.; Pardi, A. *Nat. Struct. Biol.* **1997**, *4*, 644−9.
(54) Koch, A. L. *J. Biol. Chem.* **1956**, *219*, 181−8.

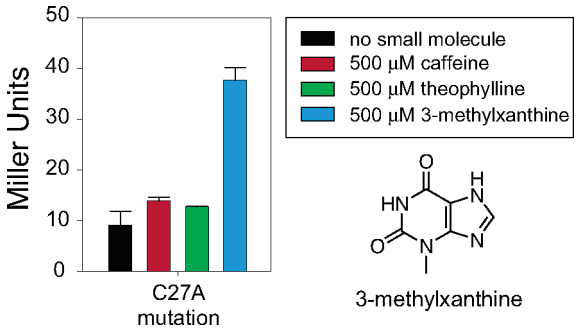


Figure 2. *β*-Galactosidase activity for the C27A mutant synthetic riboswitch in response to caffeine, theophylline, and 3-methylxanthine.

aptamer and not a generic theophylline-dependent increase in protein expression and to investigate further whether the molecular recognition properties of the aptamer determined in vitro extend to the behavior of a synthetic riboswitch in *E. coli*, we introduced a point mutation (C27A) into the aptamer sequence. In vitro, this mutation reduces the affinity of a similar aptamer for theophylline by at least 10-fold while increasing the sensitivity to 3-methylxanthine.[55] In *E. coli*, theophylline only weakly activates the C27A mutant riboswitch while 3-methylxanthine retains full activity (Figure 2). The ability to change the specificity of the synthetic riboswitch with a single base change is consistent not only with the in vitro data but also with previous studies that show that natural riboswitches sensitive to adenine and guanine differ by only a single base change.[56]

Taken together, these experiments demonstrate the potential sensitivity of the assay and suggest that the binding properties of the aptamer measured in vitro translate into an in vivo genetic system. Furthermore, the dose-dependent nature of the response suggests that synthetic riboswitches may be useful in monitoring the intracellular concentrations of small molecules, such as endogenous metabolites. Since assays for *β*-galactosidase activity may be performed in a high-throughput fashion in a plate-based format[57] and the synthetic riboswitch is capable of discriminating between closely related molecules (such as a reactant and a product in a biotransformation), we expect that synthetic riboswitch-mediated screens and selections (see below) will be particularly useful in directed evolution experiments with the goal of discovering improved biocatalysts for the production of small molecules.

Determining the Mechanism of Activation. When we began our studies we hypothesized that theophylline binding to the aptamer would increase the secondary structure of the mRNA near the ribosome binding site (RBS)[58] and that protein translation would be reduced in the presence of theophylline. Such a result would be consistent with the behavior of all known natural riboswitches that operate at the translational level[44] as well as that of a synthetic riboswitch based on the theophylline aptamer in a eukaryotic translation system.[37] However, the data clearly indicate that theophylline leads to an increase in *β*-galactosidase activity. Interestingly, a recent example of a synthetic riboswitch based on the theophylline aptamer in *Bacillus* also activates protein expression; however, in that study

(55) Soukup, G. A.; Emilsson, G. A.; Breaker, R. R. *J. Mol. Biol.* **2000**, *298*, 623−32.
(56) Mandal, M.; Breaker, R. R. *Nat. Struct. Mol. Biol.* **2004**, *11*, 29−35.
(57) Desai, S. K.; Gallivan, J. P. Unpublished results.
(58) de Smit, M. H.; van Duin, J. *J. Mol. Biol.* **1994**, *235*, 173−84.

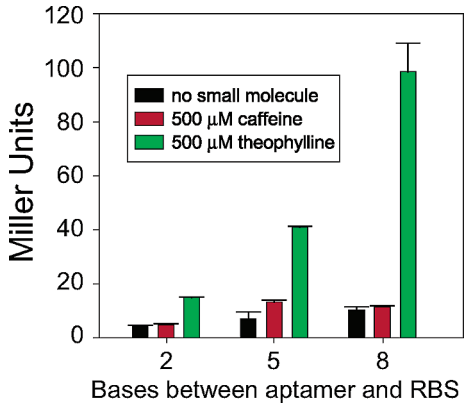


Figure 3. *β*-Galactosidase activity for synthetic riboswitches in which the aptamer is placed at various locations upstream of the ribosome binding site (five bases = wild-type). The increase in translational activation for the eight bp spacing may be due to additional purines near the ribosome binding site which favor ribosome binding.

a designed helix-slipping mechanism was proposed to account for the observed effects.[39] In our experiments we did not intentionally engineer specific interactions within the gene; the aptamer sequence was simply cloned into the 5′-UTR of the reporter gene. Indeed, when the aptamer sequence is moved either closer to or further away from the RBS, the synthetic riboswitch still functions (Figure 3), suggesting that if specific interactions between the aptamer and other regions of the gene are present, they are tolerant to changes in the location of the aptamer. The relative insensitivity of the riboswitch to the position of the aptamer led us to test whether the switch was functioning to control transcription rather than translation. A transcriptional control mechanism could explain why controlling the distance between the aptamer and the RBS is not absolutely critical and would be consistent with the mechanism of the only naturally occurring riboswitch known to activate gene expression, the adenine riboswitch, which operates by decreasing transcriptional termination in response to adenine.[56]

To determine whether theophylline is acting at the transcriptional or post-transcriptional level in this system, we created a transcriptional fusion between two genes.[59] The first gene comprised the mTCT8-4 aptamer, a ribosome-binding site, and a 61-amino acid N-terminal fragment of the IS-10 transposase followed by three in-frame stop codons. This gene was fused through a 28 base pair spacer to a second gene comprised of a ribosome binding site and the wild-type *lac*Z sequence (Figure 4A). If the theophylline-dependent increase in gene expression is controlled at the transcriptional level or is the result of an increase in mRNA stability, expression of the second gene in the fusion (*lac*Z) would be theophylline dependent. However, if the theophylline-dependent increase in gene expression is controlled at the translational level, expression of the first gene (IS10) would be theophylline dependent but expression of the second gene (*lac*Z) would be theophylline independent, as this second gene lacks the aptamer and has its own ribosome binding site (Figure 4B). Expression of this construct in *E. coli* shows that *β*-galactosidase activity is not theophylline dependent, which indicates that this riboswitch acts at the translational level (Figure 4C). The exact mechanism by which theophylline binding activates translation is being explored, and the results will be reported elsewhere.

(59) Beckwith, J. *Methods Enzymol.* **2000**, *326*, 3−7.

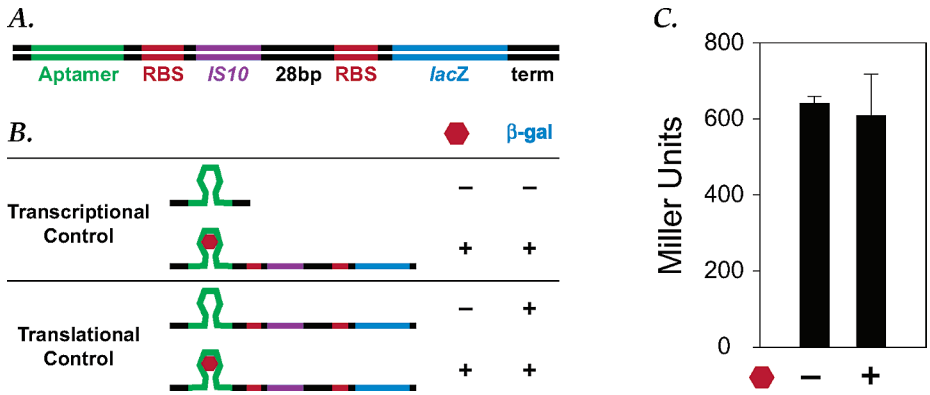


Figure 4. (A) Schematic of transcriptional fusion (aptamer = mTCT8−4, RBS = ribosome binding site, IS10 = 61 amino acid fragment from IS10 transposase, term = transcriptional terminator). (B) Expected outcomes for transcriptional and translational control when theophylline (red hexagon) is present or absent. For transcriptional control, expression of β-galactosidase is expected to be theophylline dependent; for translational control, expression is expected to be theophylline independent. (C) β-Galactosidase activity for the transcriptional fusion expressed in *E. coli*. Expression of β-galactosidase is theophylline independent, indicating translational control.

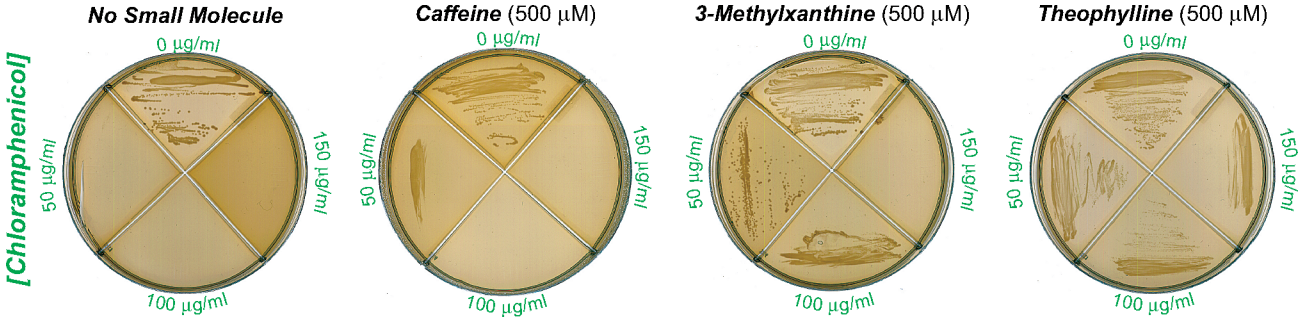


Figure 5. Plate-based selections for the presence of small molecules using the wild-type synthetic riboswitch. *E. coli* were grown in various concentrations of chloramphenicol for 18 h at 37 °C.

Metabolic Control Using a Synthetic Riboswitch: Genetic Selections for Small Molecules. One of our long-term goals is to be able to couple the life of a bacterium to its ability to access (or produce) a desired small-molecule target. The results of the experiments above suggest the possibility that a desired small molecule can be used to activate the translation of a gene that is essential to cell survival under a given set of conditions. Successful implementation of such a strategy would allow the power of genetic selection to be applied to discover enzymes capable of synthesizing nonendogenous small-molecule targets, much in the way that auxotrophic strains have been used to discover enzymes that synthesize essential metabolites.[28,29,60,61]

To determine whether a synthetic riboswitch could be used to mediate a genetic selection for the presence of a small molecule, we replaced the *lac*Z reporter gene in the riboswitch above with a gene encoding the enzyme chloramphenicol acetyl transferase (*cat*), which confers resistance to the antibiotic chloramphenicol, and we replaced the weak IS10 promoter sequence with the stronger *tac* promoter,[62] which increases transcription. We anticipated that *E. coli* cells harboring this construct would be sensitive to chloramphenicol in the absence of theophylline but would become resistant to chloramphenicol in the presence of theophylline as the translation of chloramphenicol acetyl transferase is activated. Figure 5 shows the results of plate-based selections in which *E. coli* harboring the synthetic riboswitch were grown for 18 h at 37 °C on LB/agar containing ampicillin (50 μg/mL, to maintain the riboswitch plasmid) and increasing concentrations of chloramphenicol. In the absence of a small molecule to activate translation of the *cat* gene, these cells do not survive at any concentration of chloramphenicol. In the presence of caffeine (500 μM), some growth is evident at a chloramphenicol concentration of 50 μg/mL; however, single colonies are not visible, and the surviving cells may be growing on top of a layer of dead cells and are thus not exposed to chloramphenicol. In the presence of 3-methylxanthine (500 μM), single colonies are visible at a chloramphenicol concentration of 50 μg/mL and growth is observed at a concentration of 100 μg/mL. Finally, in the presence of theophylline (500 μM), single colonies are visible at a chloramphenicol concentration of 100 μg/mL and some growth is visible at a chloramphenicol concentration of 150 μg/mL. Selections performed in liquid culture reveal a similar trend (Figure 6) and clearly demonstrate that synthetic riboswitch-mediated genetic selection can be used to detect the presence of a small molecule by simply monitoring cell growth in the presence of an antibiotic.

In addition to a genetic selection for the presence of theophylline using the wild-type synthetic riboswitch, we also performed a genetic selection for the presence of 3-methylxanthine by using the C27A mutant riboswitch to activate the translation of chloramphenicol acetyl transferase (Figure 6). In the presence of either caffeine (500 μM) or theophylline (500 μM), *E. coli* harboring the mutant riboswitch construct were not viable at concentrations of chloramphenicol above 50 μg/

(60) Rothman, S. C.; Voorhies, M.; Kirsch, J. F. *Protein Sci.* **2004**, *13*, 763−72.
(61) Kast, P.; Asif-Ullah, M.; Jiang, N.; Hilvert, D. *Proc. Natl. Acad. Sci., U.S.A.* **1996**, *93*, 5043−8.
(62) de Boer, H. A.; Comstock, L. J.; Vasser, M. *Proc. Natl. Acad. Sci., U.S.A.* **1983**, *80*, 21−5.

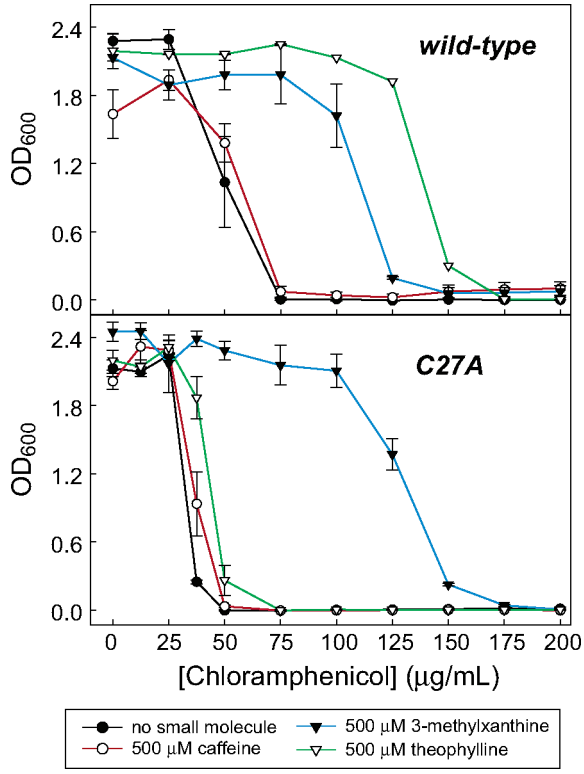


Figure 6. Genetic selection experiments for *E. coli* harboring either the wild-type (top) or C27A synthetic riboswitch (bottom) grown in liquid media with increasing concentrations of chloramphenicol. Data are recorded after 18 h of growth at 37 °C. OD_{600} is a measure of the cell density of the cultures.

mL when grown in LB media. In the presence of 3-methylxanthine (500 μM), these cells grew steadily at a chloramphenicol concentration of 100 μg/mL and remained viable even at a concentration of 150 μg/mL (Figure 6). Taken together, our results demonstrate that synthetic riboswitches can be used to genetically select for the presence of nonendogenous small molecules in *E. coli*. In essence, *E. coli* harboring these synthetic riboswitches behave as *designer auxotrophs* that require a specific, nonendogenous, small molecule to survive in the presence of the antibiotic chloramphenicol. We anticipate that these and other designer auxotrophs will be useful as selectable hosts for the cloning of biosynthetic genes and the directed evolution of biocatalysts.

Genetic Screens and Selections To Discover Synthetic Riboswitches in *E. coli*. The genetic screening and selection experiments above focused on developing synthetic riboswitches that detect the presence of a particular small-molecule target. The sensitivity and high-throughput nature of these experiments suggest that they may also be applied to the discovery of synthetic riboswitches that display new ligand specificities. By beginning with a riboswitch that responds to a particular ligand, it may be possible to create a library of mutant riboswitches and select riboswitches from this library that respond to a new ligand by monitoring activation of a reporter gene. As a proof-of-principle experiment, we asked whether a functional riboswitch sensitive to a specific ligand could be discovered against a background of riboswitches that respond to a different ligand. The wild-type riboswitch based on the mTCT8-4 aptamer (wt) activates protein translation in response to theophylline whereas the C27A mutant does not. To determine whether the wt riboswitch could be identified against a background of the theophylline-insensitive C27A riboswitch, we performed spiking experiments in which plasmid DNA encoding the wt riboswitch was serially diluted with a plasmid encoding the C27A riboswitch. We first performed screening experiments in which the riboswitches controlled the expression of the *lac*Z gene. Mixtures of plasmids encoding the wt and C27A riboswitches in varying ratios (1:1, 1:10, and 1:100) were used to transform *E. coli* by electroporation. These cells were plated onto LB/agar plates supplemented with ampicillin, X-gal, IPTG, and theophylline (1 mM) and were grown for 14 h at 37 °C followed by incubation at 4 °C to allow the X-gal hydrolysis to proceed. After 48 h, plates corresponding to the 1:1 and 1:10 dilutions revealed blue and white colonies in ratios corresponding to the dilutions of the plasmids and five blue colonies from each of these plates were picked, whereas for the 1:100 dilution, only two blue colonies were observed on the plate (which was consistent with the total number of colonies and the dilution factor) and these colonies were picked. In all cases, sequencing the plasmid DNA from the blue colonies revealed the presence of the wild-type riboswitch, as expected; thus, this assay produced no false positives. Additionally, sequencing of a corresponding number of white colonies revealed the C27A mutant riboswitch in 11 out of 12 cases; however, subsequent rescreening of the misidentified colony revealed that it was in fact blue when plated onto theophylline and X-gal. [In these experiments X-gal is spread over the surface of the plates after they are poured. This may lead to uneven coverage and produce false negatives; pouring the plates with X-gal in the medium may reduce the number of false negatives.]

Encouraged by our ability to isolate specific riboswitches in the context of a genetic screen, we turned our attention to using genetic selection to isolate riboswitches against an even larger background of related constructs. For these experiments we used the wt and C27A riboswitches to control the activation of the chloramphenicol acetyl transferase gene. Mixtures of plasmids encoding the wt and C27A riboswitches in varying ratios ($1:10^3$, $1:10^4$, $1:10^5$, and $1:10^6$) were used to transform *E. coli* by electroporation. These cells were plated at very high density onto LB/agar plates supplemented with ampicillin and were grown for 12 h at 37 °C. All colonies from the resulting lawn were scraped from the plates and cultured at 37 °C in liquid LB media supplemented with ampicillin (50 μg/mL) until the culture reached saturation. Aliquots from the saturated cultures were spread onto LB/agar plates supplemented with ampicillin (50 μg/mL), theophylline (500 μM), and chloramphenicol (100 μg/mL) and were grown at 37 °C. After 18 h, single colonies were visible on the selection plates in all cases. Five colonies from each plate were isolated, and their plasmids were sequenced. In all cases, these sequences corresponded to the wild-type riboswitch as expected. These experiments clearly show that genetic selection can be used to isolate a rare riboswitch function from a pool containing a 10^6-fold excess of synthetic riboswitches that respond to a closely related small molecule.

Conclusion

We have successfully constructed a synthetic riboswitch that activates protein translation in *E. coli* in response to a specific small molecule. We have shown that through use of an appropriate reporter gene, synthetic riboswitches can be used to perform either genetic screening or genetic selection experi-

ments to detect the presence of a specific, nonendogenous small molecule in *E. coli*, and by making a mutation within the aptamer, we demonstrated that the molecular recognition properties of an aptamer generated using in vitro selection can be translated into a sensitive genetic selection system in *E. coli*.

Taken together, these results show that it is possible to use synthetic riboswitches to create strains of *E. coli* that depend on the presence of a small molecule of our choosing for survival. Because *E. coli* grow quickly, transform efficiently, and are useful hosts for the biocatalytic production of small molecules,[4] synthetic riboswitch-mediated genetic selections have the potential to assay very large enzyme libraries to discover new biocatalysts capable of producing desired small-molecule targets. Since these selections depend only on the presence of the desired small-molecule target and not on the way in which it was made, synthetic riboswitches are not limited to monitoring a particular class of reaction (i.e., bond forming, bond breaking). As such, synthetic riboswitch-mediated genetic selections may be useful in cloning biosynthesis genes for which the products but not the mechanistic pathways are known. In addition, since powerful in vitro selection methods can be used to generate RNA aptamers that specifically recognize small-molecule targets[48] while discriminating against closely related targets (such as enantiomers[63,64]), we anticipate that in vitro selection can be used to generate aptamers and that the sensitive molecular recognition properties of these aptamers may be transferred into new synthetic riboswitches in *E. coli*. Synthetic riboswitch-mediated genetic screens and selections for small molecules based on these aptamers should provide a useful and general method for detecting biocatalytic activity in cells in the context of directed evolution experiments.

Finally, we have shown that a cell harboring a synthetic riboswitch with a particular ligand specificity can be selectively amplified from a million-fold larger pool of cells containing mutant riboswitches that respond to a closely related ligand. This proof-of-principle experiment suggests that it may be possible to use genetic selection in *E. coli* to select for synthetic riboswitches with new ligand specificities by selecting from mutant libraries of known riboswitches. Since raising a specific aptamer to a small molecule can be a time-consuming process, this selection method may be generally useful for rapidly evolving the ligand specificity of synthetic riboswitches, which may be further used to perform genetic selections for the presence of small molecules.

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Supporting Information Available: Details of plasmid construction and results of growth studies of *E. coli* in the presence of caffeine and theophylline are available. This material is available free of charge via the Internet at http://pubs.acs.org.

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(63) Famulok, M. *J. Am. Chem. Soc.* **1994**, *116*, 1698−1706.
(64) Geiger, A.; Burgstaller, P.; von der Eltz, H.; Roeder, A.; Famulok, M. *Nucleic Acids Res.* **1996**, *24*, 1029−36.