

Supplementary Information for

Reprogramming Bacteria to Seek and Destroy a Herbicide

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Supplementary Methods.

General Considerations. Synthetic oligonucleotides were purchased from Integrated DNA Technologies (Coralville, IA). Culture media was obtained from EMD Biosciences. Freeze-dried sepharose 6B was obtained from GE Healthcare. Ampicillin and chloramphenicol were purchased from Fisher. Enzymes were purchased from New England Biolabs unless otherwise noted. Purifications of plasmid DNA, PCR products, and enzymatic digests were performed using kits from Qiagen. All new constructs were verified by DNA sequencing at the Center for Fundamental and Applied Molecular Evolution at Emory University, Agencourt Biosciences (Beverly, MA), or MWG Biotech (Huntsville, AL).

Chemical Synthesis

2,4-Dichloro-6-(2-hydroxyethylamino)-s-triazine (2)^{S1}. To a solution of cyanuric chloride (1.84 g, 10 mmol) in 10 mL acetone was added 10 mL water. The resulting solution was cooled to 0 °C (ice-bath); and then with rapid stirring, a mixture of ethanolamine (0.63 mL, 10.5 mmol) in 2 mL ether was added drop-wise over 5 minutes, after which, a solution of NaHCO₃ (1.68 g, 20 mmol) in 15 mL water was added slowly. The mixture was stirred at the same temperature for 1 h, after which, the ice-bath was removed and the reaction allowed to warm to 25 °C over 2 h. Then, ethyl acetate (30 mL) and water (20 mL) were added. The layers were separated and the aqueous layer was extracted with ethyl acetate (20 mL x 4). The combined organic extracts were washed with saturated NaHCO₃, brine, and dried over Na₂SO₄. The solution was then filtered and the solvent evaporated and dried under vacuum to give a white crystalline material (0.36 g, 18%). ¹³C NMR (150 MHz, dms_o-d₆): δ = 170.0, 169.1, 166.1, 59.4, 44.1.

2-Chloro-4-isopropylamino-6-(2-hydroxyethylamino)-s-triazine (3). To a solution of 2,4-dichloro-6-(2-hydroxyethylamino)-s-triazine (0.36 g, 1.72 mmol) in 20 mL absolute ethanol were added Na₂CO₃ (0.401 g, 3.78 mmol) and isopropylamine (0.16 mL, 1.89 mmol). A reflux condenser was attached and the reaction mixture was heated to 45 °C for 8 h. After this time TLC revealed the presence of starting material, and additional isopropylamine (0.11 mL,

1.29 mmol, bp 32 °C) was added, the temperature was lowered to 35 °C, and the reaction mixture stirred for 12 h. Water (20 mL) and ethyl acetate (20 mL) were added and the layers were separated. The aqueous layer was further extracted with ethyl acetate (15 mL x3) and the organic extracts were washed with brine and dried over Na₂SO₄. The solution was filtered, concentrated and dried in vacuo to give a white solid (0.36 g, 89%). ¹H NMR (600 MHz, dms_o-d₆): δ = 7.76-7.69 (m, 1 H), 7.67-7.56 (m, 1 H), 4.70-4.66 (m, 1 H), 4.04-3.94 (m, 1 H), 3.50-3.42 (m, 2H), 3.31-3.24 (m, 2H), 1.11 (d, *J*=6.5 Hz, 3H), 1.09 (d, *J*=6.5, 3H); ¹³C NMR (150 MHz, dms_o-d₆): δ = 168.6, 166.5, 165.4, 60.28, 44.0, 43.0, 23.0. MS (ESI) calcd for C₈H₁₄ClN₅O 231.6826 (M + H); found 232.0957.

Coupling of 2-Chloro-4-isopropylamino-6-(2-hydroxyethylamino)-s-triazine to sepharose. Freeze-dried sepharose 6B (1 g) was suspended in water (10 mL), and the swelled sepharose was washed with water. Atrazine derivative **3** (3.2 mg) was dissolved in DMF (400 μL) and 2X coupling buffer (0.05 M Na₂HPO₄, 400 μL). This solution was mixed with sepharose in coupling buffer (20 mL) and the suspension was shaken gently overnight at 37 °C. The coupled-sepharose was washed with coupling buffer (100 mL), and suspended in Tris-HCl (10 mL, 1 M, pH 8). The suspension was incubated overnight at 40 °C to cap any unreacted groups. The sepharose-atrazine complex was washed with three cycles of alternating pH solution. Each cycle consisted a wash with acetate buffer (0.1 M sodium acetate, 0.5 M NaCl, pH 4). This was followed by a wash with Tris-HCl buffer (0.1 M Tris-HCl, 0.5 M NaCl, pH 8). After the last wash, the sepharose-atrazine was suspended in 10 mM Tris-HCl (pH 8) and stored at 4 °C.

In Vitro Transcription. Except transcription reactions for SELEX experiments, all transcription reactions were carried out using the Ampliscribe T7-Flash Transcription Kit (Epicentre Technologies, Madison, WI) using manufacturer's instruction. Transcription reactions were gel purified from denaturing polyacrylamide gels.

In vitro transcription for SELEX experiments. The DNA pool generated from the PCR amplification was transcribed into an initial RNA pool in a reaction containing 40 mM Tris-HCl (pH 7.9), 16 mM MgCl₂, 10 mM DTT, 2 mM spermidine, 3.2 mM of each rNTP, 70 μCi of [α -³²P]-UTP (3000 Ci/mmol, MP Biosciences), 2 U of inorganic pyrophosphatase and 10 U of T7 RNA polymerase. After overnight incubation at 37 °C, the DNA was degraded by adding 20 U of DNase (Epicentre) and incubating at 37 °C for 5 h. The transcription reaction was mixed with an equal volume of gel loading buffer (95% formamide, 20 mM EDTA, bromophenol blue). The RNA was purified on a 10% denaturing polyacrylamide gel, products were visualized by UV-shadowing and the bands corresponding to the full length RNAs were cut out. Gel slices were crushed and soaked in water and RNAs were passively eluted from the gel slices overnight at 4 °C. The resulting solution was passed through a 0.2 μm filter (VWR) to remove polyacrylamide gel particles, further purified by ethanol precipitation. Precipitated RNA was dissolved in binding buffer (20 mM Tris-HCl, 5 mM MgCl₂, 250 mM NaCl; pH 7.4) and the quantity of RNA was determined by measuring the absorbance at 260 nm.

The DNA template for structure probing was generated by using the primer pairs 5'-TTCTAATACGACTCACTATAGGGACAGGGCTAGC-3' and 5'-GAACCGGACCGAAGCCCGTGTAATTCGGG-3' with the T7 promoter sequence underlined. The resulting PCR product covers the entire 5'-UTR and the first 56 nt of the *IS10-lacZ* coding region.

For end-labeling, RNAs were dephosphorylated using calf intestinal phosphatase (CIP), phenol-chloroform extracted, and ethanol precipitated. The dephosphorylated RNAs were end-labeled using T4 polynucleotide kinase and [γ -³²P]-ATP (MP Biomedicals, Solon, OH). Labeled RNAs were gel purified on a 7% denaturing polyacrylamide gel, eluted from the gel, ethanol precipitated, and redissolved in 50 μL of water.

Transcriptional Fusion Experiment. The transcriptional fusion contained 3 stop codons after the 61st amino acid of the IS10 transposase in plasmid pJS42 (plasmid pJS42 contains the aptamer sequence) followed by a 28 bp pair spacer and then a ribosome binding site and IS10-*lacZ* fusion gene cloned from pRS415. A cassette mutagenesis strategy was used to make this construct. A PCR product (A) was generated using pJS42.1 as a template with the forward primer JPG-007 and the reverse primer SKD-134. A second PCR product (B) was generated using plasmid pRS415 as a template with the forward primer SKD-133 and the reverse primer SKD-098. PCR products A and B contained an overlapping region, and they were mixed and amplified using the forward primer JPG-007 and the reverse primer SKD-098, to give PCR product C. PCR product C was digested with *KpnI* and *SacI*, gel purified, and cloned into *KpnI* and *SacI* sites in plasmid pJS42, forming plasmid pJS58.

JPG-007

5' GCGATTAAGTTGGGTAACGCCAGGG 3'

SKD-098

5' GTTAAATTGCCAACGCTTATTACCCAGCTCGATGC 3'

SKD-133

5' **CTGACTCCTCGAGTATAAAG**ACAACAAGATGACCATGATTACGGATTCACTGGCCGTC 3'
(overlapping region, gene specific segment)

SKD-134

5' -

CTTTATACTCGAGGAGTCAGAGATCTCAGTTTATTATTAGATTTCGTTTGATGTTATGTTTTGTTCTCG
C -3'
(overlapping region, gene specific segment)

Northern blot analysis. *E. coli* TOP10F' cells harboring plasmid pJS42 were grown to mid-log-phase either in the presence or absence of atrazine (750 μ M). Cells from a 25 mL culture were harvested, total RNA was extracted using the guanidinium isothiocyanate protocol^{S2}, ethanol precipitated and redissolved in water (100 μ L). The remaining DNA was digested by adding 10 U of DNase I (Epicentre) and incubating for 1 h at 37 °C. After the DNase digest, the RNA was ethanol precipitated and redissolved in water (100 μ L).

Total RNA (~30 μ g) was loaded on a 1% formaldehyde-agarose gel. The resolved RNA was transferred to a BrightStarTM-Plus membrane (Ambion) according to manufacturer's instructions. To probe the RNA, a ³²P-labeled anti-sense RNA was prepared by in vitro transcription using a 300 bp DNA template containing the aptamer and the first 200 nucleotides of *IS10-lacZ* fusion. Pre-hybridization and hybridization were carried out at 68 °C using ULTRAhyb buffer (Ambion) following the manufacturer's instructions. Following an overnight hybridization step, the membrane was washed twice with 2 \times SSC (1 \times SSC buffer contained 150 mM sodium chloride and 15 mM sodium citrate), 0.1% SDS buffer at room temperature followed by two more washes with 0.1 \times SSC, 0.1% SDS at 68 °C. The membrane was then exposed to a storage phosphor screen and the bands were visualized using a Phosphorimager (GE Healthcare). Results are shown in Figure S4.

Chemotaxis Assays. Cells were grown to mid-log phase at 37 °C in either tryptone broth (1% tryptone, 0.5% NaCl) or M9 minimal medium containing 0.2% (v/v) glycerol, 1 µg/mL of thiamine and 20 µg/mL each of methionine, histidine, leucine. Tryptone swarm plates were prepared with 0.25% agar. Minimal swarm plates contained 0.2% agar in M9 media containing the above three amino acids (40 µg/mL) and aspartate (1 mM). Cell suspensions were diluted to $OD_{600} = 0.2$ and 3.5 µL was plated on the surface of the swarm plate near the center. Plates were incubated at 30 °C for 14 h (tryptone agar plates) or 30 h (minimal agar plates). Cells were illuminated from above with a white light, viewed against a dark background on a Gel-Doc Molecular imaging system (Bio-Rad). Plates containing cells expressing fluorescent protein were imaged on an UVP Epi Chemi II Bioimaging system (UVP, Upland, CA) using 365 nm epi-UV as the light source. The resulting picture was filtered using a Z485BP band-pass filter (Chroma Technology Corp, Rockingham, VT). False color (green) was applied using ImageJ; adjustments to brightness and contrast were performed to the whole image using Adobe Photoshop.

Cell Migration and Atrazine Metabolism. A PCR product containing the entire 5'-untranslated region and the first 187 nt of the *ISI0-lacZ* coding region was subcloned into the *KpnI* and *HindIII* sites of plasmid pSKD1248 to obtain plasmid pJS42. Plasmid pJS290 contained a fluorescent reporter gene, GFPuv, flanked by a constitutively active promoter and a transcriptional terminator. This plasmid was digested with *SapI* and was cloned into *SapI* site of pJS42 to obtain plasmid pJS292. Plasmid pMD4 was obtained from the lab of Dr. Lawrence Wackett and contained the atrazine chlorohydrolase (*atzA*) gene. Since, pJS42 and pJS292 are pUC18-derived plasmids and pMD4 is a pACYC184-derived plasmid, pJS42 or pJS292 can coexist in a cell with pMD4.

To perform cell motility and atrazine catabolism experiments, selective media with 4 mM atrazine was prepared in Petri dishes (85 mm dia). A single colony of *E.coli* JW1870 cells harboring both pJS292 and pMD4 plasmids was used to inoculate in tryptone broth, which was grown at 37 °C and grown until mid-log-phase. A diluted cell suspension (3.5 µL) was applied at center of the plate, the plates were dried in air for 10 min, and incubated at 30 °C

for 20 h. Similar experiments were conducted with JW1870 cells harboring either the pJS292 or the pMD4 plasmid.

Supplementary Results

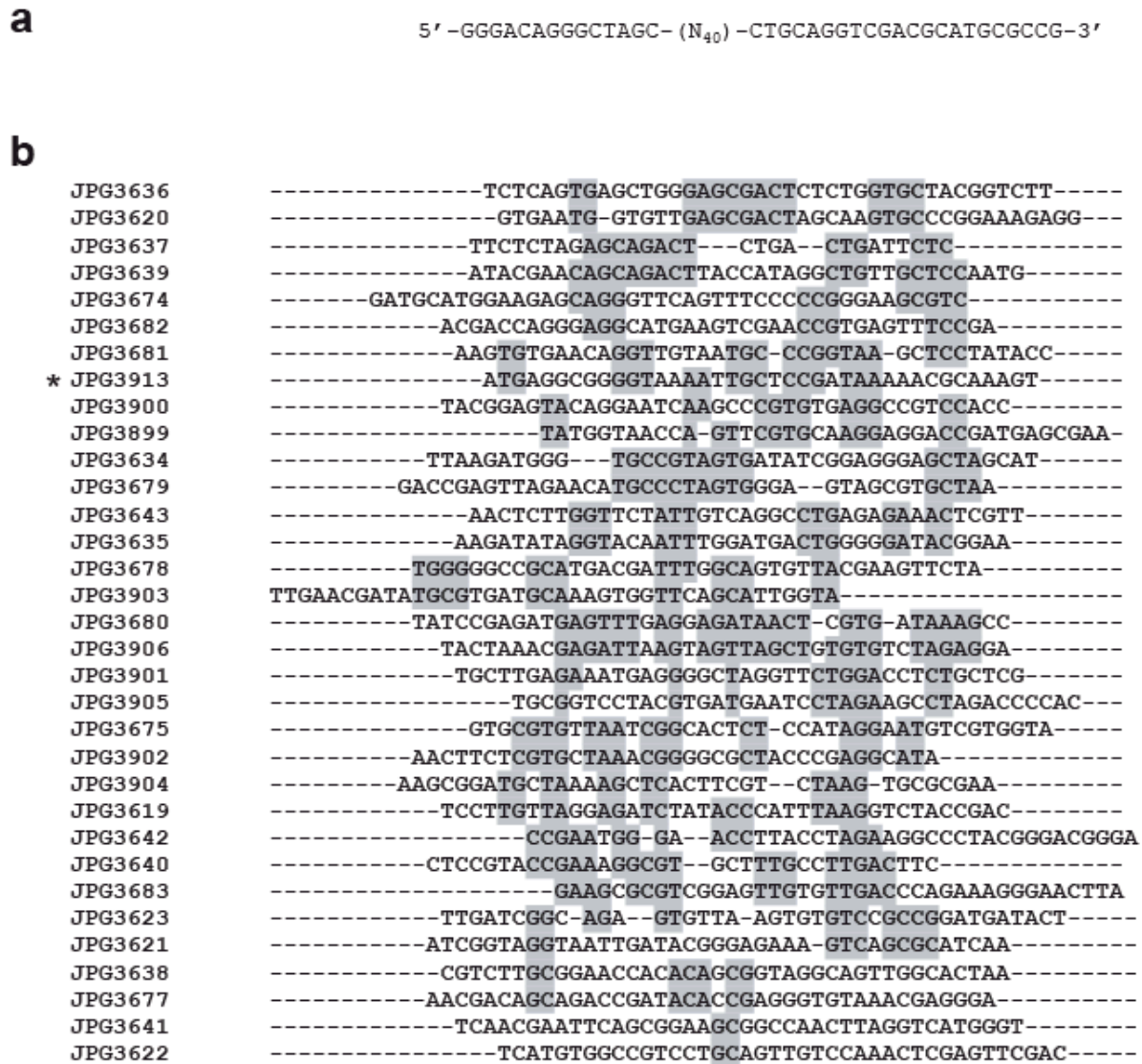


Figure S1. (a) The DNA template for generating the initial RNA pool. **(b)** Aligned sequences derived from the “N₄₀” region from clones isolated in the 12th round of the SELEX experiment. The sequences were aligned using ClustalW^{S3}. The sequence marked with an asterisk (*) is the sequence obtained through motility selection.

Verifying that the 5'-UTR is Essential for Switching.

To confirm our hypothesis that the 40 nt of the 5'-untranslated region is responsible for atrazine binding and is required for gene regulation, we tested a construct from which the 40 nt region was deleted. The construct was subcloned into *KpnI* and *HindIII* sites of plasmid pSAL172 containing the *lacZ* gene. Three separate colonies were picked, subcultured and β -galactosidase activity assayed as previously described. The results are shown in Figure S2.

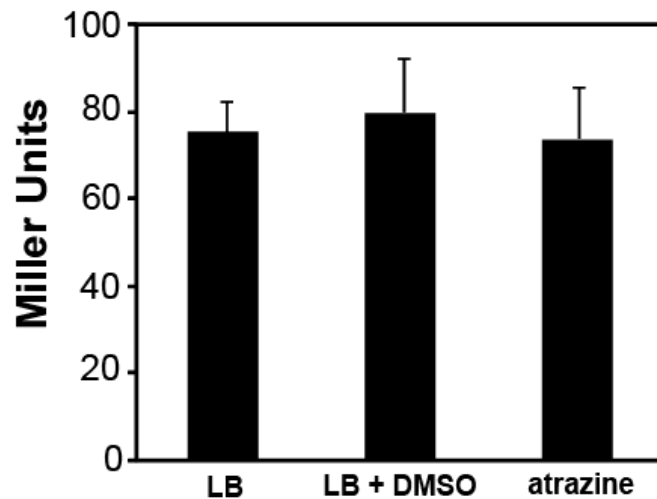


Figure S2. β -galactosidase activity (in Miller units) for a clone lacking the 5'-UTR; no switching activity is seen. LB – Luria-Bertani broth alone; LB+DMSO = LB + 4% (v/v) DMSO, Atrazine = atrazine 0.75 mM in LB + 4% (v/v) DMSO.

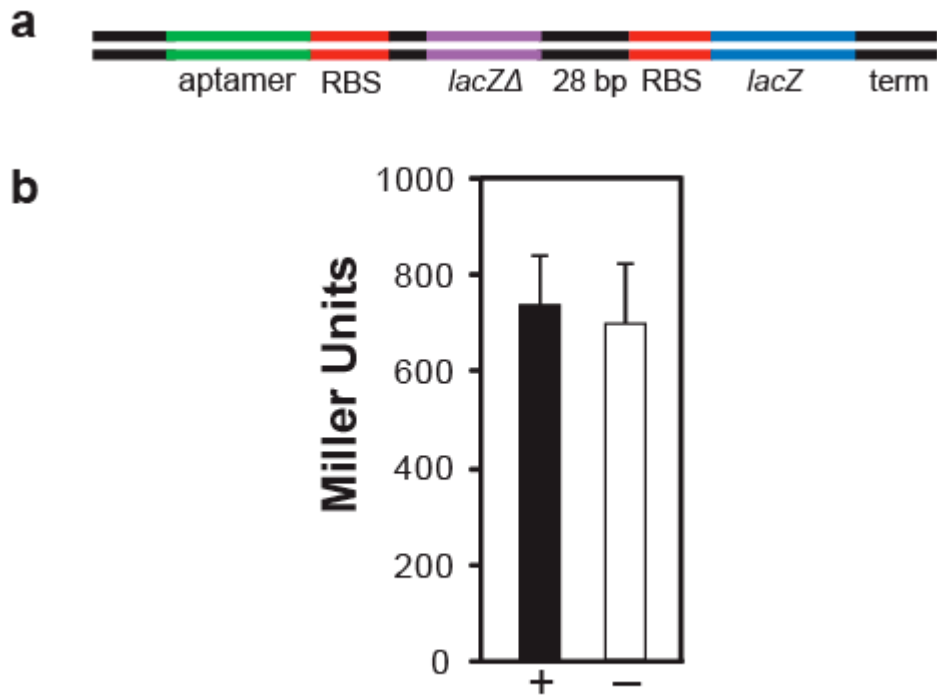


Figure S3. (a) Schematic of transcriptional fusion (aptamer = 5'-UTR obtained from in vitro and in vivo selection, RBS = ribosome binding site, *lacZ* Δ = a truncated, non-functional version of the β -galactosidase gene; *lacZ* = the β -galactosidase gene as described in the main text, term = transcriptional terminator). **(b)** β -Galactosidase activity for the transcriptional fusion expressed in *E. coli* in the presence of atrazine (750 μ M) (+) or in the absence of atrazine (-). Expression of β -galactosidase is atrazine independent, indicating translational control.

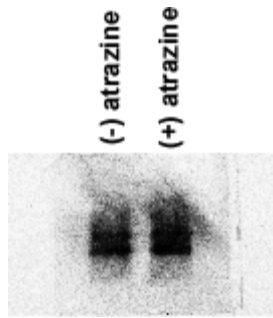


Figure S4. Northern blot analysis. Total RNA was isolated from *E. coli* TOP10F' cells harboring plasmid pJS42 and grown in the presence or absence of atrazine. The membrane was probed using ^{32}P -labeled anti-sense RNA. The results indicate that the amount of riboswitch RNA is similar in the cells grown in either the presence or absence of atrazine. This implies that atrazine does not affect transcription in vivo.

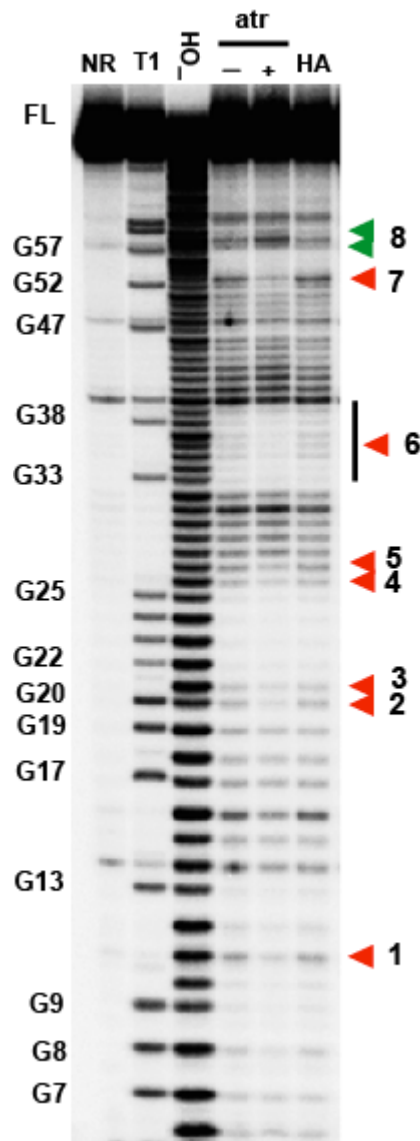


Figure S5. In-line probing of RNA aptamer reveals that it undergoes structural modulation only in the presence of atrazine. Polyacrylamide gel electrophoresis of RNA products generated by in-line probing of 5'-³²P labeled RNA. The full length RNA (FL) contained the N₄₀ region plus the flanking primer binding sequences. NR, T1, and OH represent no reaction, partial digest with RNase T1 (G-specific cleavage), and partial digest with alkali, respectively. RNA was incubated in the absence of small molecule (-), presence (+) of 1 mM atrazine or presence of 750 μM hydroxyatrazine (HA). Product bands corresponding to cleavage after G residues are numbered. Red arrowheads mark the nucleotides that react less in the presence of atrazine. Green arrowheads mark the positions that react more in the presence of atrazine.

Determining the Strengths of the Ribosome Binding Sites

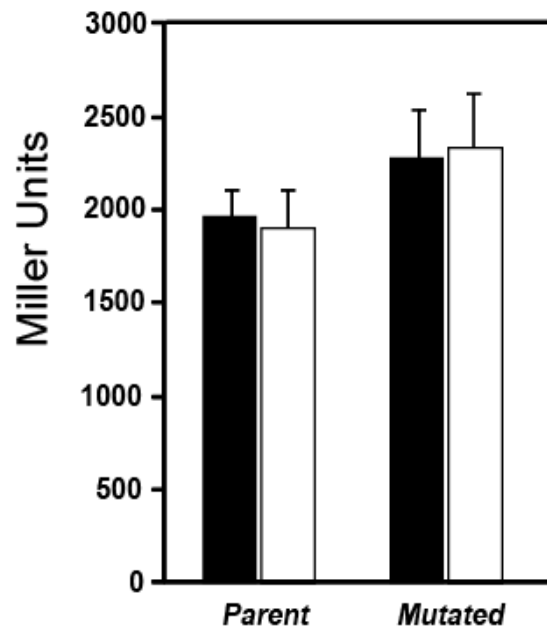


Figure S6. In vivo β -galactosidase activities of constructs in which the putative aptamer was deleted. The parent contains the N₁₀ linker sequence identified from the motility selections, the mutated version has the mutations described in the main text and shown in Figure 5c. The assays were conducted either in the presence of atrazine (750 μ M; dark bar) or the absence of atrazine (light bar). The similar levels of β -galactosidase activity suggest the mutations do not significantly affect the strength of the ribosome binding site.

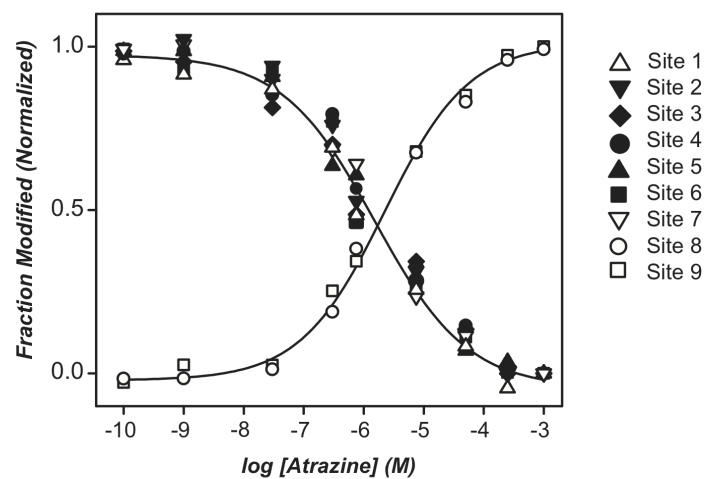


Figure S7. Plot of the normalized fraction of riboswitch cleaved versus atrazine concentration. Sites of structural modulation are as depicted in Figure 6 A and B. The data were fit assuming a single binding site model. The apparent K_d is $\sim 2 \mu\text{M}$.

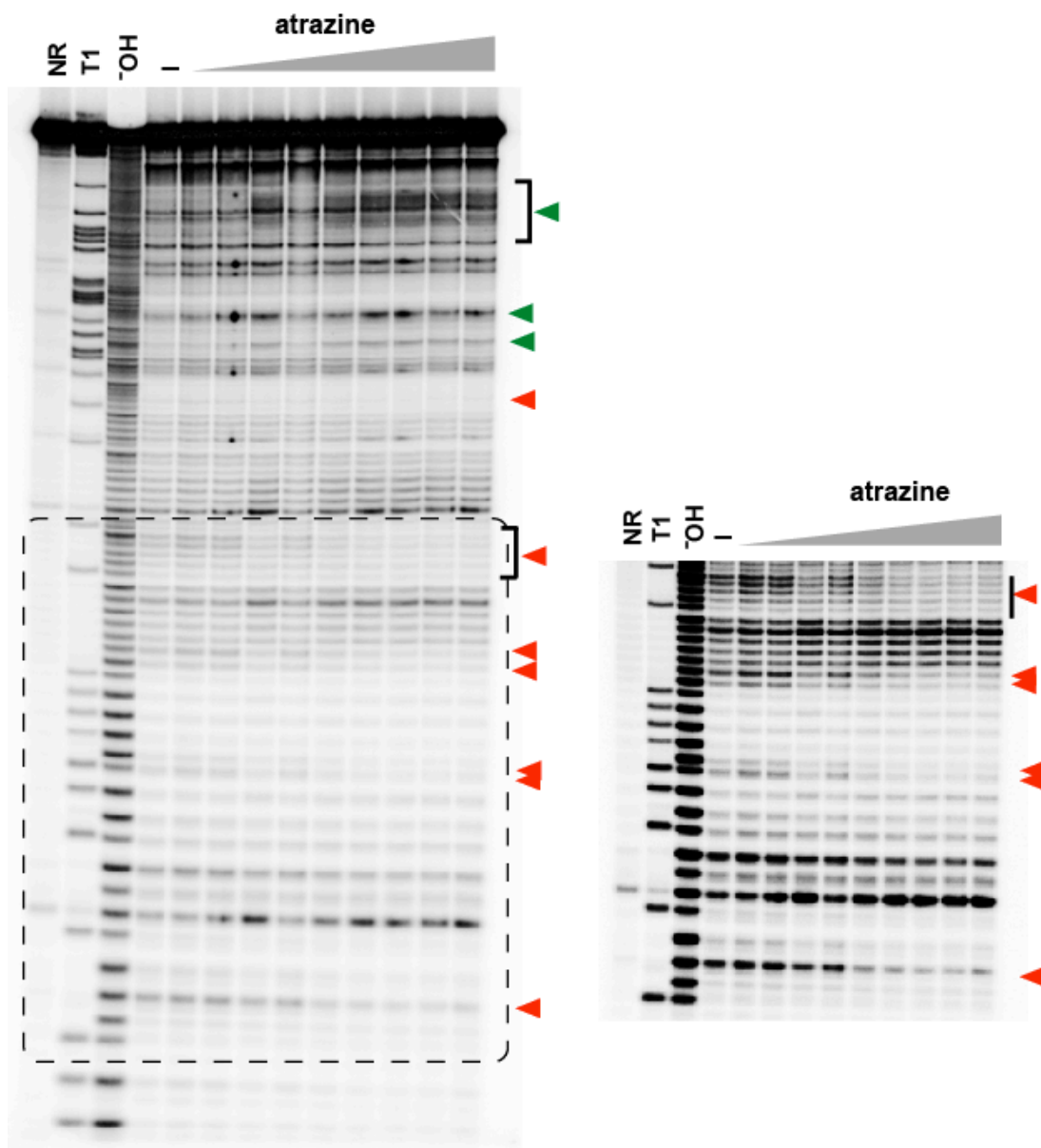
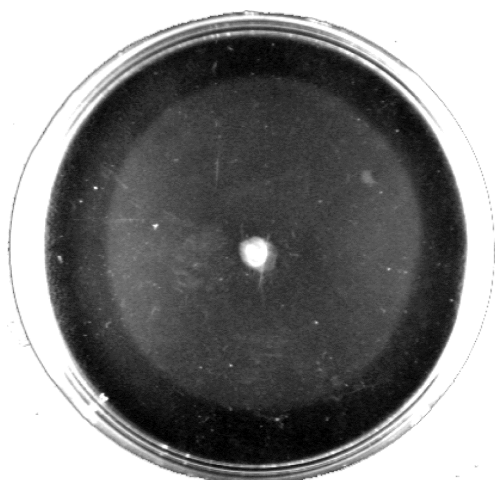
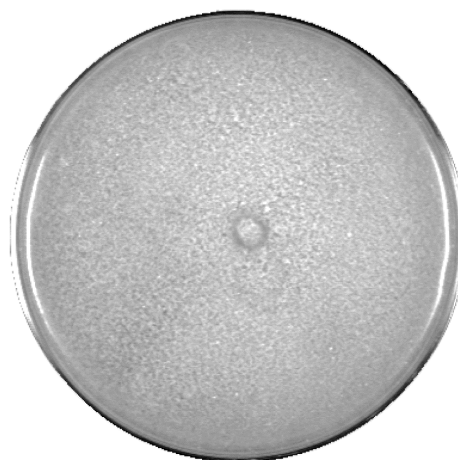


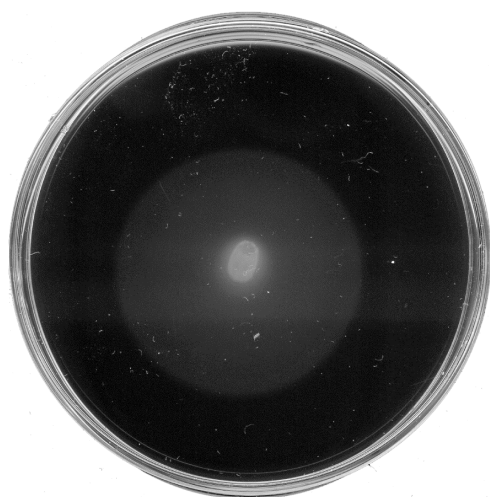
Figure S8. In-line probing reveals a concerted structural modulation of the 5'-untranslated region in the presence of atrazine. The red arrowheads mark the nucleotides that react less in the presence of atrazine. Green arrowheads mark the nucleotides that react more in the presence of atrazine. The result was summarized in Supporting Figure S7 which was derived from an average of four such independent assays. NR, T1 and OH represent no reaction, partial digest with RNase T1 (G-specific cleavage) and partial digest with alkali, respectively. The inset shows the enhanced image of the 9-40 nucleotides.



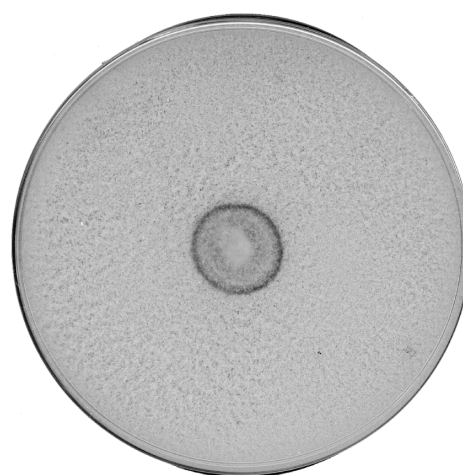
minimal media supplemented with atrazine (0.5 mM) and aspartate (1 mM)



minimal media supplemented with atrazine (4 mM) and aspartate (1 mM)



minimal media supplemented with atrazine (0.5 mM), aspartate (1 mM), and threonine (1 mM)



minimal media supplemented with atrazine (4 mM), aspartate (1 mM), and threonine (1 mM)

Figure S9. Migration experiment in minimal media. Minimal media contained $1\times$ M9 salts, 0.2% (v/v) glycerol, 1 $\mu\text{g}/\text{mL}$ of thiamine HCl, 20 $\mu\text{g}/\text{mL}$ each of methionine, leucine, and histidine. The semisolid agar plate contained 0.2% agar in M9 media, atrazine and chemoattractants (concentrations as noted in the figure). An overnight culture of a single colony of *E. coli* cells containing the plasmids were grown to saturation in tryptone broth. An aliquot of this culture (1.5 μL) was inoculated in 1.5 mL of M9 media, grown till mid-log-phase, diluted to an OD_{600} of 0.1, and 3.5 μL was applied approximately at the center of the Petri dish (85 mm dia). The plates were incubated at 30 $^{\circ}\text{C}$ for 30 h. With one chemoattractant (upper right), one dark ring indicating atrazine catabolism is present. With two chemoattractants (lower right), two dark rings are visible.

References.

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