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AMPLIFYING RIBOSWITCH SIGNAL OUTPUT USING CELLULAR WIRING

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Interim Report

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14. ABSTRACT

If fieldable riboswitch-based biological sensors are to fulfil their potential, it is necessary to increase their signal output. Here we report a novel modular amplification system using a riboswitch to initiate signaling between a sensing strain and a reporter strain of *E. coli*. A quorum sensing signaling molecule biologically wires the sensing and reporter strains together. The amplification circuit increased the amount of fluorescence generated on ligand binding compared to when the riboswitch controlled fluorescence expression directly. This had the corollary effect of increasing the sensitivity of the system, and allowed riboswitch-based reporting in *E. coli* strains that did not produce a detectable output when the riboswitch directly controlled reporter expression. The amplification circuit also reduced the time required to detect a signal output. The modularity of this amplification system coupled with the achievable increases in output can advance the development of riboswitches and biological sensors.

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Amplifying Riboswitch Signal Output Using Cellular Wiring

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Abstract

If fieldable riboswitch-based biological sensors are to fulfil their potential, it is necessary to increase their signal output. Here we report a novel modular amplification system using a riboswitch to initiate signaling between a sensing strain and a reporter strain of *E. coli*. A quorum sensing signaling molecule biologically wires the sensing and reporter strains together. The amplification circuit increased the amount of fluorescence generated on ligand binding compared to when the riboswitch controlled fluorescence expression directly. This had the corollary effect of increasing the sensitivity of the system, and allowed riboswitch-based reporting in *E. coli* strains that did not produce a detectable output when the riboswitch directly controlled reporter expression. The amplification circuit also reduced the time required to detect a signal output. The modularity of this amplification system coupled with the achievable increases in output can advance the development of riboswitches and biological sensors.

Keywords

Riboswitch, Amplification, Biological circuit, Signaling, Synthetic Biology, Biosensors.

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Riboswitches offer a method to reliably control the expression of any downstream gene in response to a diverse range of ligands.[1,2] The inherent modularity of riboswitches makes them ideal recognition elements in biological sensors since their inputs and outputs can be tailored to a specific detector. RNA riboswitches consist of a ligand-recognition aptamer, an expression platform, and a downstream gene.[3] On binding of a specific ligand, riboswitches regulate transcription or translation via a conformational change.[1,4] In riboswitches regulating translation, binding of the ligand to the aptamer results in a conformational change in the expression platform that exposes a ribosome binding site allowing translation of the downstream gene.[1,4]

However, a limitation of these riboswitches is that they are constrained to the ribosome binding site that confers switching behavior; altering the ribosome binding site sequence is likely to disrupt the functioning of the riboswitch. This limits the ability to tune the riboswitch to increase its output. Thus the efficacy of using riboswitches in sensors is reduced since their signal is usually only sufficient for detection by sensitive laboratory-based equipment, limiting their utility in fieldable sensors. Also synthetic riboswitches are developed within a specific genetic context. This becomes challenging when using a riboswitch to control a reporter gene that it was not designed and optimized for, since the sequence of the reporter may interfere with the conformation of the riboswitch, rendering it unswitchable. This can be overcome by retaining the 5' end of the gene that the riboswitch was originally optimized for and fusing it to the 5' end of the new reporter gene.[5,6] However, this is not always ideal and can lead to misfolding of the reporter. Similarly, many synthetic riboswitches are developed within a chassis specifically for enhanced transcript or protein expression.[5,7] Fieldable sensors are unlikely to utilize these cells since they do not survive well outside of controlled environmental conditions.

Biological circuits utilize molecules that connect different genetic 'components', so that the output of an upstream component serves as the input to the next component. Commonly used molecules in engineered inter-cellular biological circuits are those involved in bacterial quorum sensing.[8-10] In nature, quorum sensing bacteria produce a signaling molecule that is capable of freely crossing cell membranes. When the concentration of this molecule surpasses a threshold level, it activates the transcription of genes that have the cognate quorum sensing promoter. This process allows appropriate genes to be activated only in a high cell density environment.[11] When used in engineered biological circuits, this biological wiring allows a signal to be transmitted to surrounding cells. This has enabled synthetic biology to emulate electrical engineering to construct genetic logic gates to form genetic programs within and between cells.[8-10,12-14] We have applied biological circuitry to address the output limitations of riboswitch sensors. Wiring cells together with quorum signaling molecules allows the use of biological parts to 'tune' the output response of the sensor and, in keeping with the modular advantages of riboswitch biosensors, produce an amplification component that will be able to work with any cellular sensor that uses a riboswitch to regulate expression of a quorum signal-producing gene, as shown in Figure 1. Similarly, the circuit allows expression of any gene from a quorum signal-inducible promoter without any of the genetic context effects that can plague

changing reporters in a riboswitch.

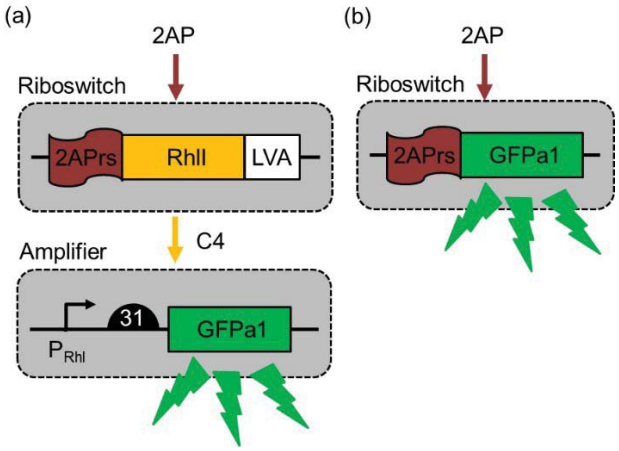

Figure 1. Schematics of DNA Architecture

A cell type containing the amplification circuit (a) is compared to a riboswitch upstream from GFP (b). The amplification circuit consists of a cell type containing a riboswitch that produces a signaling molecule ('C4') to be detected by the promoter ('PRhl') in the 'Amplifier' cell type, activating GFPa1. The riboswitch alone was tested in two different E.coli cell types, BL21 and JM109 to investigate its functionality in different chassis.

In this circuit, a riboswitch regulates production of a signaling molecule which is detected by an 'Amplifier' cell type that has a strong ribosome binding site before a reporter gene (Fig. 1a), thus amplifying its expression. The riboswitch we chose to use is responsive to 2-aminopurine (2AP) with a K_d of 2.8 nM.[6] This riboswitch was shown to have a low activation ratio (i.e., a small difference in gene expression between the inactivated and activated states) and exhibited minimal gene expression in the absence of its ligand,[15] making it an ideal candidate for signal amplification. The 2AP riboswitch was transcribed from a strong constitutive promoter listed in the Registry of Standard Biological Parts (J23104; parts.igem.org/Part:BBa_J23104). The reporter protein we have chosen is GFPa1, a green fluorescent protein from the cephalochordate *Branchistoma floridae*. This protein has been shown to exhibit extremely strong fluorescence,[16] and thus makes an ideal reporter. The quorum sensing molecule we chose to use was *N*-butyryl-homoserine lactone (C4-HSL) because it has a higher activation threshold than other quorum sensing molecules used in synthetic biology, such as *N*-3-oxo-dodecanoyl-homoserine lactone (3OC12-HSL;),[10,17,18] which will serve to insulate the reporter complex from any potential 'leakiness' in the riboswitch. C4-HSL is produced by the acyl homoserine lactone (AHL) synthetase, RhlI, and binds to its cognate transcription factor, RhlR, activating it and initiating transcription from a specific promoter, P_{Rhl}.[11] This signaling mechanism was originally

from the bacterium *Pseudomonas aeruginosa* and has no homologue in the *E. coli* chassis we are using, thus making activation by endogenous molecules unlikely. In designing this circuit we needed to tune the production of each protein to ensure that the fluorescent protein is strongly expressed on receipt of the input signal, but AHL production is kept below its threshold until the riboswitches ligand is detected. This was accomplished by modifying protein levels using different strengths of ribosome binding site and the addition of degradation tags. Degradation tags are short peptide sequences placed at the C-terminus of proteins that mark them for degradation by intracellular proteases.[19] We referenced ribosome binding sites listed in the Registry of Standard Biological Parts (parts.igem.org/Ribosome_Binding_Sites/ Prokaryotic/Constitutive/Community_Collection) and also quantified by Wang et al.,[20] and used degradation tags also listed in the registry (parts.igem.org/Protein_domains/Degradation). We placed the LVA degradation tag after RhlI to reduce riboswitch leakiness from inducing reporter gene expression without the presence of the analyte. A strong ribosome binding site (B0031) was placed before GFPa1 to serve to amplify reporter gene expression. Plasmids were used as the computational units of the cell. This allows plasmid function to be further 'insulated' by segregating them into separate cell types,[10] thus reducing the likelihood of false positives because of leaky riboswitch control of *RhlI* expression compared to if the circuit was entirely within a single cell type. An intracellular amplification circuit has been previously described,[21] but was used to increase the dynamic range of responses from promoters to a specific input rather than using riboswitches as the input receptors, and resulted in a decrease in gene expression as the reporter rather than the increase required for fieldable sensors. Plasmids forming the amplification circuit (Figure 2) were transformed into *E. coli* JM109 cells, a chassis that was not developed for enhanced protein expression and thus one that is not usually used for riboswitch experiments, but one that would therefore approximate the bacteria that might be used in fieldable sensors.

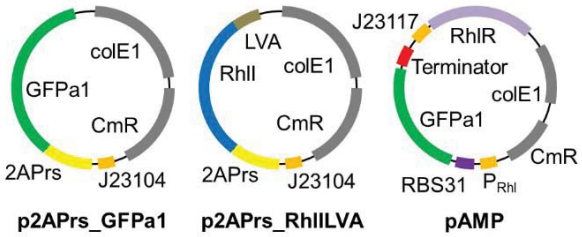

Figure 2. Schematic of the amplification circuit plasmids and the riboswitch-reporter plasmid

Components were inserted into the pJ64100 plasmid. Orange bars: promoters; yellow: 2AP riboswitch (aadA); dark purple: ribosome-binding site; green: GFPa1; blue: RhlI; taupe: degradation tag; red: terminator B0015; grey: ColEI origin of replication and chloramphenicol resistance gene.

The 2AP riboswitch amplification circuit was compared to the plasmid containing the 2AP riboswitch directly controlling GFPa1 expression (p2APrs_GFPa1, Fig. 1b.) in *E. coli* JM109 cells and also in *E. coli* cells that are usually used in riboswitch experiments because of their enhanced protein expression (BL21(DE3)).[22] No response was detected in JM109 cells containing p2APrs_GFPa1 at any of the 2AP concentrations investigated. However, in JM109 cells containing the 2AP riboswitch amplification circuit plasmids, GFPa1 expression was detected and exhibited activation ratios that were double those of BL21 cells containing GFPa1 directly controlled by the 2AP riboswitch, for all concentrations of 2AP that elicited a detectable response (Table 1). The amplification circuit in JM109 cells also exhibited a ten-fold lower limit of detection than BL21 cells containing p2APrs_GFPa1 (Figure 3). By comparison with direct C4- HSL stimulation of mono-cultures of the Amplifier cell type (Figure 1a), the fluorescence produced when the amplification circuit was exposed to 500 μM, 50 μM, and 5 μM 2AP corresponded to there being 16 μM, 2 μM, and 1 μM C4-HSL in the media, respectively. The temporal sensitivity of the riboswitch was also amplified when it was included in the amplification circuit (Figure 4). Timecourse experiments indicated that GFPa1 expression was detected sooner and increased more quickly in the amplification circuit compared to cells containing GFPa1 directly controlled by the 2AP riboswitch.

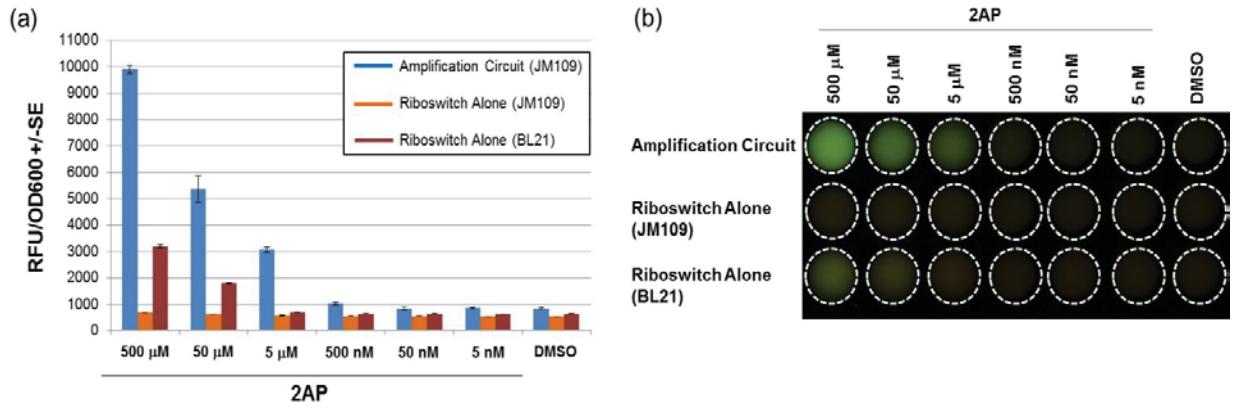

Figure 3. The amplification circuit increases riboswitch sensitivity

a) Each culture was grown for 24 h with varying concentrations of 2AP. Graph is representative of three experiments with error bars representing SE of three separate cultures. b) Fluorescence of cell types after 24 h incubation in the presence of different 2AP concentrations. Aliquot of 3 mL cultures were washed with nanopure water and loaded into a plate for visualization.

Table 1. The amplification circuit amplifies the activation ratio of the riboswitch[a]

	2AP concentration		
	500 µM	50 µM	5 µM
Amplification circuit in JM109	11.1(0.6)	6.0(0.3)	3.5(0.2)
Riboswitch alone in JM109	1.3(0.1)	1.2(0.1)	1.1(0)
Riboswitch alone in BL21	5.2(0.1)	2.9(0)	1.1(0)

[a]*Cells were inoculated into 3 mL LB-antibiotic with varying concentrations of 2AP and incubated at 37°C with shaking for 24 h. Fluorescence and OD were measured spectrophotometrically. Activation ratio was calculated by dividing the fluorescence normalized to OD of the culture at each 2AP concentration by the fluorescence normalised to OD of cultures exposed to DMSO. Mean activation ratios from three separate experiments (SE).*

By inducing a riboswitch to initiate communication to surrounding reporter cells, we have been able to amplify its signal, even when using a chassis that is not commonly used in riboswitch studies. Using this simple amplification circuit, we have expanded the utility of riboswitches as sensors by increasing the response of a 'weak' riboswitch and amplifying the response of a riboswitch when transformed into non-traditional cell types. This overcomes some of the major hurdles associated with transitioning riboswitches into sensitive fieldable biological-based sensors that will be detectable using field-based equipment. This amplification circuit increases the utility of riboswitches with low activation ratios and expression levels, and

increases the modularity of riboswitch-based sensors since, providing that the riboswitch is optimized to control AHL gene expression, changing the reporter does not have to be accompanied by the re-optimization of the riboswitch-reporter complex. This circuit will also aid in riboswitch selection, since it will identify those riboswitches that have low 'ON':'OFF' activation ratio that would be missed if the riboswitch was directly regulating the reporter gene. Selecting riboswitches in this way would also streamline their inclusion in fieldable sensors since they would already be optimized for AHL gene regulation and would plug straight into the amplification circuit.

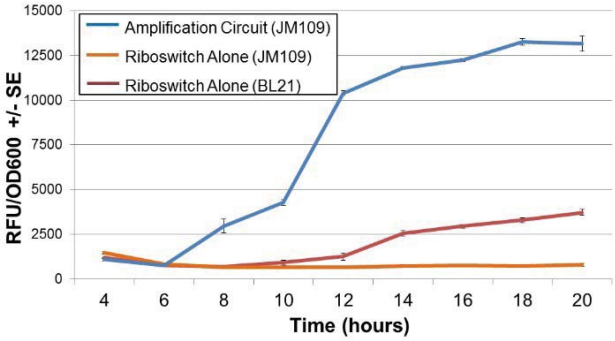

Figure 4. The amplification circuit reduces riboswitch response time

A timecourse was conducted to compare the riboswitch alone in two different E.coli cell types (BL21 and JM109) to the amplification circuit in JM109 cells. Cells were inoculated into 3 mL LB- antibiotic with 500 μM 2AP and incubated at 37°C with shaking. Fluorescence and OD was measured spectrophotometrically. Graph is representative of three experiments with error bars representing SE of three separate cultures.

METHODS

Plasmid Editing, Growth Conditions, and Data Collection. DNA plasmids were transformed into *E.coli* strains JM109 or BL21. Any changes made to plasmids, i.e. addition of degradation tag, replacement of ribosome binding site, or replacement of a gene sequence, were done using PCR, restriction digest, ligation, and/or Gibson assembly. Restriction enzymes and the Gibson assembly kit were purchased from NEB. DNA polymerase and T4 DNA ligase were purchased from Promega. All other chemicals were purchased from Sigma-Aldrich.

E.coli BL21(DE3) and JM109 cells were transformed with p2APrs_GFPa1, and *E. coli* JM109 cells were transformed with either p2APrs_RhlILVA, or pAMP (Fig. 2). Transformed cells were inoculated into Luria-Bertani (LB) broth containing 25 μM chloramphenicol and were grown to an optical density measurement (600 nm) of 0.4. These cultures were diluted 1:100

into LB-chloramphenicol media containing the stated concentrations of synthetic 2AP, or DMSO as a control. The Riboswitch and Amplifier cell types (Figure 1a) were inoculated in a 1:1 ratio and co-cultured to form the Amplification circuit. All cultures were grown at 37°C with 220 rpm shaking. At reported timepoints, 150 μL of cultures were aliquoted into 96 well flat-bottomed polystyrene plates (Costar, black with clear bottom) and optical density measurements (600 nm) and fluorescence measurements (Ex: 480 nm; Em: 510 nm) of cultures were performed using a Spectra Max M5 Molecular Devices plate reader.

Limit of detection assay. Cultures were prepared as described above. Varying amounts of 2AP were inoculated to the samples to measure efficiency at 500 μM, 50 μM, 5 μM, 500 nM, 50 nM, and 5 nM. DMSO was added to the controls. Cultures were grown for 24 hours and measured for fluorescence and optical density. Detectable signal for each culture was determined to be above the fluorescence corresponding to the mean DMSO fluorescence plus three times the standard deviation.

Timecourse assay. Cultures were prepared as described above. Synthetic 2AP was added to each sample to a final concentration of 500 μM; DMSO was added to the control samples. Cultures were measured for optical density and fluorescence every hour for 20 hours.

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