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Using a Riboswitch Sensor to Examine Coenzyme B_{12} Metabolism and Transport in *E. coli*

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SUMMARY

Small molecules play crucial roles in every major cellular process. Despite this, detecting their levels within cells remains a significant challenge. Here, we describe intracellular sensors of coenzyme B_{12} that make use of the exquisite molecular detection capabilities of a naturally occurring riboswitch. These probes sensitively detect their target using colorimetric, fluorescent, or luminescent reporters. To assess their utility in the study of biological systems, the sensors were applied to examine the synthesis and the import of coenzyme B_{12}. The sensors were able to monitor the effects of genetic deletions, recombinant expression of foreign genes, and varied growth conditions on both of these processes. These results indicate that riboswitch-based sensors can provide valuable information on intracellular small molecule concentrations that can be employed in the study of related cellular processes.

INTRODUCTION

Cells continually synthesize, degrade, import, and export a vast array of small molecule metabolites. These processes are crucial both to adapting to environmental changes and to carrying out routine cellular functions. Despite the widespread importance of small molecules to cellular physiology, monitoring changes in their intracellular concentrations remains a significant challenge. Traditionally, measuring cellular metabolite levels has been carried out in vitro, following cell lysis. While these approaches remain valuable for certain applications, they have many drawbacks as well. Efficient separation and identification of the target metabolite can require multiple steps and specialized equipment and knowledge that are not readily accessible to all researchers. Furthermore, the true intracellular concentration of the target molecule can be skewed by contamination from the growth medium or by degradation, aggregation, or adhesion to cellular debris following cell lysis. Intracellular detection of small molecules is an attractive alternative that has seen much progress in recent years (Binkowski et al., 2009; Medintz, 2006). One successful approach has made use of protein fusions between molecular receptor domains and reporter proteins such as green fluorescent protein (GFP) or firefly luciferase. Here, ligand binding to the receptor activates an otherwise inert reporter protein, which then produces a readily detectable signal (Fan et al., 2008; Paulmurugan and Gambhir, 2006). Alternatively, two fluorescent proteins attached at specific sites of the receptor undergo changes in fluorescence resonance energy transfer (FRET) upon metabolite binding (Fehr et al., 2002; Miyawaki et al., 1997; Romoser et al., 1997). To date, however, this methodology has been limited to detecting ligands with natural, well characterized, high affinity protein receptors.

Like protein, RNA has also been used effectively to detect biological agents (Cho et al., 2009). Using large oligonucleotide libraries and a selection technique known as systematic evolution of ligands by exponential enrichment (SELEX) (Ellington and Szostak, 1990; Tuerk and Gold, 1990), dozens of specific, high affinity nucleic acid receptors (dubbed aptamers) have been isolated. Artificially obtained aptamers have been successfully modified to act as in vitro sensors of target molecules using a variety of creative strategies (Navani and Li, 2006). Aptamers have also been found naturally, as sensory domains in RNA regulatory elements known as riboswitches (Mironov et al., 2002; Nahvi et al., 2002). Riboswitches are most commonly found in the 5′ untranslated regions of prokaryotic mRNAs, where they regulate the expression of downstream genes through structural changes undergone in response to the binding by a specific target molecule. Typically, ligand binding at the aptamer domain either alters ribosome access to the transcript or affects the formation of intrinsic transcriptional terminators (reviewed in Barrick and Breaker, 2007).

Intrinsically, riboswitches act as relatively simple and direct metabolite sensors that are capable both of molecular recognition and of transducing this information by altering protein expression. As depicted in Figure 1, we hypothesized that a riboswitch controlling the expression of a reporter gene could be applied as a reliable, relatively noninvasive intracellular metabolite sensor. In the present study, we construct such a sensor to monitor adenosylcobalamin (AdoCbl) concentrations in the model Gram-negative bacterium *E. coli*. The ultimate goal for any technology such as this is its application in addressing biologically relevant questions. Accordingly, this study also

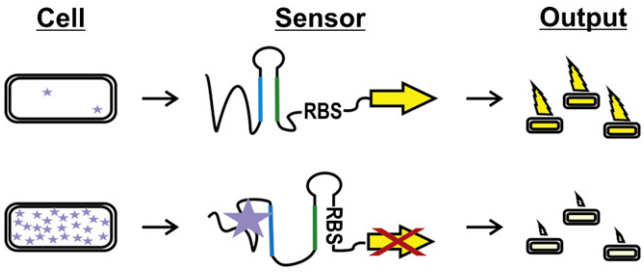

Figure 1. Depiction of Riboswitch-Based Intracellular Metabolite Sensors

In the simplified example shown, cells containing a low concentration of the target metabolite (purple star) have a higher proportion of riboswitches in the unbound state. The RNA conformation in this state features an interaction between the green and blue segments and the ribosome binding site (RBS) is unstructured. This allows for efficient translation of the reporter gene (yellow arrow) downstream and results in a high level of signal output. At higher target concentrations riboswitches adopt the bound conformation. In this state the blue segment of the riboswitch interacts with the target. This frees up the green segment and allows it to interact with the RBS. The resulting structured RBS inhibits translation initiation of the reporter gene and leads to a low signal output.

exploits the ability of these sensors to monitor target concentrations as a means to investigate the processes of AdoCbl import and metabolism.

AdoCbl, also known as coenzyme B_{12}, is a cofactor required for the catalysis of a number of isomerization reactions (Roth et al., 1996). AdoCbl is not important for growth under standard laboratory conditions for *E. coli* and related species of bacteria. Its primary importance appears to be in the metabolism of compounds that are important sources of carbon, nitrogen, and energy in specific environments. B_{12}-dependent enzymes for metabolizing glycerol, propanediol, and ethanolamine are prevalent in enteric bacteria (Abeles and Lee, 1961; Bradbeer, 1965; Toraya et al., 1979). *E. coli* lacks these enzymes for both glycerol and propanediol and thus ethanolamine utilization appears to be the paramount B_{12}-requiring process (Lawrence and Roth, 1996). A growing number of studies point to an important role for ethanolamine utilization for microbial in vivo growth and pathogenesis (Bourgogne et al., 2006; Huang et al., 2007; Joseph et al., 2006; Klumpp and Fuchs, 2007; Korbel et al., 2005; Lawhon et al., 2003; Maadani et al., 2007), providing an added layer of interest in understanding the metabolic and import processes responsible for providing cells with AdoCbl. The sensors described in this study represent valuable tools for the study of these processes as they allow for sensitive detection of cellular AdoCbl levels using fast and convenient assays.

RESULTS

Construction of Riboswitch-Based AdoCbl Sensors

The AdoCbl sensors presented here exploit the naturally occurring AdoCbl-responsive riboswitch that regulates the *E. coli btuB* gene (Nahvi et al., 2002). It has been shown to be very specific for AdoCbl, strongly discriminating against a range of structural analogs, including the other active form of vitamin B_{12}, methylcobalamin (Nahvi et al., 2002). The complete mechanism of genetic control is not fully understood for this riboswitch; however, several studies indicate that the primary mechanism involves AdoCbl-induced sequestration of the ribosome binding site (shown schematically in Figure 1) (Franklund and Kadner, 1997; Lundrigan et al., 1991; Nou and Kadner, 1998, 2000). Evidence also suggests that the beginning of the *btuB* coding region is important for a second form of expressional control that occurs at the level of transcription (Franklund and Kadner, 1997; Lundrigan et al., 1991; Nou and Kadner, 1998). We have used this entire riboswitch, including the complete 5′ untranslated region (UTR) and the first 70 amino acids of BtuB, as the sensing element for our AdoCbl sensors.

Three parallel versions of the sensor were constructed that are identical other than the reporter gene used. Each of these probes employs a different mode of detection: β-galactosidase (colorimetric), firefly luciferase (luminescent), and DsRed-Express red fluorescent protein (fluorescent). Reporter genes were fused in frame to the riboswitch and cloned into vectors derived from the pBAD series of plasmids (Guzman et al., 1995). Complete details of these plasmids and their construction are provided in Figure S1 and Supplemental Experimental Procedures (available online).

To test the ability of these constructs to detect AdoCbl they were transformed into the *E. coli* strain BW25113 (Datsenko and Wanner, 2000), henceforth referred to as wild-type (WT) cells. Reporter activity was measured for transformants grown both in media containing 1 μM of AdoCbl and in media lacking AdoCbl or a suitable metabolic precursor (referred to as "no B_{12}" conditions). All three reporter constructs showed a strong, ~100-fold repression in response of AdoCbl (Figure 2A). For each reporter, vectors featuring mutant riboswitches were also tested. These were identical to the WT sensors other than a double mutation ($A_{149}A_{150}$ > TT) to a region of the riboswitch previously shown to disrupt AdoCbl binding (Nahvi et al., 2002). None of these mutant constructs showed a detectable response to AdoCbl, indicating that the riboswitch is indeed responsible for the observed regulation.

We next sought to determine the dose response of our sensors over a broad range of target concentrations. We were able to detect AdoCbl at high picomolar media concentrations, observing a saturated response by the low-mid nanomolar range (Figure 2B). The observed sensitivity in this low concentration range is reflective of both the high affinity of the riboswitch and the ability of *E. coli* to concentrate vitamin B_{12} compounds intracellularly using an effective transport system. *E. coli* cells grown in media containing 10 nM vitamin B_{12} have been found to accumulate an intracellular concentration of ~25 μM (Reynolds et al., 1980). This suggests that the intracellular AdoCbl concentration required to saturate our sensors is in the low micromolar range. This is roughly consistent with the high nanomolar apparent K_d determined for this riboswitch in vitro (Gallo et al., 2008; Nahvi et al., 2002).

We were impressed by the observed sensitivity and signal amplitude of the sensors. However, we were keen to assess their ability to be applied in the study of biological processes that involve AdoCbl, as well as to more rigorously test their capacity to detect physiologically relevant fluctuations in AdoCbl concentrations. In the following sections we apply the riboswitch-based AdoCbl sensors to monitor the changing levels of their target in response to genetic manipulations and varying environmental

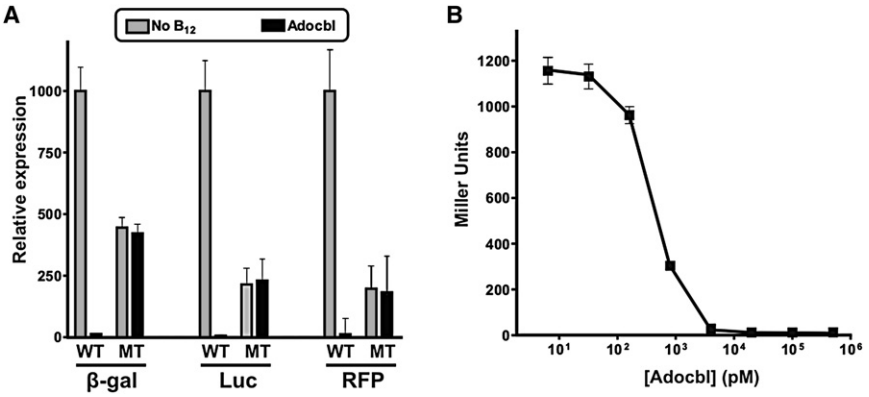

Figure 2. Intracellular Detection of AdoCbl by Riboswitch-Based Sensors

(A) AdoCbl detection using sensor constructs featuring three different reporter genes: β-galactosidase (β-gal), firefly luciferase (Luc), and the DsRed-Express RFP. For each reporter, a parallel construct featuring a double mutation that disrupts ligand binding (MT) was also tested. Assays were conducted using cells grown in medium either lacking vitamin B_{12} (no B_{12}) or containing 1 μM AdoCbl. Reporter activity was normalized for all samples by setting the values for the WT sensors in the no B_{12} conditions to 1000.

(B) Dose response of the WT β-galactosidase sensor over a wide range of AdoCbl concentrations. Error bars represent standard deviation in all graphs.

conditions. The aim of these experiments was to examine the synthesis and import of AdoCbl in *E. coli*, with an emphasis on the importance of the genetic factors involved in these processes.

Riboswitch-Based Sensors Can Be Used to Monitor AdoCbl Metabolism

AdoCbl is a large, chemically complex molecule containing a central cobalt atom that is coordinated by six ligands (see Figure 3). Four of these interactions lie in a plane with a porphyrin-like structure known as the corrin ring. The final two ligands for cobalt are an adenosyl moiety (upper axial ligand) and a nucleotide loop (lower axial ligand). *E. coli* cannot synthesize vitamin B_{12} compounds de novo because it lacks the ~20 dedicated genes required to synthesize the central ring structure. *E. coli* is, however, able to salvage incomplete corrinoid molecules from the environment by carrying out the final steps in AdoCbl synthesis that involve the addition of the upper and lower axial ligands for cobalt (Lawrence and Roth, 1996). To do so they require cobinamide (Cbi) as a minimal metabolic

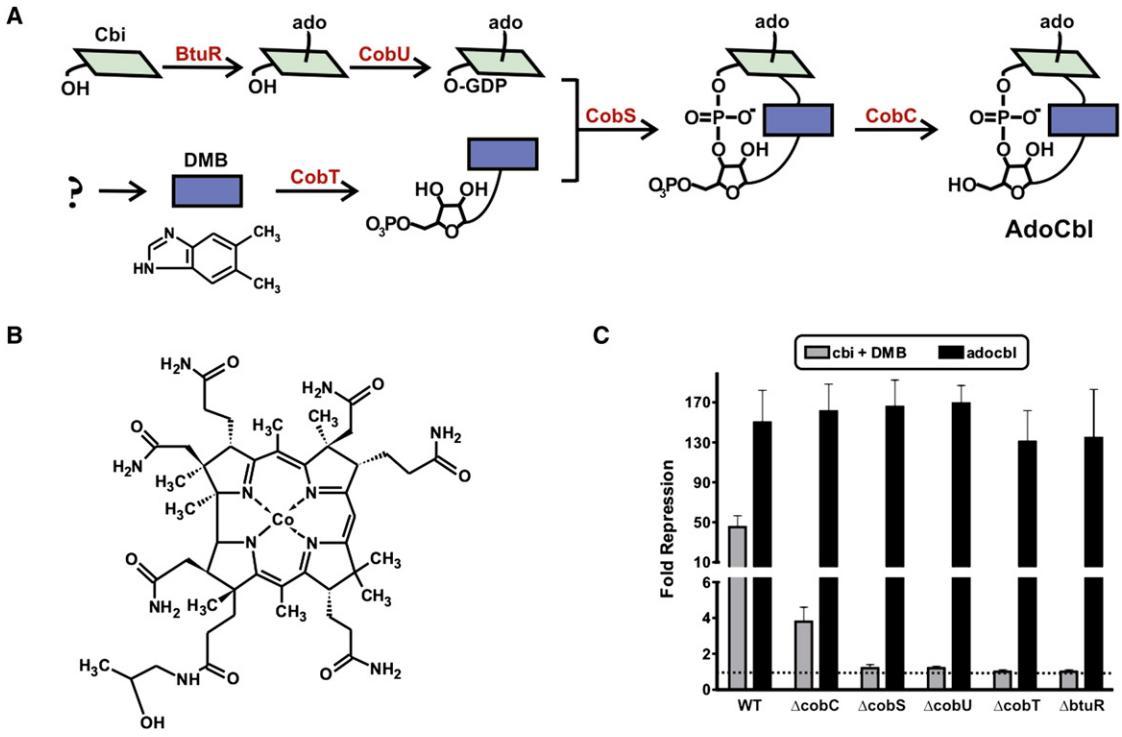

Figure 3. Using Riboswitch-Based Sensors to Probe AdoCbl Metabolism

(A) Schematic of the predicted *E. coli* AdoCbl biosynthetic pathway from minimal precursors. Ado, 5′-deoxyadenosyl.

(B) Chemical structure of Cbi.

(C) Assay probing AdoCbl concentrations in strains carrying deletions to each of the genes from the pathway shown in (A). The data is expressed as the fold repression observed for cells grown in the presence of Cbi and DMB or AdoCbl relative to the no B_{12} control. The dotted line indicates no repression and thus no detectable AdoCbl. Error bars represent the standard deviation. See also Figures S2 and S3.

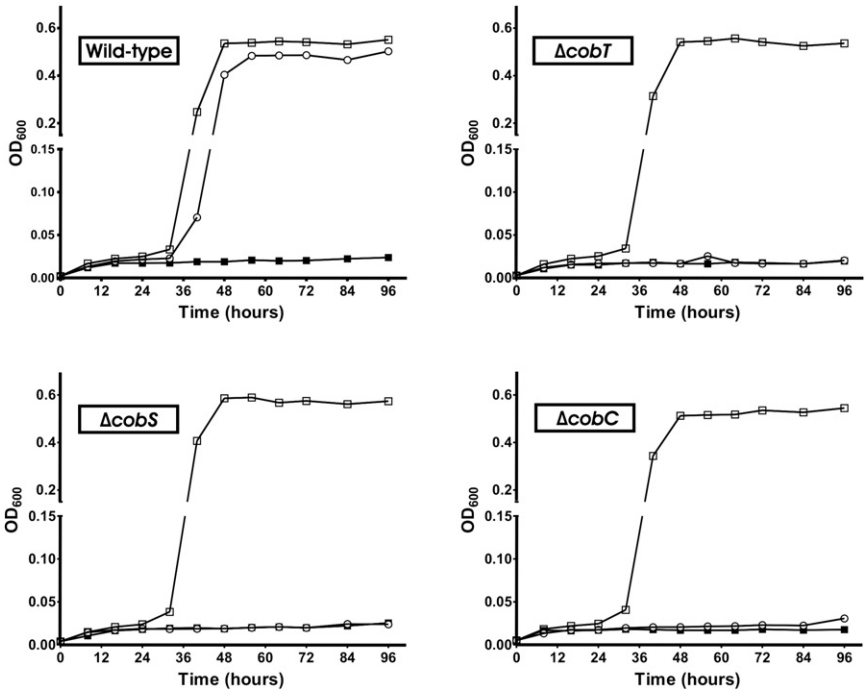

Figure 4. Growth of Metabolic Deletion Strains Using Ethanolamine as the Sole Nitrogen Source

The ability of selected strains to produce sufficient AdoCbl to sustain AdoCbl-dependent ethanolamine utilization was assessed in media containing Cbi and DMB (open circles), as well as in control media containing no B_{12} (filled boxes: negative control) and AdoCbl (open boxes: positive control). Panels are identical other than the strain used, which is indicated in the top left corner of each graph. Data points represent the mean of three biological replicates.

precursor. 5,6-Dimethylbenzimidazole (DMB), a base incorporated into the nucleotide loop of cobalamins, must also be provided exogenously to produce elevated levels of AdoCbl (see below).

Studies of vitamin B_{12} metabolism have generally focused on organisms such as *S. enterica* and *P. denitrificans* that are able to carry out the full de novo synthesis. Figure 3A shows a simplified schematic of the predicted AdoCbl biosynthetic pathway in *E. coli*, based largely on studies of homologous genes in related *Salmonella* species. To determine the importance of each of the enzymes shown in this pathway, we tested the ability of deletion strains to synthesize AdoCbl. The β-galactosidase version of the sensor was transformed into each strain and was used to monitor AdoCbl levels in cells grown in medium containing the metabolic precursors Cbi and DMB, as well as in control media containing no B_{12} (Figure 3C). For each strain, the reporter activity in the no B_{12} control was compared to the activity in the media containing Cbi and DMB to provide a fold repression. A fold repression greater than 1 implies that AdoCbl is being produced. A minor variation in the raw reporter values was observed (<2-fold) for the unrepressed expression from strain to strain as shown in Figure S2. However, this day-to-day variation was not observed for the fold-repression values, which were very consistent. Additionally, for each strain the mutant construct was tested (Figure S3). As expected, the mutant construct was not responsive to AdoCbl in any of the strains and the raw reporter activities only varied to a small extent from strain to strain, as observed for the wild-type sensor.

The reporter activities show that the Δ*cobC* strain retained a partial ability to synthesize AdoCbl, while the other deletions could not produce sufficient levels to be detected by our sensors. For *btuR*, *cobS*, and *cobU*, this is consistent with previous observations that indicate that these genes are essential for cobalamin synthesis (Lawrence and Roth, 1995; Lundrigan and Kadner, 1989; O'Toole et al., 1993). By contrast, strains lacking CobC and CobT activity maintain sufficient levels of cobalamin synthesis to grow under methylcobalamin-requiring conditions (Lawrence and Roth, 1995; O'Toole et al., 1993, 1994). While this observation has not yet been explained for *cobC*, genetic evidence from *S. enterica* suggests that the loss of *cobT* can be partially compensated for by the activity of CobB, a member of the SIR2 family of regulators (Tsang and Escalante-Semerena, 1998).

We were keen to investigate how the levels of AdoCbl detected by the sensors in these strains relate to physiological processes that involve AdoCbl in *E. coli*. Toward this end, we assessed the ability of the metabolic mutants to grow in media where ethanolamine was the sole nitrogen source. Under these conditions AdoCbl is required for growth as it is an essential cofactor for the first step in the ethanolamine utilization pathway (Bradbeer, 1965; Chang and Chang, 1975). Both metabolic mutants previously shown to be dispensable for methylcobalamin-dependent growth (Δ*cobT* and Δ*cobC*) were assayed, as were WT and Δ*cobS* cells, which served as controls. None of the tested deletion strains were able produce sufficient levels of AdoCbl to support growth under these conditions (Figure 4). When AdoCbl was provided in the medium each of the strains grew like the WT strain, confirming that these strains were experiencing a lesion in AdoCbl metabolism. These results indicate that the cobalamin synthesis that occurs in the Δ*cobT* strain, while below the detection threshold of our sensors, is also less than that required for ethanolamine utilization. This is consistent with previous results for *S. enterica* (Anderson et al., 2008). Our sensors can detect the cobalamins produced in Δ*cobC* strain, which is also unable to support growth on ethanolamine. However, it is possible that this could be due to the riboswitch detecting CobC's phosphorylated substrate (see Discussion).

Riboswitch-Based Sensors Can Detect the Availability of B_{12} Lower Axial Ligands

We next turned our attention to DMB, the base incorporated as the lower axial ligand in AdoCbl. Previous studies have shown that *S. enterica* produces DMB at very low levels from flavonoid precursors (Keck et al., 1998). These levels are sufficiently low to

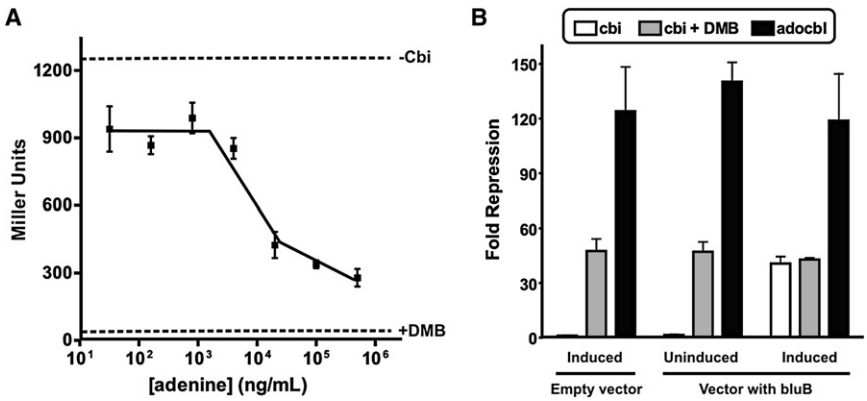

Figure 5. Monitoring the Availability of Lower Axial Ligands Using Riboswitch-Based Sensors

(A) The affect of adenine concentration on sensor response in media containing Cbi but not DMB. Dotted lines indicate the signal for controls lacking Cbi and containing both Cbi and DMB.

(B) The effect of *M. smegmatis* BluB expression on AdoCbl concentrations in the absence of exogenous DMB. BluB was expressed from pBAD18 by adding the inducer L-arabinose and compared to the empty vector and uninduced controls. The ability of BluB to produce DMB for AdoCbl synthesis was assessed in media containing Cbi alone and compared to control samples grown in media containing Cbi and DMB or AdoCbl. Error bars represent the standard deviation for all graphs.

incite the suggestion that the DMB produced by *S. enterica* is through a nonenzymatic chemical reaction or as a side product of flavin-utilizing enzymes (Maggio-Hall et al., 2003; Taga and Walker, 2008). Consistent with this, when Cbi is provided without DMB our sensors show a very low level of repression of less than 2-fold (Figure 5A). Under these conditions it is likely that some or all of the repression observed is due to the synthesis of *pseudo*-AdoCbl, a functional variant of AdoCbl in which DMB is replaced by adenine. In *S. enterica*, while DMB is used preferentially, adenine-containing cobalamins are produced using the same metabolic pathway and can be used to support B_{12}-requiring processes when DMB is not available (Anderson et al., 2008). An AdoCbl analog in which DMB was replaced with 2-methyladenine was recently found to bind the *E. coli btuB* riboswitch with a very minor reduction in affinity, indicating that this riboswitch has not evolved a strong selectivity for DMB (Gallo et al., 2008). To determine whether our sensors also detect *Pseudo*-AdoCbl, we assayed cells grown in media containing Cbi and different concentrations of adenine. We observed a dose-dependent repression of reporter activity from our sensor. This demonstrates that *pseudo*-AdoCbl is produced at an elevated level under these conditions, which can be detected by our sensors (Figure 5A).

While the source of DMB remains unclear for *E. coli*, it was recently uncovered that some organisms boast an enzyme, BluB, which catalyzes the conversion of reduced flavin mononucleotide to DMB (Campbell et al., 2006; Gray and Escalante-Semerena, 2007; Taga et al., 2007). While *E. coli* lacks an obvious BluB homolog, we were intrigued by the possibility that BluB could be recombinantly expressed in *E. coli* to provide an enhanced ability to synthesize DMB. To test this hypothesis, the *bluB* gene from *Mycobacterium smegmatis* (*M. smegmatis*) was cloned and inducibly expressed in WT *E. coli* cells also carrying the β-galactosidase sensor plasmid. Remarkably, recombinantly expressed BluB produced AdoCbl at the same level as samples where excess DMB was provided exogenously (Figure 5B). This demonstrates that a single gene from a distantly related organism can provide *E. coli* with an efficient means of synthesizing DMB, where one does not appear to exist. Furthermore, this displays the ability of the riboswitch sensors to detect genetic modifications that result in a gain of function, in addition to the loss of function detected for the metabolic deletions.

Riboswitch-Based Sensors Can Be Used to Monitor AdoCbl Transport

Vitamin B_{12} import is among *E. coli*'s most intricate and impressive transport systems. Because it is too large to permeate outer membrane (OM) porins, vitamin B_{12} is actively transported across both the OM and the cytoplasmic membrane (CM) in a process that requires at least seven genes (shown schematically in Figure 6A). To assess the importance of each of these genes in the transport process, we tested the effects of their deletion on intracellular AdoCbl levels for cells grown in media containing 10 nM and 1 μM AdoCbl (Figure 6B). Raw reporter expression of the unrepressed samples and the response of the mutant sensor for each strain were consistent with those observed for the metabolic deletion strains (shown in Figures S2 and S3). As expected, deletion of each of the transport genes had a significant impact on AdoCbl import. However, when present in the media at 1 μM, AdoCbl could be detected intracellularly in each of the strains, presumably due to a very low level of diffusion. This is consistent with previous studies that found genetically isolated mutations to *btuB*, *tonB*, *btuC*, and *btuD* could survive in vitamin B_{12}-requiring conditions when it was supplied at a sufficiently high concentration (Bassford and Kadner, 1977; Di Girolamo et al., 1971). Also consistent with these results was our observation that deletions of OM factors had a more drastic effect than those of IM factors. AdoCbl could be detected intracellularly at the 10 nM concentration for the Δ*btuC*, Δ*btuD*, and Δ*btuF* strains, likely because AdoCbl would be concentrated in the periplasm in these strains, leading to a higher level of incidental entry into the cytoplasm, as proposed previously (Roth et al., 1996). Importantly, deletions to the IM transport proteins have been shown to be unable to support growth on ethanolamine when supplied with 5 μM vitamin B_{12} (Cadieux et al., 2002). This indicates that the sensors presented here can detect AdoCbl levels lower than those required for growth on ethanolamine as the sole nitrogen source.

A far less severe phenotype was observed for deletions of the *exbB* and *exbD* genes than for any of the other five genes. This was not surprising, since it has been previously shown that *tonB* function can be partially maintained by the TolQ/TolR in the absence of ExbB/ExbD (Braun and Herrmann, 1993). Despite this compensation, the sensors can readily detect a defect in

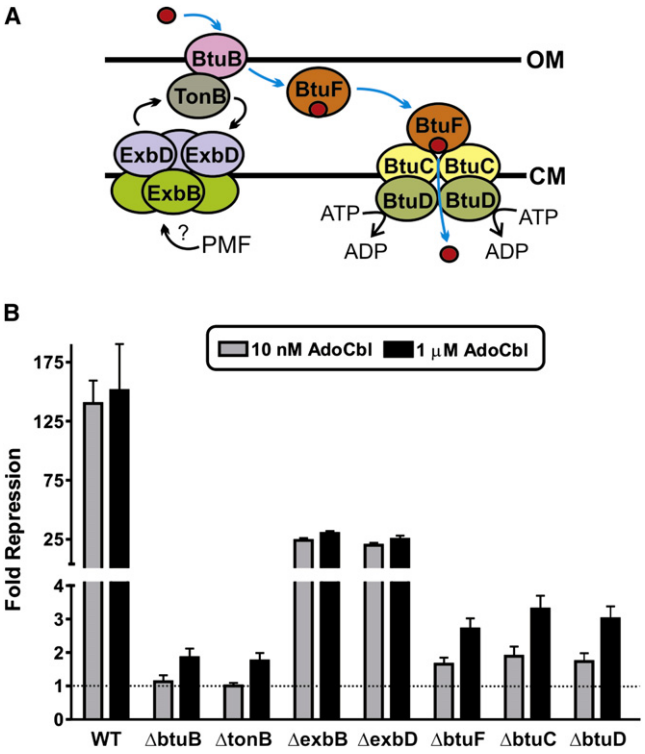

Figure 6. Using Riboswitch-Based Sensors to Probe AdoCbl Transport

(A) Schematic of vitamin B_{12} import in *E. coli*. The transport of vitamin B_{12} (small red circle) is instigated at the OM by the BtuB receptor, which binds vitamin B_{12} and a wide range of structural analogs with subnanomolar affinity. BtuB-mediated import into the periplasm requires an interaction with TonB, which harnesses the proton motive force (PMF) to energize import in conjunction with CM proteins ExbB/ExbD. Once in the periplasm vitamin B_{12} is bound by BtuF, which delivers its cargo to the CM transporter made up of BtuC/BtuD. BtuC/BtuD, an ABC transporter, uses ATP hydrolysis to pump vitamin B_{12} into the cytoplasm. Many aspects of the energetics of OM transport are unclear. The shuttling of TonB between membranes as shown is speculative, as is the stoichiometry of the active TonB/ExbB/ExbD complex.

(B) Riboswitch sensor assay monitoring AdoCbl import in strains carrying deletions to the genes shown in (A). Assays conducted as described in the caption for Figure 3C. Error bars represent the standard deviation. See also Figures S2 and S3.

these strains, showing a 5-fold change in signal compared to the WT strain.

DISCUSSION

This study demonstrates the capacity of riboswitches to be used as intracellular probes of a target metabolite. The sensors constructed here demonstrate several desirable properties. They display remarkable sensitivity, detecting AdoCbl levels at the low end of the biologically relevant range, a particularly impressive feat given the low abundance of this metabolite. Furthermore, deletions to metabolic genes showed a total lack of AdoCbl synthesis, indicating that the sensors are extremely specific with respect to Cbi and other metabolic precursor molecules that presumably accumulate under these conditions. One potential exception to this is the phosphorylated AdoCbl variant that presumably accumulates in the Δ*cobC* strain. The sensors detected a 3- to 4-fold repression in this strain when supplied with Cbi and DMB, which could reflect a detection of CobC's substrate. Alternatively, another phosphatase could partially compensate for the loss of CobC, leading to a reduced level of AdoCbl synthesis. Further experiments will be required to distinguish these possibilities.

For each of the genes predicted to be involved in AdoCbl transport and metabolism, defects were readily detected using the riboswitch-based sensors. Importantly, the extent of the resulting deficiency could also be assessed. This is most clear for the transport deletions, which showed the expected hierarchy with the OM transport genes showing the most severe phenotype, followed by the IM genes, and finally *exbB*/*exbD*, whose loss can be compensated for by the *tolQ*/*tolR* genes. An important advantage of using the sensors presented here rather than the growth phenotypes typically used to assess these mutants is that there is no selective pressure to accumulate mutations. Such suppressor mutations have been observed previously in growth experiments featuring AdoCbl mutants (Cadieux et al., 2002) and can lead to misleading data or incorrect conclusions.

We were unable to detect AdoCbl synthesis in the Δ*cobT* strain despite previous data indicating that a low level of cobalamin production is retained when *cobT* is disrupted (Lawrence and Roth, 1995; O'Toole et al., 1993). These results are based on the viability of methylcobalamin-requiring mutants, which can grow using as few as 20 cobalamin molecules per cell (Di Girolamo et al., 1971). Under conditions where cobalamins are available at extremely low quantities it is likely that a significant portion of this pool is not in the free, adenosylated form that is detected by the sensors presented here. Furthermore, for applications where the detection of an extremely low concentration range is desirable, chromosomal integration of the sensor could provide higher sensitivity. Plasmid-encoded sensors were desirable for this study to facilitate transfer between strains. However, the multicopy nature of the plasmid sensors could result in a scenario where, at sufficiently low concentrations, the target is not in excess. This is particularly applicable for cobalamins because they are required at such low levels.

One drawback of riboswitch-based sensors is that they do not directly detect the target molecule, a common limitation of intracellular detection methods. The signal measured for riboswitch-based sensors relies on the level of expression of a reporter gene, which can be influenced by varying environmental and genetic conditions. The use of these probes therefore requires proper controls to correct for factors that influence reporter expression in a nonspecific manner. In the present study, data are expressed as a fold repression in signal when different strains are assayed. This allows for samples lacking the target molecule to act as controls for any nonspecific effects that influence reporter expression. For riboswitch ligands where a "no target" control is not possible, mutant constructs that negate riboswitch binding (such as those presented in Figure 2) would serve as useful controls. The riboswitch sensors, as constructed, are meant to provide information on relative concentrations. Technologies that monitor macromolecule levels, such as western blots or oligonucleotide microarrays, can be very informative without providing quantitative information on cellular

concentrations. Applying the appropriate controls, the same is true for small molecule detection.

The sensor presented here makes use of a naturally occurring riboswitch. Natural riboswitches make ideal sensors as they presumably evolve to detect physiologically relevant target concentrations. A growing number of riboswitch classes have been identified, consisting of a diverse set of biologically important molecules and key metabolic nodes (Serganov, 2009). There is therefore a great deal of opportunity to monitor a wide range of biological processes using riboswitch sensors derived from natural sources. Importantly, we and others have shown the capacity to convert artificially isolated aptamers into riboswitches and to modify the characteristics of naturally occurring riboswitches (Desai and Gallivan, 2004; Dixon et al., 2010; Fowler et al., 2008; Lynch et al., 2009; Nomura and Yokobayashi, 2007; Weigand et al., 2008). This provides a great deal of flexibility to riboswitch-based sensing, presenting the possibility of creating custom sensors to detect a specific metabolite of interest. The development of an array of riboswitch-based sensors would open the door to multiplexing experiments where bacterial strains could be constructed that contain a number of sensors, each detecting a unique metabolite using a distinct reporter. Furthermore, riboswitch-based sensing need not be limited to prokaryotic organisms. While the majority of riboswitches that have been isolated to date have been found in prokaryotes, the thiamine pyrophospate riboswitch is also found in higher organisms, including yeast and plants (Bocobza et al., 2007; Cheah et al., 2007; Kubodera et al., 2003; Wachter et al., 2007). When this riboswitch was fused to fluorescent proteins and introduced into *Arabidopsis*, the levels of fluorescence from leaves could be controlled by the addition of exogenous thiamine (Bocobza et al., 2007; Wachter et al., 2007). This suggests there may be potential for riboswitch-based sensors as noninvasive intracellular probes in eukaryotic organisms as well, a prospect discussed in a recent review (Bocobza and Aharoni, 2008).

A major advantage of riboswitch-based sensing is its capacity for high-throughput applications. Because the readout is a simple reporter assay, this means of following small molecule concentrations could readily be scaled up to multiwell plate format to screen a large number of environmental or genetic conditions. One exciting prospect is screening single gene deletion or overexpression libraries looking for genetic factors that affect metabolite levels. Such a screen could presumably identify not only genes directly involved in the target's metabolism or import, but also genes involved in regulation, export, or in related metabolic pathways. Another intriguing possibility is screening for small molecule inhibitors of a target metabolic pathway using riboswitch-based sensors to monitor the concentration of the end product. Such a screen would have the advantage of using a living organism to provide more biologically relevant data and would also provide insight into the target of hit molecules.

SIGNIFICANCE

Simple and inexpensive methods for detecting the abundance of macromolecules such as proteins or mRNAs in biological samples have long been available to the research community. Detecting small molecule concentrations, by contrast, presents a far greater challenge. Here, we construct intracellular small molecule sensors that rely on an RNA regulatory element known as a riboswitch to detect their target. These probes emit colorimetric, fluorescent, or luminescent signals to describe the coenzyme B_{12} levels in *E. coli* cells. Importantly, the sensors were also successfully applied to study coenzyme B_{12} synthesis and transport, following target concentrations under a wide range of genetic and environmental conditions as a means to probe these processes. These experiments yielded data relevant to the understanding of vitamin B_{12} biology.

This report demonstrates that riboswitches can be used as the basis for sensors that demonstrate several attractive characteristics. The probes constructed in this study displayed very impressive specificity for their target molecule, discriminating against the complex mixture of metabolites present in the cellular environment including closely related metabolic precursors. Additionally, they were shown to be sensitive at a physiologically relevant concentration range and could detect a partial loss of function in the biological processes studied. The fast and simple nature of the reporter assays employed by these sensors makes them ideally suited for high-throughput applications, such as screening small molecule or genetic libraries. Future development of additional riboswitch sensors could make use of artificially derived or naturally occurring riboswitches to monitor metabolites of interest. The resulting probes would have a wide range of potential applications, including multiplexing experiments where multiple small molecules are detected simultaneously.

EXPERIMENTAL PROCEDURES

Plasmids and Cell Strains

All plasmids used were derived from the pBAD series of vectors (Guzman et al., 1995) and modified as described below and in Supplemental Experimental Procedures. Molecular cloning was carried out using *E. coli* NovaBlue cells (Novagen). Unless otherwise noted, all experiments shown were conducted using the *E. coli* strain BW25113 ($rrnB_{T14}$ $\Delta lacZ_{WJ16}$ *hsdR514* $\Delta araBAD_{AH33}$ $\Delta rhaBAD_{LD78}$) (Datsenko and Wanner, 2000). The single gene deletion strains were from the Keio Collection and are derived from the BW25113 parental strain (Baba et al., 2006). The Δ*cobT* and Δ*btuB* strains used were not taken from this library. The Δ*btuB* strain from the Keio collection was recently found to be subject to a duplication event such that a copy of *btuB* was retained (Yamamoto et al., 2009), presumably due to overlap with the essential *murI* gene. The Δ*cobT* strain in the copy of this library used was also found to contain a WT copy of the gene, perhaps due to an error replicating the library. Both of these strains were therefore created for the purpose of this study using the same procedure (Datsenko and Wanner, 2000), except that a cm^R cassette was used rather than kan^R. Deletions were confirmed by PCR. Primers used to create deletion strains are given in Table S1.

Molecular Cloning

Details of plasmid construction are provided in Supplemental Experimental Procedures and primer sequences are provided in Table S1. Briefly, DNA fragments for both the *btuB* riboswitch and for the individual reporter genes were obtained using PCR from a suitable template. Riboswitch-reporter gene fusions were obtained by crossover PCR of the individual fragments. The resulting fusions were cloned into a modified version of the pBAD18 plasmid (lacking all arabinose-responsive elements) using restriction digestion and ligation. The full sequence of each of these sensing plasmids is provided in the Supplemental Experimental Procedures. Synonymous constructs were

also made using the pBAD30 plasmid, a lower copy version of pBAD18. The ($A_{149}A_{150}$ > TT) mutations were made in a similar manner, using crossover PCR with mutations built into the crossover primers. The *bluB* gene from *M. smegmatis* was cloned by PCR amplification of the gene from genomic DNA, followed by digestion and ligation into the pBAD18-Kan plasmid. All constructs were confirmed by sequencing (Mobix lab, McMaster University).

Growth Conditions and Reporter Assays

For all assays described, cells were grown in rich, chemically defined medium (described in Supplemental Experimental Procedures) supplemented with the indicated concentrations of AdoCbl, Cbi (Cbi supplied as cobinamide dicyanide), and DMB (all purchased from Sigma) at 37°C with shaking. The sensor plasmid was transformed into the indicated strain by electroporation and plated on LB agar plates supplemented with 50 μg/ml ampicillin. Individual colonies were picked and grown overnight. Saturated cultures were split out into fresh media (1/1000 dilution) containing the indicated vitamin B_{12} compounds and grown for 6 hr (mid-late log phase). Cells were then assayed using the procedures described below. All data shown represent the average of at least three biological replicates, confirmed by at least two independent experiments. Fold-repression values were obtained by dividing the calculated reporter activity for the unrepressed conditions by that calculated for the repressed conditions for each sample. All reporter activity was normalized for cell density using OD_{600} measurements taken using a VERSAmax spectrophotometer (Molecular Devices).

For red fluorescent protein (RFP) assays, 1.5 ml of cells was pelleted and resuspended in 320 μl of phosphate-buffered saline (PBS). For each biological replicate, 100 μl of resuspension was added to three wells of a black, half area 96-well plate (Corning 3686) and fluorescence was read at 555/583 nm excitation/emission using a Safire fluorometer (Tecan). Measurements from the three wells were averaged to provide the value for a given biological replicate. Firefly luciferase assays were conducted using the Luciferase Assay System (Promega) and performed essentially according to the supplier instructions provided for bacterial cells. Luciferase activity was measured using the Lumat LB 9507 luminometer (Berthold Technologies). β-Galactosidase assays were performed based on the method developed by Miller (1972) modified essentially as described (Zhang and Bremer, 1995). OD_{420} measurements were taken using a Cary 100 spectrophotometer (Variant, Inc).

Assays featuring BluB (Figure 4B) were carried out using the pBAD30 version of the β-galactosidase sensor in order to allow for plasmid compatibility. The uninduced and induced samples were taken from the same overnight cultures. For the induced samples, L-arabinose (Sigma) was added to the media to a final concentration of 0.2%.

Ethanolamine Utilization Assays

For all strains tested, overnight cultures were grown in rich, chemically defined media. A total of 4.5 μl of the saturated overnight cultures was then added to 4.5 ml of growth media in which ethanolamine was the sole nitrogen source. This medium consisted of glycerol (0.2%), ethanolamine (0.1%), KH_2PO_4 (3 mg/ml), K_2HPO_4 (7 mg/ml), $MgSO_4$ (50 mM), $CaCl_2$ (100 μM), and the indicated B_{12}-related molecules at a concentration of 500 nM. The pH of the media was adjusted to ~7.2 using HCl. A total of 200 μl of these cultures was removed at the indicated time points and the OD_{600} was measured using a VERSAmax spectrophotometer (Molecular Devices).

SUPPLEMENTAL INFORMATION

Supplemental Information includes three figures, one table, and Supplemental Experimental Procedures and can be found with this article online at doi:10.1016/j.chembiol.2010.05.025.

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