Universitat de les Illes Balears

Facultat de Ciències

Memòria del Treball de Fi de Grau

btuB riboswitch from *Klebsiella pneumoniae* as a target for new antibiotics: *in vivo* study of riboswitch activation by coupling to a fluorescent protein

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Any acadèmic 2015-16

DNI de l'alumne: X1891988P

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Paraules clau del treball:
Riboswitch, *Klebsiella pneumoniae*, Cobalamin, Fluorescence

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Abstract

Riboswitches are mRNA elements that bind metabolites or metal ions as ligands and regulate mRNA expression by forming alternative structures in response to ligand binding. The *btuB* riboswitch from *Klebsiella pneumoniae,* responsible for cobalamin uptake, could be an interesting target for new antibiotics, as this bacterium has become an important pathogen in nosocomial infections. A plasmid (pJP04) has been designed and prepared by site-directed mutagenesis in order to study this riboswitch *in vivo*. The original *btuB* gene was substituted by *mCherry* gene, a red fluorescent protein, the synthesis of which was modulated by two different cobalamin derivatives so that riboswitch regulation could be visualized. As in other bacteria, whose riboswitch sequences are partially evolutionary conserved, the interaction of cobalamin with the riboswitch was shown to inhibit protein synthesis.

1. BACKGROUND

Carbapenems play a critically important role in our antibiotic armamentarium. Of the many hundreds of different ß-lactams, carbapenems possess the broadest spectrum of activity and the greatest potency against Gram-positive and Gram-negative bacteria that produce extended-spectrum ß-lactamases. As a result, they are often used as "last-line agents" or "antibiotics of last resort" when patients with infections become gravely ill or are suspected of harbouring resistant bacteria.[1] A new line of bacterial enzymes capable of inactivating carbapenems, known as *Klebsiella pneumoniae* carbapenemases (KPCs), has rapidly spread and is increasing elsewhere in the world. *Klebsiella pneumoniae*, responsible for hospital pneumonias, is the most common organism associated with KPC resistance determinants. However, KPCs are increasingly reported in other genera of the *Enterobacteriaceae* family, such as *Escherichia*, *Proteus*, *Serratia*, *Salmonella* and *Citrobacter*.[2] Treatment of an infection caused by KPC bacteria is particularly worrisome as the carbapenems are often agents of the last resort for resistant Gram-negative infections. The optimal treatment of infections caused by KPC bacteria is not well established and clinical outcome data remain sparse.[3] This leads to research in new validated cellular targets, which are needed to reinvigorate antibacterial drug discovery. One of these targets is riboswitches - messenger RNA (mRNA) structures that regulate gene expression in bacteria.

1.1. Riboswitches

Living organisms must be able to sense environmental stimuli and convert these input signals into appropriate cellular responses. These responses are generally mediated by transcription factors that bind DNA and coordinate the activity of RNA polymerase or of proteins that elicit allosteric effects on their regulatory targets. In the early 1970s, researchers began to recognize that regions of mRNA transcripts have a regulatory role in the expression of downstream gene products. Genetic regulation by RNA is widespread in bacteria. One common form of riboregulation in bacteria is the use of ribonucleic acid sequences encoded within mRNA that directly affect the expression of genes encoded in the full transcript (called *cis*-acting elements because they act on the same molecule they are coded in). These regulatory elements are known as riboswitches and are defined as mRNA elements that bind metabolites or metal ions as ligands and regulate mRNA expression by forming alternative structures in response to this ligand binding.[4]

Riboswitches are attractive targets for new drugs thanks to four main reasons. First, riboswitches evolved to recognize small molecules. Second, with the exception of the TPP riboswitch, known riboswitches occur predominantly in bacteria, not in eukaryotes. If eukaryotes employ riboswitches, it is likely that these will be distinct from those of bacteria, minimizing cross-reactivity of bacterial riboswitch-targeted ligands. Third, known riboswitches respond to omnipresent and essential metabolites and second messengers and are often associated with mRNAs encoding proteins essential for survival or pathogenesis. Fourth, riboswitches and its association with specific genes are often highly conserved across phylogeny underscoring their physiologic importance.[5]

These elements are commonly found in the 5' untranslated region (5'UTR; a stretch of RNA that precedes the translation start site) of bacterial mRNA (Figure 1). The function of riboswitches is tied to the ability of RNA to form a diversity of structures.

[1] K. M. Papp-Wallance, A. Endimiani, M. A. Taracila and R. A. Bonomo, Antimicrob. Agents Chemother. **55** (2011), 4943-4960.
[2] L. F. Chen, D. J. Anderson and D. L. Paterson, Infection and Drug Resistance **5** (2012), 133-141.
[3] E. B. Hirsch and V. H. Tam, J. Antimicrob. Chemother. **65** (2010), 1119-1125.
[4] A. L. Edwards and R.T. Batey, Nature Education **3(9): 9** (2010)
[5] K. E. Deigan and A.R. Ferré-D'Amaré, Acc. Chem. Res. **44** (2011), 1329-1338.

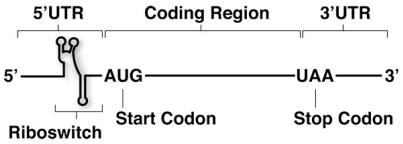

Figure 1 A typical bacterial mRNA transcript controlled by a riboregulatory element such as a riboswitch is composed of three sections: the 5' untranslated region (5' UTR), the protein-coding region beginning with the start codon (AUG) and ending with a stop codon (UAA), and the 3' untranslated region (3' UTR).[4]

Riboswitches need to form molecular architectures with sufficient complexity to carry out two main functions: molecular recognition and conformational rearrangement. The most basic of these is the double-stranded helix, similar to that found in DNA. However, since most RNAs do not need to maintain perfect Watson-Crick base pairing, they can form other types of structures. For example, single strand of RNA can fold back on itself to form a hairpin, which is composed of a helix capped by a loop. These elements of structure are called secondary structure. In larger RNAs, the helices and hairpins pack together into a specific pattern, referred to as the tertiary structure.[4]

A typical riboswitch contains two distinct functional domains. The effector molecule is recognized by an *aptamer domain*, which adopts a compact three-dimensional fold to scaffold the ligand-binding pocket. As with proteins, these RNA receptors must discriminate between chemically related metabolites with high selectivity to elicit the appropriate regulatory response. The *expression platform*, the second domain, contains a secondary structural switch that interferes with the transcriptional or translational machinery. Regulation is achieved by virtue of a region of overlap between these two domains, known as the *switching sequence*, whose pairing directs folding of the RNA into one of two mutually exclusive structures in the expression platform that represent the ON and OFF states of the mRNA.[6]

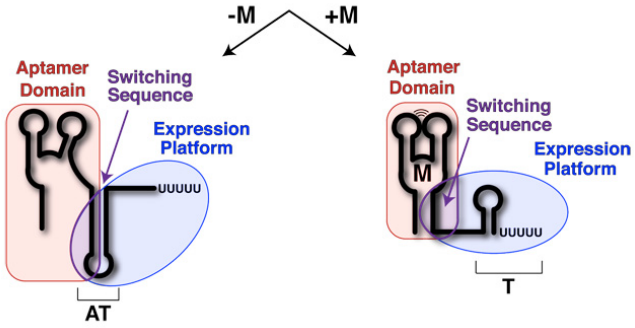

Figure 2 A riboswitch can adopt different secondary structures to effect gene regulation depending on whether ligand is bound. This schematic is an example of a riboswitch that controls transcription. When metabolite is not bound (-M), the expression platform incorporates the switching sequence into an antiterminator stem-loop (AT) and transcription proceeds through the coding region of the mRNA. When metabolite binds (+M), the switching sequence is incorporated into the aptamer domain, and the expression platform folds into a terminator stem-loop (T), causing transcription to abort.[4]

As mentioned, two mechanisms can commonly be involved in riboswitch-mediated gene control:[7]

- *Modulation of transcription termination*. Being one of the most usual mechanisms used by bacterial riboswitches, the formation of a strong stem followed by a set of uridine residues constitutes an intrinsic transcription terminator, which causes RNA polymerase to interrupt transcription and

[6] A. D. Garst, A. L. Edwards and R. T. Batey, Cold Spring Harb. Perspect. Biol. (2011), 3:a003533
[7] R. R. Breaker, Cold Spring Harb. Perspect. Biol. (2012), 4:a003566

eventually to release the DNA template and nascent RNA product. The formation of the terminator stem is controlled by ligand binging to the aptamer usually by regulating the formation of an anti-terminator or competing secondary structure.

- *Regulation of translation initiation.* Mutually exclusive base-paired structures are used by riboswitches to control ribosome access to the RBS (ribosome binding site) or Shine-Dalgarno (SD) sequence, thereby regulating translation initiation. This mechanism could be used to regulate translation from full-length mRNAs.

Because only four types of monomers are used by RNA to form selective binding pockets for target metabolites, aptamer sequences and structures tend to be strikingly well conserved over great evolutionary distances. This sequence and structure conservation serves as the basis for assigning riboswitch representatives to specific classes.

The conceptual idea that there was a period in the early history of life on Earth when RNA, or something chemically very similar, carried out most of the information processing and metabolic transformations needed for biology to emerge from chemistry, is called the RNA World. Although the exact nature of the RNA world cannot be reconstructed, riboswitches present themselves as an elegant solution to the problem of biological regulation.[7,8] Riboswitches are organized into families and classes according to two features: the type of ligand they bind, and their secondary structure.[4,9]

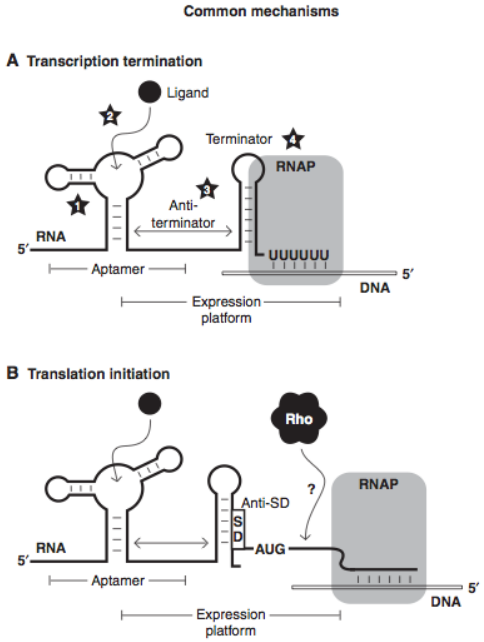

Figure 3 Established or predicted mechanisms of riboswitch-mediated gene regulation. The most common mechanisms are (**A**) transcription termination, (**B**) translation initiation. The numbers in "A" identify steps that are important for kinetically driven riboswitches. Numbers represent (1) folding of the aptamer, (2) docking of the ligand, (3) folding of the expression platform, and (4) speed of RNAP (*RNA polymerase*). In "B", *Rho* represents the transcriptional terminator protein.[7]

[8] P. G. Higgs and N. Lehman, Nature Reviews **16** (2015), 7-17.
[9] K. F. Blount and R. R. Breaker, Nature Biotechnology **24** (2006), 1558-1564.

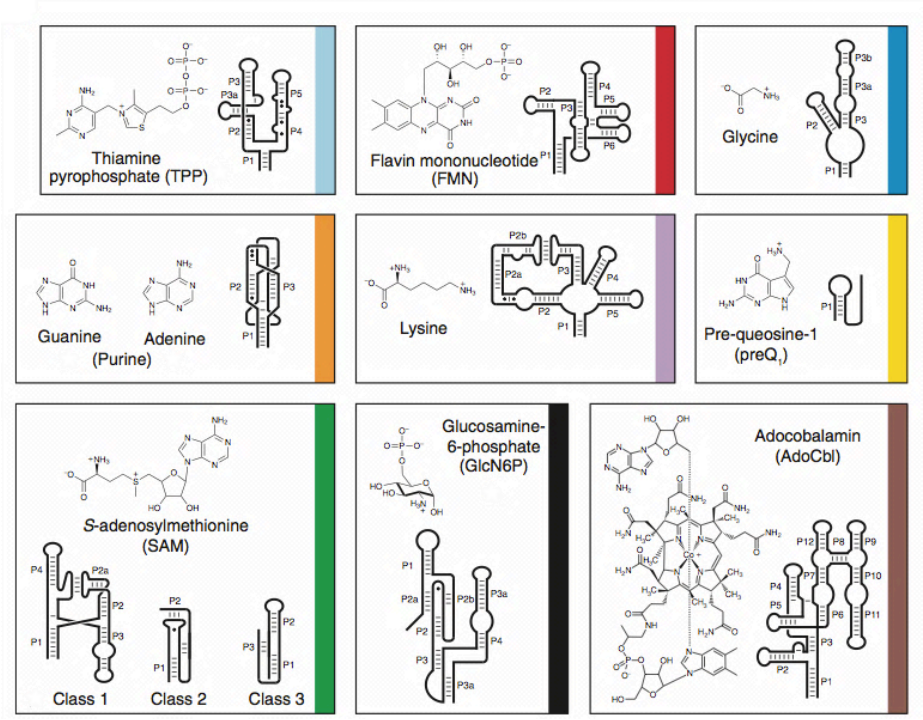

Figure 4 The secondary structure models of the receptor domains of 12 riboswitch classes and the chemical structures of the metabolites they bind. The expected protonation states at physiological conditions are shown for each functional group. Three different structural classes of riboswitches have been reported that recognize SAM. Approximately a half-dozen additional classes of conserved RNA motifs that may be riboswitches have been identified in bacteria.[9]

1.2. Coenzyme B_{12} or cobalamin riboswitches

The first riboswitch that was proven to interact directly with a cellular metabolite in the absence of proteins was a sequence responding to coenzyme B_{12} encoded upstream of the *btuB* gene in *Escherichia coli*. It was achieved by examining a 202-nucleotide fragment of the 5'-UTR, and similarly by examining the homologous mRNA fragment from *Salmonella typhimurium*.[10]

The cobalamin riboswitch family comprises two classes distinguished by peripheral extensions surrounding a common core (figure 5). The secondary structure of both classes contains a central four-way junction (P3–P6 helices) forming the core receptor domain responsible for cobalamin binding. The other shared element is a kissing-loop (KL) interaction between loop L5 of the receptor and L13 of the regulatory domain that instructs the expression machinery. For cobalamin riboswitches that regulate translation, L13 typically contains the RBS. Within the first cobalamin riboswitch class, the KL is generally linked to a downstream secondary structural switch akin to that used by most riboswitches. The classes are primarily differentiated by the presence of a large peripheral extension of P6 in one class (representing $\pm$ 95 % of the sequences of the cobalamin family) that is absent or severely truncated in the other class. Another peripheral extension between P1 and P3 further defines the classes. A unique feature of numerous cobalamin riboswitches, particularly those without the P6-extension, is that regulation seems to be achieved through tertiary structure formation (the KL interaction).[11]

Phylogenetic analyses indicate that the coenzyme B_{12} class of riboswitches is widely distributed amongst both Gram-positive and Gram-negative prokaryotes. In many instances, genes under the control of a coenzyme B_{12} riboswitch are involved in the transport of cobalamin compounds or metals, or associated with the biosynthetic pathway for the coenzyme but sometimes they control the expression of genes that are more distantly related to the coenzyme. Most striking is the involvement of coenzyme B_{12}

[10] A. Nahvi, J. E. Barrick and R. R. Breaker, Nucleic Acids Research **32** (2004), 143-150.
[11] J. E. Johnson Jr., F. E. Reyes, J. T. Polanski and R. T. Batey, Nature **0** (2012), 1-6.

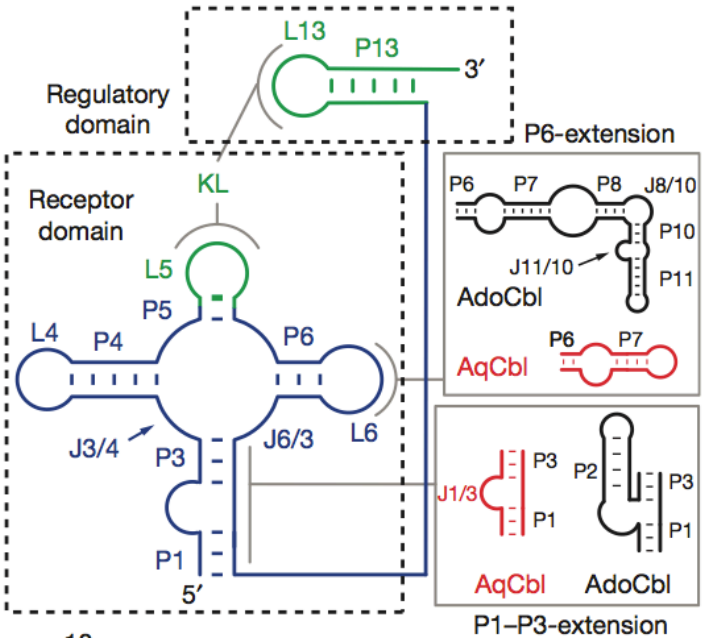

Figure 5 Secondary structure of the cobalamin riboswitch family. The conserved core is shown in blue, the KL interaction in green, and peripheral extensions distinguishing the two classes are shown in black (AdoCbl) and red (AqCbl).[11]

(adenosylcobalamin or AdoCbl) riboswitches in the control of *ribonucleotide reductases* that do not use this coenzyme.[10] Parts of the sequence of riboswitches are evolutionarily conserved, such as the B_{12}-box needed for the metabolite recognition, or the antisequestor ribosome-binding site (anti-RBS) involved in the translational regulation of the gene expression.[12]

Moreover, cobalamin riboswitches (AdoCbl riboswitch) has recently been shown to have also an influence over the expression of the ethanolamine utilization genes. It is proposed to be a subclass of the previously described classical AdoCbl-sensing riboswitch. The expression of the ethanolamine utilization genes (eutG) is activated by cobalamin in this case. Before explaining *btuB* riboswitch from *Klebsiella pneumoniae* we will first clear some aspects of *btuB* protein and B_{12} derivatives.[13, 14]

1.2.1. *btuB* protein

Gram-negative bacteria possess specialized active transport systems that function transporting organometallic cofactors or carriers, such as cobalamins, siderophores, and porphyrins, across their outer membranes, because they are too large to diffuse effectively through porin channels. The primary components of each transport system are an outer membrane transporter and the energy-coupling transperiplasmic protein *TonB*, both interacting through a conserved sequence, the "*Ton*-box" (interaction with *TonB* couples the energy of the proton motive force of the inner membrane to drive an outer membrane transport cycle). This group of *TonB*-dependent active transport proteins is abbreviated as *TBDTs*. *btuB* is the outer membrane protein required for the active transport of vitamin B_{12} (cyanocobalamin, CN-Cbl) and other cobalamins into the periplasm. Synthesis of this outer membrane transport protein is regulated in response to the availability of their substrates .[15,16]

[12] J. Palou-Mir, A. Musiari, R. K. O. Sigel and M. Barceló-Oliver, Characterization of the full-length btuB riboswitch from Klebsiella pneumoniae, J. Inorg. Biochem. (2015), http://dx.doi.org/10.1016/j.jinorgbio.2015.12.012
[13] K. A. Fox, A. Ramesh, J. E. Stearns, A. Bourgogne, A. Reyes-Jara, W. C. Winkler and D. A. Garsin, PNAS **106** (2009), 4435-4440.
[14] M. F. Del Papa and M. Perego, Journal of Bacteriology **109** (2008), 7147-7156
[15] M. D. Lundrigan, W. Köster and R. J. Kadner, Proc. Natl. Acad. Sci. USA **88** (1991), 1479-1483.
[16] D. P. Chimento, R. J. Kadner and M. C. Wiener, J. Mol. Biol. **332** (2003), 999-1014.

*TBDT*s structures are composed of two domains, a conserved N-terminal globular domain (hatch) and a 22-stranded ß-barrel (barrel). The hatch domain resides within the barrel and occludes the large (~35-40 Å diameter) pore of the large barrel domain. The hatch domains are composed of a central core of four ß-strands connected by loops. The conserved *Ton*-box is located near the periplasmic opening of the barrel and precedes the conserved hatch core. The barrels have large extracellular loops and short periplasmic turns connecting the ß-strands. *TBDT*s bind their cognate substrates using residues from hatch loops, from the interior surfaces of ß-strands in the barrel wall, and from extracellular loops of the barrel.[15]

1.2.2. Coenzyme B_{12} and other cobalamins

The corrin ring is the presumed archetype of the tetrapyrroles, a family of compounds, which exploit the special properties of the ionic forms of cobalt, iron, magnesium, and nickel. Cobalt-containing corrins, known as cobamides, have different classes of functions in organisms ranging from Archaea to humans; but they are not present in plants:[17]

- **Methyl group transfer** in the processes of methanogenesis in Archaea, acetogenesis in anaerobic bacteria and methionine synthesis in bacteria and mammals.
- **Generation of deoxyadenosyl radical** which catalyzes ribonucleotide reduction and rearrangements of amino acids in bacteria and rearrangement of *methylmalonyl coenzyme A* in bacteria and mammals.
- **Elimination***s* through *diol dehydratase* or *ethanolamine ammonia lyase*.[18]

Only some microorganisms have the capability to biosynthesize B_{12} and other natural corrinoids. Corrinoids are thus "vitamins" for other B_{12}-requiring organisms. Their functioning metabolism depends on the uptake and binding of B_{12} derivatives, their metabolic transformation into the relevant B_{12} cofactors, the controlled transport of these and the catalysis by B_{12}-dependent enzymes.[19]

The physiologically relevant vitamin B_{12} derivatives are the highly light-sensitive and chemically more labile organometallic coenzymes, coenzyme B_{12} (5'-deoxy-5'-adenosylcobalamin, AdoCbl) and methylcobalamin (MeCbl), as well as the ''inorganic'' and easily reducible B_{12} derivatives aquacob(III)alamin (H_2OCbl^+) and hydroxocob(III)alamin (HOCbl).[19]

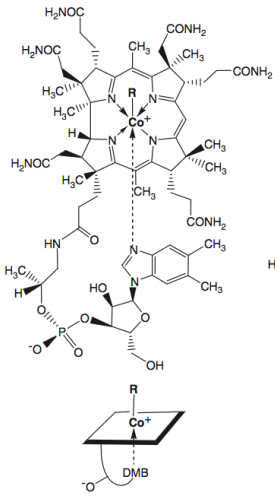

Figure 6 General structural formula of: cobalamins (Cbls=DMB-cobamides, Ado=adenosyl), vitamin B_{12} (CNCbl, R=CN), coenzyme B_{12} (R=5'-deoxy-5'-ado), methylcobalamin (MeCbl, R=CH_3), aquacobalamin (R=H_2O^+), hydroxocobalamin (HOCbl, R=HO), cob(II)alamin (B_{12r}, R=e^-).[19]

[17] J. I. Toohey, BioFactors **26** (2006), 45-57.
[18] J. S. Casas, V. Moreno, A. Sánchez and J. Sordo, Química Bioinorgánica, Editorial Síntesis, Madrid, 2002
[19] B. Kräutler, Met. Ions. Life Sci. **6** (2009), 1-51.

1.2.3. *btuB* riboswitch of *Klebsiella pneumoniae*

Our research group began studying the *btuB* riboswitch from *Klebsiella pneumoniae* because in recent years, *K. pneumoniae* has become an important pathogen in nosocomial infections, making it an interesting target for new antibiotics. Moreover, *K. pneumoniae* belongs to the same phylogenetic branch as *E. coli* and *S. typhimurium* but represents a step forward in differentiation from them.

btuB gene was located at the position 4660313-4662169 of complete genome of *K. pneumoniae* subsp. *Pneumoniae* strain MGH 78578 (from the National Center for Biotechnology Information (NCBI) (GenBank: CP000647.1)). The 5'-UTR region proposed to act as a riboswitch was located starting at position 4660061 and comprises 243 nucleotides. For characterization of the full-length *btuB* riboswitch from *Klebsiella pneumoniae*, small modifications were introduced in the riboswitch sequence: i) the 5'-end long tail was removed and a GGGA tag was engineered at the 5'-end to have a good initiation point for transcription; ii) the 3'-end was extended to contain the RBS until the initiation codon position of the gene; iii) the last four nucleotides at the 3'-end were mutated in order to introduce a restriction site for the *XhoI* enzyme (from the original $A_{240}A_{241}T_{242}G_{243}$ to $T_{240}C_{241}G_{242}A_{243}$, where positions 241–243 correspond to the start codon). The 243-nt long RNA sequence (pJP01) used in characterisation studies done by our group, which contains the aptamer and the expression platform, was:[12]

5'-GGGAC CGGUC CUGUG AGUUA AAAGG GAACC CAGUG GAAAU CUGGG GCUGA CGCGC AGCGG UAAGG AAGGU GAGAA AUGAG CGCAC UCGGU GCAGA CACUG CGGCU AGCCG UGGGA AGUCA UUAUU UCUUG AAACA GCCUC CAAGC CCGAA GACCU GCCGG AAUAC GUCGC ACUGG GUUUU AUCGU CGCGA GCAAC UGAUA AAACC UGCGG CAUCC UUUUU CUGUU UCGGA UGCUU UUACU CGA-3'.

The performed studies have proven that this RNA sequence is able to bind coenzyme B_{12} with high affinity and thus, is a functional B_{12} riboswitch. Moreover, the core structure is conserved along the bacterial speciation and B_{12} induced changes in the core are respective in the individual riboswitches. Even when B_{12} riboswitches from the phylogenetic closest taxonomic groups of bacteria present only 70 % of sequence conservation, binding of the coenzyme B_{12} and structural rearrangement mechanism take place in the same regions of the structure. It has also been proven that RBS plays a role in the switch.[12]

Obtained results were consistent with those from the well-studied B_{12}-riboswitches from *E. coli* and *S. typhimurium*. In-line probing measurements showed that affected cleavage sites appear at similar locations on secondary structures and are also consistent with the regulatory function of the sequences although different primary sequences. Comparing cleavage sites with the 8 main sites of structure modulation found by Nahvi et al. in the *btuB* riboswitch from E. coli[10], later 9 sites by Gallo et al.[20], (see proposed structures (Figures 7 and 8) based on consensus aptamer structures), it was found that 7 of the 9 main sites coincide in secondary structure location with those found in the *K. pneumoniae*. Site 1 from the *E. coli btuB* riboswitch could not be identified because of experimental limitations. Site 2 of *E. coli* is neither present in the *K. pneumoniae* riboswitch, probably because of the distinct difference in size of the P8 region. It is important to note that the *btuB* riboswitch from *K. pneumoniae* shows some additional cleavage sites not identified in the *btuB* riboswitches from *E. coli* nor *S. typhimurium* (Sites 1, 2, 3, 12 and 15).[12]

[20] S. Gallo, M. Oberhuber, R. K. O. Sigel and Bernhard Kräutler, ChemBioChem **9** (2008), 1408- 1414.

ITC was used to determine the thermodynamic parameters of the ligand – RNA association process. Results were compared to those obtained for the *btuB* riboswitch from *E. coli*.[11] The K_D=250 ± 40 nM for the wild type riboswitch from *E coli* is comparable to the one determined for *K. pneumoniae*: 210 ± 8 nM at 20 °C and 530 ± 57 nM at 25 °C.[12]

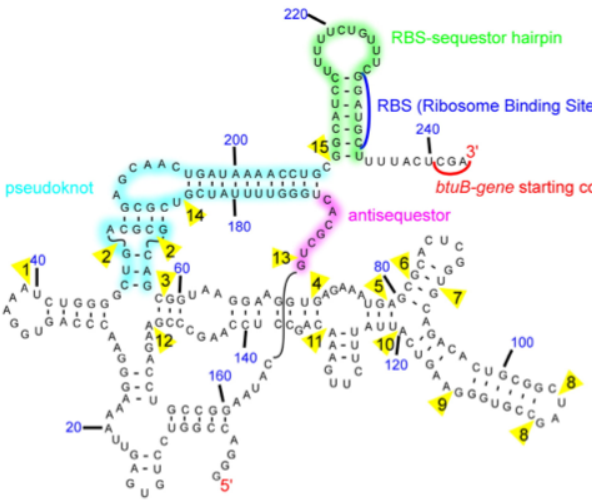

Figure 7 Proposed secondary structure for the gene-off conformation, when coenzyme B_{12} is bound, of the B_{12}-riboswitch from *Klebsiella pneumoniae* according to the aptamer consensus structure. Yellow flags indicate the cleavage sites found in "in-line probing" experiments.[12]

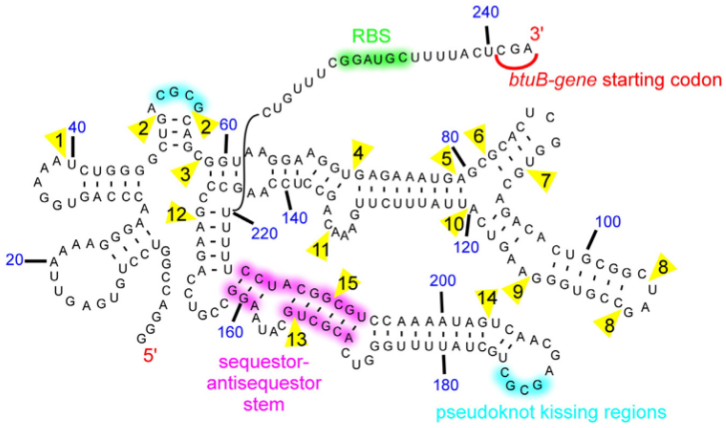

Figure 8 Proposed secondary structure for the gene-on conformation (coenzyme B_{12} not bound) of the *btuB* riboswitch from *Klebsiella pneumoniae* (strain MGH 78578).

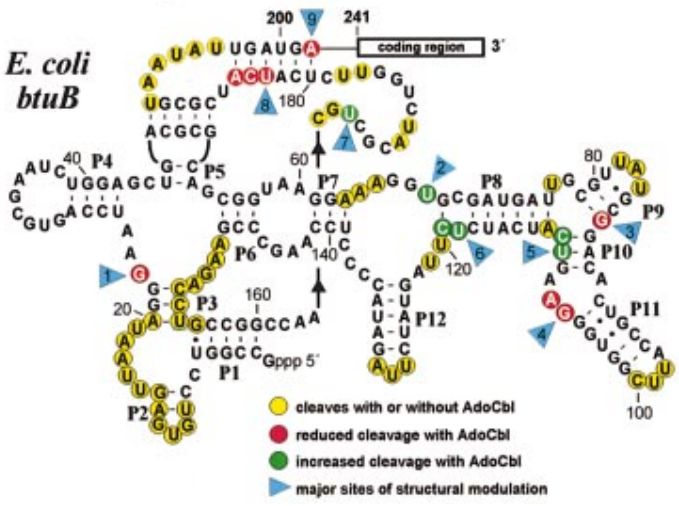

Figure 9 Sequence and revised secondary-structure model for the B_{12} riboswitch from the 5'-UTR of the *E.coli btuB* gene. Although the portion of the RNA corresponding to the most highly conserved domain throughout evolution ends at nucleotide 160, the sequences through nucleotide 202 are required for maximal binding affinity. The previously identified B_{12} box resides between nucleotides 140 and 160.[20]

1.3. *mCherry* protein and fluorescence

Fluorescent probes are one of the cornerstones of real-time imaging of live cells. They provide high sensitivity and great versatility while minimally perturbing the cell under investigation. Genetically encoded reporter constructs that are derived from fluorescent proteins are leading a revolution in the real-time visualization and tracking of various cellular events.[21] All wild type yellow to red fluorescent proteins reported so far are obligatory tetrameric and often toxic so that directed evolution has been done.

1.3.1. Fluorescence principles

Luminescence is the emission of light from any substance, and occurs from electronically excited states. Fluorescence and phosphorescence are particular cases of luminescence. The mode of excitation is absorption of a photon, which brings the absorbing species into an electronic excited state. The emission of photons accompanying de-excitation is then called photoluminescence (fluorescence, phosphorescence or delayed fluorescence), which is one of the possible physical effects resulting from interaction of light with matter. The emission rates of fluorescence are typically 10^8 s^{-1}, so that a typical fluorescence lifetime is near 10 ns (10×10^{-9} s). Fluorescence spectral data are generally presented as emission spectra. A fluorescence emission spectrum is a plot of the fluorescence intensity versus wavelength (nm) or wavenumber (cm^{-1}) and is registered in the perpendicular direction of sample excitement.[22, 23]

1.3.2. *mCherry*

mCherry is a high-performance monomeric *Anthozoa* fluorescent protein derived from *Discosoma sp.* (sea soft mushroom coral; fake anemone) fluorescent protein (*DsRed*) through extensive mutagenesis. The first true monomer derived from *DsRed* was *mRFP1*. *mRFP1* has proven to be widely useful but several properties could bear improvement. *mFruits*, including *mCherry*, are second-generation monomeric red fluorescent proteins (*mRFP*s) that have improved brightness and photostability compared to the first-generation *mRFP1*. Compared to other *mFruits*, *mCherry* offers the longest wavelengths, the highest photostability, the fastest maturation and excellent pH resistance.[24]

The ß-barrel structure of *Anthozoa* fluorescent proteins is very similar to those found in *Aequorea victoria* (crystal jellyfish) *GFP* (green fluorescent proteins) variants in terms of the general features and dimensions despite rather low correlations in the amino acid sequence homology. Unlike GFP, those obtained from *Anthozoa* feature a barrel that is more elliptical in geometry when viewed from the top or bottom, perhaps arising from the fact that most wild-type fluorescent proteins in this class were originally tetrameric in character. The *mCherry* chromophore is positioned near the central axis of the ß-barrel in a portion of the α-helix that threads its way through the centre of the structure. It is precisely indirect modification of the chromophore environment, in comparison with *DsRed*, what produces strong red-shifted variants like *mCherry*.[25,26] *mCherry* presents its absorption maximum at 587 nm and its emission maximum at 610 nm.

[21] J. Zhang, R. E. Campbell, A. Y. Ting and R. Y. Tsien, Nature **3** (2002), 906-918.
[22] J. R. Lakowicz, Prnciples of Fluorescence Spectroscopy, Third Edition, Springer, Baltimore, 2006.
[23] B. Valeur, Molecular Fluorescence: Principles and Applications, Wiley-VCH, 2001.
[24] N. C. Shaner, R. E. Campbell, P. A. Steinbach, B. N. G. Geipmans, A. E. Palmer and R. Y. Tsien, Nature Biotechnology **22** (2004), 1567-1572.
[25] X. Shu, N. C. Shaner, C. A. Yarbrough, R. Y. Tsien and S. J. Remington, Biochemistry **45** (2006), 9639-9647.
[26] http://zeiss-campus.magnet.fsu.edu/articles/probes/anthozoafps.html#fruits

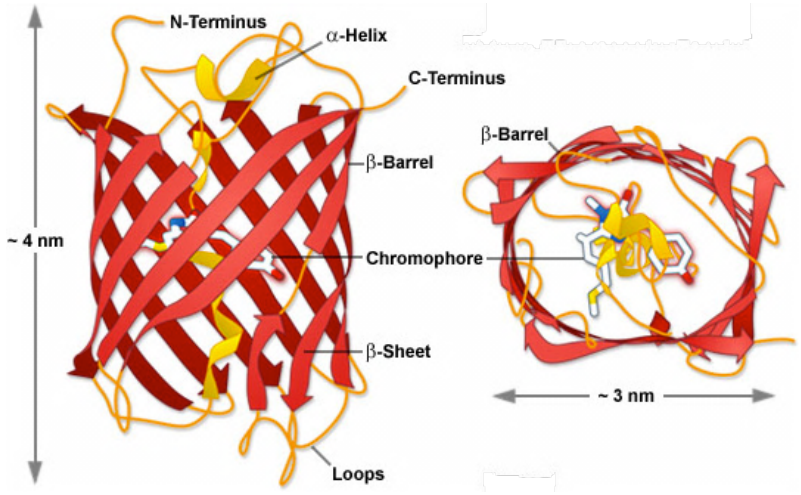

Figure 10 Architecture of *mCherry* fluorescent protein (PDB ID 2H5Q)[25,26]

1. To design a plasmid containing the *btuB* riboswitch from *Klebsiella pneumoniae* whose modulation could be displayed by spectroscopic techniques.

    1.1. To integrate an element, which can be valued by a spectroscopic method. The chosen element is *mCherry* sequence, the translation product of which is a fluorescent protein.

2. To construct the plasmid by using cloning techniques.

    2.1. To couple *btuB* riboswitch from *K. pneumoniae* with *mCherry* sequence.

    2.2. To introduce the constructed plasmid into cells, concretely, *E. Coli* bacteria.

3. To evaluate *in vivo* regulation of the mentioned riboswitch by cobalamins.

    3.1. To study *in vivo* regulation of gene expression using fluorescence measurements, pretending to prove the *btuB* riboswitch to be modulated by two cobalamin derivatives: adenosylcobalamin and cyanocobalamin.

        3.1.1. To confirm that cobalamins activate *btuB* riboswitch from *K. pneumoniae* blocking gene expression as it happens in other *btuB* riboswitches from other species such as *E. Coli*.

        3.1.2. To set *K. pneumoniae btuB* riboswitch saturation points, by adding different cobalamin concentrations to analogous cell cultures.

        3.1.3. To evaluate cobalamin uptake in living *E. Coli* cells.

3. EXPERIMENTAL SECTION

3.1. Instruments and appliances used

The polymerase chain reactions (PCR) were carried out in a Perkin Elmer GeneAmp PCR System 2400 apparatus.

Incubation of samples was performed in an Eppendorf Thermomixer Comfort and thermal shocks were performed using a Heat Block from VWR.

For sample centrifugation refrigerated 5810R centrifuge with F45-6-30 rotor (fixed angle, 45º for 6 tubes of 50 ml) and S-4-104 rotor (oscillating, for 750 ml bottles or 15 and 50 ml tubes) and refrigerated 5415R centrifuge with F45-24-11 rotor (fixed angle, 45º for 24 tubes of 1.5-2 ml) of Eppendorf, were used.

Both, the concentration of samples and optical dispersion (OD_{650}) of cultures, were measured by UV-visible spectroscopy using an Ultrospec 7000 spectrophotometer from GE Healthcare Life Sciences. For DNA samples, a 100 µl and 1 cm optical path length quartz cuvette from Hellma was used. For culture samples, 1 cm optical path length plastic fluorescence cuvettes were used. Fluorescence measurements were taken with a Varian Cary Eclipse fluorimeter.

For sterile-filtration of solutions cellulose acetate disposable, 0,2 µm porus, sterile, 30 mm filters were used attached to a sterile syringe (GE Healthcare Life Sciences).

The power source used for electrophoresis was an EC 300XL from Thermo Scientific. The horizontal agarose gels ran at 2.5 V/cm during the first 10 minutes (introduction of the sample) and then at 5 V/cm for 2 hours. The V/cm are referred to the distance between electrodes; a 16 cm electrophoresis chamber was used and size of the gels was 10 cm long and 7 cm wide. The agarose gels were soaked with ethidium bromide and displayed with a MiniBIS Pro gel documentation system from DNR Bio-Imaging Systems.

3.2. Reagents and material

Reagents were received from VWR, Merck, Prolabo, Alfa Aesar, Sigma Aldrich, Applichem and Amresco as p.a., molecular biology, biochemica or higher quality and were used without further purification. Both PCR primers and sequencing primers were ordered from Microsynth (Switzerland). 0.5 ml and 1.5 ml centrifuge microtubes were sterile PCR-clean quality from Eppendorf with low retention rate. 15 ml and 50 ml centrifuge tubes were from Corning, of polypropylene, resisting 16000 g and of medical grade quality.

- Ampicillin, 100 mg/ml: The necessary amount ampicillin was dissolved in MilliQ water and filtered sterilely. 1 ml aliquots were stored at -20ºC. Ampicillin was used in a dilution 1:1000 of the prepared aliquots, resulting in a final concentration of 100 µg/ml.

Escherichia coli bacteria were mainly cultured in 25 g/l Lysogeny Broth (LB). The solution was autoclaved for 20 min at 121ºC and once cooled to about 50ºC the antibiotic was added. For Petri dishes, the same LB medium (25 g/l) was prepared but 15 g/l of agar were also added. 50 ml of the autoclaved mixture were added to each Petri. Ampicillin (antibiotic) was spread over the Petri dish after solidification.

5X M9 salts solution contains $Na_2HPO_4 \cdot 7H_2O$ (64 g/l), KH_2PO_4 (15 g/l), NaCl (2.5 g/l) and NH_4Cl (5

g/l). The necessary amount of each salt was dissolved in MilliQ water and autoclaved 20 min at 121°C.

For M9 supplemented medium (M9 medium), sterile M9 salts were diluted to 1X final concentration and supplemented with the following nutrients that were sterilized by filtration prior to use: glucose (0,4 %), $MgSO_4$ (2 mM), $CaCl_2$ (100 μM), DL-alanine (100 μg/mL), L-arginine (22 μg/mL), glycine (100 μg/mL), L-histidine (22 μg/mL), L-leucine (20 μg/mL), L-isoleucine (20 μg/mL), L-methionine (20 μg/mL), L-proline (30 μg/mL), L-threonine (80 μg/mL), L-valine (40 μg/mL), L-lysine (88 μg/mL), L-cysteine (22 μg/mL), L-phenylalanine (20 μg/mL), L-tyrosine (20 μg/mL), L-tryptophan (20 μg/mL), L-serine (100 μg/mL), L-asparagine (100 μg/mL), L-glutamine (100 μg/mL), L-aspartic acid (100 μg/mL), L-glutamic acid (100 μg/mL), Niacin (1 μg/mL), calcium pentothenate (1 μg/mL), pyridoxine-HCl (1 μg/mL), thiamine-HCl (1 μg/mL), biotin (500 ng/mL), thymidine (40 μg/mL), thymine (40 μg/mL), uracil (40 μg/mL). The medium was also supplemented with ampicillin.

- Cobalamin solutions: 25 mg cobalamin were dissolved in 1.5 ml autoclaved and filtreted MilliQ water. The concentration was determined by measuring absorption of a 1:500 dilution of the original solution ($\varepsilon(AdoCbl)_{260}$ = 34700 M/cm,[27] $\varepsilon(CNCbl)_{309}$ = 27499 M/cm[28]).

Plasmids were extracted and purified with commercial kits: Plasmid DNA Maxi Kit from Omega Bio-Tek for large-scale cultures, the equivalent Plasmid DNA Mini Kit from Omega Bio-Tek for sequencing samples or DNA Gel extraction kit also from Omega Bio-Tek.

For DNA separation, 1 % agarose gels in TAE 1X buffer were prepared. The mixture was heated in the microwave until the solution was transparent and then 100 ml were poured into the cast.

- TAE (Tris-Acetate-EDTA) buffer 50X: 2M Tris-acetate; 50 mM EDTA pH=8.3. To prepare TAE the necessary amounts of salts were weighed, acetic acid was then added up to achieve the desired pH and flushed with water until the final volume. Finally, the solution was autoclaved.

In order to measure the size of the DNA separated by agarose electrophoresis, a measuring scale was used: the GeneRuler 1 kbp Plus DNA Ladder from Thermo Scientific. It consists of 15 fragments of individually purified (by HPLC) DNA of following size (in base pair): 20000, 10000, 7000, 5000, 4000, 3000, 2000, 1500, 1000, 700, 500, 400, 300, 200 and 75.

Agarose gels were soaked in an ethidium bromide solution (0.5 μg/ml) in 1X TAE buffer before, being photographed with UV light.

All liquid and waste materials generated during processes that involve bacteria were treated as biological material and were autoclaved in order to be further processed as normal waste. Alternatively, liquid waste can be treated with bleach (10 %).

[27] Drug Information Express,Drug R&D,Chemical Database; http://www.drugfuture.com/chemdata/Cobamamide.html
[28] J. A. Hill, J. M. Pratt and R. J. P. Williams, J. Chem. Soc. (1964), 5149-5153.

3.3. Plasmid design

In this study we have prepared a plasmid, based on pET-21b(+), where the *btuB* Riboswitch sequence is followed by the *mCherry* sequence, which replaces the native *btuB* template. The full-length riboswitch is encoded and right after that the start codon of the *mCherry* protein is placed, with the whole sequence situated in-between the promotor and the termination of the *T7 polymerase*. This plasmid also carries the ampicillin resistance gene, for selection purposes, and the *lac* operon, which allows the IPTG-activation of the protein synthesis. The replacement of the native protein with this coloured protein will allow us to detect the riboswitch-regulation with a fluorometric method.

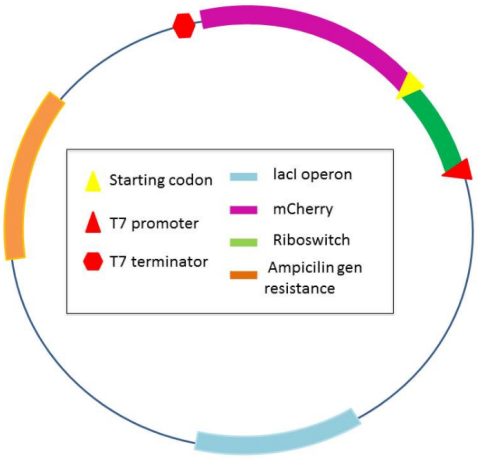

Figure 11 Plasmid pJP04 construct, showing important elements.

3.4. Plasmid construction

3.4.1. Point Mutation by PCR: *HindIII* introduction to pJP01

pJP01 plasmid, which was used in Joana Palou Mir's Master Thesis, was modified in order to introduce a *HindIII* restriction site. For this reason, a point mutation protocol was followed, technique that allows to change up to 3 base pairs. Eva Freisinger, University of Zürich, generously conceded this protocol. The method involves multiplying the whole plasmid by PCR, using complementary primers, which bear the desired mutation in the middle, followed by enzymatic digestion of the original vector and transformation of the unligated PCR product into chemically competent *E. Coli* cells. This protocol requires the following conditions:

- Mutagenic oligonucleotide primers must be designed carefully. They must have a melting point (T_m) greater or equal than 78°C (based on the following equation: $T_m = 81.5° + 0.41°(\%GC) - 675°/length$ of oligo), be made up of 25-45 nucleotides and contain the wished mutation in, approximately, the middle of the primer (shown in blue at the indicated sequences). Primers should end with one or more C or G to ensure better recognition of extremes. In addition, both primers must be complementary to each other since all the plasmid is multiplied.

- JP02_1: 5'-CTC GAG CAC CAC CA**A GCT T**AC CAC TGA GAT CC-3'
- JP02_2: 5'-GGA TCT CAG TGG T**AA GCT** TGG TGG TGC TCG AG-3'

- Vector (original plasmid) must be *dam* methylated so that it can be distinguished from the one created by PCR and digested by the restriction enzyme *DpnI*. *Dam* methylation is the addition of a methyl group by *deoxyadenosine methylase* to adenine in the 5'-GATC-3' sequence at the newly synthesized DNA. This

enzyme is found in strains of *Escherichia coli* such as DH5α and XL1blue. The plasmid cannot be greater than 8000 bp.

- Used polymerase shall be of high fidelity, with error correction; to minimize the possible apparition of spontaneous mutations while multiplying a long sequence. In this case *Pfu DNA polymerase* (enzyme coming from the hyper-thermophilic archaebacterium *Pyrococcus furiosus*) was used.

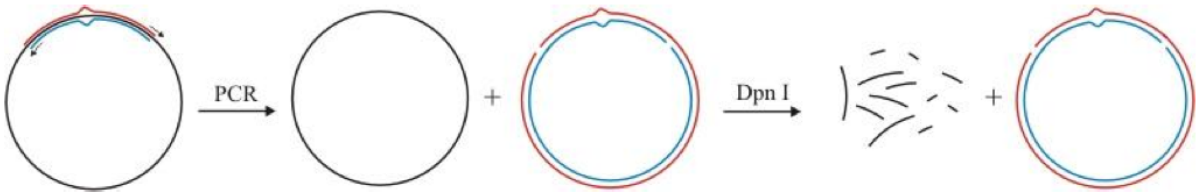

Figure 12 Process Sheme of point mutation introduction by PCR.

Duplicate reactions were prepared in PCR thin wall tubes of 0.2 ml. The final volume of the reaction mixture was 50 µl. The concentrations used in the reaction mixture were: 4,8 µg/ml of plasmid (pJP01), 0.25 µM of each of the primers (JP02_1 and JP02_2), 0.4 µM of each dNTP, 1X *Pfu* buffer, 1 mM MgSO4 and, lastly, a total of 2.5 U *Pfu DNA polymerase* enzyme.

The tubes with the reaction mixture were introduced into a thermal cycler and cycle was started following the temperature ramp shown in Figure 13: a) initialization was carried out at 95 ºC for 5 min in order to activate the *DNA polymerase*; b) double helix denaturation was performed at 95 ºC for 30 s; c) the alignment (primer binding) was fulfilled at 55 ºC for 1 min; d) elongation of the chain was performed at 68 ºC for 7 minutes (time was adapted to the length of the plasmid according to the following rule: 1 min per 1000 bp or fraction plus 1 min if the vector is large). The cycle was repeated 18 times. Once cycles finished, five more minutes at 68 ºC were added for final elongation. Last, temperature was lowered to conservation temperature, 4 ºC.

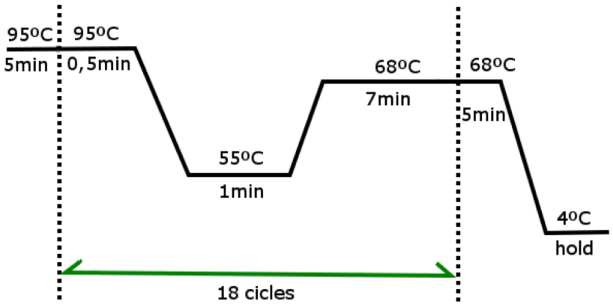

Figure 13 Temperature ramp applied to perform PCR.

Once the reaction finished, 40 U of *DpnI* were added to the mixture and then incubated for 1 h at 37 ºC without agitation to digest the original plasmid. This already digested PCR mixture, pJP02, was transformed into chemically competent cells, which were responsible, due to their own repair mechanisms, for closing the plasmid.

3.4.2. Transformation of pJP02 into chemically competent cells

DH5α cells were used due to their plasmid production fidelity. For transformation, 2 µl of PCR mixture were added into two 100 µl aliquots of chemically competent cells respectively, mixed gently

and incubated for 10 min on ice. Then a thermal shock was performed, without mixing, at 37 ºC for 3 min followed by 3 min incubation on ice. This procedure ensures that the plasmid is introduced into the bacteria and, subsequently, that cell membranes are impermeable. 500 µl of LB medium without antibiotic were then added to bacteria and incubated at 37 ºC and 400 rpm for 30 min. The tubes containing the cells were centrifuged for 2 min at 800 rpm and the supernatant removed leaving about 50-100 µl in which cells were resuspended and subsequently spread on culture plates. Petri dishes with LB-agar and the selection antibiotic (ampicillin) were plated with 50 µl cell suspension and the latter, distributed homogeneously on the plate surface. Plates were incubated at 37 ºC overnight (about 20 hours) and, after this time, colonies were counted: approximately 80 and 140 colonies were counted. The colonies were small, rounded and independent, as expected.

Two colonies were selected from each plate (selection criteria: small, rounded, isolated and opaque white) taken with a pipette tip and inoculated into culture tubes containing 5 ml LB and ampicillin. These tubes were then incubated at 37 ºC and 210 rpm for 6 h. After this time, aliquots of 1 ml cell stocks were prepared with glycerol (1:1) and stored at -80ºC (for possible uses in future). On the other hand, 500 ml LB with ampicillin were seeded with 4 ml of one of the tubes and incubated over-night at 37 ºC and 210 rpm. Afterwards, the plasmid was purified with a commercial kit in order to be able to sequence it. The sequencing was performed externally by Secugen: samples were prepared with 15 µl of 100 µg/ml plasmid and 1.5 µl 5 µM pET3' primer. Sequencing revealed that the introduction of the point mutation, introduction of a *HindIII* restriction site, worked well.

3.4.3. Point Mutation by PCR: *XhoI* introduction to pmCherry

pmCherry already carries a *HindIII* restriction site at one of the ends of *mCherry* gene. The plasmid was modified in order to introduce another restriction site at the other end of the gene sequence: a *XhoI* restriction site. The followed protocol was analogous to that followed for *HindIII* restriction site introduction to pJP01, but using different primers and vector:

The concentrations used in the reaction mixtures were: 4.1 µg/ml of plasmid (pmCherry), 0.2 µM of each of the primers (CIRERA_F and CIRERA_R), 0.4 µM of each dNTP, 1X *Pfu* buffer, 1 mM and 5 mM $MgSO_4$ and, lastly, a total of 2.5 U *Pfu DNA polymerase* enzyme.

- CIRERA_F: 5'-CGA TAA GGA TCC CGC CAC **TCG A**GT GAG CAA GGG CGA GG-3'
- CIRERA_R: 5'-CCT CGC CCT TGC TCA C**TC GA**G TGG CGG GAT CCT TAT CG-3'

The temperature ramp program was just modified at one point: elongation of the chain was performed at 68 ºC for 4 minutes instead of 7 minutes because of the shorter length of de plasmid.

3.4.4. Transformation of pmCherry into chemically competent cells

Both PCR mixtures were transformed into DH5α competent cells. The same protocol was used as for pJP02 transformation, but instead of 2 µl, 4 µl PCR mixture were added to cells. After transformation, the seeded plates were incubated at 37ºC overnight and, after this time, approximately 100 colonies were counted on the plate from the 1mM $MgSO_4$ PCR reaction (sample 1) and 30 colonies on the plate from the 5 mM $MgSO_4$ PCR reaction (sample 2).

A colony was selected of each of the plates (selection criteria: small, rounded, isolated and opaque white), taken with a pipette tip and inoculated into culture tubes containing 6 ml LB and ampicillin. These tubes were then incubated at 37 ºC and 210 rpm for 6 h. Then, two Erlenmeyer containing each 400 ml LB and ampicillin were inoculated with 4 ml bacterial culture and incubated overnight at 37 ºC

and 180 rpm (more or less 16 h). Afterwards, the plasmid was purified with a commercial kit in order to be able to sequence it, as already described above.

In the meantime, in order to check the correct mutation, the resulting plasmid was incubated with restriction enzymes (*HindIII* and *XhoI*). The original plasmid was also incubated as control. Restriction enzyme digestion takes advantage of naturally occurring enzymes that cleave DNA at specific sequences, called restriction sites. Tubes with 10 µg of plasmid, 1X R buffer and 20 U of enzyme in a total volume of 40 µl were prepared for digestion. Reaction mixtures were incubated at 37 ºC for 4 h.

Sample	Control	1X	1HX	2X	2HX
pmCherry	0.25 µg/µl				
Buffer R	1X				
dH_2O (µl)	28.49	26.49	24.49	26.97	24.97
XhoI	-	20 U			
HindIII	-	-	20 U		20 U

Table 1 pmCherry digestion conditions.

The reactions were then analysed by 1 % agarose gel in TAE, where 5 µl of each sample plus 1 µl 6X LD were charged and 1X TAE was used as electrophoresis buffer.

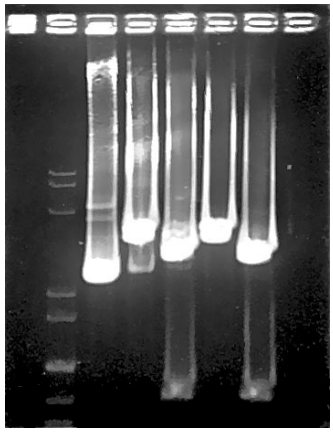

Figure 14 1 % agarose gel charged with ladder, control, 1X, 1HX, 2X, 2HX samples, from left to right.

The gel revealed that *XhoI* restriction site was correctly introduced into the plasmid for both samples, as it can be seen in figure 14. Control plasmid is undigested and ran faster because of being in the supercoiled "CCC" form. All digested plasmids are linear forms and run according to its molecular size. Obviously its mobility cannot be compared with the undigested one. Plasmids digested with one enzyme run slower than double-digested ones due to its bigger molecular size. In both double-digested lanes it is possible to observe a faster fragment, corresponding to the *mCherry* sequence, which was cut out due to the position of both restriction sites, confirming the double digestion.

Sequencing confirmed that the introduction of the point mutation, introduction of XhoI restriction site, worked.

3.4.5. Vector (pJP02) and Insert (pmCherry) digestion

In order to digest the vector, the pJP02 plasmid was first incubated with restriction enzyme *XhoI*. Tubes with 14 µg of plasmid, 1X R buffer and 50 U of enzyme in a total volume of 100 µl were incubated at

37 ºC for 4 h. After that time, the enzyme was inactivated at 80 ºC for 20 min. Then, 50 U of *HindIII* enzyme were added, and buffer R was adjusted in order to maintain 1X concentration in the mixture. The mixture was incubated overnight at 37 ºC and lastly, *HindIII* was inactivated at 80 ºC for 20 min.

The reactions were then analysed by 1 % agarose gel in TAE, where 5 µl of each sample plus 1 µl 6X LD were loaded and 1X TAE was used as electrophoresis buffer, to ensure that digestion had taken place correctly. Electrophoresis ran for 2 h. The same was done with the rest of the digestion mixture for further plasmid purification (separate plasmid from enzymes) by using a separation column included in the commercial gel extraction kit.

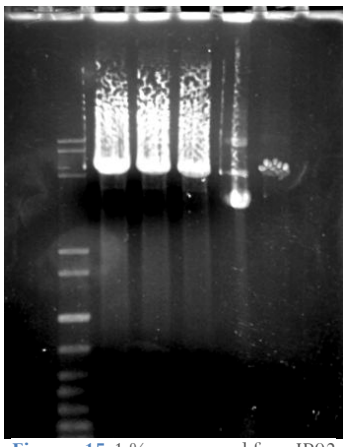

Figure 15 1 % agarose gel for pJP02 digestion verification. From left to right: ladder, 3 digested pJP02 samples and control.

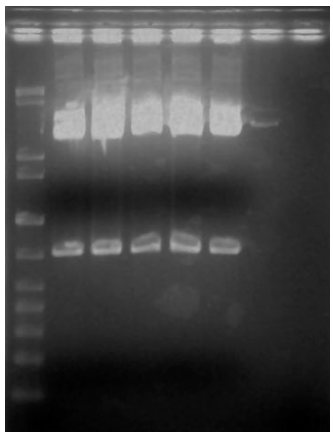

Figure 16 1 % agarose gel for pmCherry digestion verification.

For the insert digestion, 4 reaction mixtures of 40 µl were prepared, each of them carrying 10 µg of plasmid, 1X R buffer, 20 U of *Hind III* and 20 U of *XhoI*. To reach the desired final volume, dH_2O was added. The reaction mixtures were then incubated for 4 h at 37 ºC. After that time, 9 µl *FastAP* and 1 µl *FastAP* buffer were added and incubation at 37 ºC was performed for 15 more minutes. As for vector digestion, the reaction mixtures were then incubated for 20 min at 80 ºC in order to inactivate the enzymes.

To purify the DNA, 10 µl 6X LD were added to the 50 µl digestion mixture and then loaded into a 2 % agarose gel in 1X TAE. The electrophoresis ran for 2 hours. Afterwards, plasmid was purified with the commercial gel extraction kit.

3.4.6. Ligation

Ligation is the joining of two nucleic acid fragments through the action of an enzyme. It is accomplished by covalently connecting the sugar backbone of the two DNA fragments. The final step in the construction of a recombinant plasmid (pJP03) was connecting the insert (pmCherry) into a compatibly digested vector backbone (pJP02). The *T4 DNA Ligase* enzyme performed this reaction. The enzyme catalyses the formation of covalent phosphodiester linkages, which join the nucleotides permanently together. After ligation, the insert DNA was physically attached to the vector and the complete plasmid could be transformed into bacterial cells for propagation.

For pJP02 and pmCherry ligation 4 tubes with different proportions of vector and insert were prepared to ascertain the optimum ligation conditions. For control reasons, one of the tubes did not contain *T4 DNA Ligase*. Total volume for each tube was 20 µl. Tubes were then incubated for 5 min at 22 ºC.

Insert : Vector	Control	1:1	3:1	6:1
dH_2O (µl)	14.74	13.74	12.5	10.5
5X Rapid Ligation Buffer	1X			
Vector	1.8 nM			
Insert	1.9 nM		5 nM	10 nM
T4 DNA ligase	-	5 U		

Table 2 Summary of the ligation conditions.

3.4.7. pJP03 transformation into chemically competent cells

Ligation mixtures were subsequently transformed into DH5α chemically competent cells, following analogous protocol explained above. 1 colony for 1:1 mixture, 11 colonies for 3:1 mixture and 12 colonies for 6:1 mixture were counted. Colonies were small, rounded and independent. Control presented to many colonies so that they were forming a whole layer and it was impossible to count them. This last culture was discarded for this reason.

3 colonies were selected from each of the plates (selection criteria: small, rounded, isolated and opaque white) taken with a pipette tip and inoculated into separate culture tubes containing 6 ml LB and ampicillin. As there was just 1 colony in ligation mixture 1:1, only 1 culture was prepared. These tubes were then incubated at 37 ºC and 210 rpm for 6 h. DNA was then extracted and purified with a commercial kit.

The reactions were then analysed by 1 % agarose gel in TAE, where 5 µl of each sample plus 1 µl 6x LD were loaded and 1X TAE was used as electrophoresis buffer. Two control samples of pJP02 were also loaded; a digested pJP02 sample and a normal pJP02 sample. The result was that ligation mixture with 6:1 proportion of insert and vector, respectively, was the best candidate for further experiments.

Transformation of sample 6:1 was performed again following the same transformation protocol as for pmCherry. pJP03 was then extracted and purified with the commercial kit.

3.4.8. pJP03 digestion and verification

Presumably, the recombinant pJP03 plasmid should contain both, *HindIII* and *XhoI,* restriction sites. In order to verify the correct ligation of pJP02 and pmCherry, 3 digestion reactions were prepared and compared with not digested pJP02 and pJP03 samples. 5 µl of each sample plus 1 µl 6X LD were loaded into 1 % agarose gel and 1X TAE was used as electrophoresis buffer. Electrophoresis ran for 2 h.

Tube	X	H	XH
Plasmid (pJP03)	67.6 nM		
R Buffer	1X		
XhoI	50 U	-	50 U
HindIII	-	50 U	
dH_2O (µl)	30.3		25.3

Table 3 pJP03 digestion conditions.

Following observations were made from the revealed gel (figure 17): the recombinant plasmid pJP03 band ran faster than the band of pJP02 plasmid, which means that pJP03 has a higher molecular weight than pJP02; plasmid digested with both enzymes presents a band corresponding to lower molecular weight than plasmid digested with only one of the enzymes, meaning that the first one has been split into

two fragments; plasmid digested with both enzymes also presents another band at a lower molecular weight than the first band mentioned, confirming that the plasmid has been split into two fragments (vector and insert). Therefore, it can be said that ligation worked and that the obtained plasmid was de desired pJP03.

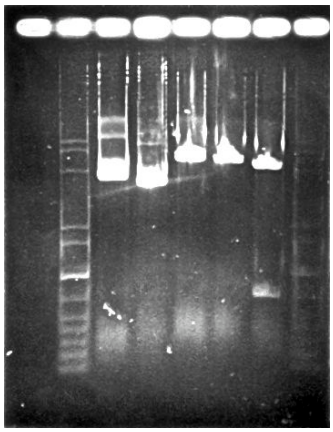

Figure 17 1% agarose gel for pJP03 digestion verification. From left to right: pJP03, pJP02, X, H and XH.

In order to verify the obtainment of pJP03, which should contain the pJP02 sequence, the *mCherry* gene, a *HindII* restriction site and a *XhoI* restriction site, the plasmid was sequenced, as already mentioned and sequencing confirmed the correct ligation.

3.4.9. Point Mutation: replacement of a restriction site for a start codon

For the fulfilment of the goals of the project, elimination of *HindIII* restriction site, between riboswitch and *mCherry* template, is needed, in order to reintroduce the original start codon. The replacement will allow the plasmid (pJP04) to be translated and, consequently, *mCherry* should be expressed according to the riboswitch modulation. For this purpose, a point mutation by PCR was performed following analogous protocol as for *HindIII* introduction to pJP01. The used primers were:

- JP04_1: 5'-GTT TCG GAT GCT TTT AC**A ATG** GTG AGC AAG GGC GAG GAG-3'
- JP04_2: 5'-CTC CTC GCC CTT GCT CAC **CAT T**GT AAA GCC ATC CGA AAC-3'

After incubation of the obtained plasmid with *XhoI* and subsequent analysis by agarose 1 % gel the point mutation was shown not to have worked. Point mutation protocol conditions, such as the concentrations of primers, plasmid or $MgSO_4$, the type of *DNA polymerase* or temperature program, were changed with no positive results. Finally, it was decided to perform the mutation externally, by Genscript, USA.

3.5. *In vivo* cobalamin modulation of protein expression and fluorescence detection

pJP04 plasmid was transformed into BL21(DE3) cells according to the above described protocol for pJP02 transformation into DH5α. The competent cell BL21(DE3) is a chemically competent *E. coli.* containing the *T7 RNA polymerase* gene controlled by the *lacUV5* promoter in its chromosomal DNA. *T7 RNA polymerase* is expressed upon addition of isopropyl-1-thio-β-D-galactopyranoside (IPTG), which induces a high-level protein expression from *T7* promoter driven expression vectors (e.g., *pET*).

Petri dishes with LB-agar and the selection antibiotic (ampicillin) were plated with 50 µl cell suspension

and the latter, distributed homogeneously on the plate surface. Plates were incubated at 37 ºC overnight. One colony was selected (the colonies were rosy and bigger than DH5α colonies) taken with a pipette tip, inoculated into separate culture tubes containing 6 ml LB and ampicillin and incubated for less than 24 h at 37 ºC and 210 rpm. The culture was then transferred into a 15 ml centrifuge tube and spun at 1000 g for 1 min. Supernatant was discarded and pellet was resuspended in 4.2 ml M9 medium.

Culture tubes containing M9 medium with different cobalamin concentrations were inoculated with 100 µl bacteria culture (M9 pellet resuspension). The total volume of each culture tube was 4.1 ml: 4000 µl cobalamin solution in M9 medium and 100 µl bacteria culture. M9 medium was used due to the absence of cobalamins. Contrarily, LB medium contains uncertain quantities of cobalamin from the yeast extract. The next table shows cobalamin concentration, adenosylcobalamin (AdoCbl) and vitamin B_{12} (CNCbl), for each tube:

Tube	**0**	**1**	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**
AdoCbl	-	100 nM	1 µM	10 µM	30 µM	50 µM	100 µM	250 µM	500 µM	750 µM
Tube	**10**	**11**	**12**	**13**	**14**	**15**	**16**	**17**	**18**	**19**
CNCbl	100 nM	1 µM	10 µM	30 µM	50 µM	100 µM	250 µM	500 µM	750 µM	1000 µM

Table 4 Cobalamin concentrations used for each tube.

Samples were incubated for 8 h at 37 ºC and 210 rpm. After 4 h incubation, 1.33 µl 1M IPTG were added to every tube.

After that time, 3 ml of each culture were transferred into plastic fluorescence cuvettes and optical dispersion was measured at 650 nm (OD_{650}) with a spectrophotometer. Neither absorption nor emission (of cobalamins or *mCherry* protein) can be appreciated at this wavelength; dispersion gives an idea about cell concentration in the medium. It was attempted to equalize the dispersion values by adding M9 culture media with the correspondent concentration of cobalamin (differences on dispersion values ranged 5%).

Finally, fluorescence was measured. Samples were excited at 560 nm and the emission spectrum was registered from 580 to 900 nm. *mCherry* presents its excitation maximum at 587 nm but as the excitation spectrum overlaps with emission spectrum, it was decided to perform excitation at a lower wavelength and minimize interferences. Fluorescent measurements were also carried out after washing (centrifuging pellets and discarding supernatant and resuspending in cobalamin free medium or water, three times) pellets and resuspending them both, in water and in M9 supplemented medium, in order to know if decrease in fluorescence intensity could be caused by cobalamins. This final experiment was done in triplicate.

4.1. General observations

Colonies transformed with pJP04 plasmid present a rosy colour, fact that confirms qualitatively that the designed plasmid has been correctly introduced into the chemically competent cells. Moreover, it can be said that the plasmid has been constructed as desired as it apparently integrates *mCherry* sequence, the translation product of which is a red fluorescent protein, the cause of colour. Fluorescence signal corroborates that the *mCherry* fluorophore is responsible for the rosy coloration of cells. Cells that have been growing in a cobalamin free medium present maximum fluorescence at 606 nm. Bearing in mind that, according to literature, emission maximum of *mCherry* is at 610 nm and that factors such as pH can shift the peak, the emission maximum at 606 nm is attributed to *mCherry* fluorescence.

In figures 18 and 19, a decrease in fluorescence intensity can be observed when both, adenosylcobalamin and cyanocobalamin, concentrations increase. The fluorescence intensity depends on the concentration of fluorophore, which is *mCherry* in this case. The concentration of *mCherry*, in turn, depends on the respective gene translation, which should be regulated by de *btuB* riboswitch. The fact that fluorescence intensity decreases while the cobalamin concentration increases is thereby attributed to the regulatory function of the riboswitch.

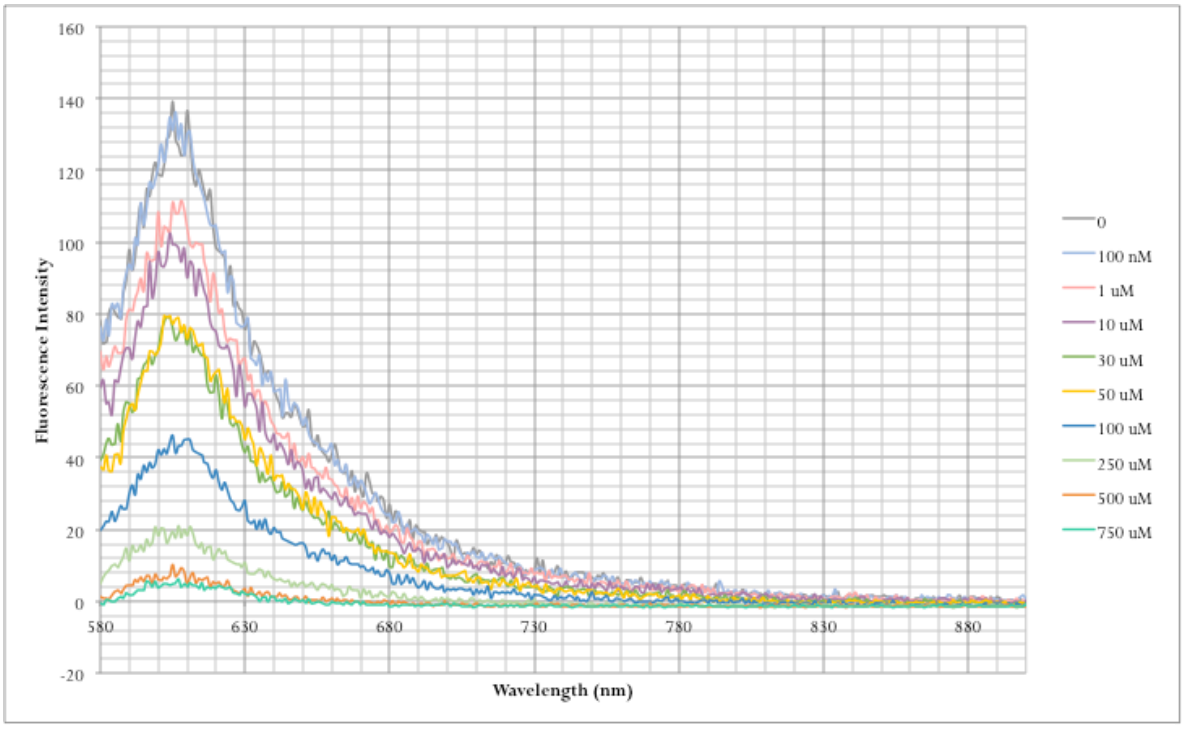

Figure 18 Emission spectrum of BL21 *E. Coli* cells containing pJP04 plasmid after 8 h incubation with different concentrations of **adenosylcobalamin**. Excitation was performed at 560 nm.

Fluorescent measurements were also carried out after washing pellets and resuspending them both, in water and in M9 supplemented medium, this is, in a cobalamin free medium. The same trend, a decrease in fluorescence intensity, could be observed as for cobalamin containing media. Thus, the decrease in fluorescence intensity is not associated with light absorption by cobalamins and confirms the regulatory function of the riboswitch.

Moreover, differences in fluorescence intensity can be noted for equal concentrations of each of the cobalamins. Fluorescence intensity diminishes more rapidly for cyanocobalamin than for

adenosylcobalamin. In other words, fluorescence intensity signal for equal concentrations is lower for cyanocobalamin. This result is not consistent considering the thermodynamic parameters of the ligand–RNA association process, this is, considering that the affinity constant for adenosylcobalamin is much higher than the one for cyanocobalamin.[12, 20] However, vitamin (cyanocobalamin) is more easily taken from the environment by bacteria, and then converted into the coenzyme (adenosylcobalamin). This would explain the differences.

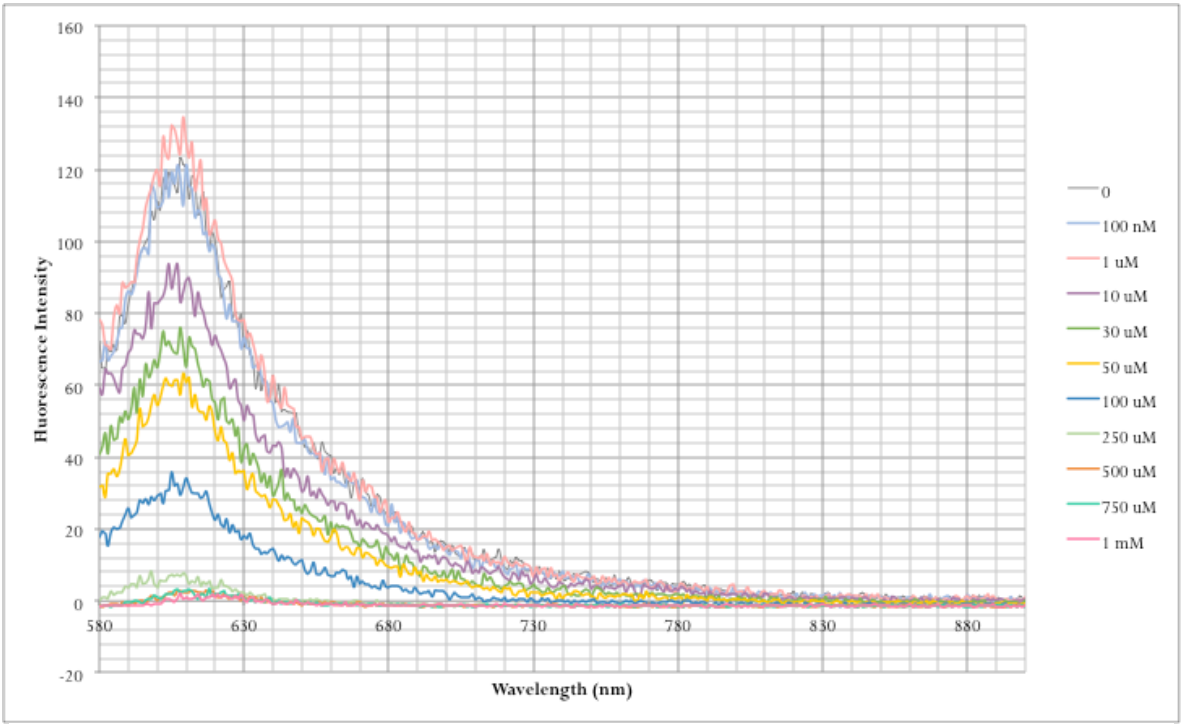

Figure 19 Emission spectrum of BL21 *E. Coli* cells containing pJP04 plasmid after 8 h incubation with different concentrations of **cyanocobalamin**. Excitation was performed at 560 nm.

4.2. Percentage of inhibition of fluorescence

Given the differences in fluorescence intensity, it was decided to calculate the percentage of inhibition of fluorescence (*%I*) and therefore, inhibition of protein synthesis (*mCherry*). The percentage is calculated by the following equation, based on fluorescence intensity at 606 nm data, where F_0 is the fluorescence intensity signal for cobalamin free cell culture and F is the fluorescence intensity signal for each sample.

$$\%I = \frac{F_0 - F}{F_0} \cdot 100 \quad (1)$$

Experiments were done in triplicate, so that for each value of cobalamin concentration three values of fluorescence intensity are available and therefore, three values of *%I*. The average of the three *%I* and the standard deviation is calculated in order to represent the percentage of inhibition in front of the decimal logarithm of the concentration of cobalamin (nM), obtaining a sigmoidal curve (figure 20).

The following tables show the obtained and used data, where FIS and SD mean fluorescence intensity signal and standard deviation, respectively:

[AdoCbl] (nM)	log [AdoCbl]	FIS Sample I	FIS Sample II	FIS Sample III	% Inhibition				SD
					#I	#II	#III	Average (Y)	
0	0	118.4	125.2	108.4	0.0	0.0	0.0	0.0	0.0
100	2.0	147.5	117.6	110.4	-	6.0	-1.9	2.1	5.6
1000	3.0	107.9	112.0	94.6	8.9	10.5	12.7	10.7	1.9
10000	4.0	101.1	92.9	77.4	14.6	25.8	28.6	23.0	7.4
30000	4.5	76.4	75.9	72.9	35.4	39.3	32.7	35.8	3.3
50000	4.7	61.0	74.1	61.3	48.5	40.8	43.4	44.3	3.9
100000	5.0	42.9	45.4	51.9	63.8	63.7	52.1	59.8	6.7
250000	5.4	20.3	19.2	19.6	82.9	84.7	81.9	83.2	1.4
500000	5.7	9.2	7.8	8.5	92.2	93.8	92.2	92.7	0.9
750000	5.9	5.1	5.1	5.1	95.7	95.9	95.3	95.6	0.3

Table 5 Adenosylcobalamin experiments data used for representation in figure 20. Value marked in red was not used.

[CNCbl] (nM)	log [CNCbl]	FIS Sample I	FIS Sample II	FIS Sample III	% Inhibition				SD
					#I	#II	#III	Average (Y)	
0	0.0	118.4	125.2	108.4	0.0	0.0	0.0	0.0	0.0
100	2.0	119.9	137.2	102.8	-1.3	-9.6	5.2	-1.9	7.4
1000	3.0	125.7	132.8	99.5	-6.2	-6.1	8.2	-1.4	8.3
10000	4.0	89.9	101.0	88.9	24.1	19.3	18.0	20.5	3.2
30000	4.5	72.6	79.7	61.0	38.7	36.3	43.7	39.6	3.8
50000	4.7	59.9	56.4	49.9	49.4	54.9	53.9	52.7	3.0
100000	5.0	32.4	37.5	42.0	72.6	70.1	61.2	68.0	6.0
250000	5.4	6.8	6.6	10.1	94.3	94.7	90.6	93.2	2.3
500000	5.7	3.0	3.4	5.2	97.5	97.3	95.2	96.6	1.3
750000	5.9	2.5	5.0	4.4	97.9	96.0	95.9	96.6	1.1
1000000	6.0	0.8	2.0	1.1	99.3	98.4	99.0	98.9	0.5

Table 6 Cyanocobalamin experiments data used for representation in figure 20.

A least squares adjustment was made for a sigmoidal curve using the SOLVER tool, included in Microsoft Excel, considering that the equation for a sigmoidal curve is:

$$y = \frac{a}{1 + e^{b+cx}} + d \quad (2)$$

The most appropriate values of a, b, c and d were calculated by minimizing the value of the sum of squared errors. Each squared error is $(y_{est}-y)^2$, where y_{est} is the estimated value for y of the curve and y is the average percentage of inhibition calculated from experimental data.

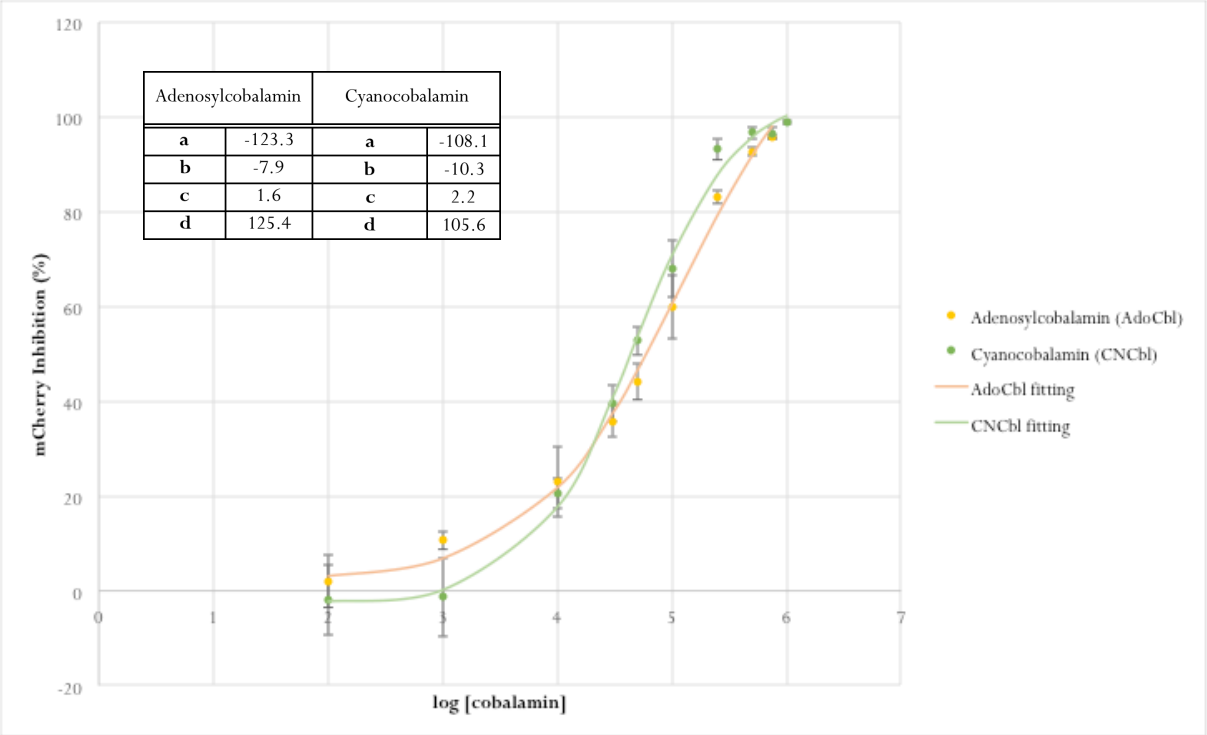

Figure 20 Representation of the percentage of inhibition in front of the decimal logarithm of the concentration of cobalamin (nM). Points are experimental values and lines are adjusted sigmoidal curves according to data. The table at the left top corner shows values *a*, *b*, *c* and *d* for the sigmoidal fitting.

With the resulting equation of the curve the value of cobalamin concentration that results in 50% inhibition (y=50) for each of the B_{12} derivatives was calculated:

$$x' = [cobalamin] = \frac{10^{\frac{ln\left(\frac{a}{y-d}-1\right)-b}{c}}}{1000}\ \mu M \quad (3)$$

To know the confidence interval for the calculated values the standard deviation of this measure was calculated by propagation of uncertainty (equation 4). The standard deviation of the fitting (of estimated y values) was calculated by making the square root of the division of the value for the sum of squared errors by *n-1*. It was considered that the uncertainty due to estimated y was the only variable uncertainty involved in the calculation of the cobalamin concentration.

$$\sigma_{x'}^2 \approx \sigma_{y_{estimated}}^2 \cdot \left(\frac{\partial x'}{\partial_{y_{estimated}}}\right)^2 \quad (4)$$

Thus, the following equation (equation 5) represents the confidence interval for cobalamin concentration.

$$\sigma_{x'} = \sqrt{\sigma_{y_{estimated}}^2 \cdot \left(\frac{-a \cdot \ln(10)}{1000 \cdot c} \cdot \frac{1}{\left(\frac{a}{y-d}-1\right) \cdot (y-d)^2} \cdot 10^{\frac{\ln\left(\frac{a}{y-d}-1\right)-b}{c}}\right)^2}\ \mu M \quad (5)$$

According to this calculations, cobalamin concentration for a 50% inhibition of *mCherry* synthesis are $[AdoCbl]_{50}$ = 60 ± 7 µM and $[CNCbl]_{50}$ = 44 ± 4 µM.

Adenosylcobalamin			Cyanocobalamin		
Y	**Y (estimated)**	**Squared error**	**Y**	**Y (estimated)**	**Squared error**
0	0	0	0	0	0
2	3	1	-2	-2	0
11	7	15	-1	0	2
23	22	2	20	18	8
36	37	2	40	40	1
44	47	5	53	54	1
60	60	0	68	71	8
83	79	15	93	88	29
93	92	1	97	96	1
96	98	6	97	99	4
			99	100	2
Squared errors sum		47	**Squared errors sum**		56
SD		2	**SD**		2

Table 7 Inhibitions, estimated inhibition and respective standard deviation values used for cobalamin concentration confidence interval calculation and sigmoidal fitting

4.3. Final discussion

Cobalamin concentrations for a 50% inhibition of *mCherry* synthesis ($[AdoCbl]_{50} = 60 \pm 7$ μM and $[CNCbl]_{50} = 44 \pm 4$ μM) confirm that protein expression is more rapidly inhibited by cyanocobalamin than by adenosylcobalamin even if the interaction of AdoCbl with the *btuB* riboswitch is more favourable than for CNCbl. These concentrations are initial cobalamin concentrations in the medium. Energy is necessary for cobalamin uptake by *btuB*, the outer membrane protein of *TonB*-dependent active transport proteins. Thus, vitamin B_{12} uptake is energetically favourable in front of coenzyme B_{12} uptake.

At the interaction between *btuB* and CNCbl, hydrogen bonds are formed primarily to the acetamide and propionamide moieties of CNCbl and most of the van der Waals interactions occur around the corrin ring and their side-chains.[16] The difference between CNCbl and AdoCbl is only an axial ligand, which is a cyano group for CNCbl and a deoxyadenosyl for AdoCbl. This way, interactions between *btuB* and AdoCbl would be mostly analogous to those for CNCbl and therefore, energy differences should be governed by the type of axial ligand. Probably the size of the ligand and consequently, the molecular weight of the cobalamin, is what determines the effectiveness of the active transport.

In this study, *mCherry* sequence was used to visualize the regulatory function of the riboswitch. Fluorescence intensity associated to *mCherry* decreases while the cobalamin concentration increases, but in not mutated cells, such as *Klebsiella pneumoniae* cells, synthesis of *btuB* protein would be regulated in response to the availability of their substrate, cobalamin.

5. CONCLUSIONS

1. A plasmid containing the *btuB* riboswitch from *Klebsiella pneumoniae* coupled to *mCherry* sequence was designed and constructed by cloning techniques.

2. The constructed plasmid (pJP04) was introduced into chemically competent *E. Coli* cells and, according to the coloration of *mCherry,* rosy colonies were obtained.

3. *In vivo* regulation of the mentioned riboswitch by cobalamins was evaluated by fluorescent measurements confirming that the *btuB* riboswitch from *Klebsiella pneumoniae* is modulated by cobalamins.

4. Fluorescent measurements were performed for two different cell growth conditions, this is, for two cobalamin derivatives: adenosylcoabalamin and cyanocobalamin. Both cobalamin derivatives showed the same trend: a decrease in fluorescence intensity for a cobalamin concentration increase.

5. It was confirmed that cobalamins activate *btuB* riboswitch from *K. pneumoniae* blocking gene expression as it happens in other *btuB* riboswitches from other species such as *E. Coli*.

6. The percentage of inhibition protein synthesis in front of the decimal logarithm of the concentration of cobalamin was represented obtaining sigmoidal curves.

7. The cobalamin uptake in living *E. Coli* cells was evaluated by comparing values for 50 % of inhibition between cobalamins, showing that cyanocobalamin uptake is more effective than adenosylcobalamin.

8. Further research is needed to get to know in depth the interaction between the noncoding mRNA fragment and cobalamin and which other factors are involved in the *in vivo* interaction.

As a final information, I would like to notice that this work was presented as a poster at the *3rd International Symposium on Functional Metal Complexes that Bind to Biomolecules – 4th Whole Action Meeting of the COST Action CM1105*. This meeting took place in Palma from April 28, 2016 to April 29, 2016. As an annex a copy of the abstract from the conference, published in the book of abstracts (ISBN: 978-84-608-7064-7; DL: PM 400-2016), and a copy of the poster presented, has been included.

Finally, we have recently been informed that this work will be part of the oral communication that Dr. Miquel Barceló will give at the 13th European Biological Inorganic Chemistry Conference – EuroBIC 13, which will take place in Budapest (Hungary) from August 28, 2016 to September 1, 2016. The talk will be entitled "A fluorescent sensor based on a riboswitch regulation mechanism for cobalamin derivatives detection", and the authors are Joana Palou-Mir, Maria Olivia Schweiss and Miquel Barceló-Oliver.

Riboswitch-based fluorescent sensor for cobalamin derivatives detection

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Riboswitches are found at the 5'-UTR of the mRNA and regulate the transcription or translation of proteins encoded downstream. We have been studying a B_{12} riboswitch from *Klebsiella pneumoniae* which regulates the translation of the *btuB* protein. This outer membrane protein, which is the first part of the B_{12} uptake system, is responsible of the active transport of vitamin B_{12} from the medium. This *btuB*-riboswitch regulation is proved to be at the translation level by masking the RBS (ribosome binding side). At low concentrations of coenzyme B_{12}, the RBS is free and the riboswitch mainly presents the gene-on conformation permitting the synthesis of the *btuB* protein. On the other hand, at high concentrations of the coenzyme the riboswitch is folded into the gene-off conformation inhibiting the translation of the protein. ITC experiments were performed in order to determine the thermodynamic parameters of the interaction between the riboswitch and the coenzyme B_{12} [1].

In this communication we present a construct where the *btuB* riboswitch sequence is followed by the *mCherry* sequence which replaces the native encoded *btuB*. The replacement of the native protein with this coloured protein will allow us to detect the regulation of the riboswitch with different amounts of coenzyme B_{12} by eye or using a fluorimetric method.

Acknowledgments
Financial support from "Govern de les Illes Balears" with FEDER funds (projects AAEE0145/09, AAEE0019/2012 and AAEE3/2014), and the COST Action CM1105 is gratefully acknowledged. Special thanks to Roger Tsien for supplying us the mCherry plasmid.

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Riboswitch-based fluorescent sensor for cobalamin derivatives detection

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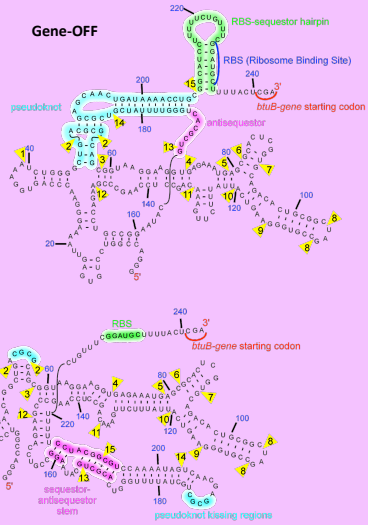
Introduction:

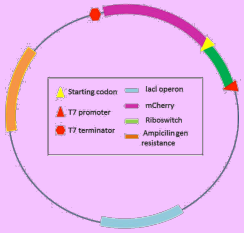
Non-codifying mRNAs have been discovered to control the expression of some genes. One kind of those regulatory elements are riboswitches, found at the 5'-untraslated region (5'-UTRs), that regulate the transcription of the RNA or the translation of proteins encoded downstream [1]. These riboswitches respond to the metabolite that is synthesized or uptaked by the downstream-encoded gene.

In the genome of *Klebsiella pneumoniae* a B_{12}-Riboswitch which regulates the translation of the *btuB* protein was identified. The computational identification of these RNA sequences is possible due to the conservation of certain domains in all the species [2]. At low concentrations of coenzyme B_{12}, the Ribosome Binding Site (RBS) is free and mainly presents the gene-on conformation, allowing the synthesis of the *btuB* protein. On the other hand, at high concentrations of the coenzyme, the riboswitch refolds yielding the gene-off conformation, in which the RBS is masked and the translation of the protein is inhibited.

In our group we have been studying the interaction of the *btuB* Riboswitch from *Klebsiella pneumoniae* with coenzyme B_{12}. Using in-line probing experiments we have proved the switch and with Isothermal Titration Calorimetry (ITC) measurements yielded the thermodynamic parameters of the interaction [3].



Construct design:



In this study we have prepared a plasmid, based on pET-21b(+), where the *btuB* Riboswitch sequence is followed by the *mCherry* sequence, which replaces the native *btuB* template. The full-length riboswitch is encoded and the starting codon of the *mCherry* protein is located right after that, with the whole sequence situated in-between the promotor and the termination of the T7 polymerase. This plasmid also carries the ampicillin resistance gene, for selection purposes, and the lac operon, which allows IPTG-activation of the protein synthesis. The replacement of the native protein with this fluorescent protein will allow us to detect the riboswitch-regulation with a fluorometric method.

Fluorescence Measurements:

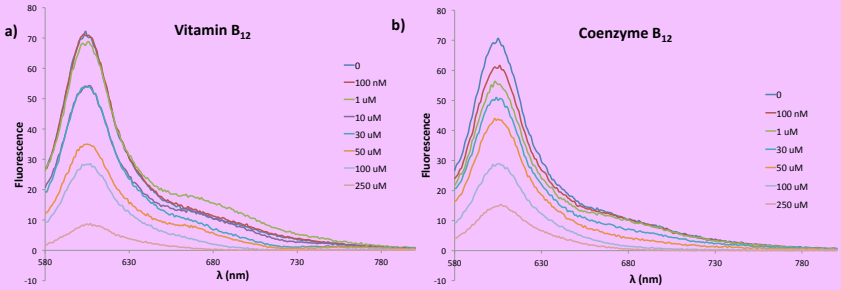
In order to avoid the absorption of the *mCherry* protein, the optical density of cells in suspension was measured at 650 nm (OD_{650}). Then the concentration of cells was normalized by dilution of the more concentrated samples with the same M9 medium.

Samples were excited at 560 nm and the emission spectrum was recorded from 580 nm to 900 nm. The following charts represent the titration experiments with different concentrations of vitamin B_{12} or coenzyme B_{12}:

Cells Culture Conditions:

The plasmid was transformed into BL21 cells. Then, cells were grown in supplemented M9 minimal medium (which doesn't contain B_{12} derivatives in its formulation) [4] with 100 μg/ml ampicillin. Different concentrations of cobalamin derivatives were added to the medium, as shown below, to study the response of the riboswitch to the different amount of vitamin B_{12} or Coenzyme B_{12} available. After growing for 4h at 37ºC and 210 rpm, IPTG was added to a final concentration of 0.33 mM and incubated for 4 additional hours.

Vitamin B_{12}	0	100 nM	1 μM	10 μM	30 μM	50 μM	100 μM	250 μM
Coenzyme B_{12}	0	100 nM	1 μM	10 μM	30 μM	50 μM	100 μM	250 μM



The representations of emission intensity vs. wavelength clearly show that when an increasing amount of vitamin B_{12} (a) or coenzyme B_{12} (b) is added, the fluorescence emission is reduced.

The correlation between cobalamin concentration and *mCherry* expression (and thus fluorescence emission) implies not only the cobalamin-riboswitch interaction. The cobalamin uptake should also be considered.

Outlook:

We have been able to detect the regulation of the translation *in vivo* using a fluorometric method: an increase of cobalamin concentration in the medium yields a reduction of the fluorescence signal due to the inhibition of the translation of the *mCherry* protein. We speculate that the different pattern that is observed using different cobalalamin derivatives is on account of a combination of the cellular absorption of the product and the affinity association constant with the riboswitch.

Acknowledgements:

The authors gratefully acknowledge financial support from the COST Action CM1105 and the "Govern de les Illes Balears" with FEDER funds (project number AAEE3/2014). The authors also thank Roland K.O. Sigel, Sofia Gallo and Anastasia Musiari from University of Zürich for all the aid and support received. Special thanks to Roger Tsien for supplying the mCherry plasmid.

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