Elsevier Editorial System(tm) for Journal of Inorganic Biochemistry

Manuscript Draft

Manuscript Number: JIB-15-0838R1

Title: Characterization of the full-length btuB riboswitch from Klebsiella pneumoniae

Article Type: SI: Metallodrugs

Keywords: RNA; btuB riboswitch; coenzyme B12; in-line probing; ITC

Corresponding Author: Prof. Miquel Barceló-Oliver, Ph.D.

Corresponding Author's Institution: University of the Balearic Islands

First Author: Joana Palou-Mir

Order of Authors: Joana Palou-Mir; Anastasia Musiari; Roland Sigel, Prof. Dr.; Miquel Barceló-Oliver, Ph.D.

Abstract: Riboswitches are cis-regulatory RNA elements on the mRNA level that control the expression of the downstream coding region. The interaction of the riboswitch with its specific metabolite, which is related to the function of the controlled gene, induces a structural change of the RNA architecture. Consequently, gene regulation is induced by un/masking of the ribosome binding site (RBS). In the genome of Klebsiella pneumoniae a sequence was identified by bioinformatics and proposed to be a B12 riboswitch regulated by coenzyme B12. Here we study this new coenzyme B12-dependent riboswitch system by in-line probing and ITC. The riboswitch sequence includes the whole expression platform as well as RBS. In-line probing experiments were performed to investigate the structural rearrangement of this 243-nt long RNA sequence while Isothermal Titration Calorimetry (ITC) yielded the thermodynamic parameters of the interaction between the riboswitch and its metabolite. The interaction of coenzyme B12 with the butB riboswitch of K. pneumoniae is an exothermic process with a 1:1 binding stoichiometry and binding affinities of log KA = 6.73 ± 0.02 at 15 °C and log KA = 6.00 ± 0.09 at 30 °C.

Prof. Miquel Barceló-Oliver
Department of Chemistry (Inorganic)
University of the Balearic Islands
Carretera Valldemossa km 7.5
E-07122 Palma de Mallorca (SPAIN)
Building: Mateu Orfila i Rotger
Room: QI-126
Phone: (+34) 971.173.199
Fax: (+34) 971.173.426
e-mail: miquel.barcelo@uib.es

December 5, 2015

Dear Professor Dawson,

Thank you very much for your email and referee reports on our manuscript. We are very grateful to the referees for the helpful comments helping us to improve our manuscript. We have now revised the manuscript according to their suggestions and have performed a series of new experiments which are also included. Please find below a detailed response to their individual points.

Reviewer #1:
1) it seems that the cleavage pattern reveals a diverse change in cleavage sites. The authors should describe more convincingly whether a single binding site exists (where?) or whether multiple interactions are possible.

>> Based on the fit and especially the ITC data, we can show that a 1:1 binding equilibtrium exists. This also fits perfectly with all B12 riboswitch studies described in the literature. We have included a paragraph explaining the expected kind of interaction as well as a brief description of the results found by X-ray with others B12 riboswitches. We hope that this part is now clearer.

2) The conclusion that the structure is conserved compared to other riboswitches with different sequence requires more substantiation, e.g. comparing putative structure from literature.

>>We have included a paragraph comparing in more detail our cleavage pattern with those found for the E. coli btuB riboswitch.

3) a linear regression through 2 points is not recommended as this always gives a good results, but small errors can lead to large deviations. At least one more ITC experiment at a temperature outside the current window should be performed.
>> This statement is certainly correct and we are very thankful. We have thus repeated our measurements at 20 °C (to have it also in triplicate) and added furthermore experiments at 15 ºC and 30 ºC. We have included Gibbs-Helmholtz and van't Hoff plots showing that indeed there is no linear correlation of Delta Cp. Instead we propose the possibility of a mechanistic change depending on temperature.

Reviewer #2:
A suggestion concerns the presentation of the data in Figure 3, where the relative maximal changes in intensity of the crucial bands from in line probing are depicted, starting with '0' - would probably be more adequate to start with 100% as the reference value.
>> Correct, one could so. However, we have prepared this Figure according to previous publications showing in-line probing experiments. Note that the intensities can either increase or decrease in dependence on ligand concentration. Hence, either a starting point of 0 or 1.0 is justified. In the end we have decided to leave it as is for consistency with previous publications.

We hope we could address all issues and are looking forward to your decision.

Thank you very much for dealing with our manuscript and with our best regards,

Yours sincerely,
Miquel Barceló-Oliver

Prof. Miquel Barceló-Oliver
Department of Chemistry (Inorganic)
University of the Balearic Islands
Carretera Valldemossa km 7.5
E-07122 Palma de Mallorca (SPAIN)
Building: Mateu Orfila i Rotger
Room: QI-126
Phone: (+34) 971.173.199
Fax: (+34) 971.173.426
e-mail: miquel.barcelo@uib.es

5 December 2015

Dear Reviewers,

Thank you very much for your reports on our manuscript. We are very grateful for the helpful comments helping us to improve our manuscript. We have now revised the manuscript according to your suggestions and have performed a series of new experiments, which are also included. Please find below a detailed response to your individual points.

Reviewer #1:
1) it seems that the cleavage pattern reveals a diverse change in cleavage sites. The authors should describe more convincingly whether a single binding site exists (where?) or whether multiple interactions are possible.

>> Based on the fit and especially the ITC data, we can show that a 1:1 binding equilibtrium exists. This also fits perfectly with all B12 riboswitch studies described in the literature. We have included a paragraph explaining the expected kind of interaction as well as a brief description of the results found by X-ray with others B12 riboswitches. We hope that this part is now clearer.

2) The conclusion that the structure is conserved compared to other riboswitches with different sequence requires more substantiation, e.g. comparing putative structure from literature.

>>We have included a paragraph comparing in more detail our cleavage pattern with those found for the E. coli btuB riboswitch.

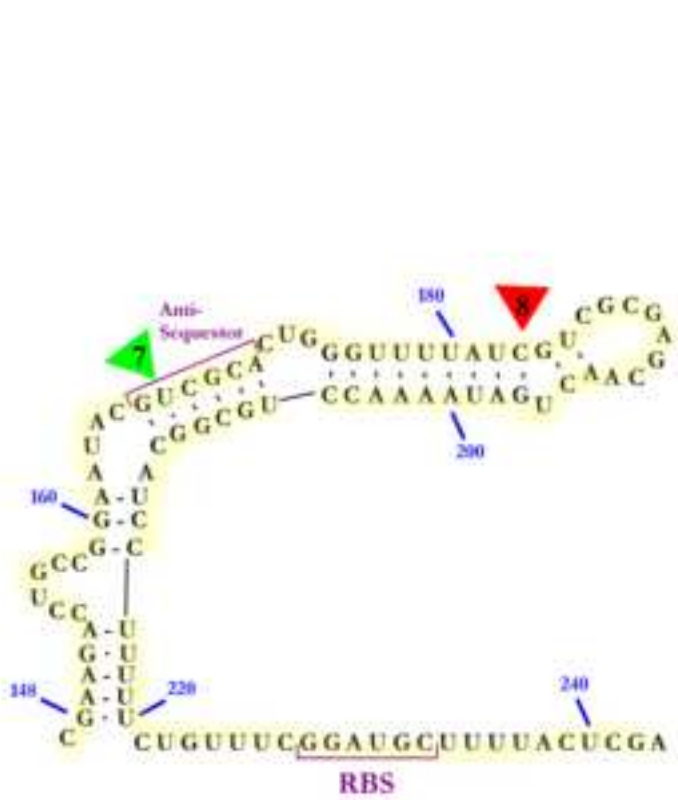
3) a linear regression through 2 points is not recommended as this always gives a good results, but small errors can lead to large deviations. At least one more ITC experiment at a temperature outside the current window should be performed.
>> This statement is certainly correct and we are very thankful. We have thus repeated our measurements at 20 °C (to have it also in triplicate) and added furthermore experiments at 15 ºC and 30 ºC. We have included Gibbs-Helmholtz and van't Hoff plots showing that indeed there is no linear correlation of Delta Cp. Instead we propose the possibility of a mechanistic change depending on temperature.

Reviewer #2:
A suggestion concerns the presentation of the data in Figure 3, where the relative maximal changes in intensity of the crucial bands from in line probing are depicted, starting with '0' - would probably be more adequate to start with 100% as the reference value.
>> Correct, one could so. However, we have prepared this Figure according to previous publications showing in-line probing experiments. Note that the intensities can either increase or decrease in dependence on ligand concentration. Hence, either a starting point of 0 or 1.0 is justified. In the end we have decided to leave it as is for consistency with previous publications.

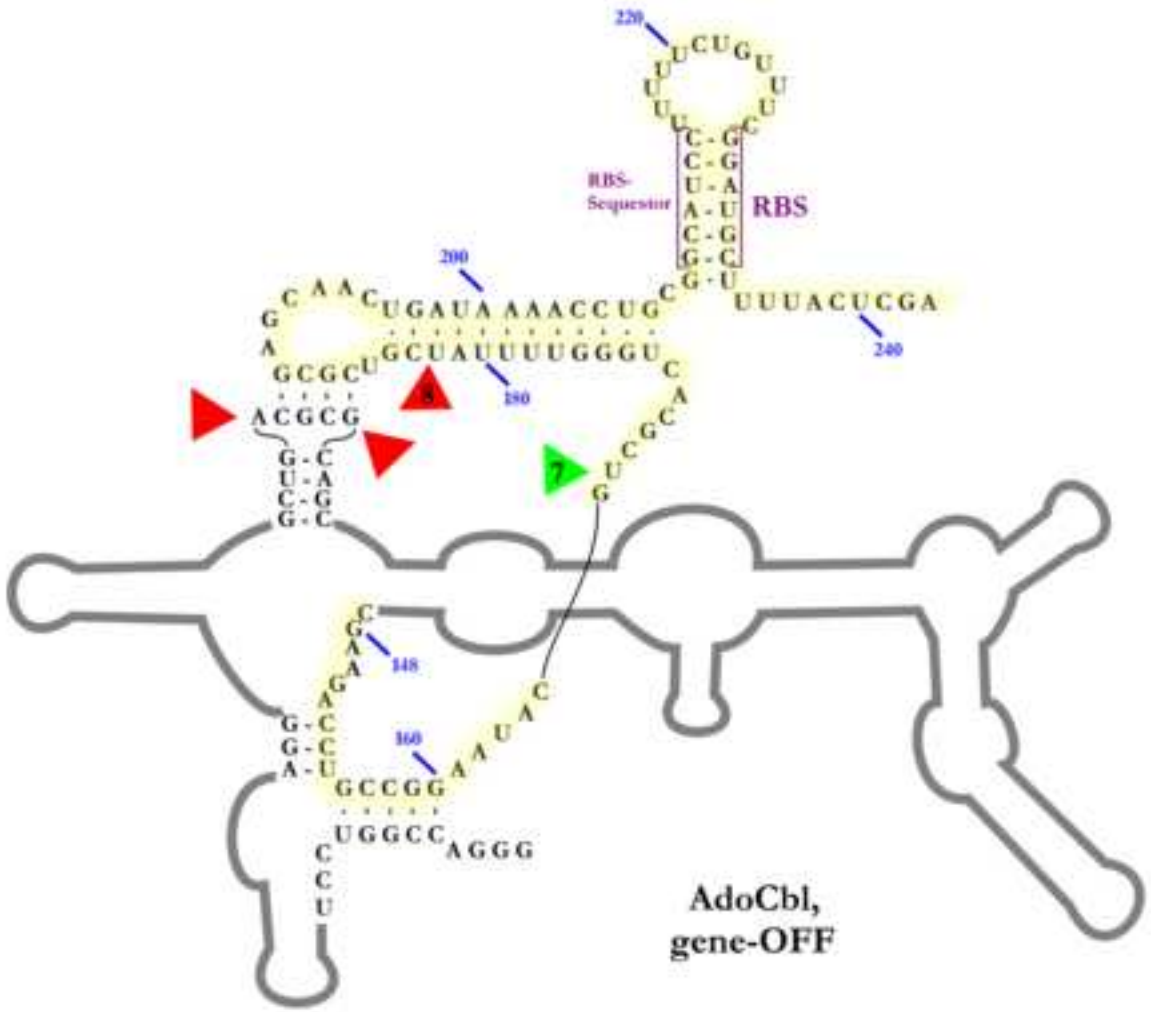
Yours sincerely,
Miquel Barceló-Oliver

*Pictogram for the Graphical Abstract

Click here to download high resolution image



NO AdoCbl,
gene-ON



AdoCbl,
gene-OFF

Graphical abstract (synopsis)

The *Klebsiella pneumoniae* genome carries a *btuB* riboswitch proposed to bind adenosylcobalamin. ITC and in-line probing experiments conducted on the whole expression platform with the ribosome binding site (RBS) have been used to study the riboswitch – cobalamin interaction.

Highlights

- the *btuB* ribowitch from *K. pneumoniae* was studied including the whole expression platform
- in-line probing proved the conformational change upon binding of the coenzyme B_{12}
- core structure B_{12}-induced changes are respective in the individual riboswitches
- ITC showed B_{12}-riboswitch interaction and refolding just involve weak interactions

Characterization of the full-length *btuB* riboswitch from *Klebsiella pneumoniae*

Joana Palou-Mir · Anastasia Musiari · Roland K.O. Sigel · Miquel Barceló-Oliver*

J. Palou-Mir · M. Barceló-Oliver

Department of Chemistry

University of the Balearic Islands

Carretera Valldemossa km 7.5

Palma de Mallorca, E-07122, Spain

A. Musiari · R.K.O. Sigel

Department of Chemistry

University of Zurich

Winterthurerstrasse 190

CH-8057 Zurich, Switzerland

* Corresponding author: e-mail: miquel.barcelo@uib.es; phone: (0034) 971.173.199; Fax: (0034) 971.173.426

Abstract

Riboswitches are cis-regulatory RNA elements on the mRNA level that control the expression of the downstream coding region. The interaction of the riboswitch with its specific metabolite, which is related to the function of the controlled gene, induces a structural change of the RNA architecture. Consequently, gene regulation is induced by un/masking of the ribosome binding site (RBS). In the genome of *Klebsiella pneumoniae* a sequence was identified by bioinformatics and proposed to be a B_{12} riboswitch regulated by coenzyme B_{12}. Here we study this new coenzyme B_{12}-dependent riboswitch system by in-line probing and ITC. The riboswitch sequence includes the whole expression platform as well as RBS. In-line probing experiments were performed to investigate the structural rearrangement of this 243-nt long RNA sequence while Isothermal Titration Calorimetry (ITC) yielded the thermodynamic parameters of the interaction between the riboswitch and its metabolite. The interaction of coenzyme B_{12} with the *butB* riboswitch of *K. pneumoniae* is an exothermic process with a 1:1 binding stoichiometry and binding affinities of log $K_A = 6.73 \pm 0.02$ at 15 ºC and log $K_A = 6.00 \pm 0.09$ at 30 ºC.

Key words: RNA · *btuB* riboswitch · coenzyme B_{12} · in-line probing · ITC

Introduction

Proteins are not the exclusive triggers of gene expression regulation. Parts of non-coding mRNAs control certain genes by base pairing with cis- or trans-encoded targets. A related kind of such regulatory elements are riboswitches, highly structured sequences situated in the 5'-untranslated region (5'-UTR) of mRNAs [1-6]. Riboswitches are highly conserved in evolution and they are present in most taxonomical groups of bacteria. In addition, riboswitches respond to the metabolite that is synthesized, transported, or taken up by the downstream-encoded proteins, inducing a structural shift in their structure in response to the binding of the metabolite. Riboswitches are composed by two main parts: the aptamer as the region where the metabolite is recognized and bound and the expression platform, which contains the RBS and which experiences structural changes in return to the changes in the aptamer region.

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 *E. coli*. The *btuB* is an outer membrane protein that transports the cobalamin into the periplasm [7-8]. Nowadays, more than fifteen types of riboswitches have been reported, responding to a wide variety of metabolites such as aminoacids, nucleobases, metal ions (Mg^{2+}), redox cofactors, and other small molecules [9-11].

The interest in the study of riboswitches is rapidly increasing because they have been postulated to be an optimal target for future antibiotics due to their regulatory function and their almost exclusive presence in bacteria [12]. Also, it has been discovered that some drugs used nowadays exhibit this mechanism of action [12]. For instance, L-aminoethyl cisteine (AEC) and DL-4-oxalysine are able to bind and activate lysine riboswitch causing the cell to cease lysine synthesis and thus the cell growth [13]. It has

also been proven that after entering the cell the antibiotic pyrithiamine is metabolized into pyrithiamine pyrophosphate, which is able to bind and activates the TPP riboswitch. Such the synthesis and import of TPP by the cell is inhibited and the cell dies as pyrithiamine pyrophosphate cannot substitute for TPP as a coenzyme [14,15]. Finally, another drug, roseoflavin is able to act in the same way on the FMN riboswitch [16].

Three of those cobalamin riboswitches have been widely studied: the *Cob* riboswitch [17-19] and the *btuB* riboswitch [18,19] from *Salmonella typhimurium*, as well as the aforementioned *btuB* riboswitch from *E. coli* [7,8,19-21]. Studies on few other B_{12} riboswitches exist [22,23], including X-ray structures of the aquocobalamin riboswitch from *Thermoanaerobacter tengcongensis* (Tte) and *environmental metagenomic env8* by Johnson Jr. & Reyes [24], as well as of the *Symbiobacterium thermophilum* B_{12} riboswitch by Peselis & Serganov [25], all of these riboswitches belonging to distant branches of the phylogenetic tree.

Parts of the sequence of these riboswitches are evolutionary 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 [19,26]. Thanks to those conserved regions, bioinformatics studies can be performed on the 5'-UTR regions of B_{12}-related genes of bacterial genomes in order to identify possible B_{12} riboswitches [26].

In the here presented work we have selected the experimentally not yet investigated *btuB* riboswitch from *Klebsiella pneumoniae* [26] for two main reasons: i) in the recent years, *K. pneumoniae* has become an important pathogen in nosocomial infections which makes it a good target for new antibiotics [27-30]; ii) *K. pneumoniae* belongs to

the same phylogenetic branch as *E. coli* and *S. typhimurium* but represents one step forward in the differentiation from them [26]. That allows us to see if a general mechanism pattern of gene-silencing is followed. The *btuB* riboswitch from *K. pneumoniae* was proposed to regulate the gene expression via inhibition of translation by sequestering the RBS [26], as already shown for *Escherichia coli* [20].

The riboswitch sequence we used here, includes both the aptamer and expression platform regions. By in-line probing we proved that our sequence acts as a riboswitch regulating the gene expression without the assistance of proteins. Further we performed Isothermal Titration Calorimetry (ITC) experiments to determine the thermodynamic binding parameters in solution for the RNA-coenzyme B_{12} interaction at two different temperatures.

Materials and methods

Materials: Coenzyme B_{12} was purchased by Sigma and used without further purification. Nucleoside 5'-triphosphates were purchased from AppliChem Life Sciences (Germany). T7 RNA polymerase used for *in vitro* transcription was produced in-house and purified by an adapted protocol from reference [31]. The water was of Milli-Q grade (Merck Millipore, USA) and was autoclaved and filtered through 0.2 μm sterile filters prior to use. All buffers, salt solutions and gels were also filtered through 0.2 μm sterile filters. All chemicals bought from Merck, Sigma-Aldrich, AppliChem, or BDH Prolabo were of at least p.a. grade and used without further purification.

Plasmid design (pJP01): The complete genome of *Klebsiella pneumoniae* subsp. pneumoniae strain MGH 78578 was downloaded from the National Center for

Biotechnology Information (NCBI) (GenBank: CP000647.1). The *btuB* gene was located at the position 4660313-4662169. The 5'-UTR region proposed to act as a riboswitch was located starting at position 4660061 and comprises 243 nucleotides [26]. For our study, 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 starting point for transcription; ii) the 3'-end was extended to contain the RBS until the starting 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 corresponds to the starting codon). The proposed secondary structure for this sequence is shown in Figure 1. The 243-nt long RNA sequence used in this study, which contains the aptamer and the expression platform, is:

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'

>> INSERT FIGURE 1

RNA preparation: The full sequence encoding the *btuB* riboswitch was synthesized by GenScript (USA) and sub-cloned into a pET-21b plasmid containing an ampicillin resistance gene. The desired sequence was placed between the T7 RNA polymerase promoter (TAAT ACGA CTCA CTAT AGGG) and the XhoI restriction site (C^TCGA_G). The plasmid pJP01 was transformed into chemically competent

XL1blue *E.coli* cells. The plasmid obtained from cultures of the transformed bacteria in LB was cleaved with the XhoI restriction enzyme prior to transcription. The RNA was produced by *in vitro* transcription with T7 RNA polymerase, purified with denaturing PAGE (10%), isolated by crush and soak at 4ºC and ethanol precipitated [31]. A final purification step was needed to avoid buffer shocks during the ITC measurements. The solvent was exchanged by repeated centrifugation of the isolated RNA against water using 10'000 MWCO Vivaspin 500 devices (Sartorius) [protocol: 500 µL H_2O, 500 µL EtOH 70% (device sterilization), 3x500 µL H_2O, sample introduction, 5x500 µL H_2O].

In-line probing experiments: The RNA was 5'-end labelled using T4-polynucleotide kinase (PNK) and ^{32}P-γ-ATP. Purification by denaturing 5% PAGE, extraction by crush and soak, and ethanol precipitation yielded the labelled RNA. The total concentration of RNA was determined by UV/Vis with a Picodrop CUBE. The labelling yield (*ca*. 10%) was checked by scintillation.

The titration experiments were performed in 50 mM Tris-HCl (pH=8.3), 100 mM KCl, 20 mM $MgCl_2$ and 26 nM RNA (total). Coenzyme B_{12} concentrations used for titration were: 0, 50 nM, 100 nM, 200 nM, 300 nM, 400 nM, 500 nM, 1 µM, 2.5 µM, 10 µM and 100 µM. Correct folding of the RNA was achieved as follows: denaturation at 90 ºC for 1 min, addition of the buffer, and folding for 20 min at room temperature prior to the final addition of the coenzyme B_{12} in the dark. For in-line probing, the samples were incubated for 40 h at room temperature in the dark. Two repetitions of the titration were performed. The intensities of the bands were established using the Image Quant TL software. Changes at each cleavage site were quantified by plotting the intensities of the bands over the coenzyme B_{12} concentration after background correction. The experimental data were fitted to a 1:1 isotherm [32] to give the binding constant (K_A) values of the different sites as an average of 2 titration experiments. The overall log K_A

value is obtained by the arithmetic mean of the K_A values of the single sites.

ITC sample preparation and measurements: All solutions were buffered in 100 mM HEPES-KOH buffer at pH=7.5, 100 mM KCl and 5 mM $MgCl_2$, being really important keeping the buffer conditions constant during the experiment. 10X ITC buffer was prepared and used to dilute the stock solutions of coenzyme B_{12} or RNA in ultrapure water to the final concentrations. RNA samples were annealed at 90 ºC for 45 s and kept on ice prior to the addition of the 10X buffer. The exact concentration of coenzyme B_{12} and RNA was checked again by UV-Vis spectroscopy prior to the ITC measurements using an Ultrospec 7000 spectrophotometer (GE Healthcare Life Sciences). Solutions were degassed for 20 minutes at the experiment temperature (15, 20, 25 and 30 ºC). All solutions containing coenzyme B_{12} were protected from light to avoid degradation.

ITC experiments were carried out using a Nano-ITC low volume instrument (171 μL cell volume) (TA Instruments) and the fitting was made using the software included with the calorimeter (NanoAnalyze Software v3.1.2, TA instruments). The calorimeter was stabilized for 1 h at the desired temperature prior to the measurements. The cells were filled with an excess of 250 μL *ca.* 15 μM RNA solution (working cell) and 250 μL 1x ITC buffer (reference cell) to avoid air bubbles. The 50 μL titration syringe was loaded with 50 μL *ca.* 100 μM coenzyme B_{12} solution (concentration 6-7 times higher than the one in the cell). The experiments were set to 17 injections of 3.03 μL with a collecting time of 300 s between injections. Once measured, raw heat is corrected for dilution effects by subtracting dilution heat (control; injection of coenzyme B_{12} into buffer in the same conditions than experiment). All the experiments were made by triplicate and the results show the mean value of them.

Results and Discussion

In-line probing measurements:

The so-called in-line probing technique probes ^{32}P-5'-end-labelled RNA structure without any chemical nor structural modification of the respective sequence. This technique is based on the differential degradation of the RNA according to its local structure: the phosphodiester bonds in the backbone are cleaved by a nucleophilic attack of the 2' oxygen on the adjacent phosphorus centre only if 2'-oxygen, phosphorus and the adjacent 5' oxygen are present an "in-line" conformation (as required for any S_N2 reaction). Consequently non-canonical local structures and single-stranded flexible regions free to adopt different conformations are more prone to be cleaved than rigid double-stranded parts [33-35]. This nucleophilic in-line attack is also catalysed by the presence of divalent metal ions such as Mg(II) or by an alkaline pH. This technique is commonly used to analyse the conformational changes on RNA constructs [35].

After incubation of the riboswitch in the presence of an increasing concentration of coenzyme B_{12} the RNA fragments were separated by denaturing PAGE and analysed. A total of eighteen bands were found to change their intensity upon addition of coenzyme B_{12} (Figure 2). The eighteen bands found were assigned to a total of fifteen sites, as some of the cleavage sites are in close proximity and show the same concentration dependent behaviour. For example, bands 2 and 2' are both situated in the same pseudoknot kissing acceptor loop (Figure 1) and are considered as a single cleavage site 2. The same applies for cleavage sites 4 and 4' as well as 8 and 8'.

>> INSERT FIGURE 2

For each cleavage site the band intensity was quantified and a background correction was applied using constant areas of the gels [35]. Corrected band intensities were fit to a

1:1 binding isotherm [32], yielding apparent affinities for each band (Table 1). With increasing amounts of coenzyme B_{12} the intensity of bands 8, 8', 10, 13 and 15 increases while for the other bands the intensity decreases (Figure 3). Due to the structural requirements of the self-cleavage reaction, those bands whose intensity has changed with the addition of coenzyme B_{12} have undergone a change in their local structure. While small structural changes are possible origin of such intensity changes, also larger changes like a switch from double-to-single stranded regions (leading to a more flexible backbone and thus an increase in band intensity) upon B_{12} binding or vice versa are feasible.

>> INSERT TABLE 1

>> INSERT FIGURE 3

The cleavage sites identified from in-line probing experiments thus allow us to postulate a secondary structure for the gene-on as well as for the gene-off conformation. Figure 1 shows the proposed secondary structure for the gene-on conformation, when coenzyme B_{12} is not bound to the riboswitch. On the other hand, Figure 4 shows the structure for the gene-off conformation, when coenzyme B_{12} is bound, which is based on the consensus structure for the riboswitch aptamer [19]. In both structures, yellow flags indicate the individual cleavage sites.

>> INSERT FIGURE 4

A comparison of both conformations explains some of the changing bands observed in the experiments, while others are probably related to direct interaction with the coenzyme B_{12} or to tertiary interactions. For example, band number 2 (and 2') is in a flexible region in the gene-on structure while fixed when forming the pseudoknot in the gene-off conformation. Accordingly, these bands decrease in intensity when coenzyme

B_{12} is added. On the other hand, the intensities of bands 13 and 15 increase upon addition of coenzyme B_{12} as these regions are base-paired in the gene-on structure while they are single stranded in the gene-off conformation.

In order to interpret the changing band pattern we compared our data with the X-ray structure of the adenosylcobalamin riboswitch from *Symbiobacterium thermophilum* [25]. Here, the whole riboswitch forms a cavity to accommodate one coenzyme B_{12} molecule bound via hydrogen bonds and van der Waals interactions spread out over the whole riboswitch sequence. Our results suggest a similar behaviour (see also vide infra) with one coenzyme B_{12} molecule inside the binding pocket and interacting with several parts of the RNA sequence. Such a distinct binding pocket can only be formed upon a major structural rearrangement, which includes melting of existing base pairing and the formation of new base-paired regions.

The results presented in this paper are consistent with those from the well-studied B_{12}-riboswitches from *E. coli* and *S. Typhimurium* [19-21]. Although being of different primary sequence, the affected cleavage sites appear at similar locations on the secondary structures and are also consistent with the regulatory function of the sequences. We compared our cleavage sites with the 8 main sites of structure modulation found by Nahvi et al. in the *btuB* riboswitch from *E. coli* [19], later 9 sites by Gallo et al. [21] (site **2** from Nahvi was split into **2** and **2b** by Gallo), and used the proposed structures based on consensus aptamer structures [19]. We have found that: i) 7 of the 9 main sites coincide in secondary structure location with those found in the *K. pneumoniae* sequence and with the same kind of intensity change (sites number **2b**, **3**, **4**, **5**, **6**, **7** and **8** from *E. coli* [21] correspond to sites number **5**, **7**, **9**, **10**, **13** and **14** for *K. pneumoniae*). ii) We cannot identify site **1** from the *E. coli* btuB riboswitch because of experimental limitations (see below). iii) Site **2** of *E. coli* is also not present in the *K.*

pneumoniae riboswitch, probably because of the distinct difference in size of the P8 region (3 bp *versus* 8 bp in *E. coli*).

This is intriguing as it implies that the local structures at these sites correspond to each other in the three riboswitches. Nevertheless, in contrast with the *btuB*-riboswitch from *E. coli* [21], we could not find any cleavage site around G26, which is directly implied in the B_{12}-box pairing. However, a clear picture is difficult to obtain as fragments shorter than 40-nt run within the buffer-front. It is important to note that the *btuB* riboswitch of *K. pneumoniae* shows some additional cleavage sites not identified in the *btuB* riboswitches of *E. coli* or *S Typhimurium* (1, 2, 3 and 12). Similarly, band 15 [21], which is part of the expression platform, is not present it the constructs studied of *E. coli* and *S. thypimurium*.

Isothermal Titration Calorimetry (ITC):

ITC measures the heat evolved or absorbed during the RNA-B_{12} interaction to report the enthalpy (ΔH), entropy (ΔS), stoichiometry of binding (n), and equilibrium association constant (K_A) from a single experiment and without labelling the individual components. Although ITC is considered one of the most accurate methods to detect equilibrium dissociation constants in the mM to nM range, it has not been widely applied to study RNA-ligand interactions [36-38].

Experiments at different temperatures, between 15 and 30 ºC, showed an exothermic process for the binding of the coenzyme B_{12} to the *btuB* riboswitch sequence of *K. pneumoniae*, as can be seen in Figure 5. Corrected peak areas were plotted *versus* concentration of coenzyme B_{12} (injection) and the resulting curve fitted to an independent process using the software included with the calorimeter (NanoAnalyze Software v3.1.2, TA instruments) (Figure 5).

>> INSERT FIGURE 5

Data from individual titrations as well as arithmetical means are shown in Table 2. The interaction follows a 1:1 stoichiometry (n = 1). ITC experiments were also performed under the same conditions used for in-line probing (20 mM Mg^{2+}), but the stoichiometric parameter n drops below 0.5, which is probably due to RNA aggregation at such high concentration of magnesium(II) [39].

>> INSERT TABLE 2

As can be seen in Table 2, our ITC experiments yield an enthalpy change at 25 ºC of –119.2 ± 2.5 $kJmol^{-1}$ and an affinity log K_A of 6.28 ± 0.05 (dissociation K_D of 530 ± 57 nM). Log K_A and dissociation constant (K_D) at the four different temperatures are summarized in Table 2 for comparison.

The thermodynamic parameters show a distinct variation with temperature, which is due to non-negligible changes in the heat capacity ($\Delta Cp = d\Delta H/dT$) [40]. In Figure 6 we have represented van't Hoff and Gibbs-Helmholtz plots to study the temperature dependence of our system: van't Hoff plot [$\ln K = -\Delta H/RT + \Delta S/R$] and Gibbs-Helmoltz plot [$\Delta G = \Delta H - T\Delta S$] are linear when ΔCp equals zero. When a curvature is found, an extra term is added to account for the ΔCp, and the curve is usually fitted with a second order polynomial function. The data shown in Figure 6A;B cannot be fit with a second order polynomial curve. Consequently, there is a more complex dependence on temperature or even a mechanistic change dependent on temperatures, as also suggested by the K_D vs. temperature graph (Figure 6A,B).

>> INSERT FIGURE 6

Nevertheless, it is possible to calculate an apparent $\Delta Cp = -5.0167\ kJ\cdot K^{-1}\cdot mol^{-1}$ (R^2 =

0.97149) from a liner fitting of the enthalpy vs. temperature graph (Figure 6C,D) although the fit does obviously not represent the complete story.

ITC was used to determine the thermodynamic parameters of the ligand-RNA association process. Although in-line probing yields an approximated value, it lies in the same order of magnitude as the value defined by ITC. We compared our ITC results to those obtained for the *btuB* riboswitch from *E. coli* [24]. The K_D = 250 ± 40 nM for the wild type riboswitch from *E. coli* is comparable to the one determined here for *Klebsiella pneumoniae* and presented in Table 2, namely 210 ± 8 nM at 20 ºC and 530 ± 57 nM at 25 ºC.

Finally, the RNA-B_{12} binding event has been shown to present a non-negligible change in the heat capacity (ΔCp), which affects stability at all temperatures, although the effect is more prominent at low temperatures. Even a small effect on stability can be biologically relevant, maybe allowing for a differential response to temperature [40]. The change of the heat capacity tells us that the binding of the coenzyme B_{12} to the riboswitch entails an important structural change.

Concluding remarks

We have investigated the full-length *btuB* riboswitch sequence from *Klebsiella pneumoniae*, which includes the expression platform and the RBS (243nt in total). In-line probing experiments showed that our RNA construct presents a distinctive cleavage pattern in the presence of 20 mM Mg(II). Addition of coenzyme B_{12} yielded a different pattern visible in the intensity changes at 15 specific sites. These facts prove that our RNA sequence is able to bind coenzyme B_{12} with high affinity and thus is a functional B_{12}-riboswitch.

The cleavage pattern is similar to the one of other B_{12} riboswitches despite their large sequence divergence. The changing bands appear in the same areas of the secondary structure. This means that the core structure is conserved along the bacterial speciation and that the B_{12} induced changes in the core are respective in the individual riboswitches. Even when the B_{12} riboswitches from the phylogenetically closest taxonomic groups of bacteria present just 70 % of sequence conservation, the binding of the coenzyme B_{12} and the structural rearrangement mechanism take place in the same regions of the structure. Finally, the presence of cleavage sites along the expression platform demonstrates that the RBS plays a role in the switch.

The quantification of the in-line probing experiments resulted in a binding affinity of log K_A of 6.77 ± 0.37 at room temperature. On the other hand, Isothermal Titration Calorimetry (ITC) confirmed a 1:1 binding stoichiometry and yielded binding affinities ranging from log K_A of 6.73 ± 0.02 at 15 ºC to log K_A of 6.00 ± 0.09 at 30 ºC. The process has been found to be exothermic with enthalpy changes ranging from –61 ± 6 kJ/mol at 15 ºC to –132 ± 5 kJ/mol at 30 ºC. Those values confirm that the interaction of the riboswitch with the cobalamin and the consequent structural change in the RNA folding does not involve breaking nor formation of covalent bonds, as it has e.g. been commonly observed in the axial positions of the cobalt(III/II) centre when binding to proteins [41]. The riboswitch activity just involves weak interactions both for the refolding and the B_{12} binding.

Both methods used in this paper resulted in dissociation constants in the nanomolar range. Also our results are of the same order of magnitude than those reported for *Escherichia coli* and *Salmonella typhimurium*, despite being a longer sequence due to the inclusion of the whole expression platform with the RBS.

Acknowledgements

The authors gratefully acknowledge financial support from the COST Action CM1105 and the SBFI (RKOS). Financial support by the "Govern de les llles Balears" with FEDER funds (project numbers AAEE0145/09, AAEE0019/2012 and AAEE3/2014) and the ERC (RKOS) is gratefully acknowledged. This work was supported by a STSM Grant (J. Palou-Mir) from the COST Action CM1105. The authors also thank Dr. Sofia Gallo for her suggestions and comments that helped to greatly improve the manuscript.

References

[1] A.R. Ferré-D'Amaré, W.C. Winkler, Met. Ions Life Sci. 9 (2011) 142-174.

[2] A.G. Vitreschak, D.A. Rodionov, A.A. Mironov, M.S. Gelfand, Trends Genet., 20 (2004) 44-50.

[3] M. Mandal, R.R. Breaker, Nat. Rev. Mol. Cell. Biol. 5 (2004) 451-463.

[4] W.C. Winkler, R.R. Breaker, ChemBioChem 4 (2003) 1024-1032.

[5] W.C. Winkler, A. Nahvi, A. Roth, J.A. Collins, R.R. Breaker, Nature 428 (2004) 281-286.

[6] A. Nahvi, N. Sudarsan, M.S. Ebert, M. Zou, K.L. Brown, R.R. Breaker, Chem. Biol. 9 (2002) 1043-1049.

[7] M.D. Lundrigan, R.J. Koster, R.J. Kadner, Proc. Natl. Acad. Sci. USA 88 (1991) 1479-1483.

[8] C.V. Franklund, R.J. Kadner, J. Bacteriol. 179 (1997) 4039-4042.

[9] M.D. Kazanov, A.G. Vitreschak, M.S. Gelfand, BMC Genomics 8 (2007) 347.

[10] J.E. Barrick, R.R. Breaker, Genome Biol. 8 (2007) R239.

[11] Z. Weinberg, J.X. Wang, J. Bogue, J. Yang, K. Corbino, R.H. Moy, R.R. Breaker, Genome Biol. 11 (2010) R31.

[12] K.F. Blount, R.R. Breaker, Nat. Biotechnol. 24 (2006) 1558-15564.

[13] K.F. Blount, J.K. Wang, J. Lim, N. Sudarsan, R.R. Breaker, Nat. Chem. Biol. 3 (2007) 44-49.

[14] N. Sudarsan, S. Cohen-Chalamish, S. Nakamura, G.M. Emilsson, R.R. Breaker, Chem. Biol. 12 (2005) 1325-1335.

[15] E. Cressina, L. Chen, C. Abell, F.J. Leeper, A.G. Smith, Chem. Sci. (2011) 157-165.

[16] E.R. Lee, K.F. Blount, R.R. Breaker, RNA Biol. 6 (2009) 187-194.

[17] S. Ravnum, D.I. Andersson, Mol. Microbiol. 39 (2001) 1585-1594.

[18] J. Vogel, Mol. Microbiol. 71 (2009) 1-11.

[19] A. Nahvi, J.E. Barrick, R.R. Breaker, Nucleic Acids Res. 32 (2004) 143-150.

[20] X. Nou, R.J. Kadner, Proc. Natl. Acad. Sci. USA 97 (2000) 7190-7195.

[21] S. Gallo, M. Oberhuber, R.K.O. Sigel, B. Kräutler, ChemBioChem 9 (2008) 1408-1414.

[22] I. Borovok, B. Gorovitz, R. Schreiber, Y. Aharonowitz, G. Cohen, J. Bacteriol. 188

(2006) 2512-2520.

[23] D.F. Warner, S. Savvi, V. Mizrahi, S.S. Dawes, J. Bacteriol. 189 (2007) 3655-3659.

[24] J.E. Johnson, F.E. Reyes, J.T. Polaski, R.T. Batey, Nature 492 (2012) 133-137.

[25] A. Peselis, A. Serganov, Nat. Struct. Mol. Biol. 19 (2012) 1182-1184.

[26] A.G. Vitreschak, D.A. Rodionov, A.A. Mironov, M.S. Gelfand, RNA 9 (2003) 1084-1097.

[27] W. Ko, D.L. Paterson, A.J. Sagnimeni, D.S. Hansen, A. von Gottberg, S. Mohapatra, J.M. Casellas, H. Goosens, L. Mulazimoglu, G. Trenholme, K.P. Klugman, J.G. McCormak, V.L. Yu, Emerg. Infect. Dis. 8 (2002) 160-166.

[28] C.T. Fang, Y.P. Chuang, C.T. Shun, S.C. Chang, J.T. Wang, J. Exp. Med. 199 (2014) 697-705.

[29] J.G. Johnson, R.R. Spurbeck, S.K. Sandhu, J.S. Matson, Genome Announc. 2 (2014) 1-2.

[30] G.V. Sanchez, R.N. Master, R.B. Clark, M. Fyyaz, P. Duvvuri, G. Ekta, J. Bordon, Emerg. Infect. Dis. 19 (2013) 133-136.

[31] S. Gallo, M. Furler, R.K.O. Sigel, Chimia 59 (2005) 812-816.

[32] R.K.O. Sigel, E. Freisinger, B. Lippert, J. Biol. Inorg. Chem. 5 (2000) 287-299.

[33] E.E. Regulski, R.R. Breaker, Methods Mol. Bio. 419 (2008) 53-67.

[34] K.M. Weeks, Curr. Opin. Chem. Biol. 20 (2010) 295-304.

[35] P.K. Choudhary, S. Gallo, R.K.O. Sigel, Methods Mol. Biol. 1086 (2014) 143-158.

[36] A.L. Feig, Biopolymers 87 (2007) 293-301.

[37] S.D. Gilbert, R.T. Batey, Methods Mol. Biol. 540 (2009) 97-114.

[38] D. Burnouf, E. Ennifar, S. Guedich, B. Puffer, G. Hoffmann, G. Bec, F. Disdier, M. Baltzinger, P. Dumas, J. Am. Chem. Soc. 134 (2012) 559-565.

[39] M. Barceló-Oliver, J. Palou-Mir, J. Biol. Inorg. Chem. 149 (2014) S769-S770.

[40] P.J. Mikulecky, A.L. Feig, Nucleic Acids Res. 32 (2004) 3967-3976.

[41] B. Kräutler, B.T. Golding, D. Arigoni (Eds.), Vitamin B_{12} and B_{12}-Proteins, Wiley-VCH, 2008, pp. 559.

Tables

Table 1. Association constants log K_A of coenzyme B_{12} binding to the 243-nt long *btuB* riboswitch from *K. pneumoniae* for each independent cleavage site. The individual values for each band correspond to the mean of two different experiments (those marked with (*) are from one titration experiment only). The arithmetic mean log K_{Aav} is given at the bottom together with the corresponding K_D value.

Cleavage site	**log K_A**
1	6.41 ± 0.18
2	7.05 ± 0.49
2'	6.45 ± 0.37
3	6.12 ± 0.00 (*)
4	6.94 ± 0.44
4'	6.65 ± 0.23
5	6.09 ± 0.00 (*)
6	7.34 ± 0.00 (*)
7	6.78 ± 0.54
8	6.98 ± 0.00 (*)
8'	6.86 ± 0.47
9	6.51 ± 0.00 (*)
10	6.34 ± 0.00 (*)
11	7.03 ± 0.49
12	6.52 ± 0.24
13	6.40 ± 0.29
14	6.44 ± 0.00 (*)
15	6.73 ± 0.17
log K_{Aav}	6.77 ± 0.37
K_D (nM)	168.67 ± 144.84

Table 2. Thermodynamic parameters as determined by ITC for the coenzyme B_{12}-riboswitch interaction at four different temperatures (15, 20 25 and 30 ºC). The errors given correspond to one standard deviation.

15 ºC	K_A **(M^{-1})**	**ΔH (kJ·mol^{-1})**	**n**	**ΔS (J·mol^{-1}·K^{-1})**
individual experiments	$5.155 \cdot 10^6$	–60.29	0.833	–80.71
	$5.405 \cdot 10^6$	–55.33	0.835	–63.13
	$5.711 \cdot 10^6$	–66.39	0.789	–101.0
mean values	$5.424 \cdot 10^6 \pm 2.785 \cdot 10^5$	–60.7 ± 5.5	0.82 ± 0.03	–81.6 ± 19.0
log K_A	6.73 ± 0.02			
K_D (nM)	184 ± 9			
20 ºC	K_A **(M^{-1})**	**ΔH (kJ·mol^{-1})**	**n**	**ΔS (J·mol^{-1}·K^{-1})**
individual experiments	$4.942 \cdot 10^6$	–83.58	0.613	–157.0
	$4.585 \cdot 10^6$	–86.59	0.923	–167.8
	$4.758 \cdot 10^6$	–80.90	1.057	–148.1
mean values	$4.762 \cdot 10^6 \pm 1.785 \cdot 10^5$	–83.7 ± 2.9	0.9 ± 0.2	–157.6 ± 9.9
log K_A	6.68 ± 0.02			
K_D (nM)	210 ± 8			
25 ºC	K_A **(M^{-1})**	**ΔH (kJ·mol^{-1})**	**n**	**ΔS (J·mol^{-1}·K^{-1})**
individual experiments	$1.729 \cdot 10^6$	–119.5	0.808	–281.3
	$1.816 \cdot 10^6$	–122.2	0.819	–290.2
	$2.117 \cdot 10^6$	–117.2	1.375	–272.1
mean values	$1.887 \cdot 10^6 \pm 2.036 \cdot 10^5$	–119.6 ± 2.5	1.0 ± 0.3	–281.2 ± 9.1
log K_A	6.28 ± 0.05			
K_D (nM)	530 ± 57			
30 ºC	K_A **(M^{-1})**	**ΔH (kJ·mol^{-1})**	**n**	**ΔS (J·mol^{-1}·K^{-1})**
individual experiments	$1.222 \cdot 10^6$	–131.2	1.002	–316.1
	$9.748 \cdot 10^5$	–128.3	0.886	–308.7
	$8.066 \cdot 10^5$	–137.4	0.853	–340.0
mean values	$1.001 \cdot 10^6 \pm 2.079 \cdot 10^5$	–132.3 ± 4.7	0.91 ± 0.08	–321.6 ± 16.4
log K_A	6.00 ± 0.09			
K_D (nM)	1000 ± 208			

Figure legends

Figure 1. Proposed secondary structure for the gene-on conformation (coenzyme B_{12} not bound) of the *btuB* riboswitch from *Klebsiella pneumoniae* (strain MGH 78578) [26]. Yellow flags indicate the cleavage sites found in the "in-line probing" experiments (vide infra).

Figure 2. In-line probing experiment of the 243-nt long *btuB* riboswitch from *Klebsiella pneumoniae*. Lane 1 (C): non-incubated RNA; Lane 2 (-): RNA incubated without coenzyme B_{12}; Lanes 3-12: RNA incubated with increasing amounts of coenzyme B_{12} (50 nM, 100 nM, 200 nM, 300 nM, 400 nM, 500 nM, 1 μM, 2.5 μM, 10 μM and 100 μM); Lane 13 (OH): alkaline hydrolysis ladder; Lane 14 (T1): RNAse T1 digestion. The cleavage bands that change their intensities upon addition of B_{12} are indicated with flags and are located at positions A39 (1), G49 (2), C53 (2'), C58 (3), U70 (4), G71 (4'), G78 (5), C81 (6), U90 (7), C104 (8), G107 (8'), G114 (9), A120 (10), A133 (11), G148 (12), G166 (13), G184 (14) and C208 (15).

Figure 3. Relative maximal changes in intensity of the individual bands. The bands assigned to a unique cleavage site have been grouped as explained before (2 and 2', 4 and 4', and 8 and 8'). The intensities shown correspond to the calculated intensities of the fully bound species, as obtained from the 1:1 binding isotherm model.

Figure 4. Proposed secondary structure for the gene-off conformation, when coenzyme B_{12} is bound, for the B_{12}-riboswitch from *Klebsiella pneumoniae* according to the aptamer consensus structure [19].

Figure 5. Evolved raw heat rate (μJ/s) *vs* time (s) and fitting of the peak areas (μJ) *vs* injection curve from the titration from the titration of the 243nt-long *btuB* riboswitch with coenzyme B_{12} at four different temperatures (15, 20 25 and 30 ºC). The data fit

well to an independent process.

Figure 6. Interpretation of ITC data. The curvatures in the Van't Hoff (A) and Gibbs-Helmholtz (B) plots indicate the presence of a non-negligible change in the heat capacity (ΔCp) with temperature. It is not possible to fit the curves to a second order polynomial fit. Similarly, the K_D vs. temperature (C) plot suggests the presence of two distinct mechanisms at low and elevated temperatures. ΔH vs. temperature plot (D) with an apparent linear fitting (R^2 = 0.97149) yielding ΔCp = –5.0167 $kJ \cdot K^{-1} \cdot mol^{-1}$. The error bars correspond to one standard deviation.

Figures

Figure 1

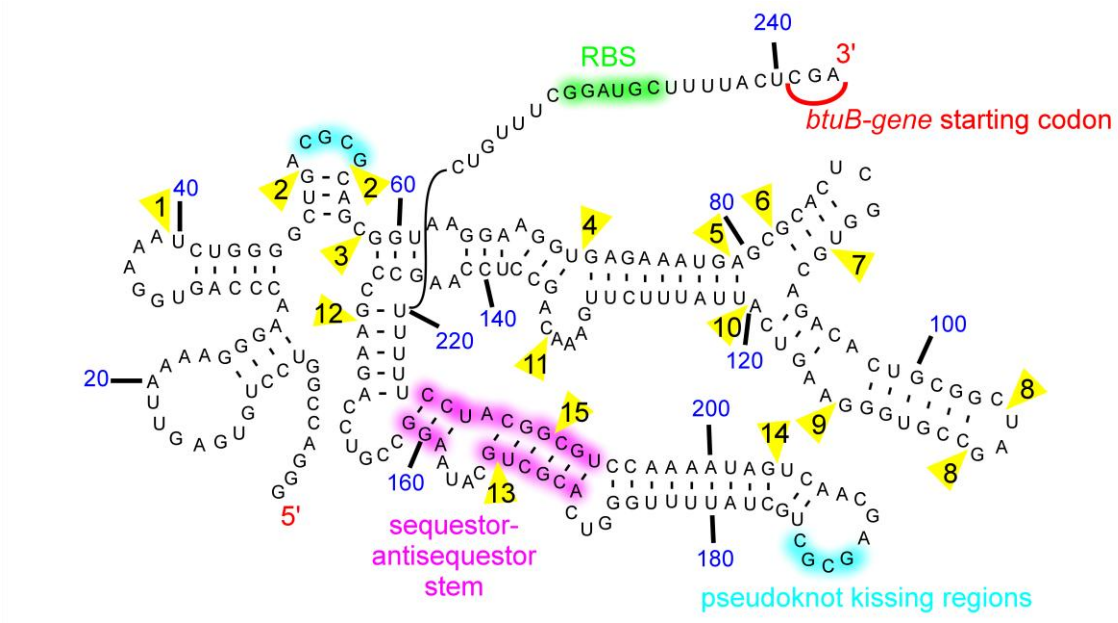


Figure 2

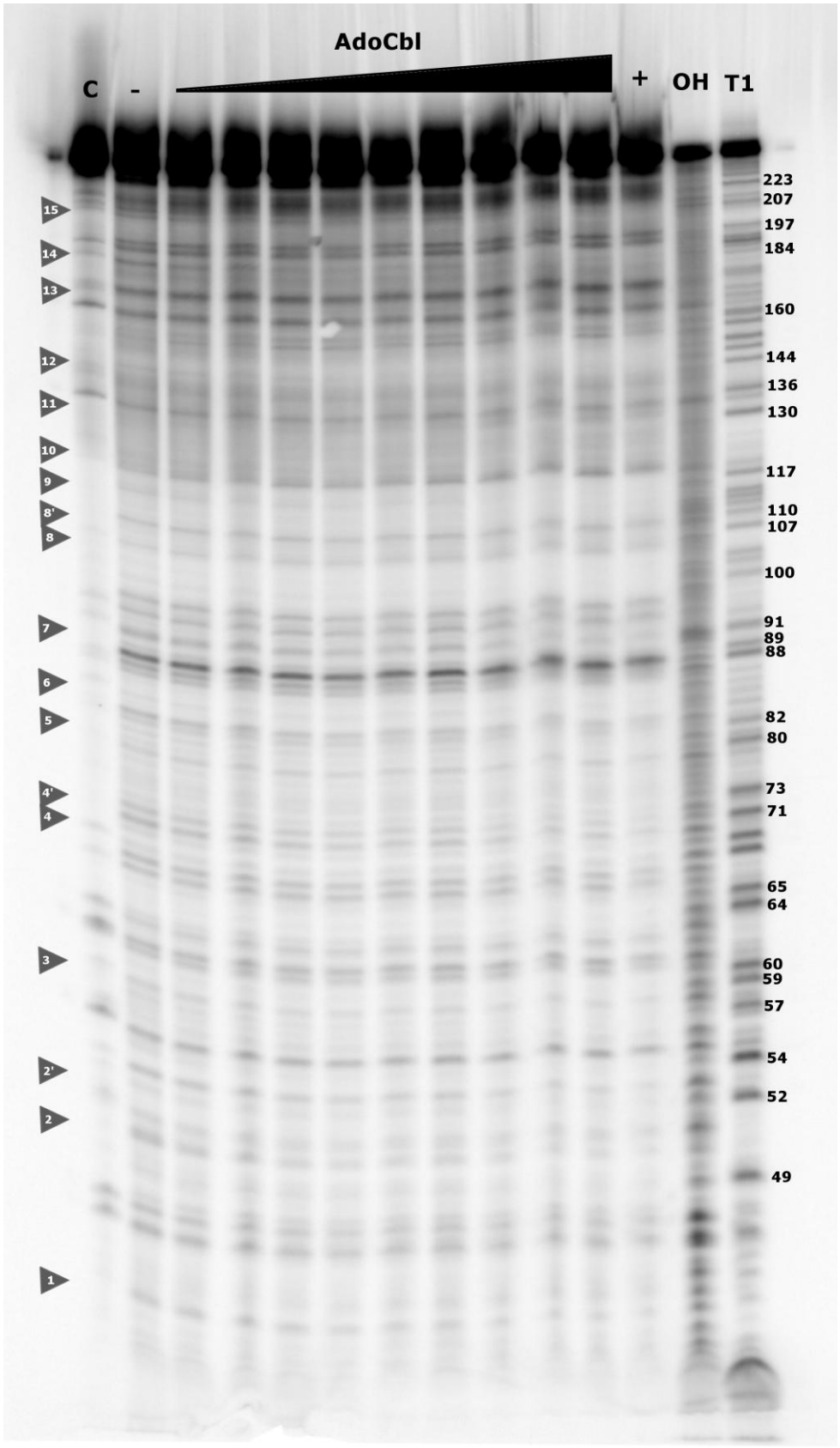

AdoCbl
C
-
+
OH
T1
223
207
197
184
160
144
136
130
117
110
107
100
91
89
88
82
80
73
71
65
64
60
59
57
54
52
49
15
14
13
12
11
10
9
8'
8
7
6
5
4'
4
3
2'
2
1

Figure 3

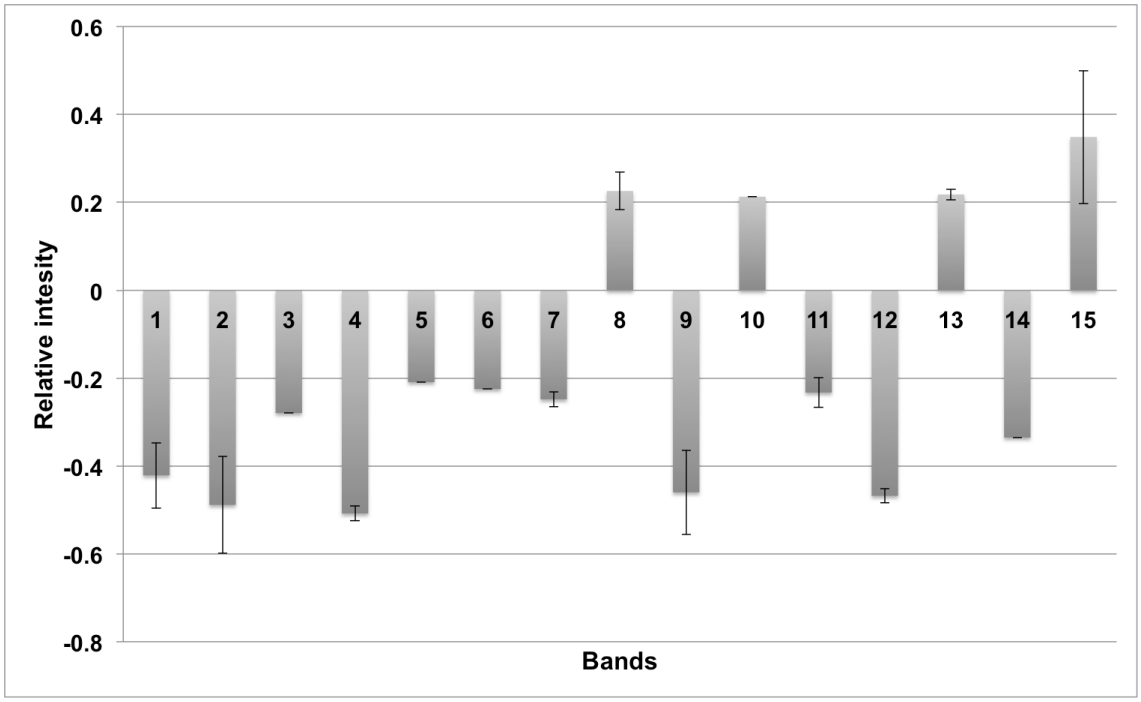

0.6
0.4
0.2
0
-0.2
-0.4
-0.6
-0.8
Relative intesity
1
2
3
4
5
6
7
8
9
10
11
12
13
14
15
Bands

Figure 4

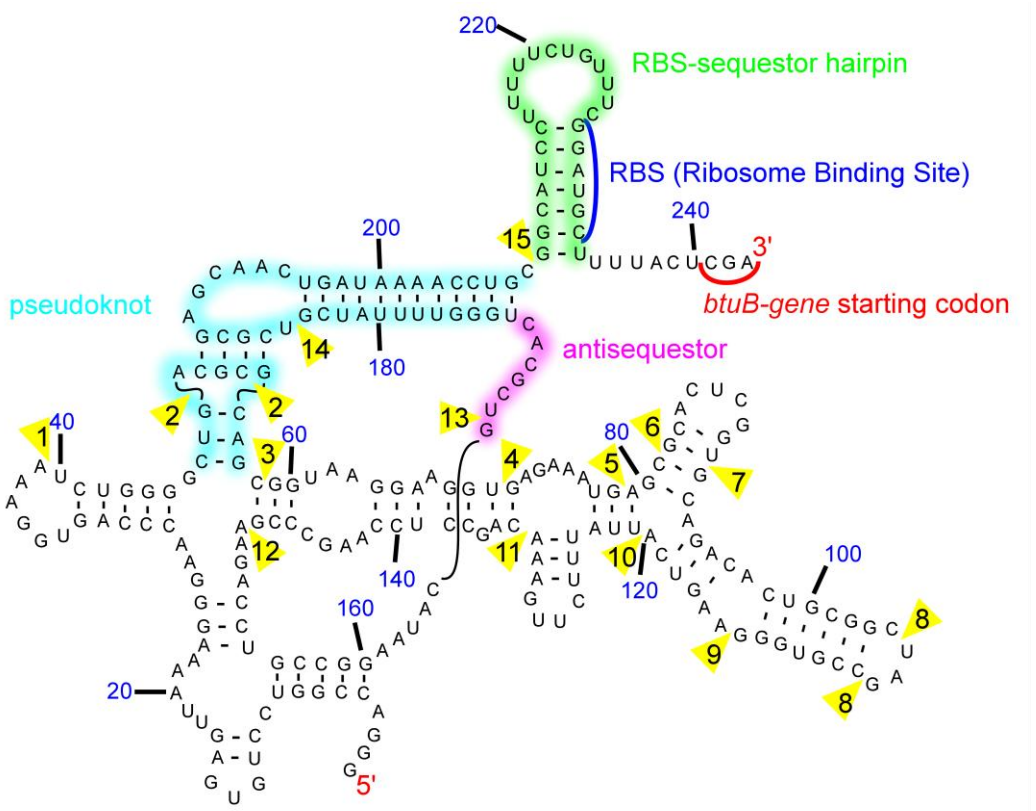

RBS-sequestor hairpin
RBS (Ribosome Binding Site)
pseudoknot
antisequestor
btuB-gene starting codon
5'
3'

Figure 5

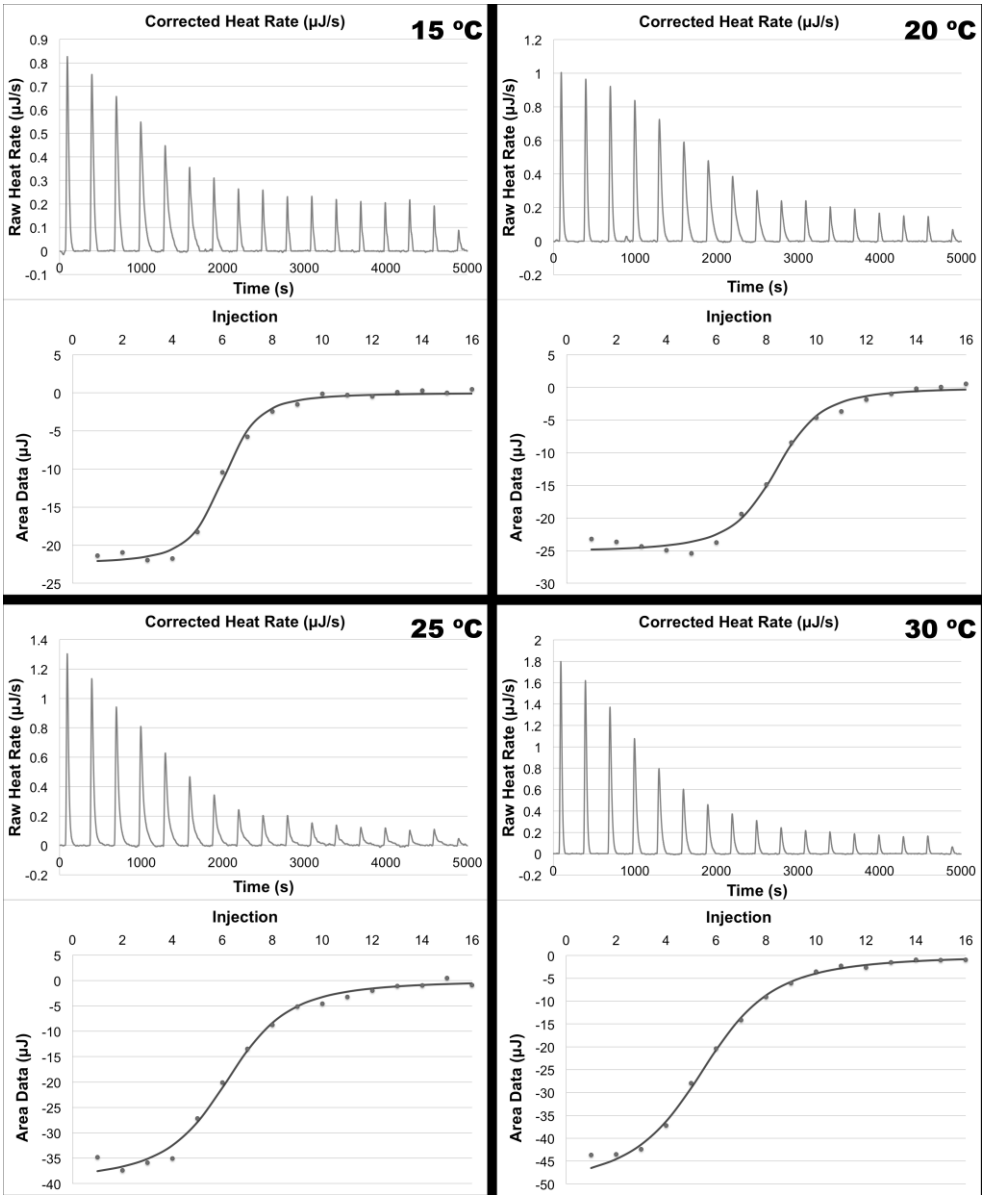
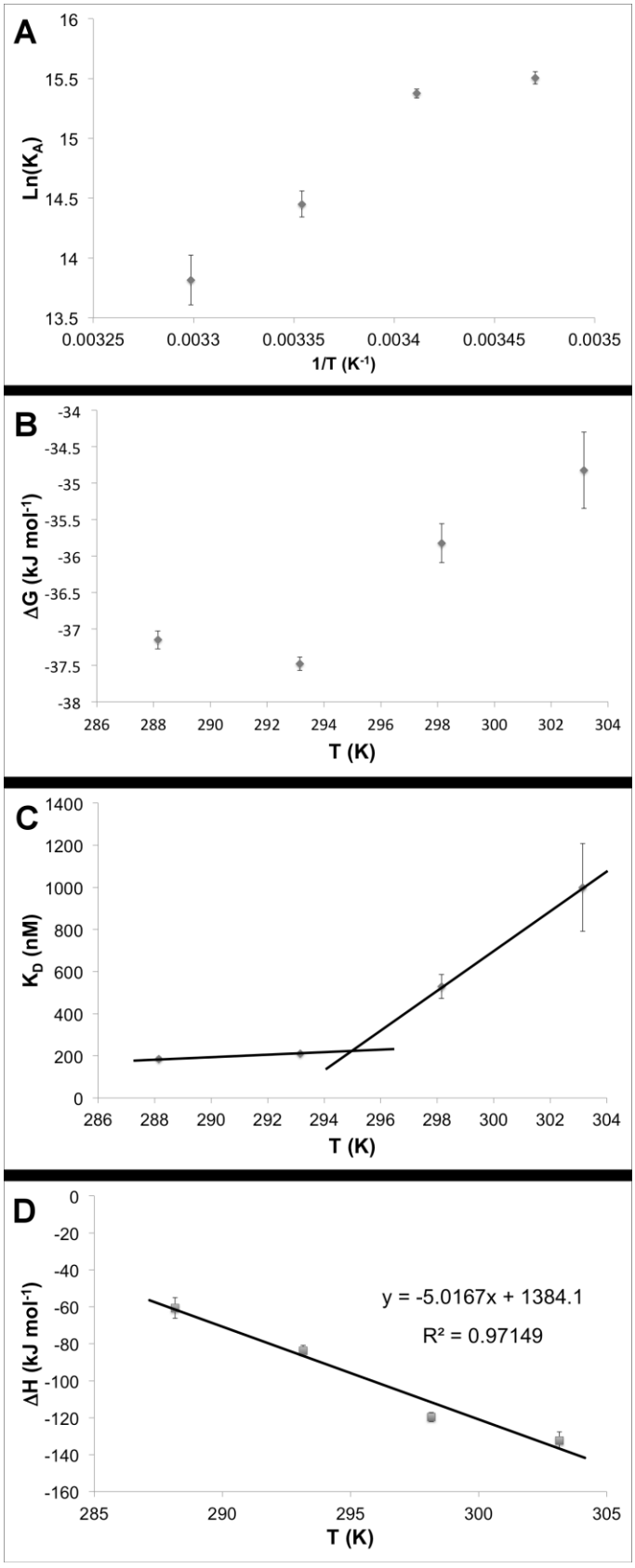
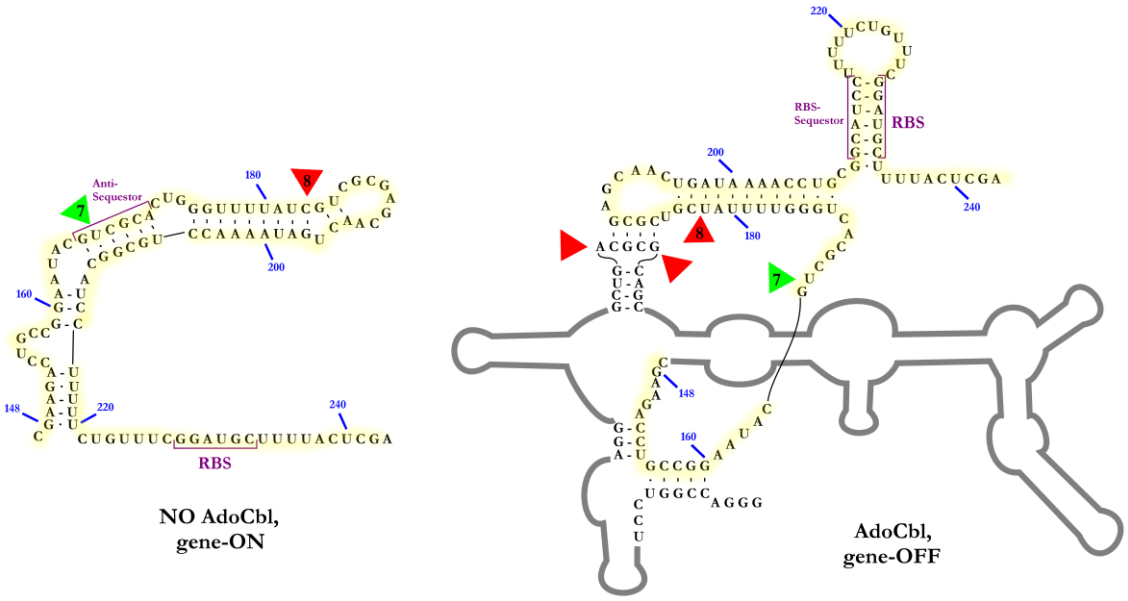

Corrected Heat Rate (µJ/s)
15 °C
Raw Heat Rate (µJ/s)
Time (s)
Injection
Area Data (µJ)
Corrected Heat Rate (µJ/s)
20 °C
Raw Heat Rate (µJ/s)
Time (s)
Injection
Area Data (µJ)
Corrected Heat Rate (µJ/s)
25 °C
Raw Heat Rate (µJ/s)
Time (s)
Injection
Area Data (µJ)
Corrected Heat Rate (µJ/s)
30 °C
Raw Heat Rate (µJ/s)
Time (s)
Injection
Area Data (µJ)

Figure 6


A
Ln(K_A)
1/T (K^-1)
B
ΔG (kJ mol^-1)
T (K)
C
K_D (nM)
T (K)
D
ΔH (kJ mol^-1)
y = -5.0167x + 1384.1
R² = 0.97149
T (K)

Graphical abstract



The *Klebsiella pneumoniae* genome carries a *btuB* riboswitch proposed to bind adenosylcobalamin. ITC and in-line probing experiments conducted on the whole expression platform with the ribosome binding site (RBS) have been used to study the riboswitch – cobalamin interaction.

Highlights

- the *btuB* ribowitch from *K. pneumoniae* was studied including the whole expression platform
- in-line probing proved the conformational change upon binding of the coenzyme B_{12}
- core structure B_{12}-induced changes are respective in the individual riboswitches
- ITC showed B_{12}-riboswitch interaction and refolding just involve weak interactions

Table 1

Table 1. Association constants log K_A of coenzyme B_{12}binding to the 243-nt long *btuB*riboswitch for each independent cleavage site. The individual values for each band correspond to the mean of two different experiments (the fitting of some bands, marked with (*) on the table are from one titration experiment only). The arithmetic mean log K_{Aav} is given at the bottom together with the corresponding K_D value.

Cleavage site	**logK_A**
1	6.41 ± 0.18
2	7.05 ± 0.49
2'	6.45 ± 0.37
3	6.12 ± 0.00 (*)
4	6.94 ± 0.44
4'	6.65 ± 0.23
5	6.09 ± 0.00 (*)
6	7.34 ± 0.00 (*)
7	6.78 ± 0.54
8	6.98 ± 0.00 (*)
8'	6.86 ± 0.47
9	6.51 ± 0.00 (*)
10	6.34 ± 0.00 (*)
11	7.03 ± 0.49
12	6.52 ± 0.24
13	6.40 ± 0.29
14	6.44 ± 0.00 (*)
15	6.73 ± 0.17
logK_{Aav}	6.77 ± 0.37
K_D (nM)	168.67 ± 144.84

Table 2

Table 2. Thermodynamic parameters as determined by ITC for the coenzyme B_{12}-riboswitch interaction four different temperatures (15, 20 25 and 30 ºC). The errors given correspond to one standard deviation.

15 ºC	K_A **(M^{-1})**	**ΔH (kJ·mol^{-1})**	**n**	**ΔS (J·mol^{-1}·K^{-1})**
individual experiments	$5.155 \cdot 10^6$	–60.29	0.833	–80.71
	$5.405 \cdot 10^6$	–55.33	0.835	–63.13
	$5.711 \cdot 10^6$	–66.39	0.789	–101.0
mean values	$5.424 \cdot 10^6 \pm 2.785 \cdot 10^5$	–60.7 ± 5.5	0.82 ± 0.03	–81.6 ± 19.0
log K_A	6.73 ± 0.02			
K_D (nM)	184 ± 9			
20 ºC	K_A **(M^{-1})**	**ΔH (kJ·mol^{-1})**	**n**	**ΔS (J·mol^{-1}·K^{-1})**
individual experiments	$4.942 \cdot 10^6$	–83.58	0.613	–157.0
	$4.585 \cdot 10^6$	–86.59	0.923	–167.8
	$4.758 \cdot 10^6$	–80.90	1.057	–148.1
mean values	$4.762 \cdot 10^6 \pm 1.785 \cdot 10^5$	–83.7 ± 2.9	0.9 ± 0.2	–157.6 ± 9.9
log K_A	6.68 ± 0.02			
K_D (nM)	210 ± 8			
25 ºC	K_A **(M^{-1})**	**ΔH (kJ·mol^{-1})**	**n**	**ΔS (J·mol^{-1}·K^{-1})**
individual experiments	$1.729 \cdot 10^6$	–119.5	0.808	–281.3
	$1.816 \cdot 10^6$	–122.2	0.819	–290.2
	$2.117 \cdot 10^6$	–117.2	1.375	–272.1
mean values	$1.887 \cdot 10^6 \pm 2.036 \cdot 10^5$	–119.6 ± 2.5	1.0 ± 0.3	–281.2 ± 9.1
log K_A	6.28 ± 0.05			
K_D (nM)	530 ± 57			
30 ºC	K_A **(M^{-1})**	**ΔH (kJ·mol^{-1})**	**n**	**ΔS (J·mol^{-1}·K^{-1})**
individual experiments	$1.222 \cdot 10^6$	–131.2	1.002	–316.1
	$9.748 \cdot 10^5$	–128.3	0.886	–308.7
	$8.066 \cdot 10^5$	–137.4	0.853	–340.0
mean values	$1.001 \cdot 10^6 \pm 2.079 \cdot 10^5$	–132.3 ± 4.7	0.91 ± 0.08	–321.6 ± 16.4
log K_A	6.00 ± 0.09			
K_D (nM)	1000 ± 208			

Figure 1

Click here to download high resolution image

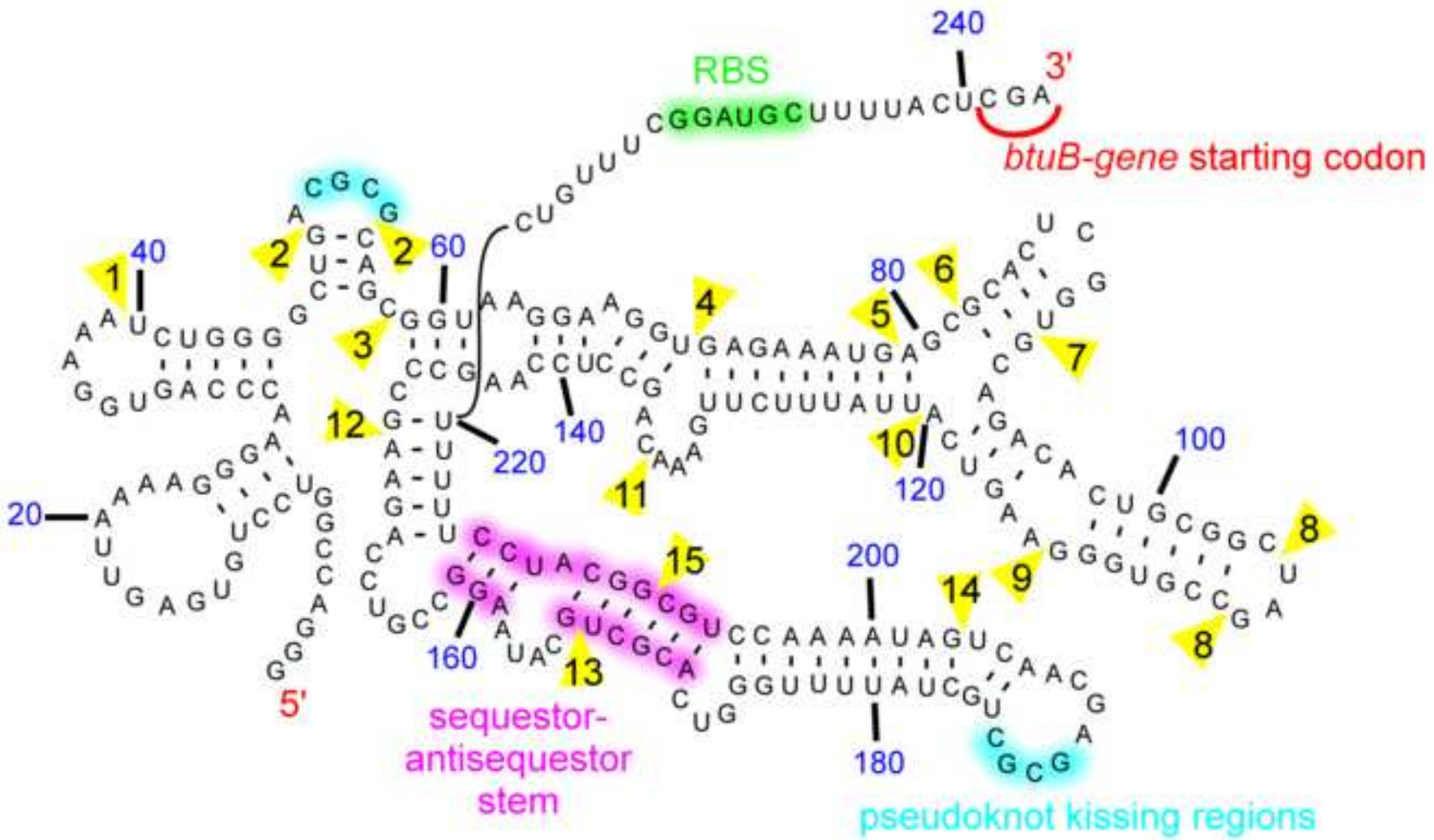


Figure 2

Click here to download high resolution image

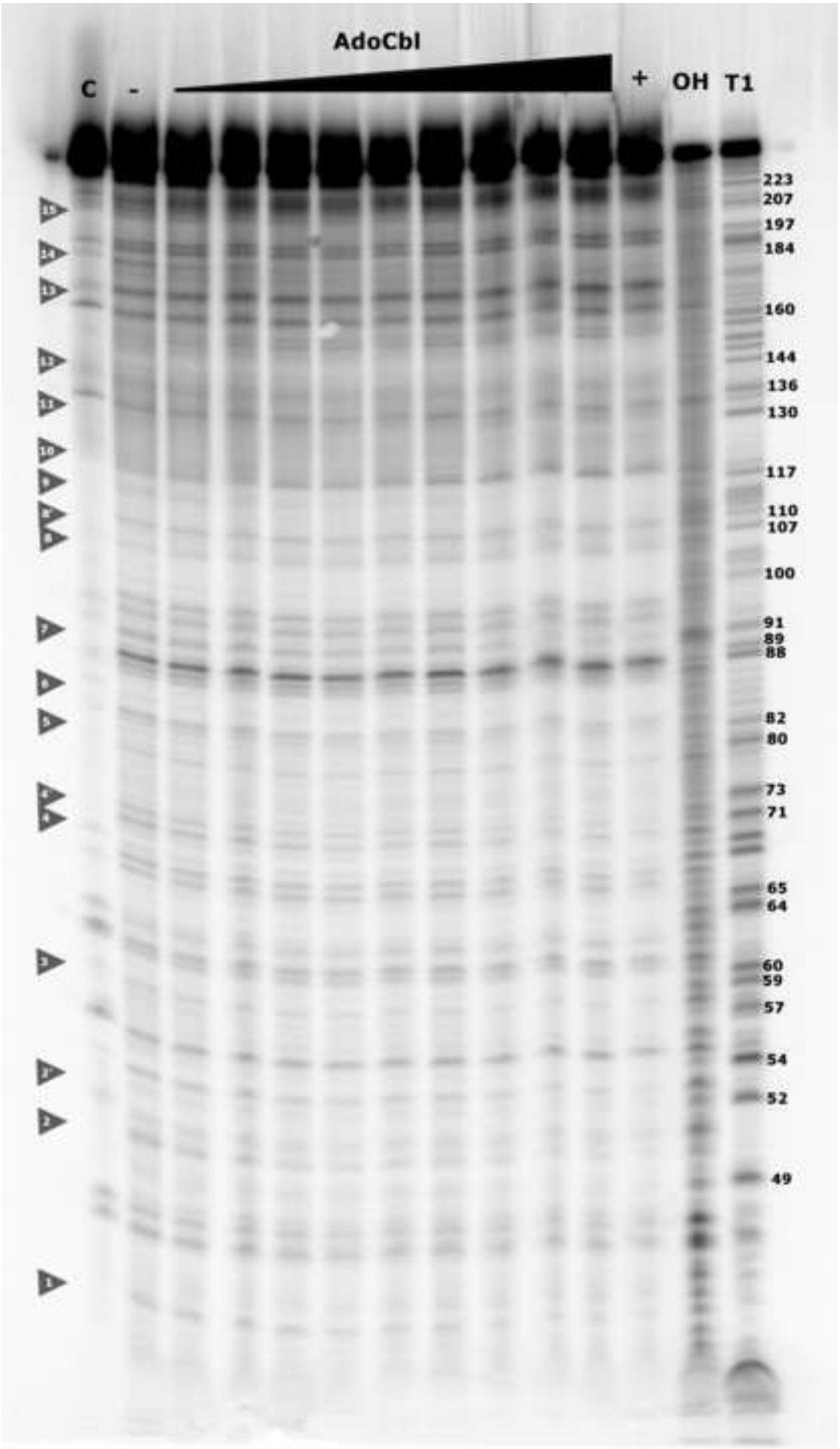


Figure 3

Click here to download high resolution image

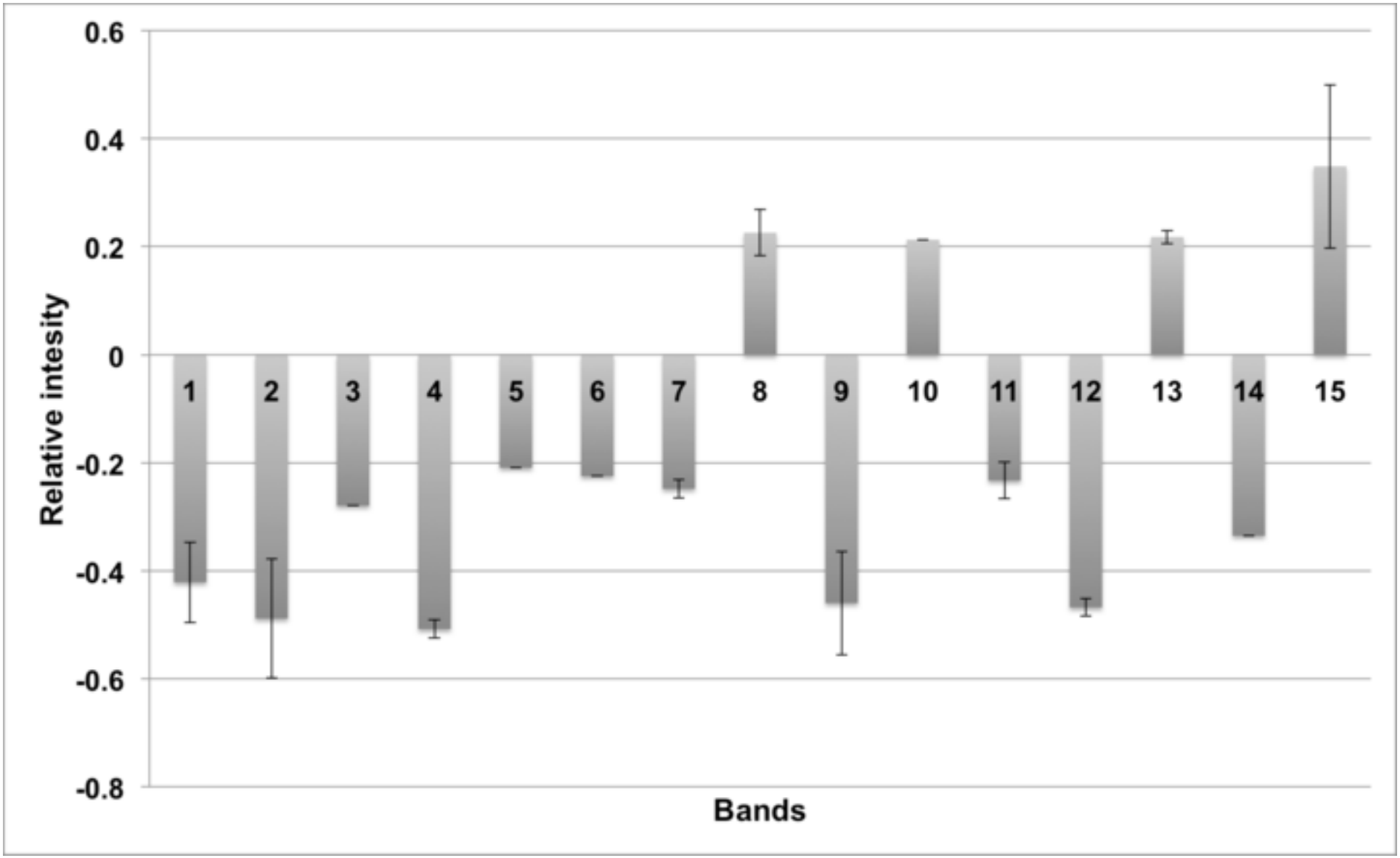


Figure 4

Click here to download high resolution image

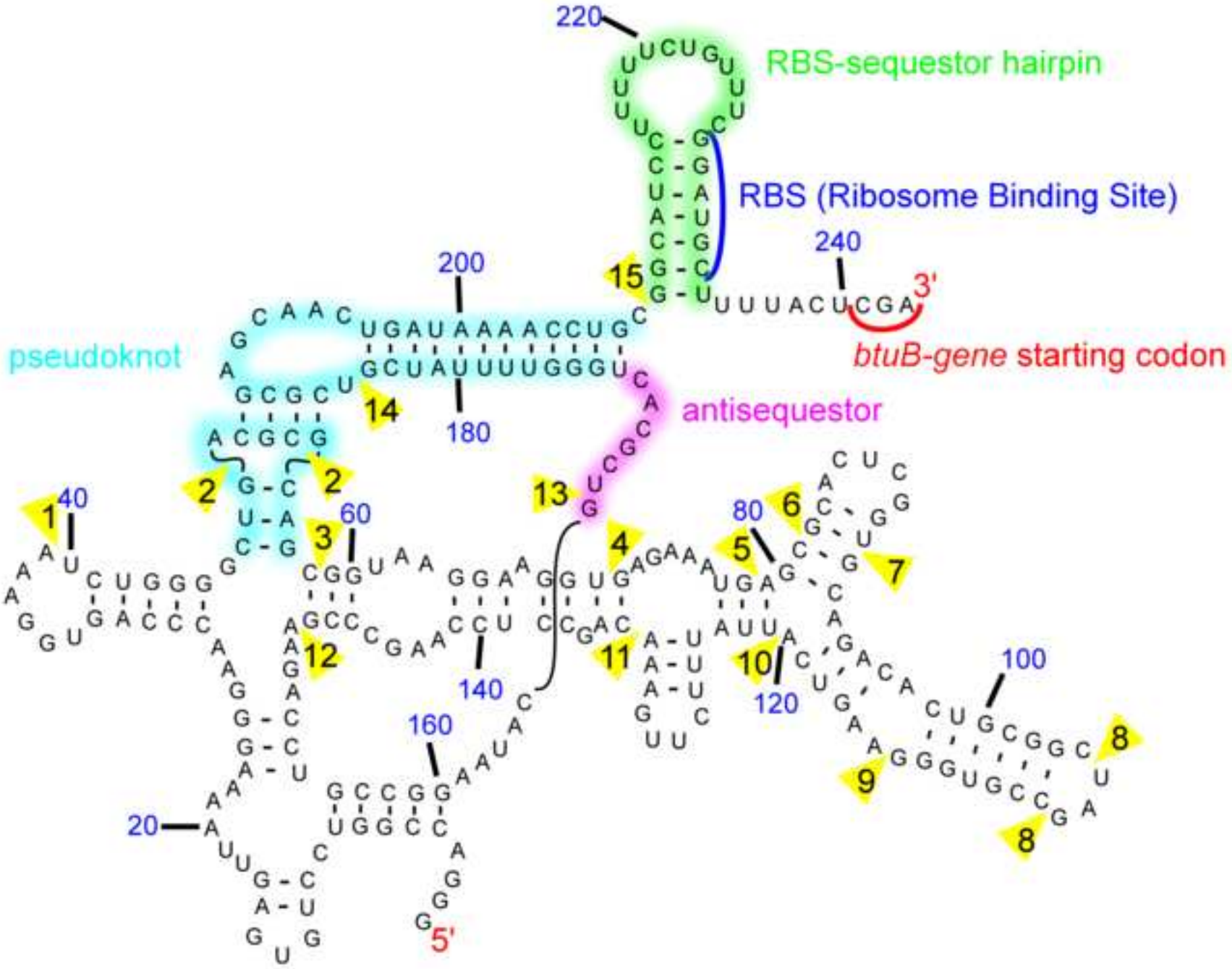


Figure 5

Click here to download high resolution image

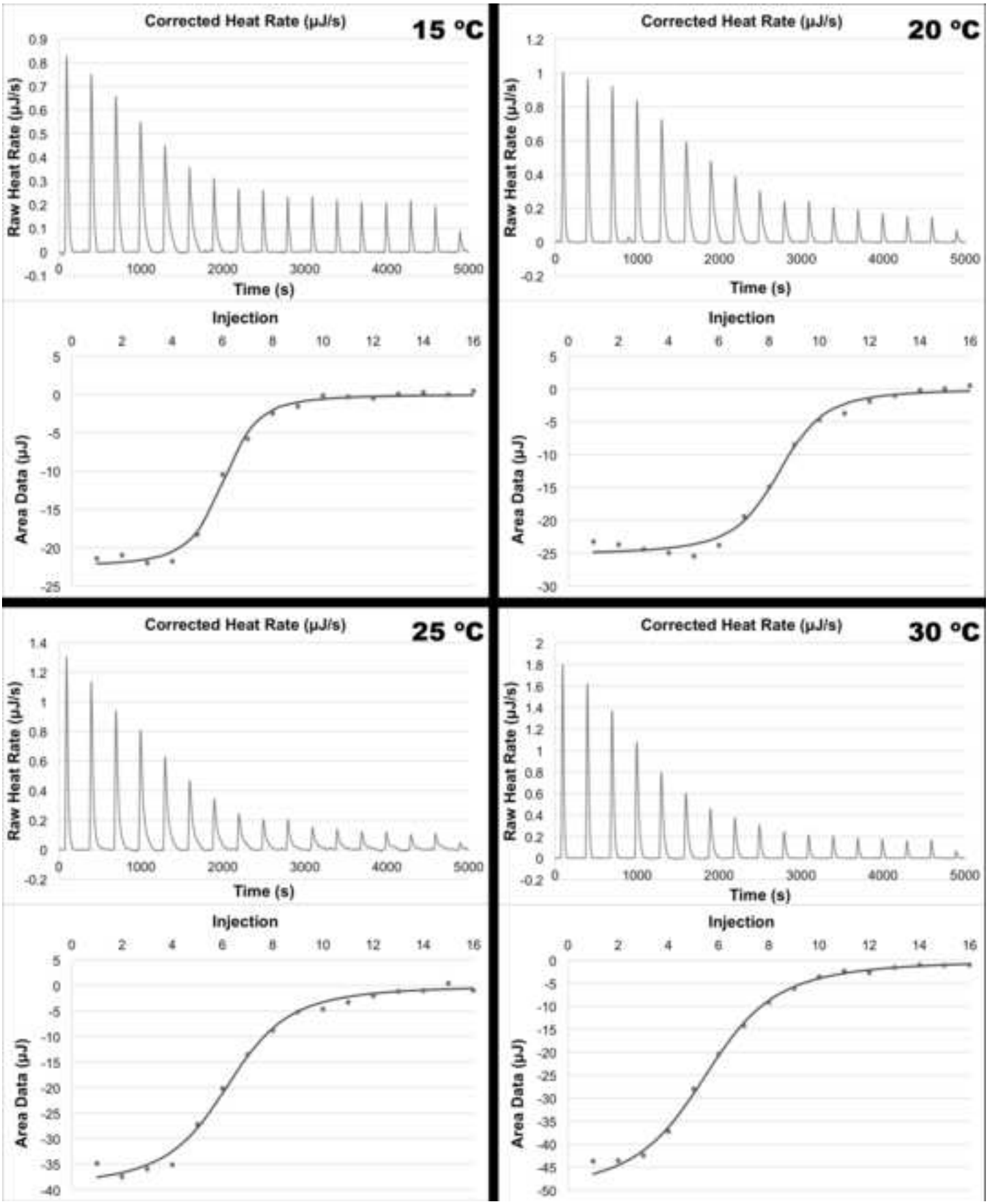


Figure 6

Click here to download high resolution image

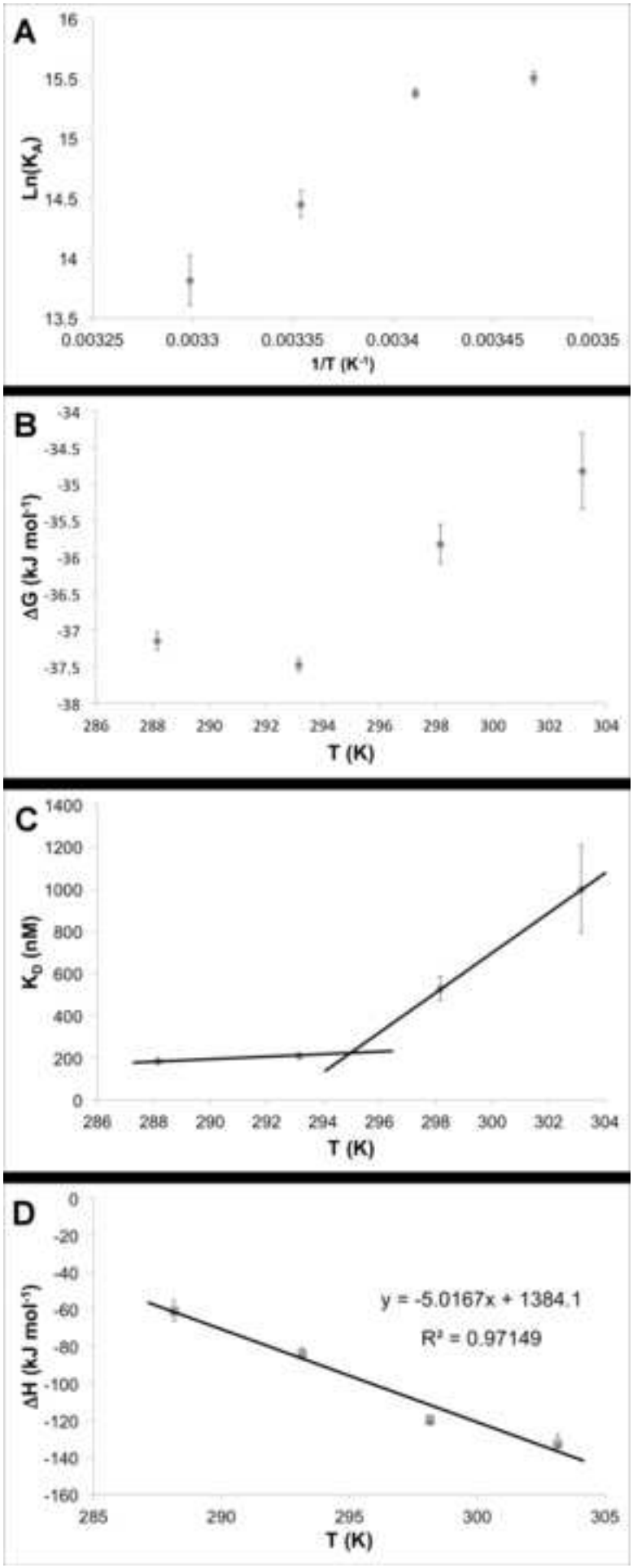


Figure 2 colour (for web)

Click here to download high resolution image

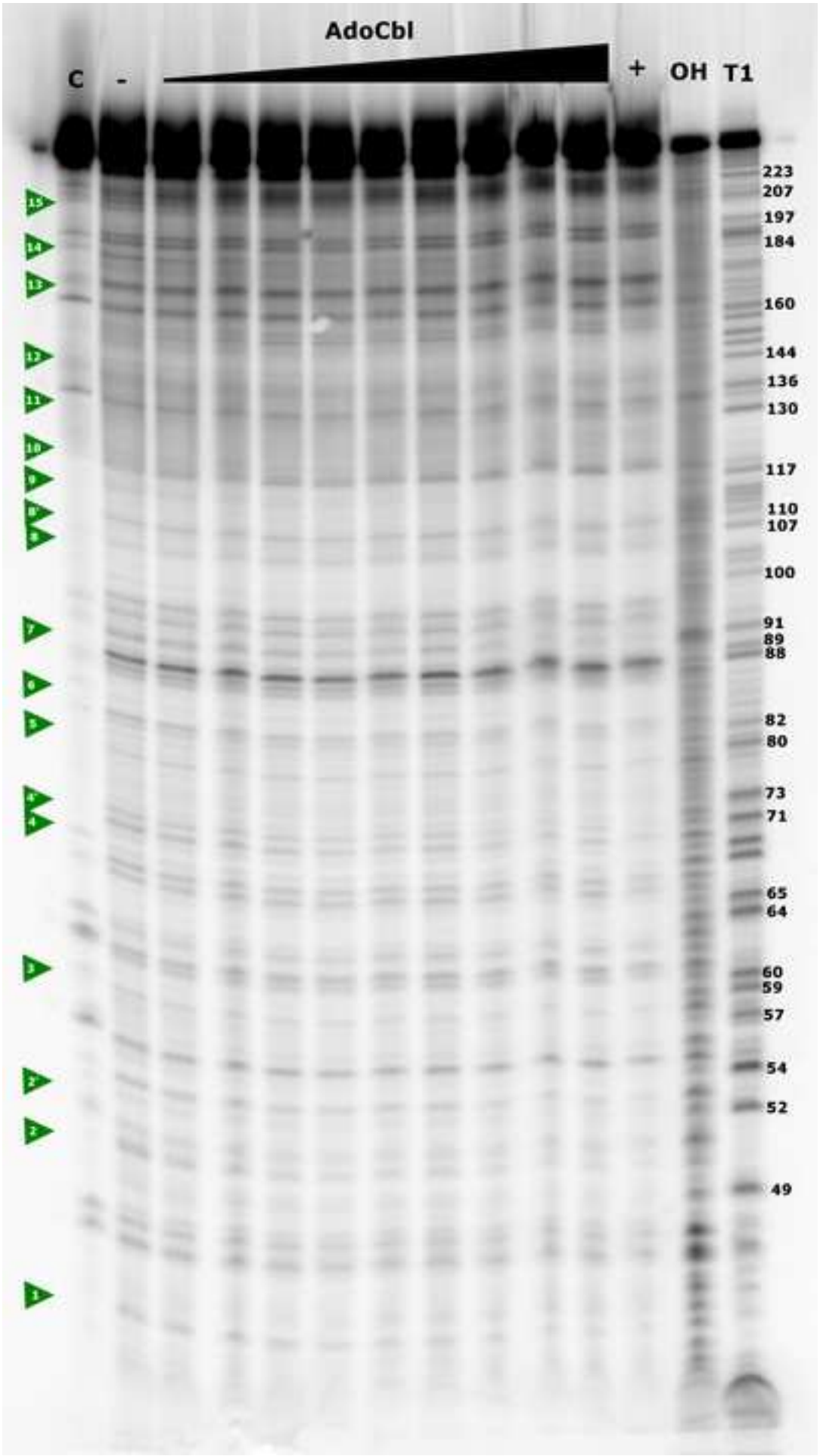


Figure 3 colour (for web)
Click here to download high resolution image

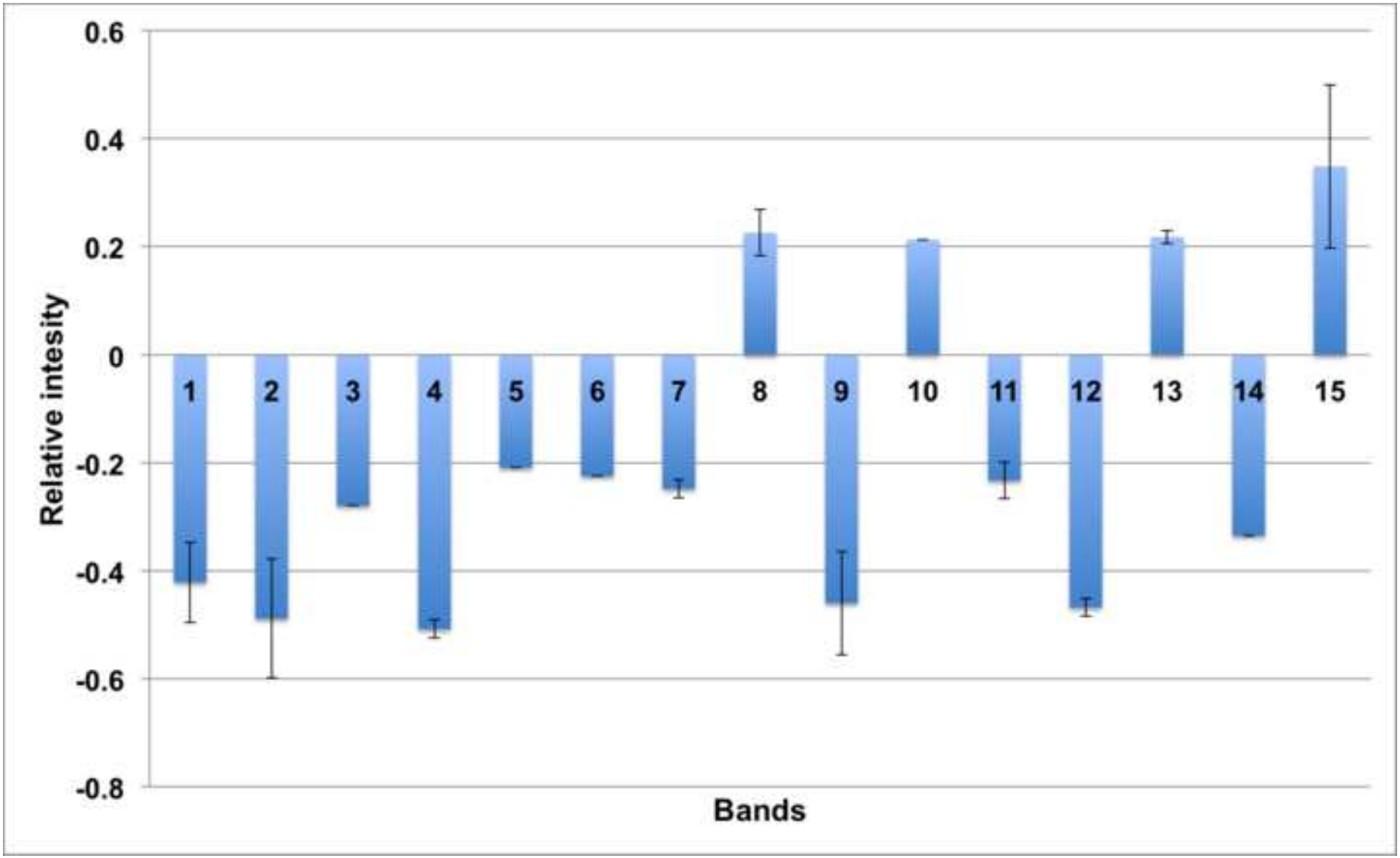


Figure 5 colour (for web)

Click here to download high resolution image

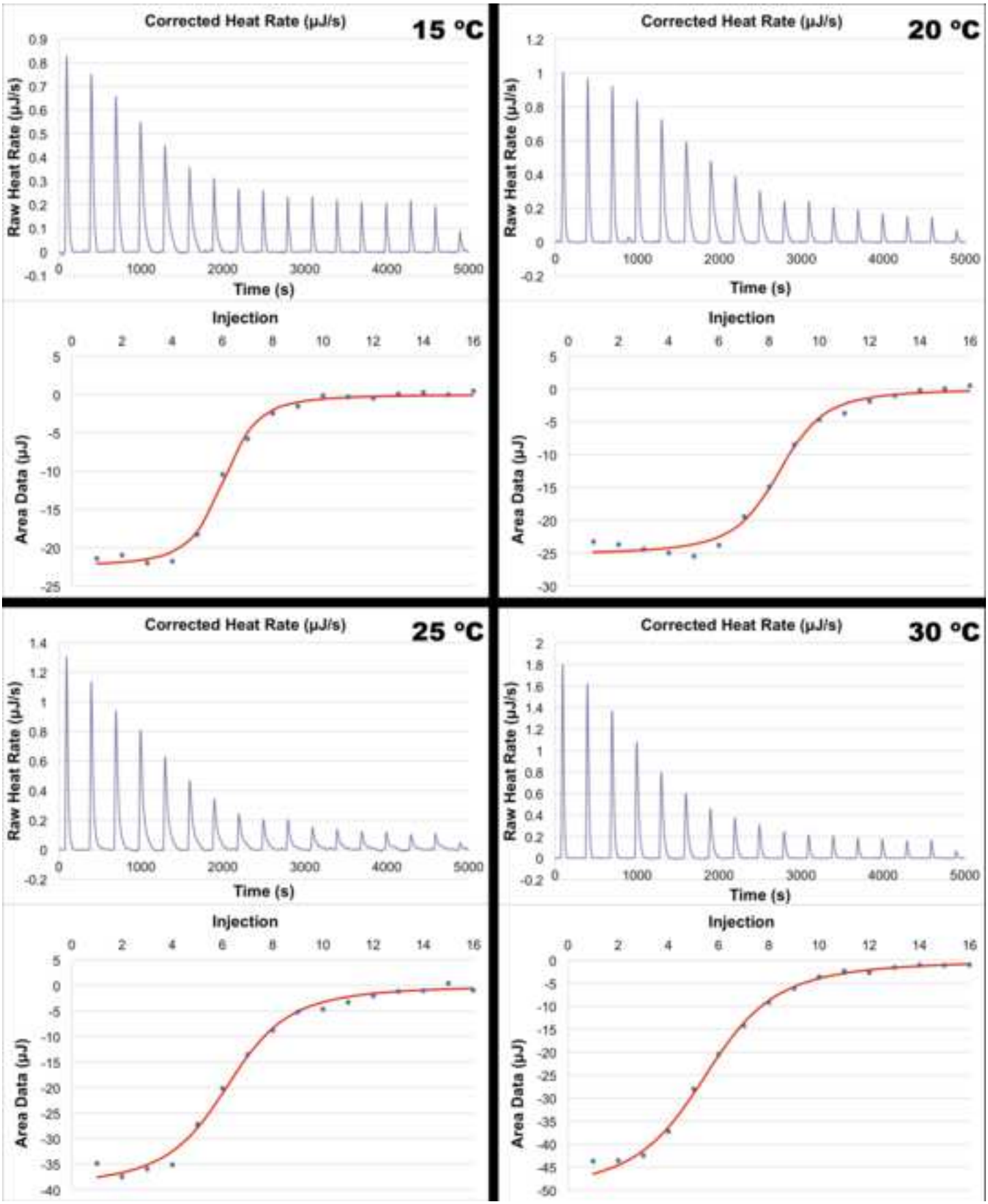


Figure 6 colour (for web)

Click here to download high resolution image