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# Potential Thermosensitive Riboswitches in the Genome of Salmonella

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**Abstract**—Currently, a number of structurally and functionally different thermosensitive elements, such as structurally and functionally different RNA thermometers, for controlling a variety of biological processes in bacteria, including virulence are known. These well-known RNA thermometers are structures, whether matched or mismatched, which are represented by either a single stretched hairpin structure or a few hairpins. Based on computer and thermodynamic analyses of 25 isolates of *Salmonella enterica* with complete genome, we have developed an algorithm and criteria to search for potential RNA thermometers, which will enable us to undertake a future search for potential riboswitches in the genomes of other socially significant pathogens. In addition to the well-known 4U RNA thermometer, another four hairpin-loop structures have been identified in *S. enterica* as new potential RNA thermometers and two of them are localized in 5'-UTR of virulence regulators *gltB* and *yaeQ*. They are highly conserved noncanonical structures and correspond to the necessary and sufficient conditions for forming RNA thermometers, since they are found in each of the 25 *S. enterica* genome isolates. We analyzed the thermosensitive motif in the pXO1 plasmid of *Bacillus anthracis*—an anthrax-causative pathogen—and visualized matched hairpins that form a cruciform structure in pUC8 supercoiled plasmid by atomic force microscopy.

*Keywords*: riboswitch, RNA thermometer, Shine-Dalgarno sequence, hairpin-loop structure, *Salmonella enterica*, atomic force microscopy (AFM)

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## INTRODUCTION

According to the central dogma of molecular biology, the role of RNA molecules is limited to being a passive intermediary between proteins and DNA molecules. It has now been established that regulatory RNAs can store not only genetic information but can also perform other functions. Some research laboratories are especially interested in the regulatory RNAs of prokaryotes, their structure and identification, as well as in their potential use in medicine and biotechnology. Regulatory RNAs have been established to exhibit a catalytic activity in the absence of cofactors, cleave phosphodiester bonds (ribozymes), and bind small molecules (ligands) and ions with a high affinity and specificity via monitoring cell metabolism and controlling gene expression, translation efficiency, transcription termination, and mRNA stability; they also regulate the translation of genes that encode heat- and cold-shock proteins, as well as virulence genes, due to temperature shifts in the surrounding environment [1–3].

Free-living microorganisms are periodically exposed to shifts in surrounding environmental conditions, such as temperature, pH, and the presence of nutrients that constantly vary. To counteract the consequences of temperature fluctuations, bacteria have developed a complex network of defense mechanisms. The potential thermosensitive elements used in nature include components from membranes to DNA, RNA, and protein molecules. Pathogenic microorganisms often respond to temperatures reaching 37°C by inducing virulence gene expression. The genes that control the sensitivity to the environment may be regulated at the transcription level through the interaction of regulatory proteins. However, a few posttranscriptional mechanisms based on RNA molecules were recently discovered [3]. Some tRNA molecules are known to be not only a substrate for ribosomes but also to include controlling elements that modulate their own expression in a condition-dependent way. Structural changes in such sensory RNAs are caused by specific surrounding shifts.

There are two principally different classes: cis-acting RNA elements with their regulatory potential in the middle of an mRNA sequence and trans-acting, small, and noncoding RNA molecules, which function through base pairing with complementary mRNA sequences localized at other loci of the genome [4].

Unlike classic attenuators that regulate the structure of an RNA leader sequence in correspondence with the translating ribosome position, the cis-acting RNAs alter their conformation in response to physical or chemical signals. The so-called riboswitches monitor the cell metabolism through binding metabolites with a high specificity and affinity. They are localized in the 5’-untranslated region (UTR) of the genes that encode biosynthesis, intake, and degradation of small metabolites and provide control over the reversible binding for these metabolic pathways.

The binding of a small molecule drives a conformational switch that alters gene expression through one of the following three mechanisms: (a) premature transcription termination, (b) translation initiation, and (c) mRNA processing. The majority of riboswitches switch off expression in a bound state; however, an insignificant number of switches have been found to switch on gene expression.

Unlike highly-specific metabolite-binding riboswitches, RNA thermometers (a closely related type of sensory mRNAs) act in response to a general physical signal, namely, to the intracellular temperature—an important parameter, which affects, in particular, the expression of both the genes that encode the heat and cold shock proteins and the virulence genes and are under the constant control of duplexes (formed, for example, by the hairpin stem of RNA thermometers). Melting at an elevated temperature is a well-known characteristic of nucleic acids. Temperature shifts are capable to modulate the conformation of regulatory RNA molecules, that is, the transition of molecule fragments from the intramolecular hairpin conformation to a single-stranded state.

There is a group of structurally and functionally different RNA thermometers that control a diversity of cellular processes. All known molecular thermometers (whether cis- or trans-acting) control translation through isolating ribosome-binding fragments, and the majority of them are localized in 5'-UTR of bacterial heat shock or virulence genes. At a low temperature, the Shine–Dalgarno sequence (SD-sequence, 5'-aaggag-3', 5'-rraggak-3' which is the consensus sequence for prokaryotes, and 5'-uygcu-3' which is the one for Gram-negative bacteria) [4] is masked (located in the middle of a hairpin-loop structure). An increase in temperature destabilizes the hairpin-loop structure, so that the ribosome binding site (the SD-sequence) becomes accessible and thus it permits translation initiation (AUG is the translation initiation start codon).

The first RNA thermometer that acts through the mechanism of melting was found in the *E. coli rpoH* gene, which encodes alternative sigma-factor $\sigma^{32}$ or RpoH [5]. The alternative RpoS sigma-factor plays a central role in the regulation of virulence-associated external surface proteins OspC and OspA in Lyme disease caused by *Borrelia burgdorferi* spirochete. Temperature is one of the RpoS-controlled surrounding environment key parameters, while $\mathrm{DsrA_{Bb}}$—a small non-coding RNA molecule—regulates the increase in the amount of RpoS, caused by a temperature shift. Lybecker et al. [6] formulated a hypothesis that $\mathrm{DsrA_{Bb}}$ is stable as a secondary structure at $t = 23°\mathrm{C}$, at which base-pairing with the rpoS transcript does not take place. At increasing the temperature, a small RNA molecule secondary structure becomes degraded due to melting which leads to the binding of the anti-SD-*rpoS* mRNA fragment. This may stimulate translation under virulent conditions (37°C) through the withdrawal of the SD sequence and the translation initiation site from the secondary structure in *rpoS* mRNA.

It is possible that the most widespread bacterial RNA thermometer is the ROSE element (Repression of Heat-Shock Gene Expression), which suppresses heat shock gene expression. This was found in many α- and β-proteobacteria, including *E. coli* and *Salmonella enterica* [7]. The ROSE-element with length from 60 to 100 nucleotides is as a rule localized in 5'-UTR of heat shock genes. Its rather complicated structure includes 2–4 hairpin-loop structures with one of them containing the SD sequence and, in some cases, also the start AUG codon. Another widespread RNA thermometer is the 4U element initially found in the *S. enterica* heat shock *agsA* gene [8]. The supposed structure contains two hairpins (four uridine residues forming complementary pairs with the SD sequence) (Fig. 1). The temperature-dependent melting has been experimentally confirmed in one of the hairpins, while the binding of the ribosome with the SD sequence takes place only at heat shock temperatures.

The 4U element is often used for controlling the heat shock and virulence genes in bacteria, since this element can bind to the 5'-agga-3' fragment of the SD sequence [8]. For example, the hypothesis regarding the control has been proven, using an RNA thermometer, for the *Yersinia* sp. *lcrF* (*virF*) gene encoding the virulence-controlling response regulator [9]. Gene translation does not occur at 26°C but is induced at $t = 37°\mathrm{C}$.

An analysis of well-known RNA thermometers and their regulatory principles has shown that for their functioning only a few nucleotides should form complementary bonds with the SD-sequence nucleotides or a flanking region to prevent the ribosome binding. It means that there have to exist in nature other still unknown types of RNA thermometers. This hypothesis has been confirmed using bioinformatic analysis. The synthesis of efficient individual RNA thermometers provided another proof of this assumption [11, 12]. Nevertheless, molecular details of the bacterial mechanisms of sensitivity to temperature shifts are still obscure in many cases.

Our computer analysis of the chromosomal DNA sequences of a variety of *S. enterica* isolates from databases has shown that the 4U thermometer analogous

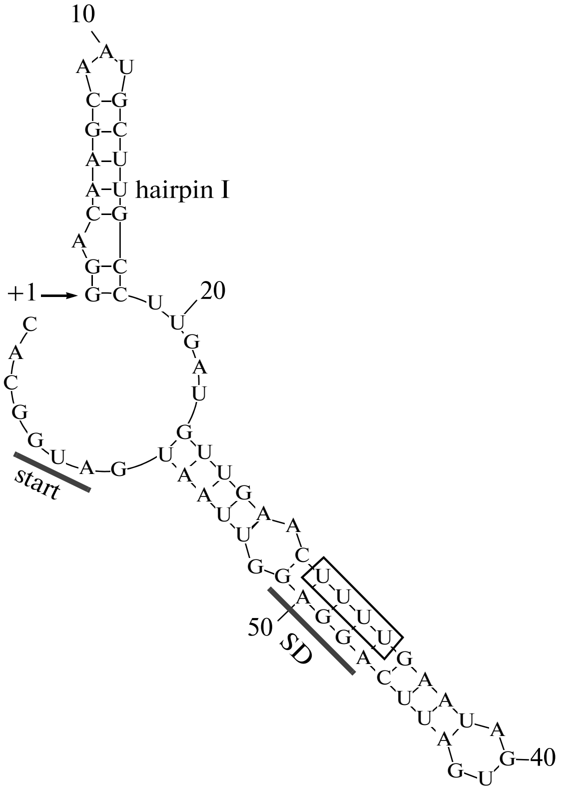

**Fig. 1.** Secondary structure that contains the predicted and confirmed 4U RNA thermometer in the *Salmonella enterica agsA* gene at $t$ = 20°C [8]. The Shine–Dalgarno sequence localized in the hairpin stem and the translation initiation site are underlined.

to the supposed thermometer for the *S. enterica agsA* gene and confirmed by *in vivo* and *in vitro* experiments by Waldminghaus et al. [8] (Fig. 1) was absent in three out of the 25 analyzed isolates (nos. CP001138, FM200053, and NC_006511). The necessity of adaptation and replication under different conditions, including temperature fluctuations, in particular, between the external environment and the host's organism, leads to the inevitable emergence in pathogens of the corresponding gene expression regulation mechanisms, where RNA thermometers belong as elements. The absence of 4U RNA thermometers in some *S. enterica* isolates indicates that they may contain certain thermosensitive elements that are different from both the 4U thermometers and, possibly, from the ROSE elements. In this paper the search of the for new earlier unknown potential RNA thermometers in the chromosomal DNA sequences of Gram-negative *Salmonella enterica* isolates was performed.

## MATERIALS AND METHODS

**Search for hairpin structures.** Chromosomal DNA sequences of 25 *S. enterica* isolates with complete genome from the GenBank database were chosen for computer analysis.

The search for chromosomal DNA fragments with a potential to form hairpin-loop structures in bacteria was performed, using the formula

$$5'\text{-aaggag}(n)_k\text{atg-}3',$$

where aaggag is the SD sequence; $n$ is any possible nucleotide; $k = 6–10$; and atg is the transcription initiation site (alternative transcription initiation sites ttg and gtg were also used).

Furthermore, the following formulas were used to search for hypothetical 4U RNA thermometers in sequenced "+" and "–" strands of the *S. enterica* chromosomal DNA

$$5'\text{-cat}(n)_7\text{ctcct}(n)_{12}\text{aaaa-}3' \text{ and}$$

$$5'\text{-tttt}(n)_{12}\text{agggag}(n)_7\text{atg-}3'.$$

The found sequences were analyzed using the established criteria.

We used the Mfold software (version 3.2) [13] for predicting the secondary structure of the found linear DNA fragments and the corresponding RNA transcripts, as well as for determining the melting temperatures of potential hairpins at physiological ionic strength ($I = 0.2$ M $Na^+$, $[Mg]^{2+} = 0.0$ mM or $I = 0.15$ M $Na^+$, and $[Mg]^{2+} = 0.2$ mM). In addition, the RNA2 program of the Gene Bee software was used for building hairpin-loop structures [14].

**Atomic force microscopy (AFM).** The supercoiled pUC8 DNA molecule (with length of 2665 bp) was visualized by Nanoscope III Multimode AFM with a D scanner (Veeco Instruments Inc., USA). AFM images of DNA were recorded in tapping mode, in the air under the "height" regime, using standard unsharpened probes (NT-MDT, Russia) with a resonance frequency of 300–360 kHz. To obtain amino mica, we used a freshly cleaved mica modified with amino groups in vapours of distilled (3-aminopropyl)triethoxysilane (Aldrich Chemical Co., United States). The modification technology has been described earlier [15].

## RESULTS AND DISCUSSION

To date, the functioning of ROSE thermometers—one of the well-known types of RNA thermometers—has been experimentally confirmed only for several of the forty ones proposed for proteobacteria [16]. The possibility of existence of the 4U element—another potential RNA-thermometer—has been established for the *Brucella melitensis dnaJ* gene and for the upstream region of the *Staphylococcus aureus groES* gene. However, it is still obscure whether they would function as RNA thermometers [8].

Analysis of specificities in the structural organization of experimentally confirmed RNA thermometers have allowed us to develop an algorithm for the search

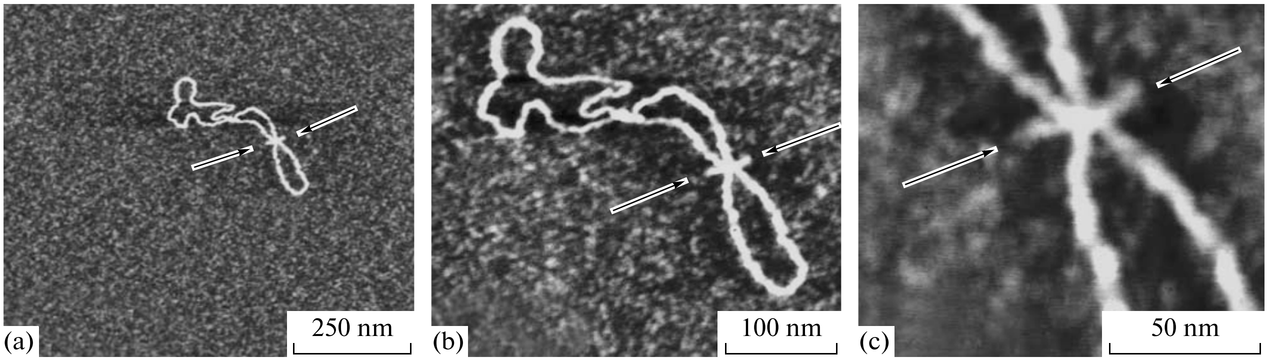

**Fig. 2.** AFM images for the supercoiled pUC8 plasmid (its length is 2665 bp) in the air. The scan size is as follows: (a) 858 nm × 858 nm; (b) 372 nm × 372 nm; and (c) 134 nm × 134 nm. The arrows show two hairpins which formed a cruciform structure.

of the corresponding fragments in the chromosomal DNA sequences in prokaryotes and RNA transcripts, one element of which is the distance between the SD sequence that is included in hairpin-loop structure and the translation initiation site, accounting for 6 (for ROSE elements) to 12 nucleotides (for 4U thermometers). We used the following main criteria for the potential RNA thermometers: (a) the melting temperature for hairpins is within the range from 32 (at ionic strength of 0.1 M $Na^+$) to 43°C (at physiological ionic strength); (b) the Shine–Dalgarno sequence is completely or partly localized in the stem of the hairpin structure; (c) the start codons are the canonical AUG codon (ATG for DNA) which ensures that more than 90% of all encoded sequences start in prokaryotes, as well as the alternative GUG and UUG codons (GTG and TTG for DNA) [17].

The well-known RNA thermometers are structures represented by either a single stretched hairpin or several hairpin-loop structures, which may be either perfect or imperfect. As the NMR research results have shown, a hairpin stem that has a ROSE element includes several noncanonical base pairs [18]; therefore, we have analyzed both perfect and imperfect hairpin-loop structures in our search of hypothetical RNA thermometers.

As has been earlier established for supercoiled DNAs that contain palindrome, it is possible under physiological conditions to form hairpins as a fragment of a cruciform structure with a stem at least 7 bp long and a loop with a size of no more than 4–5 nucleotides [19–21]. The stem of hairpins formed in 16S rRNA is on average 3–4 bp, reaching 10 bp [22], whereas a loop with 6–7 nucleotides is the most energetically preferable for RNA hairpins [23]. It has been shown by the thermal denaturation method that RNA hairpin structures are more stable, compared to the corresponding structures formed in DNA [24]. Based on literature data on both *in vivo* and *in vitro* experiments [25–28] and considering that hairpin-loop structures containing a SD sequence effectively affect translation only when their free energy is about –6 kcal/mol [29], we chose for the further analysis hairpins with loop length not exceeding eight nucleotides and a stem not exceeding 7 bp with free energy $\Delta G$ reaching approximately –2 to –6 kcal/mol. In addition, we looked into the localization of the potential hairpins relative to the *hsp* genes encoding heat shock proteins.

A combination of methods for immobilizing DNA on amino mica with the AFM advantages enabled us to visualize the hairpin-formed cruciform structure of supercoiled pUC8 DNA. An analysis of AFM images of pUC8 DNA on amino mica (Fig. 2) has shown that hairpins look like sharply expressed protrusions on DNA threads, the length of which can be directly evaluated from AFM images. An analysis of our experimental results has shown that 11–12 bp participated in the formation of a hairpin, and according to the conducted thermodynamic analysis of inverted repeats a hairpin was shown to be formed by 26 nucleotides, while its free energy $\Delta G$ was –17.8 kcal/mol. We note for comparison that the sizes of the cruciform structures that were registered, using two-dimensional electrophoresis and nuclease treatment, in DNA of φX 174 phage and pBR322, ColE1, and pAO3 plasmids are equal to 9–13 bp in the helical fragments of each of the cruciform hairpins, while their loops contain 3–5 nucleo-tides [19, 21].

Plasmid pUC8 is known to contain several palindromes that can form cruciform structures in aqueous solutions. Using the Oligo program [30], we have found that the cruci with the structure given in Fig. 3 is thermodynamically more preferable. (The concentration of plasmid pUC8 was selected so that 4–5 single supercoiled DNAs with a cruciform conformation were visualized per image of 2 μm × 2 μm in size.) One of the hairpins forming a cruci had free energy $\Delta G$ reaching –17.8 kcal/mol. The search for self-complementary fragments in the pUC8 DNA sequence, using the GeneBee software (RNA 2 program) [14], has also confirmed that the hairpin consists of 11 nucleotide model of pairs, while the loop length is four nucleotides. The model of supercoiled pUC8 DNA possessing a cruciform structure is presented in Fig. 3.

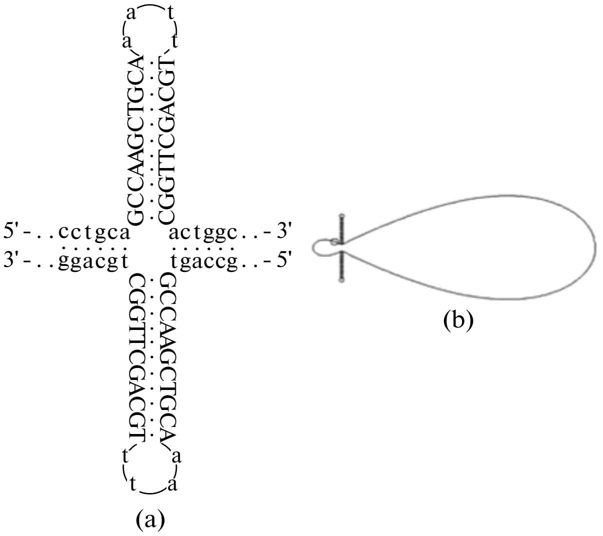

**Fig. 3.** Cruciform structure of the supercoiled pUC8 plasmid, which was obtained by GeneBee software: (a) pUC8 DNA sequence fragment (positions 273–298) that acquires a cruciform structure; (b) a cruciform structure that is formed by the pUC8 plasmid DNA inverted repeats.

There are several variants of the hairpin forming for cruci. For example, the hairpin may be formed by both complementary inverted repeats of one strand and repeats belonging to complementary DNA strands. We believe that further improvements in the AFM technology will enable researchers to determine the cruciform structure in detail, that is, what fragments of the DNA strand are complementary out of several possible formation variants of this noncanonical structure.

It is significant that RNA molecules look like condensed structures under AFM visualization. We earlier visualized RNA trascripts, immobilized on mica, which formed rod-like condensed structures 122 ± 10 nm in length with a length–width ratio of 4.5–5 [31]. We think that, for visualizing stretched uncondensed RNA molecules that contain hairpin-loop structures, it is necessary to change the surface properties of the substrate (mica). The noted morphological specificities of AFM-visualized RNA molecules can be explained by a significant effect of the surface properties of the mica where RNA transcripts are immobilized. The surface properties of the substrate are in turn determined by the hydrophobicity and density of the cations localized on the mica surface. The point is that the same mica was used for visualizing both RNA and DNA molecules, i.e., the mica possessing the hydrophobicity and surface density of cations at which linear and supercoiled double-stranded DNA molecules do not form condensed structures when immobilized on the mica surface but are characterized by an uniform distribution of DNA fragments. We have established earlier that even insignificant changes in the hydrophobicity and density of cations on the mica surface lead to significant modifications in the morphology of immobilized DNA molecules [32–33].

Since thermometers may be localized at any locus in an RNA molecule, to find the hairpins, the main components of those new potential RNA thermometers differing from the well-known 4U RNA thermometers, we at first analyzed the chromosomal DNA sequences of three *S. enterica* isolates, which did not contain any of the 4U RNA thermometers.

Our computer and thermodynamic analysis of the fully sequenced *S. enterica* genome (no. FM 200053 for GenBank) allowed us to reveal four hairpin structures (Fig. 4) that satisfy the required conditions for a potential RNA thermometer: availability of the Shine–Dalgarno sequence; the translation initiation site at a distance of no more than 15 nucleotides; the corresponding melting temperature (nearly 40–42°C) for a hairpin; and localization in the 5'-UTR. The presence of 13 known heat shock genes (with one of which the 4U RNA thermometer is bound) in isolate FM 200053 enables us to assume that there are several types of RNA thermometers in the genome of salmonella apart from the one currently known as the 4U RNA thermometer.

It is important that hairpin-loop structures (Fig. 4) do not only meet the necessary and sufficient conditions for forming RNA thermometers but also have highly conserved noncanonical structures. They are present in the genomes of all studied 25 isolates and are characterized by 100% similarity in nucleotide sequences.

The expression of virulence factors, such as hemolysin and lipopolysaccharides, is regulated in proteobacteria by transcription elongation factor RfaH. The *S. enterica yaeQ* gene was identified as a high-copy suppressor of the hemolytic defect in the deleted segment of the *rfaH* gene. Proceeding from this, Vicari et al. [34] assumed that the direct role of the *yaeQ* gene that encodes glutamate synthetase is in the transcriptional control of bacterial virulence.

It is known that different RNA thermometers have different melting temperatures.

For example, the ROSE thermometer melts at 42°C, whereas the pfrH and lcrF thermometers melt at 37°C [35]. Therefore, for consideration we selected as the potential RNA thermometers only hairpin-loop structures with a melting temperature within the range 37–42°C.

It is sufficiently difficult to predict for many 5'-UTR sequences the possibility of their functioning as RNA thermometers. To confirm the functioning of a hairpin-loop structure as a thermosensor, it is necessary to undertake some experiments, for example, *in vivo* testing of the efficiency of the reporter gene expression at various temperatures or melting the hairpin-loop structure (which contains the SD consequence). The real melting temperature of the determined RNA thermometers is measured, for example,

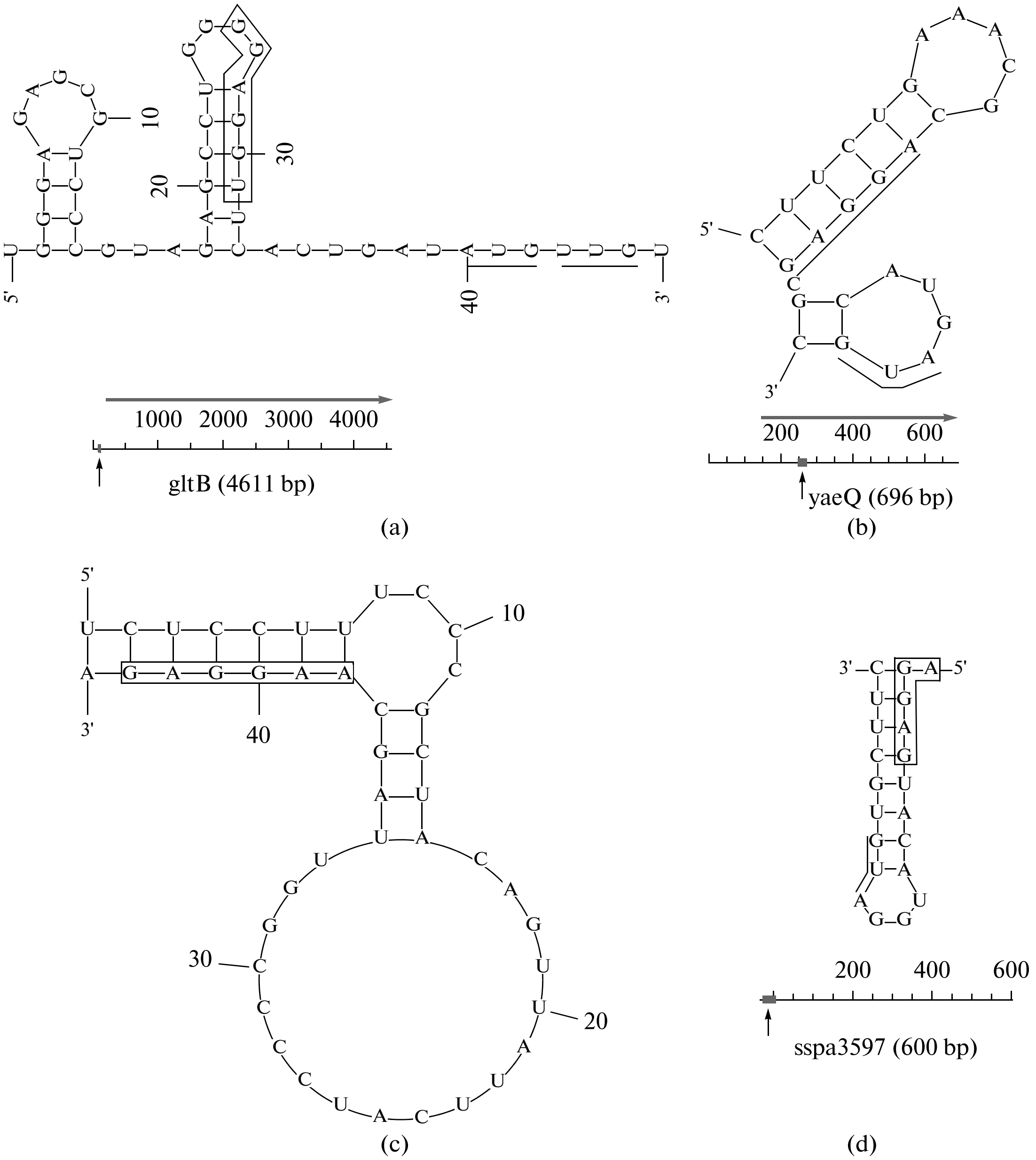

**Fig. 4.** Potential RNA thermometers containing the SD sequence in the hairpin stem (marked) and the initiation start codon (underlined): (a) the melting temperature of the hairpin ($t_{\text{melt}}$) is 43.6°C at the ionic strength $I = 0.2$ M $Na^+$. The free Gibbs energy $\Delta G = -2.0$ kcal/mol. The hairpin structure is localized in the 5'-UTR fragment of the *gltB* gene that encodes the precursor of glutamate synthetase in *S. enterica*. The line indicates mRNA; (b) $t_{\text{melt}} = 44.9$°C; $\Delta G = -1.9$ kcal/mol. The hairpin structure is localized in the 5'-UTR fragment of the gene that encodes one of the virulence regulators in *S. enterica.*; (c) the hairpin structure is localized in the 5'-UTR fragment of the *gltB* gene that encodes a hypothetical protein. $\Delta G = -9.3$ kcal/mol; (d) $t_{\text{melt}} = 42.0$°C; and $\Delta G = -2.1$ kcal/mol. The hairpin structure is localized in the 5'-UTR fragment of the gene that encodes a putative haloacid dehalogenase-like hydrolase in *S. enterica*. The indicated hairpin-loop structures correspond to the necessary and sufficient conditions for the formation of RNA thermometers and are highly conserved noncanonical structures and were found in the genomes of all the 25 analyzed *Salmonella enterica* isolates.

using circular dichroism spectroscopy or UV spectroscopy. Since the melting temperature of nucleic acids depends on the ionic strength, it is considered in assessing the thermodynamic parameters of thermometers that the concentration of $Mg^{2+}$ ions within the 1–2 mM interval corresponds to the physiological value inside bacterial cells.

The probability of the functioning of the found hairpin-loop structures as thermosensors in the *S. enterica* genome is confirmed by the fact that Neupert et al. [11] have established for the functioning of a hairpin-loop structure (fully analogous to one of the found potential RNA thermometers) as a thermosensor (Fig. 4c). A comparison of the functioning efficiencies of 12 synthetic RNA thermometers, conducted by the authors of paper [11] under physiological conditions, has shown that the indicated RNA thermometer (Fig. 4c) is one of the two RNA thermometers that most efficiently control the temperature-dependent expression of the *lacZ* gene encoding β-galactosidase in *E. coli* [11]. Our potential RNA thermometers (Fig. 4) significantly differ in their secondary structure. Although RNA thermometers are characterized by a small loop (4–5 nucleotides), the experimentally confirmed RNA thermometer (Fig. 4c) has a rather large loop, although all found hairpin structures have a perfect stem. Since it has been established in [11] that significantly differing hairpins equally efficiently switch on/off gene expression, we can expect that other hairpin structures (Fig. 4) will also be able to play the role of RNA thermometers.

To check the reliability of the algorithm for determining secondary structures, we modeled the secondary structures in a *Saccharomycetes cerevisiae* mRNA fragment. Lu et al. [36] proposed three secondary structure variations for a 74-bp fragment, each being characterized by 19 canonical base pairs. Simulation of the secondary structure, using the GeneBee software, has also confirmed the possibility of forming 19 canonical base pairs. Thus, the conducted testing has shown that the results obtained in determining the secondary structures are in good agreement with literature data; therefore, the methods for predicting potential hairpin-loop structures can be regarded as reliable.

Another evidence for the reliability of the methodology used for searching for hypothetical riboswitches in this work can be their subsequent testing. In general, any theoretical studies can be considered successful only after their experimental testing. Therefore, within a pilot project, we conducted preliminary experimental studies in the *Salmonella enterica* genome for testing a series of RNA thermometers, which we had theoretically determined before. We cloned synthesized nucleotide sequences of potential RNA thermometers into *E. coli* cells close to the 5'-end of the gene that encodes GFP and conducted several preliminary experiments with temperature shifts, observing the changes in the fluorescence of the GFP protein in *E. coli*. At temperature shifts from 22 to 37°C, a weak GFP induction was recorded but not so strong as observed for synthetic RNA thermometers during temperature-controlled gene expression in bacteria.

Thus, based on a computer and thermodynamical analysis of 25 *Salmonella enterica* isolates with complete genome, we have established an algorithm and criteria for the search for new potential RNA thermometers, which will enable us to undertake a search for potential RNA thermometers in the genomes of other socially significant pathogens. In addition to the well-known 4U RNA thermometer, hairpin-loop structures that can be new RNA thermometers have been determined for *S. enterica*. They meet the necessary and sufficient conditions for the formation of RNA thermometers and are highly conserved noncanonical structures, since they are present in the genomes of all studied *S. enterica* isolates.

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