Identification of a Small Regulatory RNA UspS Associated with the Universal Stress Protein in *Lactobacillus* Species

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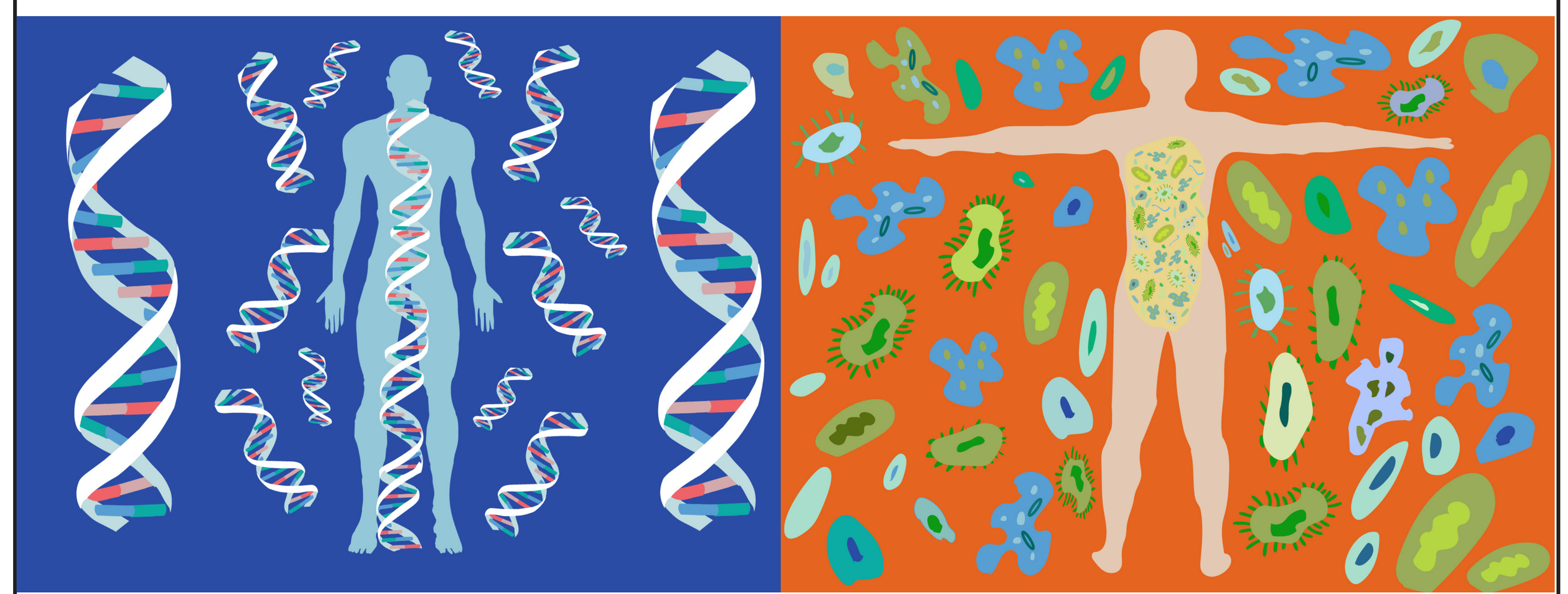
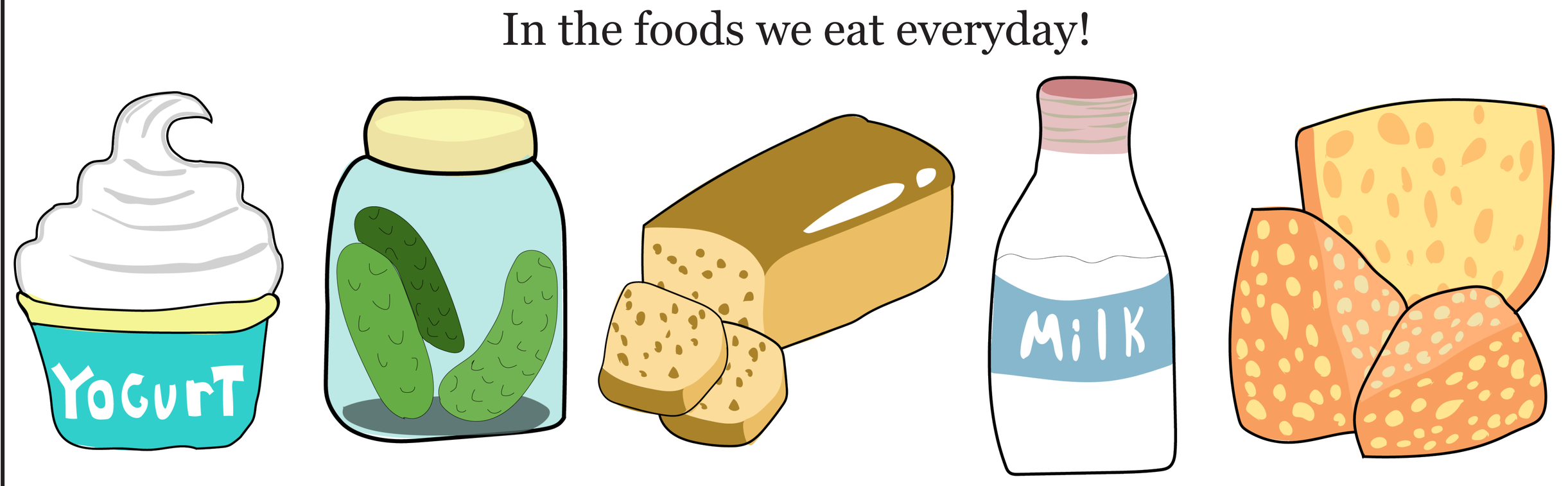
Introduction

Human Genes in your body: 22 Thousand
Bacterial Genes in your body: 8 Million

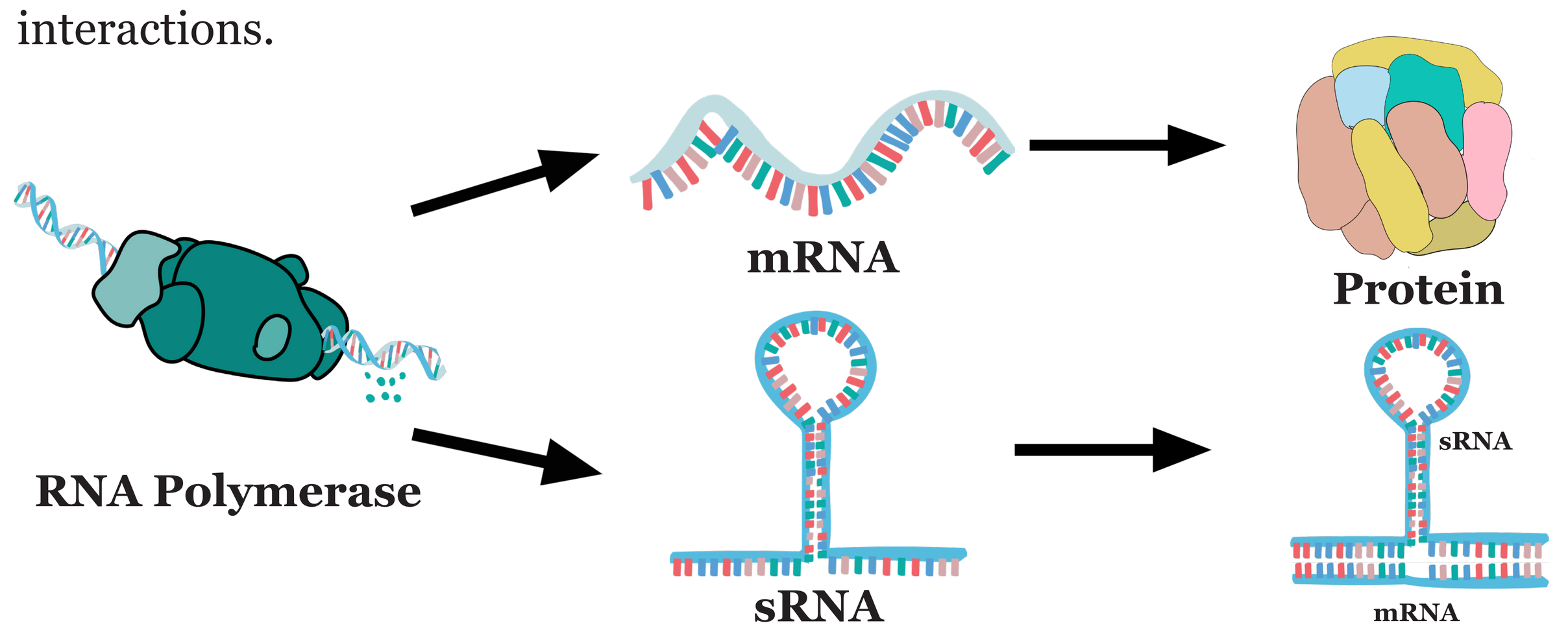


Where do we get these bacteria?
In the foods we eat everyday!



In our lab we study sRNA in Probiotic Bacteria

Goal: To identify and explore the conservation of function and structure of the sRNA, UspS, in probiotic bacteria and to further analyze its role in host interactions.



Materials and Methods

Candidate noncoding RNA sequences were chosen by searching the literature containing potential sRNAs in probiotic bacteria. A target noncoding RNA (UspS) was chosen based on a paper from 2010 by the Breaker lab.[1] The sequence was characterized using computational methods to predict the structure and function of UspS. NCBI Nucleotide Blast was used to identify the intergenic region surrounding UspS. Nucleotide sequence were taken from NCBI Nucleotide Blast.[2] A transcription start site and rho independant terminator were identified based on known *Lactobacillus* promoter sequences and uracil-rich stem loop trails.[3] mFold and RNApdbee were used to predict the secondary structure and pseudoknot sequences.[4-6] Secondary structures from mFold were input into Rosetta's FARFAR2 to create tertiary structure models.[4,7] NCBI Nucleotide Blast was used to identify UspS sequences in seventeen different species of bacteria.[2] Jalview was used to align sequences, predict a phylogenic tree, and calculate conservation between species.[8] CopraRNA and IntaRNA were used to predict protein interactions and function of UspS.[9,10,11,12]

Two types of Lactobacillaceae (*Lactobacillus acidophilus* and *Lactobacillus delbrueckii* subsp. *bulgaricus*) were prepared for growth culture inoculation by rehydration of freeze-dried bacterial cells. MRS media was used to grow both lactobacillus species under anaerobic conditions at 37 °C. Bacterial growth cultures were harvested and the DNA of *L. acidophilus*. and *L. bulgaricus* were extracted using the Wizard Genomic DNA Purification Kit (Promega). Primers were designed to amplify UspS based on NCBI's nucleotide sequences.[2] A T7 promoter and restriction sites were included in the primer design. A PCR reaction was run to target UspS in both species as a short construct without a terminator and a long construct with a terminator. Agarose gel electrophoresis confirmed the sizes of target DNA. A digest was performed using blunt end restriction enzymes DraI and SwaI. The PCR constructs and digested constructs were purified using Wizard SV Gel and PCR Clean-Up System Kit (Promega). The purified digested constructs were used for transcription using a T7 RNA Polymerase (NEB). The target RNA sizes, digest construct sizes, and DNA sizes were confirmed using polyacrylamide gel electrophoresis. Products of T7 transcripts was purified using the RNeasy MinElute clean-up kit (Qiagen). Thermal melt assays were performed using Bio-rad CFX96 Touch Real-Time PCR and analyzed using CFX Maestro.

Results

Sequence Alignment

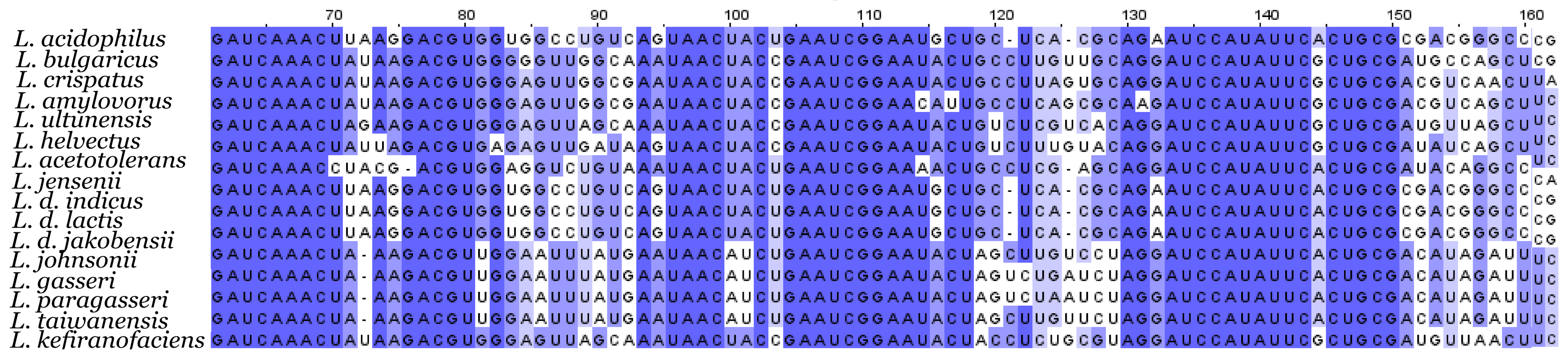


Figure 1. A sequence alignment of UspS orthologous sequences from seventeen different bacterial species. Figure was made using Jalview multiple sequence alignment[8].

Secondary Structure Comparison

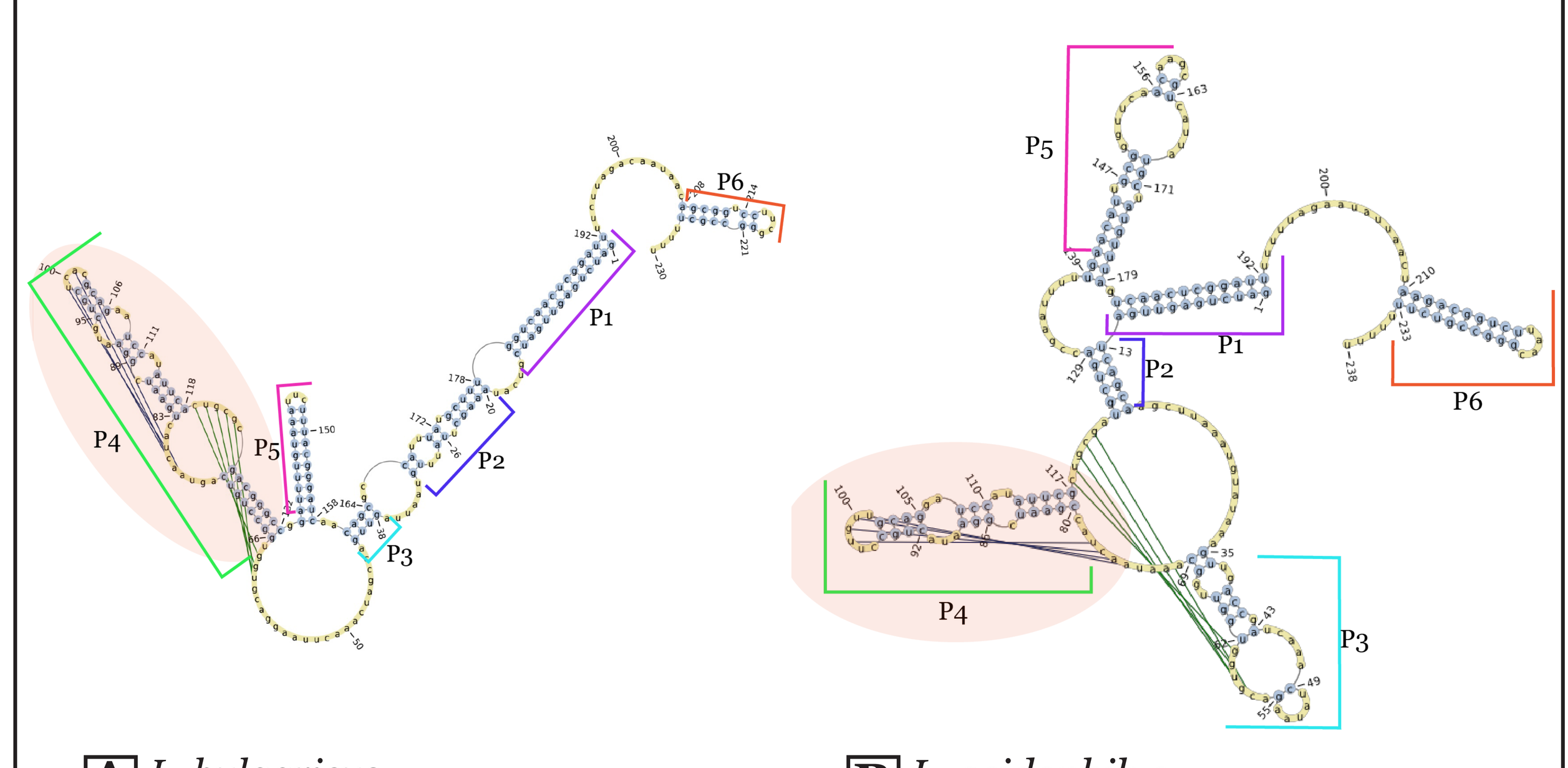


Figure 2. Secondary structures of *L. bulgaricus* (A) and *L. acidophilus* (B). Structural regions are labeled P1 through P6. The P4 regions highlighted in pink seems to be highly conserved between species. Pseudoknot sequences are identified by attaching lines on the P4 region. Figured made using mFold and RNApdbee.[4,5,6,13,14]

Conservation of UspS P4 Region

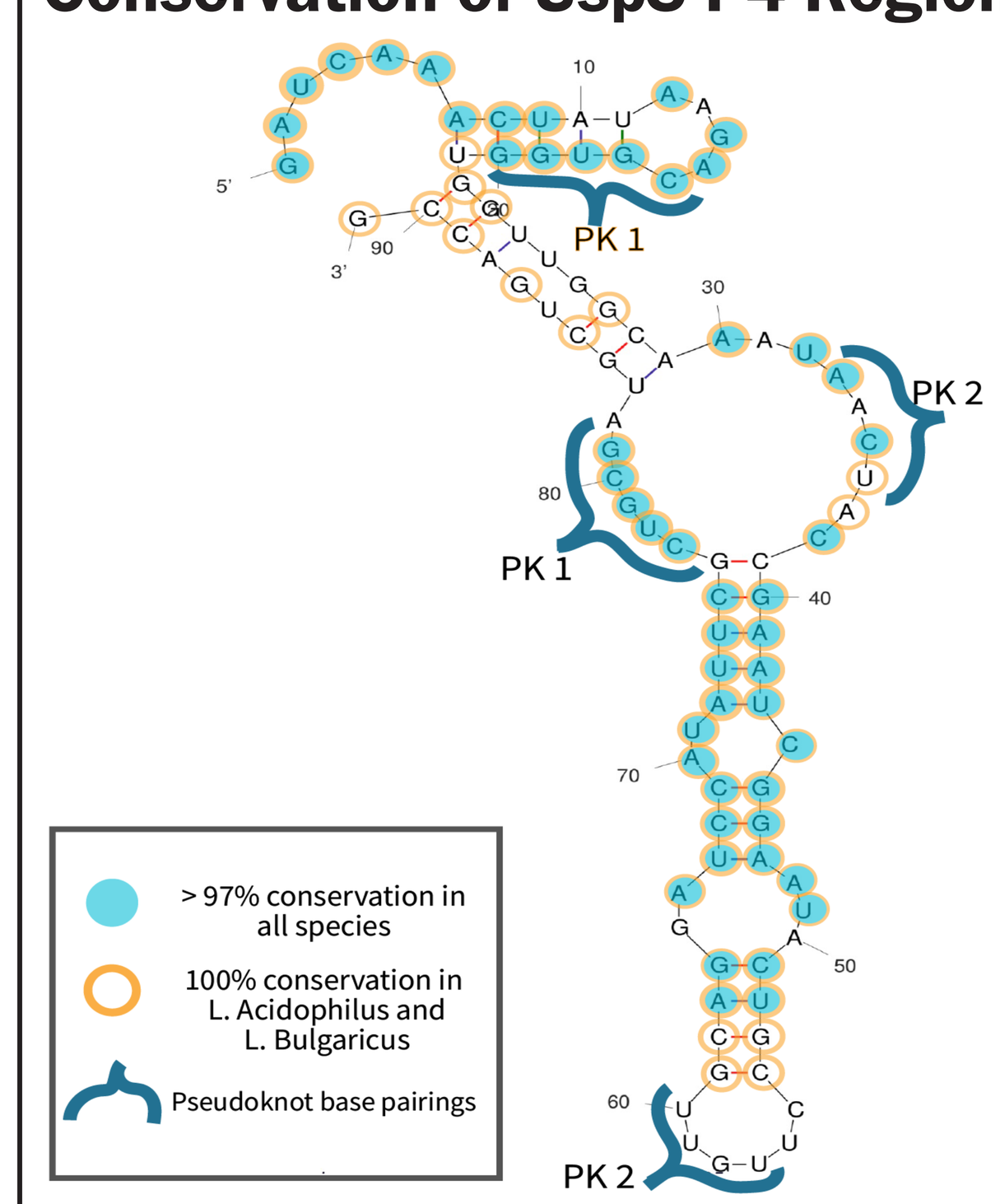
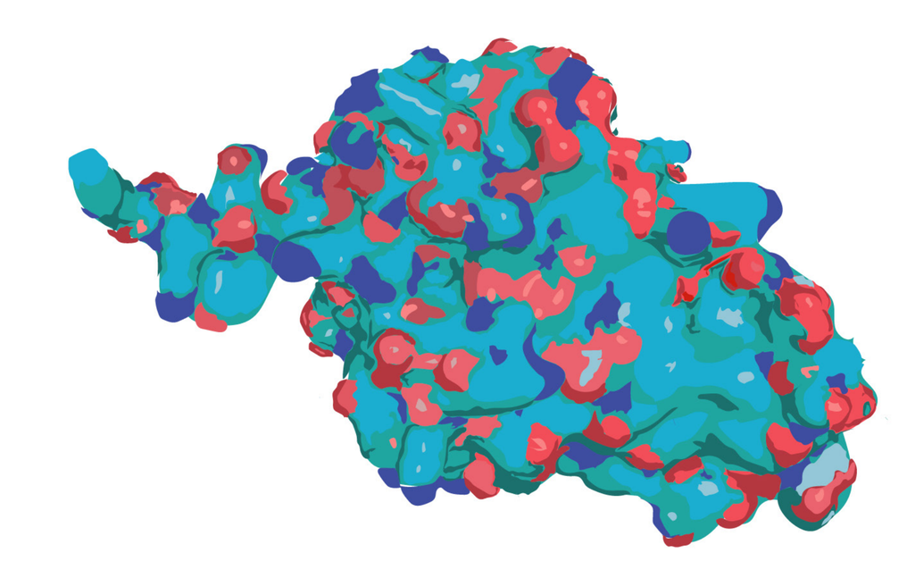


Figure 3. Conservation of UspS P4 region in seventeen species from pairwise alignment using Jalview.[8]

Protein Regulated by sRNA-mRNA Interactions



Protein: *L. bulgaricus* Universal stress protein
Function: Stress response

Figure 4. Universal stress protein associated with mRNA's predicted to interact with P4 in UspS in *L. bulgaricus and L. acidophilus*. Copra and IntaRNA were used to show interactions between P4 of UspS and mRNA encoding the universal stress protein. NCBI Blast and Uniprot were used to predict protein functions. Graphical representation prepared on Adobe Ilustrator based on Alpha Fold AF-Q1GAV8-F1-model-v4.[10,11,12,15,18,19,20]

3D Models

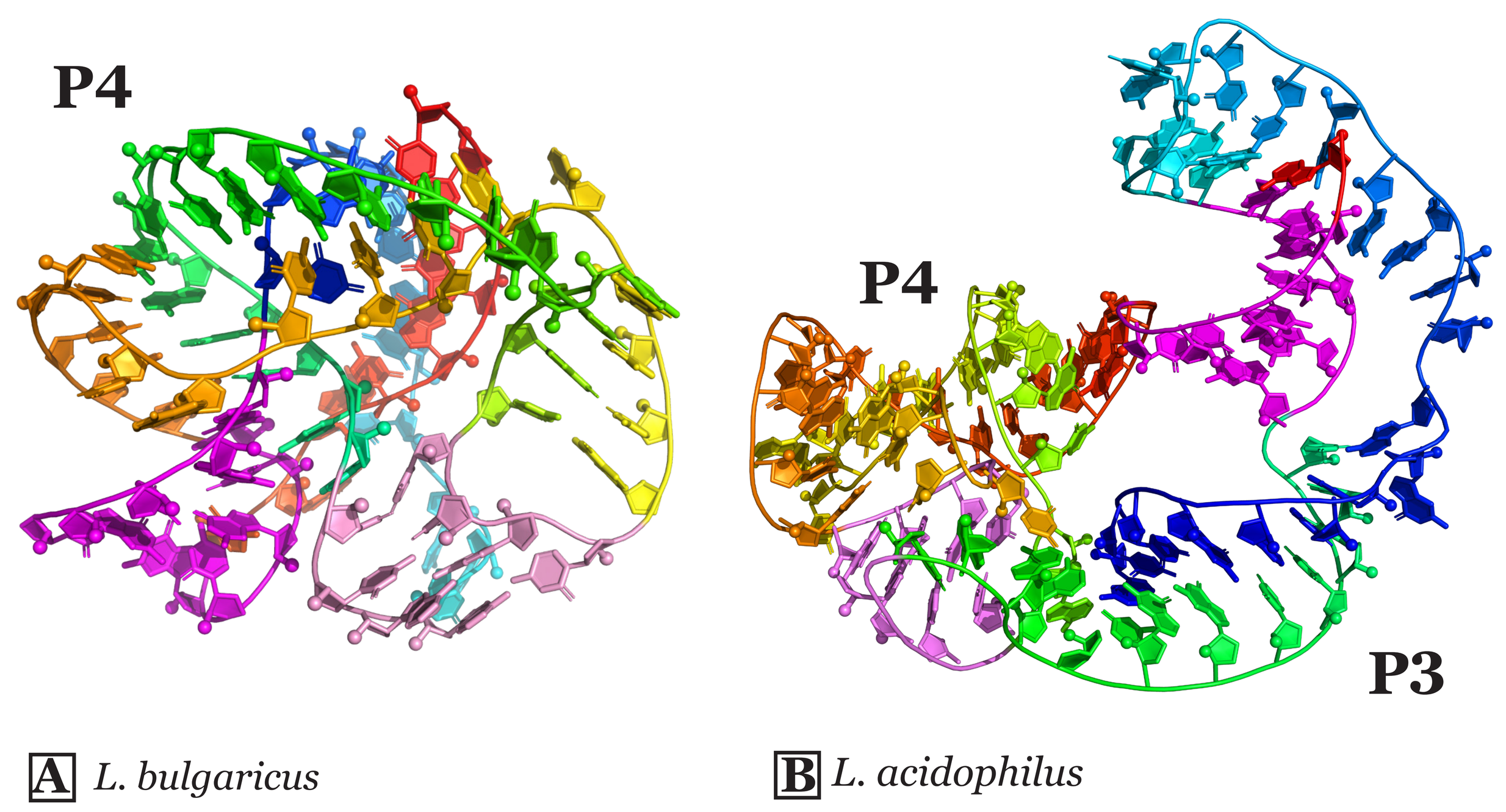


Figure 5. Tertiary structures of the P4 region of *L. bulgaricus (A)* and *L. acidophilus* (B) from Rosetta with pseudoknots highlighted in pink. Pseudoknot pair nucleotides labeled as pink and purple nucleotides.[7]

6S RNA of *E. coli*

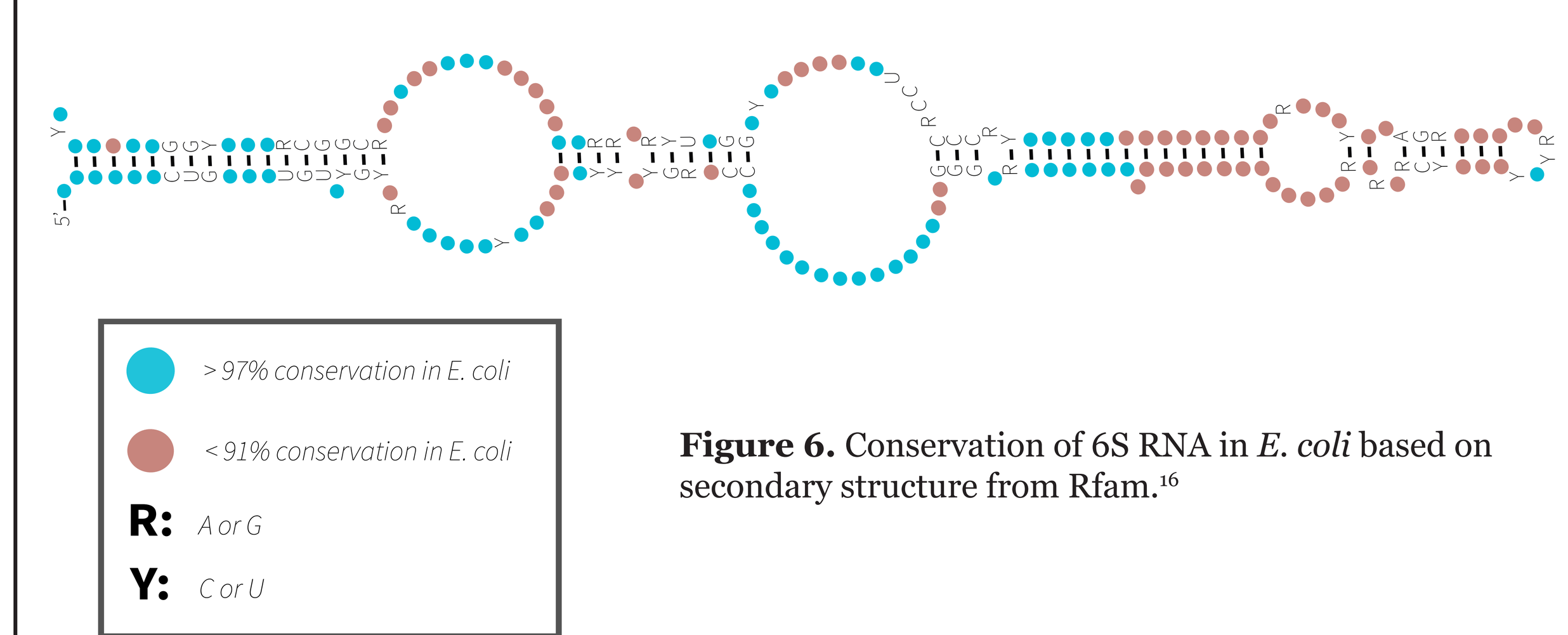


Figure 6. Conservation of 6S RNA in *E. coli* based on secondary structure from Rfam.[16]

Thermal Melt of UspS

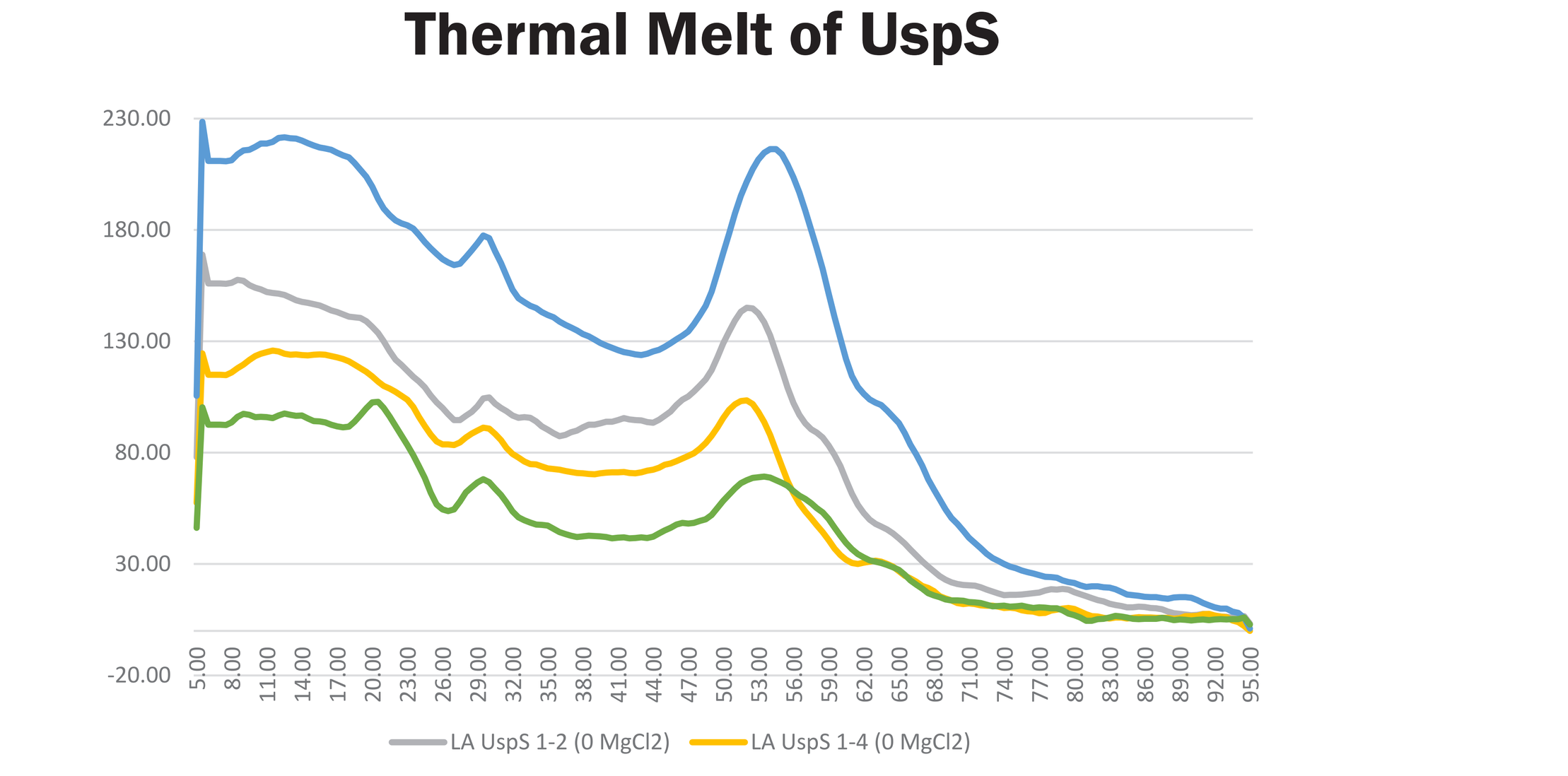


Figure 9. Thermal Melt of UspS RNA from Bacterial DNA. RNA transcripts: *L. acidophilus* P1-P6 (1-2), P1-P5 (1-4), *L. bulgaricus* P1-P6 (1-2), P1-P5 (1-4). All thermal melts performed with 0 $MgCl_2$.

Isolation of UspS RNA from Bacterial DNA

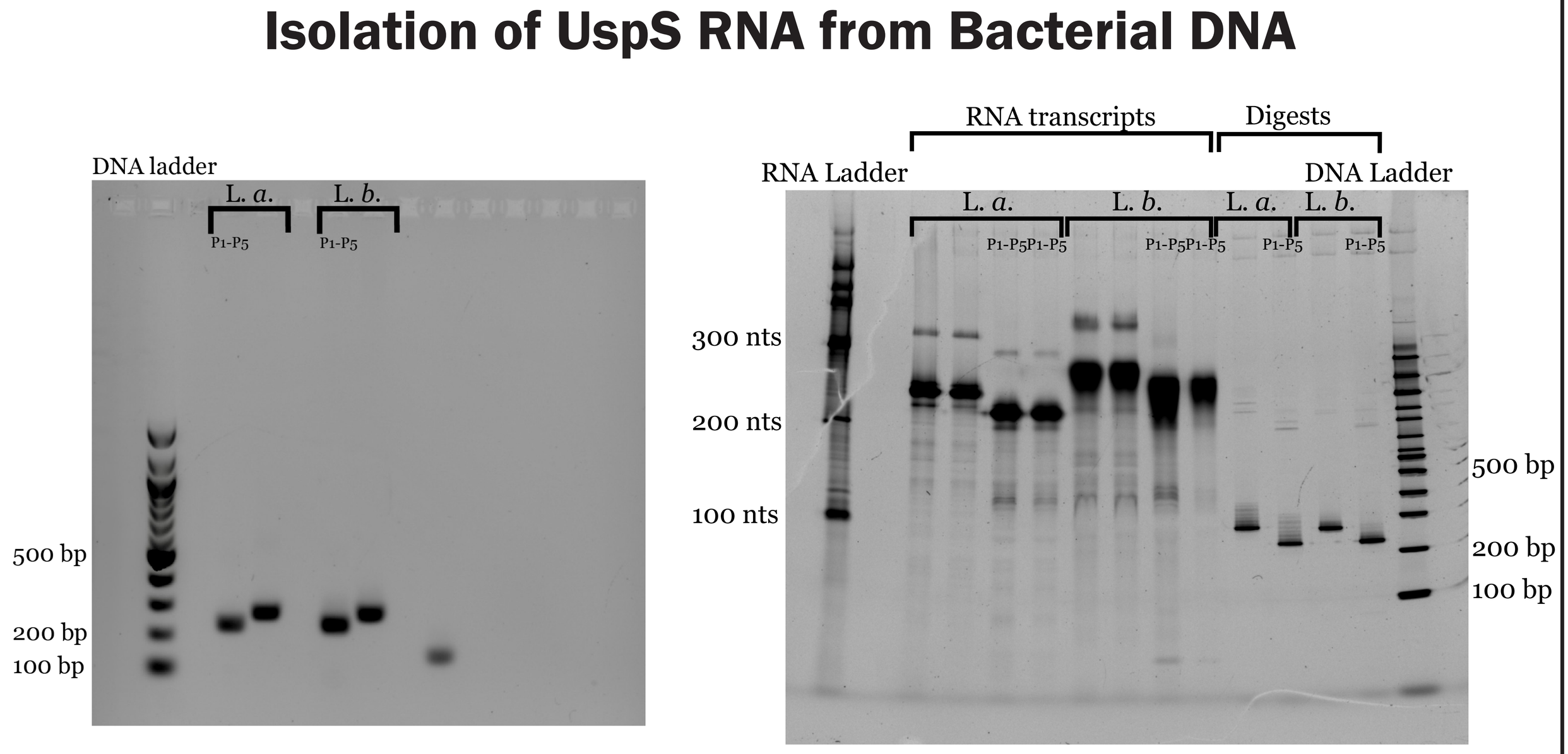


Figure 7. DNA templates of UspS. PCR products: *L. acidophilus* P1-P5, P1-P6, *L. bulgaricus* P1-P5, P1-P6. 1.5% agarose in TAE, 45 minutes at 80V, 6x loading dye, 100 bp Ladder Maker (NEB), stained with ethidium bromide.

Figure 8. PCR digests and RNA transcripts of UspS constructs. RNA transcripts: *L. acidophilus* P1-P6, P1-P5, *L. bulgaricus* P1-P6, P1-P5. All RNA transcriptions performed with 24mM and 36mM $MgCl_2$. PCR digests: *L. acidophilus* P1-P6, P1-P5, *L. bulgaricus* P1-P6, P1-P5. 4-20% Tris Glycine Gel with Native Tris Glycine buffer (Invitrogen), 45 minutes at 200V, SYBR Green II dye (Thermo Fisher), RiboRuler RNA ladder (Thermo Fisher).

Conclusion

Multiple sequence alignment shows conservation of sequences between *Lactobacillus* species suggesting this sRNA is essential for the molecular functions of lactobacilli.[8] Secondary structure predictions show structural conservation of UspS P4 between *L. acidophilus* and *L. bulgaricus* suggesting this region is a significant site for mRNA interactions.[4] The conserved structure of the P4 region suggests that UspS may correspond to 6S RNA in *Lactobacillus* species as most lactobacilli lack a predicted 6S RNA.[1,17] UspS may function similar to 6S RNA in *E. coli* by forming a complex with the sigma subunit of RNA polymerase and initiate transcription.[1,17] Three-dimensional modeling and genetic mapping allow us to see potential sites for translational control of protein synthesis.[7] The presence of a universal stress protein downstream of UspS in both *L. acidophilus* and *L. bulgaricus* infers that UspS may interact with the mRNA of a universal stress protein.[2] Other predicted mRNA interactions indicate that UspS may activate a change in the bacteria's membrane structure as a result of stress.[10-12] These interactions suggest that UspS may play a role in host interactions by changing bacterial membrane structure when exposed to stress. The confirmation of correct size bands of PCR products, digest constructs, and T7 products confirms that our methods for extracting, amplifying, and isolating target bacterial genes are a reliable method for studying sRNA in bacterial species. The thermal shift assay suggest the presence of secondary strucure of UspS in both bacterial species.

Future Work

Restriction enzymes will be used to insert UspS into the plasmid pUC18 for large scale synthesis of RNA to use in structural studies. A Electrophoretic Mobility Shift Assay will be performed to study Usp-UspS interactions *in vitro*. Future studies include confirming secondary and tertiary structures of UspS. A RNase T1 digest will be used to confirm the presence of secondary structure of UspS by observing the degradation of unpaired G residues. Different concentrations of precipitants will be tested to find optimal conditions for crystallization of RNA. Three-dimensional structures of UspS may then be observed using X-ray crystallography. Nuclear magnetic resonance spectroscopy (NMR) may be used to confirm secondary structures of UspS through observation of base pair interactions.

References

1. Weinberg, Zasha, Joy X Wang, Jarrod Bogue, Jingying Yang, Keith Corbino, Ryan H Moy, and Ronald R Breaker. "Comparative Genomics Reveals 104 Candidate Structured RNAs from Bacteria, Archaea, and Their Metagenomes." Genome Biology 11, no. 3 (2010): R31. https://doi.org/10.1186/gb-2010-11-3-r31.
2. National Center for Biotechnology Information (NCBI)[Internet]. Bethesda (MD): National Library of Medicine (US), National Center for Biotechnology Information; [1988] – [cited 2017 Apr 06]. Available from: https://www.ncbi.nlm.nih.gov/
3. McCracken, Andrea, Mark S. Turner, Phil Giffard, Louise M. Hafner, and Peter Timms. "Analysis of Promoter Sequences from Lactobacillus and Lactococcus and Their Activity in Several Lactobacillus Species." Archives of Microbiology 173, no. 5–6 (June 2000): 383–89. https://doi.org/10.1007/s002030000159.
4. Zuker, M. "Mfold Web Server for Nucleic Acid Folding and Hybridization Prediction." Nucleic Acids Research 31, no. 13 (July 1, 2003): 3406–15. https://doi.org/10.1093/nar/gkg595.
5. Zok, Tomasz, Maciej Antczak, Michal Zurkowski, Mariusz Popenda, Jacek Blazewicz, Ryszard W Adamiak, and Marta Szachniuk. "RNApdbee 2.0: Multifunctional Tool for RNA Structure Annotation." Nucleic Acids Research 46, no. W1 (July 2, 2018): W30–35. https://doi.org/10.1093/nar/gky314.
6. Antczak, Maciej, Tomasz Zok, Mariusz Popenda, Piotr Lukasiak, Ryszard W. Adamiak, Jacek Blazewicz, and Marta Szachniuk. "RNApdbee—a Webserver to Derive Secondary Structures from Pdb Files of Knotted and Unknotted RNAs." Nucleic Acids Research 42, no. W1 (July 1, 2014): W368–72. https://doi.org/10.1093/nar/gku330.
7. Watkins, Andrew Martin, Ramya Rangan, and Rhiju Das. "FARFAR2: Improved De Novo Rosetta Prediction of Complex Global RNA Folds." Structure 28, no. 8 (August 2020): 963-976.e6. https://doi.org/10.1016/j.str.2020.05.011.
8. Waterhouse, A. M., J. B. Procter, D. M. A. Martin, M. Clamp, and G. J. Barton. "Jalview Version 2--a Multiple Sequence Alignment Editor and Analysis Workbench." Bioinformatics 25, no. 9 (May 1, 2009): 1189–91. https://doi.org/10.1093/bioinformatics/btp033.
9. Wright, Patrick R., Jens Georg, Martin Mann, Dragos A. Sorescu, Andreas S. Richter, Steffen Lott, Robert Kleinkauf, Wolfgang R. Hess, and Rolf Backofen. "CopraRNA and IntaRNA: Predicting Small RNA Targets, Networks and Interaction Domains." Nucleic Acids Research 42, no. W1 (July 1, 2014): W119–23. https://doi.org/10.1093/nar/gku359.
10. Raden, Martin, Syed M Ali, Omer S Alkhnbashi, Anke Busch, Fabrizio Costa, Jason A Davis, Florian Eggenhofer, et al. "Freiburg RNA Tools: A Central Online Resource for RNA-Focused Research and Teaching." Nucleic Acids Research 46, no. W1 (July 2, 2018): W25–29. https://doi.org/10.1093/nar/gky329.
11. Mann, Martin, Patrick R. Wright, and Rolf Backofen. "IntaRNA 2.0: Enhanced and Customizable Prediction of RNA–RNA Interactions." Nucleic Acids Research 45, no. W1 (July 3, 2017): W435–39. https://doi.org/10.1093/nar/gkx279.
12. Busch, Anke, Andreas S. Richter, and Rolf Backofen. "IntaRNA: Efficient Prediction of Bacterial SRNA Targets Incorporating Target Site Accessibility and Seed Regions." Bioinformatics 24, no. 24 (December 15, 2008): 2849–56. https://doi.org/10.1093/bioinformatics/btn544.
13. Peselis, Alla, and Alexander Serganov. "Structure and Function of Pseudoknots Involved in Gene Expression Control: Structure and Function of Pseudoknots." Wiley Interdisciplinary Reviews: RNA 5, no. 6 (November 2014): 803–22. https://doi.org/10.1002/wrna.1247.
14. Antczak, Maciej, Mariusz Popenda, Tomasz Zok, Michal Zurkowski, Ryszard W Adamiak, and Marta Szachniuk. "New Algorithms to Represent Complex Pseudoknotted RNA Structures in Dot-Bracket Notation." Edited by Alfonso Valencia. Bioinformatics 34, no. 8 (April 15, 2018): 1304–12. https://doi.org/10.1093/bioinformatics/btx783.
15. Schrödinger, L., & DeLano, W. (2022). PyMOL (Version 2.5) [computer software] Schrödinger INC. http://www.pymol.org/pymol
16. Kalvari, I., Nawrocki, E. P., Argasinska, J., Quinones-Olvera, N., Finn, R. D., Bateman, A., & Petrov, A. I.(2018). Non-Coding RNA Analysis Using the Rfam Database. Current Protocols in Bioinformatics, 62(1), e51. https://doi.org/10.1002/cpbi.51
17. Wassarman, Karen M. "6S RNA, a Global Regulator of Transcription." Edited by Gisela Storz and Kai Papenfort. Microbiology Spectrum 6, no. 3 (June 2018): 6.3.06. https://doi.org/10.1128/microbiolspec.RWR-0019-2018.
18. Varadi, Mihaly, Stephen Anyango, Mandar Deshpande, Sreenath Nair, Cindy Natassia, Galabina Yordanova, David Yuan, et al. "AlphaFold Protein Structure Database: Massively Expanding the Structural Coverage of Protein-Sequence Space with High-Accuracy Models." Nucleic Acids Research 50, no. D1 (January 7, 2022): D439–44. https://doi.org/10.1093/nar/gkab1061.
19. Jumper, John, Richard Evans, Alexander Pritzel, Tim Green, Michael Figurnov, Olaf Ronneberger, Kathryn Tunyasuvunakool, et al. "Highly Accurate Protein Structure Prediction with AlphaFold." Nature 596, no. 7873 (August 1, 2021): 583–89. https://doi.org/10.1038/s41586-021-03819-2.
20. Adobe Inc. (2019). Adobe Illustrator. Retrieved from https://adobe.com/products/illustrator
21. S1chroeder, Griffin M, Debapratim Dutta, Chapin E Cavender, Jermaine L Jenkins, Elizabeth M Pritchett, Cameron D Baker, John M Ashton, David H Mathews, and Joseph E Wedekind. "Analysis of a PreQ1-I Riboswitch in Effector-Free and Bound States Reveals a Metabolite-Programmed Nucleobase-Stacking Spine That Controls Gene Regulation." Nucleic Acids Research 48, no. 14 (August 20, 2020): 8146–64. https://doi.org/10.1093/nar/gkaa546.
22. Li, Sanshu, Xue Ying Hwang, Shira Stav, and Ronald R. Breaker. "The YjdF Riboswitch Candidate Regulates Gene Expression by Binding Diverse Azaaromatic Compounds." RNA 22, no. 4 (April 2016): 530–41. https://doi.org/10.1261/rna.054890.115.
23. Wright, Patrick R., Andreas S. Richter, Kai Papenfort, Martin Mann, Jörg Vogel, Wolfgang R. Hess, Rolf Backofen, and Jens Georg. "Comparative Genomics Boosts Target Prediction for Bacterial Small RNAs." Proceedings of the National Academy of Sciences 110, no. 37 (September 10, 2013). https://doi.org/10.1073/pnas.1303248110.
24. Das Lab.(2016). Ribovis (Version 1.0) [computer software] Leland Stanford Junior University. https://ribokit.github.io/RiboVis/
25. Xiong, Zhi-Qiang, Ze-Xuan Lv, Xin Song, Xin-Xin Liu, Yong-Jun Xia, and Lian-Zhong Ai. "Recent Research Advances in Small Regulatory RNAs in Streptococcus." Current Microbiology 78, no. 6 (June 2021): 2231–41. https://doi.org/10.1007/s00284-021-02484-y.
26. Wen, Jin-Der, Syue-Ting Kuo, and Hsin-Hung David Chou. "The Diversity of Shine-Dalgarno Sequences Sheds Light on the Evolution of Translation Initiation." RNA Biology 18, no. 11 (November 2, 2021): 1489–1500. https://doi.org/10.1080/15476286.2020.1861406.
27. Cech, Thomas R., and Joan A. Steitz. "The Noncoding RNA Revolution—Trashing Old Rules to Forge New Ones." Cell 157, no. 1 (March 2014): 77–94. https://doi.org/10.1016/j.cell.2014.03.008.
28. Pappesch, Roberto, Philipp Warnke, Stefan Mikkat, Jana Normann, Aleksandra Wisniewska-Kucper, Franziska Huschka, Maja Wittmann, et al. "The Regulatory Small RNA MarS Supports Virulence of Streptococcus Pyogenes." Scientific Reports 7, no. 1 (December 2017): 12241. https://doi.org/10.1038/s41598-017-12507-z.
29. Gruber, A. R., R. Lorenz, S. H. Bernhart, R. Neubock, and I. L. Hofacker. "The Vienna RNA Websuite." Nucleic Acids Research 36, no. Web Server (May 19, 2008): W70–74. https://doi.org/10.1093/nar/gkn188.

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