Identification and Characterization of Small Regulatory RNA in *Streptococcus*

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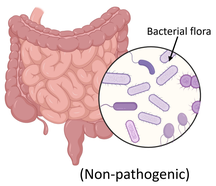
Introduction

You are never alone! - About 100 trillion bacteria in your gut.



mediates → Immune Responses, Neurological Activity, Metabolism

(Non-pathogenic)

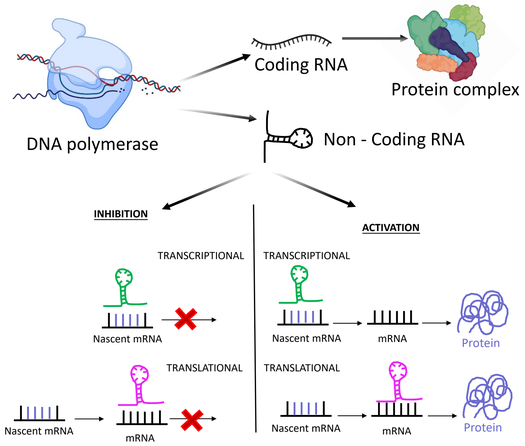
Streptococcus thermophilus
Lactobacillus acidophilus
Lactobacillus bulgaricus

(Pathogenic)

Close relative: *Streptococcus pyogenes*
Strep throat to Flesh-eating disease

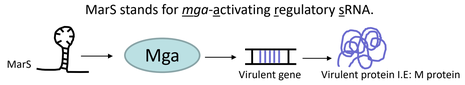
Bacterial Functions are mediated by Small Regulatory RNAs (sRNA)[1-6]

Aims: To isolate sRNA species conserved among probiotic bacteria and to study the structure and function of the isolated sRNA species.



sRNA in *S. pyogenes*[7-11]

MarS stands for *mga*-activating regulatory sRNA.

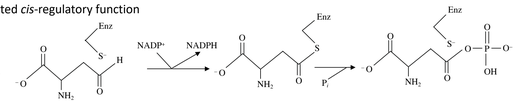


Mga -> regulator for virulent causing genes => MarS –involved in virulence

sRNA in *S. thermophilus:* Aspartate-Semialdehyde Dehydrogenase[12]

I. AsdS – 5′ UTR of the gene aspartate-semialdehyde dehydrogenase
II. Function of AsdS – Unknown
III. Postulated *cis*-regulatory function

Reaction catalyzed by Asd enzyme:



L-aspartate β-semialdehyde — Acyl-enzyme intermediate — β-aspartyl phosphate

L-aspartate 4-semialdehyde + phosphate + $NADP^+$ ⇌ L-4-aspartyl phosphate + NADPH + H^+

- Reversible
- In biological conditions: Left → Right

Gram Positive Sporulation
Bacterial quorum sensing
Bacterial cell wall cross-linking

Materials and Methods

Three types of lactic acid bacteria (*Streptococcus thermophilus, Lactobacillus acidophilus, Lactobacillus lactis*) were grown under anaerobic conditions at 37°C. The media used were: M17 for *Streptococcus thermophilus*, MRS for *Lactobacillus acidophilus*, LB for *Lactobacillus lactis*. After the bacteria reached the exponential phase, the genomic DNA of *S. thermophilus* was extracted using the Wizard Genomic DNA Purification Kit (Promega). Primers were designed to isolate the *AsdS* construct by PCR. Gel electrophoresis confirmed the presence of the correct sized DNA constructs. PCR constructs were purified before and after digestion with restriction enzymes (NEB) using a Wizard SV Gel and PCR Clean-Up System kit (Promega). The purified DNA constructs were used as templates for *in vitro* transcription reactions using T7 RNA polymerase (NEB). The transcribed RNA was characterized by agarose gel and PAGE electrophoresis to confirm the presence of the correct sized RNA.

Computational research was conducted to predict structural and functional characteristics of AsdS. The NCBI services were used to find the regions of similarity between sequences as well as aid in primer design. Jalview was used to align the sequences obtained from NCBI. Secondary structure predictions were generated using RNAfold.[12-13] Base-pairing information from RNAfold was used as input for ROSIE: FarFar2 to obtain the tertiary structure predictions.[14] Rfam was used to obtain data for noncoding RNA and KEGG was used for access to the Gene/Metabolic Pathway database. IntaRNA was used to predict putative functions of AsdS.[15] RNAalifold was used to obtain a consensus secondary structure for AsdS among *Streptococcus* species.

Results

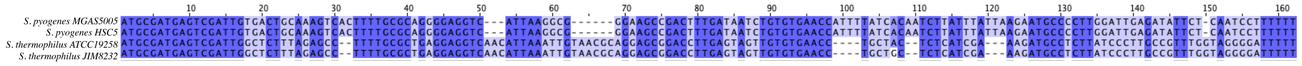
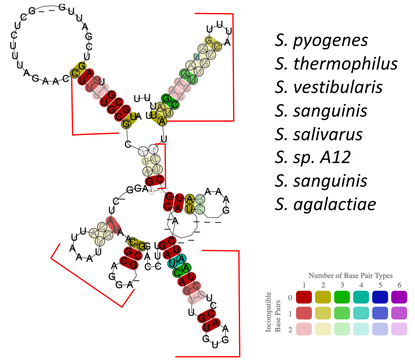


Figure 1. MarS sequences from two different *S. pyogenes* species were compared to their orthologous sequences of AsdS from two different *S. thermophilus* species. The figure was generated using Jalview.[18]

Conservation of MarS/ AsdS[13,14]



- *S. pyogenes*
- *S. thermophilus*
- *S. vestibularis*
- *S. sanguinis*
- *S. salivarus*
- *S. sp. A12*
- *S. sanguinis*
- *S. agalactiae*

Secondary Structure Comparison[13,14]

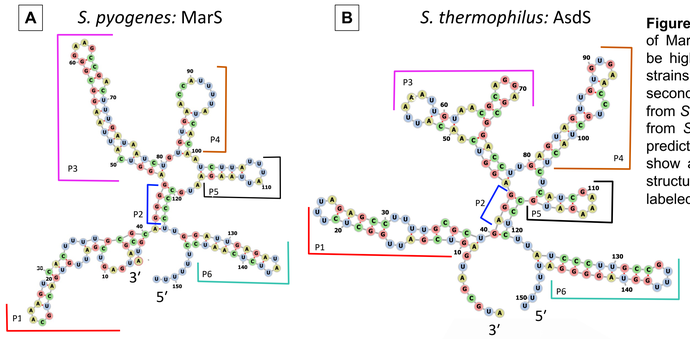


Figure 2. Secondary structure of MarS and AsdS appears to be highly conserved in various strains of *Streptococcus*. The secondary structures of MarS from *S. pyogenes* (**A**) and AsdS from *S. thermophilus* (**B**) were predicted by RNAfold[13] and show a similar arrangement of structured regions, which are labeled P1 through P6.

Genetic Mapping: Regional Analysis[15]

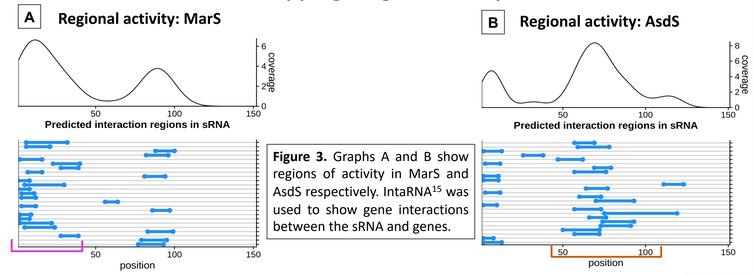


Figure 3. Graphs A and B show regions of activity in MarS and AsdS respectively. IntaRNA[15] was used to show gene interactions between the sRNA and genes.

MarS: highest activity -> P1 regions

P1 Region:
- General Metabolism
- Detoxification
- *Protein secretion
- *Virulence

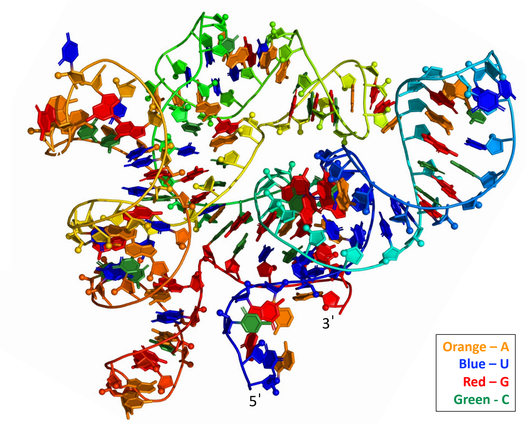
P3-P4 Region:
- Cell wall synthesis
- Virulence
- Amino acid metabolism
- RNA processing

AsdS: highest activity -> P3-P4 regions

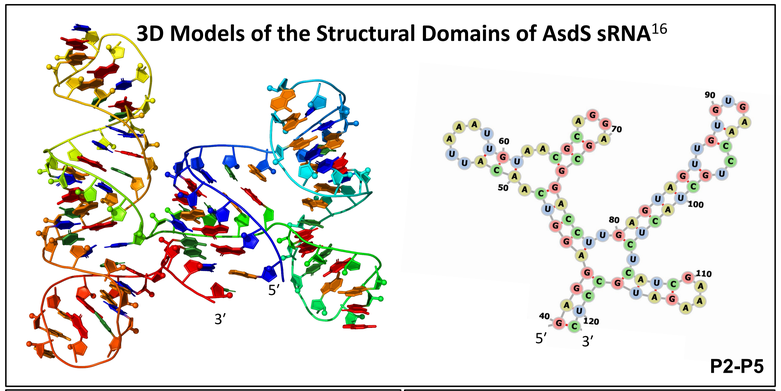
P1 Region:
- General Metabolism
- Detoxification
- Biofilm formation

P3-P4 Region
- Amino acid metabolism
- Transport
- RNA process.
- Homeostasis
- Intraspecies communication

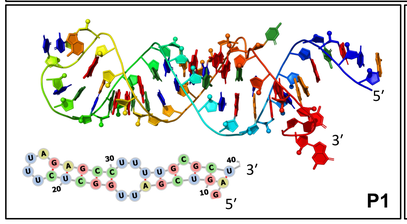
Three-Dimensional Model of P1-P5[16]



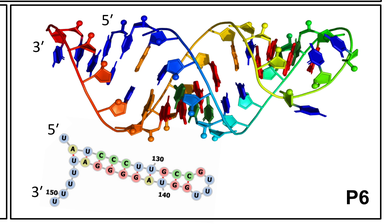
3D Models of the Structural Domains of AsdS sRNA[16]



P2-P5



P1



P6

Synthesis of AsdS RNA from DNA Templates

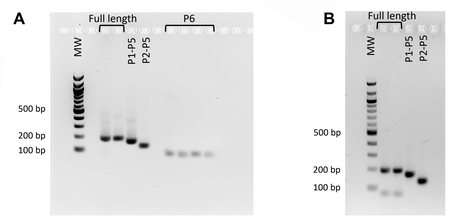


Figure 4. DNA templates for synthesis of AsdS. (4A) PCR products: P1-P6 (full-length), P1-P5, P2-P5, P6. **(4B)** PCR products: P1-P6 (full-length), P1-P5, P2-P5. 1.5% agarose in TAE, 40 minutes at 80V, 6X loading dye, 100 bp Ladder Marker (NEB), stained with ethidium bromide.

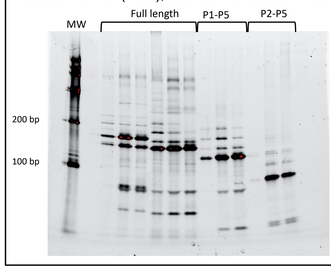


Figure 5. RNA transcripts of AsdS constructs. 4-20% Tris Glycine Gel with Native Tris Glycine buffer (Invitrogen), 40 minutes at 200 V, SYBR Green II dye (Thermo Fisher), RiboRuler RNA ladder (Thermo Fisher).

Conclusions

As a prerequisite for studying small regulatory RNA (sRNA) functions in lactic acid bacteria, we have developed methods in our laboratory to anaerobically grow *S. thermophilus, L. acidophilus,* and *L. bulgaricus* and to extract genomic DNA. Our first study focuses on AsdS sRNA from *S. thermophilus*, which was selected based on sequence homology with MarS from *S. pyogenes*. We were able to isolate the gene for the AsdS sRNA from *S. thermophilus* and design constructs of the structural domains for synthesis of RNA by *in vitro* transcription.

Secondary structure predictions show that the sequence homology between MarS and AsdS extends to the arrangement of the structured regions, P1 through P6. Three-dimensional modeling of the structural domains of AsdS sRNA allows us to see potential sites for interactions with mRNA transcripts of regulated genes. Genetic mapping with InstaRNA suggest that AsdS may regulate various processes including metabolism, detoxification, homeostasis, RNA processing, biofilm formation, and intraspecies communication.

Future Studies

In future work, the predicted secondary structure of the AsdS sRNA will be confirmed by RNase T_1 digest. Differential scanning fluorimetry will be used to find initial conditions for crystallography, with the goal of collecting X–ray diffraction data and determining the three-dimensional structure of AsdS sRNA. Alternative methods, including selective 2′ hydroxyl acylation analyzed by primer extension (SHAPE) and nuclear magnetic resonance (NMR) spectroscopy may be used to elucidate the structure and assay for interactions with mRNAs regulated by AsdS sRNA. Separately, extracellular vesicles (EVs) produced by lactic acid bacteria will be isolated with the intent of identifying the components encapsulated within them with a focus on RNA structures.

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