How Do Small Regulatory RNAs Promote Tooth Decay?

**Professor Grace Spatafora
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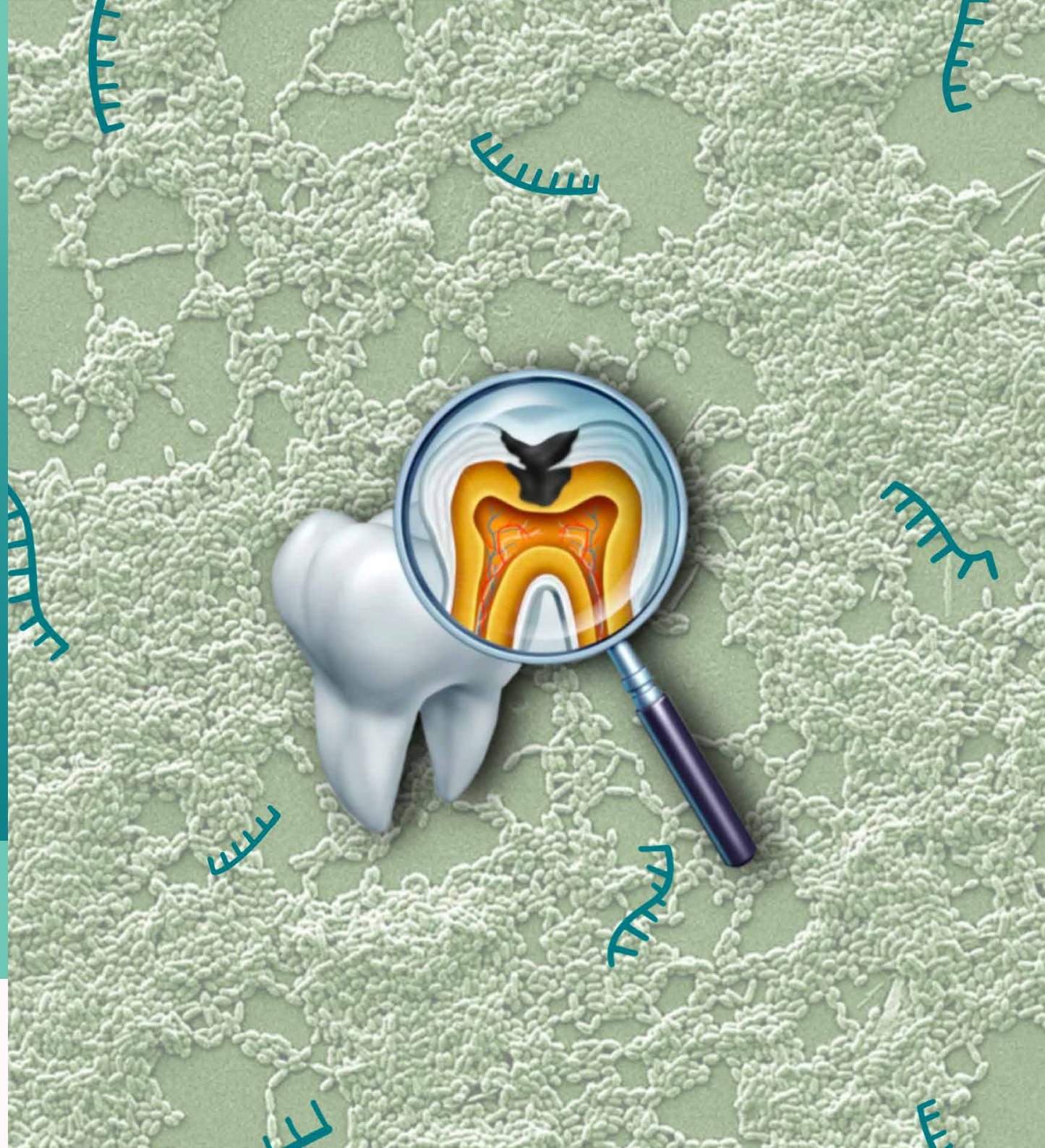
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Tooth decay (known as 'dental caries') is a global health problem. The key pathogen is *Streptococcus mutans*, a hardy tooth-colonising bacterium. An overlooked factor in caries is manganese (Mn²⁺). India Drummond and **Professor Grace Spatafora** of Middlebury College are investigating the effect of Mn²⁺ on *Streptococcus mutans* traits controlled by the SloR metalloregulator and small RNAs. Their research has important implications for dental health.

Formation of Dental Caries

One of the world's most prevalent chronic diseases is tooth decay, also known as caries. This widespread disease is driven by plaque, a multi-species biofilm on the tooth surface. Bacteria colonise teeth by secreting an extracellular polysaccharide (EPS) matrix. With a high-sugar diet, acid-forming bacteria metabolise sucrose into organic acids, and various bacteria thrive under the resulting low-pH conditions. The acidity causes enamel de-mineralisation and decay, leading to caries (cariogenesis). The main caries-forming pathogen is *Streptococcus mutans* (*S. mutans*), an oval-shaped, gram-positive bacterium with EPS-forming, acid-forming, and acid-tolerant traits.

Manganese as a Factor in Caries Formation

There is a growing focus on the importance of metal ions to caries-forming bacteria. Oral bacteria require several trace metals – including iron, zinc, nickel, and manganese – for enzymatic and structural roles. However, ion supply is variable. So, oral bacteria must limit uptake when ions are abundant (during meals) and scavenge ions when scarce (between meals). This is known as nutritional immunity.

An overlooked factor in caries formation is manganese. In fact, manganese is an absolute requirement for Streptococci. As a divalent cation, manganese (Mn²⁺) is a cofactor for several enzymes, including the antioxidant enzyme superoxide dismutase. Moreover, Mn²⁺ is involved in EPS processing needed for biofilm formation. The functions of Mn²⁺ in *S. mutans* are turning out more diverse than was thought possible. India Drummond and colleagues at Professor Grace Spatafora's laboratory group at Middlebury College, Vermont, are investigating Mn²⁺ in *S. mutans*' transcriptional gene regulation.

Probing into the *SloR* Regulon

Manganese is pivotal to an important metalloregulator, *SloR*, in *S. mutans*. *SloR* is a 25-kilodalton manganese-binding protein of the DtxR metalloregulatory family. *SloR* activity depends on the availability of Mn²⁺, so it is regarded as a manganese-centric sensor. *SloR* regulates the transcription of numerous genes, including those dictating nutritional immunity, biofilm formation, and stress tolerance.

Deploying a combination of *in silico* tools and 'wet lab' experiments, Professor Spatafora's group is probing into this diversity of *SloR*-regulated genes – known as the *SloR* regulon. The regulon includes small RNAs (sRNAs) – short (18–500 nt) non-coding transcripts. They discovered that three Mn²⁺ ions bind to *SloR*, allowing dimerisation. The *SloR* dimer N-termini then bind to the DNA, likely in the major groove, at sequences known as *SloR* recognition elements (SRE).

The Role of *SloR* in Manganese Homeostasis

S. mutans has at least two *SloR*-regulated Mn²⁺ uptake systems – *SloABC* and MntH. Modulation of the *SloABC* system by *SloR* is a major focus of the group's research. *SloABC* consists of three components expressed from a single operon: an ATP-binding protein (SloA), integral membrane protein (SloB), and lipoprotein receptor protein (SloC). The *sloR* gene, with its independent promoter, is immediately downstream of the *sloABC* operon. When Mn²⁺ is plentiful (during meals), *SloR* is bound to Mn²⁺ and binds to the sloA promoter, blocking RNA polymerase and repressing transcription. When Mn²⁺ is scarce (between meals), *SloR*, no longer bound to Mn²⁺, disengages from the *sloABC* promoter, allowing access to RNA polymerase and consequent transcription. The
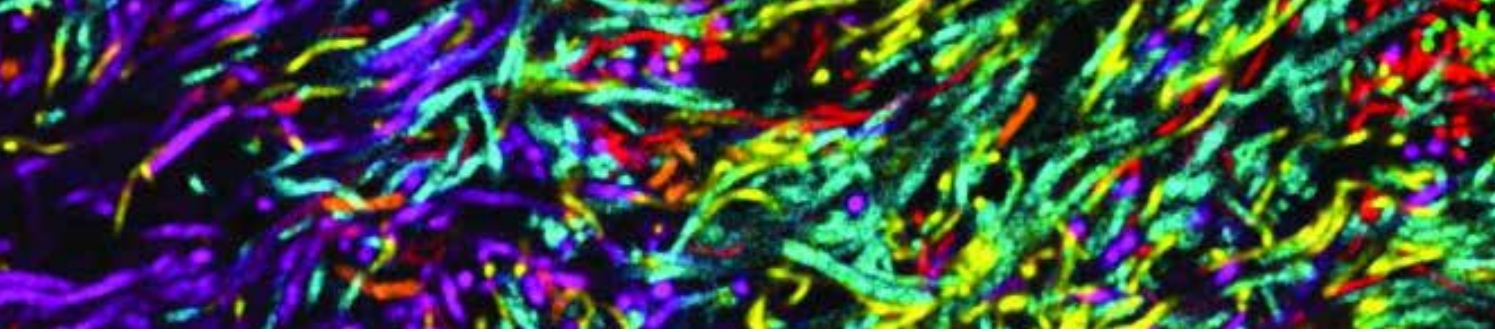
SloABC manganese-import machinery is then expressed, enabling the scavenging of Mn^{2+}. The group discovered three SREs in the sloA promoter region, indicating that *SloR* binds here.

Investigating sRNA-*SloR* Interactions

Although well-studied in eukaryotes, sRNAs are under-researched in prokaryotes. Emerging research shows that sRNAs are regulators of fitness and virulence, with particular relevance to pathogenic bacteria. sRNAs can enhance or repress gene expression via complementary base pairing, often at the 3'- or 5'-untranslated region. From a bacterial standpoint, sRNAs represent an adaptive, low-energy control system, as they are usually untranslated and can facilitate rapid responses. The group is exploring *S. mutans*' surprisingly diverse 'sRNAome.'

The Spatafora lab hypothesised that *S. mutans*' ability to cause cavities is, in part, the result of interactions between sRNAs and *SloR*-mediated gene expression. They grew two *S. mutans* strains, a wild-type (UA159) and a mutant *SloR*-deficient strain (GMS584), under high vs low manganese conditions. With RNA sequencing, they generated sRNA libraries, and compared transcript expression levels between wild-type vs *SloR*-deficient strains, and between high vs low manganese (differential expression). From these transcripts, they identified two *S. mutans* small RNAs of interest – SmsR1532 and SmsR1785. SmsR1532 is a 30-nt sRNA in an intergenic region downstream of *sloABC*R (i.e., the *sloABC* operon with adjacent *sloR* gene). SmsR1785 is a 66-nt sRNA mapping to genes encoding a toxin-antitoxin system.

## Genomics Reveal Different Ancestral Origins

The group undertook *in silico* genomics analyses of *SloR*, SmsR1532 and SmsR1785 – phylogenetics (using Anvi'o), NCBI BLASTN searching, and covariance modelling (using LocARNA). Like a time machine, these analyses reveal evolutionary history – as well as family relationships! They found that *SloR* is highly conserved across the *Streptococcus* genus. SmsR1532 emerged more recently as a common ancestor of *S. mutans* and S. troglodytae. In contrast, the analyses revealed no clear inheritance of SmsR1785, suggesting that it was acquired via horizontal gene transfer.

Elucidating Transcription with Northern Blotting

Are the sRNAs transcribed from an independent promoter (Type I)? Or are they co-transcribed as one multi-gene transcript (Type II)? The group carried out northern blotting to find out. The results revealed Type I precursor transcripts of both SmsR1532 (106-nt) and SmsR1785 (73-nt and 70-nt), indicating that they undergo post-transcription processing to yield their smaller final forms (30 and 66 nt, respectively).

Promoter-proximal and Promoter-distal – What's the Difference?

Differences in sRNA expression between the *SloR*-deficient and wild-type strains showed that *SloR* repressed SmsR1532 but enhanced SmsR1785 transcription. But how? Electromobility shift assays revealed *SloR* binding to SREs in the promoter regions of both sRNAs – showing that *SloR* binds directly to their promoters. The SmsR1532 SRE is promoter-proximal, overlapping with the -10 promoter element. This location indicates that *SloR* binds here and blocks RNA polymerase, preventing transcription. In contrast,
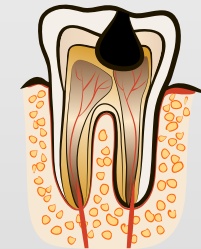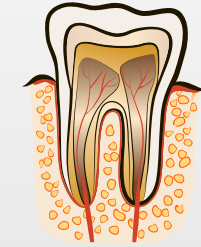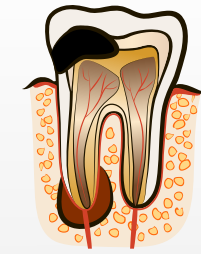
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the SmsR1785 SRE is promoter-distal, being 95 nt upstream of the transcription start site. SloR evidently encourages transcription of SmsR1785, and the group speculates that DNA bending is involved.

Predicting sRNA Behaviour with Bioinformatics

The group used *in silico* tools to predict sRNA behaviour. This analysis involved predicting sRNA secondary structure (using mFold V3.6), mRNA binding targets (using IntaRNA and TargetRNA2), and pathways potentially involving the two sRNAs (using KEGG Mapper). SmsR1532 and SmsR1785 were both predicted to form hairpin structures. Predicted targets of SmsR1532 included *sloABC*, and genes involved with sugar transport and acid tolerance. Among the predicted targets of SmsR1785 were the Fst-Sm toxin, genes involved with hydrophobicity, intracellular polysaccharides, and acid stress response. Interestingly, both SmsR1532 and SmsR1785 were predicted to target genes involved in oxidative stress, as well as EPS and biofilm formation – traits associated with *S. mutans* hardiness.

Why is This Important?

India Drummond, Professor Spatafora, and their group have demonstrated manganese-sensing by *S. mutans* via *SloR* and sRNAs. This work provides unique insights into phenotypes involved in cariogenesis. Using 'systems biology' approaches, they want to delve deeper into *S. mutans*' *SloR* regulon and sRNAome – with implications for dental health. While toothpaste and dental hygiene go so far, there is a need for effective anti-caries therapeutics. Professor Spatafora's research suggests that *SloR* is a good therapeutic target. *SloR* is expressed in bacteria and not humans, and has a druggable binding pocket. As a next step, the lab wants to carry out rational drug design to identify potential therapeutics. This research would involve investigating small molecules that bind *SloR* and facilitate its ability to repress *S. mutans* virulence.

MEET THE RESEARCHERS

Professor Grace Spatafora,
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Professor Grace Spatafora received a BS in Life Sciences from Duke University in 1980, a Master of Science from the University of Richmond in 1983, and a PhD in Biology at St Louis University in 1988. She has held academic appointments at Middlebury College since 1992, where she was appointed Distinguished Professor (endowed Chair) in 2008 and the Dean of Curriculum in 2020. Professor Spatafora's research centres around the genes involved in the Streptococcus mutans SloR metalloregulome and their involvement in dental caries. She has been supported by funding from the National Institutes of Health continuously throughout her career.

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India Drummond is a laboratory technician and post-bac associate in Professor Spatafora's Laboratory at Middlebury College. Her research focuses on investigating the role of sRNAs in the SloR regulon of Streptococcus mutans. She entered into this line of research during her senior thesis project attached to her Molecular Biology and Biochemistry major at Middlebury College, which she was awarded in 2021.

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FURTHER READING

IY Drummond, A DePaolo, M Krieger M, et al., Small regulatory RNAs are mediators of the *Streptococcus mutans* SloR regulon, *Journal of Bacteriology*, 2023, 205(9), e0017223. DOI: https://doi.org/10.1128/jb.00172-23

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