Defining the Role of RNA Secondary Modifications in the Response of Microbial Pathogens to Chemical Mediators of Inflammation

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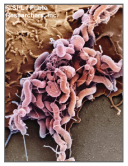
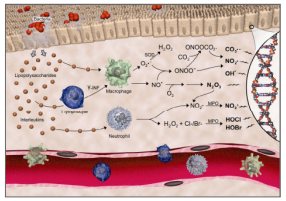
Abstract

RNA was largely ignored for much of the 20th century, including the initial phases of the development of systems-level or "omic" biology. Its transient, chemically unstable nature makes it difficult to isolate and necessitates extensive control against artifacts. Thus, the incredible diversity of RNA structures remains largely unexplored. By utilizing ultra-high sensitivity analytical methods, we are expanding this field by defining the nature and function of novel RNA species in human pathogens, beginning with *Helicobacter pylori* as a general model of an infectious species.

Helicobacter pylori & Chronic Inflammation



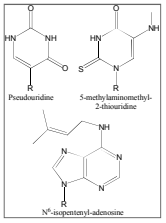
Helicobacter pylori (right) is a Gram-negative, gut-colonizing pathogen that is responsible for most cases of peptic ulcers. It is the world's most common infection, with an estimated 3 billion people (roughly half the global population) infected, and infection rates as high as 90% in some countries in the developing world. *H. pylori* is highly adapted to the environment of the human stomach, secreting large amounts of urease to neutralize local pH and relying on its flagella and helical shape to burrow into the muccoid lining. Upon colonization, *H. pylori* induces gastritis, leading to an innate immune response through recruitment of macrophages and neutrophils to the site of infection. *H. pylori* evades this

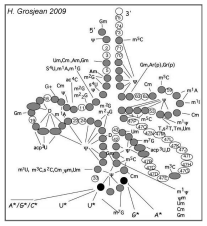


response through a host of protective enzymes, including catalase, superoxide dismutase, and various peroxiredoxins. Despite this response, most infections are asymptomatic and undiagnosed, and persist untreated for the life of the host. As illustrated to the left, the resulting chronic inflammation results in local accumulation of a host of reactive oxygen and nitrogen species, which can invade and damage surrounding tissues over time. This mechanism is thought to underlie the association between *H. pylori* infection and gastric cancer, for which it is listed as a Group 1 carcinogen by the International Agency for Research on Cancer. Its prevalence and potentially devastating consequences make *H. pylori* a major public health concern, and the increasing appearance of antibiotic resistant strains underscores the need for better understanding and novel therapeutic targets. This research seeks to address these concerns through quantitative analysis of RNA secondary modifications in *H. pylori*.

Modified RNA Nucleosides

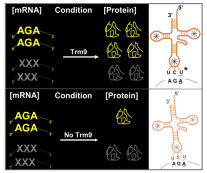
RNA research has exploded in recent years, unveiling an incredible range of functions, including catalysis, gene regulation, and tissue differentiation. Of special interest is the continued discovery of new noncanonical nucleosides, which are synthesized by enzymatic modification of the five standard nucleosides. Such secondary modifications range in complexity from simple isomerizations, methylations, and oxidations, up to the addition of chains or rings with multiple heavy atoms. Three common noncanoical nucleosides are shown to the right.





To date, more than 100 modifications have been identified from all domains of life, with any given organism typically harboring 20-30 modifications. The vast majority of these have been found in transfer RNA, where they have long been known to play a role in maintenance of the secondary structure. As seen to the left, modifications can occur almost anywhere in the tRNA structure, with a typical tRNA molecule having 8 sites modified (though this is highly variable). Despite the ubiquity of these modifications, no organism has had its complete spectrum of modified nucleosides characterized in a rigorous, quantitative manner. Applying such analysis to human pathogens could provide insights relevant to diagnosis and treatment.

RNA Modifications in Bacterial Stress Response



A new model has emerged in which tRNA modifications play an important role in translational control and stress response. Our collaborator Prof. Thomas Begley showed that in *S. cerevisiae*, loss of tRNA modifying enzyme Trm9 induces sensitivity to alkylation stress. This enzyme catalyzes a modification that frequently occurs at the wobble position of one tRNA(Arg). This modification enhances the affinity for the AGA codon, which was found to be enriched in genes for stress response proteins. In the presence of the modification, these transcripts are "pulled to the front of the line" and selectively translated, as shown to the right. Loss of Trm9 eliminates this bias and induces sensitivity to the stress. Given the number and ubiquity of known modifications, it is unlikely that this mechanism is unique to *S. cerevisiae*. Characterizing similar systems in other organisms, especially human pathogens, could lead to better understanding of disease processes and guide the development of novel tools for diagnosis and treatment.

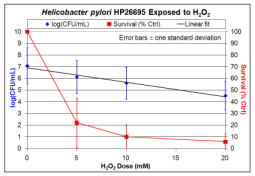
Hypothesis & Experimental Design

We hypothesize that a stress response mechanism based on translational control by modification of tRNA nucleosides exists in *H. pylori*, and that this pathway may represent a novel method of evading the immune system and/or developing antibiotic resistance. Toward the goal of elucidating such pathways, we are characterizing the spectra of modified nucleosides in response to various chemical mediators of inflammation, using the method described below.

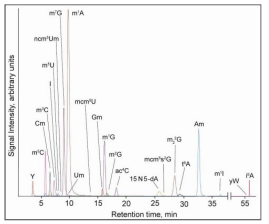
1	Generate dose-response curves for *H. pylori* HP26695 (sequenced strain) exposed to common chemical mediators of inflammation: H_2O_2, HOCl, NO, $ONOO^-$
2	Expose *H. pylori* HP26695 to toxicants at concentrations yielding 20% and 80% kill
3	Isolate tRNA with Invitrogen PureLink miRNA kit, with added antioxidants butylated hydroxytoluene (BHT), tetrahydrouridine (THU), and deferoxamine (dfx)
4	Determine tRNA quantity and quality using Agilent 2100 Bioanalyzer
5	Digest tRNA to nucleosides with nuclease P1, alkaline phosphatase, and phosphodiesterase, with added BHT, THU, dfx, and deaminase inhibitor coformycin
6	Reversed-phase HPLC with Agilent 1200 series HPLC and Thermo Hypersil Gold aQ C18 column coupled to tandem-MS with Agilent 6410 triple quadrupole
7	Record relative abundance of each modification for each toxicant, use principal component analysis to find candidate critical modifications correlated with stress

H_2O_2 Exposure Results

H. pylori was exposed in triplicate to H_2O_2 in order to generate the dose-response curve seen to the right. The results show profound sensitivity to H_2O_2, which is unexpected given the presence of catalase and the previous reports in literature. Exposures were performed on cultures obtained from frozen lab stocks, as well as from fresh ATCC stock for validation. Results were independently confirmed by a colleague, giving us confidence in the results. Using these data, we defined a low (~20%) toxicity dose of 2 mM and a high (~80%) toxicity dose of 5 mM, which was used for subsequent exposures to determine the changes induced in the spectrum of modified nucleosides.

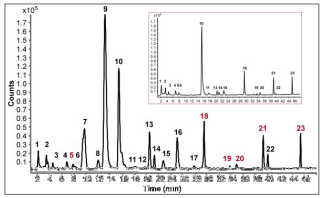


Chromatographic Method Development



A robust and sensitive reversed-phase HPLC method for the separation of modified ribonucleosides has been developed and optimized by various members of our group. Using this method, Clement Chan was able to achieve near-baseline separation of the 23 nucleoside modifications in *S. cerevisiae*, as seen to the right. This created the first complete catalog of tRNA modifications in an organism and demonstrated the feasibility of this method as a general platform for investigation. The same analysis has been applied by other members of our group to a variety of species of *Helicobacter*, *Mycobacteria*, and *Plasmodium* with similar results.

Novel *H. pylori* Modifications



Using the method described above, Dr. Kok Seong Lim from our group was able to achieve near-baseline separation of the 23 modified nucleosides in *H. pylori*, as seen to the right. This is a composite of two chromatograms; the peaks in red were not seen in the large chromatogram and were overlaid from the inset graph.

Of the 23 species identified, 17 did not occur in human TK6 cells, and 4 were only identified in the literature only in bacteria. Of special interest are 4 species whose exact mass did not match any reported nucleoside modification. These were reproducibly identified with consistent retention times in samples from *H. pylori* as well as *H. cinaedi* and *H. hepaticus*, as shown below. These species are noteworthy not only because they represent potentially novel nucleoside modifications, but also because of their potential as *Helicobacter*-specific markers. Experiments are currently underway to structurally characterize these species.

Novel Modified Nucleoside Candidates from *Helicobacter pylori*

Mass	m/z	R.T. (min)	*H. pylori*	*H. cinaedi*	*H. hepaticus*
127.0202	128.0275	7.99	+	+ + +	+ +
379.1037	380.1110	19.59	+ +	+	+ +
380.1479	381.1552	29.62	+	+	+
485.1187	486.1260	45.34	+ +	+	+

Future Directions & Conclusions

Exposure conditions for H_2O_2 have been experimentally validated, and forthcoming sample analysis will shed light on the role of modified nucleosides in survival. Exposures are ongoing to determine the dose-response relationship for HOCl, and protocols are being developed for the delivery of NO and $ONOO^-$, which pose special challenges as they are not simple aqueous solutions. Finally, a project to identify and quantify RNA modifications in biological fluids is currently in method development.

The field of RNA research has exploded recently, and each new discovery brings more questions. We are combining ultra-high sensitivity analytical techniques with hypothesis-driven experiments to define the structure and function of novel RNA species in human pathogens. Our results have the potential to have positive impacts on basic science, public health, and clinical medicine.

Acknowledgements

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