Identification and Biosynthesis of Thymidine Hypermodifications in the Genomic DNA of Widespread Bacterial Viruses

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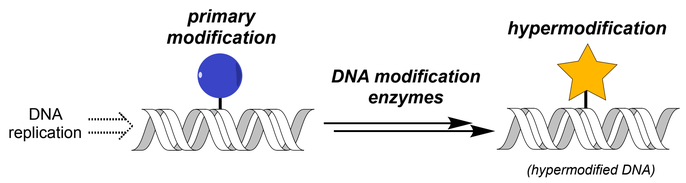
Abstract

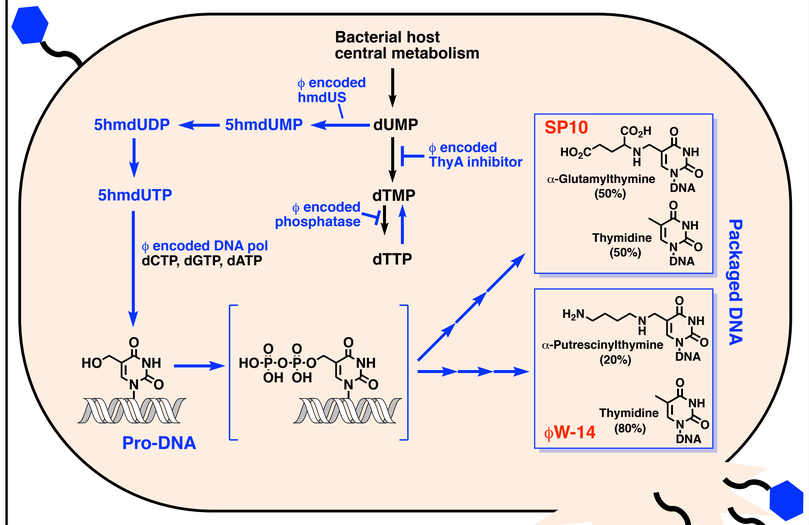
Certain viruses of bacteria (bacteriophages) enzymatically hypermodify their DNA to protect their genetic material from host restriction endonuclease-mediated cleavage. Historically, it has been known that virion DNAs from the Delftia phage ΦW-14 and the Bacillus phage SP10 contain the hypermodified pyrimidines α-putrescinylthymidine and α-glutamylthymidine, respectively. These bases derive from the modification of 5-hydroxymethyl-2'-deoxyuridine (5-hmdU) in newly replicated phage DNA via a pyrophosphorylated intermediate. Like ΦW-14 and SP10, the *Pseudomonas* phage M6 and the *Salmonella* phage VilI encode kinase homologs predicted to phosphorylate 5-hmdU DNA but have uncharacterized nucleotide content [Iyer et al. (2013) Nucleic Acids Res 41:7635–7655]. We report here the discovery and characterization of two bases, 5-(2-amino-ethoxy)methyluridine (5-*Ne*OmdU) and 5-(2-aminoethyl)uridine (5-*N*edU), in the virion DNA of VilI and M6 phages, respectively. Furthermore, we show that recombinant expression of five gene products encoded by phage VilI is sufficient to reconstitute the formation of 5-*Ne*OmdU *in vitro*. These findings point to an unexplored diversity of DNA modifications and the underlying biochemistry of their formation.

Phage DNA Hypermodification



- DNA contains non-canonical nucleotides with primary and/or secondary modification to the nucleobase (relative to "canonical" A, G, C, and T).
- Eg. substitution of C with hmdC followed by glycosylation, as seen in phage T4.

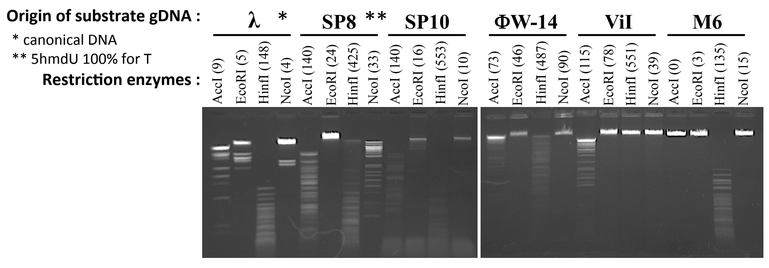
Metabolism of thymidine hypermodification



- Two stages of the biosynthesis: polymerase incorporate 5hmdU to pro-DNA, then hypermodifying enzymes further process 5hmdU-DNA to final hypermodified base.
- Examples: *Delftia* phage ϕW-14 (20% α-putrescinylthymidine replacing dT in the gDNA); *Bacillus* phage SP10 (50% dT replaced by α-glutamylthymidine in the gDNA).
- Base hypermodification reviewed in Weigele & Raleigh, *Chemical Review*, **2016**.

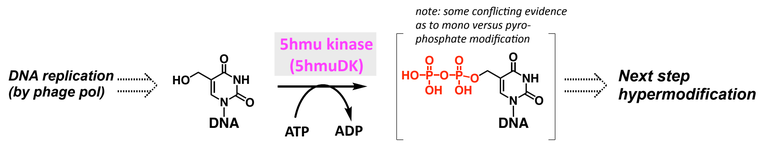
Hypermodified DNA resistant to restriction endonucleases in vitro

- **Function of hypermodification:** protect phage DNA from cleavage by the host's restriction endonuclease-based "innate immune system".

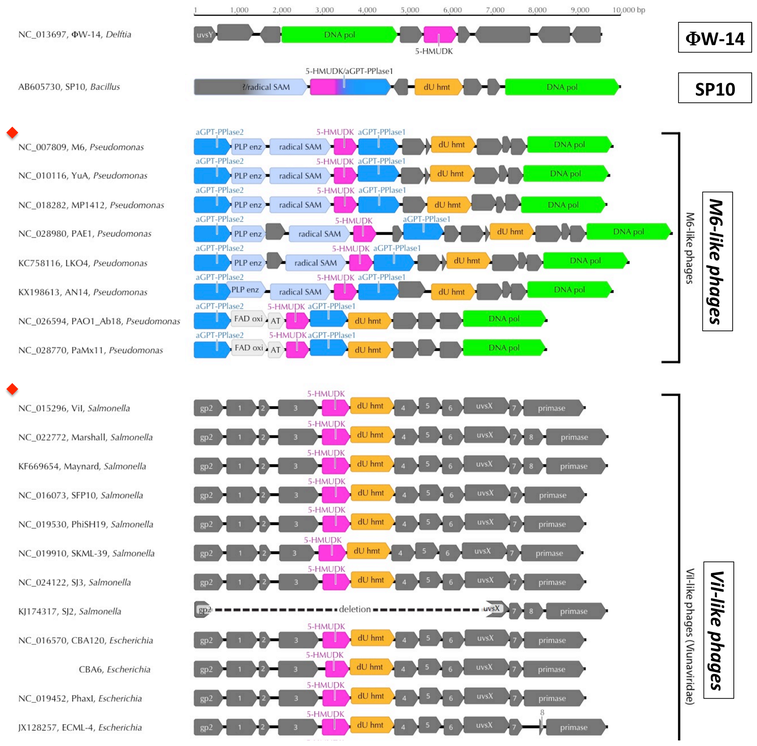


- **Fig.** Restriction digests of thymidine-modified and unmodified bacteriophage gDNAs. Phage DNAs consisted of modified nucleotide appear higher degree of resistance against restriction endonuclease digestion. Predicted numbers of RE cut sites in DNA is noted in parentheses.

5-Hydroxymethyluridine DNA Kinase (5-HMUDK) encoded in diverse phage genomes



- First step of phage DNA thymidine hypermodification is phosphorylation of DNA 5hmdU.
- DNA hypermodification derives from a transient intermediate being 5-[(hydroxymethyl)-O-pyrophosphoryl]uracil where this transformation is catalyzed by phage encoded **5-hydroxymehtyluridine DNA kinase** (5-HMUDK).



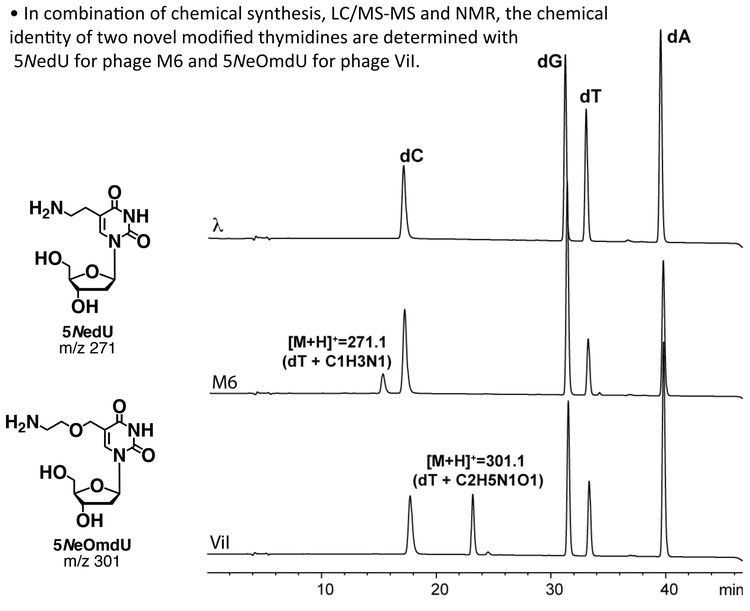
- **Figs.** *5-HMUDK* genetic neighborhood of ΦW-14, SP10, M6-likephages and VilI-like phages.

- Bacteriophages ΦW-14 and SP10 encode a 5-HMUDK, implicated as an intermediate in the biosynthesis of hypermodified thymidine derivatives found in their DNA.
- We hypothesized that other 5-HMUDK encoding phages would similarly contain thymidine hypermodifications in their DNAs.
- Genome-wide analysis of phages encoding 5-HMUDK show many share a high degree of conservation in overall gene content and organization.
- Four distinct groups of phage revealed: SP10 and ΦW-14 are unique; VilI-like phages of *Enterobacteriaceae,* and M6-like phages of *Pseudomonas*.

Discovery of two novel DNA thymidine modifications in phage gDNA

- HPLC-MS analysis (**Fig.**) of bacteriophage M6 and VilI gemonic DNA composition. Aside from four canonical nucleosides dC, dG, dT and dA, M6 and VilI show a fifty major peak corresponding to the hypermodified base.

- In combination of chemical synthesis, LC/MS-MS and NMR, the chemical identity of two novel modified thymidines are determined with 5*N*edU for phage M6 and 5*Ne*OmdU for phage VilI.



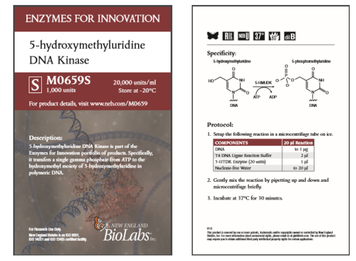
Toward deciphering the biochemistry of DNA thymidine base modification

Modification observed :

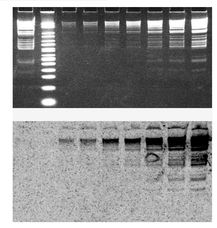
	putative activity/ function \ Phage	ΦW-14 phage	SP10 phage	VilI phage	1.255.O phage	M6 phage
Targeting on PN 5hmdU or 5pmdU	kinase (I)	✔	✔	✔	✔	✔
	kinase (II)		✔	✔	✔	
	Mag2-like (I)	✔	✔	✔	✔	✔
	Mag2-like (II)	✔		✔	✔	✔
Targeting on modifying group	PLPDE			✔	✔	✔
	rSAM enzyme		✔			✔
	glutathionylspermidine syntase	✔				

- The table summarize genes unique to thymidine hypermodifiying bacteriophages.
- The gene candidates are predicted by bioinformatic analysis using comparable analysis, genome subtraction profiling, gene neighboring association, protein folding/domain structural association (Phyre2, Swiss Model)

Biochemical characterization of 5-HMUDK activity

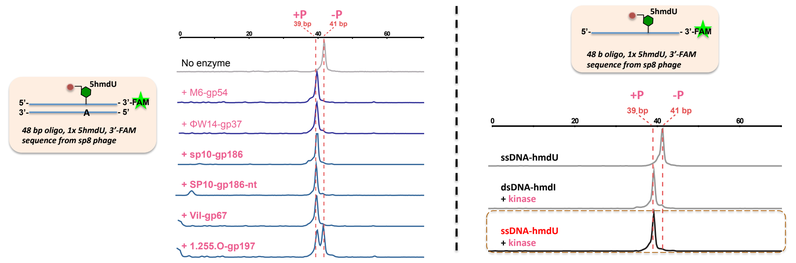


- Commercially available as NEB product in "**Enzyme for Innovation**" category.



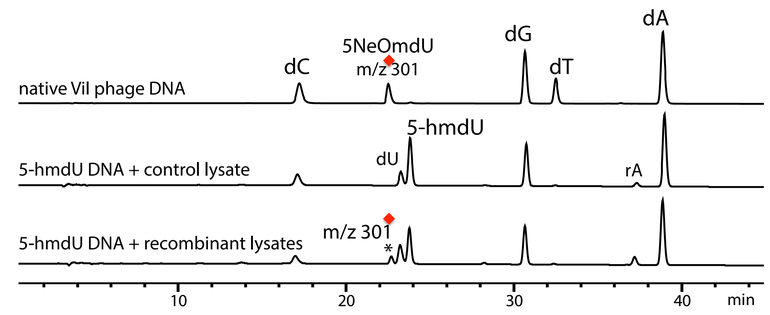
increasing amounts of XbaI predigested hmdU DNA (SP8) then treated w/ 5-HMUDK and [γ-32P]-ATP

- Phage encoded 5-HMUDK – 5-hydroxymehtyluridine DNA kinase.
- 5-HMUDK enzymatic assays show that 5-HMUDK is a 5-hydroxymethyluracil specific kinase transferring the γ-phosphate of the ATP to DNA (**right panel**), active on the 5hmdU gDNA or oligo substrates, though a single phosphate is transferred, not a pyrophosphate.



- 5hmdU oligo substrate shifts 2 bp after 5-HMUDK treatment in capillary electrophoresis assay.
- Assayed of 5-HMUDK homologues in five different thymidine modifying phages found that at least one active kinase gene product in each phage

In vitro reconstitution of 5-*Ne*OmdU from recombinant bacterial lysates



- **Fig.** Substrate DNA containing 5-hmdU (SP8 phage gDNA) incubated in mixed lysates from *E. coli* expressing VilI gp67, gp160, gp226, gp243, and gp247 contains a nucleoside product (301; denoted by the asterisk) of identical mass and retention time as the native modification of VilI.

Summary

- Two bases discovered: 5-(2-amino-ethoxy)methyluridine (5-*Ne*OmdU) and 5-(2-aminoethyl)uridine (5-*N*edU), in the virion DNA of VilI and M6 phages, respectively, where both modifications are derived from 5-hydroxymethyl-2'-deoxyuridine (5hmdU) on DNA with the enzyme hydroxymthylthymidine DNA kinase (5-HMUDK) catalyzing first step of biosynthesis.
- Recombinant-produced phage 5-HMUDK homologs were demonstrated enzymatically active on transferring γ-phosphate of ATP to 5hmdU substrate *in vitro*.
- Bioinformatic analysis of the *5-HMUDK* across various phages arose two new distinct groups with 5hmdU derived hypermodification within the phage DNA is observed. The modification genes are postulated and assayed for their activities.
- Hypermodification can be reconstituted in vitro from lysates containing five heterologously expressed phage genes.

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