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# *Mycolicibacterium smegmatis* N-methylhydantoinase modulates ClpC1P1P2 protease function

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## Abstract

**Tuberculosis is currently a leading cause of death from an infectious agent, second only to COVID-19. While advances in public health have contributed to a reduction in tuberculosis cases, the prevalence of multidrug-resistant *Mycobacterium tuberculosis* (*Mtb*) (MDR-TB) infections has created an urgent need to exploit novel drug targets. One such target is the ClpC1P1P2 protease, which degrades folded cytosolic proteins through the cooperation of the ATP-dependent unfoldase ClpC1 and the ClpP1P2 peptidase. Both protease components are strictly essential for *Mtb* viability and are validated therapeutic targets. However, efforts to develop anti-*Mtb* compounds are constrained by a limited understanding of Clp protease function and essentiality. Thus, it is crucial to identify physiological substrates and pathways regulated by this protease. More so, there is a related need to characterize regulators to understand the intricacies of modulation of the ClpC1P1P2 protease. Currently only one adaptor or regulator of ClpC1 is known, which is the adaptor ClpS.**

**In this study, we characterized N-methylhydantoinase as a novel negative regulator of the unfoldase ClpC1. This protein, which appears in a potential bicistronic operon with 5-oxoprolinase, a Clp protease substrate, affects the ATPase and unfoldase activities of ClpC1. It also impacts its ability to stimulate the peptidase activity of ClpP1P2, thus inhibiting the proteolytic activity of the ClpC1P1P2 protease. This lays the groundwork for future efforts to expound on the roles potentially played by negative Clp protease regulators in mycobacteria. Future work will be required to delineate the exact mechanism of regulation, and how this activity contributes to the physiology of mycobacteria and other actinobacterial species.**

## N-methylhydantoinase bicistronic operon

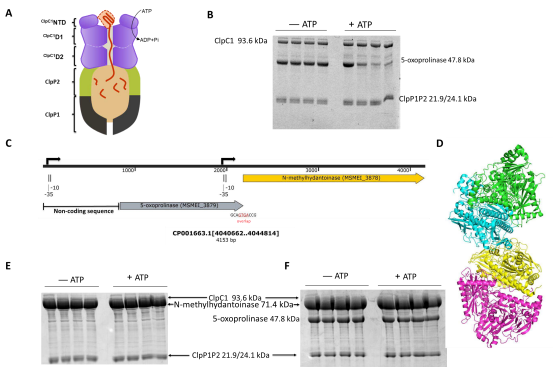



**Figure 1. *Msm* N-methylhydantoinase (Msmei_3878) gene exists proximal to ClpC1P1P2 substrate 5-oxoprolinase (Msmei_3879) in a potential operon. A)** The mycobacterial ClpC1P1P2 protease architecture, comprising of the hexameric unfoldase ClpC1 assembled with the tetradecameric peptidase ClpP1P2. **B)** 5-oxoprolinase (Msmei_3879; 47.8 kDa) is a ClpC1P1P2 substrate (Ogbonna et al., (2022). **C)** *Msm* genome (CP001663) open reading frame (ORF) showing coding sequence (CDS) of 5-oxoprolinase (Msmei_3879) and N-methylhydantoinase (Msmei_3878). **D)** N-methylhydantoinase and 5-oxoprolinase had homology to two entities in the crystal structure of ATP-dependent caprolactamase from *Pseudomonas jessenii* (PDB ID: 6YRA) (Marjanovic *et al.*, 2021). **E)** N-methylhydantoinase (Msmei_3878; 71.4kDa) was not proteolyzed by ClpC1P1P2 protease. **F)** The presence of an equimolar amount of N-

## Nmh inhibits ClpC1P1P2 protease activity

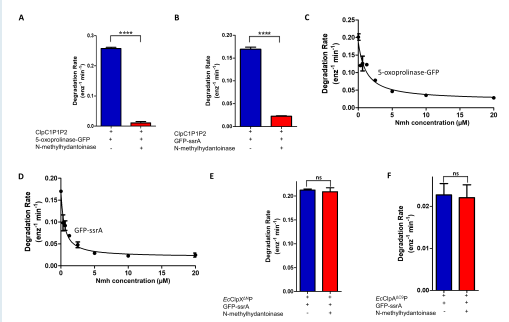



**Figure 2: N-methylhydantoinase is a novel independent ClpC1P1P2 regulator.** N-methylhydantoinase inhibits ClpC1P1P2-mediated degradation of **A)** 5-oxoprolinase-GFP and **B)** GFP-ssrA. IC50 plots were generated for the inhibition of ClpC1P1P2-mediated degradation of **C)** 5-oxoprolinase-GFP **D)** GFP-ssrA, in the presence of a two-fold dilution series of N-methylhydantoinase. IC50 values of 1.12 ± 0.56 μM and IC50 = 0.46 ± 0.11 μM were obtained for 5-oxoprolinase-GFP and GFP-ssrA respectively. **(E)** EcClpXΔNP and **(F)** EcClpAΔC9P degraded GFP–ssrA in combination with or EcClpP at similar rates with (red) or without (blue) N-methylhydantoinase. **** and ns represent p values < 0.01% and > 5% (not significant), respectively.

## Nmh attenuates ClpC1-dependent peptidase stimulation

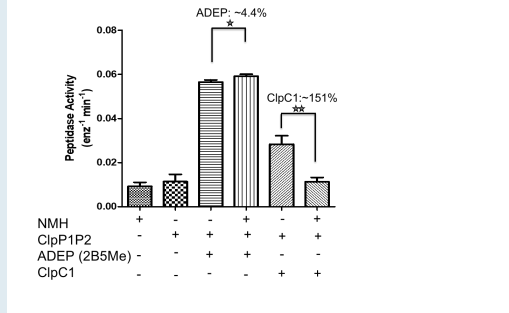
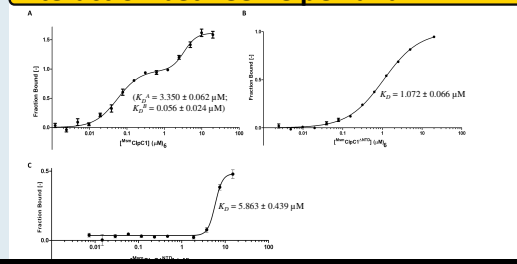



**Figure 4: Mycobacterial ClpP1P2 peptidase activity stimulated by ClpC1 and ADEP.**

## Interaction between ClpC1 and Nmh




## ClpC1 ATPase activity is modulated by Nmh

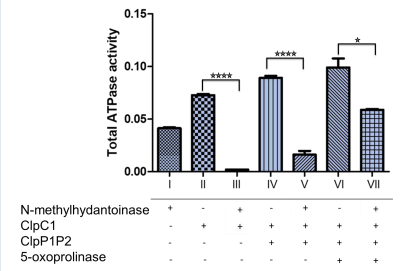



**Figure 3: N-methylhydantoinase modulates ClpC1 ATPase activity.**

## Nmh effect on ClpC1 unfoldase activity

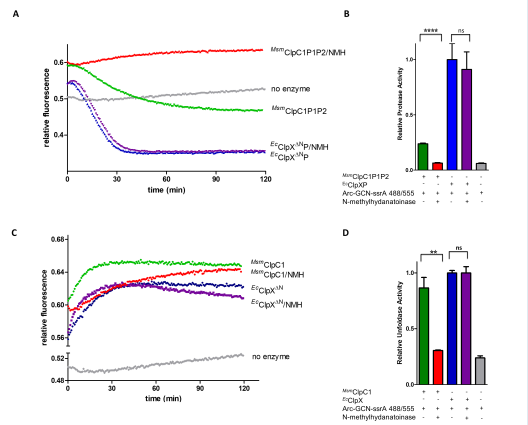



**Figure 5: *Mycolicibacterium smegmatis* ClpC1 unfoldase activity declines in the presence of Nmh.**

**A)** Protease assays of Arc-Gcn4-ssrA dimers as catalyzed by $^{Msm}$ClpC1P1P2 and $^{Ec}$ClpX$^{\Delta N}$P. **B)** Bar graph showing relative protease activity of $^{Msm}$ClpC1P1P2 — with (red) and without (green) Nmh — and $^{Ec}$ClpX$^{\Delta N}$P with (purple) and without (blue) Nmh. **C)** Unfolding assay of Arc-Gcn4-ssrA by $^{Msm}$ClpC1 in the absence (green) and presence (red) of Nmh (10 μM). *E. coli* ClpX$^{\Delta N}$ was used as a positive control in the absence (blue) and presence (purple) of Nmh. **D)** Relative rate of unfolding Arc-Gcn4-ssrA dimers by $^{Msm}$ClpC1 (in the absence (green) and presence (red) of Nmh) and by *E. coli* ClpX$^{\Delta N}$ (with (purple) and without (blue) Nmh .

## Conclusion

- N-methylhydantoinase (Nmh) was identified in the interactome of *Msm* ClpC1.
- Nmh exists in a potential bicistronic operon with 5-oxoprolinase, a known ClpC1P1P2 substrate.
- Nmh negatively regulates ClpC1P1P2 protease-mediated degradation of protein substrates
- On closer examination, Nmh inhibits ClpC1 ATPase, unfoldase and peptidase-stimulatory activities
- Direct binding between Nmh and ClpC1 NTD and core.
- Novel therapeutics mimicking Nmh ability to shut down the essential Clp protease are an interesting possibility.

## References