Structural and kinetic evidence of maleamate amidohydrolase (NicF) in the *Bacillus niacini* nicotinic acid catabolic pathway

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Background and Significance

- *N*-Heterocyclic aromatic compounds (NHACs) are pervasive environmental pollutants with structures common in herbicides, pesticides, and some pharmaceuticals. NHACs can possess carcinogenic and mutagenic properties, so it is important that strategies are developed to remove them from the environment. One promising method is bioremediation, the use of bacteria to degrade pollutants. Multiple bacterial species catabolize the NHAC nicotinic acid (NA), so its breakdown is used as a model system for NHAC degradation.

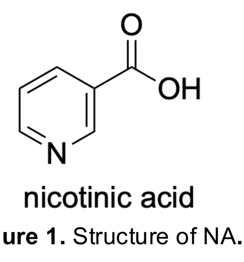


Figure 1. Structure of NA.

- *Bacillus niacini* is a soil-dwelling bacterium which degrades NA. The identification of a gene cluster upregulated in the presence of NA suggests that this operon is responsible for NA catabolism in *B. niacini*.

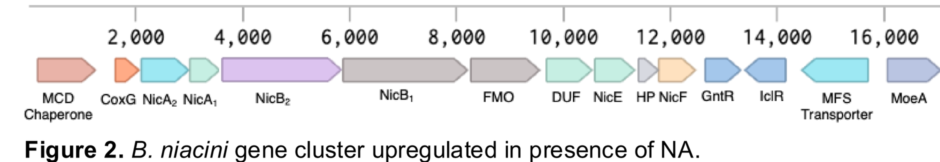


Figure 2. *B. niacini* gene cluster upregulated in presence of NA.

- The functions of several of these genes and the enzymes for which they encode remain unclear. Characterization of these enzymes and their substrates will provide a deeper understanding of the chemistry which bacteria utilize to degrade NA. This will better define the scope of NHACs broken down through bioremediation and will provide insight into potential enzyme bioengineering strategies.
- The metabolites identified thus far share similarity to those found in the NA catabolic pathways of *Pseudomonas putida*, *Bordetella bronchiseptica* and *Aspergillus nidulans*.

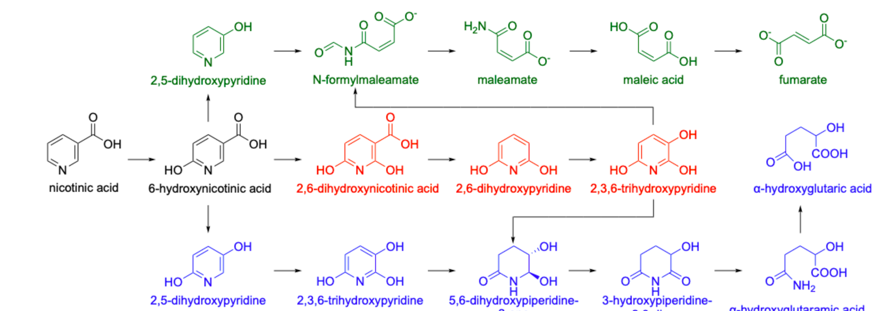
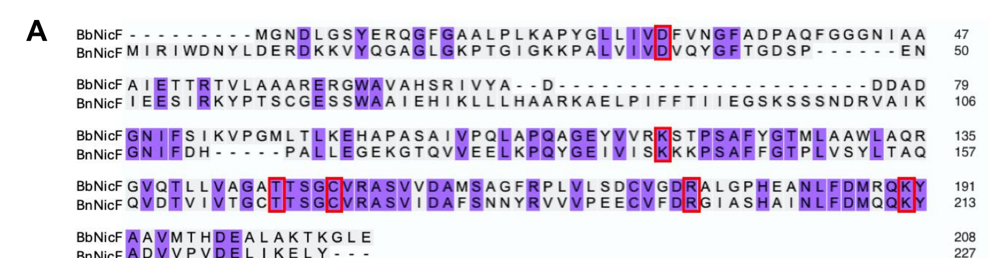


Figure 3. NA catabolic pathways known in *P. putida*/*B. bronchiseptica* (green) and *A. nidulans* (blue) and proposed in *B. niacini*.

- A *B. niacini* enzyme shares significant sequence identity with *B. bronchiseptica* maleamate amidohydrolase "NicF," including a potentially conserved catalytic triad.

A



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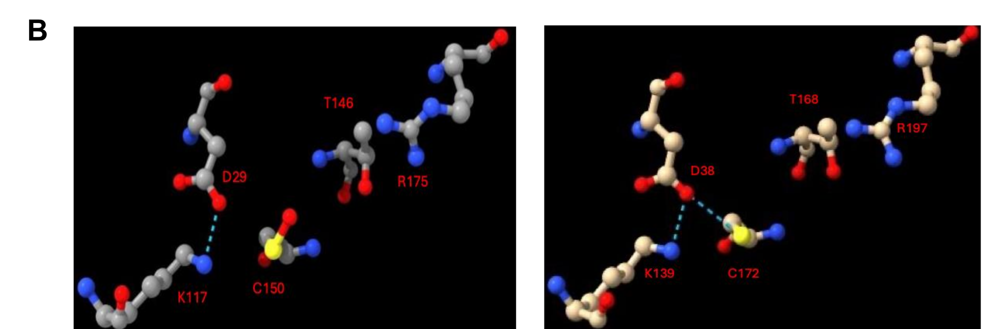

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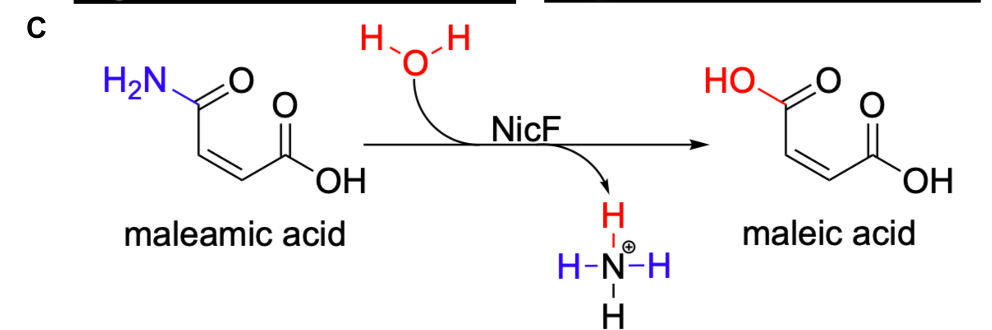


Figure 4. (A) Sequence alignment of *B. bronchiseptica* (top) and putative *B. niacini* (bottom) NicF. (B) Structural similarity of catalytic triad of *B. bronchiseptica* (left; X-ray crystallography) and B. niacini (right; AlphaFold prediction) NicF. (C) Hydrolytic deamidation of maleamic acid (MAA) to maleic acid (MA) catalyzed by *B. bronchiseptica* NicF.

Hypothesis & research objectives

Hypothesis: *B. niacini* putative NicF functions as an amidohydrolase and utilizes maleamic acid as its native substrate.

Objective 1: The nicF gene was cloned, recombinantly expressed, and purified.

Objective 2: The steady-state kinetic parameters of NicF were measured via UV-Vis spectrophotometry and isothermal titration calorimetry (ITC).

The putative nicF gene was cloned via traditional PCR approaches and UV-Vis and ITC kinetics were used to measure the steady-state kinetic parameters to confirm that maleamate is the native biological substrate for B. niacini NicF.

nicF was cloned into the pTHT vector, sequenced, expressed in Nico21 cells, and purified via Ni^{2+} affinity chromatography

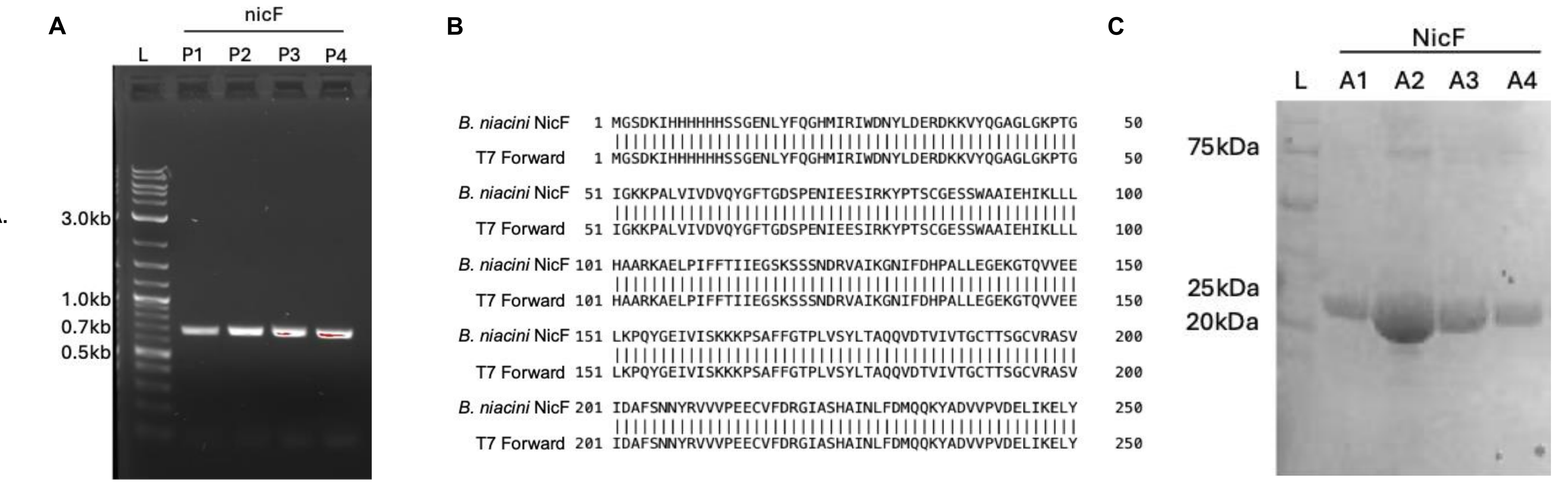


Figure 5. (A) The nicF gene was amplified from *B. niacini* gDNA via PCR. The presence of bands around 700bp in lanes 2-5 suggest that nicF was successfully amplified. (B) Alignment of known *B. niacini* NicF amino acid sequence and sequence cloned into NEB 5-alpha cells, which share 100% amino acid identity. (C) SDS-PAGE gel of purified *B. niacini* NicF. Purified protein bands between 20-25 kDa correspond to NicF (28 kDa).

AlphaFold predicted the structure of homotetrameric NicF

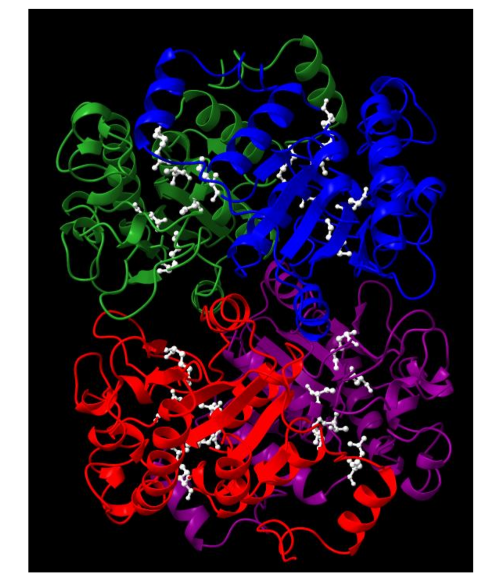


Figure 6. The AlphaFold predicted structure of tetrameric *B. niacini* NicF. Green, blue, red, and purple models represent each subunit. White residues represent the predicted critical active site residues.

The maleamate amidohydrolase activity of *B. niacini* NicF was validated by steady-state kinetics

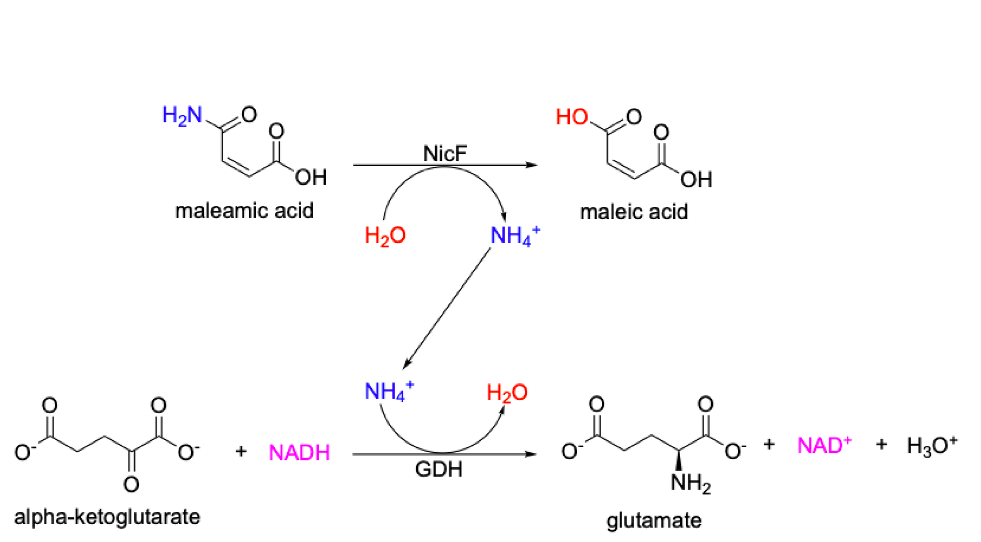


Figure 7. The glutamate dehydrogenase linked-enzyme assay used to define the NicF steady-state kinetic parameters. The oxidation of NADH was measured at 340nm by UV-Vis spectrophotometry.

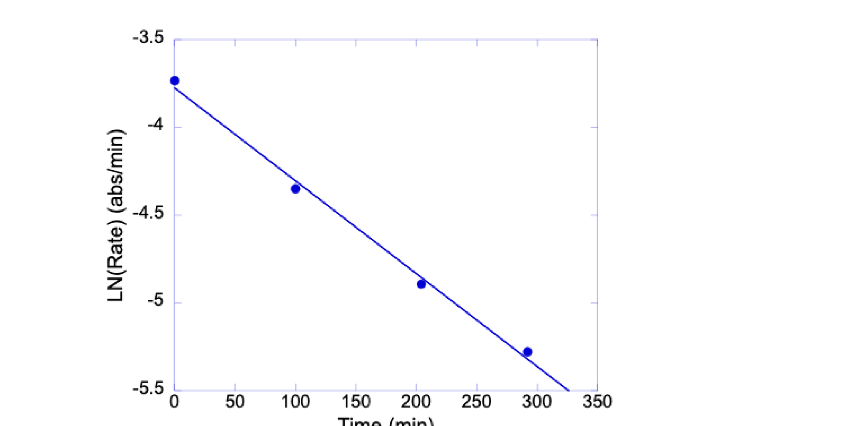


Figure 8. NicF quickly loses activity following a first order rate law. The half-life of NicF activity is 130.98 minutes. This plot was used as a correction factor in kinetic assays to account for the loss of NicF activity over time.

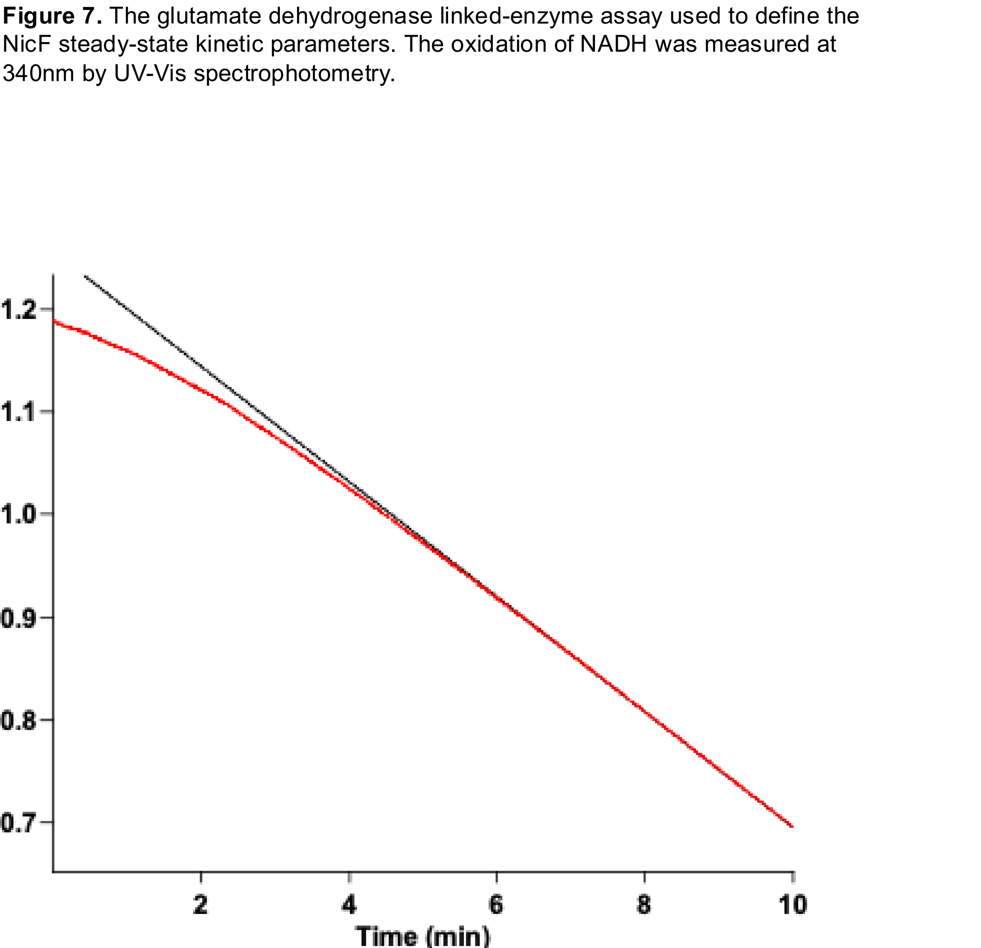


Figure 10. UV-Vis spectrophotometry initial rates data used to generate the Michaelis Menten plot in figure 13. The linear portion of the dataset was fit between 7-10 minutes.

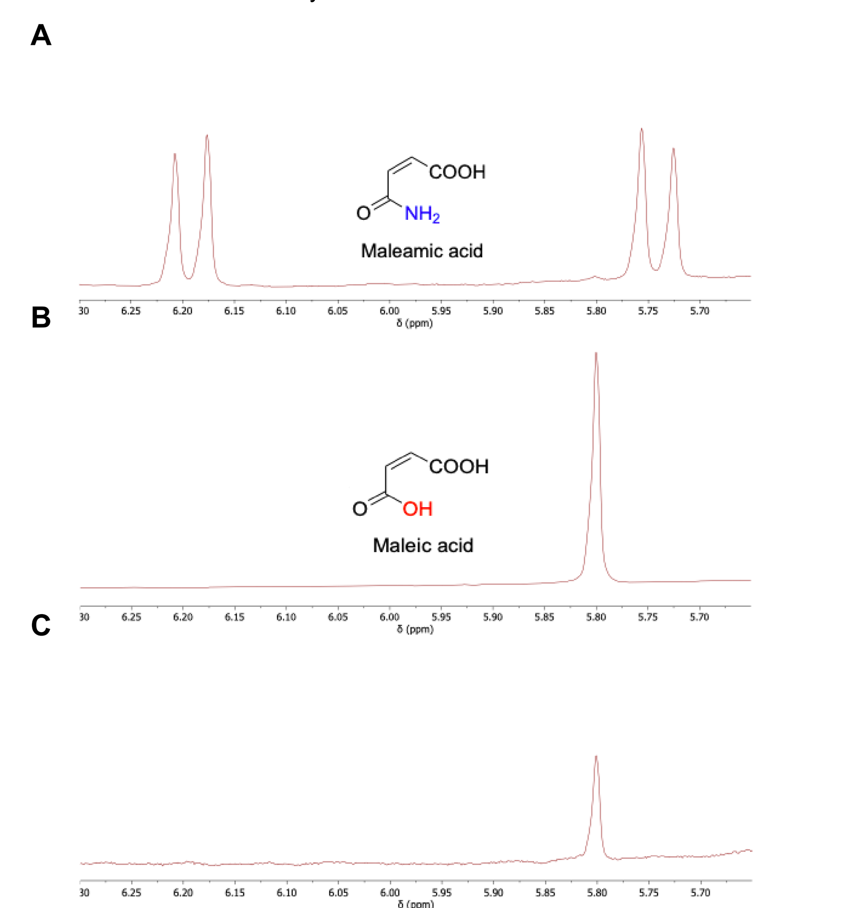
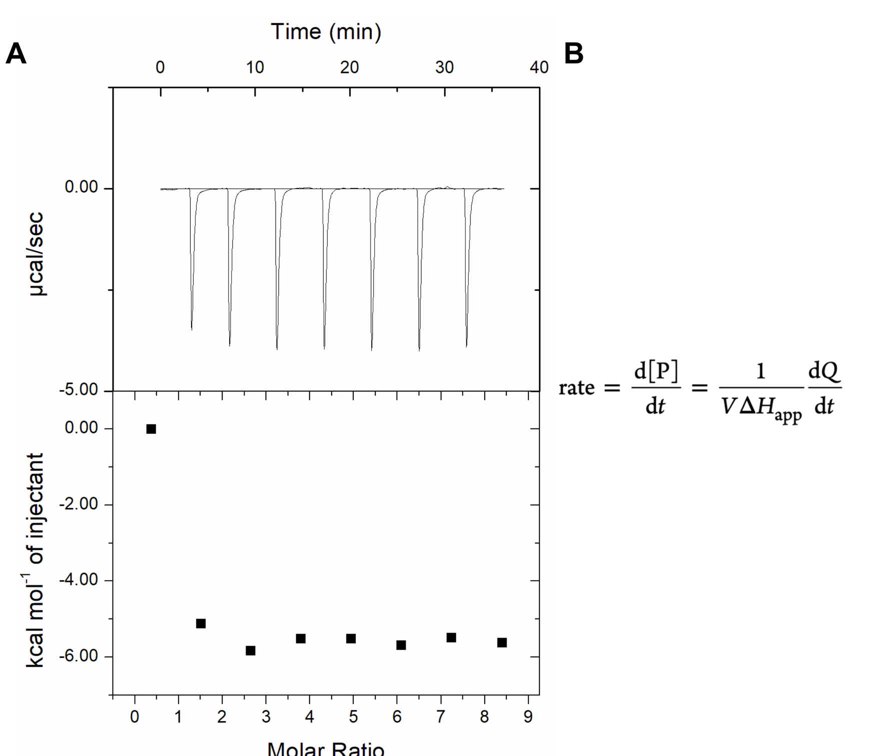


Figure 11. ¹H NMR spectra of (A) Maleamic acid standard. (B) Maleic acid standard. (C) Reaction containing NicF and maleamic acid.



$$rate = \frac{d[P]}{dt} = \frac{1}{V\Delta H_{app}}\frac{dQ}{dt}$$

Figure 9. (A) Raw ITC data used to determine the $\Delta H_{apparent}$ (-5.6 ± 0.1 kcal/mol) of the NicF catalyzed reaction in sodium phosphate buffer. (B) The equation used to convert the measured enthalpy changes to rates.

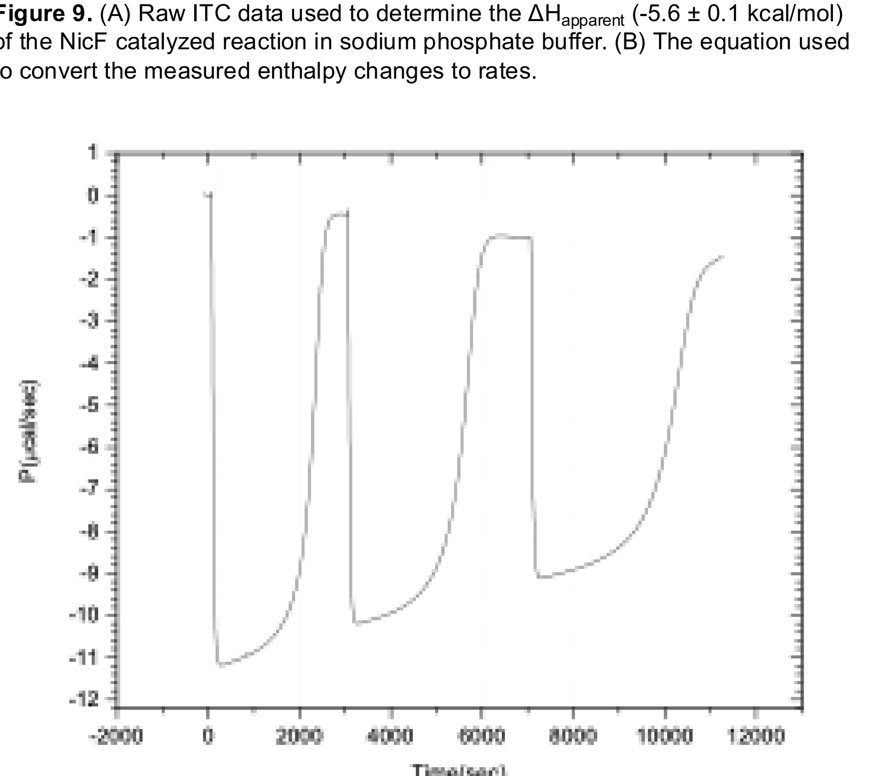


Figure 12. ITC full time course data used to generate figure 14.

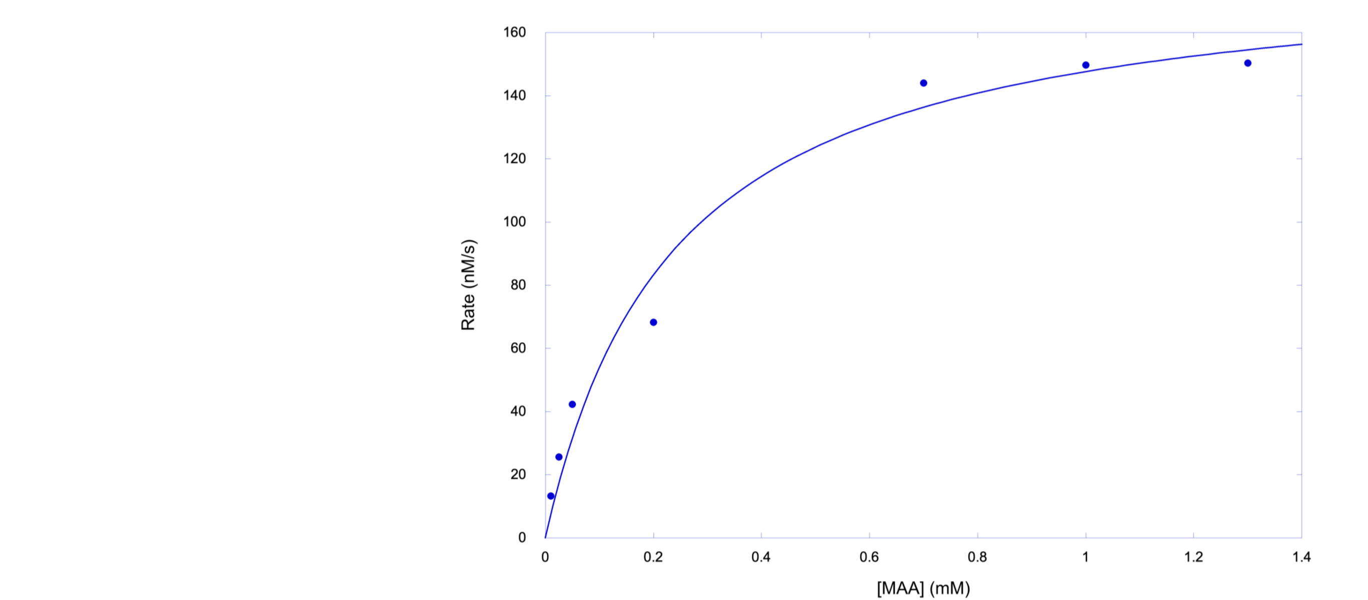


Figure 13. The Michaelis Menten plot used to define the steady-state kinetic parameters of the reaction catalyzed by NicF (5nM) via UV-Vis spectrophotometry.

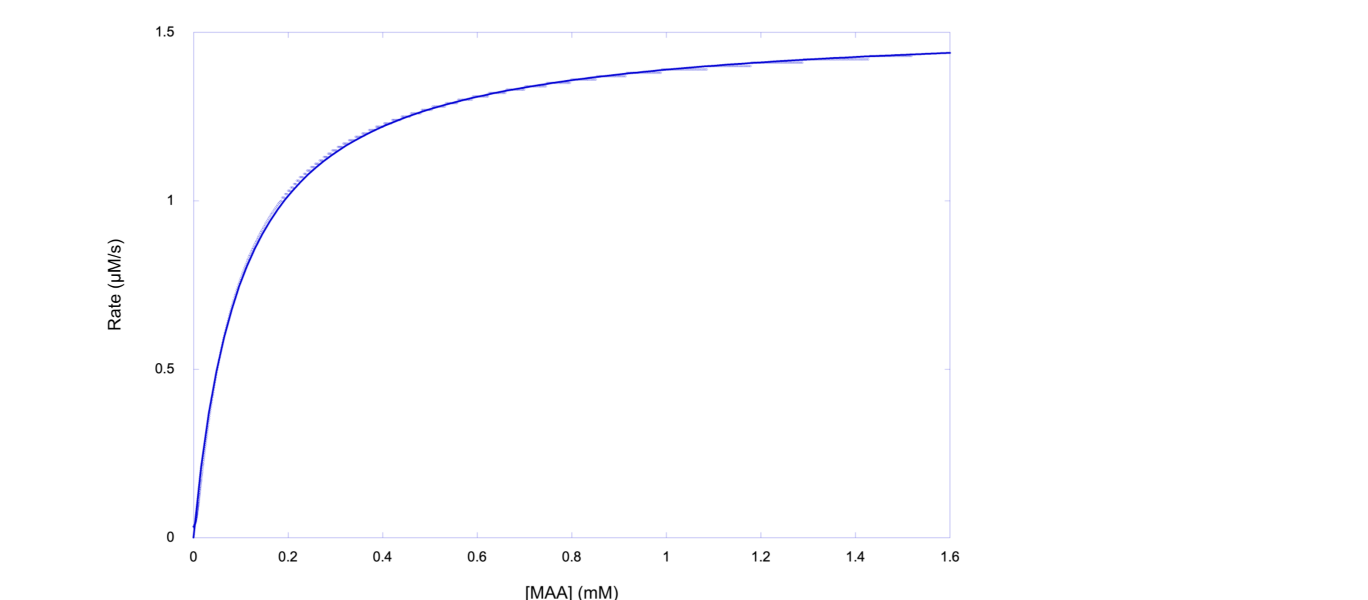


Figure 14. The Michaelis Menten plots used to define the steady-state kinetic parameters of the reaction catalyzed by NicF (100nM) via isothermal titration calorimetry.

The steady-state kinetic parameters of the NicF catalyzed reaction are physiologically relevant

Table 1. Comparison of kinetic parameters of *B. bronchiseptica* and *B. niacini* NicF

NicF Enzyme	K_M (μM)	k_{cat} (s^{-1})	k_{cat}/K_M ($M^{-1} s^{-1}$)
B. bronchiseptica	128 ± 6	11.7 ± 0.2	(9.1 ± 0.5) × 10^4
B. niacini (UV-Vis)	150 ± 80	26 ± 11	(1.9 ± 0.8) × 10^5
B. niacini (ITC)	100 ± 20	13 ± 2	(1.3 ± 0.3) × 10^5

- *B. niacini* NicF has a similar turnover rate (k_{cat}) and catalytic efficiency to *B. bronchiseptica* NicF.
- The magnitude of k_{cat}/K_M suggests that maleamic acid is likely the native substrate of *B. niacini* NicF.
- NicF, likely a homotetrameric enzyme at physiological concentrations, may lose catalytic activity due to the low concentrations needed in the kinetic assays.

DUF and HP could catalyze the ring opening and oxidation of 2,3,6-trihyroxypyridine to N-formylmaleamate

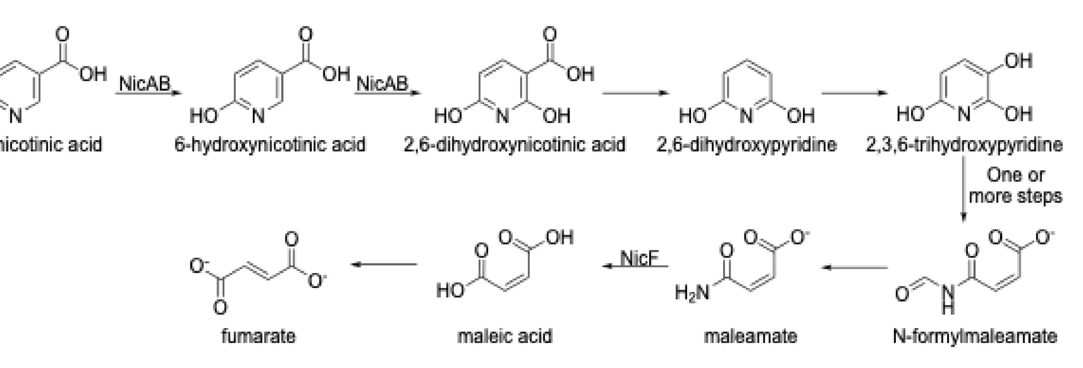


Figure 15. The proposed pathway of NA catabolism in *B. niacini* after the validation of NicF as a maleamate amidohydrolase.

- The conversion of 2,3,6-trihydroxypyridine to N-formylmaleamate requires ring-opening and oxidation reactions
- This set of reactions could be catalyzed by Domain of Unknown Function (DUF) and Hypothetical Protein (HP), which are proteins encoded for by genes within the operon responsible for NA catabolism in *B. niacini*

Future research

- Determine whether α-hydroxyglutaric acid is a viable substrate for *B. niacini* NicF.
- Establish the quaternary structure of NicF by size-exclusion chromatography or native PAGE.
- Create enzyme variants to clarify whether C172 is involved in catalysis.
- Perform cell-extract assays to identify other metabolites of NA catabolism in *B. niacini*.
- Identify potential metabolites with the same *m/z* as 5,6-dihydroxypiperidine-2-one
- Establish a kinetic protocol with higher enzyme concentration to potentially resolve the loss of NicF activity over time
- Determine the K_i of the NicF "Hilmey inhibitor."
- Determine whether NFM is a substrate of FMO, DUF, or HP

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References

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