Targeted Genome Mining Facilitates the Discovery of a Promiscuous, Hyperthermostable Amidase from *Thermovenabulum gondwanense* with Notable Nylon-Degrading Capacity

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Abstract: Plastics are ubiquitous in our ecosystems, and microplastic accumulation in the environment is an emerging global health concern. Since available recycling technologies are not economically competitive with primary plastic production, global use is expected to reach 1231 megatons by 2060, with 493 megatons leeching into the environment each year. To identify new nylon-recycling biotechnologies, targeted genome mining was used to identify thermostable enzymes capable of degrading polyamides. Here, we describe the characterization of a novel protein sourced from *Thermovenabulum gondwanense*: TvgC. TvgC is extremely stable, exhibiting a melting temperature of 93 °C and no detectable losses in hydrolytic activity after one week at 60 °C. While nylonases primarily process nylon-6, TvgC catalysed the degradation of both nylon-6 and nylon-6,6 films, exhibiting marked preference for nylon-6,6, which is considered more difficult to degrade. Finally, conversion experiments demonstrate that TvgC achieves a 1.2 wt% conversion of nylon-6 film, outcompeting the most highly engineered nylonases. This novel hyperthermostable protein represents an excellent starting point for future engineering of increasingly efficient nylonases.

Plastics are pervasive as modern society depends heavily on their use. The range of desirable properties displayed by these polymers (i.e., ductility, thermostability, high strength-to-mass ratios)[1] also pose risks to the environment from a waste management perspective. Of the 460 megatons of plastic consumed in 2019, approximately 41 megatons were recycled while the remaining 419 megatons were incinerated, landfilled, or mismanaged.[2] Plastics are generally non-biodegradable, with breakdown requiring high pressures and temperatures that make recycling costly, unsafe, and unsustainable.[3] Consequently, current recycling platforms are poorly used and effort is being directed towards developing more sustainable technologies. Biocatalytic degradation of plastics is receiving significant attention as a potential solution to this problem.[4–8]

Global plastic pollution may have allowed nature to evolve enzymes to metabolize these xenobiotics.[9] For instance, several classes of bacterial enzymes have been identified that enable organisms to metabolize polyethylene terephthalate (PET).[9] Exploiting these enzymes for biodegradation has real-world utility: in 2025, Carbios will use an engineered PETase to recycle 50,000 tons of PET annually.[10,11] Yet, biotechnologies that enable recycling of other plastics are less developed. For instance, few enzymes are known that can degrade nylon, a common polyamide used in textiles, medical appliances, fishing, agriculture, and military equipment.[12,13] Current methods to recycle nylon-6 (Fig. S1) include 200 °C hydrolysis in inorganic acids, whereas nylon-6,6 (Fig. S1) is not recycled due to poor product recovery and purification.[14,15]

To date, seven nylonases have been identified from microorganisms isolated from nylon-waste facilities.[16–22] In particular, the 6-aminohexanoate oligomer endohydrolases (NylCs) that resemble N-terminal nucleophile (Ntn) hydrolases show potential for nylon biodegradation. Unfortunately, known NylCs are neither sufficiently stable nor efficient for industrial purposes.[18,21,22] Although directed evolution has improved the thermostability and activity of nylonases, enzyme-catalyzed processes have achieved a maximal conversion of nylon-6 films of 0.67 wt%.[5] Studies on enzyme-catalyzed nylon degradation have largely focused on known NylCs from *Agromyces sp* KY5R. ($NylC_A$), *Kocuria sp*. KY2 ($NylC_K$), or *Arthrobacter sp*. K172 ($NylC_{p2}$).[6,7,21,22]

To find novel, robust NylCs, we constructed a sequence similarity network (SSN) for the S58 peptidase family, containing known NylCs (Fig. S2).[23] At an alignment score of 50, known NylCs fell into a cluster that contained one gene from the thermophilic *Thermomicrobiales bacterium* (Fig. S2). Although this gene showed 42% sequence similarity to $NylC_{p2}$, catalytic residues were not conserved. Thus, other clusters within the SSN were explored. SwissProt descriptions suggested that four genes in the largest cluster encoded putative aminopeptidases. However, no functionally annotated entries were contained therein. While many genes in this cluster came from thermophiles (Table S1), we became interested in a protein (TvgC; A0A162M3R4) from *Thermovenabulum gondwanense* that grows optimally at 65 °C

and has no known association with nylon production or waste facilities (Fig. S3, Table S2).[24–26]

Despite sharing only 31 – 33% sequence similarity with known NylCs (Table S3), further *in silico* analysis suggested that TvgC might catalyze nylon-6 degradation. As with Ntn hydrolases, known NylCs autocatalytically cleave to release the N-terminal nucleophile following translation.[6,27] The structures of full-length TvgC and NylC$_A$ were predicted and aligned to illustrate that the overall folds of these proteins are very similar (Fig. 1a; Table S3).[28–30] Critically, residues important for the autocatalytic cleavage of NylC$_A$ were conserved in TvgC (Lys154, Asn185, Asp273, Asp275, Asn233, Thr234) and coincident in the overlaid structures (Fig. 1b),[6,28–30] strongly suggesting that TvgC would undergo autoprocessing.

This suggestion was experimentally supported by attempts to overexpress TvgC (33.9 kDa). Following nickel affinity chromatography of *N*-terminally His$_6$-tagged TvgC, SDS-PAGE analysis revealed two additional bands with apparent molecular weights of ~24 kDa and ~10 kDa (Fig. S4). These bands are consistent with TvgC autocleaving to unmask Thr234 as the terminal nucleophile in the 10-kDa fragment. To drive cleavage to completion, TvgC was incubated at 50 °C for 48 hours. Over this period, the smaller bands increased in intensity while the full-length band decreased (Fig. S4). The kinetics of this process were probed using densitometry, and comparable apparent first-order rate constants of (7.1 ± 1.0) x 10^{-5} s^{-1} and (1.4 ± 0.1) x 10^{-4} s^{-1} were obtained using the full-length and the 24 kDa bands (Fig. 2). TvgC was then compared to the quaternary structure of the assembled NylC$_A$ (PDB: 3AXG).[6] Following autocatalytic cleavage of full-length length NylC$_A$, the two fragments non-covalently assemble into a tetramer of heterodimers.[27] We generated the expected cleavage products of TvgC *in silico*, docked the fragments, and aligned the resulting heterodimer with 3AXG.[28–34]

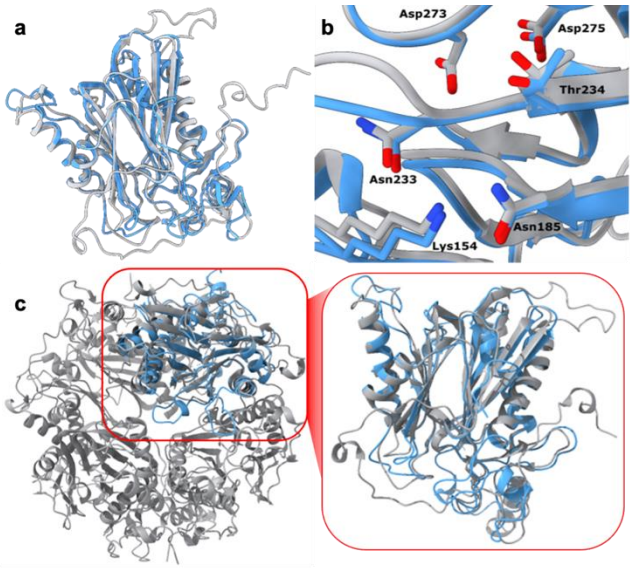


Figure 1. a) Alignment of the predicted structures of full-length NylC$_A$ (—) and TvgC (—) (RMSD of 0.88 Å). b) Autoprocessing residues are conserved and well-aligned between NylC$_A$ and TvgC. c) *In silico*-cleaved TvgC aligned with subunit of 3AXG. Isolated view in red.

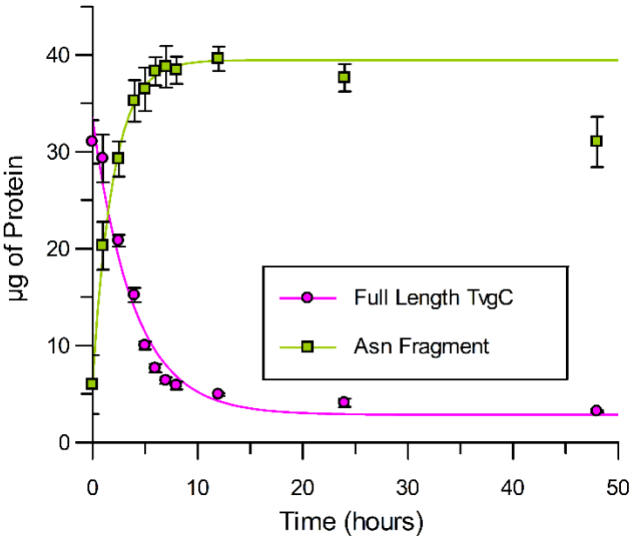
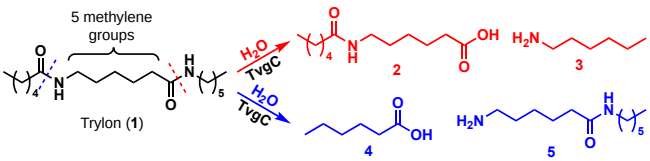


Figure 2. Time courses for autocatalytic cleavage of TvgC. First-order exponentials were used to fit all values obtained with full-length TvgC, whereas values were measured up to and including t = 12 hours for the Asn fragment.

Figure 1c demonstrates that the TvgC heterodimer aligns reasonably well with one subunit of 3AXG (Table S3). While 3AXG lacked the resolution needed to evaluate alignment of the proposed Asp-Asp-Thr catalytic triad, docking experiments using the TvgC heterodimer suggested that a nylon-6 pentamer would bind within a tunnel bearing the equivalent residues (Asp273, Asp275, Thr234; Fig. S5).[6,28–30,35]

Before probing the activity of TvgC, the melting temperature (T_m) of the purified protein was determined to be 92.8 ± 0.3 °C (Fig. S6).[36] This represents the highest T_m value for any NylC, exceeding that of highly engineered NylC variants.[6,27] For instance, NylC$_{p2}$ (T_m = 52 °C) underwent several rounds of mutagenesis before identifying a quadruple mutant (NylC$_{p2}$-TS) with a T_m of 88 °C. Bell *et al.* achieved similarly impressive T_m values of 87 °C via rational mutagenesis of NylC$_K$ and NylC$_A$.[5] Considering the relative effort required, we were enthused that genome mining delivered a hyperthermostable variant of NylC that is more thermostable than engineered variants.[27]

Initial activity tests were then conducted using small amide containing molecules. TvgC did not hydrolyze N-(4-nitrophenyl)hexanamide (Fig. S7), consistent with the suggestion that NylCs require at least three monomeric units for catalysis.[27] Subsequently, we used a "trimer" analogue of nylon-6 ("trylon", **1**; Scheme 1) and incubated this compound (96 µM) with TvgC (50 µM) for 20 minutes at 50 °C.[37] This resulted in quantitative consumption of **1** (Fig. 3). Concentrations as low as 5 µM also facilitated quantitative hydrolysis of **1** (96 µM) in under two hours (Fig. S8).



Scheme 1. The two amides in **1** are not equivalent and can be hydrolyzed to release two different sets of products

This substrate has two distinct amide bonds that would produce either **2** and **3** or **4** and **5** upon hydrolysis (Scheme 1). When fed **1**, NylC$_A$ predominantly produces **2** and **3**, indicating that NylC$_A$ selectively hydrolyzes one amide.[37] Interestingly, while TvgC exhibits selectivity for the same amide as NylC$_A$, TvgC exhibited a ~78% increased capacity to hydrolyze the other amide in **1** to release **4** and **5** (Fig. S9).

Conversion assays using substrate **1** revealed 60 °C and pH 7.5 to be the optimal conditions for TvgC catalysis (Fig. S10 and Fig. S11). Given the glass transition temperature (T_g) of nylon-6 (50-55 °C),[38] optimal activity around 60 °C would be ideal for nylon recycling applications. Furthermore, TvgC showed no loss in activity after incubation up to 70 °C and only an 18% decrease in conversion at 80 °C after 24 hours (Fig. S12). This kinetic stability extended to longer incubations, with no activity lost after incubation for one week at 60 °C (Fig. S13). TvgC even tolerates organic solvents, remaining soluble in mixtures containing up to 40%, 48%, and 54% (v/v) methanol, acetonitrile, and DMSO, respectively (Fig. S14).

After probing the amidase activity of TvgC, we evaluated nylon-degrading capacity using nylon-6 film via UPLC-MS. Incubating nylon-6 (0.38 mg enzyme/mg film) with TvgC gave rise to a peak confirmed to be 6-(6-aminohexanamido)hexanoic acid (nylon-6 dimer) (Fig 4. & Fig. S15). Over 10 days at 60 °C, TvgC (139 µM) converted 1.2 ± 0.1 wt% (mg dimer/mg film) whereas at 50 °C, TvgC (139 µM) converted 0.41 wt% of nylon-6, consistent with the observed temperature impact on activity towards **1**. Additionally, this demonstrates the utility of enzymes that function above the T_g of the plastic. Conversion was still observed using lower concentrations of enzyme (Fig. 4, Table S10). Gratifyingly, conversions obtained at 60 °C were higher than the previous benchmark: NylC$_K$-TS (a thermostable variant of NylC$_K$) achieved a 0.67 wt% conversion after 10 days at 80 °C.[5]

While the enzyme loading with TvgC is *significantly* higher than NylC$_K$-TS (0.58 mg enzyme/g nylon-6 film),[5] conversions with NylC$_K$-TS did not increase with more enzyme, suggesting some substrate-dependent limitation not observed with TvgC.[5]

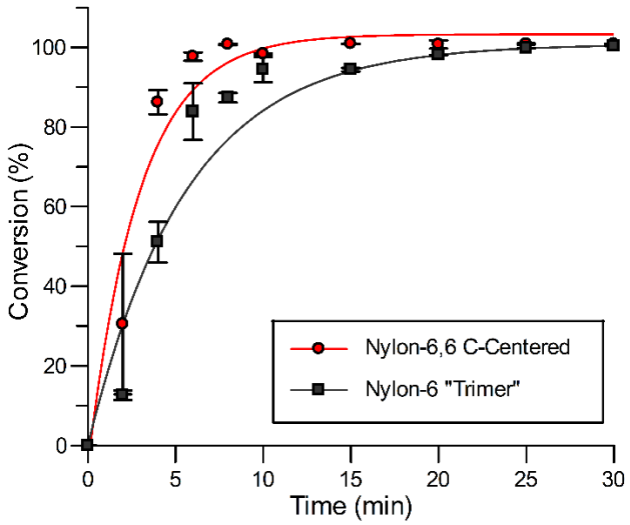


Figure 3. TvgC (50 µM) incubated with **1** (96 µM,■) and **6** (96 µM,●) for 30 minutes at 60 °C. Complete hydrolysis of **1** and **6** are observed within 20 and 8 minutes.

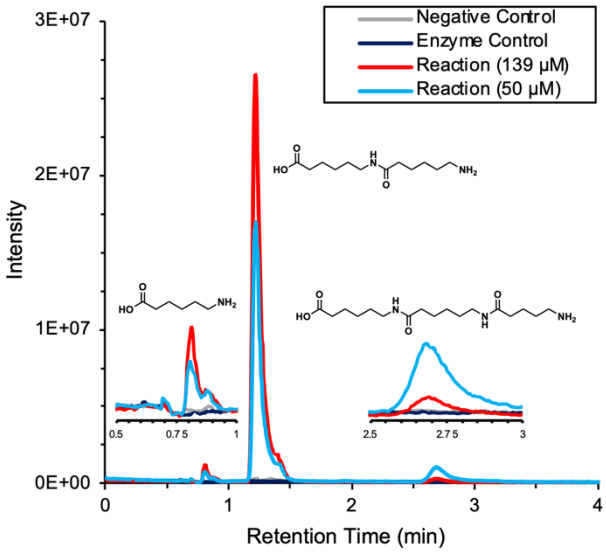


Figure 4. UPLC-MS analysis of nylon-6 (7.2 mg) incubated with 50 µM (—) and 139 µM (—) TvgC at 60 °C for 10 days. Monomer (0.81 min), dimer (1.22 min), and trimer (2.69 min) are only present in the reaction.

Fortunately, TvgC reliably overexpresses at high concentrations, so increased enzyme loadings may be possible for downstream applications. Intriguingly, small peaks corresponding to different nylon-6 degradants were observed, suggesting TvgC is non-specific, consistent with Bell's findings.[5] Despite release of monomers from the film, separate assays with nylon-6 dimer substrate showed no hydrolysis over 24 hours (Fig. 4, Fig. S16).

Given the ability of TvgC to hydrolyze either amide bond of **1**, we hypothesized that TvgC might also degrade nylon-6,6. We incubated TvgC (0.18 mg enzyme/mg film) for 10 days at 50 °C and observed 0.65 wt% conversion (Fig. 5, Fig. S17), higher than the 0.41 wt% observed with nylon-6 at this temperature. Thus, TvgC appears to be the first NylC to show preference for nylon-6,6 over nylon-6. Contrastingly, NylC$_{p2}$-TS is the only NylC reported to have activity towards nylon-6,6, albeit at 60% of that observed with nylon-6.[39] Unfortunately, efforts to thoroughly evaluate activity with nylon-6,6 were hindered by plasticizers and unknown byproducts adhering to the UPLC-MS column, which rendered it inoperable. We are developing an appropriate assay to more thoroughly evaluate nylon-6,6 film degradation by TvgC at 60 °C, as previous results suggest conversions will increase significantly at elevated temperatures.

Given issues with the nylon-6,6 film, we used two mimics as proxies: the carbonyl-centered nylon-6,6 "trimer" (**6**; Scheme 2a) and the amine-centered nylon-6,6 "trimer" (**8**; Scheme 2b).[37] We incubated TvgC (50 µM) with each mimic (96 µM) and found that it quantitatively hydrolyzes **6** to **3** and **7** in 10 minutes at 60 °C, corresponding to nearly twice the apparent rate observed with **1**. Conversion of **8** to **4** and **9** required approximately 24 hours (99.2 ± 0.2 wt% conversion) under the same conditions (Fig. 6). Interestingly, known NylCs were reported to show limited activity towards **8**.[37]

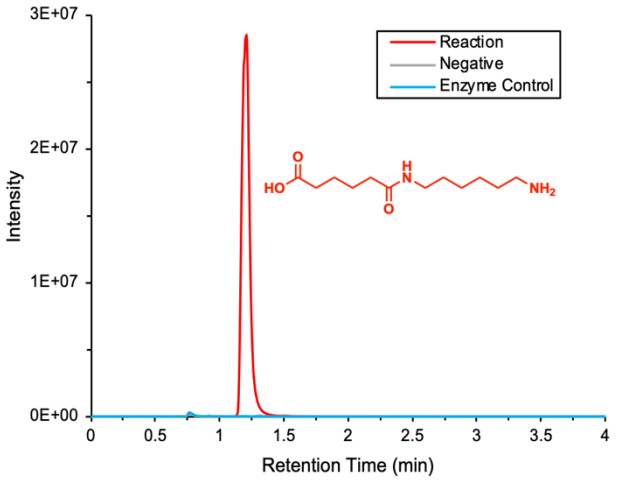


Figure 5. UPLC-MS analysis of nylon-6,6 (13 mg) incubated with TvgC (147 µM) at 50 °C for 10 days. Dimer (1.22 min) is only observed in reaction (—).

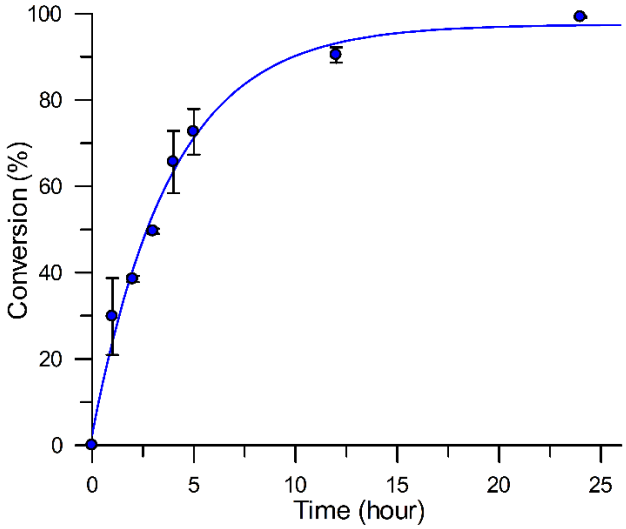
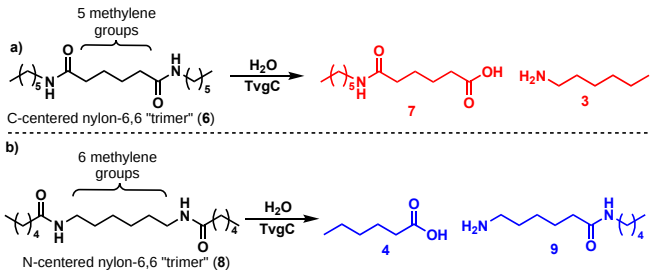


Figure 6. Time course for incubation of TvgC (50 µM) with **8** (96 µM) at 60 °C. Near quantitative conversion was observed within 24 hours.

These results suggest that the TvgC active site may accommodate various orientations for labile amides as well as increased spacing between carbonyl units of consecutive amide groups. While the leaving group amine being on the "outside" of the chain (observed in **1** and **6)** appears to improve hydrolysis, TvgC seems to accommodate the flipped amide orientation observed in **8** more so than known NylCs.[37] Moreover, apparent rates of conversion parallel the spacing between these units: **6** > **1** > **8**. While TvgC is best able to process substrates with two consecutive amide carbonyls separated by four methylene groups, this is not a strict requirement. This plasticity likely contributes to the capacity of TvgC to process both nylon-6 and nylon-6,6, as these polymers have different spacings between consecutive amides and also likely exhibit disparate amide orientations. Despite this plasticity, TvgC could not hydrolyze nylon-6,6 degradants to monomers, suggesting it cannot cleave two consecutive amide bonds, consistent with the 'endo-amidase' annotation of NylCs.

Using targeted genome mining, we have discovered and characterized a novel hyperthermostable NylC. To our knowledge, TvgC exhibits i) the highest thermostability of any nylonase, natural or engineered, ii) increased promiscuity, iii) outstanding kinetic stability, iv) a preference for nylon-6,6, and v) the highest reported conversion of nylon-6. Considering that the gold-standard nylonase (NylC$_K$-TS) was sourced from an organism exposed to nylon and subsequently engineered, it is remarkable that TvgC achieves higher levels of conversion of nylon-6. As TvgC was not discovered in nylon-exposed environments,[24–26] this work speaks to the utility of non-native enzymatic functions in biocatalysis. Moreover, the identification of this novel NylC



Scheme 2. Hydrolysis of the carbonyl-centered nylon-6,6 "trimer" (**6**) and the amine-centered nylon-6,6 "trimer" (**8**) produce two different sets of products.

expands the database of these enzymes, contributing to further bioinformatic searches for new enzymes. That said, higher concentrations of TvgC were required to achieve improved conversions. Nevertheless, TvgC represents an important development towards bio-based nylon recycling, but directed evolution to improve catalytic efficiency represents a likely next step. Several examples of directed evolution and computer-aided enzyme engineering to improve the efficiency of PET-degrading enzymes have been reported, and similar efforts could be directed to improve TvgC.[4,40–43]

Supporting Information

The authors have cited additional references within the Supporting Information.[[44–51]]

Acknowledgements

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Keywords: biocatalysis • bioinformatics • NylC • nylonase • plastic degradation

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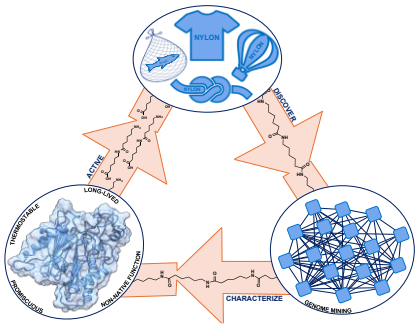

Plastic waste accumulation poses a severe threat to human and ecosystem health. As current recycling methods are inadequate, biocatalytic degradation offers a more sustainable platform. Using genome mining, we discovered TvgC, a new 6-aminohexanoate oligomer endohydrolase (NylC). TvgC has no affiliations to nylon facilities, is the most thermostable and promiscuous, and has the highest reported activity towards nylon-6, compared to known NylCs.