Characterizing the function of a novel beta-1,3-endoglucanase from hyperthermophile *Fervidibacter sacchari*, the sole isolate of class *Fervidibacteria*

Jonathan K. Covington[1], Nicole Torosian[1], Allison M. Cook[1], Marike Palmer[2], Scott G. Bryan[1], Nancy O. Nou[1], Jan-Fang Cheng[3], Miranda Harmon-Smith[3], Ian K. Blaby[3], Matthias Hess[4], Brian P. Hedlund[1,5]

[1] School of Life Sciences, University of Nevada, Las Vegas, NV, USA; [2] Department of Microbiology, University of Manitoba, Winnipeg, Canada; [3] US Department of Energy Joint Genome Institute, Lawrence Berkeley National Laboratory, CA, USA; [4] Department of Animal Science, College of Agricultural and Environmental Sciences, University of California, Davis, CA, USA; [5] Nevada Institute of Personalized Medicine, University of Nevada, Las Vegas, NV, USA

INTRODUCTION

Across the bacterial phylum *Armatimonadota*, we find evidence of polysaccharide specialization. Genes annotated as encoding glycoside hydrolases (GHs)—enzymes that degrade polysaccharides by cleaving the glycosidic linkages between sugar residues—are abundant and diverse, with some members of *Armatimonadota* possessing as many as 348 GHs across 104 GH families (1). However, only six isolates from *Armatimonadota* have been cultivated, and their saccharolytic lifestyles are under-explored despite phylum-wide specialization. *Fervidibacter sacchari* is the only isolate within the *Armatimonadota* class *Fervidibacteria* **(Figure 1)**. It is a hyperthermophile with 117 genes encoding GH domains and grows on 17 diverse polysaccharides, but the biochemical characteristics of these GHs are unknown. Here, we focus on one *F. sacchari* GH belonging to family GH50, Fsa16295Glu, and determine its substrate specificity, temperature optimum, and evolutionary history (2).

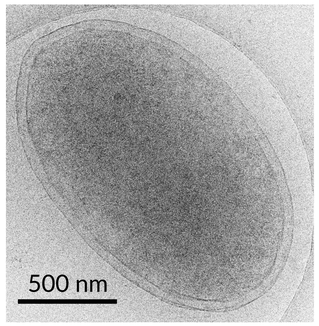

Figure 1: *F. sacchari* visualized by cryo-electron microscopy.

METHODOLOGY

Expression and purification

1. The Fsa16295Glu gene was synthesized and cloned into a pET vector for *E. coli*, which was grown to OD_{600} = 0.6 - 0.8, then induced overnight with 0.5 mM IPTG.
2. Culture pellets were resuspended in 1/5 volume lysis buffer (pH 7).
3. Cells were lysed and the enzyme was purified by heating at 80 °C, followed by centrifugation to remove cell debris.

Substrate and temperature assays

1. Substrate: Purified Fsa16295Glu was mixed 1:1 with 20 diverse polysaccharides (1% w/v) at pH 7 and incubated overnight at 80 °C.
2. Temperature: Purified Fsa16295Glu was mixed 1:1 with oat beta-glucan (1% w/v) at pH and incubated overnight at 4, 20, 30, 50, 60, 70, 80, 90, or 95 °C
3. 3,5-dinitrosalicylic acid solution was added and incubated for 20 minutes (3).
4. Absorbance was read at 570 nm (3).

Phylogenetic analysis of family GH50

1. All GH50 sequences in the CAZy database, Fsa16295Glu, and *Fervidibacteria* homologs were aligned using the MAFFT online server v. 7 (4).
2. An approximate maximum-likelihood analysis was performed using FastTree v. 2.1.11 with SH-aLRT branch support (5).
3. The phylogeny was visualized in the Interactive Tree of Life v 6.7 website.

RESULTS

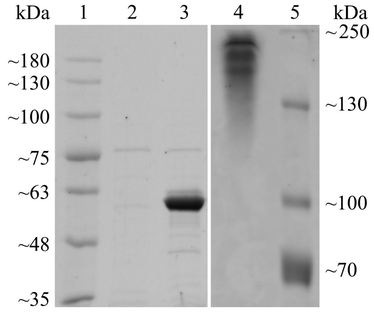

Figure 2: PAGE gels.
SDS-PAGE gel:
1) Protein ladder
2) Empty vector control
3) Native mature Fsa16295Glu

Native-PAGE gel:
4) Native mature Fsa16295Glu
5) Protein ladder

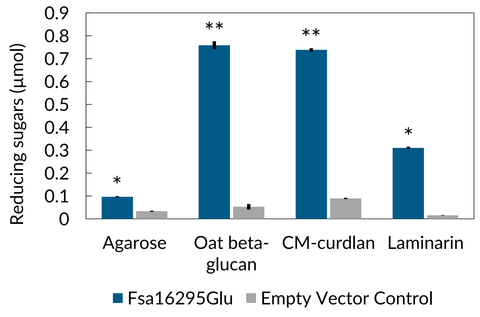

Figure 3: Substrate specificity.
Fsa16295Glu is active on agarose, oat beta-glucan, CM-curdlan, and laminarin.
* P < 0.00005
** P < 0.000005

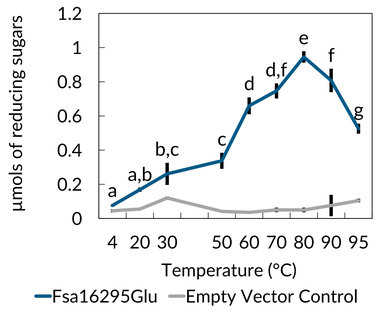

Figure 4: Temperature range and optimum. Fsa16295Glu is active on oat beta-glucan between 4 and at least 95 °C, with an optimum of ~80 °C. Shared groups are not significantly different (P > 0.05).

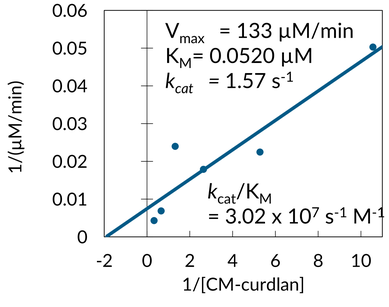

Figure 5: Lineweaver-Burk plot.
V_{max} and K_M are calculated from the reciprocals of the y and x-intercepts, respectively.

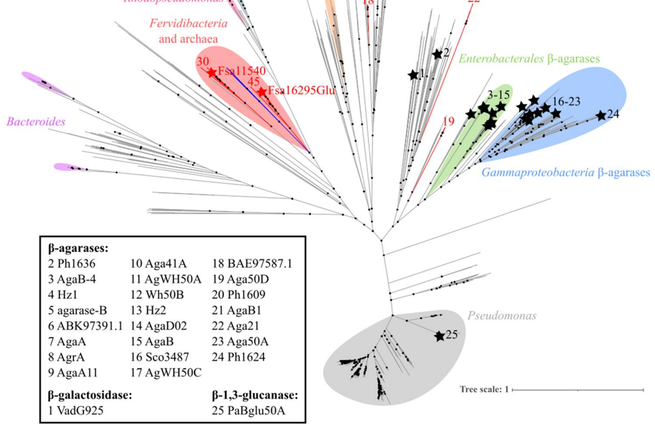

Figure 6: GH50 phylogenetic analysis. Red indicates GH50s from *F. sacchari* (stars) and *Fervidibacteria* (branches). Black stars represent characterized GH50s.

CONCLUSIONS

1. Fsa16295Glu possesses unique beta-1,3-endoglucanase activity in GH family GH50, a family of agarases **(Figure 3)** (6).
2. Fsa16295Glu is the first characterized hyperthermophilic GH50, with high activity between 60 and 95 °C, and an optimum of ~80 °C **(Figure 4)**.
3. Fsa16295Glu has a high k_{cat}/K_M of 3.02 x 10^7 s^{-1} M^{-1} compared to other GH50s **(Figure 5)**.
4. Fsa16295Glu is the first characterized representative of GH50 subfamily GH50_3 based on its unique function and distant relationship to other characterized GH50s **(Figure 6)**.

ACKNOWLEDGEMENTS

This material is based upon work supported by the National Aeronautics and Space Administration under Grants No. 80NSSC20M0043, 80NSSC17K0548, 80NSSC21M0157, and the Office of Science of the U.S. Department of Energy under Contract No. DE-AC02-05CH11231.

REFERENCES

1. Nou, N.O., Covington, J.K., Lai D., Mayali, X., Seymour, C. O., Johnston, J., et al. (2024). Genome-Guided Isolation of *Fervidibacter sacchari*, an Aerobic, Hyperthermophilic Polysaccharide-degrading Specialist. *Nature Communications*, 15, 9534.
2. Covington, J.K., Torosian, N., Cook, A.M., Palmer, M., Bryan, S.G., Nou, N.O., et al. (2024). Biochemical Characterization of Fsa16295Glu From "*Fervidibacter sacchari*," the First Hyperthermophilic GH50 With B-1,3-Endoglucanase Activity and Founding Member of the Subfamily GH50_3. *Frontiers in Microbiology*. 15, 1355444–1355444
3. Kim, J., Kwon, Y., Kim, J., Heo, S., Lee, Y., Lee, S., et al. (2014). Effective Microwell Plate-based Screening Method for Microbes Producing Cellulase and Xylanase and its Application. *Journal of Microbiology and Biotechnology*, 24(11), 1559-1565.
4. Katoh, K., Rozewicki, J., & Yamada, K.D. (2019). MAFFT Online Service: Multiple Sequence Alignment, Interactive Sequence Choice and Visualization. *Briefings in Bioinformatics*. 20, 1160–1166.
5. Price, M.N., Dehal, P.S., & Arkin, A.P. (2010). Fasttree 2--Approximately Maximum-Likelihood Trees for Large Alignments. *PLOS ONE*. 5, e9490–e9490.
6. Drula, E., Garron, M., Dogan, S., Lombard, V., Henrissat, B., & Terrapon, N. (2022). The Carbohydrate-Active Enzyme Database: Functions and Literature. *Nucleic Acids Research*, 50(D1), D571-D577.