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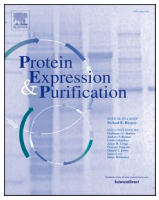

Expression and characterization of spore coat CotH kinases from the cellulosomes of anaerobic fungi (*Neocallimastigomycetes*)

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ABSTRACT

Anaerobic fungi (*Neocallimastigomycetes*) found in the guts of herbivores are biomass deconstruction specialists with a remarkable ability to extract sugars from recalcitrant plant material. Anaerobic fungi, as well as many species of anaerobic bacteria, deploy multi-enzyme complexes called cellulosomes, which modularly tether together hydrolytic enzymes, to accelerate biomass hydrolysis. While the majority of genomically encoded cellulosomal genes in *Neocallimastigomycetes* are biomass degrading enzymes, the second largest family of cellulosomal genes encode spore coat CotH domains, whose contribution to fungal cellulosome and/or cellular function is unknown. Structural bioinformatics of CotH proteins from the anaerobic fungus *Piromyces finnis* shows anaerobic fungal CotH domains conserve key ATP and Mg^{2+} binding motifs from bacterial *Bacillus* CotH proteins known to act as protein kinases. Experimental characterization further demonstrates ATP hydrolysis activity in the presence and absence of substrate from two cellulosomal *P. finnis* CotH proteins when recombinantly produced in *E. coli*. These results present foundational evidence for CotH activity in anaerobic fungi and provide a path towards elucidating the functional contribution of this protein family to fungal cellulosome assembly and activity.

1. Introduction

Anaerobic microorganisms play an important role in recycling carbon in the natural environment by breaking down recalcitrant plant biomass into sugars that feed microbial growth [1]. Many species of anaerobic bacteria and anaerobic fungi produce multi-enzyme complexes called cellulosomes that enable them to rapidly extract sugars from recalcitrant plant biomass [2,3]. Cellulosomes modularly assemble a wide array of proteins into complexes via interactions between enzyme-fused dockerin domains and a central scaffoldin protein [4,5]. While many cellulosome-incorporated proteins are carbohydrate-active enzymes (CAZymes), which participate directly in hydrolyzing plant matter, cellulosomes from both bacteria and fungi are known to encode proteins with unrelated functions, including proteases, serpins, and proteins annotated as spore coat CotH proteins, whose contributions to cellulosome function are poorly understood [5,6].

Dockerin-fused spore coat CotH proteins are an interesting class of cellulosome proteins because, across anaerobic fungal genomes [5,7], 18% of dockerin containing genes, which presumably encode cellulosome incorporated proteins, encode CotH domains (mycocosm.jgi.doe.gov). This fraction is second only to the 27% of dockerin containing genes that also encode CAZymes, suggesting an important role for CotH family proteins in fungal cellulosome biology and potential function.

CotH proteins are verified by proteomics to be present in cellulosomes from several anaerobic bacteria [8–10], but no studies have assayed the functional role this protein family plays in cellulosome biology; as non-CAZyme proteins, CotH is assumed to play some structural role in bacterial, and by extension, fungal cellulosomes.

Functional annotations of spore coat CotH proteins derive from work in *Bacillus subtilis* demonstrating that CotH proteins participate in spore coat assembly and spore germination [11]. It was later shown that members of the CotH protein family from *Bacillus* bacteria act as protein kinases and that this kinase activity is critical to proper spore germination [12]. However, no cellulosomal CotH proteins from anaerobic fungi have been experimentally characterized and their contribution to fungal cellulosome assembly and/or function remains unknown. In this

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work, we apply structural bioinformatic approaches to assess whether CotH domains from the anaerobic fungus *Piromyces finnis* conserve sequence features key to protein kinase activity. We then experimentally produce two dockerin-containing CotH proteins from *Piromyces finnis* and show that the purified proteins possess ATP hydrolysis activity. Overall, these results provide evidence that cellulosomal CotH proteins produced by anaerobic fungi act as protein kinases, an important step towards elucidating their functional contribution to fungal cellulosome assembly and function.

2. Material and methods

2.1. Molecular cloning

All spore coat CotH constructs were built using the capabilities hosted in the JGI's DNA Synthesis Platform. Nucleotide sequences for each construct are shown in Table S1 in the Supplementary Information. Celsome102 and Celsome120 were codon optimized for *E. coli* expression using BOOST (https://boost.jgi.doe.gov/ [13]), synthesized (Twist Biosciences, South San Francisco, CA, USA), and cloned into pET28a using a Gibson assembly kit (New England Biolabs, Ipswich, MA, USA). The sequences were verified using PacBio Sequel II (Pacific Biosciences, San Diego, CA, USA) The protein sequences for these proteins were obtained from the *Piromyces finnis de novo* assembled transcriptome published by Solomon et al. [14]. The closest matching genomic sequence available in public databases for Celsome102 is A0A1Y1V7V1 (Uniprot) or 245,078 (Mycocosm); Celsome120 is 100% identical to sequence A0A1Y1UXM1 (Uniprot) (GenBank **ORX42284.1**).

2.2. Protein expression and purification

E. coli strains harboring plasmids encoding each protein were cultured in baffled 500 mL shake flasks with LB broth supplemented with the appropriate antibiotic. All plasmids and strains used in this work are shown in Table 1. After inoculation at a starting $OD_{600} = 0.05$, cultures were incubated at 37 °C with shaking at 250 rpm until the cultures reached an $OD_{600} = 0.5$, at which point IPTG was added to a final concentration of 0.5 mM and the cultures were moved to a 30 °C shaker for 16–18 h of induction. Induced cultures were harvested by centrifugation at 4,000×*g* in a swing bucket rotor and cell pellets were resuspended at 1% the original culture volume in PBS (pH 7.4) containing 10 mM imidazole. 0.5 mm Zirconia-Silica (Bio-Spec) beads were added at ~10% of the solution volume and the suspension was vortexed rigorously in 50 mL conical tubes for 10 intervals of 30 s, with a 30 s rest on ice in between each interval. The soluble supernatant was recovered by centrifugation at 14,000×*g* for 10 min at 4 °C. Target proteins encoding a 6x-His tag were purified with His-Pur Ni-NTA Resin (Fisher Scientific, Waltham, MA, USA) following the manufacturer's instructions. The presence and purity of the proteins of interest in the purified sample was assessed by SDS-PAGE with SYPRO protein staining (Bio-Rad Laboratories, Hercules, CA, USA) or Western blot with HRP-conjugated anti-6x His tag antibody (Abcam ab1187) and detection with Pierce ECL Western blot substrate (Fisher Scientific, Waltham, MA, USA) (Fig. S1).

Table 1
Strains and plasmids used in this study.

Strain or Plasmid	Relevant genotype or features	Source
Strain		
E. coli BL21 (DE3)	*fhuA2 [lon] ompT gal (λ DE3) [dcm] ΔhsdS λ DE3 = λ sBamHIo ΔEcoRI-B int::(lacI::PlacUV5::T7 gene1) i21 Δnin5*	Laboratory stock
Plasmid		
pET32a	*E. coli* expression vector to create N-terminal genetic fusion to TrxA	Novagen
pET32a-XynA	DNA encoding *T. maritima* XynA in pET32a	[15]
pET28a-Celsome102	DNA encoding *P. finnis* dockerin-fused spore coat CotH (Uniprot __) in pET28a	This study
pET28a-Celsome120	DNA encoding *P. finnis* dockerin-fused spore coat CotH (Uniprot __) in pET28a	This study

2.3. Assessing kinase activity

CotH kinase activity was assayed using an Adapta Universal Kinase Activity Assay kit (Fisher Scientific, Waltham, MA, USA). A recombinant xylanase from *Thermotoga maritima* produced and purified by the same procedure as the *P. finnis* CotH proteins was used as a negative control with no kinase activity. For each *in vitro* reaction, purified CotH or XynA (negative control) was supplied at 25 μg/mL with 0.75 mg/mL substrate (if applicable) and 0.35 mM ATP in 1x kinase buffer A. Reactions were performed in triplicate at 10 μL total volume in a 384-well microplate with incubation at 30 °C for 1 h. MyeBP (sequence NKRPSNRSKYL) was procured from VWR (part no. 89143–546, Radnor, PA, USA) and H1-7 (sequence RRKASGP) was procured from Enzo Life Sciences (part no. 89160–930, Farmingdale, NY, USA).

3. Results

3.1. Structural bioinformatics suggests anaerobic fungal CotH domains are kinases

Towards elucidating the role of CotH proteins in fungal cellulosome biology, we first assessed whether CotH genes from anaerobic fungal genomes conserved sequence motifs from protein kinases critical to ATP binding and hydrolysis. Using *Piromyces finnis* as a representative anaerobic fungus, we performed a multiple sequence alignment of all annotated dockerin-containing CotH protein sequences from Mycocosm (129 sequences total) to identify potential conserved ATP binding sites. The structure of *B. subtilis* CotH, proven to be a protein kinase [12], served as a reference, highlighting a PWDXD motif that binds ATP and Mg^{2+} ions to hydrolyze ATP (Fig. 1, right). Indeed, this motif was highly conserved among the *P. finnis* CotH domains, suggesting they may also act as protein kinases (Fig. 1, left).

To assess whether *P. finnis* CotH domains could also hydrolyze ATP, we produced and purified two CotH dockerin-containing proteins, Celsome102 and Celsome120, from *E. coli* and tested their ability to hydrolyze ATP using a kinase activity assay kit that detects the generation of ADP. The two CotH proteins were purified as C-terminal 6x-His tagged proteins in *E. coli* (Fig. 2). Though sometimes protein kinases autophosphorylate, a substrate may be required to catalyze ATP hydrolysis. With no *a priori* knowledge of *P. finnis* kinase substrate specificity, we used myelin basic protein (MyeBP), a generic protein kinase substrate, and the protein kinase A substrate histone H1 phosphorylation site (H1-7) as potential CotH substrates. *Bacillus subtilus* CotH structurally conserves key active site residues from canonical protein kinase A (Nguyen et al., 2016).

The *P. finnis* CotH proteins hydrolyze ATP in the absence of substrate or in the presence of MyeBP, but not in the presence of H1-7 (Fig. 3). Apparent inhibition of CotH kinase activity by H1-7 is a surprising observation. As substrate was applied at high concentration (~1 mM), we hypothesize competitive binding between H1-7 and the natural autophosphorylation site may explain why kinase activity is observed in the absence of substrate but not in the presence of H1-7. Indeed, many natural, often arginine-rich peptides are proven protein kinase inhibitors capable of inhibiting both substrate phosphorylation and autophosphorylation [16–18]. The change in ATP hydrolysis when changing the substrate suggests that substrate phosphorylation and not just ATP hydrolysis are being performed. However, this should be confirmed by testing catalytically inactive variants of Celsome102 and Celsome120 and/or using conventional γ-^{32}P ATP to directly detect phosphate addition to kinase substrates.

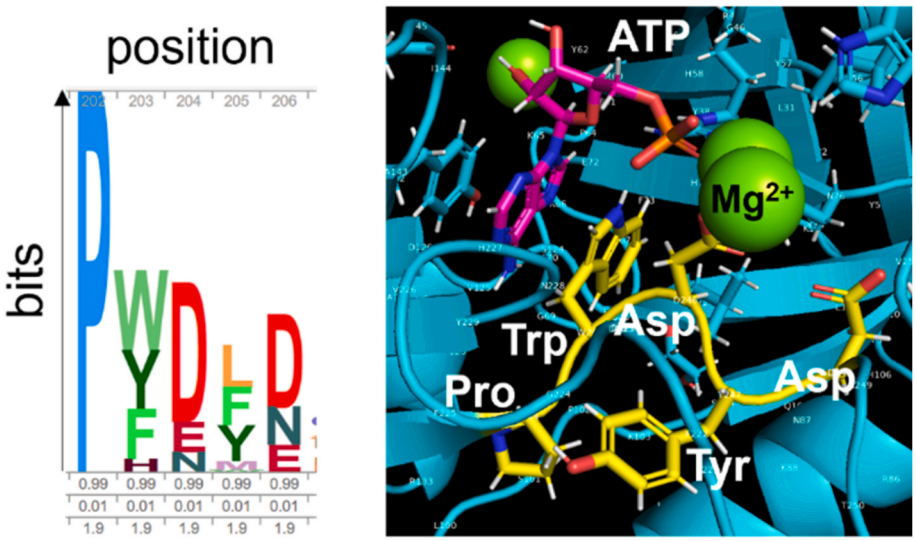

Fig. 1. *P. finnis* CotH genes conserve key sites critical to protein kinase activity. (Left) Logo plot from multiple sequence alignment of 129 CotH domain sequences from *P. finnis* showing the conserved PWDXD motif characteristic of kinases. Logo plot made with WebLogo. (Right) The PWDXD motif in B. subtilis CotH (PDB ID 5JDA) is directly involved in binding ATP and Mg^{2+} ions to mediate ATP hydrolysis.

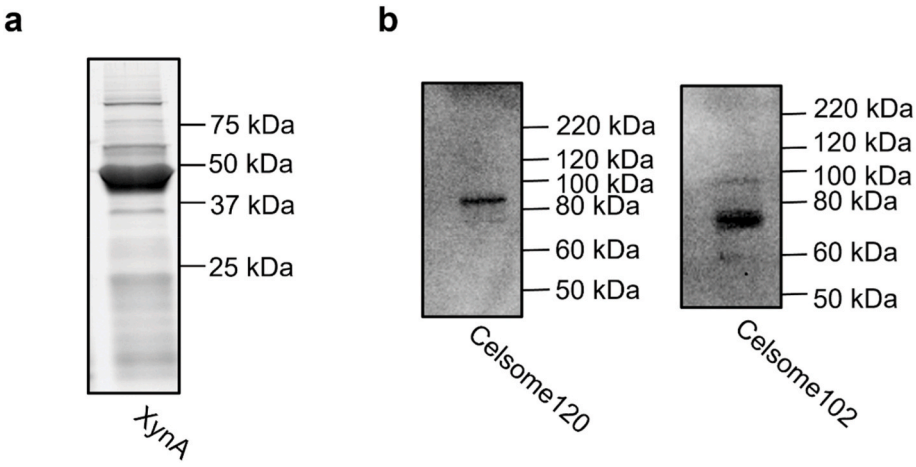

Fig. 2. Purification of *Thermotoga maritima* xylanase XynA and *P. finnis* spore coat CotH proteins from *E. coli*. A) SDS-PAGE gel of Ni-NTA purified *T. maritima* XynA (negative control for kinase assays) stained with SYPRO Ruby. B) Anti-6x His Western blot of Ni-NTA purified spore coat CotH proteins from *P. finnis*.

4. Conclusions

Spore coat CotH genes represent the second largest class of dockerin-containing genes across anaerobic fungal genomes, but their contribution to fungal cellulosome activity is poorly understood. Here we show via structural bioinformatics and experimental biochemical characterization that *P. finnis* cellulosomal CotH proteins, and likely more generally cellulosomal CotH proteins from other anaerobic fungi, hydrolyze ATP and potentially act as protein kinases. This represents an important step towards elucidating the functional role of CotH proteins in cellulosome biology, paving the way for future studies leveraging cell-free expression systems and *in vitro* biochemical assays that help elucidate the substrates that anaerobic fungal CotH kinases target. As they become available, bioimaging techniques that label CotH proteins in anaerobic fungal cultures may also prove useful in illuminating the role that CotH proteins play in anaerobic fungal biology.

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Author contribution statement

Stephen P. Lillington – Conceptualization, Methodology, Investigation, Formal analysis, Writing – Original Draft, Writing – Review & Editing, Visualization. **Matthew Hamilton** - Methodology, Investigation. **Jan-Fang Cheng** – Methodology, Investigation. **Yasuo Yoshikuni** – Conceptualization, Methodology, Resources, Writing – Review & Editing, Supervision, Project administration, Funding acquisition. **Michelle A. O'Malley** - Conceptualization, Methodology, Resources, Writing – Original Draft, Writing – Review & Editing, Supervision, Project administration, Funding acquisition.

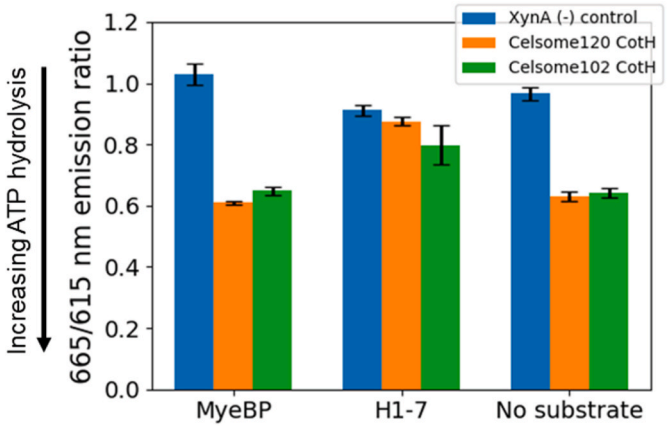

Fig. 3. *P. finnis* CotH proteins possess ATP hydrolysis activity. Production of ADP during *in vitro* kinase activity assays as measured by the Adapta kinase activity assay (Fisher Scientific). Mg^{2+} is the divalent cation supplied in these experiments. Both Celsome102 and Celsome120 autophosphorylate in the absence of substrate and potentially phosphorylate MyeBP peptide but appear not to phosphorylate the PKA substrate H1-7. XynA, a xylanase with no kinase activity, was used as a negative control. Error bars represent the SEM of technical triplicates.

Declaration of competing interest

None.

Data availability

Data will be made available on request.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.pep.2023.106323

References

[1] S.P. Lillington, P.A. Leggieri, K.A. Heom, M.A. O'Malley, Nature's recyclers: anaerobic microbial communities drive crude biomass deconstruction, Curr. Opin. Biotechnol. 62 (2020) 38–47, https://doi.org/10.1016/j.copbio.2019.08.015.

[2] L.G. Ljungdahl, H.J.M. Op den Camp, H.J. Gilbert, H.R. Harhangi, P.J. M. Steenbakkers, X.-L. Li, Cellulosomes of anaerobic fungi, in: Cellulosome, Nova Science Publishers Inc, New York, NY, 2006.

[3] C.M.G.A. Fontes, H.J. Gilbert, Cellulosomes: highly efficient nanomachines designed to deconstruct plant cell wall complex carbohydrates, Annu. Rev. Biochem. 79 (2010) 655–681, https://doi.org/10.1146/annurev-biochem-091208-085603.

[4] A.L. Carvalho, F.M.V. Dias, J.A.M. Prates, T. Nagy, H.J. Gilbert, G.J. Davies, L.M. A. Ferreira, M.J. Romão, C.M.G.A. Fontes, Cellulosome assembly revealed by the crystal structure of the cohesin–dockerin complex, Proc. Natl. Acad. Sci. USA 100 (2003) 13809–13814, https://doi.org/10.1073/pnas.1936124100.

[5] C.H. Haitjema, S.P. Gilmore, J.K. Henske, K.V. Solomon, R. de Groot, A. Kuo, S. J. Mondo, A.A. Salamov, K. LaButti, Z. Zhao, J. Chiniquy, K. Barry, H.M. Brewer, S. O. Purvine, A.T. Wright, M. Hainaut, B. Boxma, T. van Alen, J.H.P. Hackstein, B. Henrissat, S.E. Baker, I.V. Grigoriev, M.A. O'Malley, A parts list for fungal cellulosomes revealed by comparative genomics, Nat Microbiol 2 (2017) 1–8, https://doi.org/10.1038/nmicrobiol.2017.87.

[6] L. Artzi, E.A. Bayer, S. Moraïs, Cellulosomes: bacterial nanomachines for dismantling plant polysaccharides, Nat. Rev. Microbiol. 15 (2017) 83–95, https://doi.org/10.1038/nrmicro.2016.164.

[7] J.L. Brown, C.L. Swift, S.J. Mondo, S. Seppala, A. Salamov, V. Singan, B. Henrissat, E. Drula, J.K. Henske, S. Lee, K. LaButti, G. He, M. Yan, K. Barry, I.V. Grigoriev, M. A. O'Malley, Co-cultivation of the anaerobic fungus Caecomyces churrovis with Methanobacterium bryantii enhances transcription of carbohydrate binding modules, dockerins, and pyruvate formate lyases on specific substrates, Biotechnol. Biofuels 14 (2021) 234, https://doi.org/10.1186/s13068-021-02083-w.

[8] V.V. Zverlov, G.A. Velikodvorskaya, W.H. Schwarz, Two new cellulosome components encoded downstream of cell in the genome of Clostridium thermocellum: the non-processive endoglucanase CelN and the possibly structural protein CseP, Microbiology 149 (2003) 515–524, https://doi.org/10.1099/mic.0.25959-0.

[9] J.-C. Blouzard, P.M. Coutinho, H.-P. Fierobe, B. Henrissat, S. Lignon, C. Tardif, S. Pagès, P. de Philip, Modulation of cellulosome composition in Clostridium cellulolyticum: adaptation to the polysaccharide environment revealed by proteomic and carbohydrate-active enzyme analyses, Proteomics 10 (2010) 541–554, https://doi.org/10.1002/pmic.200900311.

[10] Y. Ben David, B. Dassa, I. Borovok, R. Lamed, N.M. Koropatkin, E.C. Martens, B. A. White, A. Bernalier-Donadille, S.H. Duncan, H.J. Flint, E.A. Bayer, S. Moraïs, Ruminococcal cellulosome systems from rumen to human, Environ. Microbiol. 17 (2015) 3407–3426, https://doi.org/10.1111/1462-2920.12868.

[11] G. Naclerio, L. Baccigalupi, R. Zilhao, M. De Felice, E. Ricca, Bacillus subtilis spore coat assembly requires cotH gene expression, J. Bacteriol. 178 (1996) 4375–4380, https://doi.org/10.1128/jb.178.15.4375-4380.1996.

[12] K.B. Nguyen, A. Sreelatha, E.S. Durrant, J. Lopez-Garrido, A. Muszewska, M. Dudkiewicz, M. Grynberg, S. Yee, K. Pogliano, D.R. Tomchick, K. Pawłowski, J. E. Dixon, V.S. Tagliabracci, Phosphorylation of spore coat proteins by a family of atypical protein kinases, Proc. Natl. Acad. Sci. USA 113 (2016) E3482–E3491, https://doi.org/10.1073/pnas.1605917113.

[13] E. Oberortner, J.-F. Cheng, N.J. Hillson, S. Deutsch, Streamlining the design-to-build transition with build-optimization software tools, ACS Synth. Biol. 6 (2017) 485–496, https://doi.org/10.1021/acssynbio.6b00200.

[14] K.V. Solomon, C.H. Haitjema, J.K. Henske, S.P. Gilmore, D. Borges-Rivera, A. Lipzen, H.M. Brewer, S.O. Purvine, A.T. Wright, M.K. Theodorou, I.V. Grigoriev, A. Regev, D.A. Thompson, M.A. O'Malley, Early-branching gut fungi possess a large, comprehensive array of biomass-degrading enzymes, Science 351 (2016) 1192–1195, https://doi.org/10.1126/science.aad1431.

[15] S.P. Gilmore, S.P. Lillington, C.H. Haitjema, R. de Groot, M.A. O'Malley, Designing chimeric enzymes inspired by fungal cellulosomes, Synth Syst Biotechnol 5 (2020) 23–32, https://doi.org/10.1016/j.synbio.2020.01.003.

[16] C. House, B.E. Kemp, Protein kinase C contains a pseudosubstrate prototope in its regulatory domain, Science 238 (1987) 1726–1728.

[17] T. Nishi, R.J.A. Budde, J.S. McMurray, N.U. Obeyesekere, N. Safdar, V.A. Levin, H. Saya, Tight-binding inhibitory sequences against pp60 c−src identified using a random 15-amino-acid peptide library, FEBS Lett. 399 (1996) 237–240, https://doi.org/10.1016/S0014-5793(96)01329-4.

[18] E. Ekokoski, O. Aitio, K. Törnquist, J. Yli-Kauhaluoma, R.K. Tuominen, HIV-1 Tat-peptide inhibits protein kinase C and protein kinase A through substrate competition, Eur. J. Pharmaceut. Sci. 40 (2010) 404–411, https://doi.org/10.1016/j.ejps.2010.04.013.