3D structure of *Geobacillus* GtfC: GH13 or GH70?

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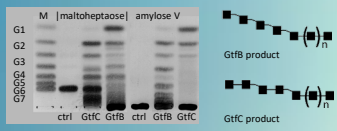
Background & goal

GtfC-type 4,6-α-glucanotransferases (α-GTs) are of interest for modification of starch into low-glycemic food ingredients[1]. They have been classified in Glycoside Hydrolase family 70 (GH70), together with glucansucrases and GtfB-type α-GTs from lactic acid bacteria (LAB). However, GtfC-type α-GTs:

- occur in *non*-LAB
- share *low* sequence identity
- *lack* circular permutation

GtfCs therefore have been proposed as evolutionary intermediates between starch-degrading GH13 α-amylases and α-glucan synthesizing GH70 GtfBs[2].
We determined the first crystal structure of a GtfC, GbGtfC-ΔC from *Geobacillus* 12AMOR1[3], compared its 3D structure, and performed a phylogenetic and structural analysis of 63 putative GtfC-type α-GTs.



Previous characterization of GbGtfC products[3,4] showed that the enzyme synthesizes a linear α-1,4/α-1,6 alternating α-glucan, while GtfB products contain consecutive α-1,6 linkages.

Structure determination

A His-tagged and C-terminally truncated construct of *Geobacillus* 12AMOR1 GtfC (GbGtfC-ΔC, residues 33-738)[2] was crystallized by vapor diffusion at 293 K using 1.07-1.14 M $(NH_4)_2SO_4$, 0.1 M MES-NaOH, pH 6.5, 0.4 M Na_3Citrate.

The GbGtfC-ΔC crystal structure was determined by molecular replacement.

Parameter	*overall*	*highest shell*
Resolution (Å)	131.3-2.25	2.31-2.25
R_{pim}	0.030	0.245
Unique observations	59408	4359
Multiplicity	1.9	1.9
I/σ	20.5	4.2
Completeness (%)	99.3	95.0
$CC_{1/2}$	0.999	0.795

Phylogenetic analysis of GtfCs

An NR-BLASTp search with the *Geobacillus* 12AMOR1 sequence was performed; only hits showing a complete catalytic domain (presence of GH70 homology motifs I-IV) and circular permutation were selected.

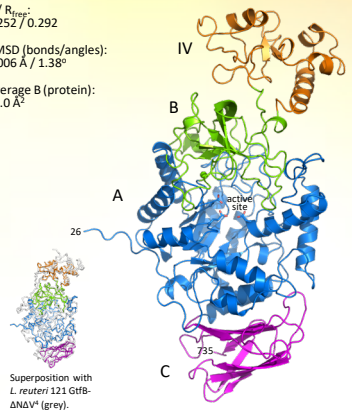
A phylogenetic tree was constructed including other GH70 enzymes (glucansucrases, GtfBs, GtfDs) as well as GH13_5 α-amylases. Before alignment, permuted sequences were first 'depermuted'.

GbGtfC crystal structure

R / R_{free}:
0.252 / 0.292

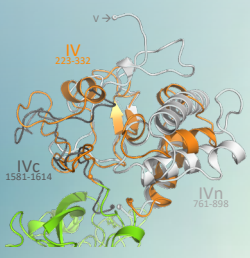
RMSD (bonds/angles):
0.006 Å / 1.38°

Average B (protein):
55.0 Å²

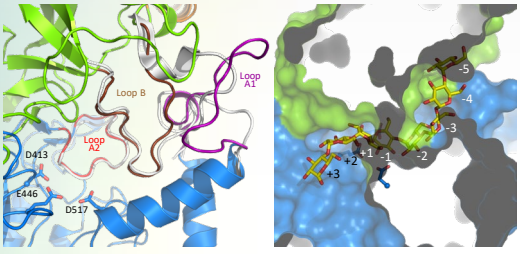


The GbGtfC-ΔC crystal structure showing the canonical GH70 domain arrangement but with a non-permuted catalytic domain A. The GH13/GH70 core consists of domains A+B+C; the auxiliary domain IV is a 110-residue single-segment insertion in domain B.

Comparison with GtfB-type α-GTs

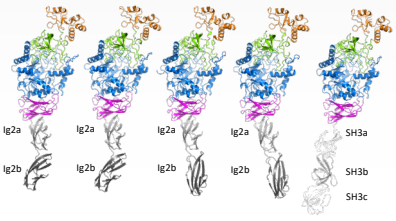


Domain IV of GbGtfC is a 110-residue single segment insertion in domain B, partially overlapping with that of the dual segment domain IV of the GtfB-type 4,6-α-GT from *Lactobacillus reuteri* 121[4] (shown in grey).

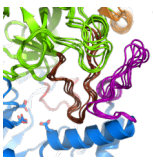


The catalytic triad of GbGtfC contains D413 (nucleophile), E446 (acid/base) and D517 (transition state stabilizer). Three loops near the active site (loops A1, A2 and B) create a tunneled binding groove in GbGtfC, similar to *L. reuteri* 121 GtfB[4,5]. A starch fragment (maltooctaose) was modeled in the binding groove based on crystal structures of other GH70 4,6-α-GTs and GH13 α-amylases.

Modeling other GtfC-type α-GTs



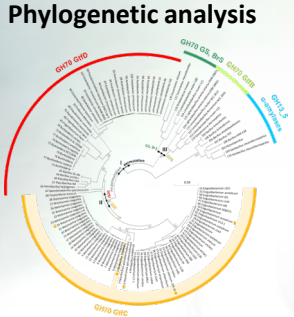
Geobacillus 12AMOR1	*Heyndrickxia sporothermodurans*	*Exiguobacterium sibiricum*	*Exiguobacterium acetylicum*	*Weizmannia coagulans*
	seq id =76.3%	seq id = 56.3%	seq id = 56.5%	seq id = 72.6%
pLDDT = 94.8	pLDDT = 94.9	pLDDT = 94.6	pLDDT = 94.3	pLDDT = 94.8

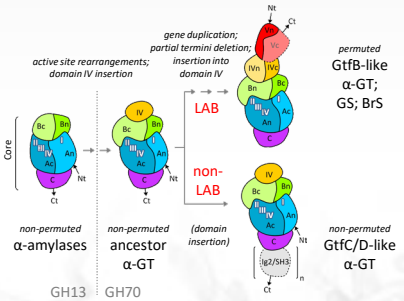


AlphaFold[6] models of selected GtfC-type α-GTs; N-terminal residues with pLDDT <60 are not shown. C-terminal domains have Bacterial Immunoglobulin type 2 (Ig2)-like or SRC Homology 3 (SH3) folds.

Superposition of the active sites with the loops A1, A2 and B show that each of these GtfCs feature a tunneled binding groove like GbGtfC.

Phylogenetic analysis





Unrooted phylogenetic tree and proposed evolutionary pathways for GH70 and GH13 sequences. GH13 α-amylases (present in all kingdoms of life) acquired transglycosylation specificity by changing their active site, and insertion of domain IV resulted in a (still non-permuted) GH70 ancestor α-GT. From the ancestor, two branches evolved: in non-LAB, GtfC- and GtfD-type α-GTs remained non-permuted and acquired C-terminal Ig2- or SH3-domains. The second branch evolved in LAB: GS (glucansucrases) and BrS (branching sucrases) became circularly permuted, acquiring different auxiliary domains (e.g. domain V).

Conclusions & Outlook

We determined the first crystal structure of a GtfC-type 4,6-α-glucanotransferase (GbGtfC-ΔC). Its core domain topology and active site architecture is highly conserved with most GtfB-type 4,6-α-GTs found in LAB, despite low sequence similarity, despite the absence of circular permutation in the catalytic domain, and despite the fact that GtfC- (and GtfD-)type starch-converting enzymes evolved in non-LAB.

GbGtfC represents the so far 63 putative GtfCs found in databases, with a tunneled architecture of the binding groove. We thus expect that GtfC-type 4,6-α-GTs synthesize linear α-glucans. The so far unique specificity of GbGtfC, leading to alternating α-1,4/α-1,6 linkages in its products, is currently under investigation through mutation and docking studies.

1. Gangoiti, J. *et al.*, Crit. Rev. Food Sci. Nutr. (2020), 60, 123-146. **2**. Gangoiti, J. *et al.*, Appl. Environm. Microbiol. (2015), 82, 756-766. **3**. Te Poele, E.M. *et al.*, J. Agric. Food Chem. (2021), 69, 9859-9868. **4**. Bai, Y. *et al.*, Structure (2017), 25, 231-242. **5**. Pijning, T. *et al.*, J. Agric. food Chem. (2021), 69, 13235-13245. **6**. Jumper *et al.*, Nature (2021), 596, 583-589.