Structural enzymology of *Cellvibrio japonicus* Agd31B reveals α-transglucosylase activity in Glycoside Hydrolase Family 31

Johan Larsbrink\textsuperscript{1=}, Atsushi Izumi\textsuperscript{2=}, Glyn R. Hemsworth\textsuperscript{2}, Gideon J. Davies\textsuperscript{2,*}, Harry Brumer\textsuperscript{1,3,*}

\textsuperscript{1} Division of Glycoscience, School of Biotechnology, Royal Institute of Technology (KTH), AlbaNova University Centre, 106 91 Stockholm, Sweden.

\textsuperscript{2} York Structural Biology Laboratory, Department of Chemistry, the University of York, York YO10 5DD, United Kingdom

\textsuperscript{3} Michael Smith Laboratories and Department of Chemistry, University of British Columbia, 2185 East Mall, Vancouver, BC, V6T 1Z4, Canada

Running title: *A bacterial α-transglucosylase from glycoside hydrolase family 31*

\textsuperscript{=} The equal contribution of these authors is highlighted.

\* To whom correspondence should be addressed: Harry Brumer, Tel: +1 604 827 3738, Fax: +1 604 822 2114, e-mail: brumer@msl.ubc.ca; Gideon J. Davies, Tel. +44 1904 328260, Fax: +44 904 328266, e-mail: gideon.davies@york.ac.uk

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**Background:** Transglycosylases are important enzymes in bacterial glycogen metabolism.

**Results:** The tertiary structure and function of a novel α-tranglucosylase have been defined.

**Conclusion:** In addition to previously known activities, glycide hydrolase family 31 (GH31) contains a group of enzymes with 1,4-α-glucan 4-α-glucosyltransferase activity.

**Significance:** This gives new insight into bacterial glycogen utilization and will inform future bioinformatic analyses of (meta)genomes.

**SUMMARY**

The metabolism of the storage polysaccharides glycogen and starch are of vital importance to organisms from all domains of life. In bacteria, utilization of these α-glucans requires the concerted action of a variety of enzymes, including glycoside hydrolases, glycoside phosphorylases, and transglycosylases. In particular, transglycosylases from glycoside hydrolase families GH13 and GH77 play well-established roles in α-glucan sidechain (de)branching, regulation of oligo- and polysaccharide chain length, and formation of cyclic dextrans. Here, we present the biochemical and tertiary structural characterization of a new type of bacterial 1,4-α-glucan 4-α-glucosyltransferase from glycide hydrolase family 31 (GH31). Distinct from 1,4-α-glucan 6-α-glucosyltransferases (EC 2.4.1.24) and 4-α-glucanotransferases (EC 2.4.1.25), this enzyme strictly transferred one glucosyl residue from α(1→4) glucans in disproportionation reactions. Substrate hydrolysis was undetectable for a series of malto-oligosaccharides, except maltose, for which transglycosylation nonetheless dominated across a range of substrate concentrations. Crystallographic analysis of the enzyme in free, acarbose-complexed, and trapped 5-fluoro-β-glucosyl-enzyme intermediate forms revealed extended substrate interactions across one negative and up to three positive subsites, thus providing structural rationalization for the unique, single-
monosaccharide transferase activity of the enzyme.

Glycogen is a highly branched, mixed-linkage α(1→4)/α(1→6)-glucan polymer that serves as a readily accessible, osmotically neutral, cellular energy reserve in all domains of life (1-3). Glycogen is structurally related to amyllopectin which, together with the linear polysaccharide amylose (α(1→4)-glucan), comprises the plant storage reserve starch (4). Prokaryotic and eukaryotic glycogen biosynthesis and degradation is a complex, highly conserved, and tightly controlled process involving a myriad of enzymes and regulatory factors (2,3). In bacteria such as Escherichia coli, glycogen is synthesized from ADP-glucose by the combined action of glycogen synthase, which builds linear α(1→4)-glucan chains, and glycogen branching enzyme, which catalyzes chain re-arrangement via α(1→4)-to-α(1→6) transglycosylation, thereby yielding polydisperse molecules with molar masses of up to 10^7-10^8 Da (2,3). In turn, catabolism under carbon-limited conditions occurs via the sequential action of glycogen phosphorylase and debranching enzyme to yield glucose-1-phosphate.

In bacteria, glycogen metabolism is closely linked to the metabolism of storage maltodextrins, which involves the build-up and re-arrangement of linear α(1→4)-gluco-oligosaccharides by transglycosylation (5). This process has been well-described in E. coli, in which the amylomaltsase MalQ, a member of Glycoside Hydrolase Family 77 (GH77, 6), catalyzes the transfer of a 4-α-glucanosyl fragment from the non-reducing end of malto-oligosaccharide donor substrates, and possibly the disaccharide maltose, to malto-oligosaccharide acceptors (4-α-glucanotransferase activity, EC 2.4.1.25) (5,7,8). The bacterial amylomaltsases are structurally and functionally related to the plant disproportionating enzymes (“D-enzymes”) of GH77, which transfer maltosyl and longer 4-α-glucanosyl units from maltotriose and higher congeners (9). Likewise, certain thermophilic bacterial 4-α-glucanotransferases of GH13 catalyse the disproportionation of maltotriose (10,11), maltotetraose (12,13), and longer 4-α-glucan chains.

Indeed, transglycosylation reactions leading to the re-arrangement of α-glucans are widespread among bacteria. Glycogen branching and debranching aside, diverse enzymes with 4-α-glucanotransferase activity (α(1→4)-glucan:α(1→4)-glucan transferase activity) can produce a range of linear and cyclic maltodextrin products via freely reversible disproportionation and cyclization reactions, respectively (9,14). The production of 6-, 7-, and 8-membered α(1→4)-linked cyclodextrins by the cyclodextrin glucanotransferases (CGTases, EC 2.4.1.19) of Glycoside Hydrolase Family 13 (GH13) represents an especially important process in industrial starch valorization (15). Analogous α(1→6)-linked cycloisomaltooligosaccharides are the main products of some GH66 enzymes (16-18). Likewise, certain bacterial members of GH77 catalyze the production of large cyclic α-glucans (degrees of polymerization ≥ 22) through intramolecular transglycosylation (9). The GH31 enzymes ctsY and ctsZ from Arthrobacter and Sporosarcina species generate commercially interesting cycloalternan tetrasaccharides (cyclo[→6]-α-D-Glcp-(1→3)-α-D-Glcp-(1→6)-α-D-Glcp-(1→3)-α-D-Glcp-(1→4) from α(1→4)-glucans via a remarkable three-step, coupled reaction involving α(1→4)-to-α(1→6) transglycosylation, intermolecular isomaltosyl transfer, and cyclization (19,20). In addition to these dominant transglycosylases, a number of retaining α-glucose hydrolases with degrees of transglycosylation ability have also been identified ((21-23), and references therein).

The Gram-negative soil saprophyte Cellvibrio japonicus is best known for its ability to efficiently utilize a plethora of plant cell wall polysaccharides as energy sources (24). Additionally, the genome sequence of this
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organism has revealed a large number of predicted α-glucan active enzymes. In total, the C. japonicus genome encodes 22 enzymes from GH families 13, 15, 31, 57 and 77 (25), that may be predicted to act on starch and/or glycogen. However, none of these have been biochemically or structurally characterized (6,25).

GH31, in particular, is one of the major α-glucosidase-containing GH families. This family is however functionally diverse; it also contains α-xylanases and α-glucan lyases, in addition to the aforementioned ctsY and ctsZ α-transglycosylases. A phylogenetic analysis has recently been presented that partially delineates these activities in clades, although sequence-based functional prediction is not absolute (26). The generally exo-acting GH31 enzymes, which are members of Clan GH-D together with GH27 and GH36, have been suggested to share a common ancestor with members of clan GH-H, which comprises generally endo-acting α-glucan-active enzymes of GH13, 70, and 77 (27).

Building upon our interest in the post-genomic characterization of GH31 enzymes from C. japonicus (26), we present here a detailed structural enzymology study of CjAgd31B, whose coding sequence resides within a gene cluster encoding predicted α-glucan-active enzymes and sugar transporters. Biochemical analysis revealed that CjAgd31B is a predominant transglucosylase with strict α(1→4)-linkage specificity, which represents a previously undiscovered activity in GH31. Crystallography of the enzyme in free, acarbose-complexed, and trapped 5-fluoro-α-D-glucopyranosyl-enzyme intermediate forms has highlighted the structural basis for the strict transfer of a single glucosyl residue and preference for maltotriose and longer substrates. Taken together, the data suggest a biological role for CjAgd31B in glycogen or maltodextrin metabolism that may be complementary to that predicted for the GH77 homologue CjMal77Q.

EXPERIMENTAL PROCEDURES

Curve fitting and processing of kinetic data were performed using Origin 8 software (OriginLab). p-Nitrophenyl (pNP) α-glycosides, sucrose, D-maltose and starch from corn were purchased from Sigma. Maltooligosaccharides (maltotriose to maltohexaose), isomaltose, melibiose and acarbose were purchased from CarboSynth. α-glucosyl fluoride and 5-fluoro-α-D-glucopyranosyl fluoride (5FαGlcF) were kind gifts from Professor Stephen Withers (Department of Chemistry, University of British Columbia, Canada). Ultrapure water was used in all experiments and refers to water purified on a Milli-Q system (Millipore) with a resistivity, ρ, > 18.2 MΩ-cm.

Cloning of CjAgd31B – The open reading frame encoding CjAgd31B (GenBank accession number ACE84782.1) was amplified by PCR from genomic DNA of C. japonicus Ueda107 using Phusion polymerase (Finnzymes) and the following primers (Thermo Fischer Scientific):

5′-CACCATGAATCCGGTCAAACG-3′ and 5′-ATGCAACCTGAGGTTAAGCGCTTC-3′

with the forward primer incorporating the CACC overhang needed for TOPO cloning and excluding the predicted signal peptide (cleavage site between amino acid residues 24-25). The PCR product was cloned into the pENTR/SD/D-TOPO entry vector (Invitrogen) and recombined into the pET-DEST42 destination vector (Invitrogen) as described previously (26).

Gene expression and protein purification – Plasmids harboring the CjAgd31B gene were transformed into E. coli BL21(DE3) by electroporation, and the gene was expressed and purified by immobilized metal affinity chromatography (IMAC) following an established protocol described in (26). Analysis by SDS-PAGE showed the protein to be electrophoretically pure. LC-ESI-MS was used for protein molar mass determination as described previously (28). For crystallography studies, the protein was further purified by size exclusion chromatography and ion exchange chromatography. The eluted protein solution was
concentrated to 5 ml by a Vivaspin 20 concentrator (Sartorius Stedim Biotech) and loaded onto a HiLoad 16/60 Superdex 200 pg column (GE) equilibrated with 20 mM Tris (pH 8.0), 300 mM sodium chloride. The eluted protein solution was dialyzed into 20 mM Tris (pH 8.0) at 4°C for 16 hours. The dialyzed protein solution was loaded onto Resource Q column (GE) equilibrated with 20 mM Tris (pH 8.0) and eluted with a linear gradient of 20 mM Tris (pH 8.0), 400 mM sodium chloride. Two major peaks of CjAgd31B were obtained and the peak eluted in lower salt concentration was collected and used for protein crystallization. Protein concentrations were determined from $A_{280}$ values of suitably diluted samples using an extinction coefficient of 139245 M$^{-1}$·cm$^{-1}$, as calculated by the ProtParam tool on the ExPASy server (29).

Thin layer chromatography (TLC) – TLC was performed using normal phase silica on aluminum plates, eluted with acetonitrile–water (2:1). Analytes were visualized by immersion in 8% H$_2$SO$_4$ in ethanol followed by charring.

High Performance Anion-Exchange Chromatography with Pulsed Amperometric Detection (HPAEC-PAD) – Oligo- and monosaccharides were analyzed on a Dionex ICS-3000 HPLC system operated by Chromelion software version 6.80 (Dionex) using a Dionex Carbopac PA200 column. Solvent A was water, solvent B was 1 M sodium hydroxide and solvent C was 1 M sodium acetate.

Gradient A: 0 to 5 min, 10 % B, 2 % C; 5 to 12 min, 10 % B and a linear gradient from 2-30 % C; 12 to 12.1 min, 50 % B, 50 % C; 12.1 to 13 min, an exponential gradient of B and C back to initial conditions; 13 to 17 min, initial conditions.

Gradient B: 0 to 4 min, 10 % B, 5 % C; 4 to 8 min, 10 % B and a linear gradient from 5-25 % C; 8 to 8.1 min, 50 % B, 50 % C; 8.1 to 9 min, an exponential gradient of B and C back to initial conditions; 9 to 13 min, initial conditions.

Gradient C: 0 to 4 min, 10 % B, 6 % C; 4 to 17 min, 10 % B and a linear gradient from 6-25 % C; 17 to 17.1 min, 50 % B, 50 % C; 17.1 to 18 min, an exponential gradient of B and C back to initial conditions; 18 to 22 min, initial conditions.

Gradient D: 0 to 4 min, 10 % B, 6 % C; 4 to 10 min, 10 % B and a linear gradient from 5-25 % C; 10 to 10.1 min, 50 % B, 50 % C; 10.1 to 11 min, an exponential gradient of B and C back to initial conditions; 11 to 15 min, initial conditions.

Gradient E: 0 to 4 min, 10 % B, 6 % C; 4 to 15 min, 10 % B and a linear gradient from 5-25 % C; 15 to 15.1 min, 50 % B, 50 % C; 15.1 to 16 min, an exponential gradient of B and C back to initial conditions; 16 to 20 min, initial conditions.

pH-rate profile – Measurements of the pH-dependence of CjAgd31B were carried out using maltose as substrate an HPAEC-PAD-based transglycosylation assay, described below. The buffers used (50 mM) were: sodium citrate (pH 3–6.5), sodium phosphate (pH 6.5–8), glycylglycine (pH 8–9) and glycine (pH 9–10) (Supplemental Figure S1).

Enzyme assays – Activity on pNP glycosides was analyzed by a stopped assay as previously described (26), using an enzyme concentration of 6.5 μM.

The transglycosylation activity of CjAgd31B on various oligosaccharides was performed in 100 μl reactions at 25 °C in 50 mM citrate buffer (pH 6). For initial-rate saturation kinetics experiments, CjAgd31B was added to a final concentration of 1.6 μM for maltose and 270 pM for maltotriose, maltotetraose and maltopentaose. Reactions proceeded for typically 10 min and were stopped by addition of 4 μl 5 M sodium hydroxide. HPAEC-PAD was used for product analysis, using Gradients A, B, D and E for reactions on maltose, maltotriose, maltotetraose and maltopentaose, respectively. Commercial maltooligosaccharides (maltose to maltohexaose) were used as standards.

To test different acceptors, starch was used as a glucosyl donor. Starch from corn was dissolved to 1 % (w/v) in water followed by dialysis in deionized water using a 5 kDa cutoff membrane to
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remove monosaccharides and small oligosaccharides. 50 µl reactions containing 0.4 % starch as donor, 1 mM of acceptor (glucose or isomaltose) and 2 µM enzyme were incubated at 25 °C for 10 min, and terminated by addition of 2 µl 5 M sodium hydroxide. Products were analyzed by HPAEC-PAD using Gradient C.

**IC<sub>50</sub> measurements** – The inhibition of CjAgd31B by acarbose (0-1050 µM) was determined by using maltotriose (100 µM) as a substrate in reactions as described above. Product formation was analyzed by HPAEC-PAD (Gradient B).

**Crystallization and Data Collection** – CjAgd31B was stored in 5 mM Bis-Tris-Propane (pH 8.5) and concentrated to 7 mg/ml by using a Vivaspin 20 concentrator. In an initial crystal screening using Crystal Screen HT, Index HT, SaltRx HT (Hampton Research) and modified Newcastle Screen prepared at YSBL, small single crystals were obtained in several conditions. Well-diffracting crystals were obtained after 3-4 days in 1.8 M ammonium sulfate, 0.1 M HEPES (pH 7.0), 2 % PEG400 at 20°C by the sitting drop vapor diffusion method. The structure was solved using experimental phasing with an iodine derivative. This was prepared by placing approximately 1 µl of a 0.25 g/ml potassium iodide solution into a 2 µl crystallization droplet to allow slow diffusion of iodine into the crystal. Crystallization droplets with iodine solution were left at 20°C for 16 hours prior to freezing and data collection.

For the complex structures, crystals were soaked in 1.8 M ammonium sulfate, 0.1 M HEPES (pH7.0), 2 % PEG400 and either 5 mM 5-fluoro-α-D-glucopyranosyl fluoride (5FαGlcF) or 5 mM acarbose for 1 hour at 20°C.

All crystals were cryoprotected by 2.0 M lithium sulfate, 0.1 M HEPES (7.0), 2 % PEG400. The X-ray data for the free enzyme and iodine-soaked crystals were collected at 100 K on ADSC Q315 CCD detector at BL-ID14-4 and BL-ID29 at the European Synchrotron Radiation Facility (ESRF), respectively. The details of the data collections are listed in Table 1. All data were processed using iMOSFLM (30) and programs from the CCP4 suite (31) unless otherwise stated. The statistics of the data processing and structure refinement are listed in Table 1.

Experimental phasing was performed by Single Wavelength Anomalous Diffraction (SAD) methods at a wavelength of 1.8 Å. Heavy-atom sub-structure solution and initial phasing was performed on the 2.9 Å resolution iodine-derivatized crystal data with autoSHARP (32) followed by phase extension with the 1.9 Å free-enzyme data set using DM (33). The 1.9 Å data were used as a starting point for automatic model building using ARP/wARP (34) (Table 2). Structure refinement, including TLS refinement of molecular motions, was performed using PHENIX (35) used interspersed with manual rebuilding using COOT (36). Complex structures were solved by molecular replacement using MOLREP (37) with the free enzyme structure as a search model and refined as above.

**RESULTS**

**Bioinformatics analyses** – CjAgd31B is found among putative receptors, transporters and α-glucan-active enzymes in the genome of C. japonicus (Supplemental Figure S2). Notably, these genomic neighbors include a predicted α-amylase, cyclomaltodextrin glucanotransferase, 6-phospho-β-glucosidase, and a glucokinase. CjAgd31B has a predicted secretion signal peptide, and is thus likely to be localized in the periplasm or to be secreted extracellularly. In addition to CjAgd31B, the genome of C. japonicus encodes two other GH31 members, the putative α-glucosidase CjAgd31A, and the biochemically and structurally characterized α-xyllosidase CjXyl31A (26). Although all are members of GH31, CjAgd31B has a low sequence
similarity to both CjAgd31A and CjXyl31A, with amino acid identities of 28 and 27 % and similarities of 43 and 45 %, respectively. From our recent phylogenetic analysis (26), the biochemically characterized member of GH31 most similar to CjAgd31B is YihQ from Escherichia coli (sequence identity 28 %, similarity 44 %). E. coli YihQ has been annotated as an α-glucosidase, based on a weak ability to hydrolyze the artificial substrate α-glucosyl fluoride, although the enzyme was impotent toward a range of other α-glucosides (38).

**Gene expression** – A gene construct encoding CjAgd31B, with a C-terminal hexahistidine tag and lacking the predicted native signal peptide, was expressed in E. coli BL21(DE3) cells. The protein product was purified by IMAC for kinetic analyses and additionally by SEC and ion exchange for crystallization studies; purity in both cases was confirmed by SDS-PAGE (data not shown). The molar mass of CjAgd31B, corresponding to the C-terminal His-tagged enzyme starting at N25 (the natural site of signal peptide cleavage), was verified by LC-ESI-MS (expected, 94478.8 Da; observed, 94478.1 Da. Supplemental Figure S3). The overall yield was typically around 100 mg/L of culture broth.

**Transglycosylation activity on malto-oligosaccharides** – Based on membership in GH31, the substrate specificity of CjAgd31B was initially tested using pNP-α-glucoside and pNP-α-xyloside; the enzyme showed no apparent hydrolysis of either of these substrates after extended incubation (1 mM substrate, 6.5 µM enzyme, up to 4 hours incubation). The enzyme also displayed no detectable activity on sucrose, melibiose, isomaltose, or α-glucosyl fluoride (a substrate for E. coli YihQ (38)). On maltose [Glcpa(1→4)Glc], however, formation of both glucose and longer oligosaccharides could be observed using TLC (data not shown). The transglycosylation potential of CjAgd31B was confirmed by HPAEC-PAD following incubation of 6.5 µM enzyme with 10 mM maltose for 30 minutes, which led to a build-up of malto-oligosaccharides. Products with a degree of polymerization (d.p.) of up to 14 glucose residues could be detected (Figure 1A).

To further analyze the catalytic properties of CjAgd31B, HPAEC-PAD was used to measure product formation from reactions on malto-oligosaccharides. Under conditions of low substrate conversion conditions (< 10 % of substrate consumed) the enzyme was shown to transfer a single glucose moiety from a donor to an acceptor molecule. Incubation of the enzyme with linear malto-oligosaccharides (maltotriose to maltopentaose) exclusively yielded Glcₙ₋₁ and Glcₙ₊₁ products via transglycosylation (Figure 1B). On maltotriose, maltotetraose, and maltopentaose, the production of glucose, which would indicate competing substrate hydrolysis, was not observed under initial-rate conditions. Thus, apparent Michaelis-Menten kinetic parameters for these substrates could be directly determined from plots of vₐ/[E] versus [Glcₙ] (Figure 2A), where the rate of transglycosylation product formation is given by Equation 1 (in this case, the rate of Glcₙ₋₁ formation can also be used). The best substrate for the enzyme was maltotriose with a (kₖₐₜ/Kₘ)ₚₚ value of 196 s⁻¹·mM⁻¹, while maltotetraose and maltopentaose displayed comparable (kₖₐₜ/Kₘ)ₚₚ values of 72 and 58 s⁻¹·mM⁻¹, respectively (Table 3).

\[ v_{\text{transglycosylation}} = \frac{d[Glc_{n+1}]}{dt} \]  

Hydrolysis could, however, be detected when maltose was used as a substrate. Here, the production of Glc was measurably higher than the 1:1 stoichiometric ratio of Glc to Glc₂ expected for disproportionation (Glc₂ → Glc₃ + Glc). In this case, the velocity of the transglycosylation reaction was directly measured according to Equation 1 by quantifying the maltotriose produced. Determination of the hydrolytic rate required subtraction of amount of glucose co-produced by disproportionation from that arising
from hydrolysis, including accounting for the stoichiometry of glucose release by hydrolysis (2 mol Glc/mol maltose), Equation 2. From the specific transglycosylation activity on 0.5 mM maltose, the pH profile was found to be broad, with an optimum of pH 6.5 (Supplemental Figure S).

$$v_{\text{hydrolysis}} = \frac{d([\text{Glc}]-[\text{maltotriose}])}{dt}$$  (2)

Figure 2B shows plots of $v_o/[E]_t$ versus [Glc] for both the hydrolytic and transglycosylation reactions, which clearly indicate the predominance of transglycosylation over a wide range of substrate concentrations. The $(k_{cat}/K_m)_{\text{app}}$ value determined for the transglycosylation reaction was 39 s$^{-1}$·M$^{-1}$ (Table 3). Whereas the $(k_{cat})_{\text{app}}$ value for maltose transglycosylation is actually higher than that of maltotriose, the lower $(k_{cat}/K_m)_{\text{app}}$ value on the disaccharide is due to a high $(K_m)_{\text{app}}$ value. This high value may reflect the observation of up to four enzyme subsites (-1 to +3, nomenclature according to (39)) in the crystal structure (vide infra), of which only two would be occupied by maltose acting as a donor substrate.

Across the range of maltose concentrations examined (50 µM to 50 mM), the rate of hydrolysis was consistently low (Figure 2B). At 50 µM maltose, the rate of hydrolysis was equal to the rate of transglycosylation ($v_o/[E]_t = 2$ s$^{-1}$), after which the hydrolytic rate increased to a maximum of 10 s$^{-1}$ at 2 mM substrate. Nonetheless, the hydrolytic rate was only 13% of the transglycosylation rate at this maximum. The rate of hydrolysis steadily decreased to approximately 8 s$^{-1}$ at 50 mM maltose as the transglycosylation rate continued to increase, resulting in a relative hydrolysis rate of < 2% at this concentration.

Starch and isomaltose as an alternate donor/acceptor pair – Starch was also tested as a high d.p. donor substrate with glucose and isomaltose as alternate acceptor substrates. Despite dialysis to reduce the content of short maltooligosaccharides, the commercial starch sample contained a minor amount of $\alpha$(1→4) glucans with d.p. 2 and higher). When CjAgd31B was incubated with starch alone, the amounts of shorter maltooligosaccharides (maltose to maltotetraose) still present in the substrate mixture decreased significantly while longer maltooligosaccharides (maltopentaose to maltododecaose) increased, in keeping with previous observations on purified maltooligosaccharides during kinetic analyses (Figure 3A). In contrast, when the reaction was supplemented with 1 mM glucose, a large increase of maltooligosaccharides (maltose to maltododecaose) could be observed (Figure 3B). Here, glucose (which, being a monosaccharide, cannot act as a glycosyl donor) acted as viable acceptor substrate in the breakdown of the glycosyl-enzyme intermediate formed via initial attack of starch chain-ends. This reaction is the reverse of glycosyl-enzyme formation when maltose acts as a donor substrate, consistent with the principle of microscopic reversibility. The large increase in medium-length maltooligosaccharides compared with the glucose-free reaction both demonstrates how the non-reducing ends present in starch are utilized as glucose donors, and also suggests that glucose moieties are mainly “shuffled” between the non-reducing ends of the starch molecules when no additional acceptor is included in the reactions.

The disaccharide isomaltose $\text{Glc}(1\rightarrow6)$-Glc on its own was not a substrate for CjAgd31B, which indicated that the enzyme was unable to utilize $\alpha$(1→6)-linked glucosides as donor substrates. Glycogen and starch both contain $\alpha$(1→6)-linked branch points, which prompted us to test whether such branches might be extended by CjAgd31B, using isomaltose as a model. Indeed, the enzyme transferred glucosyl moieties from starch to isomaltose (1 mM), as indicated by the appearance of an alternate series of peaks on the chromatogram, each slightly preceding the corresponding all-$\alpha$-linked congener, which...
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suggests the formation of Glc(a1→4)-Glc-α(1→6)-Glc saccharides (Figure 3B). As such, these data indicated that the +2 subsite of the active site is not strictly specific for α(1→4) linked sugars, but will accommodate α(1→6) linked isomers.

**Inhibition by acarbose** – The inhibitory effect of the pseudotetrasaccharide acarbose, a common α-glucanase and α-glucosidase inhibitor, was assayed using maltotriose as the substrate at a fixed concentration of 100 µM. The IC₅₀ value was determined to 75.1 ± 3.4 µM by plotting the relative activity versus the concentration of acarbose and fitting Equation 3 by nonlinear regression (Figure 4). With reactions performed at a substrate concentration (100 µM), which is much lower than the apparent Kₘ value (1.2 mM), the IC₅₀ value is approximately equal to the Kᵢ value. The Kᵢ may be more accurately calculated using Equation 4 (40), which yielded 81.4 µM.

\[ k_{obs} = \frac{k_{max} [I]}{1 + IC_{50}} \]  
\[ K_i = \frac{IC_{50}}{1 + [S] / K_m} \]  

The calculated Kᵢ value of CjAgd31B is similar to Kᵢ values for acarbose with other GH31 enzymes, such as the human maltase-glucoamylase N-terminal subunit (62 µM, (41)) and sucrase N-terminal subunit (14 µM, (42)), while it is significantly higher than the Kᵢ values for the human maltase-glucoamylase C-terminal subunit (1.72 µM (43)), the *Gracilaria lemaneiformis* α-glucan lyase (0.02 µM, (44)), and the *Thermoplasma acidophilum* AglA α-glucosidase (2.99 µM, (23))

**Tertiary structures of CjAgd31B** – Three tertiary structures of CjAgd31B were obtained at 1.9, 2.0 and 1.85 Å resolution, respectively: the free enzyme, a non-covalent complex with the inhibitor acarbose, and a trapped 5-fluoro-β-D-glucopyranosyl-enzyme intermediate. All crystals contained one molecule in the asymmetric unit. The CjAgd31B structures consisted of a typical GH31 fold comprised of four domains, with two insertions (Figure 5A): the N-terminal domain (N-terminal; residues 35-240), the catalytic (β/α)₈ domain (residues 241-586) with insertion domain 1 (Insert 1; residues 345-384) and insertion domain 2 (Insert 2; residues 415-435), the C-terminal proximal domain (C-proximal; residues 587-667) and the C-terminal distal domain (C-distal; residues 668-817). In all structures, the electron density map of the ten N-terminal residues from 25 to 34 and C-terminal residues from 818 to the end (859) including the V5-epitope and His-tag provided by the expression vector were disordered. The free and acarbose structures had a disordered region from 137 to 140 and the 5FβGlc-enzyme structure had a disordered region from 139 to 140. The visible secondary structures of the four domains, including the insertion domains, in CjAgd31B were well conserved with the human sucrase isomaltase (PDB ID 3lpp) of GH31, with an r.m.s.d. of 1.9-2.4 Å.

The free enzyme structure reveals a water-lined pocket where the conserved catalytic aspartic acid residues (D412 and D480) are located (45,46). The pocket was 318 Å³, as calculated by the Pocket-Finder server (47), with a depth of approximately 11 Å. The active site pocket was composed of residues Y179, F271, D299, L300, M311, I341, E343, Y376, F377, W410, D412, L413, E417, R463, W477, D480, D509, F513, R538, H540, and Q542 (Supplemental Figure S4). To define enzyme-substrate interactions, two complexes – with the inhibitory tetrasaccharide acarbose *(vide supra)* and the covalent 5-fluoro-β-D-glucopyranosyl-enzyme intermediate – were obtained.

**Structure of the Acarbose Complex** – The CjAgd31B complex with acarbose revealed clear, unambiguous density for the inhibitor in the -1 to +3 subsites (Figure 5B, subsite nomenclature according to (39)). In the -1 subsite, the enzyme-derived nucleophile, D412, is indeed poised for nucleophilic attack, lying 3.2 Å “above” the pseudo-anomeric carbon of acarbose and with a
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nucleophile-C1-NH angle of 164.1 degrees. The catalytic acid, D480, lies 2.5 Å from the “integlycosidic” nitrogen of acarbose, as expected. The hydrophobic residues L300, I341, W410, W477, F271, and F513 all lie within 4 Å distance from the -1 subsite pseudo-sugar of acarbose (Supplemental Figure S4).

The +1 subsite contains the 6-deoxyglucosyl moiety of acarbose. Hydrogen bonds are made to R463, E417 and a water molecule, and enzyme-substrate distances suggest van der Waals contacts to F377. In most other solved structures of GH31, the hydrogen bonds provided here by E417 (Insert 2) are made instead by a aspartate residue from a loop in the N-terminal domain (42,48-50); the other exception to this is found in CjXyl31A, in which a PA14 domain insert in the N-terminal domain extends the active site (26,42,48-50).

The substrate-interacting residues in the -1 and +1 subsites of CjAgd31B structure are essentially homologous to those in the human maltase-glucoamylase (MGAM) and the Ro-αG1 α-glucosidase from Ruminococcus obeum, with the exception of F271, L300, F377 and L413, which instead are W, I, W and M in the MGAM and Ro-αG1 structures, respectively (Supplemental Figure S5; (48,49)). These side chains all make van der Waals contacts to acarbose, as well as 5FβGlc (vide infra). It is possible that F271 of CjAgd31B, which corresponds to W169 and Y299 in Ro-αG1 and MGAM, respectively, contributes to substrate specificity, as wild type Ro-αG1 prefers isomaltose to maltose as a substrate while the W169Y mutant inverts this preference (49).

Notable features of the +2 and +3 subsites are the tyrosine clamp of Y179 (part of a long loop extending from the N-terminal domain) and Y376 of Insert 1, which together form van der Waals contacts to the internal glucose moiety (third ring) of acarbose (Figure 6B, cf. Supplemental Figure S4). A similar hydrophobic clamp has not been found in other GH31 structures, apart from the PA14-mediated protein-sugar interaction in CjXyl31A (26).

Structure of the trapped covalent 5-fluoro-β-glucosyl-enzyme intermediate – To assess factors that may lead to the strict transglycosylation activity of CjAgd31B, a near-mimic of the covalent glycosyl-enzyme intermediate was accessed using a classic “Withers” reagent, 5-fluoro-α-D-glucopyranosyl-fluoride (5FoGlcF).

The electron density map clearly reveals the trapped 5-fluoro-β-D-glucopyranosyl-enzyme, observed in 1S1 skew boat conformation (conformational aspects of catalysis are reviewed in (51)) formed via covalent linkage to the OD2 atom of D412 with 1.34 Å distance (Figure 6A). The majority of interactions of this sugar are the same as previously observed for the -1 subsite of the acarbose pseudo-tetrasaccharide (Supplemental Figure S4), but in addition, two water molecules (W1 and W2 in Figure 6B) bind to OD2 atom of D480 the acid/base residue. The water molecules W1 and W2 form a hydrogen bond network with D480, R463, E417 and a third water molecule (W3) binding to Q542. Although transglycosylation is always kinetically favored over hydrolysis (52), what is unusual about CjAgd31B and indeed other transglycosylases is how they overcome the thermodynamically favored hydrolysis reaction in 55 M water, as discussed below.

DISCUSSION

Through a combination of enzymological and structural analysis, we have revealed that CjAgd31B from the soil saprophyte Cellvibrio japonicus possesses the ability to exclusively transfer single glucosyl units from α(1→4)-glucans to the non-reducing-terminal 4-OH of glucose and α(1→4)- and α(1→6)-linked glucosyl residues; weak hydrolysis activity is only observed on the disaccharide maltose. As outlined in the Introduction and discussed below, this type of transglycosylase has not previously been...
described in GH31, nor any other CAZyme family, to our knowledge.

GH31 enzymes utilize a double-displacement mechanism involving a covalent glycosyl-enzyme intermediate, which, as was the case here for CjAgd31B, can be trapped and directly observed using kinetic probes derived from fluorosugars (46). In the natural reactions catalyzed by GH31 members, the glycosyl-enzyme is most commonly decomposed by water, yielding substrate hydrolysis. However, this intermediate can also be intercepted by saccharide acceptor substrates to generate transglycosylation products with varying efficiencies, in a substrate- and enzyme-dependent manner (53,54). Indeed, several members of GH31 have been shown to possess transglycosylation ability, although yields are typically low due to dominating hydrolytic reactions (21-23,55,56). In this context, the strict transglycosylating activity on maltooligosaccharide substrates with d.p. ≥ 3 is particularly noteworthy.

CjAgd31B is also distinct from homologues of bacterial ctsY and ctsZ gene products, which are the only other predominant transglycosylases to have been identified in GH31 thus far. Working in concert, ctsY and ctsZ generate cycloalternan tetrasaccharides from α(1-4)-glucans via a three-step reaction (19,20). In the first step, ctsZ acts as an α(1→4)-to-α(1→6) transglucosylase to generate isomaltosyl moieties at the end of α(1→4)-glucan chains. Our HPAEC-PAD data indicate that, in contrast, CjAgd31B effects α(1→4)-to-α(1→6) transglycosylation, and moreover, cannot address (1→6)-linkages: isomaltose is not a donor substrate. In the second and third steps of cycloalternan tetrasaccharide synthesis, ctsY catalyzes an intermolecular isomaltosyl transfer to yield a α-D-Glc(1→6)-α-D-Glc(1→3)-α-D-Glc(1→6)-α-D-Glc(1→4)-α-glucan structure, followed by intramolecular cyclization to yield cyclo[α(1→3)-α-D-Glc(1→3)-α-D-Glc(1→6)-α-D-Glc(1→4)-α-D-Glc(1→3)-α-D-Glc(1→2)]. These two reactions are clearly distinct from that catalyzed by CjAgd31B. Unfortunately, the three-dimensional structures of both ctsZ and ctsY are currently unknown, which precludes comparison with CjAgd31B to understand the structural basis for these divergent transglycosylation activities in GH31.

The reactions catalyzed by CjAgd31B bear some similarity to, but are again distinct from, those catalyzed by 4-α-glucanotransferases (EC 4.2.1.25) of GH13 and GH77. GH13 encompasses a huge diversity of α-glucan hydrolyzing and transglycosylating enzymes (57), of which the Thermotoga spp. 4-α-glucanotransferases are perhaps the most relevant to the present study (12,13,58). T. maritima and T. neapolitana 4-α-glucanotransferases catalyze disproportionation reactions of maltooligosaccharides, utilising maltotetraose as the smallest donor substrate (12,13). The structure of the T. maritima enzyme has revealed that the active site is an open cleft comprised of at least 5 subsites (-2 to +3), which provides clear rationalization for the ability of the enzyme to randomly transfer longer α-glucan chains. In contrast, the T. maritima maltosyltransferase (MTase), also of GH13, strictly transfers maltosyl (Glc₂) units from maltotriose and longer maltooligosaccharides to the 4-position of α-glucan acceptor substrates (58), due to the presence of a unique protein motif that blocks the active-site cleft (59).

GH77 enzymes are structurally related to GH13 enzymes in clan GH-H, and thus generally possess open cleft-shaped active sites (60-62) that confer specificity for longer glucan donor substrates (9). For example, the eukaryotic S. tuberosum (potato) starch disproportionating (“D”) enzyme has such an extended active-site (PDB ID 1x1n), transfers extended α-glucan chains, and does not use maltose as a donor substrate(14,63,64). The disaccharide is also not a substrate for the Thermus aquaticus amylomaltase of GH77, which is distinguished by its propensity to form large cyclic α-glucans from long α(1→4)-
glucan donors (14,60). In the context of bacterial maltooligosaccharide metabolism, the *E. coli* GH77 amylomaltase MalQ appears to favor the transfer of longer α-glucan chains, although there appears to be some debate whether this enzyme can utilize maltose as a donor, thereby transferring a single glucosyl residue to longer congeners.

*Cj*Agd31B thus occupies a unique catalytic place as a 4-α-glucosyltransferase among the broader spectrum of 4-α-glucanotransferases. As a member of GH31, *Cj*Agd31B belongs to clan GH-D, which is also comprised of glycoside hydrolase families GH27 and GH36. Clan GH-D and GH-H members are built on a common triose-isomerase (β/α)8 barrel scaffold and may share a distant evolutionary relationship (27). However, in contrast to the clefted clan GH-H members, clan GH-D members are typified by shallow, pocket-shaped active sites comprised of only one negative subsite accommodating a monosaccharide residue, the glycosidic bond of which undergoes catalysis. *Cj*Agd31B likewise presents an active-site pocket, as revealed by acarbose and 5-fluoro-glycosyl-enzyme complex structures, which allows speculation as to its strict glucosyltransfer capacity.

The inherent challenge for transglycosylases is overcoming the thermodynamic preference for water as a nucleophile versus saccharide acceptors substrates. It has long been established by Withers, through analysis of the reactivation of trapped intermediates (52), that transglycosylation is kinetically favored over hydrolysis. Crystallographic analysis of the trapped covalent glycosyl-enzyme intermediate of *Cj*Agd31B suggests that the hydrogen-bonding scheme does not place a water molecule with appropriate geometry nor interaction with the catalytic acid/base residue D480 to facilitate hydrolysis of the intermediate. Instead, two water molecules (one of them disordered) appear to lie on either side of the position expected of a catalytically competent nucleophile. The interglycosidic nitrogen of acarbose does, however, interact with the acid/base (and with its O3 and C5 groups binding in positions corresponding to the observed waters of the trapped intermediate).

This solvent hydrogen-bonding arrangement suggests that the *Cj*Agd31B active site has evolved to avoid deprotonation and activation of water, while optimization of hydrogen bonds to O6 and O3 of acceptor glucosides in the +1 subsite allows a favourable placement of the O4 atom for deprotonation and concerted electrophilic migration of C-1 of the β-glucosyl-enzyme. In this process, Glu417 and Arg463 play particularly important roles in binding the O3 of the +1 sugar and legislating against a water molecule positioned to enable hydrolysis. A caveat is that the trapped intermediate observed is that of a 5-fluoro glycoside, so it is possible that the observed solvent network is perturbed by the unnatural 5-fluoro substituent. However, the solvent network of the free enzyme structure is similar to that of the trapped intermediate complex, especially in context of the O6- and O3-mimicking water molecules, where there is no suitably poised “nucleophilic” water molecule bound to D480.

It is currently unclear what role(s) *Cj*Agd31B might play in the biology of *C. japonicus*. Interestingly, the gene encoding *Cj*Agd31B is located among a cluster of genes predicted to encode α-glucan active enzymes (α-amylase, cyclomaltodextrin glucanotransferase, 6-phospho-β-glucosidase, glucokinase) and transporter proteins (TonB-dependent receptors and ABC transporters; Supplemental Figure S2). Whether these genes are co-regulated or comprise an operon is currently not known. This genomic association, together with the observation that *Cj*Agd31B is encoded with a native secretion signal peptide, hints toward a role in glycogen or starch metabolism in the periplasm. Indeed, *Cj*Agd31B could possibly have a function similar to the GH77 amylomaltase MalQ from *E. coli*, which creates longer α-glucan chains from shorter maltooligosaccharides as substrates for maltodextrin phosphorylases; these...
phosphorylases require maltopentaose as a minimal substrate to generate \( \text{Glc-1-P} \), for further metabolism (5,65,66). *C. japonicus* does, however, possess a predicted GH77 homologue (*CjMal77Q*, CIA_1882, (25)), which is located elsewhere in the genome and in proximity to other predicted glycogen/starch-active enzymes. This would suggest that *CjAgd31B* and *CjMal77Q* most likely have independent, or perhaps complementary, functions.

Another possible clue to the physiological function of *CjAgd31B* can be gleaned from analysis of potential GH31 orthologs. The closest biochemically characterized GH31 member to *CjAgd31B* is the \( \alpha \)-glucosidase YihQ from *E. coli* K-12 MG1655 (38). Biological data on *E. coli* YihQ are currently lacking, however a reverse genetics analysis of a YihQ orthologue in *Salmonella enterica*, serovar Enteriditis, indicates that \( \Delta \text{yihQ} \) mutants are deficient in capsular polysaccharide formation (67). Notably, the LPS of this organism consists of a repeating core glycan comprised of tyvelose, L-rhamnose, galactose, and mannose that is appended with extended \( \alpha(1\rightarrow4) \)-glucan chains. It is therefore tempting to speculate that YihQ, and by extension *CjAgd31B*, may act as a transglucosylase to extend or restructure these chains. In this context, it is interesting to note that *E. coli* YihQ has previously been designated as an \( \alpha \)-glucosidase based on a weak activity on \( \alpha \)-glucosyl fluoride but no other \( \alpha \)-glucosides (38). A reassessment of YihQ activity both *in vitro* and *in vivo*, in light of the transglycosylation capacity of GH31 enzymes demonstrated in the present study, may well be warranted.

In conclusion, the detailed enzyme structure-function analysis of Agd31B from the model soil bacterium *Cellvibrio japonicus* presented here, which has defined a previously unknown \( \alpha \)-transglucosylase activity in GH31, will inform future functional genomics studies in bacteria and other microorganisms.
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REFERENCES

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A bacterial α-transglucosylase from glycoside hydrolase family 31


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FOOTNOTES

The abbreviations used are: 5FoGlcF, 5-fluoro-α-d-glucopyranosyl-fluoride; d.p., degree of polymerization; GH, glycoside hydrolase family; HPAEC-PAD, high performance anion-exchange chromatography with pulsed amperometric detection; IMAC, immobilized metal affinity chromatography; LC-ESI-MS, liquid chromatography electrospray ionization mass spectrometry; pNP, p-nitrophenyl; SAD, single wavelength anomalous diffraction; SDS-PAGE, sodium dodecyl sulfate polyacrylamide gel electrophoresis; SEC, size exclusion chromatography; TLC, thin layer chromatography.

FIGURE LEGENDS

FIGURE 1. HPAEC-PAD chromatograms of reaction products of maltooligosaccharides, catalyzed by CjAgd31B. A: Incubation with maltose (10 mM, 6.5 mM enzyme, 30 min). Shoulder peaks are assumed to be transglycosylation products arising from contaminants in the commercial maltose preparation. B: Incubation with maltotetraose (0.5 mM, 10 min reaction). Solid line: commercial maltotetraose sample prior to addition of enzyme; dotted line: enzyme reaction, indicating production of maltotriose (M3) and maltopentaose (M5) by disproportionation. Commercial maltooligosaccharides were used as standards to determine retention times.

FIGURE 2. A: Initial rate kinetics curves fitted to the Michaelis-Menten equation for maltotriose (black squares), maltotetraose (grey circles), and maltopentaose (black triangles); B: Initial rate kinetics curves fitted to the Michaelis-Menten equation for maltose. Black squares represent formed glucose, grey circles maltotriose and black triangles the rate of hydrolysis.

FIGURE 3. HPAEC-PAD chromatograms of donor/acceptor specificity experiments using starch, isomaltose (IM), and glucose: A: Starch blank (0.4 % w/v; solid line) and following incubation with CjAgd31B (dotted line), showing a reduction of short maltooligosaccharides (M to M5) and an increased amount of medium-length maltooligosaccharides (M7 to M12); B: Starch (0.4 %) following incubation with CjAgd31B with no additional acceptor substrate added (solid line), with added isomaltose (1 mM; dashed line), and with added glucose (1 mM; dotted line). With both alternate acceptor substrates, there is a clear increase in the amount of short- to medium-length oligosaccharides formed compared to when starch is used alone.
FIGURE 4. Inhibition of CjAgd31B by acarbose. Maltotriose was used as substrate (100 µM, 10 min reaction) and the inhibitory effect of acarbose on transglycosylation (formation of maltotetraose) was calculated by non-linear regression, fitting Equation 3 to the data.

FIGURE 5. The 3-D structure and ligand binding site of CjAgd31B. A: 3-D structure of CjAgd31B as a protein cartoon. The N-terminal β-sandwich domain is colored red, the central catalytic (β/α)_8 domain is shown in blue with inserts 1 and 2 colored purple and pink respectively, the C-terminal proximal β-sandwich is orange and the C-terminal distal β-sandwich domain is green. B: Observed electron density for the acarbose complex of CjAgd31B (map is at 1σ in divergent stereo). The nucleophile, D412, acid-base D480 and hydrophobic “clamp” residues (discussed in text) are shown. This figure was drawn with CCP4MG (66).

FIGURE 6. The covalent intermediate of CjAgd31B. A: Observed electron density for the trapped covalent 5-fluoro-β-glucosyl-enzyme intermediate of CjAgd31B (map is contoured at 1 σ in divergent stereo). The nucleophile, D412, acid-base D480 and a pair of solvent water molecules are shown. B: Overlay of the -1 and +1 subsite of the covalent intermediate complex (pink, waters as red spheres) with the acarbose (yellow) complex (Figure 5B). Of particular note is that neither (nearby) water of the intermediate complex is in appropriate position for nucleophilic attack of the intermediate by hydrolysis, implying that hydrogen-bonding is optimized to prevent hydrolysis and facilitate transglycosylation. This figure was drawn with CCP4MG (66).
Table 1. X-ray data collection and refinement statistics.

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Table 2. Iodine-SAD phasing statistics

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Table 3. Transglycosylating activity of CjAgd31B on various substrates.

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<th>((K_m)_{app}) (mM)</th>
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<th>(\Delta\Delta G^\dagger) (kJ mol^{-1})</th>
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\(\Delta\Delta G^\dagger\) was calculated using the formula: \(\Delta\Delta G^\dagger=RT\ln(k_{cat}/K_m[\text{maltotriose}]/k_{cat}/K_m[\text{maltooligo}])\)

No activity was detected on pNP-α-Glc, pNP-α-Xyl, α-glucosyl fluoride, isomaltose, sucrose or melibiose.
A bacterial α-transglucosylase from glycoside hydrolase family 31

FIGURE 1

A bacterial α-transglucosylase from glycoside hydrolase family 31
A bacterial α-transglucosylase from glycoside hydrolase family 31

FIGURE 2

A

[Graph showing enzyme activity vs. substrate concentration (S) in mM.]

B

[Graph showing substrate inhibition by maltose (mM).]
FIGURE 3
FIGURE 4

A bacterial α-transglucosylase from glycoside hydrolase family 31
A bacterial α-transglucosylase from glycoside hydrolase family 31

FIGURE 5
FIGURE 6

A bacterial α-transglucosylase from glycoside hydrolase family 31
Structural enzymology of *Cellvibrio japonicus* Agd31B reveals α-transglucosylase activity in glycoside hydrolase family 31

Johan Larsbrink, Atsushi Izumi, Glyn R. Hemsworth, Gideon J. Davies and Harry Brumer

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